An amicable separation
Chick’s way of doing EMT

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Epithelial to mesenchymal transition (EMT) is a morphogenetic process in which cells lose their epithelial characteristics and gain mesenchymal properties, and is fundamental for many tissue remodeling events in developmental and pathological conditions. Although general cell biology of EMT has been well-described, how it is executed in diverse biological settings depends largely on individual context, and as a consequence, regulatory points for each EMT may vary. Here we discuss developmental and cellular events involved in chick gastrulation EMT. Regulated disruption of epithelial cell/basement membrane (BM) interaction is a critical early step. This takes place after molecular specification of mesoderm cell fate, but before the disruption of tight junctions. The epithelial cell/BM interaction is mediated by small GTPase RhoA and through the regulation of basal microtubule dynamics. We propose that EMT is not regulated as a single morphogenetic event. Components of EMT in different settings may share similar regulatory mechanisms, but the sequence of their execution and critical regulatory points vary for each EMT.

EMT is a concept proposed in cell biological studies of tissue morphogenesis during embryonic development. It involves changes of inter-cellular organization from a two dimensional epithelial type to a three dimensional mesenchymal one. The earliest EMT in animal development occurs during gastrulation, a process that forms mesoderm and endoderm tissue layers from the ectoderm. In amniotes, which include reptiles, birds and mammals, ectoderm is specified as an epithelial sheet. EMTs in gastrulation and other developmental settings take place with spatial and temporal constraints, and are coupled with complex patterning and cell fate specification events, most of which are not directly related to EMT. Molecular mechanisms underlying EMT are therefore difficult to be teased out. Furthermore, EMT is often defined in literature to include morphogenetic events within the epithelial sheet and mesenchymal cell population, which in strict sense should not be regarded as such. The descriptive power of EMT has been based on detailed cell biological studies, which may also be critical for current molecular studies of EMT. Here we use chick as an example to discuss the developmental, morphogenetic and cellular aspects that set the stage for gastrulation EMT.

Before formation of mesoderm and definitive endoderm, chick embryo is composed of two layers, epiblast and hypoblast, in area pellucida from which all intraembryonic tissues develop (Fig. 1A). The hypoblast is a part of primitive endoderm and is formed by cells segregated from an earlier multilayered epiblast before its epithelialization into a single-cell-thick layer. During gastrulation, the epiblast is stretched tight as a result of adhesion and outward migration of its marginal cells in area opaca over acellular vitelline membrane. In the area pellucida, epiblast cells are also covered by the vitelline membrane, but without obvious adhesion or interaction between them. Epiblast integrity is maintained mainly by tight junctions present in every epiblast cell. Mesoderm cells form through a de-epithelialization process, i.e., EMT, from the epiblast. Where, when and how EMT takes place to generate mesoderm are influenced by a number of developmental factors that are not directly related to the regulation EMT. The primitive streak (streak), the site of EMT, is a dynamic structure. It is specified at posterior part of the area pellucida by an earlier dorsal/ventral patterning event at stage HH1. Elongation of the streak anterior-ward occurs from HH2 to HH4, regulated by “global” tissue morphogenetic movement within the epiblast plane. The streak then retracts as development proceeds, and by HH9-10, regresses to a small region at the posterior end of the embryo. Throughout its elongation and retraction, the streak continues to generate mesoderm cells, but general morphology of the streak and its physical relationship with other developing tissues change, which affects how EMT takes place at the cellular level. In addition, mesoderm cell formation involves their concurrent patterning. Specification of different mesoderm lineages along the dorsal/ventral axis of the embryo is reflected in relative anterior/posterior locations of precursors in the streak, and a separate anterior/posterior patterning event is reflected in the timing of precursor cell ingress on within each lineage. These patterning events lead to variations of EMT even within the narrow definition of chick gastrulation. Moreover, the streak also serves as a signaling center in patterning neighboring ectoderm and in defining ectoderm/mesoderm boundary together with ectoderm derived signals. Thus cells located in and immediately near the streak are making constant decisions of whether to go in and become mesoderm or to
stay out in the ectoderm either as neural or epidermal cells.\textsuperscript{8}

The dynamic developmental setting for gastrulation EMT in chick suggests that molecular studies of any given EMT should take biological context into consideration. In contrast to this biological complexity, however, overall cell biological description of chick gastrulation EMT is relatively straightforward. Epithelial morphology of epiblast cells is maintained by tight junctions, epithelial adherens junctions and cell/BM interactions. They move toward the streak, break down cell/BM interaction, disrupt apical tight junctions and apical/basal polarity, switch to mesenchymal adherens junctions and move away from the streak (Fig. 1C and D). The key to understanding molecular regulations of EMT, however, may not lie in how to achieve EMT, which can be relatively easily triggered by a few exogenous signals in both culture and embryonic settings, but rather in how to execute these component events of EMT in a sequential and coordinated fashion in the context of other developmental processes and with spatial and temporal constraints often unique for each EMT.

In chick, mesoderm cell fate is specified in and around the streak when mesoderm precursors are still located in the epiblast. The streak, while being easily distinguishable as an overall structure, does not have a morphologically distinct lateral borders. Brachyury expression, marking mesoderm precursor fate (Fig. 1B), starts quite far away from the streak midline when seen in sections. The earliest sign of EMT is the initiation of basement membrane (BM) breakdown 5–10 cells away from the midline (Fig. 1C and D),\textsuperscript{1,3,9-11} although it is possible that BM breakdown is regulated by mesoderm specifiers like Brachyury. In gastrulation stage chick embryo, the BM underneath the epiblast is very thin and barely visible in electron microscopy (EM) analysis. Molecular components of the BM, such as laminin, fibronectin and perlecan, however, can be readily detected with immunohistochemistry (Fig. 2A). The BM has been considered to be crucial, together with tight junctions, for maintaining epiblast integrity in the chick embryo.\textsuperscript{3} However, during gastrulation, BM breakdown takes place early during EMT, whereas tight junctions are seen in every epiblast cells (Figs. 1D and 2A), including streak midline cells with only a narrow stalk connected to the apical surface (Fig. 2B). Chick epiblast is under mechanical tension, being pulled tight by outward migration of cells at its margin, without which embryonic development largely ceases.\textsuperscript{4,5} The epiblast also has transport and barrier functions to mediate the passage of water from the albumen to the developing embryo.\textsuperscript{12} It is therefore conceivable that these constraints make it necessary for the chick embryo to maintain tight junctions throughout the epiblast and to initiate EMT by modulating the basal side of epiblast cells first.

Our recent analyses on molecular regulation of BM breakdown (Fig. 1D) indicate that the activity of a small GTPase, RhoA, in the basal compartment of epiblast cells plays a critical role in this process. The loss of basal RhoA activity, regulated by a Rho specific guanine nucleotide exchange factor, Net1, is coincident with BM breakdown,
EMT in chicken gastrulation breakdown remains unclear. Interestingly, in cultured ovarian cancer cells, RhoA activation and microtubule stabilization were reported to mediate TGFβ1 signal induced secretion of extracellular matrix protein TGFBI.13 Conversely, secreted or membrane anchored metalloproteases were shown to promote BM degradation during EMT both in vitro and in vivo.14-16

After BM breakdown, epiblast cells engage in extensive morphological changes in preparation for leaving the epithelium. Apical surface narrows progressively, basal surface widens and establishes contacts with underlying mesoderm cells, and cells become significant taller due to apical/basal elongation. These changes result in bottle-shaped cells in streak midline (Fig. 2B), with main cell body residing in mesenchymal territory and with a long and thin cytoplasmic neck connected to the apical surface. This morphology often gives the false impression of these cells as mesenchymal in sections. As mentioned earlier, apical tight junctions are still very prominent for these bottle shaped cells. Basal contacts with mesenchymal cells appear to involve a different type of cell/matrix interaction, as extracellular matrix proteins such as tenascin and fibrillin-2 are strongly upregulated there (Fig. 2A). It is unclear whether these contacts play a more important role in the migration of mesoderm cells or in the de-epithelialization of midline epiblast cells. The loss of BM and basal microtubules, and the tight anchoring of apical junctions with the rest of the epithelium may partially account for the overall morphological changes, although distribution of microtubules and Golgi complex in these cells may also suggest an active regulation in membrane vesicular trafficking similar to what have been described in culture cells.17,18 In addition, de-epithelialization may involve cell-cell communication, as some neighboring streak epiblast cells have been reported to form rosette shaped cell groups during ingression.19 Conceptually, epiblast cells after the initiation of BM breakdown but before tight junction disruption may be defined as an intermediate state of EMT, similar to the “hybrid cell” suggested in several cancer EMT studies.20,21 The “hybrid cell” has both epithelial and mesenchymal traits judging from EMT molecular markers, such as E-cadherin, keratin, vimentin and MMPs. In our situation, the intermediate state cell expresses generally defined markers for both epithelial (E-cadherin) and mesenchymal (N-cadherin and vimentin) states (Fig. 2A).10

and exogenous activation of either RhoA or Net1 leads to BM retention in midline epiblast and ingressed mesoderm cells. RhoA signaling has been widely demonstrated to regulate cytoskeletal dynamics. In the context of regulating epiblast cell/BM interaction, RhoA appears to achieve this by regulating basal microtubule stability. Treatment with nocodazole, a microtubule destabilizing chemical, led to prominent ectopic BM breakdown. Direct cause for BM breakdown remains unclear. Interestingly, in cultured ovarian cancer cells, RhoA activation and microtubule stabilization were reported to mediate TGFβ1 signal induced secretion of extracellular matrix protein TGFBI.13 Conversely, secreted or membrane anchored metalloproteases were shown to promote BM degradation during EMT both in vitro and in vivo.14-16

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After ingression, mesoderm cells move away from the streak and follow semi-stereotyped migratory routes according to their
dorsal/ventral fate. Due to limitations in high resolution imaging in vivo, however, it is currently unclear how individual cells make each migratory step. Newly formed mesenchymal cells fill in the space, between the epiblast and endoderm, that lacks the extracellular matrix. These cells therefore have to gradually build up matrix proteins around them and at the same time engage in active migration. The traction force for early mesoderm migration has been postulated to be generated from three types of contact: with basal side of the ectoderm, with basal side of the endoderm, and with each other. These contacts have been well-documented in EM studies, although it is unclear what roles each type of contact plays. In cell culture studies, directional migration has been shown to involve proper orientation of the Golgi complex and centrosome relative to the position of the nucleus. Polarized positioning of the Golgi and centrosome close to the direction of migration facilitates membrane vesicular trafficking along microtubules to and from dynamic cellular processes at the leading edge. In our immunohistochemical studies (Fig. 2C) and in live cell imaging with fluorescent labeled nucleus and Golgi complex (unpublished data), however, we have observed a consistent positioning of Golgi complex at the rear end of migrating mesoderm cells that are not in direct contact with either the ectoderm or endoderm, similar to what has been described in EM studies. Prior to mesoderm cell ingression, Golgi complex in epiblast cells is always positioned apical to the nucleus (Fig. 2C). Thus newly formed mesoderm cells, although having lost apical/ basal polarity prominent in all epiblast cells, seem to have inherited this "basal forward, apical behind" organization.

From cell biological perspective, however, this organization may underlie fundamental mechanisms involved in regulating cell migration in vivo. Using GFP-EB1 fusion protein (unpublished data), we have observed dynamic microtubule formation and dissociation from MTOC in migrating mesoderm cells, with a periodicity in the range of seconds. These microtubules often also reach cellular processes at cell periphery. A correlation could be seen with the dynamic changes in microtubules on the one hand and those of cellular processes on the other, although the latter seem to be more abundant than the former and their causal relationship is unclear. It is noteworthy that mesoderm cells are not very motile immediately after ingestion, and only pick up speed gradually. This is consistent with the fact that E-cadherin is still expressed in newly generate mesoderm cells, its downregulation and switch to N-cadherin dominated adhesions interaction take place gradually as these cells initiate active migration. Signals regulating "step by step" migration of these cells are not understood. Over a longer time period and at the level of cell populations, signals derived from streak cells, such as Wnt and FGF molecules, were shown to mediate chemo-repulsion and promote migration of cells away from the streak. It will therefore be interesting to see how such signals influence cytoskeletal organizations and inter-cellular interactions during mesoderm cell migration.

In summary, mesoderm formation during chick gastrulation offers a rare opportunity in which a number of cell biological phenomena and concepts can be investigated in vivo. We propose that most EMT processes, like in the case of chick gastrulation, are not regulated as a single morphogenetic process. Individual components, such as disruption of BM, dissociation of tight junction, switch of adherens junction mediated interaction and directional cell migration, may share similar molecular regulatory mechanisms.

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References

1. Hay ED. Organization and fine structure of epithelium and mesenchyme in the developing chick embryo. In: Fleischmajer R, Billingham RE, eds., Epithelial-mesenchymal interactions; 18th Hahnemann symposium. Baltimore: Williams & Wilkins 1968; 31-55.
2. Hay ED. The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev Dyn 2005; 233:706-20.
3. Bellairs R. The primitive streak. Anat Embryol (Berl) 1986; 174:1-14.
4. Bellairs R, Bromham DR, Wylie CC. The influence of the area opaca on the development of the young chick embryo. J Embryo Exp Morphol 1967; 17:195-212.
5. New DA. The adhesive properties and expansion of the chick blastoderm. J Embryo Exp Morphol 1959; 7:146-64.
6. Voiculescu O, Bertocchini F, Wolpert L, Keller RE, Stern CD. The amniote primitive streak is defined by epithelial cell intercalation before gastrulation. Nature 2007; 449:1049-52.
7. Iimura T, Pourquie O. Collinear activation of Hox genes during gastrulation is linked to mesoderm cell ingestion. Nature 2006; 442:568-71.
8. Sheng G, dos Reis M, Stern CD. Churchill, a zinc finger transcriptional activator, regulates the transition between gastrulation and neurulation. Cell 2003; 115:603-13.
9. Sanders EJ, Prasad S. Epithelial and basement membrane responses to chick embryo primitive streak grafts. Cell Differ 1986; 18:233-42.
10. Nakaya Y, Sukowati EW, Wu Y, Sheng G. Rhoa and microtubule dynamics control cell-base- ment membrane interaction in EMT during gastrulation. Nat Cell Biol 2008; 10:765-75.
11. Trebst RL, Hay ED, Revel JD. Cell contact during early morphogenesis in the chick embryo. Dev Biol 1967; 16:78-106.
12. Stern CD, Manning S, Gillespie JL. Fluid transport across the epiblast of the early chick embryo. J Embryo Exp Morphol 1985; 88:365-84.
13. Ahmed AA, Mills AD, Ibramim AE, Temple J, Benkiron C, Viau M, et al. The extracellular matrix protein TGFβ induces microtubule stabilization and sensitizes ovarian cancers to paclitaxel. Cancer Cell 2007; 12:514-27.
14. Hotary K, Li XY, Allen E, Stevens SL, Weiss SJ. A cancer cell metalloprotease triad regulates the basement membrane transmigration program. Genes Dev 2006; 20:2673-86.
15. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001; 17:463-516.
16. Egblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002; 2:161-74.
17. Mach M, Micromotubule organization and function in epithelial cells. Traffic 2004; 5:1-9.
18. Rodriguez-Boulan E, Kretzir G, Mach M. Organization of vesicular trafficking in epithelia. Nat Rev Mol Cell Biol 2005; 6:233-47.
19. Wagastr IT, Bellert G, Mogensen MM, Munsterberg A. Multicellular rosette formation during cell ingestion in the avian primitive streak. Dev Dyn 2008; 237:91-6.
20. Brabletz T, Jung A, Reu S, Porzner M, Hlahef K, Kallinor F, et al. Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc Natl Acad Sci USA 2001; 98:10356-61.
21. Lee JM, Dedhar S, Kallur R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development and disease. J Cell Biol 2006; 172:973-81.
22. Duband JL, Thiery JP. Distribution of fibronectin in the early phase of avian cephalic neural crest cell migration. Dev Biol 1982; 93:308-23.
23. Kupfer A, Louvard D, Singer SJ. Polarization of the Golgi apparatus and the microtubule- organizing center in cultured fibroblasts at the edge of an experimental wound. Proc Natl Acad Sci USA 1982; 79:2603-7.
24. Jaappe AB, Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 2005; 21:247-69.
25. Yang X, Chrisman H, Weijer CJ. PDGF signalling controls movement patterns of cardiac progenitors and requires RhoA function. Development 2008; 135:1029-37.