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Mesoderm Layer Formation in *Xenopus* and *Drosophila* Gastrulation

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Abstract

During gastrulation, the mesoderm spreads out between ectoderm and endoderm to form a mesenchymal cell layer. Surprisingly the underlying principles of mesoderm layer formation are very similar in evolutionarily distant species like the fruit fly, *Drosophila melanogaster*, and the frog, *Xenopus laevis*, in which the molecular and the cellular basis of mesoderm layer formation have been extensively studied. Complementary expression of growth factors in the ectoderm and their receptors in the mesoderm act to orient cellular protrusive activities and direct cell movement, leading to radial cell intercalation and the spreading of the mesoderm layer. This mechanism is contrasted with generic physical mechanisms of tissue spreading that consider the adhesive and physical properties of the cells and tissues. Both mechanisms need to be integrated to orchestrate mesenchymal morphogenesis.

Potential forces driving mesoderm layer formation

During gastrulation, the basic body plan of multicellular animals is established (Leptin, 2005). In its course, the mesoderm and endoderm move to the interior of the embryo, whereas the ectoderm remains on the outside. After its internalization, the mesoderm typically forms a mesenchymal cell layer between ectoderm and endoderm, before it breaks up into its various derivative structures. Mesoderm layer formation has recently been studied in some detail in *Drosophila* (McMahon *et al.*, 2008; Murray and Saint, 2007; Schumacher *et al.*, 2004) and in *Xenopus* (Damm and Winklbauer, 2011; Winklbauer and Schurfeld, 1999). In both systems, the mesoderm is internalized in a narrow region of the embryo, the ventral furrow or the blastopore lip, respectively. Once inside, the coherent mesodermal cell mass advances with a free leading edge across the ectodermal substratum, while its other end remains at the site of internalization. In this way, the mesoderm spreads out into a thin layer underneath the ectoderm (Figs. 1, 2A).

Mesoderm layer formation depends on the adhesive properties and the motility of cells. In mesenchymal morphogenesis, motility can be oriented or random, and both types of movements can in principle create forces that drive mesoderm layer formation (Winklbauer, 2009). The coordinated movement of globally oriented cells plays a key role in the remodelling of mesenchymal tissues, for example in vertebrate convergent extension (Keller *et al.*, 2008), or in *Drosophila* border cell migration (Montell, 2003). Random cell motility, on the other hand, is ubiquitously present, at levels ranging from small membrane fluctuations to random protrusive activity. Any morphogenetic cell rearrangement also requires that cell-cell adhesion is flexible in the tissues involved. The combination of

random motility and flexible adhesion makes mesenchymal tissues behave in some respect like liquids, and consequently, surface or interfacial tensions can be main driving forces for tissue-level movements (Steinberg, 1978; Graner, 1993; Foty *et al.*, 1994; Beysens *et al.*, 2000; Jakab *et al.*, 2008). These forces are sufficient to bring about morphogenetic shape changes of tissues in vitro, such as the engulfment of one tissue by another (Steinberg, 1970; Phillips and Davis, 1978; Graner, 1993; Steinberg and Takeichi, 1994; Foty *et al.*, 1996). Moreover, the tension-derived forces are of the same magnitude as those generated by oriented cell movement, for example during convergent extension (Ninomiya and Winklbauer, 2008). The initial version of this concept, postulating specific affinities of tissues for each other, was developed by Holtfreter in the context of amphibian germ layer formation (Holtfreter, 1939, 1944; Townes and Holtfreter, 1955). Later, it was refined by Steinberg (Steinberg, 1963, 1970) into the Differential Adhesion Hypothesis, and it was used to explain the positioning of the mesoderm between ectoderm and endoderm in the amphibian gastrula (Holtfreter, 1944; Townes and Holtfreter, 1955; Phillips and Davis, 1978). A contribution of cortical tension to cell sorting and tissue positioning was recently described, using zebrafish gastrula tissue as a model (Krieg *et al.*, 2008; Manning *et al.*, 2010).

In treating tissues as liquids, the spreading of a cell aggregate on a substratum can be described as a movement towards an equilibrium between tissue-substrate adhesion and tissue cohesion, where the degree of spreading depends on the strength of cell-cell adhesion relative to cell-substratum attachment (Martz *et al.*, 1974; Ryan *et al.*, 2001; Xu *et al.*, 2009). Spreading generally increases with lower cell-cell adhesion and higher cell-substrate adhesion. However, strong substrate adhesion can adversely affect cell movement, and modeling suggests an optimum substrate adhesiveness for spreading (Xu *et al.*, 2009). Explants of *Xenopus* dorsal mesoderm spread indeed on an artificial fibronectin-coated substratum (Reintsch and Hausen, 2001). Lamellipodia are present mostly on the free margin of such an explant, suggesting that the outward movement of the peripheral cells over available free substratum drives spreading. At the same time, more and more interior cells come in contact with the substratum by passive cell rearrangement behind the leading edge, until an equilibrium is reached after a few hours (Winklbauer *et al.*, 1992; Reintsch and Hausen, 2001). Given the apparent simplicity of this process, mesoderm layer formation may seem straightforward, consisting of the spreading of the initially compact mesodermal mass on the free ectoderm surface. However, both in *Drosophila* and in *Xenopus*, an intricate pattern of cell orientation is observed in the internalized mesoderm, suggesting that oriented cell migration underlies mesoderm layer formation.

Mesoderm layer formation in *Xenopus* and *Drosophila*

In *Drosophila*, the formation of the mesoderm layer can be divided into several stages: epithelial-mesenchymal transition (EMT), flattening, dorsal-lateral migration, and monolayer formation (Murray and Saint, 2007; McMahon *et al.*, 2008; Schumacher *et al.*, 2004; McMahon *et al.*, 2010) (Fig 1). During EMT and flattening the internalized mesoderm loses its epithelial polarity and attaches as a multilayered aggregate of mesenchymal cells to the basal surface of the ectoderm along the ventral midline (Fig. 1A). The outward movement of the mesoderm in the dorsal-lateral direction is associated with a reorganisation of cells in this direction, which becomes obvious in cell shape changes and the formation of lamelliform and filiform protrusions at the free dorsal edges of the migrating cells (Schumacher *et al.*, 2004; Klingseisen *et al.*, 2009; Clark *et al.*, 2011). This protrusive activity is transient and ceases when all cells have made contact with the ectoderm, forming a monolayer (Schumacher *et al.*, 2004). Inner cells of the mesoderm aggregate reach the ectoderm by cell intercalation (Clark *et al.*, 2011; Murray and Saint, 2007; McMahon *et al.*, 2008). Mesoderm layer formation is independent of mitotic divisions, although two mitotic

waves occur during the process, one during EMT and one preceding monolayer formation (Campos-Ortega and Hartenstein, 1997; Leptin and Grunewald, 1990; Bate, 1993).

In the anterior mesendoderm of the *Xenopus* gastrula, which leads the advancing mesodermal mass, cells in contact with the ectoderm are oriented in the direction of overall mesoderm translocation towards the animal pole of the embryo (Fig. 2C). Cells are unipolar, and extend lamelliform and filiform protrusions such as to underlap cells ahead of them. Deeper within the tissue, cells are oriented obliquely towards the ectoderm (Fig. 2C). Behind the leading edge mesendoderm, the prechordal mesoderm cells are likewise unipolar, but are strictly oriented perpendicular to the ectoderm, i.e. they point with their protrusions towards it (Fig. 2B,C). Further posterior, the unipolar chordamesoderm cells are oriented in opposite directions, with some cells pointing towards and some away from the ectoderm (Fig. 2C). The cells of the endodermal vegetal cell mass deep to the mesoderm are elongated along the animal-vegetal axis, i.e. in parallel to the ectoderm (Keller and Schoenwolf, 1977; Damm and Winklbauer, 2011).

In *Drosophila*, the radial intercalation movement of mesoderm cells towards the ectoderm, which is suggested by cell orientation, has been observed directly (Clark *et al.*, 2011; Schumacher *et al.*, 2004; Murray and Saint, 2007; McMahon *et al.*, 2008). The movement involves the extension of dynamic protrusions from the mesoderm towards and between ectoderm cells. Whether this radial protrusive activity drives radial cell intercalation which in turn promotes layer formation is not clear. The available imaging technology is unable to detect inner mesoderm cells with sufficient details. In addition to dorsal lateral movement, the mesoderm also extends in the anterior-posterior (AP) direction. The ectoderm substrate extends in this direction actively in the process of germ band elongation (Zallen and Blankenship, 2008). The relative segmental positions of the mesoderm and ectoderm cells are maintained during mesoderm layer formation (Bate, 1993) and live imaging suggests that both germ layers translocate with similar dynamics along the AP axis (McMahon *et al.*, 2008). Thus during flattening and dorsal migration, the mesoderm follows the ectoderm substrate in register along the AP axis and at the same time spreads out perpendicular to this movement in a dorsal-lateral direction. The interaction between mesoderm and ectoderm must be able to direct cells both along the anterior-posterior and the dorsal-ventral axis.

In *Xenopus*, direct observation of interior cells is difficult. However, movement according to cell orientation has been reconstituted in vitro, and deduced to occur in the embryo (Fig. 2B,C). First, the leading edge mesendoderm translocates across the BCR during gastrulation, and this directional migration can be reproduced on in vitro deposited ectodermal extracellular matrix (Winklbauer and Nagel, 1991; Nagel and Winklbauer, 1999; Nagel *et al.*, 2004). Second, radial intercalation of prechordal mesoderm cell is inferred from the reduction of the thickness of this mesoderm layer during mid-gastrulation from 2-3 cells to a single cell layer (Fig. 2B,C) (Damm and Winklbauer, 2011), and a corresponding expansion of the area occupied by anterior mesoderm (Keller and Tibbetts, 1989). Moreover, radial intercalation in this region was observed in explants (Damm and Winklbauer, 2011). Third, radial intercalation in the chordamesoderm, with its bidirectionally oriented cells, has also been observed in explants from this region (Wilson and Keller, 1991; Wilson *et al.*, 1989).

Together, these observations suggest that in *Xenopus*, mesoderm layer formation is mainly driven by oriented cell movements. The main component is an interdigitation in a radial direction, with cells moving over each others surface and wedging between each other until they arrive at the ectodermal layer. This radial cell intercalation promotes the thinning and spreading of the layer in the prechordal mesoderm, until a monolayer is formed. The directional migration of the leading edge supports or at least complements this spreading. Thus, the global organization of cell motility and the orientation of protrusive activity is an

essential mechanism. To what degree, if any, random motility and differential adhesion (which prefers mesoderm-ectoderm over mesoderm-mesoderm adhesion) contribute to layer formation remains to be determined.

One reason for a mechanism based on oriented cell movement might be that mesoderm layer formation in *Xenopus* must adapt to regional differences which are imposed by the spherical geometry of the embryo. Thus, the leading edge mesendoderm starts out from the equator of the embryo, and then converges from dorsal, lateral and ventral sides as it advances anteriorly towards the animal pole (Davidson *et al.*, 2002) (Fig. 2A). Therefore, spreading of this region would be counterproductive. Orienting all the substrate-apposed cells in the direction of mesoderm translocation should prevent spreading of the cell mass, but at the same time promotes its translocation as a whole, since all cells move simultaneously forward. In contrast, for the prechordal mesoderm extensive spreading is required as it moves along the surface of the spherical embryo from a narrower to a wider circumference (Keller, 1976) (Fig. 2A). Radial intercalation should be an effective means to achieve the spreading of this region. A second reason could be that in *Xenopus*, translocation of the mesoderm relative to the ectoderm is accompanied by cell-cell repulsion between the two tissues (Wacker *et al.*, 2000), Rohani *et al.* submitted). This mutual repulsion may be incompatible with tissue spreading driven by a preference for substrate adhesion over tissue cohesion.

Oriented protrusive activity is also involved in *Drosophila* mesoderm layer formation. A more general reason for the implementation of such a mechanism is that a narrow optimum may exist for the balance between adhesive parameters to generate sufficient spreading in the required time, and fixing substrate adhesiveness or cell-cell cohesion at the required values may interfere with other functions. The rate of mesoderm spreading on fibronectin *in vitro*, estimated from the data in Reintsch and Hausen (Reintsch and Hausen, 2001), is about 3 times slower than the velocity of mesoderm translocation in the embryo, which we measured at 2 $\mu\text{m}/\text{min}$ (Winklbauer, unpublished results). This suggests that spreading based on differential adhesiveness might be too slow for the velocities required *in vivo*. In agreement with this, it has been estimated for the zebrafish embryo, that interfacial tensions would not be sufficient to drive mesoderm layer formation during gastrulation at its normal rate (Schotz *et al.*, 2008).

Molecular control of mesoderm layer formation

The molecular underpinnings of mesoderm layer spreading have been studied in *Drosophila*, and in *Xenopus* for the prechordal mesoderm. Interestingly, the same basic principle seems to be employed in these two systems: growth factors secreted by the ectoderm regulate mesodermal morphogenesis, in particular by acting as chemoattractants. In both cases, a complementary expression of respective tyrosine kinase receptors in the mesoderm and of the ligands in the ectoderm is found. In *Drosophila*, the FGF receptor Heartless (Htl) is expressed in the mesoderm and its two FGF8-like ligands, Pyramus (Pyr) and Thisbe (Ths) are expressed in the ectoderm (Gryzik and Müller, 2004; Beiman *et al.*, 1996; Shishido *et al.*, 1993; Stathopoulos *et al.*, 2004). In *Xenopus*, PDGF-A is expressed in the ectoderm and the receptor, PDGFR- α , in the mesoderm (Ataliotis *et al.*, 1995).

In *Drosophila*, FGF signaling is required for several phases of mesoderm layer formation (McMahon *et al.*, 2010; Schumacher *et al.*, 2004; Wilson *et al.*, 2005; Clark *et al.*, 2011). In mutants affecting the FGF ligands or the receptor, the mesoderm fails to attach to the ectoderm symmetrically relative to the ventral midline, indicating that FGF signaling is essential to promote the mutual attachment of the germ layers (McMahon *et al.*, 2010; Schumacher *et al.*, 2004; Klingseisen *et al.*, 2009). Phenotypic analyses suggest that the

requirement of FGF for mesoderm-ectoderm attachment relies upon two functions. First, FGF directs protrusive activity during mesoderm-ectoderm attachment (Wilson *et al.*, 2005; Clark *et al.*, 2011). The absence of mesoderm cell protrusions extending towards the ectoderm will reduce the probability to form adhesions to the ectoderm. Secondly, recent data show that in the absence of FGF signaling the epithelial structure of the internalized mesoderm is maintained, which might compromise efficient interaction with the ectoderm (Clark *et al.*, 2011). During dorsal migration the two FGF8-like ligands are differentially required. While *Ths* is dispensable for dorsal migration and protrusive activity of the dorsal leading edge, *Pyr* is essential for this process (Klingseisen *et al.*, 2009). Several pieces of evidence suggest that *Pyr* provides a chemotactic signal that directs mesoderm cells from lateral to dorsal ectodermal positions. *Pyr* is expressed in a dynamic fashion; during EMT and flattening its expression overlaps with *Ths*, but during dorsal migration, *Pyr* expression becomes restricted to the dorsal most domain of the ectoderm and in a segmentally repeated pattern in the ventral ectoderm (Gryzik and Müller, 2004; Klingseisen *et al.*, 2009) (Fig. 1B). Ubiquitous expression of *Pyr* compromises directional protrusive activity and dorsal lateral migration, and lack of *Pyr* results in loss of dorsal protrusive activity, while lack of *Ths* does not affect dorsal protrusions and (Klingseisen *et al.*, 2009).

Quantitative data show that both *Pyr* and *Ths* are required for radial cell intercalation during dorsal migration and consequently for monolayer formation (McMahon *et al.*, 2010). The genetic requirements for radially oriented protrusive activity are less well understood. While mutants for *htl* do not exhibit radial protrusions, both *pyr* or *ths* mutants still do, although in reduced numbers (Clark *et al.*, 2011). This indicates that both ligands are required for radial movements, suggesting that they are acting in a redundant fashion, although their expression patterns are only partially overlapping (Fig. 1B).

For *Xenopus*, it has been shown that PDGF-A orients mesoderm cells toward the ectoderm (Damm and Winklbauer, 2011). When PDGF-A synthesis in the ectoderm or PDGFR- α function in the mesoderm are inhibited, prechordal mesoderm cells are no longer oriented toward the ectoderm, and radial intercalation is arrested. Interestingly, cells become not randomly oriented in inhibited embryos, but parallel to the ectoderm, similar to the endoderm cells deep to them. In an explant system, PDGF signaling is required not only for prechordal mesoderm cell orientation, but also for directional migration. Moreover, PDGF-A signaling was shown to be sufficient to redirect migration of these cells, and to act as an instructive cue (Damm and Winklbauer, 2011). Thus, the mechanism of radial intercalation in the prechordal mesoderm appears to consist of the chemoattractive reorientation of cells towards the PDGF-A emitting ectoderm, and the intercellular migration of cells over each. Once they reach the source of PDGF-A, they are repelled by an ephrinB/EphB-dependent tissue separation mechanism which prevents them from invading the ectoderm (Rohani *et al.* submitted). Therefore, cells accumulate at the ectoderm-mesoderm boundary, constituting an example of a boundary capture mechanism.

In both *Drosophila* and *Xenopus*, the dorso-laterally or animally directed vs the radial movements are controlled by the differential expression of closely related growth factors. In *Drosophila*, the dorsal movement is dependent on *Pyr*, while *Ths* is dispensable, and both FGF8-like ligands are required for radial intercalation (McMahon *et al.*, 2010; Clark *et al.*, 2011). In *Xenopus*, these movements are controlled by the expression of two splice variants of PDGF-A, a long and a short form (Damm and Winklbauer, 2011). The long form contains a matrix binding site near its C-terminus and becomes immobilized at the cell surface or in the extracellular matrix, whereas the short form is diffusible (Smith *et al.*, 2009; Andersson *et al.*, 1994; Raines and Ross, 1992). It is this short form which attracts prechordal mesoderm cells to the ectoderm, over a distance of up to 5 cell diameters. In contrast, the long form, which associates with the fibronectin matrix of the ectoderm (Smith *et al.*, 2009),

provides directional information to the anterior mesendoderm. The orientation of cells in this region toward the animal pole, and their directional migration on the ectodermal matrix *in vitro* and *in vivo*, depend on an interaction with the long form PDGF-A (Nagel *et al.*, 2004). Interestingly, high doses of short form PDGF-A are able to reorient these cells towards the ectoderm, similar to prechordal mesoderm cells (Damm and Winklbauer, 2011). This suggests that the leading edge mesendoderm is less sensitive to PDGF-A signaling, in agreement with a decreased PDGF receptor expression.

Scales of understanding: From genes to tissue movements

The concept of “generic physical mechanisms” (Newman and Comper, 1990) refers to the observation that physical forces and properties which govern the behavior of inanimate materials such as liquids can also determine, and are perhaps the evolutionary origin of, morphogenetic processes in embryos. Mesoderm layer formation, seen as the simple spreading of a cell mass on an external substratum, would seem to be a prime candidate for such a generic physical mechanism. However, both in *Xenopus* and in *Drosophila*, elaborate oriented cell behavior, and signaling from the ectoderm to the mesoderm to control this behavior, are associated with this seemingly simple process. On the other hand, physical properties such as tissue surface or interfacial tensions based on random motility and flexible cell-cell adhesion seem unavoidable attributes of tissues, and the forces they generate have to be reckoned with. Future studies will probably reveal that both genetically programmed cell behavior and generic physical processes contribute to mesoderm spreading. The relationships of such physico-genetic mechanisms and their evolution have recently been discussed in more general terms for the morphogenesis of multicellular systems (Newman *et al.*, 2006; Newman and Bhat, 2008).

It has to be kept in mind though that physical properties can also act to attenuate a process, and the respective forces have to be overcome by regulated cell behaviors (Ninomiya and Winklbauer, 2008). Whereas routine approaches are being applied to decipher signaling networks and motile behaviors that affect morphogenesis, the study of tissue mechanical properties by genetic or other experimental manipulations of these properties seems to be more challenging. To gain such understanding, genetic and biophysical analyses will have to be used in complementary fashion. Experimental designs will aim for situations in which the two mechanisms, programmed cell behavior and generic physical processes, can be genetically separated. However, since these two classes of mechanisms are closely interconnected at the molecular level, the experimental outcomes might suffer from the high degree of pleiotropy present in the system. For example, cell adhesion depends on the interaction of adhesion molecules with the cortical cytoskeleton, which in turn is essential for cell motility; changes in any of the respective components affects the others in complex ways. Thus, modulating tissue surface tension by altering cell adhesion molecule expression or functionality, without simultaneously affecting active cell motility, may not always be possible. The identification of critical nodes in such regulatory networks will therefore remain a necessary and promising undertaking in the future.

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Figure 1. Mesoderm layer formation during *Drosophila* gastrulation

(A) Schematic drawing of sagittal sections of *Drosophila* embryos during mesoderm (Me) layer formation. The mesoderm (pink color) forms in the ventral (V) domain of the embryo and is internalized beneath the ectoderm (Ec). In the posterior (P) domain the endoderm primordium is formed by the posterior midgut invagination (pmg). In the anterior (A) domain the cephalic furrow (cf) separates the head from the trunk segments. During layer formation, the mesoderm extends in anterior-posterior direction and at the same time spreads into a thin cell sheet along the dorsal-ventral axis. (B) Histological cross sections through embryos during layer formation; cell membranes are visualized by immunolabeling the membrane protein Neurotactin (red) and the mesoderm cells (Me) are indicated in green by staining against the nuclear mesoderm marker Twist. Schematic drawings indicate the dynamic expression of the FGF8-like growth factors Pyr and Ths and the expression of the FGF receptor Heartless (Htl). Arrows indicate the movement of cells as determined by live cell imaging and tracking.

Figure 2. Mesoderm layer formation during *Xenopus* gastrulation

(A) Schematic drawing of sagittal sections of *Xenopus* embryos during mesoderm layer formation between early and late gastrula stages. Leading edge mesendoderm (LEM), prechordal mesoderm (PCM) and chordamesoderm (CM) on the dorsal side, and Xbra expressing ventral mesoderm are indicated. Ec, ectoderm; En, endoderm; BC, blastocoel cavity; AC, archenteron cavity. After Damm and Winklbauer (2011). (B) High magnification view of prechordal mesoderm in an early mid-gastrula, fractured sagittally. Dashed, red line indicates the boundary between endoderm and prechordal mesoderm. (C) Overview of dorsal mesoderm regions in a late mid-gastrula, fractured sagittally. Arrows indicate cell orientation. Abbreviations in (B,C) as in (A).