REVIEW SUMMARY

Epithelial Plasticity: A Common Theme in Embryonic and Cancer Cells

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Background: During embryonic development, cells often travel long distances to form new tissues and organs. To be able to migrate, embryonic cells undergo a process known as epithelial to mesenchymal transition (EMT). Once migratory embryonic cells reach their destination they undergo the reverse process, mesenchymal to epithelial transition (MET) to later differentiate into multiple cell types. This reveals a high degree of cell plasticity, referring to the ability of cells to reversibly change phenotype, a common feature of embryonic cells. Research indicates that the EMT program is reactivated in cancer cells in the delamination from a primary tumor, the first step towards the colonization of distant organs to form secondary tumors (metastasis). It is the dissemination of cancer cells and the subsequent formation of metastasis that is responsible for the vast majority of cancer-associated deaths. Like EMT, recent advances show that cancer cells rely on the reactivation of developmental programs through MET for the localization and proliferation of disseminating cells. The embryo provides clues to understand the complex cell biology of EMT and MET in cancer and move towards improved therapeutic strategies.

Advances:

Due to the importance of the EMT/MET programs in normal development for the generation of tissues and organs as well as its role in cancer, stringent regulatory mechanisms are needed. Multiple extracellular signals converge in the activation of transcription factors (EMT-TFs) that can trigger the full EMT program. In addition, epigenetic and splicing programs as well as microRNAs regulatory networks regulate epithelial plasticity towards EMT or MET. As differentiated normal and cancer cells can

re-enter an undifferentiated stem-like state, another level of cell plasticity has become apparent, helping to understand complex cell behaviors and interactions.

Outlook: Technical advances in noninvasive in vivo imaging of embryos will help define cell behavior and plasticity in normal development, fundamental to understand congenital malformations. This knowledge will undoubtedly facilitate the study of tumor progression in animal models of cancer. Cancer cells can also be directly interrogated about their plastic states in molecular terms after purifying and analyzing circulating or disseminated single cells from animal models and also from patients, helping in the design of improved therapies. With respect to antimetastatic therapies, inhibiting EMT may be counterproductive in tumors that disseminate early, as rather than preventing metastasis, it could favor the formation of secondary tumors from already disseminated cells. Strategies aimed at targeting cancer stem cells are very promising but it is important to bear in mind that new cancer stem cells can be produced from differentiated non-stem bulk tumor cells.

ARTICLE OUTLINE

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EMT in embryonic development and the delamination of cancer cells form primary tumors

Reversal of developmental EMTs for organ formation

Reversal of EMTs for metastatic colonization

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EMT/MET in cancer: Conflicting data or just plasticity?

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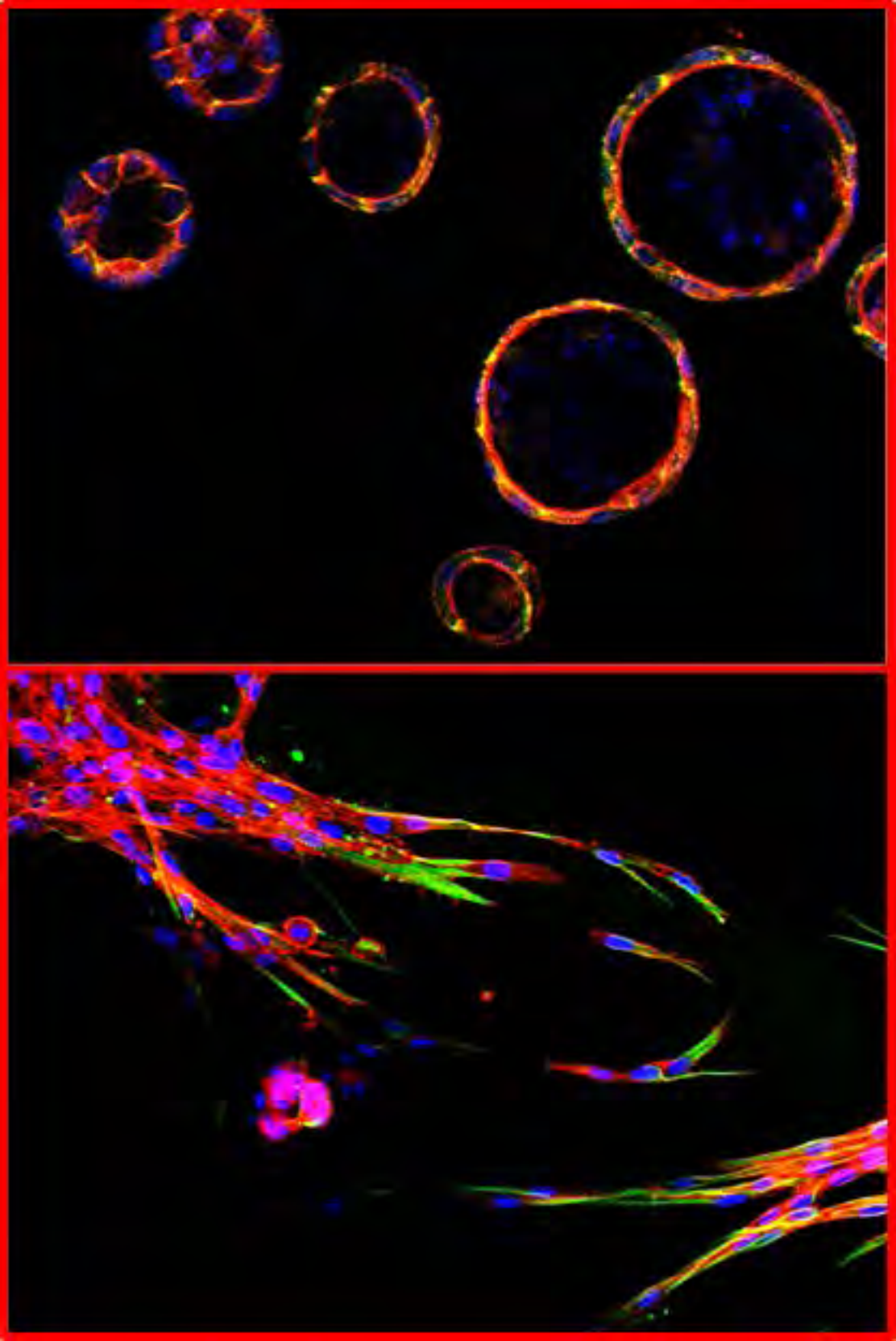
Figure Legend

Epithelial plasticity in 3D cultures. Epithelial cells (MDCK) form ducts when grown on 3D-matrices resembling the in vivo microenvironment (top). When grown under identical conditions, MDCK cells expressing Prrx1 (an EMT inducer) form networks of mesenchymal cells (bottom). Note the dramatic phenotypic change that is accompanied by the acquisition of motility and invasive properties. Blue, nuclei revealed by DAPI staining; Red, Actin filaments as seen after phalloidin binding; Green, E-cadherin (epithelial marker) in top panel and Vimentin (mesenchymal marker) in bottom panel.



Epithelial plasticity: a common theme in embryonic and cancer cells

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During embryonic development, many cells are born far from their final destination and must travel long distances. To become motile and invasive, embryonic epithelial cells undergo a process of mesenchymal conversion known as epithelial to mesenchymal transition (EMT). Likewise, EMT can be seen in cancer cells as they leave the primary tumor and disseminate to other parts of the body to colonize distant organs and form metastases. In addition, through the reverse process (MET), both normal and carcinoma cells revert to the epithelial phenotype to respectively differentiate into organs or form secondary tumors. The parallels in phenotypic plasticity in normal morphogenesis and cancer, highlight the importance of studying the embryo to understand tumor progression and to help in the design of improved therapeutic strategies.

Cellular plasticity refers to the ability of cells to reversibly change their phenotype. An example is seen in early metazoan embryogenesis, where epithelial cells take on mesenchymal characteristics, a process termed epithelial to mesenchymal transition (EMT). The EMT implies the conversion of an epithelial cell into a mesenchymal cell with the ability to migrate and invade adjacent tissues. Through the associated changes in adhesion and behavior cells can move into the interior of the embryo, travel long distances and participate in the formation of internal organs (1). Importantly, the latter implies the reversibility of the EMT, as when cells arrive to their destination they usually revert to the epithelial phenotype undergoing a mesenchymal to epithelial transition (MET) to settle, proliferate and differentiate into different organs (2) (Figure 1).

The hallmarks of the EMT can be summarized as follows: loss of cell-cell junctions, loss of apico-basal polarity and acquisition of migratory and invasive properties (3) (Figure 1). This phenotypic change is accompanied by a dramatic change in cell behavior (see Suppl. Movies) and is triggered in response to extracellular signals

by the activation of one or several transcription factors belonging to different families including Snail, Twist, Zeb and others (4), generally termed EMT-TFs (5). These EMT-TFs elicit the EMT program by repressing the epithelial phenotype, enhancing mesenchymal traits including motility and inducing the ability to degrade the basement membrane and the extracellular matrix in general. In addition, they also impinge into other basic cellular processes that help maintain the mesenchymal phenotype, the efficacy of migration and to survive in adverse environments. As such, EMT-TFs attenuate proliferation and protect from cell death activating survival signaling cascades (2) (Figure 1). Due to the pivotal role of E-cadherin loss, EMT-TFs are sometimes referred to as E-cadherin repressors and also, a decrease in functional E-cadherin is often associated with the activation of EMT. However, it is important to bear in mind that the EMT program and EMT-TFs are much more than E-cadherin transcriptional repressors, that E-cadherin downregulation (or endocytosis) is not necessarily associated with the EMT program and that E-cadherin re-expression is not sufficient to revert the fibroblastic phenotype (6).

When cells that escape from carcinomas are converted into mesenchymal cells with migratory and invasive properties, this can be considered an EMT (2, 3) and many EMT-TFs have been characterized that operate during tumor progression (7). However, the relevance of the EMT in cancer biology has been a matter of debate (8). The application of sophisticated imaging techniques to animal models and the characterization of circulating tumor cells (CTCs) from patients as cell with an EMT signature have provided evidences that the EMT also occurs during the dissemination of cells from a primary tumor (9-12).

Since the EMT can also endow cells with stem cell properties, and given that cancer can be initiated, maintained and propagated by stationary and motile cancer stem cells (CSC; 5, 13), a new field of study has emerged that requires the concerted effort of developmental biologists and cancer researchers. However, even though developmental and pathological EMTs have many common features they also have obvious differences, leading to the classification of three different EMT types (14). Type 1 refers to developmental EMTs, type 2 to those related to wound healing, tissue regeneration and organ fibrosis and type 3 refers to the EMT associated with cancer.

Developmental EMTs imply the conversion of epithelial into mesenchymal cells, but embryos lack inflammatory responses, typical of Type 2 and 3 EMTs that occur in the adult (14, 15). Type 2 EMT is also characterized by the appearance of myofibroblasts with the ability to secrete an excess of extracellular matrix molecules in response to inflammation (14). In contrast to the situation in embryos, in cancer cells and during regeneration, the acquisition of a mesenchymal state in fibrosis can be considered as an end stage, which leads to organ degeneration and failure (Figure 1). During cancer progression, tumor cells undergo type 3 EMT, which in addition to invasion and motility involves intravasation of delaminated cells into lymphatic and blood vessels, and their subsequent extravasation. The intravasation and extravasation steps do not occur in fibrosis and do have obvious parallels with the migratory processes undergone by embryonic cells. In this review I will focus on the similarities, in particular between embryonic and cancer cells, and how the latter rely upon developmental programs, not only to delaminate from the initial tumor but also, to later colonize distant sites. The common theme of epithelial cell plasticity unifies both events.

Global cellular programs regulate epithelial plasticity

EMT is triggered by many extracellular signals and agents both in embryos and in cancer cells. The most potent inducers are members of the TGFβ/BMP family, but also Wnt, Notch, epidermal growth factor (EGF), fibroblast growth factor (FGF), hypoxia, obesity, alcohol, nicotine, UV light and others (reviewed in 2). Such pathways and agents may act alone or in combination, and the response is dependent on the cell context (2). The response to such signals converges on the activation of the EMT-TFs, which can cooperate to induce or maintain the mesenchymal phenotype (4, 16). The existence of many EMT-TFs provides robustness to the system ensuring the implementation of the EMT program during embryonic development (1, 17). Analyzing regions where EMT is induced in embryos indicates that Snail expression normally precedes the expression of other factors, which are more relevant for the maintenance of the mesenchymal phenotype (2, 4). This temporal hierarchy of EMT-TF activation and cooperation and also seem to operate in tumors, as observed for Snail factors and

Twist in breast epithelial cells and breast cancer progression (16, 18-20). Once activated, EMT-TFs can, in turn, activate the same signaling pathways to generate positive regulatory autocrine loops that help maintain the mesenchymal phenotype both in normal and transformed breast stem cells (21).

Since EMT may be extremely deleterious when aberrantly activated in the adult (3) it needs to be very tightly regulated to ensure epithelial homeostasis. Besides the transcriptional level, there is also post-transcriptional, post-translational and epigenetic control, including a splicing program specifically associated with the epithelial phenotype and regulation by non-coding RNAs (recently reviewed in 22-24, Figure 1). The epigenetic, splicing and microRNA networks operating during EMT are now being defined at the whole genome level, both in embryos and cancer cells (25-28), identifying global regulatory programs that control the EMT. Such programs provide specificity and robustness through their phenotypic impact on each aspect of the EMT both in the embryo and in cancer cells (reviewed in 17, 24) (Figure 1).

EMT in embryonic development and the delamination of cancer cells from primary tumors

The internalization of cells from the surface of the embryo to form organs often involves an EMT. Cells disrupt cell-cell adhesion contacts within the primitive epithelial layer and ingress. The best studied examples are the ingression of mesendodermal precursors at the primitive streak in amniotes and the delamination of the neural crest (Figure 2), but it is worth noting that many additional EMT processes exist in the embryo, as with the exception of the anterior central nervous system and the epidermis, all vertebrate adult tissues originate from cells that underwent at least one EMT. The best evidence of EMT in cancer comes from the observation of single cells delaminating from primary tumors (9) and from the finding that circulating and disseminated tumor cells from patients (CTCs and DTCs, respectively) show EMT features and a high degree of epithelial plasticity (10,11) (Figure 2).

EMT in development and disease is not necessarily an "all or nothing" transition, but partial EMT can occur, where cells share epithelial and mesenchymal traits. Partial EMTs occur during *Drosophila* gastrulation, the early migration of groups of neural crest cells in anamniotes (amphibian and fish). These cell movements are sometimes referred to as collective migration to indicate that cells move in a coordinated manner while maintaining some cell-cell contacts (29). However, in terms of molecular mechanisms, during these three processes cells have engaged into the EMT program, downregulating *E-cadherin* transcription and losing apico-basal polarity upon activation of EMT-TFs (3). Partial EMTs are also observed in the epithelial component of carcinosarcomas (30) and at intermediate states during the progression of organ fibrosis, when parenchymal (epithelial) cells have activated the EMT program and show both epithelial and mesenchymal markers (31).

Cell movements can also occur in the absence of an EMT, when cells move while maintaining epithelial integrity. Examples of this type of movement include the migration of the lateral line primordium in the zebrafish embryo (Figure 2) and that of border cells in the *Drosphila* egg chamber (32). Cells do not express EMT-TFs, maintain cell junctions (i.e. including E-cadherin expression) and normally also maintain apico-basal polarity. These movements can also occur in cancer, when tumor cells invade without segregating from the invasive front (Figure 2), and these are able to colonize lymph nodes. However, in contrast to cancer cells that have undergone an EMT, they are not able to intravasate into blood vessels, to later develop blood-borne distant metastasis (33).

Reversal of developmental EMTs for organ formation

As noted above, developmental EMTs are reversible (34). As such, the cells that go through so called primary EMTs in the embryo later undergo the reverse process, MET, in order to differentiate into multiple cell types. Early mesodermal cells, generated by EMT and located at different medio-lateral levels of the embryo (axial, paraxial, intermediate and lateral plate mesoderms), condense into transient epithelial structures through MET to give rise to the notochord, the somites, the primordia of the urogenital system, and the splanchopleura and somatopleura, respectively (Figure 3).

In the majority of these structures a second round of EMT is completed. For example, the dorsal somite converts into the dermal and myotomal mesenchyme, which will give rise to components of the dermis and the muscle satellite cells. The ventral part will become the mesenchymal sclerotome that later generates the vertebrae, the ribs and the tendons (Figure 3). In turn, precursor cells of endodermal organs such as the pancreatic endocrine cells migrate upon undergoing EMT and revert this migratory phenotype through a MET to form the Langherham islets. A striking example of epithelial plasticity is the development of the cardiac valves, for which the initial mesenchymal precursors are specified at gastrulation stages and they must undergo three consecutive rounds of EMT/MET (2).

Although many signaling pathways have been identified for EMT (2), the signals involved in the induction of MET have not been well characterized in embryonic development. The most studied and amenable processes are those associated with the epithelialization of the paraxial and intermediate mesoderm to form the somites and the precursors of the renal system, respectively. An increasing gradient of BMP signaling along the medio-lateral axis patterns the different mesoderms, with lower concentration specifying the paraxial mesoderm. BMP7 counteracts TGFβ signaling and is the most prominent known epithelializing agent, with examples seen during kidney development (35), during the reprogramming of fibroblasts to induced pluripotent stem cells (iPSC; 36), in mouse models of renal fibrosis (37) and in cancer cells (38). Eph/Ephrin signaling is also involved in the epithelialization of somites (39) and Wnt signaling is required for MET during nephron development (40).

For nearly all MET processes, there is a concomitant downregulation of the corresponding EMT-TFs (i.e. 41, 42). This downregulation is accompanied by the appearance of specific epithelial markers that are associated with differentiation into the distinct tissues and organs. In the case of the epithelialization of the somitic and pronephric tissues, MET is associated with the induction of Paraxis and Pax2, respectively.

Reversal of EMTs for metastatic colonization

Despite the observations above, it is not universally accepted that tumor cell dissemination is related to a change in cell identity (i.e. a requirement for EMT, see 8) but rather, it might rely on mutations that weaken the cell-cell adhesion of cancer cells. Nevertheless, it is clear that carcinoma metastases usually adopt a well-differentiated epithelial phenotype. Ironically, this is one of the arguments used to dismiss the importance of the EMT in cancer progression. However, the differentiated phenotype of metastases reflects the epithelial plasticity implicated in tumor progression, in order words, the reversion of EMT firstly suggested by Brabletz and colleagues (43) and again resembling the situations described during embryonic development (34).

As in the embryo, MET concurs with the downregulation of EMT-TFs in cancer cells. MET not only implies a reversion to the epithelial phenotype but also an increase in cell proliferation, important for the growth of the secondary tumor. Several transcriptional repressors have been recently identified that can repress EMT-TFs, including Kruppel-like factor 17 (KLF17, 44), E74-like factor 5 (ELF5, 45) and Single-minded 2s (SIM2s, 46). Both ELF5 and SIM2s repress *Snail2* expression. Conversely, Snail represses *Single-minded* during *Drosophila* gastrulation (47), likely revealing an additional regulatory loop in the control of epithelial plasticity.

Complex regulatory loops also control epithelial plasticity post-transcriptionally, as numerous microRNAs are integrated into EMT/MET regulatory networks (22, 24). Double-negative feedback loops have been described between EMT-TFs (Snail, Zeb and Gata3 factors) and several miRs, including members of the miR-200 family and miR-34a (48-50), which promote MET and protect the epithelial phenotype (Figure 4). Recently, an additional microRNA has been added to this network, as miR-22 promotes EMT and cancer progression by indirectly repressing miR-200 (51) and miR-506 has been identified as a new node promoting MET (28) (Figure 4).

While TGFβ is a potent inducer of EMT-TFs, the tumor suppressor p53 activates miR-34a and miR-200 (50, 52) (Figure 4). The latter can be antagonized by the opposing role of mutant p53, which activates Zeb1 trough the repression of miR-130 (53). Another p53 family member, p73 and its truncated antagonistic from DNp73, also

play opposing roles in tumor progression as while p73 behaves as a tumor suppressor, DNp73 induces EMT (54). Going back to the mentioned microRNAS, although much less is known about their function during embryonic development, it is clear that miRs help the establishment of embryonic territories (55) and that a double-negative regulatory loop is established between miR-200 and Sox2 transcription factor during neuronal differentiation (56). The expression pattern of miR-200 in developing embryos is compatible with its role in preserving the epithelial phenotype and promoting MET.

MET induction by miR-200 was shown to promote breast cancer metastasis (57) in agreement with earlier findings indicating that MET facilitates bladder cancer metastasis (58) and the observation that myeloid cells induce a MET in the metastatic niche (59). However, until very recently a requirement for epithelial plasticity and the reversion of EMT had yet to be directly assessed *in vivo*.

In an elegant study using a spontaneous squamous cell carcinoma model in mice carrying skin-specific inducible Twist, it was shown that Twist-mediated EMT is sufficient to promote the dissemination of cancer cells into the bloodstream. Yet more importantly, it was shown in this model that Twist needs to be downregulated for metastasis to occur (60). Further in vivo evidence for epithelial plasticity and the requirement of MET has been found in the progression of breast cancer to the metastatic state. EMT mediated by an EMT-TF, the homeobox and paired-related Prrx1 factor, needs to be reverted, and Prrx1 loss is required for metastatic colonization (16). Therefore, a reversible EMT seems to be necessary for the metastasis of primary carcinomas, revealing a developmental program that is reactivated by cancer cells in order for them to successfully complete the final step of the metastatic cascade, the formation of macrometastasis.

Uncoupling EMT and stemness

It has been found that EMT can confer stem-like properties on cells (61, 62). This is consistent with the concept of “migratory cancer stem cells” (63) and provides a link between the EMT program and the characteristics associated with cancer stem cells

(CSC), i.e. self-renewal and the capacity to seed secondary tumors and produce differentiated non-stem cells (5). However, the relationship between EMT and stemness is another controversial issue in tumorigenesis, as it was later shown that fibroblasts must undergo a MET to complete the initial reprogramming of fibroblasts to iPSCs (36, 64). Again, help may come from development, although CSCs differ from canonical embryonic stem cells (ESCs) in that they only revert to the phenotypes of the primary tumor and therefore, they have a more restricted potency than both ESCs and iPSCs (65).

The need for a MET during reprogramming is consistent with the epithelial nature of ESCs. In fact, the "Yamanaka factors" (Sox2, Klf4, Oct4 and Myc) act together with BMP7 to repress TGFβ signaling and EMT-TFs to revert fibroblasts to the epithelial phenotype (64). In agreement with this, both Snail1 and Prrx1 are downregulated during reprogramming before cells acquire pluripotency genes (65). In line with the incompatibility of a stable EMT phenotype and metastatic colonization, Celià-Terrasa and colleagues (67) have shown that EMT can suppress tumor-initiating abilities (TICs) in epithelial cells. Together, these results link the MET with stem-like properties, rather than EMT with stemness as proposed previously. A plausible explanation for this apparent contradictory data may be that epithelial plasticity and stemness are regulated independently. Indeed, the MET undertaken during reprogramming of fibroblasts requires continuous presence of the Yamanaka factors and it does not initially involve the acquisition of stemness, which represents a later event (36). In addition, one of the EMT-TFs, Snail2, cooperates with Sox9 in the acquisition of the mesenchymal mammary stem cell state, although it seems that Snail2 is mainly involved in the induction of EMT and Sox9 is responsible for the entry into the stem cell state (68). Further evidence of such independent regulation comes from the analysis of Prrx1, which unlike "classical EMT-TFs" (Snail, Twist, Zeb factors) induces EMT without concomitantly inducing CSC properties. By contrast, it is the loss of Prrx1 that facilitates self-renewal and mammosphere-forming ability in breast cancer cells (16), simultaneously inducing MET and tumor-initiating capacity, both favoring metastatic colonization.

How Prrx1 downregulation is achieved at the metastatic niche and how tumor initiating capacity is maintained upon the downregulation of EMT-TFs linked to stemness (Snail-type) is a matter of future investigation (Figure 5). Another pending issue is whether the two EMT types are parallel or exclusive processes in a particular tumor. On the one hand, Prrx and Snail do not show any correlation in datasets from patients. However, Prrx1 and Twist are correlated, and in these cases, Prrx1 prevails (16) as PRRX downregulation is sufficient to induce MET and CSC properties. It is possible that both types (Snail and Prrx) could co-exist, as depicted in Figure 5, but information is still lacking. On the other hand, isolated CTCs depict both EMT and CSC traits (10, 11), suggesting that they might not include the Prrx1 type. This has not been specifically addressed but it is likely that in purified CTCs Prrx-positive cells are underrepresented, as they are fully mesenchymal and might have escaped isolation with current purification protocols, which use epithelial markers to identify CTCs (10, 11).

In summary, data accumulated over the last few years indicate that EMT-TFs are developmental factors that when aberrantly activated in tumors initiate the invasion-metastatic cascade. This finding also favors the cancer stem cell versus the clonal evolution model by which successive mutations accumulate in a tumor providing a defined phenotype (69). However, two additional issues need to be discussed here. On the one hand, stemness is also a plastic state during tumor progression whereby both stationary and migratory CSCs can co-exist (13).

On the other hand, there are different subsets of EMT-TFs that while all able to induce the mesenchymal phenotype, decrease proliferation and promote invasion, they can either provide or repress CSC properties (classical EMT-TFs vs Prrx1, respectively), or in other words, they can either promote or repress tumor initiating capacities. The bottom line is that (i) EMT-TFs need to be downregulated for metastasis to form, which implies reversion to the epithelial phenotype and increased proliferation (Figure 5) and (ii) the regulation of stem cell properties is independent from that of epithelial plasticity. Indeed, malignancy by cancer cells require the reversion to the epithelial phenotype and the maintenance of a “stemness” state (70), which may be achieved when the Prrx1-type of EMT-TFs is repressed (16) and/or when

Connective Tissue Growth Factor (CTGF), recently described as both MET inducer and stem-like properties enhancer, is activated in cancer cells (71) (Figure 5).

EMT/MET in cancer: Conflicting data or just plasticity?

The high degree of epithelial plasticity observed during cancer progression together with the independent regulation of EMT and stemness has several implications. EMT-related invasion plus dissemination is required but it is not sufficient for completing the metastatic cascade, and therefore invasive and metastatic may not be equivalent terms.

In agreement with the requirement for invasion and dissemination for metastasis formation, the presence of CTCs has prognostic and predictive value in breast cancer patients (72). Accordingly, abrogating EMT-TFs such as Snail factors in cancer cells, make these cells less prone to invade and later generate metastasis when injected in mice (18). However, constitutive expression of Snail or Twist also suppresses metastasis formation (60, 67) as EMT-TFs must be downregulated for metastasis to form (60, 16). Rather than representing a paradox, these data simply reflect the plasticity of epithelia and the dynamics of the whole metastatic process, resembling that of embryonic cell migration to populate distant territories. Thus, while the EMT is associated with the early steps in metastasis, i.e. delamination, invasion and intravasation, it is not associated with metastatic colonization. Indeed, there is no association between the expression of Snail or Twist in primary tumors and relapse-free survival in patients with breast or lung squamous carcinomas (16). This should be born in mind when referring to the role of different elements in tumor progression and, consequently, avoid the use of the term “invasive and metastatic” for factors involved in the acquisition of invasive properties by cancer cells. A similar situation applies when we contemplate embryonic development. The EMT-TFs are expressed in the embryo wherever EMT processes take place. However, EMT-TFs are not expressed in the derivatives of these migratory populations, since once embryonic cells reach their destination to differentiate into distinct tissues, the EMT inducers are repressed. Thus, EMT-TFs are related to cell behavior rather than to cell fate and hence, their

expression is only transient. Accordingly, EMT-TFs cannot be used as markers of differentiated cell populations, the equivalent of differentiated distant metastases.

The description of signals that downregulate EMT-TFs at the metastatic sites awaits further investigation but it is clear that interactions between CSCs and the tumor microenvironment dictate both EMT/invasion of the primary tumor (73, 74) and colonization at the metastatic niche (57, 58, 74). The EMT program also seems to help recently extravasated cells to extend filopodium-like protrusions (FLPs), to interact productively with the niche, promoting homing and proliferation (75). Cytokines are important to modify both microenvironments as they are fundamental to recruit mesenchymal stem cells (MSCs) and inflammatory cells. Indeed, the stroma influences metastasis formation and recent data point to the regulation of TGFβ/BMP signaling in both the primary tumor and the metastatic niche in colorectal and breast carcinomas (33, 76, 77).

Towards improved metastatic therapeutic strategies

Cell plasticity and heterogeneity in tumor biology also has an impact on the design of therapeutic strategies (69). Classical cytotoxic chemotherapy has proven beneficial in patients with carcinoma. However, one of the main caveats is the appearance of tumor recurrence, associated with acquired multidrug resistance and concomitant with the appearance of signs of EMT and stemness in the residual or relapsed tumor after chemotherapy. Indeed, resistance to cell death is a property of embryonic migratory cells upon undergoing EMT, implemented to promote their arrival to their destination (78). A similar strategy is used by cancer stem cells and therefore, efforts are being devoted to fighting not only proliferation but also EMT and stem cell-like properties to prevent the metastatic disease in cancer patients.

To target the EMT, the strategy has been to inhibit some of its inducing signaling pathways including those of EGFR, PDGFR and TGF-β. Recent data indicate that inhibiting the TGF-β pathway might be beneficial in colorectal cancer metastatic disease (77). But even if the bulk of the tumor can be significantly decreased, chemoresistant stem cells will support the subsequent growth of the tumor.

Therefore, different strategies have been used to directly target CSCs, including inhibitors of additional pathways such as those triggered by the developmental factors Notch, Wnt and Shh, using cytoprotective agents and/or small molecules identified in high-throughput screenings.

Recent data indicate that not only stem cells can generate differentiated cells but also that adult normal and (non-stem) cancer cells can re-enter the stem state, pointing to a bidirectional conversion between stem and non-stem populations (79, 80) (Figure 5). This additional plasticity seems to be dependent on the chromatin state of the *Zeb1* promoter, which is in a bivalent state in non-CSCs so that it can be rapidly activated in response to EMT inducing signals to help the conversion of non-CSCs into CSCs (81) (Figure 4). Thus, there is a need to adjust therapeutic strategies to this superimposed level of cellular plasticity, as combating CSC is unlikely to be sufficient as new stem cells can be generated from the remaining differentiated tumor cells. The idea is to combine classical chemotherapy with anti-CSC drugs to simultaneously hit non-stem cells and CSC in a tumor.

Finally, it is clear that fully preventing delamination from the primary tumor will impede metastasis, which is the principle that has inspired efforts by academia and pharmaceutical companies to block the EMT. A recent report shows that this seems to be a valid strategy, as observed in a mouse model of ovarian carcinoma (28). Nonetheless, in the light of recent data on epithelial plasticity during metastatic colonization, and the need for cancer cells to revert to the epithelial phenotype through a MET (16, 60), rather than preventing metastasis, inhibiting EMT could favor the formation of secondary tumors from already disseminated cells. This is particularly important in carcinomas where EMT is a very early event in tumorigenesis, where cancer cells have already disseminated before the tumor is diagnosed, as in pancreatic and breast carcinomas (82, 83). In contrast, as EMT reversion does not happen during the development of the fibrotic disease, therapeutic intervention inhibiting EMT is a promising strategy to ameliorate fibrosis and organ degeneration (37).

In summary, recent advances reveal an unanticipated degree of cell plasticity during the progression of carcinomas to the metastatic state and in the generation of

CSCs, reflecting the complex biology of cancer. This concept will undoubtedly influence the strategies for therapeutic intervention. Further complexity may arise when considering other tumor types. An example is found in melanomas, where recent data show that ZEB1 and TWIST1 promote dedifferentiation and are associated with late-stage melanoma and poor prognosis whereas SNAIL2 and ZEB2 do not behave as classical EMT inducers, as they are expressed in normal melanocytes and work as tumor suppressors (84). Fortunately, the process of morphogenesis provides clues as to how cell plasticity may occur in cancer, including the mechanisms used in the maintenance and differentiation of CSCs. Embryos together with cancer animal models such as “avatar” mice (carrying cancer cells from individual patients) will help define the best therapeutic strategy even on a personalized manner. In general terms, although inhibiting EMT may be counterproductive, strategies aimed at targeting cancer stem cells are very promising, keeping in mind that new cancer stem cells can be produced from differentiated non-stem bulk tumor cells.

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Figure legends

Figure 1. The control of epithelial plasticity in development and disease. Embryonic epithelial cells undergo EMT to migrate and then revert to the epithelial phenotype at their destination to give rise to different tissues and organs. After injury, epithelial cells undergo a partial EMT to heal the wound. Later undergo a MET to maintain epithelial homeostasis. Likewise, disseminated cancer cells need to return to the epithelial state during metastatic colonization, accompanied by the recovery of a high proliferation potential for the establishment of a secondary tumor. A MET is also required during reprogramming of fibroblasts to iPSCs. The development of organ fibrosis also involves EMT, which does not revert and leads to organ degeneration and failure. Both developmental and pathological EMTs induce not only morphological changes but also provide motility and invasive properties together with the ability to overcome safe-guard mechanisms, leading to cell survival and chemotherapy resistance, particularly relevant in cancer patients. Global cellular programs, including epithelial-specific splicing, epigenetic mechanisms and microRNA regulatory networks are in place to protect epithelial homeostasis. EMT, epithelial to mesenchymal transition; MET mesenchymal to epithelial transition.

Figure 2. Cell migration in embryos and tumors. Individual migration occurs after epithelial cells undergo EMT, as for neural crest migration during normal embryonic development and the delamination of breast cancer cells from the primary tumor. Partial EMT can also occur when cells activate the EMT program but maintain some contacts with their migrating neighbors. Integrated tissue migration implies a decrease in cell-cell adhesion forces but the maintenance of epithelial traits including cadherin expresion and apico-basal polarity. This type of migration is observed in the developing zebrafish lateral line and in the invasive front of some carcinomas. Cells at the invasive front can also activate the EMT program, in which case they can be seen delaminating as individual or loosely

organized groups of cells. Blue, epithelial cells; Yellow, mesenchymal cells (after EMT).

Figure 3. Reversibility of EMT during embryonic development. Physiological EMTS are reversible. Adult vertebrate tissues with the exception of the epidermis and part of the central nervous system are the result of one or several rounds of EMT/MET. The figure follows a temporal developmental sequence. 1. The precursors of the endoderm and the mesoderm ingress at the primitive streak, a prototypical example of primary EMT. 2. The mesodermal precursors migrate to occupy different positions along the medio-lateral axis of the embryo to give rise to axial, paraxial, intermediate and lateral mesoderm, which upon undergoing MET will generate notochord, somites, the urogenital system and the splanchno- and somatopleura, respectively. 3. The majority of these epithelial derivatives undergo a second round of EMT. The figure exemplifies secondary EMTs showing those occurring in the somites (see text). Blue, epithelial cells; Yellow, mesenchymal cells after EMT.

Figure 4. Epithelial plasticity and microRNA regulatory networks. A-D. Signaling pathways mediated by TGF-β or the Notch ligand Jagged induce the expression of EMT-TFs (Zeb, Snail, Prrx or Gata3 factors), which are downregulated by a series of microRNAs (miR-200, miR-130, miR-34a and miR-506) to protect epithelial integrity. Double negative-feedback loops are established in which microRNAs and the TFs behave as reciprocal repressors. The tumor suppressor p53 prevents EMT by activating miR-200 and miR-34a. In contrast, mutant p53 and miR-22 activate EMT by repressing miR-130 and miR-200, respectively. Epigenetic regulation of the *Zeb1 and miR-200* promoters impinges into their feedback loop. *Zeb* promoter bivalent state of allows its rapid activation, EMT and re-entry into the CSC state by increasing the expression of the surface marker CD44. In contrast, the TET complex demethylates *miR-200* promoter, thereby favoring MET. Sirtuin 1 (Sirt1) favors EMT by cooperating with EMT-TFs in the repression of E-cadherin expression. miR-

506 is a newly described EMT regulator able to repress several EMT-TFs including Snail2 and Prrx-1. Blue: MET related molecules; Yellow: EMT related molecules; Pink: CSC markers.

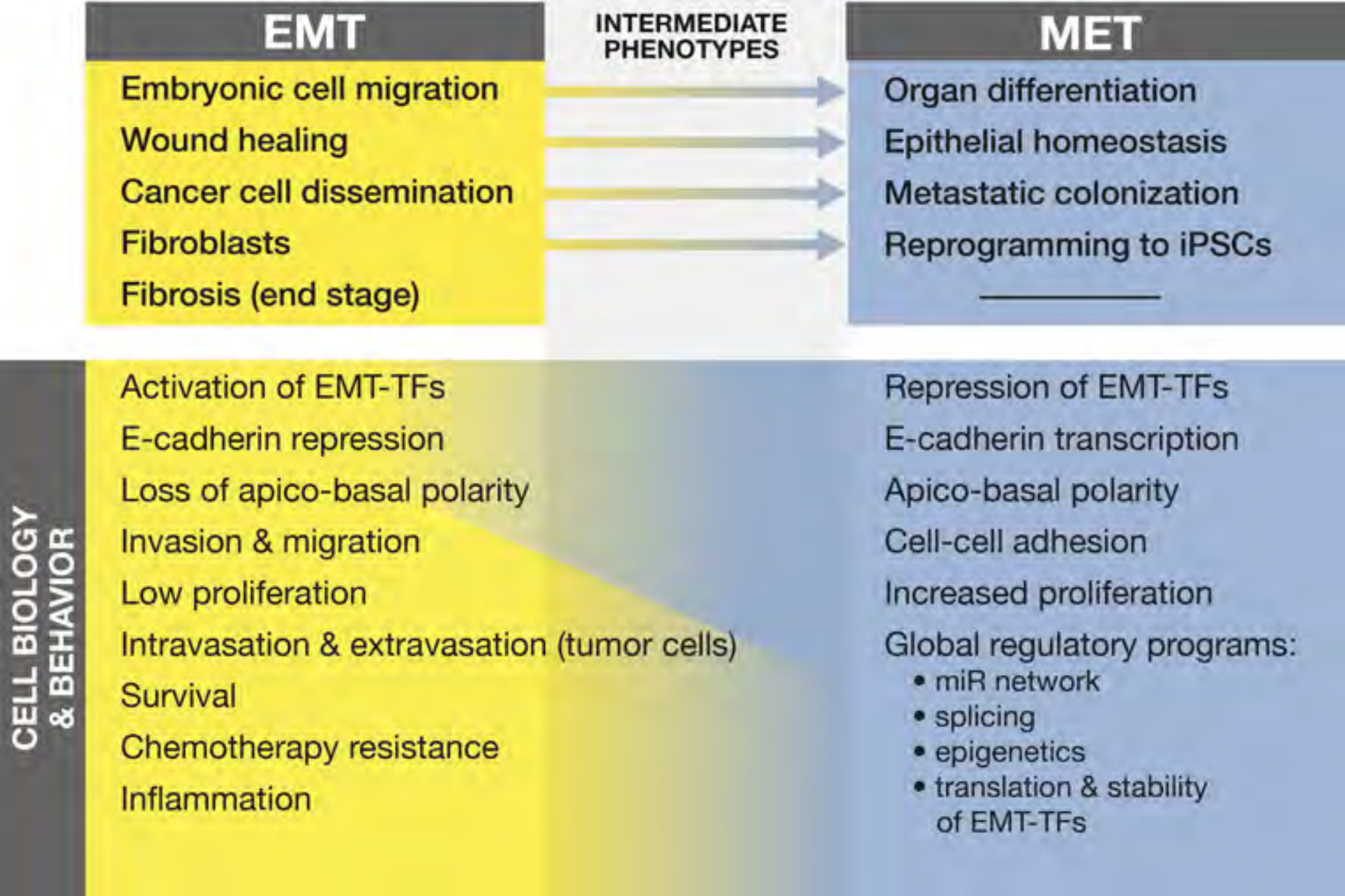
Figure 5. Reversibility of EMT during metastatic colonization. Cancer cells delaminate from the primary tumor expressing EMT-TFs, which endow them with invasive properties to migrate through the extracellular matrix and enter the lymphatic and blood vessels. Those migratory cancer cells expressing classical EMT-TFs (Snail, Twist and Zeb factors) present CSC properties whereas those expressing Prrx1 do not. These two classes of CTCs are depicted although whether the two types of EMT occur in parallel or are exclusive for individual tumors has not been shown. After extravasation, disseminated tumor cells can colonize distant organs, undergoing a MET upon downregulation of the EMT-TFs, which reverts them to the epithelial state and increase proliferation. Further work is necessary to characterize the signals that downregulate the EMT-TFs while maintaining CSC properties. CTGF has been shown to induce MET concomitantly with CSC traits in cancer cells, a global effect similar to that produced after Prrx1 downregulation. Stationary cancer stem cells can be converted into differentiated tumor cells and dedifferentiation of non-CSC can generate cells with tumor initiating capacity. This bidirectional conversion (white double arrows) adds a high degree of phenotypic plasticity in tumors that requires the design of improved therapeutic strategies.

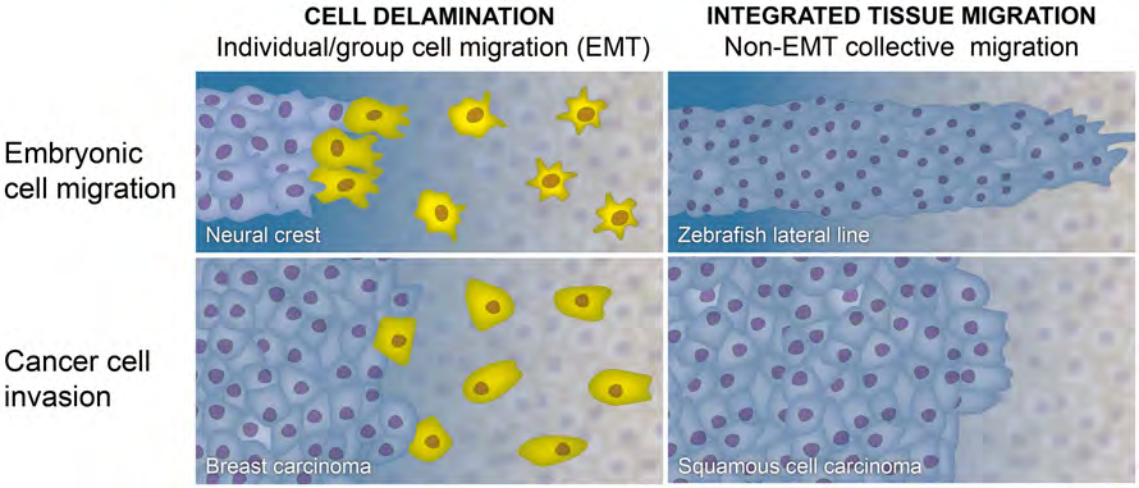
Supplementary movies legends

Movie S1. Epithelial cells are unable to degrade extracellular matrix and migrate.
Kidney Epithelial (MDCK) cells were suspended in Matrigel (BD Biosciences):Media (1:1) and a drop of the mixture was placed onto a glass-bottom culture Petri dish (MatTek). The drop was covered with culture medium and incubated in a chamber at 37ºC and 5% CO_2 (Life Imaging Services, Basel, Switzerland) that surrounds an inverted confocal Laser Scanning Spectral Confocal Microscope TCS SP2 AOBS (Leica

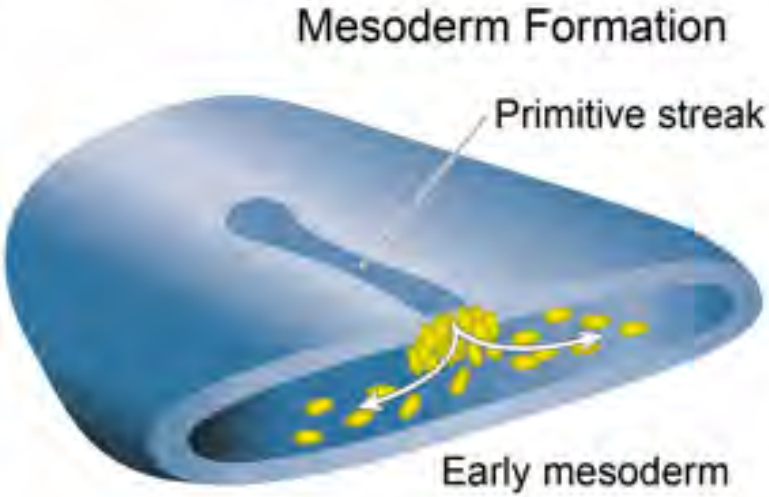
Microsystems, Heidelberg GmbH). For time-lapse movies, one image was captured every 10 min for a total of 17 hr. Movies were assembled using the ImageJ software (http://rsbweb.nih.gov/ij/).

Movie S2. Epithelial cells stably transfected with Prrx1 undergo a full EMT, degrade extracellular matrix and migrate. For time-lapse movies, one image was captured every 10 min for a total of 17 hr.
MDCK-Prrx1 cells were cultured and images processed as control cells (see Movie S1 legend).

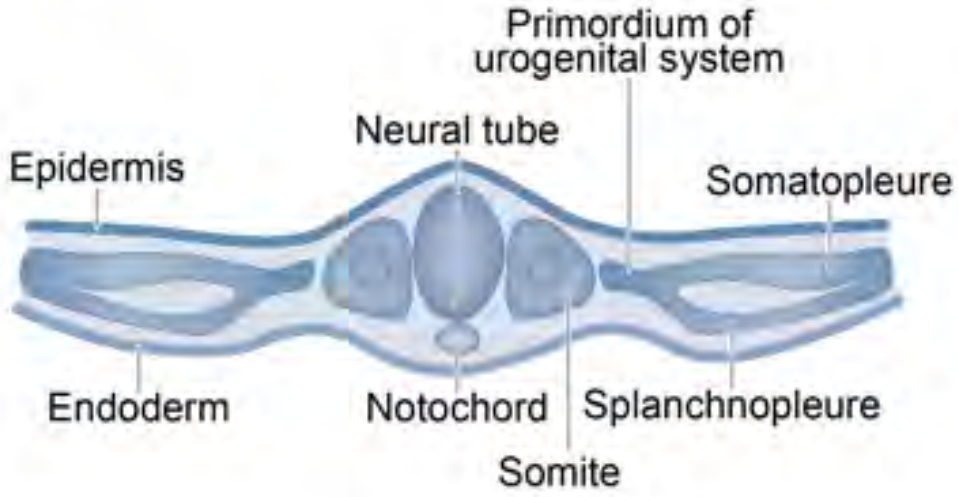

EMT
INTERMEDIATE PHENOTYPES
MET
Embryonic cell migration
Wound healing
Cancer cell dissemination
Fibroblasts
Fibrosis (end stage)
Organ differentiation
Epithelial homeostasis
Metastatic colonization
Reprogramming to iPSCs
CELL BIOLOGY & BEHAVIOR
Activation of EMT-TFs
E-cadherin repression
Loss of apico-basal polarity
Invasion & migration
Low proliferation
Intravasation & extravasation (tumor cells)
Survival
Chemotherapy resistance
Inflammation
Repression of EMT-TFs
E-cadherin transcription
Apico-basal polarity
Cell-cell adhesion
Increased proliferation
Global regulatory programs:
• miR network
• splicing
• epigenetics
• translation & stability of EMT-TFs


CELL DELAMINATION
Individual/group cell migration (EMT)
INTEGRATED TISSUE MIGRATION
Non-EMT collective migration
Embryonic cell migration
Cancer cell invasion
Neural crest
Zebrafish lateral line
Breast carcinoma
Squamous cell carcinoma

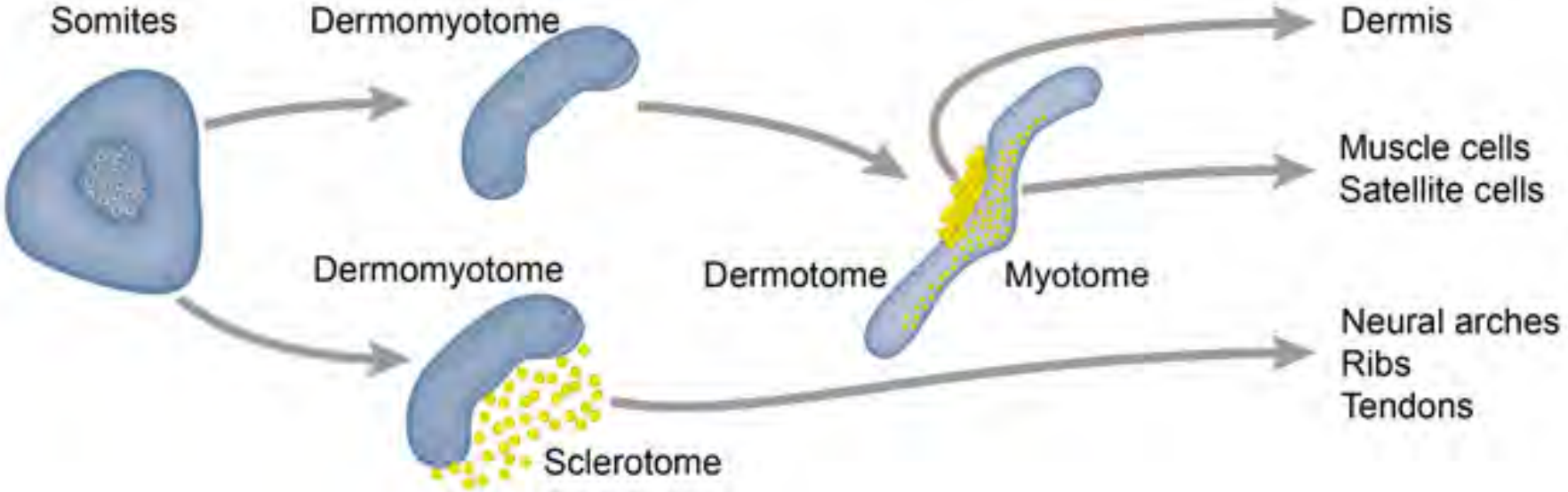
1. Primary EMT

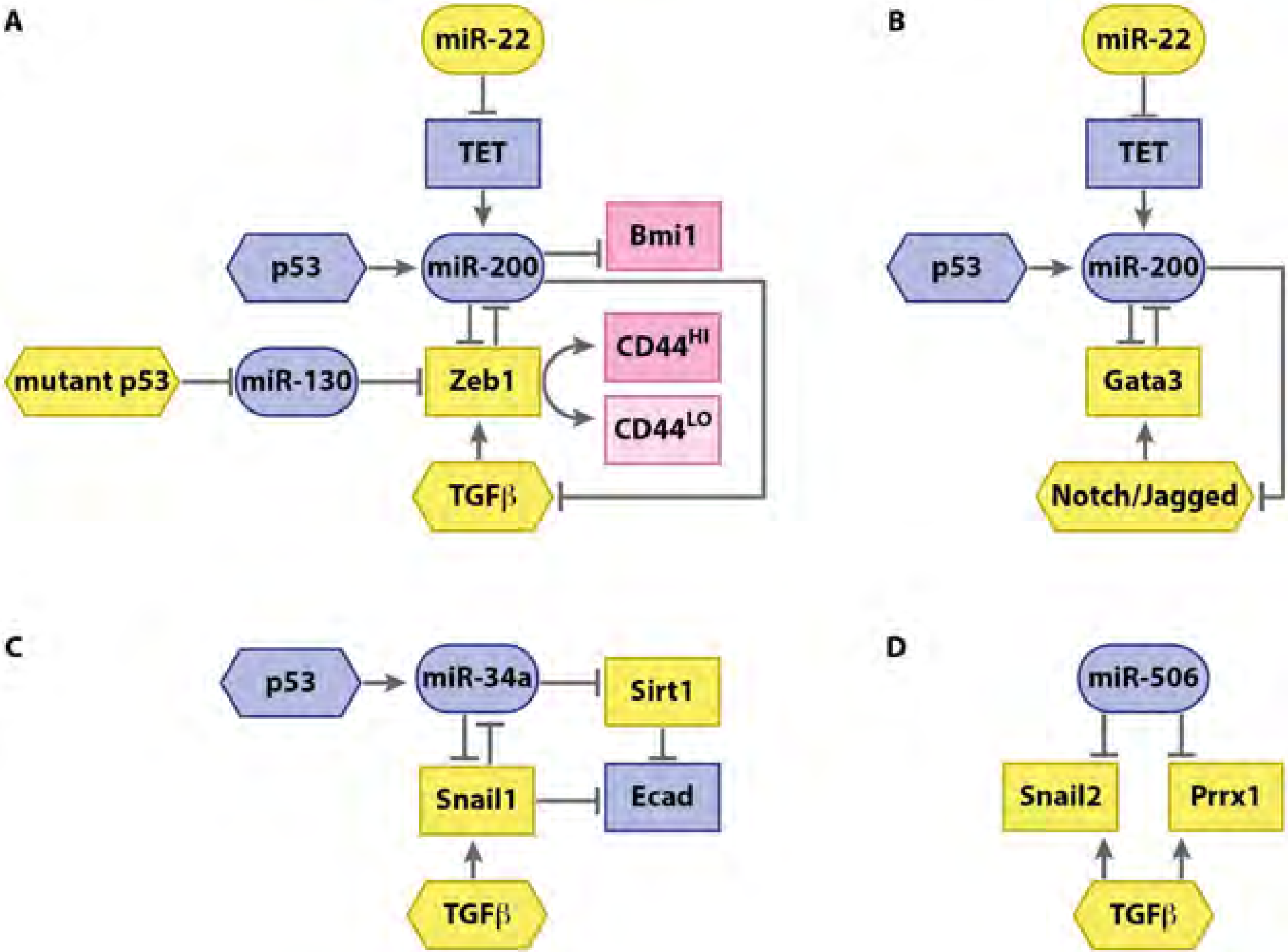


2. MET



3. Secondary EMT




A
miR-22
TET
p53
miR-200
Bmi1
mutant p53
miR-130
Zeb1
CD44HI
CD44LO
TGFβ
B
miR-22
TET
p53
miR-200
Gata3
Notch/Jagged
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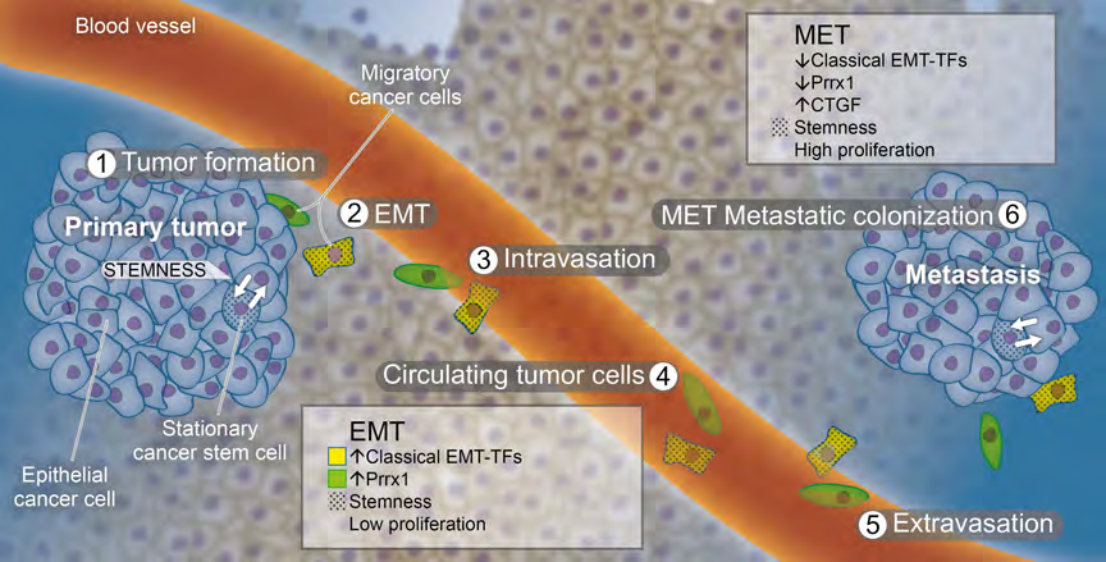
TGFβ
D
miR-506
Snail2
Prrx1
TGFβ


Blood vessel
Migratory cancer cells
MET
↓Classical EMT-TFs
↓Prrx1
↑CTGF
Stemness
High proliferation
1 Tumor formation
Primary tumor
STEMNESS
2 EMT
3 Intravasation
MET Metastatic colonization 6
Metastasis
Circulating tumor cells 4
Stationary cancer stem cell
Epithelial cancer cell
EMT
↑Classical EMT-TFs
↑Prrx1
Stemness
Low proliferation
5 Extravasation