Complex networks orchestrate epithelial–mesenchymal transitions

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Abstract | Epithelial–mesenchymal transition is an indispensable mechanism during morphogenesis, as without mesenchymal cells, tissues and organs will never be formed. However, epithelial-cell plasticity, coupled to the transient or permanent formation of mesenchyme, goes far beyond the problem of cell-lineage segregation. Understanding how mesenchymal cells arise from an epithelial default status will also have a strong impact in unravelling the mechanisms that control fibrosis and cancer progression.

Mesoderm
In animals with three tissue layers, the mesoderm is the middle layer of tissue, lying between the ectoderm and the endoderm. In vertebrates, it forms the skeleton, muscles, heart, spleen, kidney and other internal organs.

Endoderm
The innermost germ layer of the developing embryo. It gives rise to the lungs, digestive tract, thyroid, thymus, liver and pancreas.

Phyla
Large groups of species that share the same body plan. The animal kingdom is composed of about 30 phyla including Porifera, Cnidaria, Arthropoda, Echinodermata, and Chordata, which includes the Vertebrata as a subphylum.

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Epithelial and mesenchymal cells differ in various functional and phenotypic characteristics (FIG. 1). Epithelial cells form layers of cells that are closely adjoined by specialized membrane structures, such as tight junctions, adherens junctions, desmosomes and gap junctions. In addition, epithelial cells have apical–basolateral polarization, which manifests itself through the localized distribution of adhesion molecules such as cadherins and certain integrins, the organization of cell–cell junctions as a lateral belt, the polarized organization of the actin cytoskeleton, and the presence of a basal lamina at the basal surface. Epithelial cells are motile and can move away from their nearest neighbours, while remaining within the epithelial layer[1]. However, cells do not detach and move away from the epithelial layer under normal conditions.

Mesenchymal cells, on the other hand, do not form an organized cell layer, nor do they have the same apical–basolateral organization and polarization of the cell-surface molecules and the actin cytoskeleton as epithelial cells. They contact neighbouring mesenchymal cells only focally, and are not typically associated with a basal lamina. In culture, mesenchymal cells have a spindle-shaped, fibroblast-like morphology, whereas epithelial cells grow as clusters of cells that maintain complete cell–cell adhesion with their neighbours (FIG. 1). Cultured mesenchymal cells tend be highly motile, but this is not necessarily the case *in vivo*[2]. Indeed, there is plasticity in the way that mesenchymal cells migrate — they might migrate together as chains, or as individual cells that exhibit either cyclic extension–adhesion–retraction translocation or amoeboid-type crawling[3].

Epithelial cells can convert into mesenchymal cells by a process known as the epithelial–mesenchymal transition (EMT; FIG. 2). The term EMT describes a series of events during which epithelial cells lose many of their epithelial characteristics and take on properties that are typical of mesenchymal cells, which require complex changes in cell architecture and behaviour (FIGS 1,2). The transition from epithelial- to mesenchymal-cell characteristics encompasses a spectrum of inter- and intracellular changes, not all of which are always seen during EMT. EMT does not therefore necessarily refer to a lineage switch. The precise spectrum of changes that occur during EMT is probably determined by the integration of extracellular signals the cell receives, although this is still unclear. The reverse process, known as mesenchymal–epithelial transition (MET) has also been reported[4–7].

During embryogenesis, a number of extracellular signals can convert epithelial cells into mesenchymal cells by triggering EMT (FIG. 2). Mesenchymal cells do not derive exclusively from the mesoderm primary germ layer[8], epithelial endodermal cells can also produce mesenchymal cells[9]. EMT regulates important processes during the early stages of development of most organisms, except the two phyla of Porifera and Cnidaria (BOX 1); in the absence of EMT, development cannot proceed past the blastula stage. MET occurs during somitogenesis, kidney development, and coelomic-cavity formation[4–7]. Interestingly, EMT also has a role in adult organisms, as it has been shown to contribute to the pathology of certain diseases[10].

EMT did not receive molecular analysis until the early 1980s. Since then, a number of molecular differences have been observed between mesenchymal and epithelial cells (FIGS 1,2). For example, mesenchymal cells do not express epithelial (E)-cadherin, whereas epithelial cells do[11]. The constitution of intermediate filaments is also different, with vimentin being typical of mesenchymal cells and different types of cytokeratin

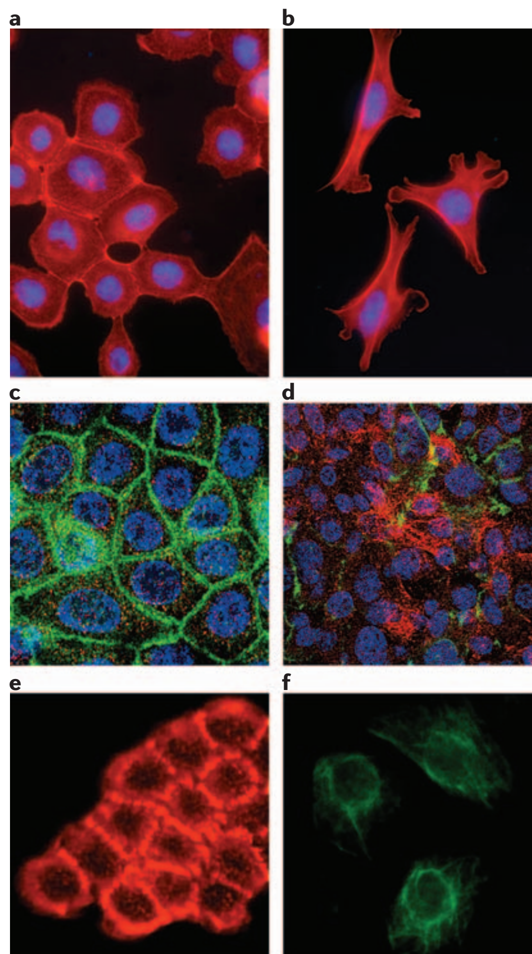

Figure 1 | **Images of EMT.** SCp2 murine mammary cells (**a**) were treated with matrix metalloproteinase-3 (**b**) to induce epithelial–mesenchymal transition (EMT)[10]. In both panels, cells were stained with anti-phalloidin antibodies to visualize the actin microfilaments and 4′,6-diamidino-2-phenylindole (DAPI) to visualize DNA. Pictures courtesy of D. Radisky (Mayo Clinic Cancer Center, Jacksonville, Florida, USA). Eph4 V12-transformed murine mammary cells (**c**) were treated with transforming-growth factor-β (**d**) to induce EMT. Cells were stained with anti-E-cadherin (green) and anti-vimentin (red) antibodies. Pictures courtesy of H. Beug (Institute of Molecular Pathology, Vienna, Austria). EMT in rat bladder carcinoma NBT-II cells. Pre-EMT cells were stained with anti-desmoplakin antibodies to visualize desmosomes (**e**), whereas post-EMT cells were stained with anti-vimentin antibodies to visualize vimentin expression (**f**).

Porifera
The most primitive phylum of the animal kingdom, it includes sponges.

Cnidaria
Radially symmetrical animals that form a phylum that includes jellyfish, corals, hydra and anemonies.

Blastula stage
An early-stage embryo that is composed of a hollow ball of cells.

being characteristic of epithelial cells. However, recent insights into the network of signals that regulate EMT and their role and integration during embryological processes indicated that several mechanisms are involved in the initiation and execution of EMT in development, and that the molecular mechanisms that regulate EMT are considerably overlapping with those that control cell adhesion, motility invasion, survival and differentiation. It is anticipated that our understanding of the role of EMT in development and also diseases such as fibrosis and cancer will be unravelled at the molecular level within the next decade. Given the recent rapid advance in our knowledge of the signalling strategies that orchestrate EMT, we discuss here the current understanding of the molecular basis of EMT and also the central importance of the signalling pathways that are involved in EMT in various developmental processes. Finally, we turn our attention to discuss pathological situations in which the same molecular pathways that regulate developmental EMT have an important role.

Signalling EMT

Tissue-culture studies have been instrumental in defining the molecular regulation of EMT. These studies show that several extracellular activators can trigger EMT, that extensive crosstalk exists between the signalling pathways that activate and repress EMT, and that EMT-inducing signalling pathways have many common endpoints, including downregulation of E-cadherin expression and expression of EMT-associated genes (FIG. 3). On the other hand, *in vivo*, each EMT event is regulated by a particular subset of EMT activators and repressors. For example, transforming growth factor-β2 (TGFβ2) regulates EMT in the atrioventricular canal, whereas TGFβ3 regulates palate-fusion EMT[12]. Furthermore, the effect of a given inducer on EMT is context dependent — scatter factor/hepatocyte growth factor (SF/HGF) induces EMT during somitogenesis, but it inhibits EMT in other processes[13]. The triggers and the signalling pathways that regulate EMT have been previously reviewed[14–16], so here we give a brief overview of the cascades that are involved in this process and highlight some recent important findings.

EMT is triggered by an interplay of extracellular signals, including components of the extracellular matrix (ECM), such as collagen and hyaluronic acid[17], as well as soluble growth factors, such as members of the TGFβ and fibroblast growth factor (FGF) families, epidermal growth factor (EGF) and SF/HGF (FIG. 3). Receptor-mediated signalling in response to these ligands triggers the activation of intracellular effector molecules, such as members of the small GTPase family — Ras, Rho and Rac — and members of the Src tyrosine-kinase family. These effectors orchestrate the disassembly of junctional complexes and the changes in cytoskeletal organization that occur during EMT. The activation of signalling pathways also results in the activation of transcriptional regulators such as snail (now known as SNAI1 (REF. 18)) and slug (now known as SNAI2), which regulate the changes in gene-expression patterns that underlie EMT.

A central target of these transcriptional regulators is the repression of the E-cadherin gene, an important caretaker of the epithelial phenotype. Downregulation of E-cadherin (or mutations that occur in cancer) has several important consequences that are of direct relevance to EMT. E-cadherin levels become limiting, which results in the loss of E-cadherin-dependent intercellular epithelial junctional complexes, and E-cadherin-mediated sequestering of β-catenin in the cytoplasm is abolished. As a result, β-catenin localizes

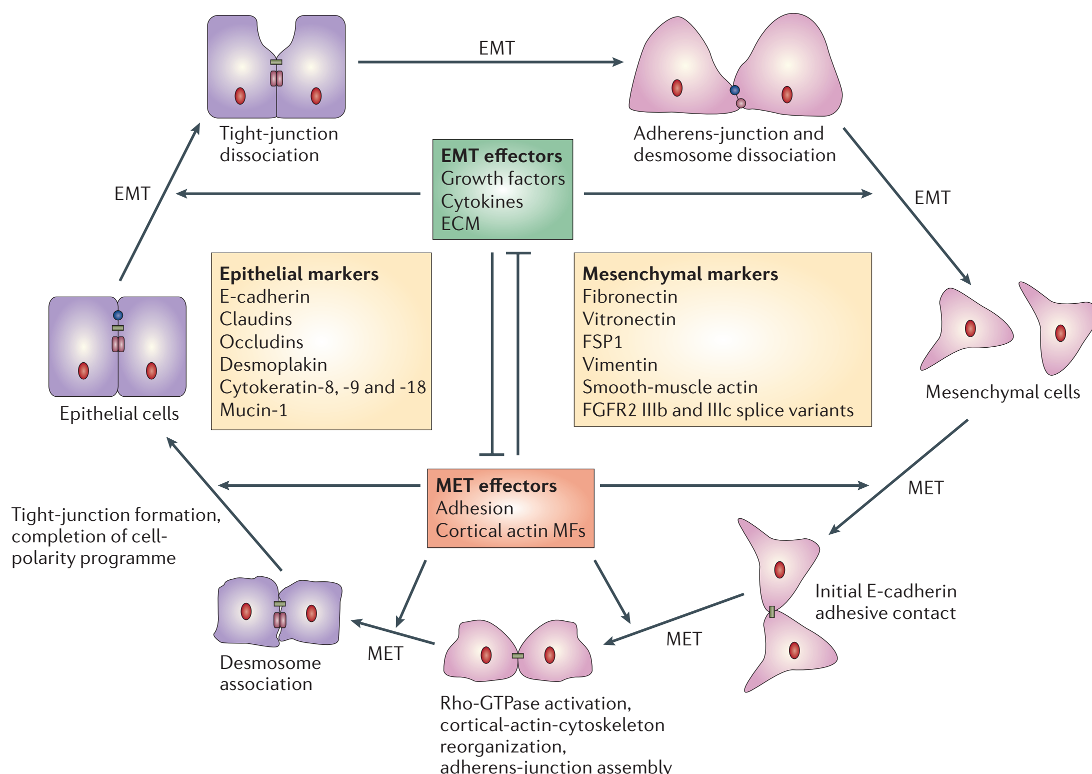

Figure 2 | **The cycle of epithelial-cell plasticity.** The diagram shows the cycle of events during which epithelial cells are transformed into mesenchymal cells and vice versa. The different stages during EMT (epithelial–mesenchymal transition) and the reverse process MET (mesenchymal–epithelial transition) are regulated by effectors of EMT and MET, which influence each other. Important events during the progression of EMT and MET, including the regulation of the tight junctions and the adherens junctions, are indicated. A number of markers have been identified that are characteristic of either epithelial or mesenchymal cells and these markers are listed in BOX 1 and BOX 2. E-cadherin, epithelial cadherin; ECM, extracellular matrix; FGFR2, fibroblast-growth-factor receptor-2; FSP1, fibroblast-specific protein-1; MFs, microfilaments.

to the nucleus and feeds into the Wnt signalling pathway by activating transcriptional regulation through LEF/TCF4 (lymphoid-enhancer-binding factor/T-cell factor-4).

The role of SNAI1 regulation. The central role of SNAI1 in the regulation of EMT has been underscored by recent studies that show the complex regulation of SNAI1 stability, subcellular localization and function through different phosphorylation events. The activity of glycogen-synthase kinase-3β (GSK3β) is inhibited by the AKT/PKB (protein kinase B), Wnt and Hedgehog pathways, each of which regulate EMT[19,20]. GSK3β has been shown to phosphorylate a number of transcription factors — such as p53, Myc and nuclear factor of activated T cells (NFAT) — which causes its export from the nucleus[21]. GSK3β phosphorylates two Ser residues on SNAI1, one of which targets SNAI1 for ubiquitination and degradation, whereas the other promotes its nuclear export[22]. Mutations in SNAI1 that prevent GSK3β-mediated phosphorylation result in a stabilized form of SNAI1 that localizes in the nucleus and induces EMT. As expected, inhibition of GSK3β activity led to enhanced cellular levels of SNAI1 with concomitant downregulation of E-cadherin.

It has also been shown that activators of EMT, such as EGF, signal to SNAI1 through p21-activated kinase-1 (PAK1)[23]. PAK1 phosphorylates SNAI1 on a different Ser residue from GSK3β, which results in the accumulation of SNAI1 in the nucleus and subsequent SNAI1-mediated transcriptional repression of target genes. Mutation of the phosphorylation site on SNAI1 or knockdown of PAK1 expression resulted in cytoplasmic accumulation of SNAI1 and ablation of its transcriptional-repressor activity. These studies show how different signalling pathways that regulate EMT impinge upon SNAI1 function, by regulating its activity through phosphorylation of different sites that alter its stability and subcellular localization.

Crosstalk between integrins and cadherins. In addition to dissociating cell–cell adhesions, the cells that undergo EMT have to regulate the integrin-mediated contacts with the ECM. Extensive crosstalk between integrin signalling and pathways that regulate EMT has been observed. For example, the TGFβ signalling through p38 MAPK that is required for EMT is dependent on signalling by β1 integrin[24]. Conversely, TGFβ-induced expression of disabled-2 (DAB2), an adaptor molecule

Box 1 | EMT in evolution and development

Mesenchymal cells are a great invention of metazoans that allow the shaping of the embryo through gastrulation. These cells are able to move and settle at sites of critical epithelial–mesenchymal interactions or they can differentiate into new structures. The formation of mesenchymal cells during evolution occurred more than 600 million years ago as a critical step in the establishment of the three germ layers. Sponges have no mesoderm, nor any structured layers, and therefore they cannot form tissues and organs. The Cnidarians are derived from the primitive colonial protozoans, but represent diploblastic species with ectoderm and endoderm, which allow the establishment of more elaborate tissue structures. However, they lack mesoderm. The importance of epithelial–mesenchymal transitions (EMTs) in morphogenesis was considerably augmented with the appearance of the Vertebrata, a subphylum of Chordates. Somite morphogenesis and neural-crest ontogeny are two important acquisitions of vertebrates.

The detailed strategy for EMT differs at various sites in a given embryo and is species-dependent. However, the effector molecules are evolutionarily conserved and include Wnts, fibroblast growth factors (FGFs), snails, nuclear factor (NF)-κB and E-cadherin. A plethora of molecules can induce SNAI1 during development, many of which are conserved throughout evolution, whereas a few are restricted to vertebrates. SNAI1 is expressed in the endoderm of Cnidarians, which is formed by the invagination of the ectoderm. Wnts and SNAI1 were already utilized in primitive species for induction of epithelial-cell plasticity prior to the acquisition of EMT during evolution. This situation is reminiscent of SNAI1 expression in the invaginating mesoderm in the fruitfly (*Drosophila melanogaster*)[18]. It is now clear that SNAI1 is not a primary inducer of mesoderm or neural crest, but, rather, contributes to epithelial-cell plasticity and EMT through modulation of adhesive interactions, which allow cell movement. The anti-apoptotic function of SNAI1 might be important in embryos, in which epithelial cells need protection from cell death during the dramatic morphological changes that occur during EMT.

that binds to β1 integrin, is required for integrin activation, the formation of focal adhesions and cell survival during EMT[25]. Similarly, α2β1 integrin is required for aspects of collagen and FGF1-induced EMT[26].

Recent work has uncovered a fascinating level of cross-talk between integrins and E-cadherin that coordinates the switch from cadherin- to integrin-mediated adhesions during EMT. Downregulation of E-cadherin has been shown to signal to integrins, because endocytosis of E-cadherin results in the activation of the small GTPase Rap1 — a protein that regulates the cytoplasmic activation of integrins and that is required for focal-adhesion formation[27]. On the other hand, integrins can cause the downregulation of E-cadherin in a number of ways. Integrin-linked kinase (ILK) interacts with the cytoplasmic domain of β1 and β3 integrins, and it is activated through cellular interactions with the ECM and growth factors. ILK downregulates E-cadherin expression[28], and is required for TGFβ-induced EMT[29]. β1 integrins also activate RhoA and Rac1, which leads to the disruption of cadherin-mediated adhesions[30]. Integrin-mediated focal adhesion kinase (FAK) activation can transiently down-regulate Rac1 in epithelial HeLa cells at sites of formation of N-cadherin-mediated cell–cell contacts[31,32]. These findings are in agreement with the observation that constitutive Rac1 activation does not allow the establishment of cell–cell contacts[33]. In addition, constitutively active Src induces EMT and internalization of E-cadherin in KM12C cells that is dependent on signalling by αvβ1 integrin as well as on Src-dependent FAK phosphorylation[31]. Together, these studies illustrate the cross-regulation that exists between E-cadherin and integrins.

Primitive colonial protozoans
Single-celled organisms that live in colonies — they might be the organisms from which Porifera developed.

Diploblastic
Animals that are composed of two cell layers. They belong to the phylum Cnidaria.

Integrated approaches to EMT study

Classic molecular- and cell-biology techniques have helped us to understand some of the molecular mechanisms that cells use to regulate EMT. However, EMT is a dynamic process that involves many overlapping regulatory pathways as well as intra- and intercellular events, and interdisciplinary approaches are required to understand this complex regulation. For example, the dynamic formation and dissolution of junctional complexes is a central process during EMT (FIGS 2,4), and biophysical approaches together with high-throughput screening have recently shed light on the EMT-related modulation of junctional-complex integrity.

The fully polarized epithelial phenotype requires the assembly of adherens junctions, tight junctions and desmosomes[34]. The initiation of desmosomes is poorly understood, but it is clear that the formation of adherens and tight junctions are closely linked. Signalling pathways that control EMT converge on the control of E-cadherin, the prototypic epithelial adhesion molecule in adherens junctions. Small focal adherens junctions first appear at the tips of protrusions during the initial stages of epithelial-cell contact. These contain E-cadherin, immunoglobulin (Ig)-superfamily adhesion molecules (nectins and junctional adhesion molecules (JAMs)), as well as zonula occludens-1 (ZO-1; REF. 35; a tight-junction protein, which interacts with the actin cytoskeleton). Initial contacts are E-cadherin-mediated and mature into adherens junctions in cooperation with nectins[36]. These contacts promote the formation of tight junctions: JAMs nucleate clusters of two partitioning-defective proteins (PAR3 and PAR6, which are components of a metazoan polarity complex) and atypical protein kinase C (aPKC) together with claudin and occludin, which anchor the actin cytoskeleton through ZO-1, -2, and-3 (FIG. 4a). Nectin-mediated adhesion also cooperates at this early phase, perhaps through the ability of these proteins to nucleate cadherin–catenin complexes[37].

Mechanical forces during adhesion formation. The force of separation of cell doublets that express different levels of E-cadherin has recently been measured quantitatively[33,38]. The force that developed during the initial contact between two E-cadherin-expressing cells in suspension is on the order of a few nano-Newtons (nN) after 30 seconds. A rapid increase in adhesion strength is observed between 30 seconds and 30 minutes, which is followed by a slower increase up to 1 hour later, reaching a force of more than 200 nN. The initial contact does not require connection to the actin cytoskeleton, which is subsequently absolutely required for the strengthening of cell adhesion. The force of separation of cell doublets that express type II cadherin-7 and -11 is much weaker than the force necessary to detach type-I-E-cadherin-mediated cell adhesion. A switch from type I cadherins to type II cadherins is usually observed during EMT, which leads to the formation of migrating mesenchymal cells[38].

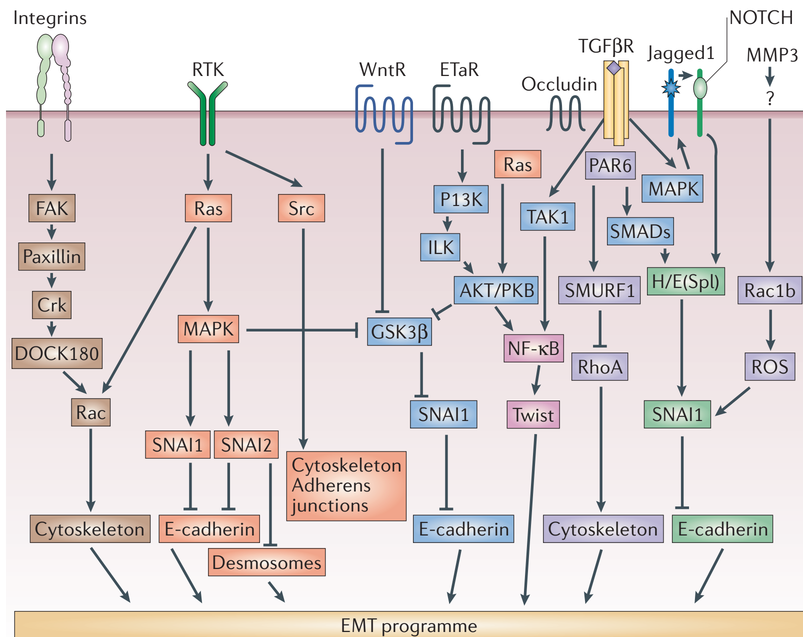

Figure 3 | **Overview of the molecular networks that regulate EMT.** A selection of the signalling pathways that are activated by regulators of EMT and a limited representation of their crosstalk is illustrated. Activation of receptor tyrosine kinases (RTKs) is known to induce EMT in several epithelial cell types and *in vivo*, but it is now clear that the EMT process often requires co-activation of integrin receptors. The role of transforming growth factor-β (TGFβ) signalling in EMT is established for a limited number of normal and transformed cell lines, whereas *in vivo* data has indicated a mutual regulation of the TGFβ and NOTCH pathways during EMT. There is now increasing evidence that other signalling pathways could have an important role in EMT, including G-protein-coupled receptors. Matrix metalloproteinases (MMPs) can also trigger EMT through as-yet-undefined receptors. ETaR, endothelin-A receptor; FAK, focal adhesion kinase; GSK3β, glycogen-synthase kinase-3β; H/E(Spl), hairy/enhancer of split; ILK, integrin-linked kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PAR6, partitioning-defective protein-6; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase-B; ROS, reactive oxygen species; TAK1, TGFβ-activated kinase-1; TGFβR, TGFβ receptor; WntR, Wnt receptor.

Ectoderm
The outermost of the three primary germ layers of the embryo, from which the skin, nerve tissue and sensory organs develop.

Mesenchyme
Embryonic tissue that is composed of loosely organized, unpolarized cells of both mesodermal and ectodermal (for example, neural crest) origin, with a component-rich extracellular matrix.

High-throughput mapping of the TGF network. The dissolution of tight junctions is an early step during EMT, and recent high-throughput protein–protein-interaction screens have revealed the molecular mechanisms through which TGFβ signalling regulates this process[39,40]. In these LUMIER (luminescence-based mammalian interactome mapping) studies, fluorescently tagged TGFβ receptor-1 (TGFβR1) was co-transfected with a FLAG-tagged prey library into HEK293T cells and the interaction of the prey protein with TGFβR1 was assessed. Several candidate TGFβR1-interacting proteins were identified, and subsequent bioinformatic and molecular- and cell-biology analyses led to the identification of pathways that regulate tight-junction dissolution (see below). Importantly, the LUMIER approach also allowed the dynamic changes in the network of protein–protein interactions in response to TGFβ to be documented[39].

Occludin, a structural component of tight junctions, was shown to interact with TGFβR1 (REF. 39). Mutation of the TGFβR1-binding site on occludin showed that the TGFβR1–occludin interaction is required for the localization of TGFβR1 to tight junctions, which is critical for TGFβ-mediated tight-junction dissolution during EMT. Furthermore, PAK1 (REF. 23; see above) was also found to physically associate with TGFβR1, although the significance of this interaction on PAK1 activity was not assessed.

PAR6 was also found to interact with TGFβR1 (REF. 40). PAR6 functions as a scaffold for the assembly of polarity-regulating proteins such as Rho, aPKC and PAR3, and orchestrates the assembly of tight junctions. In NMuMG cells, PAR6 forms a member of the TGFβR1–occludin complex. After treatment of cells with TGFβ, TGFβR2 is also recruited to this complex and phosphorylates PAR6. PAR6 localizes to dynamic protrusions of polarized migratory cells together with SMURF1, an E3 ubiquitin ligase that ubiquitylates RhoA and controls local RhoA degradation[40,41]. Moreover, both SMURF1 and RhoA ubiquitination were found to be necessary for tight-junction dissolution (FIG. 4b). Mutation of the Ser that is phosphorylated by TGFβR2 on PAR6 caused abrogation of tight-junction dissolution, but did not interfere with TGFβ-dependent SMAD activation or aspects of TGFβ-induced EMT such as vimentin expression. This observation indicates that PAR6 phosphorylation in response to TGFβ represents only one of the many pathways that are regulated by TGFβ during EMT.

EMT is required for gastrulation

EMT is an integral part of the tissue remodelling that occurs during embryogenesis. Epithelial sheets can remodel through distinct processes, which include cell intercalation, invagination, evagination, branching and multi-layering (FIG. 5). Epithelial-cell plasticity has been maintained in all metazoans, and epithelial sheets can convert reversibly or irreversibly into mesenchymal cells through EMT. This process is extremely critical as it often allows the formation of the three-layered embryo through gastrulation. It is also responsible for the formation of other structures, particularly in the vertebrates, such as the vertebrae, the cardiac valves, the craniofacial structures, the neural derivatives and the secondary palate, as well as mediating the disappearance of the male mullerian duct.

Gastrulation (the formation of the gut) is a primordial process of development in most metazoans. Following cellularization or cleavages of the egg, the newly formed blastula undergoes a dramatic reorganization, which leads to the establishment of the three primary germ layers: ectoderm, mesoderm and endoderm.

ERK activation regulates EMT in the sea urchin. A first EMT at the vegetal pole leads to the formation of the primary mesenchyme, whereas the secondary mesenchyme forms at the tip of the invaginating endoderm after a second EMT. Although the primary and secondary mesenchymes differ notably in terms of cell lineage, they undergo a similar EMT, which is characterized by the activation of the extracellular signal-regulated kinase (ERK) pathway[42,43]. Inhibition of the activity of MAPK and ERK kinase (MEK) abrogates the formation of the

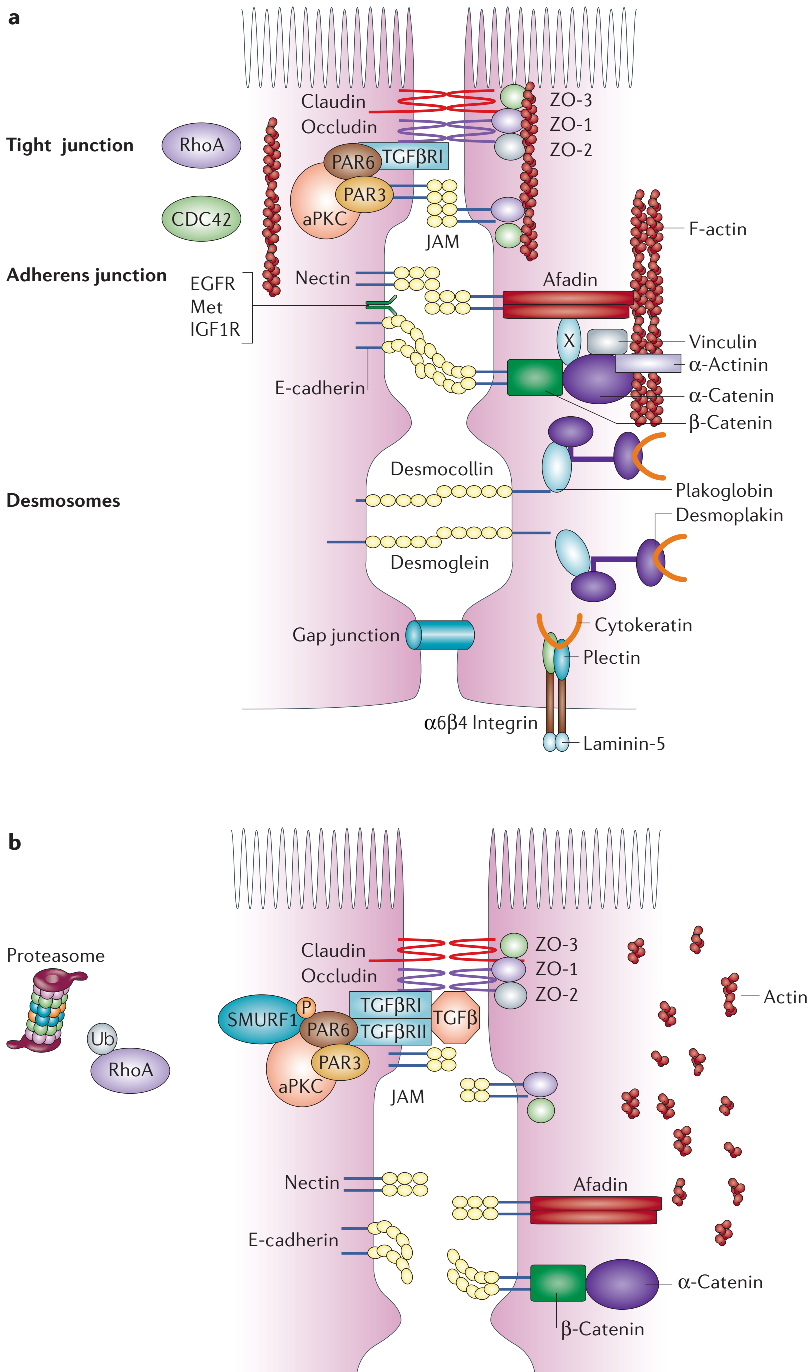

Figure 4 | **Molecular events in junctional complexes during EMT.** In response to various epithelial–mesenchymal transition (EMT) inducers — such as signalling through the epidermal growth factor receptor (EGFR), Met and the insulin-growth-factor-1 receptor (IGF1R) — intact junctional complexes in epithelial cells (**a**) are dissociated (**b**) as a consequence of several molecular events, including transcriptional downregulation of important components, such as E-cadherin. Activation of these receptor tyrosine kinases has an important impact on the assembly and stability of adherens-junction components. Transforming growth factor-β (TGFβ) can induce tight-junction disassembly through degradation of RhoA and subsequent depolymerization of filamentous (F)-actin. RhoA is ubiquitylated by SMURF1, a ubiquitin ligase that is recruited to the TGFβ receptor (TGFβR) complex that is assembled as part of the tight junction. This finding supports the idea that the EMT signalling pathways alter the stability of junctional complexes by destabilizing the cortical cytoskeleton. JAM, junctional adhesion molecule; αPKC, atypical protein kinase C; PAR3/6, partitioning-defective protein-3/6; ZO-1/2/3, zonula occludens-1/2/3.

primary mesenchyme. The temporal inhibition of ERK indicates that this pathway is not involved in the initial specification of mesodermal cells, but, rather, mediates their ingression and subsequent engagement in skeletogenesis. The transcription factors ETS1 and ALK1 are among the main targets of ERK that are required for EMT. Overexpression of ETS1 results in an excessive number of primary mesenchymal cells, whereas ALK1 directly promotes skeletogenesis. The ERK-mediated formation of primary mesenchyme is under the control of Wnt–β-catenin signalling, whereas the NOTCH–delta pathway contributes to the formation of the secondary mesenchyme. Receptor tyrosine kinases (RTKs) might also be activated early in the primary and secondary mesenchymal cell progenitors. The resulting activation of ERK pathways contributes to the transcription of mesodermal regulatory and differentiation genes that are required for EMT.

A complex network orchestrates EMT in fruitflies. Gastrulation in the fruitfly (*Drosophila melanogaster*) initiates at the completion of the cellularization step in a stripe of ventral cells, which invaginate as an epithelium, but subsequently undergo an EMT. The newly formed mesenchymal cells migrate dorsally along the ectoderm before differentiating into visceral and somatic mesoderm. Snail (the orthologue of vertebrate SNAI1) and the transcription factor Twist are expressed in the presumptive mesoderm and in the invaginating cells. Twist and Snail mutants have a defective gastrulation, which indicates that these genes are essential for gastrulation. These genes belong to a regulatory network, which is controlled by Dorsal (nuclear factor (NF)-κB). Dorsal is involved in the establishment of the dorso–ventral axis, and therefore controls the specification of mesodermal and ectodermal lineages prior to gastrulation. Snail downregulates the transcription of Shotgun (the orthologue of vertebrate E-cadherin) in mesodermal cells that undergo invagination at the ventral furrow[44].

Pebble, a Rho guanine nucleotide-exchange factor, which was originally characterized as an essential component of cytokinesis, is also expressed in the invaginating mesodermal cells[45]. Pebble mutants have defective EMT, as mesodermal cells remain as aggregates and fail to migrate dorsally. Pebble probably has a role in the reorganization of the actin cytoskeleton through activation of Rho1 or Rac1, thereby promoting the downregulation of fruitfly E-cadherin and the locomotion of mesenchymal cells. These small GTPases have already been shown to control the remarkable shape changes in the invagination of the ventral furrow[45], where the epithelial mesoderm undergoes a dramatic elongation along its apico–basal axis.

EMT occurs in the zebrafish organizer. The zebrafish (*Danio rerio*) organizer is the site for EMT of axial mesendoderm cells, which subsequently migrate forward along with non-axial cells that undergo convergence–extension movements. Liv1, a seven-spanning transmembrane receptor that is involved in Zn^{2+} transport[46] is downstream of signal transducer and activator

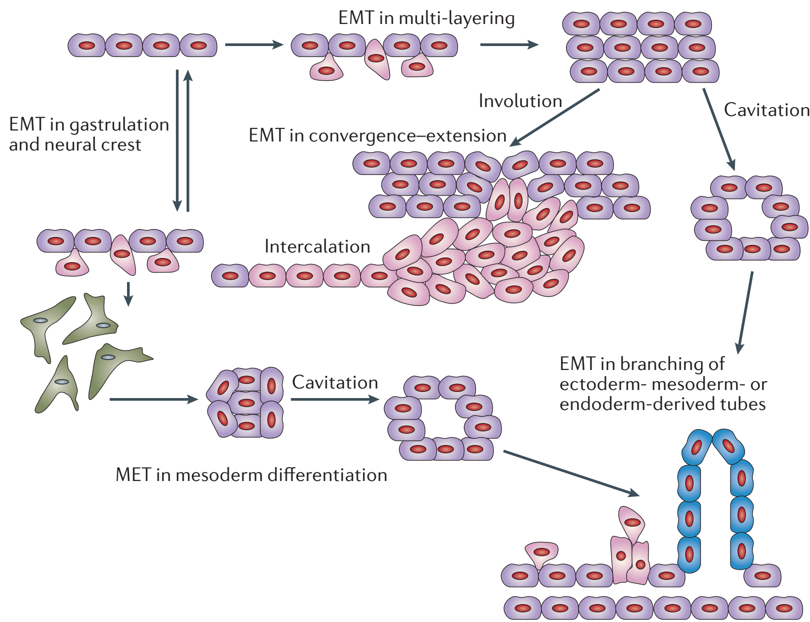

Figure 5 | **Tissue remodelling and EMT.** During embryonic development, epithelial cells (purple cells) undergo partial or complete epithelial–mesenchymal transition (EMT) (pink cells). EMT is an important component of various morphogenic events, such as gastrulation, neural-crest formation and branching of the three germ-layer-derived tubes (blue cells). The reverse process, during which mesenchymal cells (green) undergo mesenchymal–epithelial transitions (METs), also has an important role during embryonic development. Most animals gastrulate using a full EMT, including fruitflies (*Drosophila melanogaster*), most amphibians (such as urodeles), fish, birds, reptiles and mammals. However, there are some notable exceptions, such as the African clawed frog (*Xenopus laevis*) — in this organism individual mesenchymal cells are not formed during gastrulation, but rather a mass of epiblast cells penetrate the blastocoel cavity (involution) and converge–extend to form the axial mesoderm (notochord and somites). This mechanism, however, implies that gastrulating cells must exchange rapidly with their neighbours to form an elongated structure.

of transcription-3 (Stat3), and is able to mediate all the cell-autonomous functions of Stat3 in axial mesendodermal cells. In Liv1-depleted embryos, mesendodermal cells remain cohesive within the organizer, and similar defects are observed in Snail-defective mutants, although Liv1 and Snail are regulated independently. Nonetheless, Liv1 activity depends on its promotion of Snail nuclear localization in organizer cells. Snail might be activated by a RTK–MAPK pathway, and possibly cooperates with a cytokine-receptor–Stat pathway to downregulate E-cadherin. However, it is likely that other effectors of the Stat3 pathway contribute to the restriction of the specific migration of axial mesendodermal cells.

Multiple pathways contribute to EMT in mouse gastrula. In murine (*Mus musculus*) gastrulation, EMT is responsible for the formation of mesoderm and definitive endoderm. FGF receptor-1 (FGFR1) signalling controls the expression of SNAI1, which inhibits E-cadherin transcription[47]. Inactivation of SNAI1 leads to complete inhibition of EMT, although some mesodermal markers, such as brachyury, appear in cell clusters at the site of ingression[48]. The Wnt signalling pathway is also involved in this process, as expression of a truncated β-catenin construct in oocytes induced a premature EMT in the epiblast, concomitant with SNAI1 transcription[49]. The prematurely formed mesenchymal cells lost E-cadherin expression and gained several mesodermal markers, including brachyury and LEF1, which are direct targets of β-catenin.

Multiple pathways contribute to EMT in mouse gastrulation. However, the role of individual effectors such as β-catenin must be evaluated more extensively because the Wnt–β-catenin canonical pathway regulates the formation of the node. The node is the most critical transient embryonic structure that serves as an organizing centre for subsequent development, including the formation of the primitive streak, the anterio–posterior axis[50] and the somites[51].

Organizer
A small dorsal region of the vertebrate gastrula-stage embryo that has the remarkable capacity to organize a complete embryonic body plan. Hilde Mangold and Hans Spemann first identified the organizer in amphibian embryos using tissue transplantation.

Mesendoderm
Cells that form early during gastrulation in the vertebrate and are destined to give rise to mesodermal and endodermal derivatives.

Rostrocaudal
The anterior–posterior (head to tail) polarity of animals.

Lessons from neural-crest EMT

After gastrulation in vertebrates, epidermal and neural territories are progressively defined along the rostrocaudal axis. The neural crest is a transient population of cells that forms at the boundary between these two territories. Neural-crest cells undergo an EMT within the dorsal neural epithelium and subsequently migrate, before giving rise to many different derivatives. Neural-crest cells are certainly one of the best model systems to study EMT, as many of the newly formed mesenchymal cells migrate over long distances, and the induction of the neural-crest territory and the subsequent programme of migration and differentiation are under the control of overlapping signalling pathways. However, there are significant differences in the mechanisms that induce neural-crest formation in different vertebrates, and a unique molecular scheme for neural ontogeny is therefore unlikely to be found.

BMPs in the African clawed frog and zebrafish. A gradient of bone morphogenetic proteins (BMPs) has been shown to be critical in the African clawed frog and zebrafish embryos for the induction and refining of boundaries between neural tissues, the neural crest and the adjacent ectoderm — these are characterized by high, medium and low BMP expression, respectively[52]. High BMP concentrations indirectly repress neural markers and induce epidermal cytokeratins. Intermediate concentrations induce neural-crest-cell markers such as Snail, Snail2, Sox9, (a member of the SoxE subgroup of the high mobility group (HMG)-box-containing transcription factors), transcription factor AP2 and the winged-helix transcription factor Foxd3. Other signals include FGF and Wnt.

The current proposal is that these neural-crest inducers function through Zic (Zinc-finger transcription factor), Pax3/7 (paired-box proteins -3 and -7), Dlx5 and Msx1/2 to specify the dorsal neural tube and neural-crest territory[52] (FIG. 6; *Dlx* genes encode homeobox transcription-factor orthologues to the fruitfly *Distal-less* gene; Msx proteins are homeobox transcription factors that are related to *Msh* (the fruitfly muscle-segment homeobox gene)). Genes that are induced by these transcription factors include

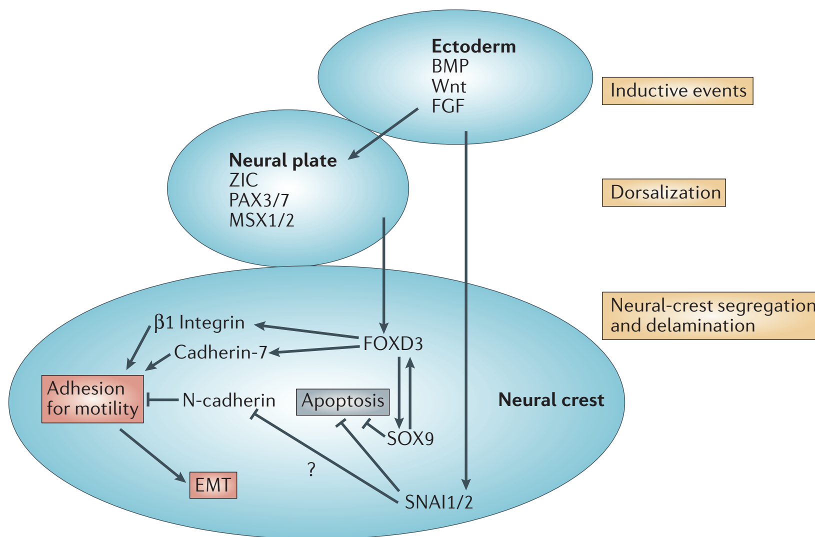

Figure 6 | **Different signalling pathways cooperate to regulate EMT during neural-crest determination and segregation.** The neural-crest territory is progressively determined in a rostrocaudal gradient along the neural axis of the vertebrate embryo at the interface between the neural tube and the lateral ectoderm. This simplified scheme does not reflect the formidable complexity of the signalling pathways that regulate the epithelial–mesenchymal transition (EMT) during neural-crest determination and segregation. The pathways that are involved differ from species to species and at different axial levels. Although this figure depicts three distinct steps with a different set of molecules for induction, dorsalization and delamination, these events occur as a continuum in time and space. EMT itself occurs during a certain period of time at each axial level, concommitantly with initial lineage segregation and protection from apoptosis. Factors that execute EMT in the context of neural-crest determination remain to be identified. Altered cell-adhesion properties have an important role in the migration of neural-crest cells. BMP, bone morphogenetic protein; FGF, fibroblast growth factor; MSX1/2, homeobox transcription factors related to *Msh* (the muscle-segment homeobox gene in fruitflies); PAX3/7, paired-box proteins 3/7; ZIC, zinc-finger transcription factor.

Snail, *Snail2*, *Sox9*, *Sox10*, *AP2*, *Foxd3* and *Myc* in the neural-crest territory[52]. In the African clawed frog, *Snail2*, which is an EMT-inducing gene in the neural crest, responds directly to Wnt signalling through its Tcf/Lef-binding site[53,54]. The relative interdependence of these transcription factors and their unconserved hierarchy at the head and trunk level and in different species is a remarkable feature in the control of neural-crest ontogeny[55,56].

Insights from chick and mouse experiments. A series of complementary experiments in chick (*Gallus gallus*) and mouse embryos have now allowed the definition of some epistatic relationships in neural-crest EMT[55]. In chick embryos, Foxd3 and Sox10 inhibit N-cadherin expression and promote expression of β1 integrin and type II cadherin-7, which are normally expressed in migratory neural-crest cells. However, Foxd3 requires the concomitant expression of Sox9 and Snail2 to induce EMT in mouse embryos. Sox9 has a dual role as it inhibits cell death and specifies the neural-crest-cell lineages. Snail1 can also inhibit cell death; this could explain why loss of Sox9 induces cell death, as Sox9-null cells lack Snail1 expression. However, none of these genes has proven to be the master gene of EMT, and the important effectors of EMT remain to be discovered in the context of neural-crest formation. Furthermore, the proposed connection with the genes that specify the neural-plate border and neural crest in terms of EMT has not been established.

Neural crest
A transient embryonic structure of vertebrates that appears in the ectoderm at the junction between the neural plate and lateral ectoderm. This structure gives rise to many distinct derivatives following precise migratory routes at each axial level. The derivatives include cranio–facial structures (cartilage, bone, muscles), melanocytes, adrenal medulla, and cells of the sensory and autonomic nervous systems.

EMT regulates heart-valve formation

Endocardial EMT is regulated through the TGFβ and NOTCH signalling pathways. TGFβ functions through the activation of the transcription factors SMAD2, -3 and -4, and the subsequent activation of SNAI1[57], which represses transcription of E-cadherin. NOTCH genes are transmembrane receptors for the delta and serrate/jagged families. Ligand binding results in a γ-secretase-mediated cleavage of the cytoplasmic tail of the NOTCH proteins. The cleaved NOTCH tail translocates to the nucleus and regulates gene expression through binding to the transcription factor RBPJk/CBF1/Su(H), which activates genes of the hairy/enhancer of split (Hes) family. Recent studies provided a fascinating insight into how the TGFβ and NOTCH pathways interact to orchestrate EMT. Analysis of RBPJk and NOTCH1 mutant embryos indicated that NOTCH-induced TGFβ2 transcription regulates the endocardial EMT that is required for cardiac-valve development[58]. Disruption of NOTCH signalling was associated with loss of SNAI1 expression, and inappropriate vascular endothelial (VE)-cadherin expression. Importantly, TGFβ-family members are known to promote endocardial EMT[59], and the reduced transcription of TGFβ2 that was observed in the outflow tract and atrioventricular-canal myocardium of RBPJk- and NOTCH1- mutant embryos, indicated that NOTCH-induced TGFβ2 expression has an important role in EMT that occurs during the formation of the cardiac valve. TGFβ-induced EMT in cultured epithelial cells was also found to induce the Hes-family member HEY1 and the NOTCH ligand JAG1 (REF. 60). HEY1- and JAG1-knockdown or inhibition of NOTCH blocked TGFβ-induced EMT. TGFβ-induced HEY1 expression is biphasic, the initial phase being dependent on SMAD3 but independent of JAG1, whereas the second phase is JAG1-dependent. These data are consistent with a model in which TGFβ induces expression of HEY1 and JAG1 through SMAD3. JAG1 then activates NOTCH, which leads to a second wave of HEY1 expression. Taken together, these observations indicate a close mutual regulation of the TGFβ and NOTCH signalling pathways during EMT.

The roles of EMT in disease

EMT has an important role in the development of many tissues during embryogenesis, but similar cell changes are recapitulated during pathological processes, such as fibrosis and cancer (BOX 2). A common spectrum of EMT-associated changes in morphology and gene-expression patterns are associated with these events, and recent studies have shown a remarkable similarity between the signalling pathways that regulate EMT in these pathological processes.

Box 2 | **Evidence supporting a role for EMT during tumour progression**

Phenotypic

- Expression of proteins that are characteristic of mesenchymal cells (for example vimentin, fibroblast-specific protein-1 (FSP1/S100A4), SNAI1 and SNAI2, nuclear β-catenin and stromelysin-3) and loss of epithelial markers (for example E-cadherin[11,15]) correlates with tumour progression and poor prognosis[18,93].
- Invasion of adenocarcinomas is accompanied by the release of single cells through an epithelial–mesenchymal transition (EMT) process[15].

Genetic

- Sarcomatoid carcinomas contain tumour-cell populations that have mixed carcinoma and sarcoma phenotypes. Both populations arise from a common epithelial precursor, which indicates that EMT is involved in this transformation.
- Genetic analysis indicates that carcinoma cells contribute to the stromal-fibroblast population in tumours[94].

Functional

- FSP1/S100A4 is a lineage marker for mesenchymal cells. Depletion of FSP1/S100A4-positive cells in tumours suppresses metastasis[95].
- Manipulation of E-cadherin expression causes both change of phenotype and invasive behaviour[11,15,96].
- Conditional expression of ERBB2 (also known as HER2/neu) induces the formation of tumours that regress upon withdrawal of ERBB2 expression. The regressed tumours can relapse and metastasize through spontaneous induction of SNAI1. These tumours consist of mesenchymal cells[93].
- Claudin-1, a tight-junction component, is upregulated in colorectal tumours. Expression of claudin-1 in colonic carcinoma cells induces phenotypic and gene-expression changes that are characteristic of EMT, and promotes metastasis. Inhibition of claudin-1 expression in metastatic cells suppresses metastasis[97].
- Transforming growth factor-β (TGFβ) induces EMT in H-Ras-transformed mammary epithelial cells. Inhibition of NF-κB activity reverses the TGFβ-induced EMT and also abrogates the metastatic potential of these cells *in vivo*[98].
- Twist is expressed in invasive lobular breast carcinomas. Suppression of twist expression in tumour cells reduces their metastatic potential *in vivo*. On the other hand, ectopic expression of twist promotes EMT, expression of mesenchymal markers, increased motility and downregulation of E-cadherin[99].

SNAI2 regulates re-epithelialization. During re-epithelialization of wounded skin, keratinocytes undergo a series of changes that are reminiscent of EMT, including loss of polarity and alteration of the actin cytoskeleton, disruption of cell–cell contacts, and partial or complete breakdown of the basement membrane[61,62]. The cells also change their motile properties and migrate from the wounded edge of the epithelium into the de-epithelialized area. However, not all features of EMT are seen. For example, the migrating keratinocytes remain part of a cohesive cell sheet, as they retain some intercellular junctions. The keratinocytes subsequently regain their epithelial characteristics during the final stages of re-epithelialization.

Recent studies indicated that SNAI2 has an important role in orchestrating this process. SNAI2 is expressed in keratinocytes at wound margins, consistent with a possible role in early wound re-epithelialization. Importantly, targeted deletion of *Snai2* in mice dramatically reduces epithelial outgrowth from skin explants, whereas constitutive expression of SNAI2 in keratinocytes resulted in accelerated re-epithelialization[63].

A complex network controls EMT during fibrosis. Chronic kidney disease is characterized by cumulative tissue fibrosis, which results in the progressive loss of kidney function. Fibrosis is characterized by increased numbers of myofibroblasts that deposit interstitial ECM, and a significant fraction of these fibroblasts are thought to arise through EMT of the tubular epithelial cells[64]. Inflammatory processes in response to injury in the kidney lead to increased levels of factors such as TGFβ, EGF and FGF2 that can induce EMT of tubular epithelial cells[64,65]. In addition, animal models of kidney disease indicate that EMT occurs in tubular epithelial cells[66–68], although these models do not always accurately reflect human disease processes. However, similar observations have been made in studies of human renal fibrosis[69,70]. Genetic tagging of renal tubules in experimental animals has shown that, in the fibrotic kidney, 36% of the fibroblast-specific protein-1 (FSP1)-positive fibroblasts arise from tubular epithelial cells[71]. Furthermore, targeted deletion of tissue-type plasminogen activator (tPA) blocked tubular-epithelium EMT but not activation of interstitial fibroblasts after obstructive kidney injury, which results in reduced fibrosis[72]. This observation also underscores the possible importance of tubular epithelium EMT for kidney fibrosis.

EMT could be responsible for fibrosis in other organs. The emerging paradigm is that inflammatory mediators that are produced in response to injury cause EMT, which can lead to fibrosis. In the peritoneal cavity, renal dialysis causes injury to the mesothelial lining. This results in EMT of mesothelial cells, expression of SNAI1 and the onset of fibrosis[73], probably as a result of TGFβ production[74]; these events have now been confirmed in animal models[75]. TGFβ levels are also increased in the lungs of patients with fibrotic pulmonary diseases such as idiopathic pulmonary fibrosis[76,77]. TGFβ induces

Basement membrane
An extracellular-matrix structure that can be visualized by light microscopy and lines the basal side of epithelia.

EMT of alveolar epithelial cells[78,79] and fibroblastic cells that express both epithelial and mesenchymal markers can be detected in human biopsies[79]. Furthermore, a significant body of evidence points to TGFβ-induced EMT of lens epithelial cells as an important event during cataract formation and injury-induced lens-capsule fibrosis[80]. The recurring theme of the involvement of TGFβ in the pathogenesis of EMT-dependent fibrotic diseases is highlighted by the finding that *Smad3*$^{-/-}$ mice are resistant to the induction of several fibrotic diseases[81]. Similarly, injury-induced lens-capsule fibrosis requires SMAD3 (REF. 82), and is inhibited by gene-therapy-mediated expression of SMAD7, which inhibits TGFβ signalling[83].

EMT and cancer. Numerous observations support the idea that EMT has a central role in tumour progression. During progression to metastatic competence, carcinoma cells acquire mesenchymal gene-expression patterns and properties. This results in changed adhesive properties, and the activation of proteolysis and motility, which allows the tumour cells to metastasize and establish secondary tumours at distant sites[84]. In tissue culture this progression is accompanied by partial or complete EMT, and induction of EMT in many carcinoma cell lines results in the acquisition of metastatic properties *in vivo* (BOX 2). It has recently been suggested that the acquisition of mesenchymal markers and properties during tumour progression simply reflects genomic instability and that EMT does not occur in tumours[85]. However, it is highly unlikely that the coordinated and complex gene-expression patterns that are required to endow tumour cells with the mesenchymal properties that are required for metastasis could arise through random mutations as a result of genomic instability. Rather, it is more likely that genomic instability changes the expression of important factors that regulate EMT. SNAI1, for example, regulates the expression of many EMT-associated genes in colorectal carcinoma cells[86]. In this regard, it is striking that the same signalling pathways that regulate developmental EMT are also activated during tumour progression. It is also clear that the EMT that is observed in cultured tumour cells converts into increased metastatic potential *in vivo* (BOX 2), arguing against the idea that EMT in tumour cells is a tissue-culture artefact without biological significance.

Future perspectives

EMT *in vivo* is embedded in context-dependent inductive and differentiation events. This finding reflects the fact that all morphogenetic processes must be tighly controlled and integrated with the other processes that occur during embryonic development. At each site of EMT in the embryo, the previous developmental history has to be taken into account. *In vivo* studies are invaluable for studying EMT, but suffer from the drawback that they are much more demanding than the *in vitro* studies, particularly in mice. High-throughput studies[39,40], however, have the potential to unravel the different facets of EMT rapidly. In addition, the new extensive screening of RNA interference (RNAi) libraries in the fruitfly and morpholino oligonucleotide libraries in zebrafish should help to identify the critical nodes of the interaction networks that regulate EMT.

Due to their simplified context, *in vitro* studies allow the relatively easy identification of pathways that are involved in the morphological conversion of epithelial cells, and several pathways that can induce EMT have been discovered. However, further analysis is required in a more global context. *In vitro* studies are limited, because very few immortalized epithelial cell lines can undergo EMT. The induction of EMT by TGFβ in immortalized NMuMG cells is almost unique, whereas Eph4 cells need to be Ras transformed, otherwise they undergo apoptosis when they are exposed to TGFβ. Moreover, most cell lines are not fully polarized and often lack tight junctions owing to the culture conditions or their status of transformation, which limits their utility for the study of EMT. Another problem consists of the kinetics of EMT conversion, which vary considerably from hours to a week. Some of the molecular events of EMT are embedded in other cellular programmes, and it is therefore not a surprise to see very few informative studies on differential gene expression during EMT.

Better *in vitro* models are clearly required to study EMT, and those obtained in three-dimensional cultures where epithelial cells can polarize and form functional glandular structures hold particular promise[87–90]. In these models, EMT needs to be induced transiently using inducible constructs. The use of reporter genes under the control of EMT-regulated promoters will also be important to indicate the critical steps in the execution of the EMT programme. The FGFR2 IIIb and IIIc splice variants, for example, could be used as one of the markers of the transition[91]. SNAI1, E-cadherin and vimentin are also possible candidates, although the promoters that have been defined so far have not yet provided specific tools *in vivo*, because their temporal and spatial expression is not restricted to sites of EMT. Specific gene expression that is driven by the tissue-plasminogen-activator promoter in murine neural-crest cells can constitute a new marker of late-phase EMT[92]. More work is therefore required to develop appropriate promoter constructs. These could also be used for imaging *in vivo* or the specific inactivation of candidate genes that might be involved in EMT proccesses.

Additional studies are required to understand how the molecular machines that control epithelial-cell polarity are assembled and how they can be altered during the early stages of EMT. One of the crucial issues is to understand the crosstalk between cadherin- and integrin-mediated adhesion. Intriguingly, these two adhesive complexes share numerous structural and enzymatic components, and they are both associated with tyrosine-kinase surface receptors, most of which have been implicated in EMT. These problems require an interdisciplinary approach in which cell- and molecular-biology methods are combined with emerging techniques in biophysics and imaging, and particularly with global high-throughput analyses. Together, these studies hold the promise of elucidating the complex molecular strategies that regulate EMT.

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