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Fibroblast growth factor receptor-dependent morphogenesis of the *Drosophila* mesoderm

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The *Drosophila* fibroblast growth factor (FGF) receptors Heartless and Breathless are required for the morphogenesis of the mesoderm and the tracheal system. In this article we discuss a number of questions relating to the morphogenesis of these tissues and speculate on poorly understood aspects of the underlying mechanisms. As yet a ligand has not been identified for Heartless, but in the case of Breathless the ligand may in some situations act as a chemotactic signal. We consider it unlikely that release of a distant chemotactic signal plays a role in the morphogenesis of the mesoderm. Instead we propose that the change in the mesoderm from an invaginated epithelium to a single layer of cells spread out on the ectoderm could be a result of the mesodermal cells trying to maximize their contact with the ectoderm. Exactly how the activation of the FGF receptors affects cell behaviour and leads to cell movement is not understood. The signal could simply be permissive, causing cells to become motile, or it could act as a directional signal for cells that are already motile, or perhaps provide both functions. Furthermore, it is unclear how signal transduction is coupled to morphological change. It seems unlikely that activation of transcription targets is essential for cell migration and it is possible that FGF signalling may have a direct effect on the cytoskeleton independent of the activation of the mitogen-activated protein kinase cascade. Analysis of the function of *dof*, which encodes a cytoplasmic protein required for FGF signal transduction may provide an insight into these issues.

Keywords: fibroblast growth factor (FGF); Heartless; Dof; signalling; tyrosine kinase; migration

1. INTRODUCTION

Cell rearrangement and migration are important morphogenetic processes that shape the developing organism and reshape parts of the adult during wound healing, angiogenesis and regeneration. Cell movement is controlled at various levels. The differentiation state of the cells, and specifically the transcription factors present, determines which receptors and components of signal transduction pathways are expressed and hence how the cells respond to their environment. The behaviour of differentiated cells is affected by different cues in the environment. There is good evidence that the extracellular matrix, neighbouring cells, growth factors and chemotactic factors all influence cell movement. When a cell receives a signal from the environment it is relayed to the actin cytoskeleton by the small GTP-binding proteins Rho, Cdc42 and Rac (Chant & Stowers 1995; Ridley & Hall 1992). These molecules can modulate the actin cytoskeleton of cultured cells in different ways, resulting in the appearance of filopodia, membrane ruffles or lamellipodia. However, little is known about how these structures influence or are employed *in vivo* in the morphogenic processes of multicellular organisms.

One type of receptor for extracellular signals that has been shown to be necessary for cell migration in *Caenorhabditis elegans* and *Drosophila* is the receptor for fibroblast growth factor (FGF), which is a receptor tyrosine kinase (RTK). Like other receptors of this class, the FGF receptor activates the mitogen-activated protein kinase (MAPK) cascade via the adaptor Grb2/Drk and the small GTPase Ras (Wassarman *et al.* 1995). However, unlike other RTKs, it does not bind directly to Grb2 (see Klint *et al.* 1995; Kouhara *et al.* 1997). One of the unsolved problems is therefore how the signal from the FGF receptor is transmitted to Grb2 and Ras. Other proteins must exist that help in establishing a link between the FGF receptor and the MAPK module. Two candidates, FRS2 and Dof, have been found (Kouhara *et al.* 1997; Vincent *et al.* 1998; Wang *et al.* 1996). FRS2 was identified biochemically in vertebrates as a protein that is phosphorylated upon stimulation of cells by FGF, and forms a complex with Grb2 and Sos. Dof was found in *Drosophila* and shown to be essential for the transmission of the FGF signal to MAPK, acting downstream of the receptor, but upstream of Ras (Imam *et al.* 1999; Michelson *et al.* 1998; Vincent *et al.* 1998).

2. FGF RECEPTOR SIGNALLING DURING *DROSOPHILA* DEVELOPMENT

The two known FGF receptors in *Drosophila*, Heartless and Breathless, are required for the morphogenesis of different tissues. The gene *breathless* encodes the FGF receptor expressed in the respiratory system of the fly, the tracheae, and is needed both for the establishment of the tracheal tree and for its remodelling in response to changes in oxygen requirement (Jarecki *et al.* 1999;

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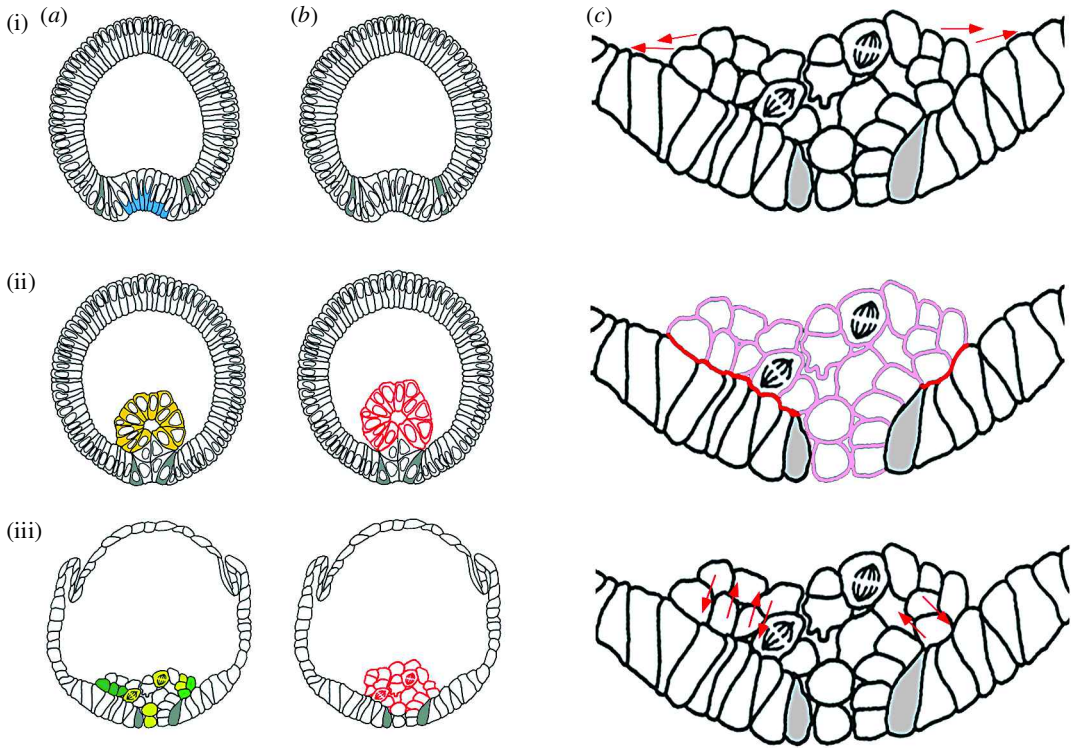

Figure 1. (*a*) Stages of mesoderm morphogenesis in the *Drosophila* embryo. Drawings of cross-sections of embryos at three successive stages of mesoderm invagination. Cells in which specific changes are observed are marked in colour. The mesectodermal cells, which border the mesoderm on each side, are filled in with grey. (i) The invagination begins with the formation of the ventral furrow by apical constriction of the most ventral cells (constricted apical sides of cells in blue). (ii) When the mesoderm has invaginated, its central part, still an epithelial tube, begins to make contact with the ectoderm. Yellow: cells expressing the FGF receptor Heartless and its downstream signalling mediator, Dof. MAPK activity begins to be detectable in the first cells that have already contacted the ectoderm (not marked here). (iii) As the mesodermal tube continues to flatten against the ectoderm under the influence of FGF receptor signalling (dark green: cells in which the MAPK cascade has been activated), cell division begins throughout the mesoderm primordium (light green). (*b*) Defects in *heartless* and *dof* mutant embryos. (i) The first steps of mesoderm morphogenesis are unaffected. The ventral furrow forms and the mesoderm invaginates as an epithelial tube. (ii) Once the tube is fully internalized, the establishment of the tight contact between the mesoderm and the ectoderm is lost. No MAPK activity is detectable. (iii) The mesoderm loses its epithelial structure and its cells divide. Now some cells are in contact with the ectoderm but most remain in the centre near the site of invagination. Activation of MAPK is still not observed. (*c*) Possible mechanisms by which the mesodermal cell layer is established. (i) The cells at the leading edge of the mesoderm migrate towards a chemotactic signal at the dorsal edge of the ectoderm. (ii) The cells of the mesoderm have a low affinity for each other (pink) and a high affinity for the ectoderm (red) and seek to maximize their contact with the ectoderm. (iii) Convergent extension in the mesoderm reduces the mesoderm to a single-cell layer and extends it dorsally. For convergent extension to occur in the direction indicated by the arrows it would be necessary to establish a difference between the external and more internal cells.

Klämbt *et al*. 1992; Reichman-Fried *et al*. 1994). The gene *heartless* encodes an FGF receptor expressed in the embryonic mesoderm and was first identified because of its essential role in the development of one of the mesodermal derivatives, the heart (Beiman *et al*. 1996; Gisselbrecht *et al*. 1996; Shishido *et al*. 1993, 1997).

The development of the tracheal system begins with the invagination of the epithelial primordium. The anlage of the tracheal tree consists of ten segmentally arranged pairs of deep ectodermal invaginations of approximately 20 cells each. Each of these invaginated sacks undergoes a stereotyped sequence of cell rearrangements that converts the epithelial invagination into a branched structure (Samakovlis *et al*. 1996). Further cell rearrangements extend and bifurcate the branches, a subset of which eventually fuse to create a continuous tracheal system. Finally, fine tertiary branches grow into tissues, directed by the oxygen requirement of the target tissues (Jarecki *et al*. 1999). In *breathless* mutants, the epithelial invagination of the tracheal primordium is unaffected, but the later steps of tracheal morphogenesis fail to occur. However, at least part of the differentiation programme of the cells in the unbranched invaginations continues, as judged by the expression of several late tracheal differentiation markers (Klämbt *et al*. 1992; Reichman-Fried *et al*. 1994; Samakovlis *et al*. 1996).

The ligand for the tracheal FGF receptor has been identified as the product of the *branchless* gene (Sutherland *et al*. 1996). The *branchless* gene, which codes for an FGF homologue, shows the same mutant phenotype as *breathless*. Interestingly, *branchless* is expressed near the tips of the growing tracheal branches, fading in the region which a branch has just reached, and being activated at the next point towards which the branch is destined to extend. This has led to the suggestion that Branchless acts as a chemotactic signal (Sutherland *et al*. 1996). The chemotactic properties of Branchless are best demonstrated by its ability to attract the growth of the fine tertiary branches in larvae towards oxygen-starved cells (Jarecki *et al*. 1999).

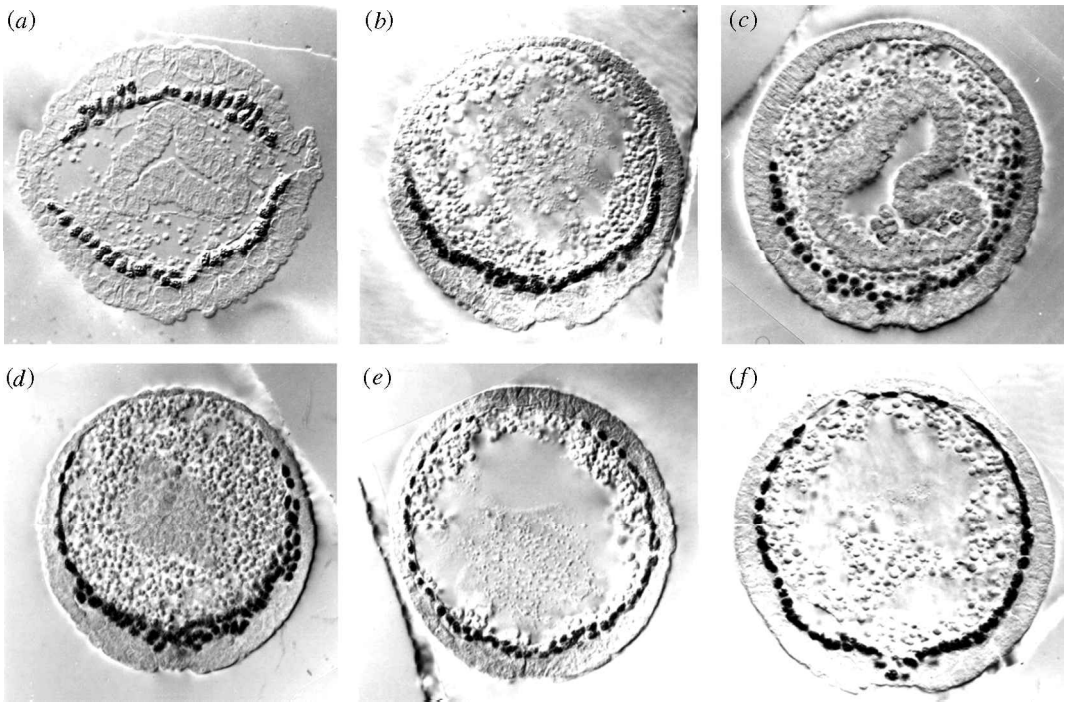

Figure 2. The consequence of changing ectodermal cell fates upon the migration of the mesoderm. Embryos in which the fates of ectodermal cells are changed due to mutations in zygotic ventralizing genes were used to test whether the fates of the underlying ectoderm cells influence the migration of mesodermal cells (*a*) *wt*; (*b*) *sog*; (*c*) *tsg*; (*d*) *srw*; (*e*) *tld*; (*f*) *dpp*. Transverse sections at about 50% egg length of embryos at the fully extended germ-band stage are shown, which have been stained with antibodies directed against Twist to visualize the mesoderm. The phenotypes of the mutant embryos are variable, those with strong phenotypes (i.e. where migration was furthest towards the dorsal midline) are shown.

The mesoderm also invaginates as an epithelial layer, driven by cell shape changes under the control of two transcription factors, Twist and Snail, which also control all subsequent steps of mesoderm development, including the expression of the FGF receptor Heartless and Dof. When the central part of the mesoderm is fully internalized, its cells make contact with the ectoderm (figure 1*a*). Adherens junctions that had been established in the blastoderm are now lost (Oda & Tsukita 1999) and the epithelium dissociates into single cells which spread out to cover the ectoderm as a single-cell layer (figure 1*a*). The loss of the epithelial state does not depend on cell division, although the cells divide during this process, since it also occurs in *string* mutants, in which no division takes place (Leptin & Grunewald 1990). One possibility is that this state is caused or facilitated by mesodermal cells switching from expression of ectodermal E-cadherin to N-cadherin. The change from E- to N-cadherin is under the control of the two key transcription factors that regulate development of the mesoderm. Snail represses E-cadherin in the mesoderm, while Twist activates the expression of N-cadherin (Oda *et al*. 1998).

The function of the FGF signalling pathway is needed immediately after mesoderm invagination. In *heartless* and *dof* mutants the mesoderm primordium fails to make contact with the ectoderm and spread out as a single-cell layer, and initially remains near the site of invagination (figure 1*b*). Mesodermal cells only begin to distribute over the ectodermal surface after a long delay. The resulting mesoderm varies in thickness and is narrower than in the wild-type. One effect of this is that cells at the edge of the mesoderm, which are normally induced by a signal from the ectoderm to develop as visceral musculature and heart, fail to reach the regions in which they can receive this signal, and the primordia of these tissues are therefore reduced. In wild-type embryos the mesodermal cells that establish the initial contact between the mesoderm and the ectoderm accumulate the diphosphorylated form of extracellular signal-regulated kinase (Erk) (Gabay *et al*. 1997). The activation of the MAPK cascade in these cells is absolutely dependent upon FGF-mediated signalling, since activation of Erk does not occur in either *heartless* or *dof* mutants (Gabay *et al*. 1997; Vincent *et al*. 1998). It is not exactly clear how Dof acts within the signalling pathway or with which other proteins it interacts. Dof is a cytoplasmic protein that unlike most intracellular signalling components, which are expressed ubiquitously, is only expressed in cells that express the FGF receptors. The protein contains an ankyrin repeat, a coiled-coil structure and many tyrosines within environments that suggest that if phosphorylated they could act as binding sites for the SH2 domains of proteins such as Grb2/drk, Csw/Shp2, Ras-GAP and PI3-kinase (Vincent *et al*. 1998). Hence, Dof may be needed to assemble the FGF receptor or allow it to autophosphorylate, it may be an adaptor that links the receptor to Ras, or it may perhaps be involved in distributing the signal to different downstream targets.

3. HOW DOES FGF SIGNALLING CONTROL CELL MOVEMENT?

How cell behaviour is affected by the inability of the mesoderm to receive an FGF signal in *heartless* and *dof*

mutants is not clear. Activation of the FGF receptor may simply play a permissive role in migration of the mesoderm causing cells to become motile, so that they can move towards a second, directional signal. Alternatively, it might function in an instructive fashion as a directional signal for cells that are already motile; or it could even provide both functions, induction of motility and spatial cues. When Branchless, the ligand for the FGF receptor Breathless, is secreted by oxygen-starved cells it clearly has the ability to attract the extensions of tracheal cells (Jarecki *et al.* 1999). However, it is less clear that this situation is relevant to cell migration in the mesoderm, since a ligand for Heartless has not yet been identified and it is not clear that a ligand must act as a chemoattractant for mesodermal cells.

There are several reasons that make it unlikely the dorsalward spreading of the mesoderm is directed by a distant chemotactic signal originating from the dorsal part of the ectoderm (figure 1*c*). First, when the width of the mesoderm primordium is reduced and fewer mesodermal cells invaginate, these cells spread out only until they have flattened into a single-cell layer (Maggert *et al.* 1995). If there was directed cell movement towards a chemotactic signal, one would expect cells to migrate to the dorsal edge even when the mesodermal primordium consisted of a small number of cells. However, the leading edge of the mesodermal cell layer does not continue to move to the region at the dorsal edge of the ectoderm and no gaps appear in the mesodermal cell layer. Second, we have analysed mesoderm migration in mutant embryos in which the fates of ectodermal cells had been changed. No matter what changes in the fate of the ectoderm were introduced, the mesodermal cells always spread dorsally (figure 2). Therefore, there is no specific ectodermal cell population required to direct the movement of mesodermal cells dorsally.

There are two other mechanisms which might create a single-cell layer from an epithelial tube. The mesodermal cells could either seek to maximize their contact with the ectoderm, or they could seek to intercalate between each other (figure 1*c*). If mesodermal cells had a propensity to try to increase their contact with the ectoderm, this would initially produce overall directional movement towards the ectoderm. Cells already in contact with the ectoderm would not move much at all, but the other mesodermal cells would have to insert themselves between these cells, perhaps by using filopodia to find the ectoderm and pull themselves towards it, pushing the cells that made the initial contact with the mesoderm aside. Eventually this would result in a net movement of the mesodermal cell mass away from the site of invagination until the cells covered the area of the mesoderm required for each one to adhere to the ectoderm. This is precisely the situation seen when the number of mesodermal cells is reduced. While there is no evidence for such a process in the mesoderm, long filopodial extensions of imaginal disc cells towards sources of FGF have indeed been observed (Ramirez-Weber & Kornberg 1999), although at the moment their significance is not clear. It is possible that FGF might act as an instructive signal, emanating from the ectoderm and serving as an attractant towards the underlying ectodermal cells, rather than towards a specific part of the ectoderm lying some distance from the cells. A process of convergent extension or cell intercalation among mesodermal cells, which would result in a single-cell layer apposed to the ectoderm, is more difficult to envisage. In this instance FGF might alter the properties of cells already in contact with the ectoderm cells and hence provide the spatial information necessary for convergent extension or cell intercalation to form a single-cell layer apposed to the ectoderm. However, it is equally possible that in either situation, the FGF signal might act simply as a permissive signal, stimulating motile activity or extension of filopodia or lamellipodia, while other cell adhesion or recognition molecules are used to guide movement toward the correct target.

It is debatable whether all aspects of cell behaviour controlled by FGF signalling are dependent upon one another. Migration might be regulated via the transcriptional activation of genes by FGF signalling, or by a direct effect of FGF signalling on the cytoskeleton. One of the transcriptional targets in the tracheae is the *Drosophila* homologue of the serum response factor (SRF), which indeed controls the development of the fine tertiary tracheal branches (Montagne *et al.* 1996). In this case the morphogenetic signal of FGF therefore seems to be mediated by transcriptional activation of SRF. However, recent experiments in tissue culture cells indicate that the activation of SRF in the trachea could be a consequence of depletion of G-actin within these cells (Sotiropoulos *et al.* 1999). At earlier stages of development of the tracheal tree, no such links are known, and it is therefore conceivable that FGF signalling might affect cell behaviour not via transcription of genes but by a more direct mechanism. Also in the mesoderm a morphogenetic mechanism independent of transcription appears more likely since there is not sufficient time between the activation of the MAPK cascade via the FGF receptor and detectable morphogenetic activity to allow transcription of new genes. In fact, it is not clear that the observed activation of MAPK is a prerequisite for, or a consequence of, establishing contact between ectoderm and mesoderm. It may be required for the spreading of the mesoderm on the ectoderm, or a parallel process necessary for the activation of genes required at a later stage. MAPK activation can only be demonstrated by the time the mesodermal cells have already contacted the ectoderm. An important question to address to understand how FGF signalling influences cell migration will be whether the signalling pathway downstream of the FGF receptor branches or is directed only to Ras and from there to the MAPK cascade, and at what level an incoming signal might be directed to different subcellular targets to cause reorganization of the cytoskeleton.

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