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Mechanisms of Development 120 (2003) 1351–1383

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Mechanisms, mechanics and function of epithelial–mesenchymal transitions in early development

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Accepted 29 June 2003

Abstract

Epithelial–mesenchymal transitions (EMTs) are an important mechanism for reorganizing germ layers and tissues during embryonic development. They have both a morphogenic function in shaping the embryo and a patterning function in bringing about new juxtapositions of tissues, which allow further inductive patterning events to occur [Genesis 28 (2000) 23]. Whereas the mechanics of EMT in cultured cells is relatively well understood [reviewed in Biochem. Pharmacol. 60 (2000) 1091; Cell 105 (2001) 425; Bioessays 23 (2001) 912], surprisingly little is known about EMTs during embryonic development [reviewed in Acta Anat. 154 (1995) 8], and nowhere is the entire process well characterized within a single species. Embryonic (developmental) EMTs have properties that are not seen or are not obvious in culture systems or cancer cells. Developmental EMTs are part of a specific differentiative path and occur at a particular time and place. In some types of embryos, a relatively intact epithelium must be maintained while some of its cells de-epithelialize during EMT. In most cases de-epithelialization (loss of apical junctions) must occur in an orderly, patterned fashion in order that the proper morphogenesis results. Interestingly, we find that de-epithelialization is not always necessarily tightly coupled to the expression of mesenchymal phenotypes.

Developmental EMTs are multi-step processes, though the interdependence and obligate order of the steps is not clear. The particulars of the process vary between tissues, species, and specific embryonic context. We will focus on 'primary' developmental EMTs, which are those occurring in the initial epiblast or embryonic epithelium. 'Secondary' developmental EMT events are those occurring in epithelial tissues that have reassembled within the embryo from mesenchymal cells. We will review and compare a number of primary EMT events from across the metazoans, and point out some of the many open questions that remain in this field.
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Keywords: Epithelial–mesenchymal transition; De-epithelialization; Ingression; Morphogenesis

1. Introduction

1.1. Overview

Epithelial organization is a defining characteristic of the metazoa, in that most multi-cellular organisms have epithelial sheets on their outer surfaces for nearly all their life cycle (Nielsen, 1991). An embryonic cell sheet is considered epithelial if the cells have an apical free (non-adhesive) surface on one side, and face embryonic tissue on the other, basal–lateral side (e.g. Hay, 1968). Epithelia serve several fundamental, related purposes. First, they serve as a barrier to the external environment, allowing the embryo to create an internal, physiologically controlled environment free from other organisms and debris. Second, epithelia potentially regulate patterning by forming barriers and limiting the diffusion of signaling molecules within the embryonic cavity. They may also serve to 'planarize' signaling and patterning within a two-dimensional sheet, through the planar cell polarity (PCP) pathway (Adler, 2002). Their apparent coherence and involvement in early patterning events give the impression that epithelial sheets are more efficient or precise in patterning. But this opinion should be tempered by the fact that cells of epithelial sheets (Keller, 1978), even the most cohesive and physiologically impermeant (Keller and Trinkaus, 1987) can exchange neighbors, often as much as deep, mesenchymal cells (Keller et al., 1992), and mesenchymal cells are capable of very precise patterning as well, as seen in the precise segmentation of the somites from the segmental plate

Abbreviations: EMT, epithelial–mesenchymal transition; PMC, primary mesenchyme cell; WT, wild type; ICM, inner cell mass; FGF, fibroblast growth factor; FGFR, FGF receptor; TGFβ, transforming growth factor β.

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doi:10.1016/j.mod.2003.06.005

(Pourquie, 2001) and the progressive, cell by cell onset of mediolateral cell intercalation motility in amphibians (Shih and Keller, 1992). Third, cells organized as an epithelium may provide more mechanical integrity than mesenchymal cells in the early embryo, and thus serve as the first line of defense of the embryo against mechanical disruption. The importance of an integrated epithelial sheet in early embryogenesis is highlighted by the fact that free-living amphibian embryos immediately, during their initial cleavage, form circumferential tight junctions and adherens junctions, sequester a controlled internal environment, and polarize their cells along the apical–basal axis, with a non-adhesive outer membrane and an adhesive basolateral domain (Fig. 1A–D) (reviewed by Danilchik et al., 2003; Fleming et al., 2000). Mouse embryos, despite developing inside the more controlled environment of the uterus, also epithelialize fairly early, after three or four cleavages, during compaction (Fig. 1E–H). But it is difficult to make a complex organism from one layer of cells, or even two.

Evolution of epithelial–mesenchymal transition (EMT) allowed morphogenesis of much more complex embryonic structure. Internalization of cells provided a mechanism for generating a new cell type, the mesenchymal cell, and allowed an increase in embryonic complexity from diploblastic to triploblastic grades of organization. EMT, the breakdown of the epithelium, along with ingression, the movement of the cells into the interior, probably evolved from mechanisms for internalizing gametes or dividing cells (see Buss, 1987). Release from the epithelial layer may have eventually allowed the evolution of the phenotypic plasticity of the mesenchymal cell type, in particular its ability to actively migrate individually, and thereby allowed further evolutionary innovations (Perez-Pomares and Munoz-Chapuli, 2002). One advantage mesenchymal cells have over epithelial cells is that they are free to rearrange or move about in three dimensions. Embryonic epithelial cells, despite the fact that they can actively change shape, actively or passively intercalate during convergence and extension, and crawl during wound healing (reviewed by Keller et al., 2000, 2003; Keller and Hardin, 1987) are constrained in these activities to a two-dimensional cell sheet. In modern triploblastic metazoans, EMT is used as a mechanism for reorganizing groups of progenitor cells into a more complex set of juxtaposed tissues, thereby allowing new inductive interactions (e.g. Behringer et al., 2000; Tam et al., 2001). For this reason it is important that EMTs occur in the correct place, at the right time and in the right sequence, such that progenitor cells come to lie in the appropriate pattern.

We consider EMT to be the entire series of events involved in the transition from an epithelial to a mesenchymal phenotype. In general terms, we mean the phenotypic transition of a cell that is integrated into a coherent sheet with apical–basal polarity to an association with a less coherent, more individually motile group of cells without apical–basal polarity. The epithelial state of organization may vary, and the specifics of developmental EMTs differ from case to case, depending largely on whether the starting state is an epithelial sheet or a sheet of cells with epithelial properties. In some cases, the cell layer involved is a proper epithelium with organized apical tight and adherens junctions and a basal lamina, as during primary mesenchyme cell (PMC) ingression in the sea urchin. But in other cases, such as in teleost or nematode (*Caenorhabditis elegans*) gastrulation, the situation is much more ambiguous; the only epithelial feature in *C. elegans* appears to be the differentiation of the apical vs. basolateral membrane domains, and, in the fish, coherence as a cell sheet; there are no circumapical tight or adherens junctions (Hogan and Trinkaus, 1977; Krieg et al., 1978). The differences between these 'epitheloid' cell populations with mesenchymal properties and the various states of mesenchymal organization, including migrating cell 'streams' (Davidson et al., 2002b; Trinkaus, 1984), coherent arrays of intercalating mesenchymal cells (see Keller et al., 2000), and migration of individual cells, are poorly defined. Therefore, removing a cell from an epithelium or an epitheloid array is a highly diverse process and may involve very little or a lot of remodeling of junctions, polarity, motility, and adhesive properties.

Taken one step at a time, a primary EMT, which is one that occurs in the primary embryonic epithelium, is more complex a process than it first appears (Fig. 1I–K). Necessarily, at some point it must involve loss of epithelial phenotype, or de-epithelialization, which would by itself leave non-epithelial, or nominal 'mesenchymal' cells, in place of what was an epithelium, and also leave a surrounding epithelium with free edges where the process of de-epithelialization had stopped (Fig. 1J). Epithelial sheets abhor a free edge and predictably respond with wound healing behavior, the marginal and sub-marginal cells advancing over the wound site until meeting the cells from the other side (see Davidson et al., 2002a; Radice, 1980; Trinkaus, 1984; Wood et al., 2002). This behavior alone would not necessarily insure inclusion of the de-epithelialized cells within the embryonic body. Therefore evolution of mechanisms of de-epithelialization must have gone hand-in-hand with evolution of mechanisms of ingression, the movement of de-epithelialized cells into the embryonic body (Fig. 1K). The evolution of the mesenchymal phenotype, which insures the proper association of ingressed cells within the internal cavity and confers the ability to migrate as individual cells, may have evolved somewhat independently of de-epithelialization and ingression. In addition, mechanisms have evolved that minimize the loss of mechanical and physiological integrity of the primary embryonic epithelium; in the example shown, de-epithelialization and ingression are localized and preceded by apical constriction of the de-epithelializing cells, thus minimizing the task of re-sealing the epithelium (pointers, Fig. 1K). Additional mechanisms will be discussed below, including bridging junctions that perhaps form new sealing junctions before old ones are

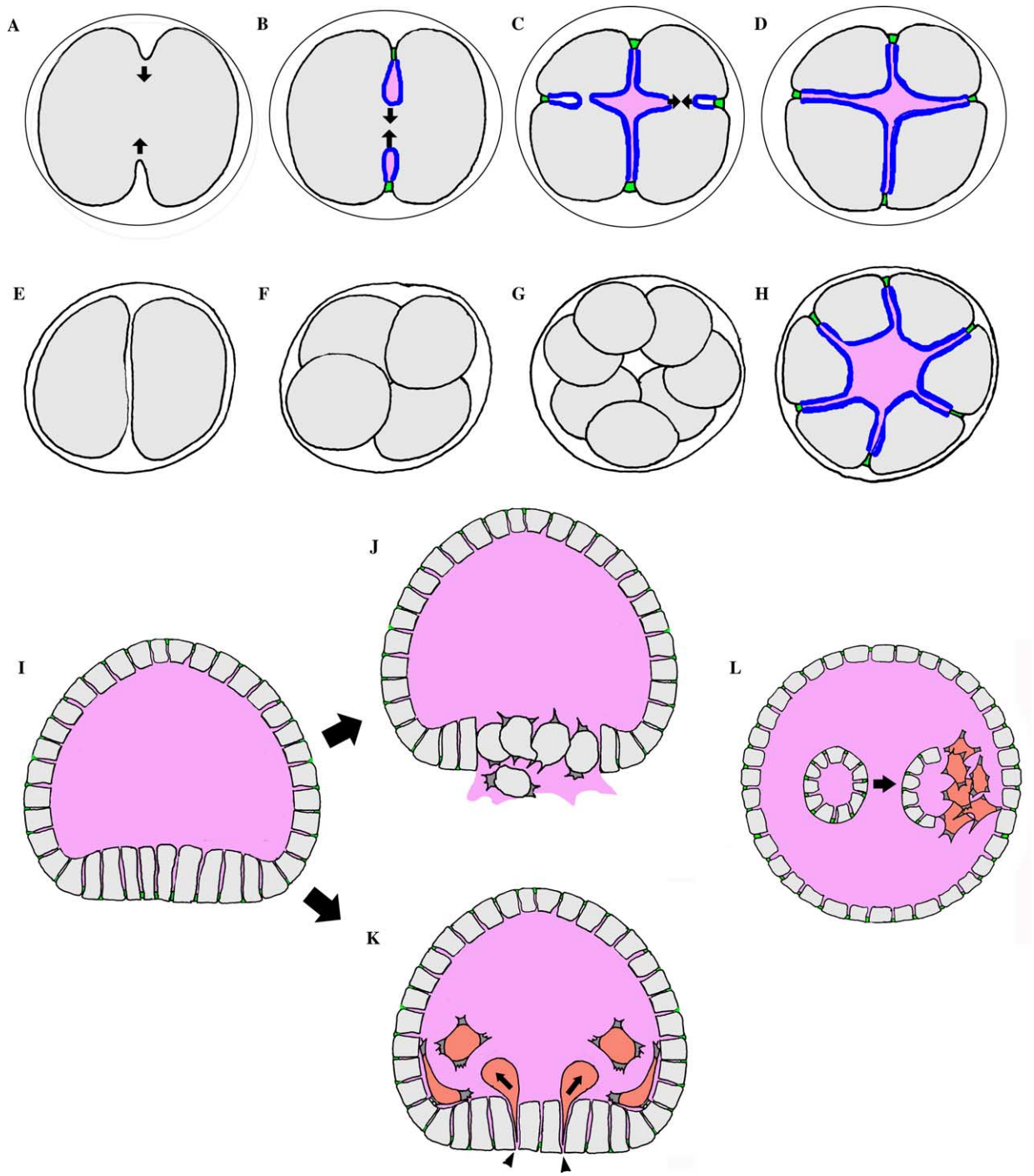

Fig. 1. Formation of the embryonic epithelium in early development. The embryonic epithelium may form during the first cleavage of the zygote, as in *Xenopus* (A–D). During the first and subsequent cleavages, a shallow furrow is formed in the primary or oocyte membrane (arrows, A), and then new or secondary membrane (blue) is added to the walls of the furrow by exocytosis to deepen it to completion (B–D), thus immediately generating an apical–basal polarity in the type of membrane. A circumferential, apical junctional complex (green) forms at the juncture of the old, oocyte membrane and the new, added membrane as the furrow deepens, immediately sequestering an internal, physiologically controlled environment, and providing mechanical integrity. In contrast, other organisms not so dependent on controlling the internal environment, such as the mouse embryo (E–H), undergo cleavage of the zygote to the 8- or 16-cell stage (E–G), and only then do the blastomeres adhere to one another, form circumferential junctions, begin physiological control of the internal environment, and become polarized in the apical–basal axis ('compaction', H). If primary embryonic epithelial cells simply transformed into mesenchymal cells, a large wound would result, and the mechanical and physiological integrity of the embryo would be compromised (I–J). Mechanisms have evolved that allow EMT and ingression of cells out of the epithelium to occur with minimal disruption of the embryonic epithelium (I–K). In contrast, internalized, secondary epithelial undergo EMT within a protected environment (L).

disassembled, and invagination of a tube prior to its de-epithelialization.

Primary developmental EMTs are one of the morphogenic mechanisms driving germ layer reorganization of the initial primary embryonic epithelium (Fig. 1I,K) during gastrulation, neurulation and neural crest formation. Examples include endoderm ingression in *C. elegans*, PMC ingression in sea urchins, and ingression of mesoderm from the surface of the amphibian gastrula or epiblast of the chick. Secondary developmental EMT involves cells that have secondarily adopted an epithelial organization and then undergo an EMT during organogenesis (Fig. 1L). These include ventral somite de-epithelialization to form the sclerotome (e.g. Brand-Saberi et al., 1996; McGuire and Alexander, 1992), EMT of cells in the endocardial endothelium to form the endocardial cushions in the atrioventricular canal of the heart (e.g. Markwald et al., 1996) and EMT of border cells in the ovarian follicles of the fruit fly (e.g. Abdelilah-Seyfried et al., 2003; Bai et al., 2000; Montell, 2001). Secondary EMTs occur largely within the embryonic environment and may not involve maintenance of epithelial integrity or ingression. Other developmental EMT events occur within extra-embryonic tissues, for example trophoblastic EMT in mammals (reviewed by Sutherland, 2003). There are also many mesenchymal-to-epithelial transitions (METs) (reviewed by Barasch, 2001) that play an important role in organogenesis.

Here we will discuss selected EMTs in early development. The literature on regulation of various EMTs in embryos (reviewed by Hay, 1995; Ip and Gridley, 2002; Locascio and Nieto, 2001) and in cell culture (reviewed by Boyer et al., 2000; Martinez Arias, 2001; Savagner, 2001) is large and will not be reiterated here. We will focus on the mechanisms of EMT, particularly the cell biological steps involved and their morphogenic function, and identification of unresolved issues, which include the following. The starting state of the 'epithelium' in a surprising number of EMTs in early embryos is poorly characterized in terms of its state of organization, including the types of junctions (tight, adherens, desmosomes), apical and basolateral membrane differentiation, and whether or not the epithelium is a physiologically resistant and mechanically coherent sheet, rather than an 'epitheloid' sheet. Also, the steps involved in epithelial cells detaching from one another, the mechanisms for down-regulating junctions of one type, and up-regulating others, the mechanics of ingression and the process of resealing the hole or 'wound' left by removal, or preventing such a lesion in the first place, are poorly understood in cases where mechanical and physiological integrity is important. Finally, experimental perturbations suggest that developmental EMTs do not necessarily follow a standard series of phenotypic changes that are obligately linked or ordered. Again, some EMTs involve more stringent requirements than others for the maintenance of an intact epithelium, which may influence the order of events and even their necessity. We will examine several of these cases, in an attempt to understand the functional interrelationship of the steps, and their dependence on context.

1.2. Events comprising EMTs

Cells undergoing a primary EMT generally go through some or all of the following steps:

- *Specification to differentiate into a type of cell that will go through EMT*. Specification toward a mesenchymal phenotype initiates many important changes in gene expression and protein function that must all work in concert for a developmental EMT to occur correctly. This will direct the subsequent steps and may require stopping cell division so that the cytoskeleton can be used to drive the cell shape changes and motility needed for EMT.
- *Temporal and spatial patterning of the progress of the EMT within the area destined to undergo EMT*. Patterning is important in that large areas of epithelium destined to undergo EMT usually do so progressively from a restricted zone, which allows both a necessary maintenance of physiological and mechanical continuity of the remaining epithelium and the spatial regulation of morphogenesis.
- *Move, or be moved, to the site of EMT, generally through epithelial morphogenesis*. Movement of cells to the correct position is not always a requirement, as they may initially lie there to begin with (as in the sea urchin), but in other cases it is clearly required, as in the chick or mouse primitive streak or the urodele amphibian, where large areas of epithelium are moved to a local site of ingression. The mechanism behind these movements is poorly understood in nearly all cases.
- *Alteration or disruption of the basal lamina*. Ingressing cells often move past or through a basal lamina, which may mechanically impede their ingression and therefore must be disrupted prior to ingression, presumably by the ingressing cells. The mechanism behind this is again poorly understood. Matrix metalloproteases are thought to be important in, among other things, remodeling or degrading the extracellular matrix during organogenesis, later tissue remodeling events, and cancer (Sternlicht and Werb, 2001), and perhaps cell migration during gastrulation (Suzuki et al., 2001) but evidence for a role in primary developmental EMTs is lacking so far (see Page-McCaw et al., 2003).
- *Change in cell shape, generally by an apical actinmyosin contractile mechanism and/or changes in adhesion*. Ingressing cells often but not always go through a bottle-shaped stage, which may have two functions: by constricting their apices cells may displace much of their intracellular contents basally and initiate movement out of the epithelium. Perhaps

more important, apical constrictions reduce the amount of non-adhesive apical membrane and circumferential, apical junctions that must finally be broken upon ingressing. It would also reduce the size of the hole left in the epithelium. It is generally thought that apical constriction is driven by an actinmyosin-based contraction, while the apical membrane is reduced by endocytosis. Changes in adhesion may also contribute to cell shape change on EMT. Cell behaviors in echinoderm gastrulation are consistent with the possibility that cells round up by loss of basolateral adhesion (Gustafson and Wolpert, 1963).

- *De-epithelialize*. We define de-epithelialization as the loss of the coherent contact between neighbors that characterizes a particular epithelium, and the eventual loss of an apical membrane domain. This involves a loss of the extensive circumferential apical junctions, specifically the circumapical tight and adherens junctions, in the case of epithelia that are physiologically and mechanically very impermeant and coherent, but it can also involve loss of the junctions accounting for the apical coherence of less coherent and resistive epitheloid sheets, a state of 'epithelialness' that is poorly characterized. How these processes occur is not understood. The evidence suggests that targeted endocytosis of epithelial junctions and adhesion molecules may be important and the apical membrane may eventually be completely eliminated by endocytosis.
- *Ingress*. We define ingression simply as the withdrawal of the ingressing cell's apex from the epithelial layer and into the deep layer. It differs from de-epithelialization in that a cell could de-epithelialize and not move out of the sheet. Normal ingression is associated with de-epithelialization (see above) and adoption of basal mesenchymal characteristics (see below), including an active motility and strong traction on deep tissues or structures, to pull the cell out of the epithelium. The cell might also be squeezed out of the remaining epithelium by virtue of the fact that loss of apical coherence is likely to stimulate wound healing (Radice, 1980).
- *Maintenance of epithelial integrity*. Ingression nominally would leave a hole or wound in the epithelium, a wound that would have to be healed, given that the primary embryonic epithelium is the embryo's physiological and mechanical barrier with the outside world. The evidence below suggests at least two ways that this could be done. The first is 'wound healing'; a small hole is left in the epithelium, due to apical constriction and zipping together of the adhesions of adjacent cells as their contact with the apically constricting cell is diminished; this small opening is then quickly sealed by zipping up of adhesions of adjacent cells as the ingressing cell turns loose. The second is that adjacent cells form extensions that arch over the top of the ingressing cell and form additional, bridging junctions above the ingressing cell, thus sealing the epithelium before the ingressing cell breaks its own junctions with these cells.
- *Differentiate cell behavior and organization characteristic of a mesenchymal phenotype*. This process begins prior to de-epithelialization, continues through ingression, and is not yet complete in recently ingressed cells. Ingressed cells often retain markers of their apices shortly after ingression, such as remnants of tight junctions. Cells must continue the process of turning off epithelial characters and turning on mesenchymal characters. This requires a major reorganization of the cell, including completely dismantling the apical junctional 'scaffold' that is thought to regulate discrimination between apical and basal–lateral (e.g. Rashbass and Skaer, 2000) by vesicular traffic, and organization of the cytoskeleton. This, with the removal of the apical membrane, results in the loss of the cell's apical–basal polarity. The basal–lateral membrane also must be remodeled, including the removal of epithelial adhesive molecules, perhaps by endocytosis, and replacement by mesenchymal-type adhesion molecules (cadherins, for example) and matrix receptors (integrins). The cytoskeleton must be remodeled, from what we imagine is a static, structural epithelial configuration to a dynamic, migratory configuration, a process that involves change from epithelial cytokeratins to mesenchymal vimentins, and probably substantial changes in regulation of actin polymerization, microtubule dynamics and myosin function to allow protrusive activity, all poorly understood phenomena in embryonic EMTs.

We will discuss a number of examples of primary developmental EMTs with these issues in mind and summarize general conclusions at the end.

2. Primary mesenchyme cell ingression in the sea urchin

Research on sea urchins has been done on a wide variety of species (e.g. *Arbacia punctulata, Lytechinus pictus, Strongylocentrotus purpuratus*), but the results are often assumed to apply equally to all species. Where discrepancies have been found, we have indicated the species in question, but the reader should keep in mind that most of the comments below should be taken as generalizations, at best.

2.1. Morphogenesis of PMCs during ingression

At the onset of gastrulation the sea urchin is a single-layered epithelial sphere surrounding a blastocoel (Fig. 2A). Gastrulation begins as the PMCs undergo EMT and ingress into the blastocoel (Fig. 2B). The PMCs are derived from the micromeres and form a ring of cells around

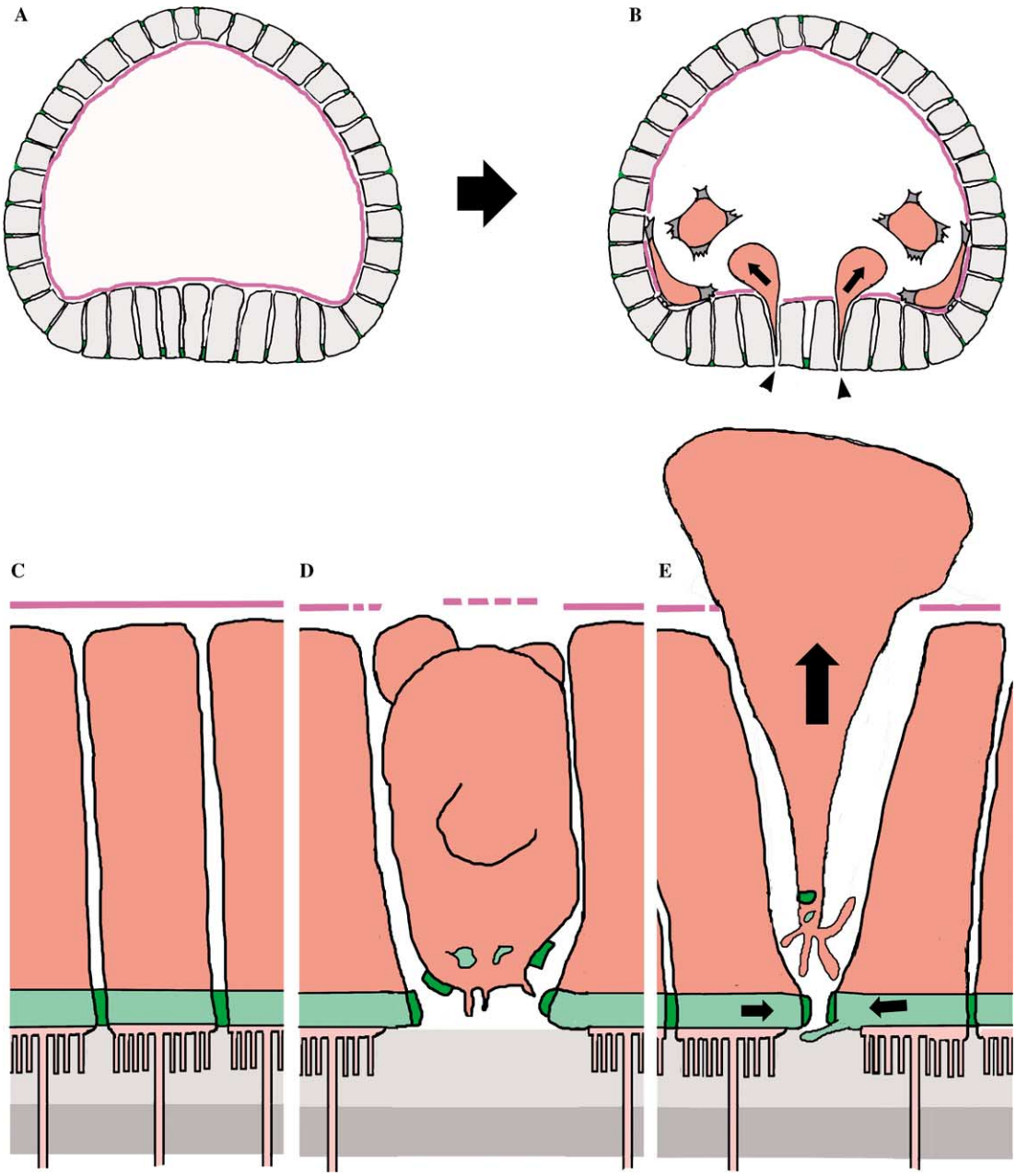

Fig. 2. Key features of the EMT of sea urchin primary mesenchyme cells are illustrated. The blastula consists of a single-layered epithelium (A). Primary mesenchyme cells differentiate in the vegetal region of the blastula and undergo EMT and ingression (red cells, B), after which they migrate to specific sites and form the larval skeleton (not shown). The epithelial cells are underlain by a basal lamina (magenta line, A–E), and are attached to an extra-embryonic matrix, the hyaline layer (gray, C–E) by microvilli; in most species, each cell bears a cilium that extends through the hyaline layer. The cells are joined circumferentially at their apices by a junctional complex (green), which segregates the cell surface into a basolateral (red) and an apical (pink) domain. EMT involves breakdown of the apical junctions and the connection to the hyaline layer, appearance of holes in the basal lamina, rounding and blebbing of the cells, and ingression (C–E). Temporary holes that appear where cells have left the epithelium are quickly healed (arrows, E).

the thickened vegetal plate (Katow and Solursh, 1981, 1980; Okazaki, 1975). They are bounded by a basal lamina-like matrix on the inside (magenta line, Fig. 2A,C), and they are attached to one another by circumferential, apical junctions (green, Fig. 2C) and to an extra-embryonic matrix, the hyaline layer (gray, Fig. 2C), by microvilli. Prior to ingression, PMCs are attached to each other by apical tight junctions (Balinsky, 1959) and to the hyalin layer covering the apical face of the epithelium (Katow and Solursh, 1980). At the time of ingression, PMCs lose their affinity for hyalin and echinonectin in this extra-embryonic matrix and for other cells of the blastular epithelia and gain affinity for fibronectin, which is found in the basal matrix layer (Burdsal et al., 1991; Fink and McClay, 1985; McClay and Fink, 1982). PMCs, but not their non-ingressing neighbors, constrict their apices, narrow their neck, and expand their basal ends, becoming 'bottle cells'. Apical junctions between PMCs and neighboring cells become

reduced or absent in ingressing cells, and microprotrusions are seen in the vicinity of the remnants of these junctions (Gibbins et al., 1969; Katow and Solursh, 1980). Prior to the onset of PMC ingression, the basal lamina, which is thin and discontinuous (Katow and Solursh, 1979), disappears under ingressing cells (Katow and Solursh, 1980) (Fig. 2C), suggesting digestion or mechanical disruption by the PMCs. The PMCs then withdraw their neck from the epithelial layer (ingress), and they are finally 'shed' into the blastocoel cavity as rounded up cells (Fig. 2C–E). In *L. pictus*, PMCs, but not their non-ingressing neighbors, lose their apical cilia prior to ingression, whereas PMCs in *Mespilia* may never form cilia on their apical surface prior to ingression (citations in Katow and Solursh, 1980). Ingression occasionally results in holes in the epithelium through which material can pass; apparently epithelial integrity is not strictly required, at least in the short term (Fig. 2D,E). However, both the ingressing cells and their neighbors make many protrusive contacts with adjacent cells from their apical ends (Katow and Solursh, 1980), suggesting that protrusive activity may be involved in temporarily blocking and/or resealing the hole left by the ingressing cell. These protrusions are filled with actin microfilaments. In *Mespilia*, ingressing cells leave behind a fragment of their apical domain, but this was not seen in *Arbacia* or *Lytechinus* (Katow and Solursh, 1980, and references therein).

2.2. The role of changing adhesion in PMC ingression

The disassembly of adherens junctions is associated with and may be required for PMC ingression. Cadherin (LvG Cadherin) and catenin (Lvβ-catenin) staining are localized to apical junctional regions prior to ingression, but in PMCs that have ingressed, cell surface staining is strongly reduced and instead staining appears in intracellular aggregates, suggesting that adherens junctions are endocytosed during the process of ingression (Miller and McClay, 1997a,b). It is not clear whether endocytosis directly reduces adhesion by removing functional adherens junctions, and thereby breaks the connection with other cells, or whether only non-functional adherens junctions are endocytosed; that is, the components of the junctions are removed only after they have been made ineffective in some other fashion and have broken contact with adjacent cells. In the latter case, junction endocytosis may occur long after the cell has ingressed. It is also not known if junctional endocytosis is a necessary component of ingression, which it might be if it is directly involved in reducing adhesion rather than just as a shuttle of already compromised junctional components to degradation or turnover pathways.

Endocytic processing of junctions requires protein synthesis. Presumptive PMCs in embryos treated 4 h prior to ingression with the translation inhibitor cordycepin show apical constriction but fail to ingress (Anstrom and Fleming, 1994). Cadherin staining remains in the junctional region in these embryos and there is less intracellular staining than in controls (Miller and McClay, 1997a,b), suggesting that protein synthesis-dependent endocytosis of junctional components may be necessary for ingression. However, a specific causal link between adherens junction disassembly and ingression has not been shown.

Ingression is associated with a change in PMC adhesive preference from the epithelial cells to the underlying basal lamina. αSU2 integrin is expressed basally in the embryonic epithelium from the mid-blastula stage and appears to be involved in binding laminin in the basal lamina (Hertzler and McClay, 1999). At the time of PMC ingression, αSU2 expression becomes discontinuous in the region of ingression, and is no longer detectable in PMCs that have ingressed. But whether loss of αSU2 expression precedes and is essential for ingression is not known. Ingressed PMCs do show reduced adhesion to laminin (Hertzler and McClay, 1999) and increased adhesion to fibronectin, which is also a component of the basal lamina (Burdsal et al., 1991; Fink and McClay, 1985; McClay and Fink, 1982), relative to ectodermal cells, which remain epithelial. This suggests that the expression of αSU2 is replaced by a yet-uncharacterized fibronectin-binding integrin (e.g. Marsden and Burke, 1998).

The behavior of PMCs appears to be autonomous: their loss of affinity for the extra-embryonic matrix and for other cells of the blastular epithelia and their gain of affinity for fibronectin occur when PMCs are cultured in isolation (Burdsal et al., 1991; Fink and McClay, 1985; McClay and Fink, 1982). And PMCs still ingress when grafted heterotopically (Wray and McClay, 1988).

2.3. Mechanical forces due to active basolateral traction do not appear to contribute significantly to PMC ingression

The mechanism driving PMC ingression is unknown, but appears to rely largely on changes in the adhesive properties described above, rather than mechanical forces generated by the PMCs or their neighbors. PMCs show no basal filopodial or lamellipodial protrusions while ingressing (Katow and Solursh, 1980), indicating that active traction by the PMCs probably does not play a role. But they do show a circus or blebbing movement (Gustafson and Kinnander, 1956), which may be involved in 'jostling' them loose from the adjacent cells (McClay et al., 1995) (Fig. 2D).

2.4. Role of the cytoskeleton

Microtubules are found along the long axis of PMCs prior to ingression (Anstrom, 1989; Gibbins et al., 1969; Katow and Solursh, 1980), and in neighboring blastomeres, on the sides facing the ingressing PMC (Katow and Solursh, 1980). Based on these observations, microtubules were suggested to be responsible for driving the shape changes seen associated with ingression (Gibbins et al., 1969; Tilney and Gibbins, 1969). Disrupting microtubules with colchicine and hydrostatic pressure or stabilizing them with D2O prevents the ingression of PMCs in *Arbacia punctulata*

(Tilney and Gibbins, 1969); however this is likely due to other toxic effects of these treatments, including the prevention of the cell division that occurs to produce the PMCs (Anstrom, 1989) in this quickly developing species. Treatment of *Strongylocentrotus purpuratus* with colchicine, β-lumicolchicine (a control which does not affect microtubules), nocodazole or taxol did not prevent bottle cell formation and ingression at the normal time (Anstrom, 1989). But it did disrupt development, including subsequent PMC differentiation as in *Arbacia*, suggesting that microtubules are not required for cell shape change to the elongated 'bottle' shape with a bulbous basal end, nor ingression of the PMCs.

On the other hand, apical constriction seems to have some role in ingression, although it is not absolutely essential or sufficient. An inhibitor of actin–myosin based contraction, papaverine, reduces apical constriction in PMCs and delays ingression, but prevents neither bottle cell formation nor eventual ingression (Anstrom, 1992). Perhaps reduction of adhesion is facilitated by the fact that apical constriction reduces apical, junctional perimeter of the cell. But apical constriction alone is not sufficient for ingression (Anstrom and Fleming, 1994). Vesicular transport, and hence membrane addition to the basal surface, is also not required for shape change or ingression (Anstrom and Raff, 1988).

2.5. How is specification of PMCs related to specification of EMT?

Some progress has been made in understanding how the signaling that specifies EMT is related to that which specifies PMC differentiation. In most cases characterized so far, failure of cells to execute EMT are due to failure to specify the PMC fate, rather than failure of downstream aspects of EMT such as ingression or de-epithelialization. PMCs of embryos injected with a morpholino against Alx1, a homeodomain protein controlled by maternal βcatenin, show no sign of differentiation and also do not go through any aspect of EMT (Ettensohn et al., 2003). Ets1, a transcription factor involved in PMC fate specification (Kurokawa et al., 1999), operates independently of Alx1 (Ettensohn et al., 2003). Ets genes are also associated with EMT of the chick epicardium and endocardium, during emigration of neural crest cells and dispersion of somites into the mesenchymal sclerotome in the chick, and neural crest migration in the mouse (Fafeur, 1997; Macias, 1998; Vlaeminck-Guillem, 2000). Ets genes may be involved in regulating serine protease urokinases (Majka and McGuire, 1997), which may function to remodel ECM, promote cell migration by regulating cell–matrix interactions, and activate growth factors (reviewed by Thery and Stern, 1996). The major question these results raise is how are the signal transduction pathways specifying cell fate related to those specifying events in EMT? Many different cell types undergo very similar EMTs, implying that a regulatory module, or modules, specifying one or the other aspect of EMT, or perhaps multiple events in EMT, is integrated into different cell fate determining pathways. One of these modules may be downstream of Ets signaling. The nature of this integration and its variation from case to case within and between species, should be studied in depth.

3. Ingression of presumptive axial and paraxial mesoderm in amphibians

The amphibians also show a diversity of morphogenic mechanisms, especially in relation to which tissues ingress, and when and where this ingression occurs. We have indicated the specific species in many cases; results should be taken as generalizations otherwise.

3.1. Morphogenesis, morphology and cell biology of superficial mesoderm ingression

Amphibian embryos begin with a portion of their presumptive mesoderm in the superficial epithelial layer, whereas the rest of it originates from deep mesenchymal layers. These cells contribute to the axial (notochordal, hypochordal), paraxial (somitic), and lateral–ventral mesoderm of the tadpole (Bose, 1964; Delarue et al., 1994, 1992; Lofberg and Collazo, 1997; Minsuk and Keller, 1996, 1997; Shook et al., 2002; Vogt, 1929). In anuran amphibians (frogs), these cells ingress from the roof of the gastrocoel (primitive gut cavity) during neurulation (Fig. 3A), whereas in urodele amphibians (salamanders), the presumptive somitic and lateral ventral cells ingress during gastrulation, just inside the blastopore (Fig. 4); their notochordal and hypochordal cells ingress in the second half of neurulation from the gastrocoel roof. These superficial presumptive somitic and notochordal cells of some anurans, such as *Xenopus laevis* and *Ceratophrys ornata*, generally (Lundmark, 1986; Purcell and Keller, 1993; Shook et al., 2002) undergo EMT by constricting their apices, elongating along the apical–basal axis, and then ingressing (Fig. 3B–D). The epithelium maintains continuity and the constriction of the apices probably pulls the cells toward the zone of ingression (Fig. 3B–D), but other factors may also be involved. In other anurans, the presumptive somitic mesoderm undergoes EMT and internalizes by a process called 'relamination' (Minsuk and Keller, 1996; Shook et al., 2002) (Fig. 3E–G). The basal ends of the presumptive somitic cells become integrated into and appear to join the deep, mesenchymal somitic cells (Fig. 3E,F). At this point, the lateral endodermal cells appear to move across the relaminated superficially derived presumptive somitic cells (Fig. 3F,G). How relamination occurs is not known. One possibility is that the apical membrane of the relaminated presumptive somitic cells loses its epithelial character and becomes mesenchymal-like (i.e. adhesive)

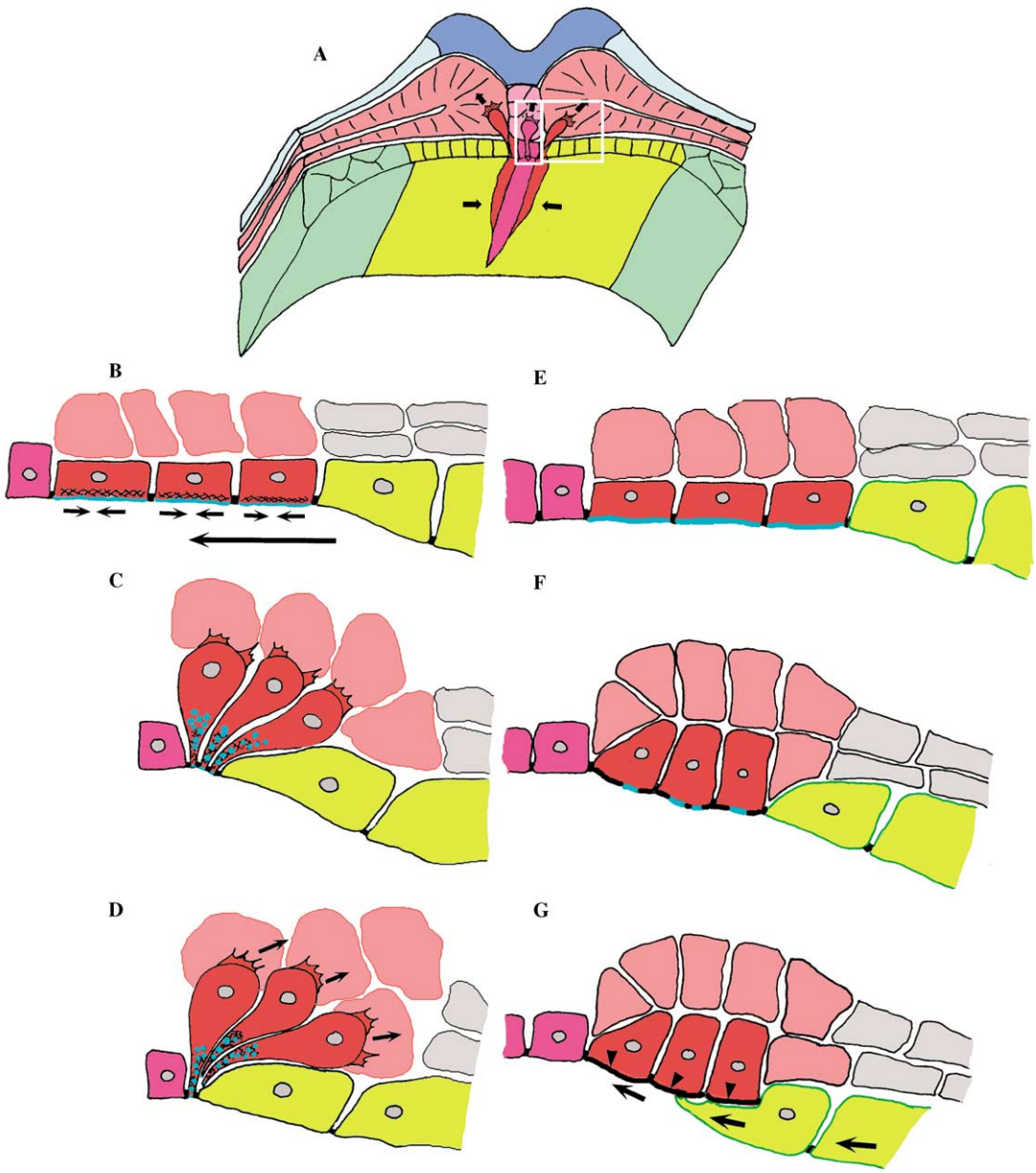

Fig. 3. Different modes of EMT occur during removal of presumptive mesoderm from the gastrocoel roof of amphibians (A). Presumptive endoderm is shown in yellow, deep, mesenchymal presumptive somitic mesoderm in light red, and superficial, epithelial presumptive somitic mesoderm in dark red. In the anurans *Xenopus laevis* and *Ceratophrys ornata*, the epithelial prospective somitic cells (dark red) undergo apical constriction, become bottle-shaped, and ingress to join the deep somitic mesoderm (light red) (cross-sectional view, B–D). In *Hymenochirus boettgeri*, an anuran closely related to *Xenopus*, the epithelial prospective somitic cells are internalized by a process called 'relamination' (sectional views, E,F). The epithelial presumptive somitic cells associate with the deep somitic mesoderm at their basal–lateral aspect and become part of the deep somitic tissue without leaving the epithelium (E,F). The endoderm cells (yellow) lateral to the somitic cells then somehow move across their apical surface and cover them over, making them part of the deep region where they become mesenchymal (F,G). This may involve remodeling of the apical membrane of the superficial somitic cells, in place, with out apical constriction, from a non-adhesive epithelial phenotype (blue, E) to an adhesive deep, mesenchymal cell phenotype (black, F,G). The superficial notochordal cells of *Xenopus* ingress as bottle cells to join the deep notochordal component, similar to the behavior of the epithelial somitic cells (B–D).

prior to ingression, and therefore the endodermal cells see it as a substrate and migrate across it (Fig. 3F,G).

After gastrulation, at least part of the presumptive notochord, the amount varying with the species, is present in the superficial epithelial layer of the gastrocoel roof (Fig. 3). The superficial presumptive notochordal mesoderm of the anurans, *Xenopus* and *Ceratophrys*, undergoes EMT in the same manner as the superficial presumptive somitic mesoderm; that is, they undergo apical constriction, form bottle cells, and ingress. The presumptive notochordal mesoderm of *Ambystoma*, a urodele amphibian, undergoes apical constriction but it is not clear whether all of these cells ingress directly, or if some of them are first covered by the adjacent endoderm, and then de-epithelialize. Little more is known about this process and it is long overdue for more study.

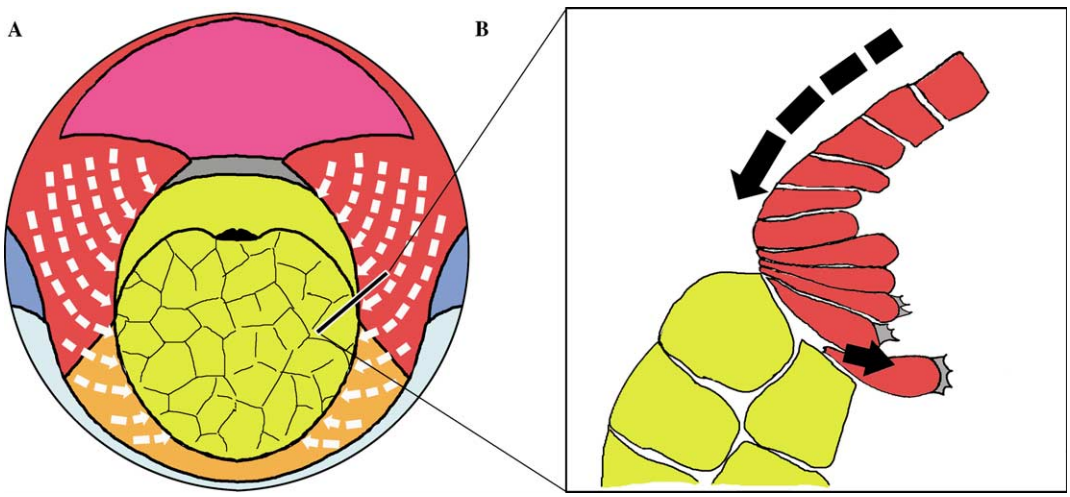

Fig. 4. The urodeles, *Ambystoma mexicanum, A. maculatum, and Taricha torosus* have large areas of superficial presumptive somitic (red) and lateroventral mesoderm (orange) in the early gastrula (A), which is removed just inside the blastopore, next to the endoderm (yellow), during gastrulation by EMT and ingression. At the vegetal edge of the somitic area, the presumptive somitic cells undergo apical constriction, de-epithelialize, and ingress (cross section shown in B); as these cells undergo apical constriction and ingression, the remaining epithelial cells move vegetally and sequential undergo these processes (white arrows, A; black arrows, B). Eventually, the lateral edge of the presumptive notochord (magenta, A) is pulled into apposition with the endoderm (yellow, A).

Ingressing cells, particularly those that ingress as bottle cells, appear to endocytose much of their apical membrane (Lofberg, 1974; Shook et al., 2002; Shook, unpublished data). Whether cadherins or other elements of adherens junctions or tight junctions are also being endocytosed is unresolved. Cingulin, an intracellular component of tight junctions, is expressed at a uniform level per apical circumference of all cells that are in the region of ingression in *Ambystoma*, suggesting that tight junctions are intact through the point of ingression (Shook et al., 2002). Cingulin is withdrawn down the neck of ingressing bottle cells, but whether this reflects endocytosis of excess tight junction components, simple basally directed transport of cingulin, or a lengthening of the tight junction zone along the neck of the ingressing cells is not known.

3.2. Somitic and lateral–ventral mesoderm of the urodele undergo progressive, localized EMT at a restricted zone, a bilateral, amphibian 'primitive streak'

The urodeles *Ambystoma mexicanum, A. maculatum and Taricha granulosa*, have a massive amount of presumptive somitic and lateralventral mesodermal cells in the marginal zone of the early gastrula, and they are internalized by ingressing at a localized site adjacent to the presumptive endoderm, just inside the blastopore, known as the subduction zone (Shook et al., 2002) (Fig. 4A,B). The events occurring in the subduction zone are very similar to those seen in the primitive streak of amniotes, discussed below. The presumptive mesodermal cells in the epithelial layer undergo apical constriction next to the presumptive endoderm, and as they do so, the remaining presumptive mesoderm moves, or is pulled, toward the presumptive endoderm as an epithelial sheet, probably by virtue of the apical constriction (dashed arrow, Fig. 4B). The apically constricted cells then ingress, and leave the epithelium adjacent to the endodermal cells (arrow Fig. 4B). As successive mesodermal cells approach the subduction zone, they too undergo apical constriction and ingress adjacent to the epithelial endoderm. The apices of the ingressing cells constrict to a greater or lesser degree as they approach the subduction zone, but with few exceptions, remain integrated into the epithelium as they approach the endoderm and ingress. As presumptive mesodermal cells ingress, the presumptive endodermal and mesodermal cells on either side must somehow form a new epithelial seal, as the continuity of the epithelium does not appear to be broken.

In explants of the superficial presumptive mesoderm in *Ambystoma*, in which cells are prevented from ingressing and are separated from the tissue adjacent to which they normally ingress, the cells will sequentially apically constrict and de-epithelialize, in the same temporal and spatial pattern as in intact embryos, although about 2 h later than they would ingress in intact siblings (Shook et al., 2002). Therefore they appear to either be pre-programmed for sequential ingression, or they are able to organize their collective behavior that way without outside influence. Ingression in the intact embryo and de-epithelialization in explants both occur progressively, such that the cells that are, or would be, closest to the endoderm express these behaviors sequentially, as shown (Fig. 5A–C,J–M). The apparent integrity of the subducting presumptive mesodermal epithelium and the continuous expression of cingulin around the apices of cells about to ingress, suggests that de-epithelialization may occur only after ingression has begun; that is, circumferential junctions are maintained even as the apex is pulled below the surface, and the remaining epithelial cells bridge over it (Shook et al., 2002).

Clearly, however, the ingressing cells must go through some change that encourages at least the adjacent endoderm to seek to form new junctions with cells beyond the ingressing cell.

3.3. Maintaining mechanical continuity during ingression

The behavior of epithelially derived presumptive somitic cells in the urodele suggests some mechanisms that allow ingression of large areas of cells without disrupting the epithelial layer. First, all the cells fated to ingress do not undergo EMT at once, but in sequential order, in a localized zone, thereby minimizing the area over which the ingression is occurring. Second, their behavior suggests that the properties of the cells are tightly regulated in space and time, as follows, such that cells are drawn into the zone of EMT and then undergo EMT and ingression while maintaining continuity of the sheet. Cells far from the subduction zone are fated to become mesoderm and to ingress but have not yet begun apical constriction (gray cells, #3–5, Fig. 5A); nevertheless, they are drawn toward the subduction zone (dashed arrow, Fig. 5A) by apical constriction of a limited number of cells, in this case two cells (cells #1 and #2, Fig. 5A), the first of which is nearing completion of apical constriction and is about to ingress. However, the apically constricting cells maintain their apical junctional adhesions with one another, with the endodermal cell, and with the cells not yet undergoing apical constriction (Fig. 5A), and thereby maintain the observed mechanical and physiological continuity. The linkage of the cells also assures that apical constriction will be a prime force in bringing cells toward the subduction zone (dashed arrow, Fig. 5A). Finally, the apically constricted cell next to the endoderm enters a new state and loses its adhesion to all of its neighbors and begins ingression (cell #1, Fig. 5B). Just after, during, or immediately preceding this event, the endodermal cell and the next apically constricting cell in the sequence must make adhesions in order to maintain continuity. The first possibility is that the ingressing cell turns loose first, and immediately thereafter, its former neighbors, the endodermal cell and the next apically constricting cell form junctions and heal the opening (Fig. 5C). During apical constriction, the junctional perimeter is dramatically reduced (Fig. 5D,E), and when the ingressing cell detaches (Fig. 5F), there is only a small wound to cover over (Fig. 5G–I). The second possibility is apical protrusions of the cells on one or more sides bridge above the ingressing cell and form a new junctional complex above the ingressing cell before it detaches (Fig. 5J), and only then does the ingressing cell detach and ingress (Fig. 5K). There is evidence for this sort of bridging in amniotes, discussed below, and it has the potential of maintaining junctional continuity through the process of ingression. However, as we discuss below, this is a more complicated mechanism than first appears in sectional view. In any case, the endodermal cell and the mesodermal cell just beyond the ingressing cell must be able to make junctions, despite the fact that in a few moments, this mesodermal cell will also enter the non-junctional state (Fig. 5K–M). The effect of not having a carefully progressive, sequential order of change in junctional capacity and adhesion is illustrated by imagining that greater numbers of cells simultaneously enter the ingressing state; if, for example, cells #1–5, would simultaneously de-epithelialize, a large wound would form, and physiological and mechanical continuity would be destroyed. The deep mesenchymal cells would be exposed to low osmolarity pond water and resulting in swelling and probably lysis of the deep cells (see Holtfreter, 1943). Mechanical integrity of the sheet is also probably necessary, as it seems likely that the tension generated in the epithelial sheet by apical constriction and ingression functions in convergence and blastopore closure (see Keller et al., 2003; Shook et al., 2002).

The bridging protrusions, however, must form junctions that are integrated into the existing apical junctional complex to form a continuous seal. As constriction reduces the apical area of the ingressing cell, small protrusions could indeed bridge over it, forming new junctions where they meet one another, and zipping up to close the channel above the soon-ingressing cell (Fig. 5N–P). However, viewed in 3D perspective, the forming protrusions must either build the junctional contacts with adjacent protrusions from their origin (black arrows, Fig. 5Q), or extend the protrusions, form the junctional complex at contacts with other protrusions, and then extend the junction back toward the original junctional seal between the cells (white arrows, Fig. 5Q). Regardless of whether or not these mechanisms are used in amphibians or in amniotes (see Section 5.3.), leakage does appear to occur as electric currents can be measured at the blastopore of amphibians (Hotary and Robinson, 1994; Metcalf et al., 1994) and at the chick primitive streak (Jaffe and Stern, 1979).

3.4. Do the 'epitheloid' cells of the deep involuting marginal zone of Xenopus undergo a type of EMT during involution?

The deep region of the involuting marginal zone (IMZ) of the anuran amphibian, *Xenopus laevis*, shows epitheloid characteristics although it clearly is not a tight-junctioned epithelium, as is the superficial, truly epithelial layer above it. The behavior of this layer raises questions about the definition of the epitheloid state of organization, and whether the behavior of these layers represents an EMT or not. Beginning early in embryonic cleavage, at stage 7, the superficial epithelial cells of *Xenopus*, which are bound circumferentially at their apices with tight and adherens junctions, undergo radial cell divisions, giving rise to a layer of deep cells, with no apical domain (Chalmers et al., 2003). Derivatives of these cells will give rise to the deep presumptive mesodermal cells in the IMZ, the deep cells of the double layered neural plate, and the deep layer of

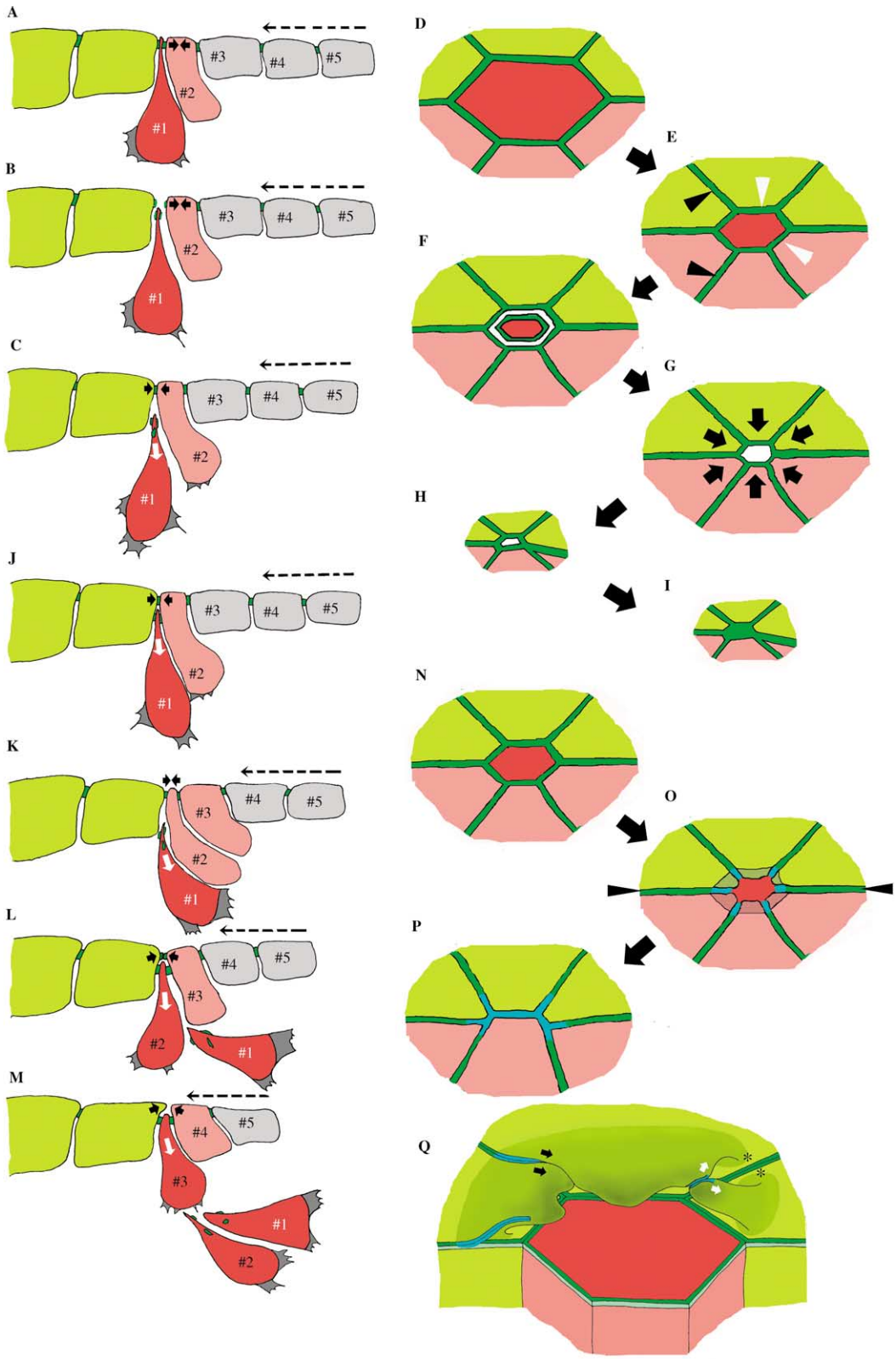

Fig. 5. An example from the ingression of presumptive somitic cells of the urodele amphibian gastrula illustrates that EMT and ingression of cells can generate a pulling force or tension that can pull the epithelial sheet toward the site of EMT and ingression while maintaining epithelial integrity. The presumptive endoderm is shown in yellow, the ingressing presumptive somitic cells in dark red, deep presumptive somitic cells in light red, and those being towed toward the ingression zone in gray. The endoderm cells serve as an anchor and a boundary next to which most presumptive somitic cells ingress (A). An ingressing cell (#1, A) is about to leave the epithelium, and the next cell to ingress is undergoing apical constriction (#2, arrows, A). As it does so, it pulls the remaining epithelial somitic cells (gray, #3–5) toward the ingression zone (dashed arrow, A). The process of ingression could occur in two ways. In the first, the apical

the epidermis. We have generally considered these cells mesenchymal in nature because they are connected to one another by localized adhesions at the ends of filiform and lamelliform protrusions, and do not have circumferential adhesions or free apices. However, they perhaps could be considered epitheloid in that they are part of a multi-layered epithelium with a true tight-junctioned epithelial layer on one side and a fibronectin-rich matrix beneath them. Moreover, they express cytokeratin intermediate filament protein, generally considered to be an epithelial marker, albeit less than the superficial layer (Klymkowsky et al., 1992). On the other hand, vimentin, usually characteristic of mesenchymal cells, does not appear until neural tube closure although a vimentin-like protein does appear in the early neurula (Dent et al., 1989; Herrmann et al., 1989; Torpey et al., 1990). During epiboly of the animal cap, and during the initial extension and thinning of the IMZ, these cells actively intercalate radially to form a thinner array of greater area, prior to involution (Keller, 1980; Marsden and DeSimone, 2001). And after involution some of these cells will migrate (Davidson et al., 2002a,b; Winklbauer and Keller, 1996; Winklbauer et al., 1996), while others will undergo mediolaterally polarized motility and cell intercalation, during convergence and extension of the mesodermal and neural tissues (see Keller, 2002; Keller et al., 2000). Interestingly, this polarized behavior is in part controlled by components of the PCP pathway (reviewed by Keller, 2002), which was originally described as operating in epithelial systems in *Drosophila* (reviewed by Adler, 2002).

As the deep mesodermal cells of the IMZ involute, they interact differently in regard to the fibronectin-rich mat of ECM under the pre-involution layer and the pre-involution cells themselves; they no longer integrate themselves into the pre-involution array but spread on and migrate on its inner surface (Wacker et al., 2000; Winklbauer and Keller, 1996; Winklbauer et al., 1996; Winklbauer and Schuerfeld, 1999). These behavioral changes are accompanied by cytoskeletal changes (Selchow and Winklbauer, 1997). Thus, this population of cells is undergoing a transition similar to that seen in stereotypical EMTs, except that they lack the initial apical–basal membrane differentiation and circumapical junctions of true epithelial cells.

At least a sub-population of the presumptive deep mesodermal cells expresses Snail, a transcription factor, throughout gastrulation (Linker, 2000, p. 2115; Aybar et al., 2003). Snail is commonly associated with developmental EMT events in many other systems (e.g. Aybar et al., 2003; Carver et al., 2001; Ciruna and Rossant, 2001; LaBonne and Bronner-Fraser, 2000; Oda et al., 1998), and while it is sometimes simply associated with mesoderm formation, its ancestral function is thought to be in directing developmental EMTs (Lespinet et al., 2002, and references therein), suggesting that it might be playing a similar role in involuting deep presumptive mesoderm in *Xenopus*.

3.5. Molecular control of mesodermal EMT in amphibian gastrulation

There are some examples of molecular determinants that will induce ingression but neither the underlying mechanism nor the relationship of this mechanism to normal ingression are understood. Ectopic expression of the homeobox-containing gene Xanf-1 in the ventral marginal zone (VMZ) causes bottle cell formation, and the formation of a secondary axis (Zaraisky et al., 1995). Over-expression of Xanf-1 in the dorsal marginal zone causes massive ingression of cells at early gastrula stage; these cells joined head mesoderm and endoderm. Similar results were obtained when goosecoid RNA was injected into animal cap blastomeres, it also caused ectopic ingression (Blumberg et al., 1991; Niehrs et al., 1993). But it did not induce a second axis or any additional mesodermal marker expression there (Xanf1 and goosecoid are mesendoderm markers themselves, and perhaps determinants). This suggests that goosecoid might induce the cell behavior independent of the cell type. Ingression may thus be a side product of inducing a migratory cell type.

4. Ingression from the amniote epiblast: the chick

Cells ingress from the chick epiblast in two stages. First, scattered cells throughout the epiblast ingress to form the primary hypoblast immediately below the epiblast (Eyal-Giladi and Kochav, 1976; Weinberger et al., 1984). These cells give rise to extra-embryonic tissues. Second, cells within the primitive streak go through EMT and ingress to form the endodermal and mesodermal tissues of the embryo (Bellairs, 1986).

junctions break, leaving a gap in the epithelium (B), which is immediately healed (C). But epithelial continuity is maintained; apical constriction reduces the size of the potential wound (D–F), which can be healed easily (G–I). In the second mechanism, as the apex of the ingressing cell begins to drop below the surface, the endoderm cell and the newly constricting somitic cell bridge above the ingressing cell and form a junctional complex, which maintains mechanical continuity (J). The ingressing cell then detaches and ingresses (K), and the process repeats itself progressively (L,M). A surface view of an ingressing cell provides another view of mechanical contiguity (N–P). As the apical region constricts, bringing the boundaries of the endodermal and mesodermal cells surrounding the ingressing cell into proximity, protrusions are extended across the ingressing cell (shaded, O), and they contact one another to form a second layer of junctions above the ingressing cell (P, and in sectional view, J). Note that a three-dimensional view shows that in order to maintain a continuous seal, the second apical junctional array would have to extend from the original (black arrows, Q), or extend from sites of formation on the protrusions toward the original array (white arrows, Q).

4.1. Hypoblast polyingression

Polyingression, as the scattered ingression of cells to form the primary hypoblast is called, begins posteriorly and progresses anteriorly (Bancroft and Bellairs, 1974; Harrisson et al., 1991; Weinberger et al., 1984). A basal lamina is present below the epiblast by the time of laying, which is before the formation of the streak but while polyingression is still going on, and ingression occurs through this basal lamina (Sanders, 1979). It is interrupted by a large number of blebs in the region of polyingression (Harrisson et al., 1991), and rounded holes are found in the ventral surface of the epiblast, with hypoblast cells extending processes through the holes into the epiblast layer (Lawson and Schoenwolf, 2001b). Some polyingression may continue from the portion of the epiblast anterior to the tip of the primitive streak even as cells begin to ingress through the streak (Harrisson et al., 1991). Cells ingress as bottle cells, which apparently have active apical contraction, judging from the indented 'crypts' they sometimes produce in the epiblast (Bancroft and Bellairs, 1974; Weinberger and Brick, 1982; Weinberger et al., 1984).

Although the polyingression of hypoblast cells is progressive from posterior to anterior, it is unlike the highly sequential ingression in the urodele amphibian, discussed above, and in the primitive streak, discussed next, in that the cells are scattered and it appears that most ingress as individuals, from a surrounding intact epithelium. Again, there is no large area of cells undergoing EMT at the same time, and thereby generating a large wound in the epithelium.

4.2. Primitive streak morphogenesis

The embryonic endoderm and mesoderm, as well as some extra-embryonic mesoderm is formed by ingression of epiblast cells through the primitive streak, which forms from the posterior margin of the epiblast and elongates anteriorly (see Lawson and Schoenwolf, 2001a). The streak is composed of cells extending (Lawson and Schoenwolf, 2001a) and growing by polarized division (Wei and Mikawa, 2000) from the posterior margin of the blastoderm. Cells anterior to the streak are also incorporated into the streak as it elongates (Lawson and Schoenwolf, 2001a). Cells of the presumptive endoderm and of the extra-embryonic, lateral, and somitic mesoderm ingress through the streak from the epiblast. Epiblast cells forming these tissues tend to express HNK-1 (Stern and Canning, 1990), a carbohydrate antigen that serves as a marker for cells in the primitive streak (Canning and Stern, 1988) and neural crest (Bronner-Fraser, 1987). The endoderm cells are inserted into the hypoblast, pushing it from beneath the forming embryonic body (Lawson and Schoenwolf, 2003), and the mesoderm cells form the mesoblast between the endoderm and the epiblast (Fig. 6) (Bellairs, 1986). As the cells within the streak ingress, they are replaced by cells lateral to the primitive streak that move medially into it (Lawson and Schoenwolf, 2001a). Most, but not all the cells within

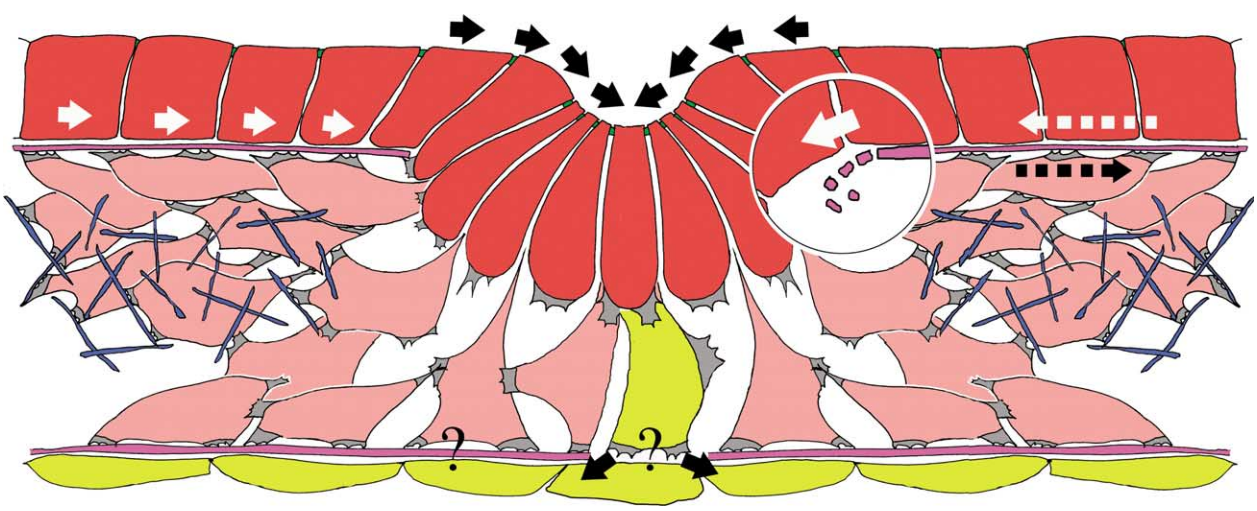

Fig. 6. A diagram shows possible force-generating processes during EMT and ingression of prospective mesodermal cells from the epiblast during chick gastrulation. A progressive process of apical constriction and removal of cells by ingression, while maintaining mechanical continuity, pulls the lateral epiblast cells medially (black arrows) where they then also sequentially undergo EMT and ingression. In this mechanism, the basal lamina at the basal sides of the cells (magenta) would probably be towed along passively toward the midline with the movement of the epiblast cells, where a localized breakdown from the medial end (inset circle, right). Alternatively, the basal surfaces of the epiblast cells might actually crawl on the basal lamina and leave it behind (white arrows, left), in which case it would not be broken down except at the onset of ingression at the basal surface of the epiblast in the primitive streak region. Another possibility is that the ingressed cells form a cohesive stream of cells that exert traction on the underside of the basal lamina (black dashed arrow, right side), thus pulling it and the overlying epiblast medially (white dashed arrow, right side). Other possibilities include the freshly ingressed cells moving through a three-dimensional matrix (blue) or on the upper surface of the endoderm (yellow) or any matrix on its surface (magenta, ?). The process by which the earlier ingressing endodermal cells (yellow), undergo EMT, enter into the endodermal epithelial layer (yellow) and re-epithelialize is not well understood (black arrows, ?).

the primitive streak ingress as they move into it (Lawson and Schoenwolf, 2001b); those that do not ingress (or substantially delay ingression) may be cells that have been inappropriately moved into the streak, or may be cells that have some patterning effect on ingressing cells. Interestingly, midline cells in the streak appear to die, and this phenomenon is essential for left–right patterning (Kelly et al., 2002). This provocative report raises many questions about the dynamic, micropatterning of cells within an EMT/ingression zone, an issue that deserves further attention. Cells continue to ingress through the streak as it regresses. Most but not all ingressed cells migrate away from the primitive streak ipsilaterally (Levy and Khaner, 1998), perhaps in response to repulsive FGF signals from the primitive streak itself (Yang et al., 2002). Because the presumptive fates of cells in epiblast overlap substantially, it is clear that there is some cell mixing that occurs as cells migrate toward, through and away from the streak, but it is not clear at what point this mixing occurs (Hatada and Stern, 1994; Lawson et al., 1991).

4.3. Morphology and behavior of ingressing cells

Epiblast cells prior to ingression are closely packed and columnar (Revel et al., 1973). They progressively become elongated from lateral to medial in the primitive streak, with bottle cells found predominately in the medial region (Lawson and Schoenwolf, 2001b). The bottle cells loose their regular, single-layered columnar epithelial organization and begin to show extensive protrusive activity, both apically and basally, based on morphological studies (Balinsky and Walther, 1961), while underneath them the basal lamina breaks down. Related to this apical protrusive activity, adjacent cells eventually cover the apical ends of ingressing bottle cells. In electron micrographs, protrusions reaching over cell junctions to contact the apical surface of the adjacent cell form apical 'vacuoles' bridging across ingressing cells (Balinsky and Walther, 1961). These observations offer the strongest support for the mechanism of bridging across ingressing cells (Fig. 5). However, the three-dimensional aspect of this process has not been described, and exactly how cells might accomplish this feat is unknown, especially how the cells might integrate new, supra-apical junctions with previous ones (Fig. 5Q). Live imaging and correlated serial electron microscopy should be done to confirm these impressions from static evidence. The apices of cells near the primitive streak show few microvilli, while those more peripheral show numerous microvilli (Bancroft and Bellairs, 1975); this is converse to the situation in amphibians where the apically constricted bottle cells have progressively more microvilli or microfolds on their surfaces. Like the cells undergoing polyingression, those in the primitive streak have strong 'blebbing' activity at their basal surfaces (the basal aspect of the epiblast); these blebs are generally correlated with the disappearance of the basal lamina underneath the cell, and thus are thought to presage ingression (Harrisson et al., 1991; Vakaet, 1984). It has been suggested that these blebs are involved in convergence of cells toward the primitive streak and cell shape changes prior to de-epithelialization (Vakaet, 1984), discussed below. The basal ends of the bottle cells within the streak have filopodial protrusions which contact subjacent, already ingressed mesenchymal cells (Balinsky and Walther, 1961; Solursh and Revel, 1978).

The bottle cells appear to form by apical constriction. In electron micrographs there appears to be a denser, putative 'contractile' layer at the apical end of the bottle-shaped cells at the level of cell junctions (Balinsky and Walther, 1961). Curiously, these apices bulge above the level of the cell junctions, suggesting that the contractile apparatus is connected around the circumference of the apices, but not to their faces (Balinsky and Walther, 1961). These bottle cells are thought to form by apical constriction and produce an amphibian-type local invagination (see Hardin and Keller, 1988; Vogt, 1929) in the primitive streak (the primitive groove), making the primitive streak analogous to the amphibian blastopore (Balinsky and Walther, 1961). Like the ingressing echinoderm and amphibian cells, the ingressing epiblast cells appear to endocytose apical material. Endocytosis vacuoles in cells ingressing from the primitive streak appear to be the product of 'pinocytosis', perhaps representing endocytosis of the apical membrane (Balinsky and Walther, 1961). The pinocytosis seen in chick is similar to that found in the frog (Balinsky, 1961).

4.4. Remodeling of junctions

Ingressing primitive streak cells appear to maintain most of their junctions right up to the point of ingression and show fragments of some on post-ingression mesenchyme cells. At pre-streak stages, epiblast cells have extensive tight junctions (Bellairs et al., 1975), but no desmosomes (Bellairs et al., 1975; Sanders, 1973). After streak formation the epiblast has good tight junctions consisting of multiple, anastomosing 'strands' circumapically (Revel et al., 1973) and gap junctions (Bellairs et al., 1975); desmosomes are found peripherally, but not centrally, in or near the streak (Overton, 1962). Otherwise, cell–cell contacts at the apices of the streak cells are similar to those in the epiblast outside the streak; there are no intercellular gaps between the cells, and tight junctions are still present (Balinsky and Walther, 1961). Junctions in Hensen's node are similar but the tight junctions appear as misplaced or fragmented sections of the multi-strand organization seen in the epiblast, or as single to a few interconnected strands, and they do not form a complete belt around the cell. Gap junctions in the node are smaller, perhaps not as well organized. Underlying mesenchyme cells have both tight and gap junctions on their surfaces, and 'isolated islands' or individual strands of tight junction appear on the cell body and the gap junctions appear on both the cell body and filopodia (Revel et al.,

1973). There is no evidence for endocytosed tight junctions, as there is for desmosomes (Burdett, 1993; Overton, 1968); it appears that as cells become bottle-shaped, their tight junctions become disrupted and as they ingress, bits of tight junction still link cells together (Revel et al., 1973).

4.5. Changes in the basal lamina

Fibronectin (Sanders, 1982) and laminin (Zagris et al., 2000) are found in the basal lamina before the appearance of the primitive streak (Lawson and Schoenwolf, 2001b). With the appearance of the streak, however, these glycoproteins become depleted and the basal lamina appears disrupted, as seen by SEM, in the region of the primitive streak but not elsewhere (Lawson and Schoenwolf, 2001b).

Primitive streak cells can break down the basal lamina and can continue to do so after the initial formation of the streak and the initial lesion in the basal lamina; bits of exogenous primitive streak transplanted ectopically between the hypoblast and the native primitive streak disrupt the basal lamina (Sanders and Prasad, 1986). This capacity is maintained by the ingressed mesenchymal cells, which invade and disrupt basal lamina matrix (Matrigel) in culture, whereas epiblast cells immediately adjacent to the primitive streak, and hence presumably fated to ingress, but not yet ingressing, penetrated the gel as thin tongues of cells without disrupting it (Sanders and Prasad, 1989a). Therefore EMT in the primitive streak seems to involve capacity to disrupt matrix and show invasive behavior, along with apical constriction, junctional remodeling and other aspects of EMT. Plasminogen activator, thought to be involved in digesting matrix and allowing invasion (e.g. into Matrigel) (Erickson and Isseroff, 1989; Marotti et al., 1982; Valinsky and Le Douarin, 1985), is not active in the primitive streak after its formation (Sanders and Prasad, 1989b); it is not known whether it is active prior to this time.

Removing chondroitin sulfate from the basal lamina of the chick epiblast using a sub-lethal dose of chondroitinase results in ectopic sites of ingression and other disruptions of normally ordered primitive streak formation, and sometimes even additional primitive streak-like structures (Canning et al., 2000). One interpretation of this result is that chondroitin sulfate stabilizes the epiblast cell behavior in the non-ingressing mode, although one always worries about specificity of activity in enzyme experiments.

The basal lamina could be static and thus only need to be disrupted once, by the cells initially forming the streak at the onset of ingression (left side, Fig. 6), or it could move medially with the epiblast cells approaching the streak, and be disrupted by each epiblast cell prior to its ingression (right side, inset, Fig. 6). The evidence suggests that the epiblast cells carry intact basal lamina to the primitive streak with them, and cells within the primitive streak appear to have a continuing ability to disrupt the basal lamina. Based on labeling with the ultrastructural marker concanavalinA-ferritin, Sanders (1984) argued that the underlying basal lamina was moved toward the streak with the epiblast cells, and then degraded in the streak. But if a piece of epiblast with glucosamine-labeled basal lamina is transplanted into another embryo, the labeled basal lamina is found later both underneath and lateral to the final position of the transplanted (and still superficial) epiblast (Bortier et al., 2001; Van Hoof and Harrisson, 1986). The presence of labeled material underneath the transplanted epiblast is consistent with movement of the basal lamina with the epiblast, but its presence lateral to the transplanted piece could mean that the epiblast cells actually move medially on the basal lamina but carry components of it with them. Alternatively, the label could be carried laterally by the underlying, previously ingressed mesodermal cells moving in this direction on the under-surface of the basal lamina. However, label is found lateral to the final position of the transplant, even when transplanted to a region not overlying mesoderm (Bortier et al., 2001), making this interpretation unlikely. The hypoblast may also have some earlier role in shaping the basal lamina, thus further confounding the issue (Harrisson, 1993). The turnover of basal lamina components in regard to the epiblast and mesodermal cells, and the motility of these cells with respect to the basal lamina should be investigated further.

4.6. Post-ingression migration

The ingressed mesodermal cells take on a mesenchymal morphology and migrate laterally to form the mesoderm. Cells immediately below the primitive streak are round and closely packed, but more lateral, the (migrating) cells flatten out (Solursh and Revel, 1978). The ability of ingressed cells to bind fibronectin is required for their migration away from streak, but not for EMT or ingression through the streak (Harrisson et al., 1993). During and after de-epithelialization, hyaluronate is synthesized and accumulates on ingressing cells (Sanders, 1979; Sanders and Prasad, 1986; Solursh et al., 1979). Hyaluronate fills or perhaps generates extracellular spaces, and it has a number of effects on cell motility, including both support and inhibition of migration, in a large number of morphogenic processes in embryogenesis and organogenesis (Toole, 1982, 2001). However, its function in the mesodermal region of the chick is not understood.

4.7. Forces bringing the cells to the streak

Because of the integrity of the epithelial epiblast during apical constriction of the streak cells, it is likely that this event has the morphogenic function of bringing the epiblast cells toward the site of ingression (black arrows, Fig. 6), much as this behavior is thought to do the same thing in urodele amphibians (Fig. 4). In this case, the epiblast cells would be passively displaced and it is likely that the basal lamina would be brought along with the epiblast cells (as illustrated on the right side, white dashed arrow, Fig. 6).

ECM fibrils radiate radially from the chick primitive streak region (England, 1981). This radial organization appears to be the result of tension (Kucera and Monnet-Tschudi, 1987). Part of the tension of apical constriction in the streak and node could be transmitted by this matrix as well as by the cells themselves. The basal surfaces of the epiblast cells might also migrate actively and medially on the basal lamina toward the site of ingression (white arrows, left side, Fig. 6). However, there is not much evidence for this mechanism. Vakaet (1984) suggested that the blebs at the basal ends of the epiblast cells might be involved in convergence of the cells toward the streak. Another possibility is that the ingressed mesoderm cells exert traction on the inner surface of the basal lamina of the epiblast (black, dashed arrow, right side, Fig. 6), and tend to pull it and the attached epiblast medially (white, dashed arrow, Fig. 6), while they pull themselves laterally. In any case, the epiblast cells are moved medially, ingress, and then the majority move laterally on the same side (Levy and Khaner, 1998), describing an involution movement that is likely a result of multiple morphogenic processes, including apical constriction, EMT, ingression, and migration.

The epiblast epithelium maintains its continuity throughout its approach toward the streak, and the cells seem to retain their junctional complex right up to and perhaps past the point of initiating ingression. This argues that the chick, like the urodele amphibian, uses both the progressive patterning of the EMT and ingression processes, such that the cells accomplish these events in roughly serial order, without generating large lesions in mechanical and physiological continuity of the epiblast (Fig. 5). Comparison of the behavior of the urodele and chick EMT and ingression process argues that the primitive streak of the bird and the lateroventral lip of the urodele are essentially functionally equivalent structures and that the urodele, in fact, has a 'bilateral primitive streak' (Lundmark, 1986; Shook et al., 2002).

4.8. Molecular control of EMT in the primitive streak

Hepatocyte growth factor/scatter factor (HGF/SF) induces behavior resembling some aspects of the intrinsic EMT in the chick embryo, but it is not clear that it is the native effector of EMT. HGF/SF is a plasminogen-related growth factor without plasminogen's serine protease activity. It is closely related to HGFl/MSP (HGFlike/macrophage stimulating protein). HGF/SF's receptor is c-met, a tyrosine kinase receptor (Thery et al., 1995), known to mediate EMTs in a number of systems. When applied to chick blastoderm, HGF/SF generates local ectopic structures resembling primitive streaks and/or neural plates (Stern et al., 1990). Local accumulation of cells in the mesodermal layer is often found when HGF was applied, perhaps representing cells ingressed from the induced primitive streak-like structure (Stern et al., 1990). Alternatively, it could be that the HGF source (cells or bead) placed between epiblast and hypoblast de-epithelializes and attracts hypoblast cells, which then induce something like a primitive streak (see Mitrani and Eyal-Giladi, 1981). It is not at all clear why HGF should induce axial structures. In pre-streak stages, HGF/SF mRNA is expressed in the posterior marginal zone (Streit et al., 1995), where the cells that will give rise to the initial primitive streak reside at this stage (Khaner, 1998; Lawson and Schoenwolf, 2001a). Once the primitive streak has formed, HGF/SF mRNA is expressed at the anterior tip of the streak, in Hensen's node (Streit et al., 1995). HGF/SF can induce neural phenotypes in culture (Streit et al., 1995). HGF/SF induces cultured (pre-streak) epiblast cells to down-regulate E-cadherin and up-regulate N-cadherin (DeLuca et al., 1999), a change that normally occurs as these cells ingress through the primitive streak (Edelman et al., 1983; Hatta and Takeichi, 1986). Curiously, more cells from the lateral epiblast responded to HGF/SF by changing from E- to N-cadherin, than did medial epiblast cells, and cultured lateral epiblast cells produce more HGF/SF (by immuno assay) than medial epiblast cells (DeLuca et al., 1999), which is the opposite of what one would expect if high levels of HFG/SF were inducing ingression. In the mouse, primitive streak ingression occurs in the absence of HGF/SF or c-met; mouse embryos null for HGF appear to gastrulate (Uehara et al., 1995).

Activin, FGF, and Wnt-1 are all present in the streak-stage chick embryo, but none of them will produce the E- to N-cadherin switch or myogenesis, both of which are induced by HGF/SF in cultured epiblast of pre-streak embryos (DeLuca et al., 1999). This may be explained if the epiblast cells are not yet competent for some other reason, because activin can produce a full axis if added to intact embryos. Activin applied to the epiblast of intact embryos induces an exogenous primitive streak or, when applied in a scattered pattern, it induces scattered sites of ingression (Cooke et al., 1994; Mitrani et al., 1990; Stern et al., 1995; Ziv et al., 1992), including cells that appear to be ingressing (Cooke et al., 1994). At the very least, activin mimics the induction of the differentiative pathway that leads to ingression and EMT. There is, however, no clear correlation between activin application and the expression of HNK-1, a marker of ingressing cells (Canning and Stern, 1988), in newly formed streaks or sites of ingression. However, HNK-1 expression seems to be limited to cells that are presumptive anterior mesendoderm, a cell type not induced by activin alone (Cooke et al., 1994), suggests that it may not have a direct role in ingression. Antagonizing endogenous activin by ectopic application of follistatin protein causes the partial dissolution of the primitive streak and node, both morphologically and as assayed by loss of expression of molecular markers (Levin, 1998). However, cell ingression through the node following follistatin application is normal, suggesting that it does not depend on the pit-like morphology of the wild-type node.

TGFβ1 is expressed in the epiblast and hypoblast cells under the node of streak stage embryos, and ingressed,

lateral mesoderm cells express TGFβ1 more strongly than overlying epiblast (Sanders and Prasad, 1991). How TGFβ1 is related to EMT is unresolved, but may be related to its ability to induce snail, at least in cultured cells (Peinado et al., 2003). In cultured epiblast and mesoderm cells, TGFβ1 regulates the epithelial–mesenchymal state, the synthesis and deposition of fibronectin and laminin, and the expression of matrix receptors in complex ways depending on the cell type (mesoderm or epiblast) and the substrate (laminin or fibronectin) used for culture (Sanders and Prasad, 1991). This work suggests a complex molecular ecology involving matrix-dependent regulation of growth factor responses, and additional work should be done to determine how this system works in vivo.

5. Ingression through the amniote primitive streak: the mammal (principally the mouse)

Mammals also gastrulate by ingressing cells through a primitive streak, much like the chick (Fig. 6). The time, order and site of cellular ingression through the primitive streak influence the destination of the mesodermal and endodermal cells in the craniocaudal and dorsoventral body axes (Tam et al., 2001). This and the broadly overlapping fate maps of presumptive tissues suggest that cells are not specified for a particular fate prior to ingression but instead are induced during or shortly after ingression. As in the chick, a primitive streak forms from the medial posterior margin of the epiblast, but in the case of the mouse, the epithelial epiblast is curved into a cup, with the apical surface inward. The epiblast is derived from the inner cell mass, a mesenchymal tissue delaminated by polarized, radial divisions from the freshly compacted, epithelial blastomeres (see Sutherland et al., 1990). Thus it is secondarily epithelialized. At full extension of the streak, the node lies at the bottom of the cup and the streak lies up one side of the cylindrical epiblast. As in the chick, cells move to the streak, become bottle-shaped and ingress through the primitive streak and node, penetrate a basal lamina, and migrate away from the streak. At the outset of EMT, the epiblast is underlain by the primitive endoderm, the tissue analogous to the chick primary hypoblast. After undergoing EMT at the node/streak, presumptive endoderm cells are inserted into this primitive endoderm to form the embryonic endoderm, and the mesoderm, as in chicks, migrates laterally between the endoderm and overlying epiblast to form the mesodermal layer.

5.1. Primitive endoderm formation

The primitive endoderm in mammals is derived from the ICM (Matta, 1991). It is unknown whether the primitive endoderm is formed by delamination from the ICM, or by ingression from the epiblast prior to streak formation, as in the formation of the primary hypoblast from the epiblast in the chick. Some cells are thought to move from the anterior pre-streak epiblast directly into the primitive endoderm (Tam and Beddington, 1992), but the mechanism behind this is also unknown (Tam et al., 2001).

5.2. Epiblast morphology and streak morphogenesis

Primitive streak formation and movements of the cells toward and through the streak in the mouse (Tam et al., 1993), rat (Solursh and Revel, 1978) and rabbit (Viebahn et al., 1995) are similar to that in the chick described above. Pre-primitive streak epiblast cells have tight junctions but no gap junctions (Batten and Haar, 1979). The primitive streak first appears as an area of local epithelial disorganization at the posterior margin of the epiblast (Hashimoto and Nakatsuji, 1989; Tam et al., 1993), with a breakdown of the basal lamina and an increase in intracellular space. As the primitive streak forms, there is an increase in the number of cell–cell junctions (Batten and Haar, 1979), until cells within the epiblast are organized tightly into an epithelium by elaborate intercellular junctions (Tam et al., 1993). Cells closer to the primitive streak are more elongated, and bottle cells are found within the streak (Solursh and Revel, 1978; Tam et al., 1993). In the rat, the bottle cells are connected apically to adjacent cells by tight junctions, and at deeper levels by gap junctions (Solursh and Revel, 1978). These shapes suggest that apical constriction probably occurs in the mouse in a fashion similar to the chick. Spaces are seen between the lateral surfaces of cells in the streak; this and blebbing suggest reduced cell adhesion between these cells at deeper levels (Hashimoto and Nakatsuji, 1989), in contrast to the tight association of these cells in the chick. The mouse primitive streak is also much broader than the chick primitive streak, relative to the size of their epiblasts, suggesting that the localization of EMT is perhaps less tightly regulated.

5.3. Vaulting or bridging junctions may bridge across ingressing cells and maintain epithelial continuity

The most direct evidence for the bridging of neighboring cells across ingressing cells (Fig. 5) comes from work in the rabbit. In the rabbit, adherens junctions are found between the protrusions of the apical domains of ingressing cells and adjacent cells, in addition to their usual circumapical zonula adherens junctions (Viebahn et al., 1995). Transient tripartite zonula adherens-type junctions are found apically between ingressing mesoderm cells and two neighboring epiblast cells. The neighbors of the ingressing cell form two sets of junctions. They form a continuous junction, more apically with each other; then just basal to this junction, they form a junction complex with the apex of the ingressing bottle cell, similar to the bridging junctions described in Fig. 5 (see also Enders et al., 1986). This suggests that the primitive streak cells are capable of forming, apical junctional belts at two levels. Thus when the cell actually

ingresses and releases its apical seal with adjacent cells, the epithelial integrity would be uninterrupted because of these 'vaulting' or bridging junctions between the neighboring cells (Viebahn et al., 1995).

5.4. Junctional remodeling and adoption of mesenchymal characters

In the rabbit, ingressed cells show punctate adherens junctions (Viebahn et al., 1995), but these may be desmosomal junctions, connected to intermediate filaments (Garrod and Fleming, 1990). Epiblast cells express desmoplakins and have desmosomal junctions and cytokeratins from some time prior to the formation of the primitive streak (Franke et al., 1982; Jackson et al., 1981). After ingression through the streak, mesenchymal cells express vimentin and have no desmoplakins (Franke et al., 1982).

5.5. The basal lamina is broken down as in birds

Initially there is a 'thin and coarse' basal lamina covering the outside of the epiblast of the pre-streak mouse embryos, and this basal lamina becomes fragmented at the basal surface of the forming streak (Hashimoto and Nakatsuji, 1989). In the rabbit, basal endocytic pits are seen in regions of disrupted basal lamina, suggesting that the ingressing cells are actively removing laminar components (Viebahn et al., 1995). Disruption of the basal lamina and basal cell blebbing are seen at the center of the medial portion of the streak (Hashimoto and Nakatsuji, 1989).

5.6. Ingressed cells migrate laterally

In both mouse and rat, the bottle cells in the streak have lamellipodial as well as filopodial protrusions from their basal ends, which contact subjacent cells (Hashimoto and Nakatsuji, 1989; Solursh and Revel, 1978; Tam et al., 1993). Cells immediately below the primitive streak that have lost their apical connection to adjacent cells in the streak are round and closely packed, forming a streak 2–3 cell layers thick by midstreak stage. More lateral cells flatten out and adopt a stellate, migratory morphology (Solursh and Revel, 1978; Tam et al., 1993). Unlike the chick, where fibronectin seems to be necessary for lateral migration of the ingressed cells, fibronectin does not appear to be required for any aspect of EMT in the mouse, but only in the final differentiation of the tissue. Explanted epiblast cells only adhere well to fibronectin, whereas explanted mesodermal (primitive streak) cells adhere well to fibronectin as well as laminin, type IV collagen and vitronectin (Burdsal et al., 1993). Both fibronectin and α5 integrin-null embryos differentiate notochordal and somitic lineages (George et al., 1993; Georges-Labouesse et al., 1996; Goh et al., 1997), at least a subset of which appear to migrate to the correct location but then fail to differentiate the morphologically mature structure of these tissues. α5 Integrin-null mice have similar but less severe phenotypes, primarily in the posterior somitic tissues (Yang et al., 1993). Presumably the cells are able to migrate using some other component of the basal lamina as a substrate.

5.7. Expression of cadherins and their regulation by snail

Loss of E-cadherin adhesivity is often associated with EMT in cultured cells (Batlle et al., 2000; Cano et al., 2000) as well as with ingression through the primitive streak in the mouse (Ciruna and Rossant, 2001; Damjanov et al., 1986) and the chick (Edelman et al., 1983). When cultured with antibodies that disrupt E-cadherin-mediated adhesion, epiblast cells explanted onto fibronectin undergo EMT and migrate outward, turn off E-cadherin, express vimentin, and change their predominant adhesive interactions from cell–cell to cell–matrix (Burdsal et al., 1993). But turning off E-cadherin expression is apparently not strictly required for cells to ingress through the streak, or even to migrate. Embryos null for the transcription factor Snail, form a mesodermal layer, but the cells appear to retain many epithelial characteristics (apical–basal polarity, adherens junctions), and continue to express E-cadherin mRNA and protein (Carver et al., 2001). Although Snail is known to down-regulate E-cadherin in a number of situations (Batlle et al., 2000; Cano et al., 2000), E-cad levels in mesoderm of Snail-null embryos are lower than in their embryonic ectoderm. Hence some event, other than or in addition to Snail expression, is repressing E-cadherin during ingression. These results suggest that either the decrease in E-cadherin expression (and presumably the weakening of the adherens junctions) is enough to allow ingression in mouse, or that ingression is regulated by something other than, or in addition to, Snail and the loss of E-cadherin. It may be that association with mesenchymal cells is more important than dissociation from epithelial cells in driving ingression. For example, N-cadherin will promote motility in cultured cells, regardless of E-cadherin expression (Nieman et al., 1999). Apparently, cell motility (ingression and migration) is to at least some degree independent of cell de-epithelialization. That is, an aggressive association with and migration into the deep mesenchymal environment might be enough to mechanically tear the cells out of the epithelium without undergoing all the steps in de-epithelialization. It will be very interesting to find out what other cellular changes usually associated with EMT do or do not take place in Snail-null embryos.

In this regard, the contemporary mouse literature often states that mutant or otherwise experimentally manipulated cells have gone through an EMT if they are found beneath the streak (e.g. Russ et al., 2000; Sun et al., 1999), but it is often not clear how many of the steps of an EMT these cells have accomplished in experimental situations where the composite of what normally comprises EMT may be separated. In some cases the cells ingress and/or round up, but as they are not migrating away from the streak,

the apparently have not adopted the normal mesenchymal phenotype. In many cases they have not lost all their epithelial markers (e.g. E-cadherin, Carver et al., 2001; Ciruna and Rossant, 2001). More work should be done to characterize the expression of junctional components and the structure of tight and adherens junctions, as well as others, in the cells beneath the streak in order to better define their ambiguous state of EMT.

5.8. Regulation of primitive streak ingression by FGF and FGF receptors

FGFR1 influences EMT and morphogenesis of mesoderm at the primitive streak by controlling Snail, and thus, E-cadherin expression (Ciruna and Rossant, 2001). But whereas cells null for Snail are able to migrate away from the streak and form axial and paraxial mesodermal tissues, FGFR1-null cells tend to accumulate in the primitive streak and fail to traverse it. Instead they remain as a columnar epithelium, apparently fail to undergo EMT, and are deficient in contributing to paraxial mesodermal tissues (Deng et al., 1994; Yamaguchi et al., 1994). In embryos chimeric for WT and FGFR1-null cells, FGFR1-null cells are under-represented in endodermal and mesodermal tissues (Ciruna et al., 1997). When WT epiblast and primitive streak cells are cultured on fibronectin, epiblast cells stay put, whereas primitive streak cells migrate away (Burdsal et al., 1993). FGFR1-null primitive streak cells migrate as well as wild type cells on fibronectin, indicating that this is not the reason for their failure to migrate away from the streak. E-cadherin is normally down-regulated in cells lying under the primitive streak and not expressed in mesoderm (Ciruna and Rossant, 2001; Damjanov et al., 1986), but in FGFR1-null embryos, E-cadherin expressing cells are seen under the primitive streak (Ciruna and Rossant, 2001), suggesting that FGFR1 is responsible for down-regulating E-cadherin. This probably occurs through down-regulation of Snail; Snail expression is reduced in FGFR1-null embryos, suggesting that FGFR1 represses E-cadherin by turning on Snail. Since Snail expression is known to promote EMT in cultured cells (Batlle et al., 2000; Cano et al., 2000), this could explain the failure of cells to ingress through the primitive streak. Regulation of Snail and thus E-cadherin, however, is probably only a small part of FGFR1's function; it may act more generally in regulating paraxial and posterior mesoderm specification and morphogenesis. (Ciruna et al., 1997). Thus in FGFR1-null embryos, cells normally fated to form paraxial mesoderm may simply fail to ingress through the streak because they are no longer differentiating on a mesodermal pathway.

In FGF8-null embryos, cells apparently ingress (move beneath the superficial epiblast), but do not migrate away from the primitive streak (Sun et al., 1999). It is not known whether these cells are competent to migrate. FGF8-null embryos also fail to express FGF4 in the streak. In the absence of both FGF8 and FGF4, epiblast cells move into the streak and undergo what the authors consider an EMT, but most cells then fail to move away from the streak and no embryonic mesoderm- or endoderm-derived tissues develop, although extra-embryonic tissues form (Sun et al., 1999). FGF-8 is potentially a repulsive signal that drives migration out of the streak (Yang et al., 2002), which might explain the failure of FGF and FGFR mutants to migrate away from the streak, even though at least FGFR mutants are still competent to migrate in culture, whereas Snail mutants are able to migrate away from the streak.

5.9. Control of EMT by Brachyury

In embryos null for Brachyury, cells tend to accumulate in and ventral to the streak (Wilson et al., 1995, 1993), suggesting that they are less efficient than normal cells at ingression through or migration away from the streak. This could be due to a failure to correctly modulate the expression of adhesion molecules, from cell–cell to cell–substrate based adhesion. If cells from the primitive streak of a Brachyury-null embryo are cultured on ECM substrate, they move moderately but significantly slower than wild type cells (about 60–80% the wild type speed) (Hashimoto et al., 1987), but this alone does not seem enough to explain their accumulation in the streak. Brachyury is required for normal posterior axial and paraxial mesodermal differentiation (Wilson and Beddington, 1997), and that failure to turn on the appropriate differentiative pathway in Brachyury-null cells probably prevents them from joining the respective tissue due to failure to express some adhesion molecule (e.g. axial protocadherin for the notochord), or failure to respond to some other signals that normally direct them to join these tissues. There is a population of cells in the streak, and especially in the node, that do not show the normal time-course of ingression but instead remain within the streak or node for extended periods of time (Nicolas et al., 1996; Tam and Beddington, 1987; Wilson and Beddington, 1996). This suggests that passage through the node or streak is a regulated event rather than a simple stochastic mechanical event. Transit time in the streak may be regulated by different levels of Brachyury. Expression of Brachyury within the node is variable, and cells expressing high levels of Brachyury tend to leave the streak earlier than normal (Wilson and Beddington, 1997). The fact that cells can migrate out of the streak into the anterior but not posterior mesoderm and endoderm in Brachyury-null mutants suggests that it is not just a failure of motility, but rather a failure to join specific tissue 'streams' that is defective in these embryos.

Eomesodermin (Eomes) appears to be required for the migration of epiblast cells toward or perhaps through the primitive streak, but not for mesodermal patterning. In embryos that were null for Eomes in their ICM, but not in trophoblastic tissues (which require Eomes for proper differentiation), the primitive streak failed to elongate and no morphologically distinguishable node was detectable

(Russ et al., 2000). At the posterior margin of the epiblast (where the streak originates), however, there was a thickening of the epiblast, with rounded cells underneath that were no longer part of the epiblast epithelium, suggesting that at least some ingression had occurred, but the cells had failed to leave the streak region. Cells do not migrate away from this position, judging by the lack of embryonic or extra-embryonic mesodermal tissue. Initial mesoderm specification appears to occur normally, as Brachyury and FGF-8 are expressed as in pre-streak embryos, but epiblast cells do not move toward the streak. Russ et al. (2000) imply that an active movement of the presumptive mesoderm cells in the epiblast toward the streak has failed, suggesting either that Eomes is required autonomously to turn on movement toward the streak, or is required non-autonomously to turn on a signal directing these cells toward the streak. Alternatively, failure to move toward the streak could be the result of a simple mechanical blockage, as cells do not move away from the streak, thus preventing further advance toward the streak. Mesoderm specification is initiated, but markers for the anterior portion of the streak and the node and for neurectoderm do not come on. *mml*, a mouse Mix/Bix homolog (targets of Xbra and VegT) is normally expressed in the primitive streak (Pearce and Evans, 1999) but not in Eomes-null embryos (Russ et al., 2000) (but is in Brachyury-null embryos). mml also appears to play a role in ingression through the streak, at least for some tissues (see below, Hart et al., 2002). In embryos with ICMs chimeric for Eomes-null cells and wild type cells, the wild type cells move through the streak normally, whereas Eomes-null cells do not (Russ et al., 2000). It is not clear whether wild type cells distant to streak can move toward the streak, just that some cells, perhaps those already resident in the streak do successfully ingress.

The mouse gene *mml* may be involved in modulating nodal signaling for the specification of axial mesodermal and endodermal tissues. Like Eomes-null embryos (see above, Russ et al., 2000), *mml*-null embryos again have a thickened posterior streak and fail to form a node; however, the streak does apparently elongate somewhat (Hart et al., 2002). *mml*-null embryos are deficient in axial mesoderm, heart and gut tissues. The progenitors of all these tissues are present in the pre-streak epiblast, but fail to go through morphogenesis appropriately (Hart et al., 2002).

6. EMT during neural tube closure

In vertebrate species, the nervous system is formed when cells in the neural plate undergo apical constriction and invagination as an epithelium to form a transient 'tube' followed by the fusion of its edges to form a continuous outer epithelial ectodermal layer, and the at least partial transient de-epithelialization of the neural cells facing the lumen, which later re-epithelializes and reforms (Aaku-Saraste et al., 1996; Davidson and Keller, 1999) (Fig. 7).

The mechanism of epithelial fusion of the edges of the neural plate is unknown, but must either involve the loss of the junctions between these cells and their neural neighbors, so that they can form new junctions with their apposing

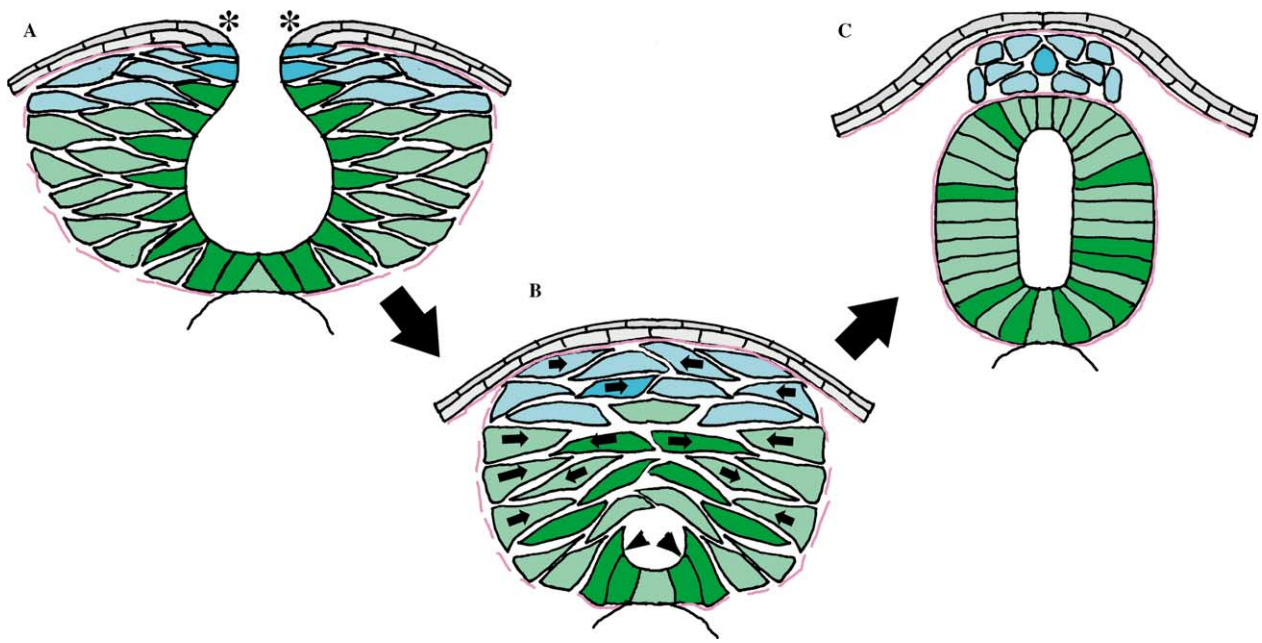

Fig. 7. Steps in neural tube closure: neural fold fusion, de-epithelialization and restoration of the lumen of the neural tube, as seen in *Xenopus laevis*. The edges of the neural folds come into apposition and fuse (A,B), such that the ectodermal epithelial cells adjacent to the neural tissue (∗ s) form new epithelial junctions with each other. After the tube de-epithelializes, the formerly superficial cells (bright green) undergo radial intercalation of with the deep neural cells (light green) (B,C). After intercalation, a lumen reforms (C).

neighbors (∗ s, Fig. 7A), or they may form a second set of junctions with these neighbors, prior to dissociating their junctions with the neural cells that have been internalized, similar to the formation of bridging junctions, discussed above.

In the chick, once the neural tube has closed, the cells facing the lumen of the tube no longer express occludin, a tight junction component, and no longer form an impermeable seal, suggesting tight junction function has been lost (Aaku-Saraste et al., 1996). However, they actually increase expression of ZO1, a component of both tight and adherens junctions, as well as N-cadherin (Aaku-Saraste et al., 1996), suggesting that the adherens junctions are retained in these cells. In the frog, once the neural folds have fused, the dorsal half of the prospective neural tube de-epithelializes (Fig. 7B) (Davidson and Keller, 1999) and tight junctions are lost and replaced by gap junctions (Decker and Friend, 1974). As the lumen reforms, cells intercalate with their deep neighbors to form a single layered tube (Davidson and Keller, 1999) (Fig. 7B,C). Interestingly, neural plates that are held open during the time their siblings close their tubes fail to undergo radial intercalation of the deep neural cells into the superficial neural plate, perhaps because they also fail to de-epithelialize (Poznanski et al., 1997).

An important feature of this mode of internalization is that it provides a way to quickly bring a fairly large area of epithelium inside, sealed off from the exterior environment, where it can undergo EMT without breaking the physiological barrier in a massive way and generating a huge wound surface. This mechanism is also used to internalize the mesoderm in the very rapidly developing *Drosophila* embryo, discussed below, possibly because it allows the rapid removal of a large area. An alternative mode, the sequential ingression seen in the superficial presumptive somitic tissue of urodeles, requires a cell-by-cell approach to a zone, and ingression only in that limited zone (see Section 3.2).

7. Primary developmental EMTs in *Drosophila*

In *Drosophila*, the mesoderm forms by the invagination of the ventral epidermis to form the ventral furrow, which subsequently pinches off (Leptin and Grunewald, 1990; Sweeton et al., 1991), much like the formation of the neural tube, described above, except of course that a lumen does not re-form. The invaginated mesodermal cells then go through an EMT and migrate dorsolaterally to form the mesodermal tissues. The invagination of these cells appears to be driven by apical constriction, and their choreographed changes in cell shape and junctional positioning (Ip and Gridley, 2002) and biomechanics (see Keller et al., 2003) are very interesting. Their apical constriction is perhaps related to ingression events in other species but is not directly related to the EMT per se. In absence of concertina (cta), which encodes a heterotrimeric G protein, the cell shape changes in the ventral furrow are uncoordinated and proceed slowly, but nevertheless the mesoderm is internalized, arguing that at least some internalization can occur without the shape changes proceeding normally (Parks, 1991). In absence of DRhoGEF, a RhoGTPase exchange factor, neither apical constriction nor internalization occur (Barrett et al., 1997; Hacker and Perrimon, 1998), suggesting that this Rho activator and Rho are involved in apical constriction and perhaps additional steps in EMT.

Interestingly, mitosis must be blocked in order for the shape changes and invagination of the ventral furrow to take place. Mitosis in the ventral epidermis of the fly just prior to ventral furrow invagination is delayed with respect to the highly synchronized divisions in the rest of the embryo (Edgar and O'Farrell, 1990). The mesodermal cells, which normally divide just after undergoing EMT and before they migrate, are not internalized if they are forced to divide prior to ventral furrow formation by early cdc25 (String) activity (Foe et al., 1993). The gene Tribbles is also involved in delaying this mitosis, by its interaction with String (Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). In embryos mutant for Tribbles, mitosis again occurs early, before the onset of ventral furrow formation, and the apical constriction and elongation of the cells that would form the ventral furrow, and its invagination, does not occur. Snail also plays a role in mediating this delay in mitosis, apparently as part of its function in regulating mesodermal morphogenesis (Grosshans and Wieschaus, 2000). These results suggest that cells cannot undergo the shape changes, or some other differentiative event, necessary for ventral furrow formation while dividing. Whether EMT subsequently fails to occur in these cells as a direct result of their early cell division, or as an indirect result, for example because some specification event fails because they are dividing, or because they are not invaginated, is not clear.

In regard to changes in cell adhesion, E-cadherin transcription ceases but the protein is expressed in the invaginated mesodermal cells until after they have de-epithelialized and begin to migrate away, at which time N-cadherin protein expression begins (Oda et al., 1998). Thus most of the mesodermal internalization and EMT occurs when E-cadherin is present but N-cadherin is not. Coated vesicles were found frequently near the apical junctions in invaginating mesoderm, suggesting that endocytosis may play a role in reducing E-cadherin mediated cell–cell contact in the mesoderm. As invagination proceeds, the adherens junctions break down and E-cadherin expression becomes discontinuous. The onset of N-cadherin expression in the mesoderm cells was found to depend on the expression of Twist, but not Snail, whereas the repression of E-cadherin depended on the expression of Snail but not Twist. In absence of the either, the presumptive mesodermal cells did not undergo EMT, but instead they remained in the epithelial layer (see Kosman et al., 1991; Leptin, 1991; Oda et al., 1998).

8. Primary developmental EMTs in other invertebrates

8.1. Caenorhabditis elegans

Gastrulation in *C. elegans* consists of the internalization of two endodermal precursor cells at the 26 cell stage. These cells constrict their apical domain and are subsequently covered by adjacent cells. The cells are not epithelial, and have no adherens or tight junctions (Krieg et al., 1978), although they do appear to be adhesive. They do, however, show apical–basal polarity (Nance and Priess, 2002), in that Par-3 is expressed only on cell surfaces with no other contacts (those facing the outside of the embryo). Par-3 organizes myosin across the apical cortex, which is responsible for the actin–myosin based apical flattening and contraction (Lee and Goldstein, 2003; Nance and Priess, 2002). The apical contraction of the endodermal cells appears to both push these cells inside the embryo and draw the neighboring cells over them (Lee and Goldstein, 2003). This may represent a primitive mechanism for ingression, used by embryos that have not yet achieved an epithelial state of organization.

In *C. elegans* laminins form a basal lamina that regulates the separation and maintenance of epithelial and mesenchymal tissues. Laminins are expressed toward the end of gastrulation; laminin αβ is expressed just as the cells finish ingressing (Huang et al., 2003). Knock out experiments with the laminins suggest that they are involved in regulating the adhesive separation of different germ layers and tissues as some of them epithelialize, by forming basement 'membranes' between them. *C. elegans* thus seems to have taken the strategy of putting cells in the right place, and only then forming proper epithelia, as they become mechanically necessary.

8.2. Hydrazoans

The hydrazoan *Phialidium* shows unipolar ingression of bottle-shaped cells (Byrum, 2001). This suggests that apical constriction and bottle cell ingression is a primitive feature of ingression in metazoans. A small amount of delamination to form an internal cells layer also occurs in this species. Delamination is also thought to be an ancestral character of the Cnidarians (see Byrum, 2001). No basement membrane (basal lamina) had formed by the time of ingression, suggesting that basement membranes may not be a primitive feature of metazoans. After cells ingressed, they lost their cilia, and began to express filopodial protrusions. Experiments with explanted cells show that cells from oral regions (where cells were ingressing from) of the pre-ingression embryo acquire significantly higher adhesive affinities than those from aboral regions of the pre-ingression embryo by post-gastrulation, but not before. This suggests that differential adhesion is not a mechanism for driving ingression in this species.

While this is an incomplete survey of EMT mechanisms in invertebrates, it makes clear that the further study of less complex metazoans will be instructive in understanding how developmental EMTs, and their regulation, have evolved. This will allow us to understand the essential elements of EMT, and the advantages of the nuances added by more complex species.

9. Internalization of cells in the blastoderm in fish

During gastrulation in the teleost fish, which begins with the onset of epiboly in Fundulus (Trinkaus, 1996), or shortly after the onset of epiboly in zebrafish (Kimmel et al., 1995), cells at the margin of the blastoderm begin to move deep with respect to the other blastoderm cells, forming a hypoblast, where they begin to show protrusive activity and undergo migratory behaviors (Kane and Adams, 2002; Trinkaus et al., 1992) and eventually go on to give rise to endodermal and mesodermal tissues. The superficial layer of the blastoderm (the epiblast) does not appear to be an epithelium in the usual sense of the word. The teleost blastoderm is covered by an extra-embryonic epithelial sheet, the 'enveloping layer', with apical junctions (Lentz and Trinkaus, 1971), which serves the protective barrier function of an embryonic epithelium in the fish. But there is no evidence for epithelial-type apical junctions between the cells of the blastoderm (Hogan and Trinkaus, 1977). By late blastula to early gastrula stages in the zebrafish, the epiblast is moving as a tightly packed, coherent sheet (Concha and Adams, 1998). As cells ingress into the hypoblast, they become less coherent with their neighbors (D'Amico and Cooper, 1997). In Fundulus, cells within the blastoderm become more adhesive with each other at late blastula to early gastrula stages, but again show no indication of the sort of epithelial tight or adherens junctions seen in the enveloping layer, nor any desmosomes or extensive interdigitations of apposed plasma membranes (Hogan and Trinkaus, 1977; Trinkaus and Lentz, 1967). They do however form extensive non-junctional appositions with each other, and gap junctions (Hogan and Trinkaus, 1977).

Cells are able to ingress as individuals in both zebrafish (Carmany-Rampey and Schier, 2001; Shih and Fraser, 1995), and in Fundulus (Trinkaus, 1996), although they appear to do so as relatively coherent groups of cells in smaller, more densely cellularized teleosts (Kane and Adams, 2002). This is in any event an 'involution' movement, in that generally speaking, cells within the epiblast move toward its vegetal margin as a more-or-less coherent sheet, move inward, and then advance away from the margin, or at least the point of involution. Arguably, this may be less strictly true in Fundulus, where cells often move away from the margin before ingressing (Trinkaus, 1996), but the overall movement is still the same.

The mechanism of ingression in teleosts is poorly understood. In Fundulus, the apices of the cells constrict

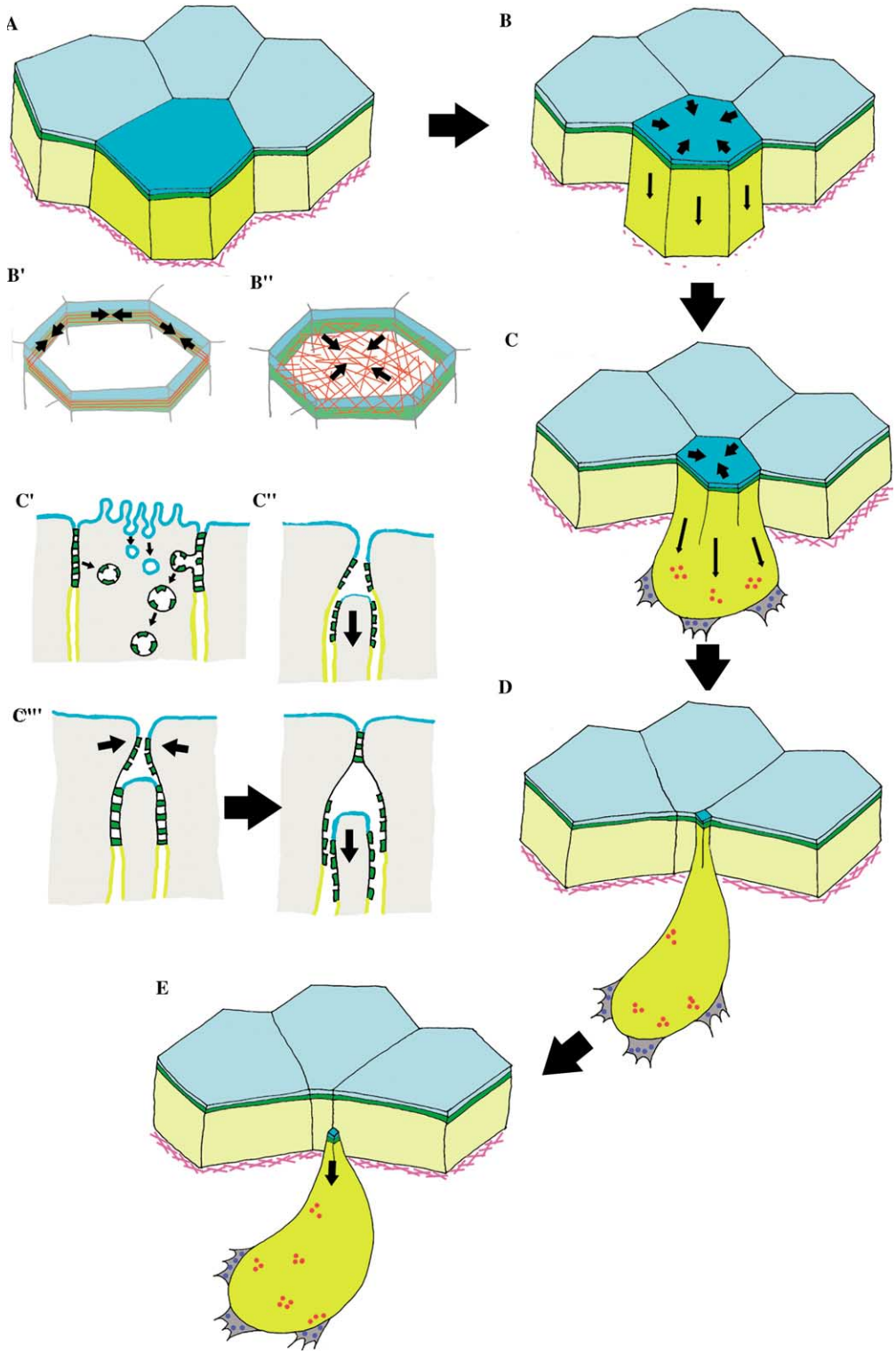

Fig. 8. Steps in the process of epithelial-to-mesenchymal transition are shown schematically. First, inductive or other specification events occur, committing the cell to an EMT (dark blue, highlighted cell, A). Generally but not always, the cell undergoes a constriction of its apical region (small thick arrows, B,C), a process which probably involves either a circumferential contractile cytoskeleton (B′) or a contractile cytoskeletal meshwork spanning the apices (B″). Coincident with the apical constriction, the cell often begins to elongate the apical–basal axis as cytoplasm is pushed basally (small skinny arrows, B,C). The cell also begins to break down the basal lamina (magenta, A–C). Other changes may include formation of protrusions at the basal ends (gray, C,D), down-regulation of epithelial cell adhesion and cell–extracellular matrix adhesion receptors, and expression of mesenchymal adhesion molecules (basolateral spots, C,D). Epithelial cell adhesion molecules are down-regulated, and as the apical region of the cell shrinks, the apical junctions decrease in circumference and in strength, and eventually the cell pulls itself, or is pulled or pushed beneath the surface and out of the epithelium (C–E). In some cases the apical membrane is thrown into microvilli or microfolds as the apical region of the cell decreases in area, and membrane may be internalized (C′). Molecules or whole junctions of

(Trinkaus, 1996), but there are no clues as to the mechanisms behind this. Nothing is known about the change in cell–cell associations in any teleost, either from tight and coherent in the epiblast to migratory in the hypoblast, or from association with epiblast cells to association with hypoblast cells. Cells appear to ingress autonomously, and probably require mesendodermal specification to become competent to ingress (Carmany-Rampey and Schier, 2001).

Studies should be done in the teleost fish to learn the state of the epithelial organization of the outer layer of the blastoderm. Is it epitheloid, without a circumferential junctional complex? Perhaps so, given the fact that the enveloping layer forms a high resistance physiological and mechanical barrier outside the blastoderm.

Ingression has also been found in the chondrostean fish, the white sturgeon, Acipenser transmontanus (Bolker, 1993), but of a much different type. Chondrostean fish have embryos much more like those of amphibians than teleosts, and use a distinctly amphibian style of gastrulation. This includes the ingression of a set of presumptive mesodermal cells from the roof of the gastrocoel (Bolker, 1993), strongly resembling the same process in anuran amphibians (see above). Cells appear to be ingressing as bottle cells into the presumptive deep somitic and notochordal regions, where they presumably become mesenchymal. Otherwise, nothing is known about the mechanism of this ingression event and the associated EMT.

10. Conclusions

10.1. General features of EMTs in early development

We can make some generalizations about the events of primary developmental EMTs at this point (Fig. 8). First, most but not all EMTs begin with an apical constriction, which reduces the surface area, the apical boundary length with adjacent cells, and tends to push the cytoplasm to the basal region of the cell, thereby causing a shape change (Fig. 8A–D). All these features could aid and abet removing the cell from the epithelium, which is probably why it is a widely distributed phenomenon, but it is not an essential feature of all EMTs. Apical constriction appears to involve a circumferential contractile apparatus in some cases and a contractile, apex-spanning meshwork in others (see Keller et al., 2003) (Fig. 8B′,B″). If a basal lamina is present, it is broken down by mechanisms that are poorly understood in primary developmental EMTs (Fig. 8B). Mesenchymal-type cell–cell and cell–matrix adhesion molecules are up-regulated (Fig. 8C), and coordinate with this process, epithelial-type adhesion molecules, junctions and junctional components, as well as apical membrane are internalized by endocytosis, particularly in EMTs involving apical constriction (Fig. 8C′). Eventually, the combination of down-regulation of adhesion at the apex and increasingly strong, competing adhesions at the basal ends results in detachment of the apex and ingression, followed by sealing of the wound left by the withdrawal of the apex (Fig. 8D,E,C″). In many but not all cases, this ingression involves a mesenchymal-type, active migration at the basal ends of the ingressing cells. In some EMTs, small lesions are generated when ingression occurs, and these are quickly healed over, but in other systems, there appears to be a mechanism for sealing over the ingressing cell with another tier of junctions prior to detachment and ingression, thereby maintaining epithelial integrity (Figs. 5N–Q,8C‴). There are a number of significant unresolved issues about these events in primary EMTs.

10.2. Is it an EMT if it is not from a 'proper' epithelial sheet?

Some of the events we have described involve cells that do not originate from an epithelial sheet with a free apical surface, circumapical tight junctions and apical–basal polarity. We have included them because they nonetheless involve a transition, at the least, to a more mesenchymal state, from a state involving at least some epithelial characters. Thus, many of the mechanisms for these transitions may be shared by and relevant to more proper EMTs. Which is to say, we are interested in the steps the cells are taking to modulated their epithelial-mesenchymal phenotype, and are not particularly concerned with whether the starting and ending point match particular preconceived notions of 'epithelial' or 'mesenchymal'.

10.3. Maintenance of epithelial continuity

Cells undergoing EMT from a primary, single-layered epithelium that faces the external environment, and acts as both a physiological barrier and a mechanical integrator of embryonic structure, such as the sea urchin and amphibian, are under stringent constraints. EMT in this case is organized to minimize the disruption to the epithelium. EMT is patterned such that cells only ingress from a specific, fairly

the junctional complex may also be removed from the cell surface and internalized as vesicles (C′). We envision two ways of removing the cell from the epithelium. The apical junctional complex breaks, the contiguity of the cell with epithelium is broken, and it leaves the epithelium (ingression) and a hole in its place (C″). Alternatively, the adjacent cells might bridge over the ingressing cell, form a junctional complex above it, and provide physiological and mechanical contiguity while the cell ingresses (C‴). Disarrayed patches of junctions are often found on freshly ingressed cells (C″,C‴). Other cytoskeletal changes also occur. Vimentin containing intermediate filaments are formed in favor of the cytokeratin intermediate filaments of epithelial cells, and the regulation of the cytoskeleton, protrusive activity, and contact and guidance behavior is altered to the mesenchymal pattern by as yet poorly understood mechanisms.

restricted location, as single cells or a single row of cells, and the junctions left unattached can be quickly resealed. The process of patterning large areas of cells to undergo nearly sequential EMT is poorly understood. Other epithelia or epitheloid sheets that do not face the external environment (the mouse, and especially the fish) can perhaps be more disorganized about where and how cells ingress.

Not only is patterning of ingression important for maintaining epithelial integrity, but also, at least in some cases, for the ordered addition of progenitor cells to the deep mesodermal layer. This may explain the involution-type behavior associated with ingression in the vertebrates where cells move toward a locus of ingression, then inside, and finally away from the point of ingression, while maintaining order.

Maintenance of epithelial integrity in the face of ingression may involve formation of 'bridge' junctions over the ingressing cells, or the point by point 'unzipping' of junctions between the ingressing cells and its neighbors and the 'rezipping' of those junctions between the remaining neighbors. But whether these mechanisms actually occur, and how, is still unresolved. There are two other mechanisms for moving epithelial cells into the interior of an embryo. Delamination by radial cell division is a mechanism commonly used by the Cnidarians, the Porifera and other invertebrates to produce mesenchyme (Kume and Dan, 1988). It is also used in vertebrates, such as the radial division of the epithelial cells to form the deep blastomeres in *Xenopus*, for example, but whether this really constitutes a transition to a mesenchymal phenotype, rather than to part of a multi-layered epithelium, is unclear. Delamination as a mechanism for formation of mesenchyme seems less prevalent in more complex metazoans. It would be interesting to know why. Finally, invaginating an epithelial sheet, pinching it off, and then de-epithelializing the internalized cells, as in neural tube de-epithelialization in vertebrates and ventral furrow formation in *Drosophila*, is another mechanism for rapidly moving large areas of epithelial cells inside an embryo. The subsequent transition to a mesenchymal phenotype is then uncomplicated by restrictions normally placed on external epithelia. The question remains though, as to how the invaginated tube pinches off; the epithelial cells at the point of pinching must still form new junctions with their new neighbors.

10.4. Specification of the phenotype

Specification of cells to a phenotypic pathway that includes EMT is obviously an important first step in a successful EMT. Unless the entire pathway is turned on at the right time and place, cells fail to undergo a complete EMT (Ciruna and Rossant, 2001; Ciruna et al., 1997; Russ et al., 2000; Sun et al., 1999; Wilson et al., 1995, 1993); this is probably not the result of the failure to regulate one or two specific molecules, but a failure to turn on the entire EMT pathway, as part of the specification event for a particular type of mesenchymal cell. Some of the receptors that may mediate developmental EMTs [e.g. FGFR and c-met (the HGF receptor)] are tyrosine kinase receptors, which have been shown to be involved in turning on a wide range of cell processes important for EMT (reviewed by Savagner, 2001), and turn on the Brachyury transcription factor (reviewed by Smith, 1997), as well as Snail (Ciruna and Rossant, 2001). The Snail and Slug transcription factor family is also clearly involved in regulating EMT (Ip and Gridley, 2002). Snail and Slug seem to be involved in the disassembly of the adherens junctions by repressing E-Cadherin translation (Batlle et al., 2000; Bolos et al., 2003; Cano et al., 2000), disassembly of desmosomes (Savagner et al., 1997) and tight junctions (Ikenouchi et al., 2003) by unknown mechanisms. Snail and Slug play other roles as well, including in turning on MMPs and vimentin (Yokoyama et al., 2003), and the Wnt pathway by releasing β-catenin from adherens junctions (Ciruna and Rossant, 2001). All of these roles may be directly related to repressing E-cadherin expression (Ciruna and Rossant, 2001), as simply blocking E-cadherin binding will also cause an EMT (Burdsal et al., 1993). E-cadherin thus appears to be an important central regulator of EMT, by modulating adhesion, cytoskeletal anchoring, junctional scaffold stabilization, and sequestration of β-catenin. Snail/Slug potentially have many more direct or indirect targets, however, and their functions, and functional relationship to one another, in various processes and in different species is a key issue for further work (see Nieto, 2002).

10.5. Regulation of cell division

Cells apparently need to stop dividing in order to be able to undergo EMT. Tribbles is involved in blocking mitosis during ventral furrow formation and failure to do so prevents ventral furrow invagination (Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). Whether this also directly, or only indirectly blocks EMT is not clear. Snail may also be involved in blocking mitosis, allowing cells to go through an EMT (Grosshans and Wieschaus, 2000). FGFR1 also appears to be involved in decreasing proliferation (Hebert et al., 2003), perhaps through its activation of Snail and subsequent inhibition of mitosis. Stopping cell division may be important for allowing cells to differentiate toward a mesenchymal cell type, or for allowing cells to make use of their cytoskeleton to drive some aspect of EMT, or both.

10.6. Patterning EMT

In some systems, it may be that cells must be in the right place in order to go through EMT, possibly because they require a local signal. This may be the case with cells null for Eomes (Russ et al., 2000), perhaps because they require some signal within the primitive streak, which is lacking in absence of Eomes, to initiate the ingression and de-epithelialization program. In other systems however, this

is not the case; both sea urchin (Burdsal et al., 1991; Fink and McClay, 1985; McClay and Fink, 1982; Wray and McClay, 1988) and amphibian cells (Shook et al., 2002) fated to go through EMT will do so when isolated from the cells adjacent to which they would normally ingress and de-epithelialize. It is clearly important to constrain the expression of EMT to a local region at any one time in cases where the embryonic epithelium must be kept reasonably intact and the area fated to undergo EMT is large. Onset of EMT could be done by pre-programming a temporal–spatial pattern, by proximity to a particular site, or by whether or not the cell ahead in the progression has executed a particular step in EMT.

10.7. The basal lamina

It appears to be generally true that the basal lamina is disrupted prior to cells ingressing through it, but how this occurs, and even whether it is entirely necessary is not clear. It does however appear to be important in regulating the epithelial–mesenchymal state of the cells resting on it, as its disruption can cause an ectopic EMT (e.g. Canning et al., 2000).

10.8. Cell shape change

Shape change, in particular apical constriction, may not be an essential feature of EMT, as in some cases EMT will proceed without it (Anstrom, 1992; Minsuk and Keller, 1996). Presumably it improves the efficiency of ingression and better preserves the integrity of the epithelium from which the cell is ingressing. Apical constriction acts to minimize the problems in maintaining epithelial integrity, by minimizing the size of the hole that ingression may leave, and by minimizing the amount of circumapical junctions that are supporting the mechanical integrity of the epithelium. This is important in conjunction with the proper spatial and temporal regulation of ingression, as if too many cells ingressed over too large an area, and/or left too large a hole in the epithelium, the resulting wound might be further expanded by the mechanical tension surrounding the site of ingression, thus significantly delaying or perturbing morphogenesis, and might allow too great an exchange between the external and internal physiological environments, further disrupting development. The amount of circumapical junctional material that must be dissociated from adjacent cells and the associated scaffold that must be disassembled is also minimized. The minimization of the apex and its junctions is also facilitated by the endocytosis of apical membrane, and adherens junctions (e.g. Miller and McClay, 1997a,b) and perhaps desmosomes as well (Burdett, 1993), although this has not yet been seen in a developmental context. Apical constriction generally appears to be driven by the contraction of an actin–myosin mesh across the apical surface, anchored to the circumapical junctional scaffold (Lee and Goldstein, 2003; Nance and Priess, 2002; Young et al., 1991).

10.9. Changes in adhesion

While shape change may prime a cell for ingression, it does not appear to drive ingression; rather changes in adhesive affinities appear to be responsible for de-epithelialization and withdrawal (ingression) from the epithelial surface. However, a clear example showing that blocking any part of this change in adhesion blocks ingression is still lacking. Changes in cadherin expression to one expressed by other deep cells are the usual suspect here, but the expression of integrins must also play a role. The expression of cadherins and integrins allowing interaction with other deep cells and the matrix in the deep layer may be important for pulling cells out of the epithelial layer, and is clearly important for migration away from the site of ingression and proper association with the appropriate deep tissue. And in order to allow ingression and de-epithelialization, tight junctions must be dissociated and presumably desmosomes and gap junctions must be endocytosed or otherwise disassembled. How the tight junctions are finally disassembled is still a mystery.

10.10. Mechanisms of integrating the various steps in EMT

The dissociation of the different steps of EMT, and the consequences thereof are quite illuminating. That mouse cells null for Snail, and thus unable to properly repress E-cadherin expression, can still ingress and migrate (Carver et al., 2001) shows that turning off the adhesion molecules for association with the epiblast is not a strict requirement, but rather that the movement of cells into the deep layer is instead regulated by some other molecule. Likewise, neural crest cells or epiblast cells that lose their epithelial attachments, fail to express the migratory aspect of a mesenchymal phenotype if they are not able to bind to fibronectin, at least in the chick (Harrisson et al., 1993; Testaz and Duband, 2001). Thus the aspect of EMT that mediates dissociation from epithelial cells may be somewhat independent of the aspect that mediates association with the mesenchymal cells. Failure to turn off E-cadherin does have consequences however, in that ingressed cells fail to separate normally, and morphogenesis is consequently impeded. And failure to turn on an appropriate mesenchymal adhesion molecule, while turning off the epithelial adhesion molecule can lead to apoptosis (Testaz and Duband, 2001). This may be a method to prevent cells from becoming completely independent of association with other cells, i.e. metastatic. There are also examples of cells that appear to have ingressed at the mouse primitive streak, but not to have truly de-epithelialized or undergone other steps of their normal EMT (Ciruna et al., 1997; Hart et al., 2002; Russ et al., 2000; Sun et al., 1999; Wilson et al., 1995, 1993).

The partial independence of these EMT steps, e.g. to ingress and migrate without truly de-epithelializing, or to de-epithelialize without adopting a mesenchymal phenotype suggest that at least some of these steps evolved as mechanism for driving primitive internalizations of cells, and that other steps have subsequently been tacked on to refine the process, or to simply regulate the behavior of cells once internalized. Thus when some of the steps are eliminated, some aspects of EMT and/or internalization may still operate but very sloppily.

We hope this review serves as a useful reference for, and provokes further investigations into developmental EMTs. It is necessarily an incomplete and biased view of the field and we apologize to those authors whose work we were unable to include.

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