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Review

Epithelial to mesenchymal transition during gastrulation: An embryological view

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Gastrulation is a developmental process to generate the mesoderm and endoderm from the ectoderm, of which the epithelial to mesenchymal transition (EMT) is generally considered to be a critical component. Due to increasing evidence for the involvement of EMT in cancer biology, a renewed interest is seen in using *in vivo* models, such as gastrulation, for studying molecular mechanisms underlying EMT. The intersection of EMT and gastrulation research promises novel mechanistic insight, but also creates some confusion. Here we discuss, from an embryological perspective, the involvement of EMT in mesoderm formation during gastrulation in triploblastic animals. Both gastrulation and EMT exhibit remarkable variations in different organisms, and no conserved role for EMT during gastrulation is evident. We propose that a 'broken-down' model, in which these two processes are considered to be a collective sum of separately regulated steps, may provide a better framework for studying molecular mechanisms of the EMT process in gastrulation, and in other developmental and pathological settings.

Key words: Cell biology, epithelial to mesenchymal transition, gastrulation, mesoderm.

Introduction

During embryonic development, a single fertilized egg eventually gives rise to an organism with hundreds of different cell types. The diverse cell types in complex tissues and organs, however, can be loosely categorized into two general types: those cells with a two-dimensional organization with their neighbors (epithelial) and those with a three-dimensional organization (mesenchymal). The two-dimensional organization of an epithelium is relative, of course, as it can fold into topologically complex structures, and epithelial cells often interact with other cells outside the epithelial sheet. Nevertheless, this general categorization has provided an important framework for the description and understanding of tissue morphogeneses during animal development. Any morphogenetic process can be viewed conceptually as cell organizational changes either within the epithelial or mesenchymal state, or a transition between these two states (EMT for epithelial to mesenchymal transition or MET for mesenchymal to epithelial transition).

The concept of EMT/MET, since first proposed four decades ago in cell biological studies of chick embryos (Trelstad *et al*. 1966, 1967; Hay 1968), has been used to describe diverse biological phenomena from trophectoderm formation in developing mammalian embryos (Collins & Fleming 1995; Morali *et al*. 2005; Eckert & Fleming 2008) to tumor metastasis and invasion (Savagner 2005; Yang & Weinberg 2008). Broad-scope discussions of EMT/MET in development and disease can be found in several recent review articles (Hay 1995; Shook & Keller 2003; Thiery 2003; Huber *et al*. 2005; Savagner 2005; Zavadil & Bottinger 2005; Thiery & Sleeman 2006; Baum *et al*. 2008; Yang & Weinberg 2008). This review will focus on EMT during gastrulation, which is regarded as the earliest and most typical EMT event during animal development.

Key concepts: epithelium, mesenchyme, gastrulation and germ layers

Mesenchymal cells generally have more irregular morphology and higher motility than epithelial cells. It is, however, not easy to define these cells based on any cell biological criterion. Instead, mesenchymal cells are

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often regarded as cells that do not have an epithelial morphology (Trelstad *et al*. 1967; Hay 1968). It is therefore necessary to go through what are typically considered to be the characteristics of an epithelium. A two-dimensional epithelial structure is generally marked by the presence of (i) an apico-basal polarity; (ii) a trans-epithelial barrier (tight junctions or septate junctions); (iii) an epithelial specific cell–cell adhesion mediated by adherens junctions; and (iv) a basement membrane-like extracellular matrix. These characteristics are not present in all epithelial structures and none of them is unique for epithelial cells. When introducing the concept of EMT/MET, Hay (1968) provided a much more general definition for the epithelium as a tissue layer with a free surface. This free surface is the side facing the exterior of the embryo for the surface ectoderm, the gastrointestinal lumen for the gut endoderm, and a cavity for mesoderm-derived epithelia such as somites, blood vessels and nephritic tubules. This definition also has its limitations. For instance, the stratification of an epithelium often results in epithelioid tissue organization without a free surface, such as in deep layer ectoderm cells in *Xenopus* embryos and in basal epithelial layer of skin epidermis. Therefore, the question about what criteria one should use to properly define an epithelial cell remains to be one of the most debated in the EMT/MET field.

Gastrulation means the formation of the gut. This developmental process gives rise to endoderm cells in diploblasts (animals with two germ layers), and to mesoderm and endoderm cells in triploblasts (vast majority of metazoans). In either diploblastic or triploblastic animals, pre-gastrulation blastula cells (or epiblast cells in amniotes) are generally considered to have an epithelial morphology. Endoderm formation in diploblasts can take place either through an ingression/delamination process, which would involve an EMT-like event, or through an invagination/involution process, which does not involve EMT (Byrum & Martindale 2004). In triploblasts, mesoderm formation often, but not always, as we will discuss later, involves EMT-like morphological changes. Endoderm formation in triploblasts, like in diploblasts, may or may not involve EMT. In addition, gastrulation, a loose term describing the sum of morphogenetic processes leading to the formation of three germ layers (ectoderm, mesoderm and endoderm), is also used to include morphogenetic processes within individual germ layers either prior to or after EMT. Our discussions will not cover these topics. Instead, we will first discuss in some detail about how EMT is involved in mesoderm formation in several experimental organisms, and then discuss recent findings on molecular regulations of gastrulation EMT in mouse and chick embryos. We will also provide some suggestions on how to view the gastrulation EMT from an evolutionary perspective, and on how the studies on gastrulation EMT in avian embryos may provide useful insight for EMT studies in general.

EMT in mesoderm formation during gastrulation

Drosophila

In *Drosophila*, the invagination of mesoderm precursor cells within the ventral blastoderm starts immediately after the completion of cellularization (Leptin 2004) (Fig. 1A). No basement membrane has been described so far either for the cellularized blastdermal epithelium or for the invaginating mesoderm precursors. Other epithelial characteristics are present at the onset of invagination, including markers for epithelial adherens junctions, septate junctions (functionally equivalent to tight junctions as transepithelial barriers) and apico-basal polarity (Oda *et al*. 1998; Tepass *et al*. 2001; Pellikka *et al*. 2002; Lecuit 2004; Kolsch *et al*. 2007). The invagination process can be viewed as a topological rearrangement event of an intact epithelium. Indeed, before mesoderm cells adopt a mesenchymal morphology, all three epithelial characteristics are still present in invaginated mesoderm precursors. The transition from epithelial-shaped mesoderm precursors to mesenchymal-shaped mesoderm cells is rapid, and appears to take place simultaneously for all precursors (Leptin 2004). Localized expression of adherens junction and septate junction markers, as well as other non-junctional polarity markers, are lost in dissociated mesoderm cells. The loss of DE-cadherin is followed by the cytoplasmic export of DN-cadherin mRNA, which has been present but restricted to the nucleus prior to mesoderm precursor dissociation (Oda *et al*. 1998), marking a shift to the migratory behavior of dissociated mesoderm cells. Molecules involved in fibroblast growth factor (FGF) signaling, including FGF receptor *heartless,* sugar modifying enzymes involved in mediating FGF signaling, *sugarless* and *sulfateless,* and intracellular mediators *dof*, *ras* and *pebble* have been reported to control aspects of this EMT process (Beiman *et al*. 1996; Gisselbrecht *et al*. 1996; Shishido *et al*. 1997; Michelson *et al*. 1998; Vincent *et al*. 1998; Lin *et al*. 1999; Smallhorn *et al*. 2004).

Sea urchin

Mesoderm cells in sea urchins form in two phases (Fig. 1B). The primary mesenchymal cells, which give rise to the larval skeleton, ingress from the vegetal plate in the blastula stage embryo prior to the formation of archenteron (the primitive gut). The secondary mesenchymal

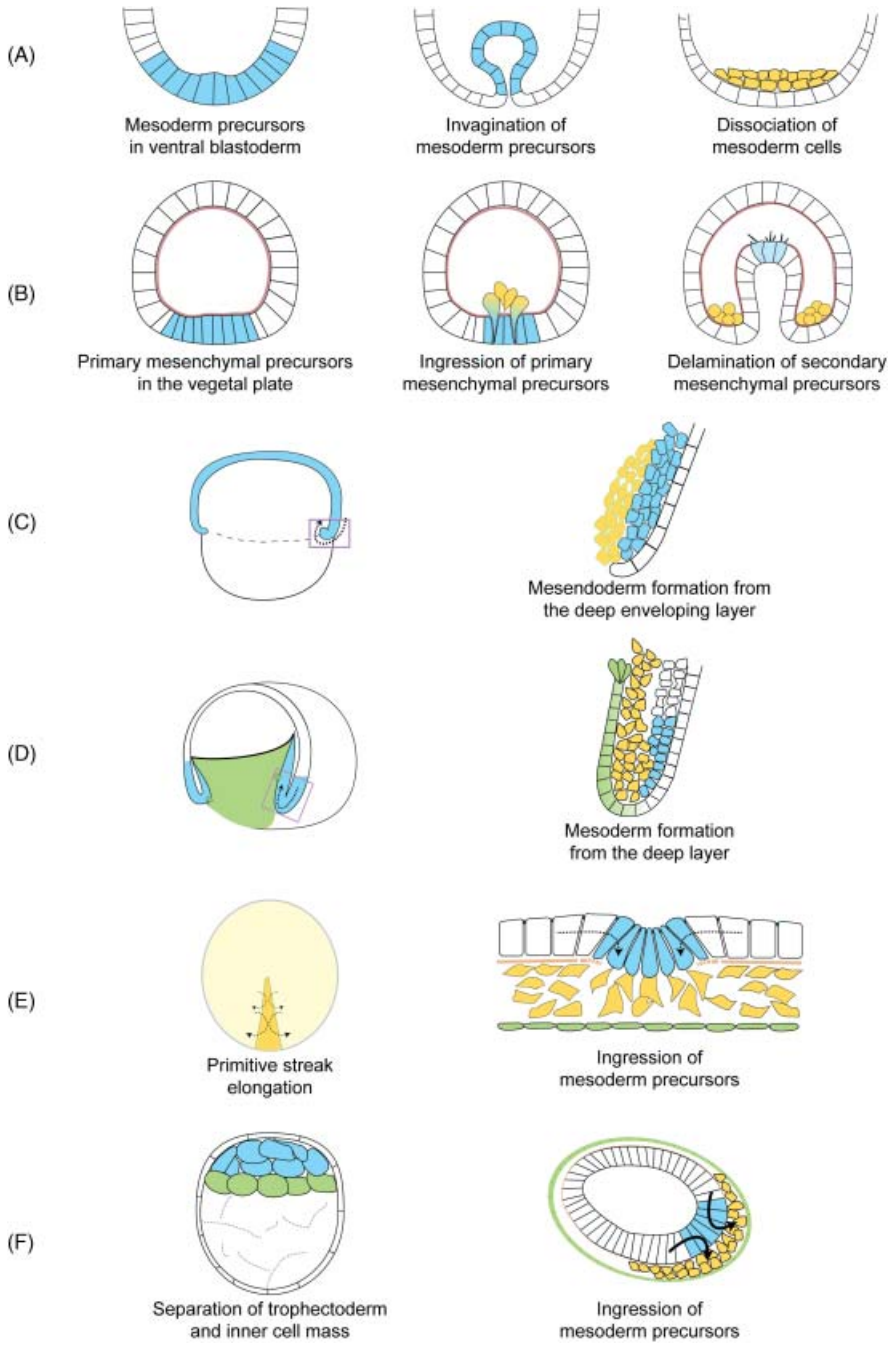

Fig. 1. Processes of mesoderm formation in several experimental triploblastic animals. (A) Drop-shophila; (B) sea urchin; (C) zebrafish; (D) xenopus; (E) chicken; (F) mouse. Blue, mesoderm precursors located in the ectoderm; yellow, mesoderm; green, endoderm; light brown, basement membrane.

cells form from the tip of the archenteron during the latter half of the invagination process. The ingression of primary mesenchymal cells is generally considered as a typical EMT process. Prior to the ingression, the vegetal plate cells are of an epithelial morphology, with septate junctions, adherens junctions, basement membrane and apico-basal polarity (Balinsky 1959; Wolpert & Mercer 1963; Gustafson & Wolpert 1967; Katow & Solursh 1979, 1980; Spiegel & Howard 1983; Andreuccetti *et al*. 1987; McClay *et al*. 2004; Itza & Mozingo 2005; Wessel & Katow 2005). Sea uchin blastula cells have an additional feature in that apical membranes establish prominent interactions with the hyaline layer, an extracellular matrix covering the apical surface of blastula epithelium (Fink & McClay 1985). The loss of interactions between the apical membrane processes and the hyaline layer constitutes the earliest morphological sign of the initiation of EMT. Subsequently, ingressing primary mesenchymal cells lose junctional interactions with its neighbors and break through the basement

membrane. The loss of junctional interaction is a multistep process. EM studies (Katow & Solursh 1980) revealed that when the basal end has broken through the basement membrane, apical interactions with neighboring cells are still present. Ingression of primary mesenchymal cells takes place individually, and is accompanied by extensive cell morphological changes including apical constriction, although the ingression and apical constriction have been postulated to involve different mechanisms (Anstrom & Fleming 1994). Loose basement membrane matrix covering the vegetal plate cells shows discontinuity underneath ingressing cells, and is resealed as a continuous layer after ingression of all primary mesenchymal cells. The delamination of secondary mesenchymal cells from the tip of invaginating archenteron is less characterized. Similar to *Drosophila* mesoderm formation, archenteron invagination in sea urchins can be viewed as a topological rearrangement of an intact epithelium, and invaginated endoderm cells retain epithelial junctional interactions, polarity markers and the basement membrane (Miller & McClay 1997; McClay *et al*. 2004; Wessel & Katow 2005). The endoderm cell/basement membrane interaction is weaker than that in the ectoderm (Hertzler & McClay 1999), and adherens junctions are modulated to accommodate the planar cell rearrangement within the archenteron epithelium (Ettensohn 1985; McClay *et al*. 2004). Secondary mesenchymal precursor cells, located at the tip of the invaginating archenteron, maintain apical epithelial interactions with neighboring endoderm cells. Half-way during the invagination process, these cells lose the basement membrane and send out long basal filopodia. These filopodia make contact with the basement membrane of animal pole cells and the force generated by this interaction is responsible for at least the last third of archenteron invagination (Hardin 1988). During this process, basal filopodia also make extensive contacts with the extracellular matrix (instead of the basement membrane) around the secondary mesenchyme cells. These cells eventually delaminate from the archenteron and contribute to other mesoderm lineages, such as muscle cells. The precise timing of apical junction dissociation has not been investigated.

Zebrafish

Before the onset of epiboly, blastoderm cells in teleosts can be separated into a surface enveloping layer and a deep enveloping layer (Fig. 1C). The former forms a protective layer with squamous epithelial morphology (Betchaku & Trinkaus 1978; Keller & Trinkaus 1987; Warga & Kimmel 1990), but does not give rise to either mesoderm or endoderm cells. The deep enveloping layer is the equivalent of the ectoderm/epiblast and will give rise to the mesoderm and endoderm layers after gastrulation. The deep enveloping layer is about four- to five-cells-thick prior to gastrulation. During the involution movement at the blastoderm margin, the deep enveloping layer flattens to two- to three-cells-thick due to radial cell intercalation (Warga & Kimmel 1990). No epithelioid structure is evident, however, either in the multi-cell-thick deep enveloping layer or in the involuted mesendoderm (Montero *et al*. 2005), suggesting that gastrulation in zebrafish does not involve the EMT process in the traditional sense. Instead, differential cell adhesion mediated by cadherins and differential tensile force in cells of different germ layers have been shown to contribute to germ layer segregation (Kane *et al*. 2005; Montero *et al*. 2005; Shimizu *et al*. 2005; Krieg *et al*. 2008).

Xenopus

Prior to involution, the blastoderm in the marginal zone is composed of two layers: a superficial layer and a deep layer (Keller 1980; Keller & Shook 2004, 2008; Shook & Keller 2008) (Fig. 1D). The superficial layer, similar to the surface enveloping layer in zebrafish, serves as a protective barrier for the developing embryo and can be considered as a continuous epithelium with tight junctions, adherens junctions and apico-basal polarity. Although the superficial layer has a relatively smooth basal surface facing the deep layer in sections and EM analyses, no basement membrane has been reported for it. Unlike the surface enveloping layer in zebrafish, however, the superficial layer in *Xenopus* participates in the involution and contributes to the endoderm. This process can be viewed as not to involve EMT, for the epithelial sheet is somewhat retained in the involuted endoderm. It is, however, unclear whether these cells still retain tight junctions. Some of the involuted cells located in the endoderm layer will later on contribute to the mesoderm through an EMT process. Underneath the superficial layer, the deep layer cells undergo a similar involution process in the marginal zone. Prior to the involution, the deep layer cells intercalate radially and adopt, from multi-layered mesenchymal cells, an epithelioid morphology with a single-cell-thick layer and a thin basement membrane, but without tight junctions. These cells involute and form the majority of the mesoderm lineage. This can be viewed as an incomplete EMT process. Many earlier involuting deep layer cells contributing to the anterior mesendoderm, however, never have the ‘opportunity’ to form this epithelioid structure, and are instead pushed inside by the forces that initiate the blastopore formation (apical constriction of bottle cells and vegetal endoderm rotation). Formation of these mesoderm cells therefore does not involve an EMT process.

Chick

Prior to primitive streak formation, the epiblast in chick embryos becomes epithelioid by late Stage HH1 (Eyal-Giladi Stage IX) (Bellairs *et al*. 1975, Eyal-Giladi & Kochav 1976; 1978; Kochav *et al*. 1980; Fabian & Eyal-Giladi 1981). The hypoblast cells form by a combination of the segregation of initially multilayered blastoderm disc before the formation of single cell-thick epiblast and the poly-ingression process during epithelioid epiblast formation (Kochav *et al*. 1980; Fabian & Eyal-Giladi 1981). These hypoblast cells aggregate as islands initially and, during primitive streak formation, spread out and adopt the morphology of a loose mesenchymal sheet (Stern 2004). As the primitive streak is being formed, deep layer cells in the posterior marginal zone (including within the Koller's sickle) contribute further to the hypoblast and endoblast, whereas the superficial layer will contribute to definitive endoderm and mesoderm lineages (Bellairs 1986; Stern 1990; Bachvarova *et al*. 1998; Lawson & Schoenwolf 2001; Callebaut 2005; Voiculescu *et al*. 2007). Formation of the primitive endoderm, the hypoblast and endoblast therefore can be considered not to involve the EMT process. Some of the mesoderm cells are derived from the middle layer of the posterior marginal zone that has never adopted an epithelial morphology (Bachvarova *et al*. 1998; Stern 2004; Callebaut 2005). Formation of these mesoderm cells therefore also does not involve the EMT process. Most of the mesoderm cells are formed by the convergence of epithelial-shaped epiblast cells towards the forming primitive streak, which subsequently undergo EMT and ingress to adopt a mesenchymal morphology (Fig. 1E). These mesoderm precursor cells located in the epiblast have all the characteristics of epithelial cells (Trelstad *et al*. 1967; Hay 1968; Nakaya *et al*. 2008).

Mouse

Trophectoderm is the first epithelial structure to form in mammalian embryos, with all associated epithelial characteristics (Vestweber *et al*. 1987; Collins & Fleming 1995; Ohsugi *et al*. 1996, 1997; Fleming *et al*. 2000a and b; Sheth *et al*. 2000; Flechon *et al*. 2003, 2007; Maddox-Hyttel *et al*. 2003; Morali *et al*. 2005; Moriwaki *et al*. 2007; Eckert & Fleming 2008; Nishioka *et al*. 2008). The inner cell mass cells, which are of mesenchymal morphology, soon give rise to two epithelial structures, the epiblast and the primitive endoderm (Fig. 1F). This is generally considered as a primary epithelialization process like the formation of trophectoderm, although in some published reports it is viewed as a secondary epithelialization process as some inner cell mass cells are derived from polarized division of already epithelialized morula cells (Shook & Keller 2003). Some similarity can be drawn between this and the stratification of already epithelialized blastoderm cells in *Xenopus* (Chalmers *et al*. 2003; Ossipova *et al*. 2007) in that the ectoderm is separated into two cell populations with different functions: an outer population maintains the epithelial morphology and serves as a barrier, and an inner population serves as cell source for three germ layers. The primitive endoderm differentiates into visceral endoderm and parietal endoderm, with the former maintaining epithelial morphology and the latter becoming mesenchymal (Enders *et al*. 1978; Hogan & Newman 1984; Verheijen & Defize 1999; Rivera-Perez *et al*. 2003; Perea-Gomez *et al*. 2007; Gerbe *et al*. 2008). Similar to chick, the mesoderm and definitive endoderm cells in mammals are derived from the epithelial-shaped epiblast cells through the EMT process (Tam & Beddington 1987, 1992; Lawson *et al*. 1991; Tam *et al*. 1993; Tam & Gad 2004).

Evolutionary considerations

The diverse mechanisms used for gastrulation and for mesoderm formation, as exemplified above in several model organisms and in many other triploblastic animals investigated (Arendt & Nubler-Jung 1999; Stern 2004; Solnica-Krezel 2005; Shook & Keller 2008), would tend to suggest that there are few evolutionarily shared mechanisms for the process of EMT in mesoderm formation. Some generalized understanding, however, can be achieved by viewing it from the perspective of physical and physiological constraints imposed on embryonic development throughout evolution. The physical constraint is reflected in the effect of yolk content on cleavage, gastrulation and epiboly processes (Arendt & Nubler-Jung 1999; Shook & Keller 2008). The physiological constraint is reflected in the difference between an embryo's internal physiochemical properties and those of the environment, including water, osmotic pressure and ion concentrations (Fesenko *et al*. 2000; Fleming *et al*. 2000b; Kiener *et al*. 2008). The ectoderm, prior to the generation of mesoderm and endoderm layers, can be considered to have three main roles: (i) as a source of cells for the mesoderm and endoderm; (ii) as a protective layer between the developing embryo and the environment; and (iii) as a protective layer for the nutritious substance of the embryo. The first is of course generally regarded as the primary role of pre-gastrulation ectoderm. This role, however, is often de-coupled with the second role. In such cases, there may be no particular reason for the pre-gastrulation ectoderm (as a cell source for the other two germ layers) to adopt an epithelial morphology. The epithelialization of the ectoderm in post-gastrulation embryos can therefore be a secondary process, after the generation of

the mesoderm and endoderm. Examples of this can be found in the separation of zebrafish ectoderm into the superficial enveloping layer and deep enveloping layer, in the stratification of *Xenopus* ectoderm into surface layer and deep layer, and in some sense in the separation of trophectoderm and inner cell mass in mouse embryos. The de-coupling of the first and the third roles is prominent in embryos with a significant amount of yolk substance deposited in the fertilized egg. Whether cellularized by holoblastic cleavages or syncytialized by meroblastic cleavages, yolky cells are part of developing embryos and will eventually become part of the endoderm. A large amount of yolk in many vertebrate embryos therefore requires the de-coupling of the epiboly-like process, as a means of internalizing these yolky cells, and the formation of the mesoderm and endoderm from the ectoderm in the traditional sense of gastrulation. Due to this de-coupling, mesoderm and endoderm formation often takes place before gastrocoel formation and is shifted from a circumblastoporal process such as in sea urchin, to dorsally favored as in *Xenopus* and Zebrafish, and to dorsally restricted as in amniotes (Voiculescu *et al*. 2007; Shook & Keller 2008). These de-coupled roles of embryonic ectoderm suggest that EMT, as a cellular morphogenetic process, is required for mesoderm formation when and only when mesoderm precursor cells are located in the ectoderm (and in some cases internalized mesendoderm) layer with an epithelial morphology, which does not have to be the case as we have discussed above.

Genetic dissection of EMT in mesoderm formation

Nevertheless, EMT was first proposed as a cellular mechanism for mesoderm formation in avian embryos (Trelstad *et al*. 1967; Hay 1968), and EMT/MET processes have since been used to describe a large variety of other developmental processes, including neural crest formation from the border of neural/non-neural ectoderm (Duband *et al*. 1995; Newgreen & McKeown 2005; Sakai *et al*. 2005; Duband 2006; Sakai *et al*. 2006), somitogenesis and sclerotome formation from epithelialized somite (Duband *et al*. 1987; Nakaya *et al*. 2004; Hay 2005; Takahashi *et al*. 2005; Takahashi & Sato 2008) and cardiac valve development from endocardium (Person *et al*. 2005; Runyan *et al*. 2005). This is not surprising as all cells in an organism can in principle be grouped into either an epithelial or mesenchymal category. Changes in tissue organization during normal and pathological development will often involve EMT or MET. As in the case of mesoderm formation, EMT in other developmental processes often comes with evolutionary variations. EMT as a descriptive term therefore would be much less useful if there are no shared molecular mechanisms governing diverse EMT processes. Indeed, uncovering and understanding these mechanisms has been the focus of EMT-related research in the past decade. With regard to EMT during gastrulation, genetic and molecular studies have been primarily focused on the mouse system.

As we have discussed earlier, the majority of mesoderm cells in amniotes (reptiles, birds and mammals) form by a process of ingression during gastrulation. This ingression is a typical EMT process. Pre-ingression mesoderm precursor cells have all the characteristics of epithelial cells, whereas post-ingression mesoderm cells are mesenchymal. Genetic experiments in mice have revealed that several extracellular signals, mediated by transforming growth factor-β (TGF-β), Wnt and FGF receptors, contribute to the EMT process in generating mesoderm cells (Deng *et al*. 1994; Yamaguchi *et al*. 1994; Sun *et al*. 1999; Ciruna & Rossant 2001; Kemler *et al*. 2004; Ben-Haim *et al*. 2006; Arnold *et al*. 2008), although platelet-derived growth factor (PDGF), Notch, Hedgehog and nuclear factor-κB (NF-κB) pathways have also been implicated in other EMT processes, with their roles in gastrulation EMT not carefully examined yet (Huber *et al*. 2005; Baum *et al*. 2008; Van Den Akker *et al*. 2008). Change of adherens junction types, from E-cadherin mediated epithelial interactions to N-cadherin mediated mesenchymal interactions, is generally considered as a critical step in this process (Hatta & Takeichi 1986; Cano *et al*. 2000; Zohn *et al*. 2006). FGF signaling in the primitive streak has been shown to regulate gastrulation EMT in mouse (Yamaguchi *et al*. 1994; Sun *et al*. 1999; Zohn *et al*. 2006). FGF signals downregulate E-cadherin expression to promote EMT during gastrulation by regulating *snail* gene expression (Ciruna & Rossant 2001). Snail, a zinc-finger transcription factor, directly binds to E-boxes in the promoter region of E-cadherin gene and represses its transcription in cancer cell lines (Batlle *et al*. 2000; Cano *et al*. 2000). Mouse embryos deficient in *snail* function exhibit abnormal mesoderm cell morphology with apico-basal polarity and epithelial type adherence junctions, and E-cadherin expression is retained in the mesoderm of *Snail*$^{-/-}$ mutants (Carver *et al*. 2001). In a number of tumor cell lines, it has been suggested that Wnt and receptor tyrosine kinase mediated signals also promote Snail stabilization and its nuclear import, and subsequently enhance EMT. The relationship between Snail and signaling pathways other than FGF has yet to be clarified at cellular levels *in vivo*. Furthermore, as a Snail independent pathway, it has been demonstrated that p38 mitogen-activated protein kinase (MAP kinase) and p38-interacting protein and/or EPB4.1L5 (Band 4.1 super family) are required for the downregulation of E-cadherin

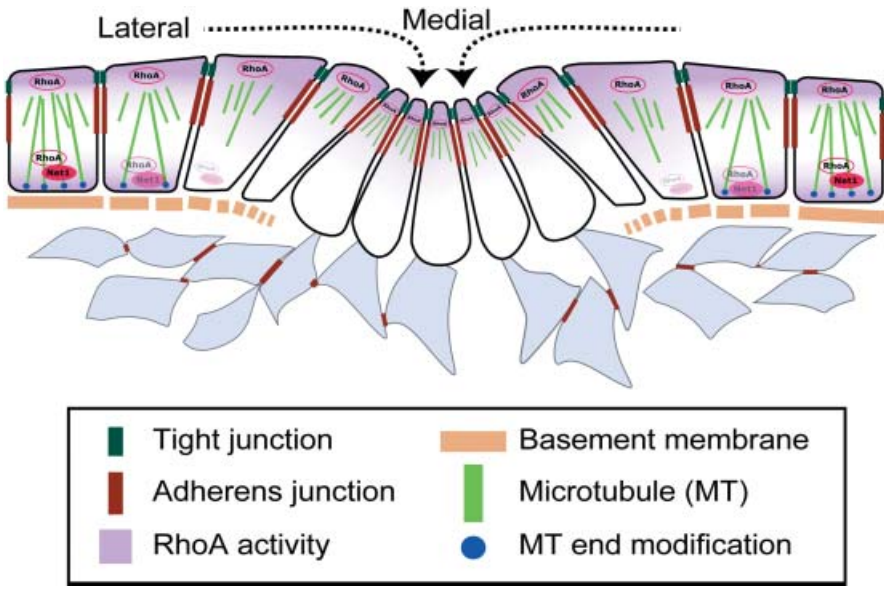

Fig. 2. Multi-step epithelial to mesenchymal transition (EMT) process in mesoderm formation during chicken gastrulation. In the primitive streak where mesoderm ingression takes place, precursor cells first express mesoderm markers (e.g. *brachyury*), then break down the basement membrane. These cells leave the epiblast after disrupting the tight junctions and apical/basal polarity, followed by a shift from E-cadherin- to N-cadherin-based adherens junctions.

protein at the post-transcription level during EMT in mouse gastrulation (Zohn *et al*. 2006; Lee *et al*. 2007). In addition to the mouse model, Snail gene family members have been shown to play crucial roles in the induction of EMT in other systems. For instance, Lv-snail, a member of the Snail family in sea urchin *Lytechinus variegatus*, is required for the EMT process in primary mesenchyme formation by downregulating cadherin expression and promoting cadherin endocytosis (Wu *et al*. 2007). Interestingly, Snail and cadherin regulated EMT has also been proposed for mediating mesoderm formation in zebrafish (Yamashita *et al*. 2004; Montero *et al*. 2005; Krieg *et al*. 2008), an organism that does not have a characteristic epithelial ectoderm as we have discussed above. Overall, these studies have provided the groundwork for investigating the molecular basis of EMT.

Basement membrane breakdown, a novel aspect in regulating gastrulation EMT

It has been well recognized that the EMT process involves the breakdown of the epithelial basement membrane. In her earliest description of EMT, Hay (1968) considered basement membrane degradation during mesoderm formation in chick embryos as one of the most prominent features of EMT. Molecular mechanisms of basement membrane breakdown during gastrulation EMT have not received careful investigation. A recent report indicated that this step is also critically regulated (Levayer & Lecuit 2008; Nakaya *et al*. 2008) (Fig. 2). Breakdown of epithelial basement membrane, marked by fibronectin and laminin, was shown to be the earliest event for gastrulation EMT in chick embryos, taking place prior to the breakdown of tight junctions and apico-basal polarity, whereas the shift in adherens junction types was seen to take place after the ingression. These observations indicate that EMT during gastrulation may take place as a multi-step process, each of which is controlled by a distinct set of signals. Supporting this model, it was shown that basally localized small GTPase RhoA and its activator Net1, a RhoA GEF (Guanine Nucleotide Exchange Factor), control the interaction between the basement membrane and the basal membrane of ectoderm cells. In ectoderm cells lateral to the primitive streak, RhoA protein and activity are detected both apically and basally, whereas in ectoderm cells within the primitive streak, basal RhoA and Net1 (Net1 is detected only basally in lateral regions) are downregulated, with apical RhoA still intact. Ectopic expression of RhoA did not affect normal ectoderm lateral to the streak, but resulted in the failure of basement membrane breakdown in medial streak and in ingressed mesoderm cells. Interestingly, many mesoderm precursor cells overexpressing RhoA still manage to finish the ingression process and move to the mesoderm layer, indicating that other EMT related events, such as the downregulation of tight junctions and the switch of adherens junction types, are not obviously affected. Furthermore, it was shown that the regulation of basement membrane breakdown mediated by RhoA takes place in the context of dynamic cytoskeletal changes, in particular the destabilization of basal microtubules, during the process of ingression. These observations further support the idea that EMT is a collective process composed of distinct cellular steps that are separately regulated. It is so far still unclear however, what the

biochemical mechanisms for the degradation of basement membrane proteins are, and how this process is linked to mesoderm fate specification marked by Brachyury on the one hand, and to the later disruption of tight junctions and apico-basal polarity, and the switch of adherens junction types on the other.

Given the diversity of EMT, the sequence and timing of these cellular steps have been reported to vary in a number of other EMT processes that have been investigated in some detail. During the formation of endocardial cushion mesenchymal cells, it appears that the loss of intercellular junctions takes place prior to the invasion through lamina densa of endocardial epithelium (Person *et al*. 2005). Sclerotome formation is another example in which downregulation of adherens junctions takes place before basement membrane disruption (Duband *et al*. 1987). In the case of neural crest cell delamination, while basement membrane breakdown is a necessary step for cranial crest cells, at the trunk level, the delamination does not involve the basement membrane (Nichols 1981; Newgreen & Gibbins 1982; Martins-Green & Erickson 1986). Moreover, no desmosomes or functional tight junctions are present in pre-delamination crest cells (Erickson *et al*. 1987; Aaku-Saraste *et al*. 1996), and breakdown of cadherin mediated adherens junctions triggers immediate delamination (Newgreen & Gooday 1985). A recent study with induced EMT in cultured epithelial cells suggested that tight junction dissociation occurs prior to adherens junction dissociation (Ozdamar *et al*. 2005; Thiery & Huang 2005).

These differences highlight the complex nature of context-dependent coordination of cellular components in different EMT processes. It is thus not surprising that, in terms of signals regulating EMT, no clear consensus has emerged as to whether a few 'master regulatory' molecules control the entire EMT (i.e. one decision point triggers a procession of cellular events), or an individual cellular event takes distinct signaling cues. Observations in gastrulation EMT favor the latter hypothesis, which is also favored by recent *in vitro* studies on independent regulations of tight junctions and adherens junctions (Hollande *et al*. 2003; Ozdamar *et al*. 2005). Nevertheless, signaling cues controlling individual cellular events likely crosstalk, resulting in both the complexity for any given EMT and the diversity of how EMT can be executed in different *in vivo* settings.

Future perspectives

In the four decades since EMT was first proposed, a large number of descriptive and experimental studies have been reported in literature, providing a rich resource of both *in vitro* and *in vivo* data. Proper understanding of EMT will obviously require the integration of knowledge from both fields. EMT studies using culture systems remain to be an irreplaceable alternative for detailed molecular analyses. However, one should not lose sight of the fact that EMTs in development and disease often take place in a much more dynamic and complex context. In addition, the relationship between EMT and differentiation is increasingly being appreciated in the stem cell research field. It has recently been reported that the induction of EMT in human mammary epithelial cells (HMLEs) by Twist, Snail or TGF-β1 leads to morphological change of these cells to a mesenchymal shape with the upregulation of stem cell markers (Mani *et al*. 2008), pointing to a possible direct link between EMT and pluripotency. This would not be unexpected from the embryological perspective, as the first sign of cellular differentiation during embryogenesis is often manifested as the epithelialization of the ectoderm cells. Furthermore, new molecular mechanisms, such as through the investigation of microRNAs, are being proposed for the regulation of EMT (Burk *et al*. 2008; Cano & Nieto 2008; Gregory *et al*. 2008; Korpal *et al*. 2008; Park *et al*. 2008). By evaluating the expression of 207 microRNAs in the many cancer cell lines, miR-200 family was identified as a general marker for E-cadherin positive and vimentin negative cancer cells (Park *et al*. 2008). This and other recent reports also show that miR-200 family targets both ZEB1 and ZEB2 to inhibit E-cadherin repression, and altering the levels of miR-200 in established cancer cell lines caused changes consistent with either EMT or MET induction (Burk *et al*. 2008; Cano & Nieto 2008; Gregory *et al*. 2008; Korpal *et al*. 2008; Park *et al*. 2008). Taken together, with a combination of novel tools and new perspectives, the EMT research has entered an interesting time. This, in return, will undoubtedly contribute to the understanding of how germ layers are formed during gastrulation, which remains one of the most fascinating questions for embryologists.

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