Hindgut visceral mesoderm requires an ectodermal template for normal development in *Drosophila*

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Accepted 7 November; published on WWW 21 December 2000

**SUMMARY**

During *Drosophila* embryogenesis, the development of the midgut endoderm depends on interactions with the overlying visceral mesoderm. Here we show that the development of the hindgut also depends on cellular interactions, in this case between the inner ectoderm and outer visceral mesoderm. In this section of the gut, the ectoderm is essential for the proper specification and differentiation of the mesoderm, whereas the mesoderm is not required for the normal development of the ectoderm.

Wingless and the fibroblast growth factor receptor Heartless act over sequential but interdependent phases of hindgut visceral mesoderm development. Wingless is required to establish the primordium and to enhance Heartless expression. Later, Heartless is required to promote the proper differentiation of the hindgut visceral mesoderm itself.

Key words: *Drosophila*, Embryo, Visceral mesoderm, Hindgut ectoderm, Cell signalling

**INTRODUCTION**

In the *Drosophila* embryo, the mesoderm becomes subdivided soon after gastrulation into populations of cells that will give rise to the various mesodermal derivatives (Bate, 1993). Although we now have a good understanding of the mechanisms by which mesodermal cell fates are allocated in the trunk (or segmented region) of the embryo (Baylies et al., 1998; Frasch, 1999), little is known about how the terminal mesoderm is organised, or the mechanisms by which it is patterned.

Visceral muscle forms the outer layer of the gut. It arises from clusters of cells in both the trunk and terminal mesoderm and develops in concert with the inner layer of the gut, which is composed both of endodermal cells (midgut) and ectodermal cells (foregut and hindgut). Two subtypes of visceral muscle envelop the midgut endoderm, the circular visceral muscle, which arises from the caudal mesoderm (Georgias et al., 1997; Broihier et al., 1998; Kusch and Reuter, 1999). To emphasise their distinct origins, the subtypes of visceral mesoderm have been referred to as the trunk visceral mesoderm and caudal visceral mesoderm (Broihier et al., 1998; Kusch and Reuter, 1999). Since the hindgut visceral muscle also arises from the caudal mesoderm and the longitudinal visceral muscle eventually ends up in the trunk of the embryo, we feel that this terminology could lead to confusion. For these reasons, we would like to propose a simple and logical nomenclature for the different subtypes of visceral mesoderm in the *Drosophila* embryo. We have designated the circular visceral mesoderm (which gives rise to the circular visceral muscle) as the CVM, the longitudinal visceral mesoderm (which gives rise to the longitudinal visceral muscle) as the LVM and the hindgut visceral mesoderm (which gives rise to hindgut visceral muscle) as the HVM. Finally, we suggest the abbreviation of FVM for the foregut visceral mesoderm, though consideration of this subtype of visceral mesoderm is beyond the scope of this paper.

Most of our understanding of visceral mesoderm development has centred on the CVM. Several observations indicate that distinct mechanisms govern the development of the HVM. First, whereas the CVM is derived from 11 metamerically repeated clusters of cells on either side of the embryo (Tremml and Bienz, 1989; Azpiazu and Frasch, 1993), the HVM arises from a single cluster of cells that migrates en masse over the hindgut ectoderm. Second, unlike the CVM, cells of the HVM primordium express relatively high levels of Twist (Dunin Borkowski et al., 1995; Baylies and Bate, 1996).

One feature of the CVM is its role in promoting the development of the midgut endoderm. The endoderm is derived from anterior and posterior embryonic cells that invaginate during gastrulation (Skaer, 1993; Campos-Ortega and Hartenstein, 1997). To form the midgut endoderm, these cells migrate over a considerable distance before they meet in the centre of the embryo. The CVM serves as a substrate on which the endodermal cells migrate and later undergo a mesenchymal-epithelial transition (Reuter et al., 1993; Tepass and Hartenstein, 1994). In addition, the CVM provides the endoderm with anteroposterior (A/P) cues. Thus, signals from the visceral mesoderm are required for the expression of genes in discrete domains within the endoderm (Bienz, 1994; Bienz, 1995; Dunin Borkowski et al., 1995; Baylies and Bate, 1996; Azpiazu and Frasch, 1993; Tremml and Bienz, 1989; Kusch and Reuter, 1999; Broihier et al., 1998; Georgias et al., 1997; Baylies et al., 1998; Frasch, 1999).
The best known example of this is the regulation of labial expression in the endoderm by signals (Decapentaplegic (Dpp), Wingless (Wg) and Vein (Vm)) derived from the visceral mesoderm.

Unlike the midgut endoderm, the hindgut ectoderm maintains its tubular structure throughout embryogenesis and is intrinsically patterned, as seen by the restricted expression of a number of segmentation genes (Skaer, 1993; Bienz, 1994; B. S. M. and M. B., unpublished observations). This leads us to suggest that one of the features distinguishing the development of the CVM from the HVM might be the direction of instructional interactions between the visceral mesoderm and inner epithelial layer of the gut.

Here we show that, unlike midgut endoderm, the hindgut epithelium is essential for the proper specification and differentiation of the HVM. In addition, hindgut ectoderm can develop largely wild-type characteristics in the absence of visceral mesoderm. Of the three signalling molecules known to be expressed in the hindgut ectoderm, (Wg, Hedgehog (Hh) and Dpp; Hoch and Pankratz, 1996), only Wg is indispensable for the formation of differentiated HVM. Wg is required to enhance Twist expression in the HVM, mirroring its role in promoting the development of the somatic mesoderm in the trunk by maintaining enhanced levels of Twist expression (Bayles and Bate, 1996; Riechmann et al., 1997). Further, we show that a fibroblast growth factor (FGF) receptor, Heartless (Htl), is also essential in the development of the HVM.

Wg and Htl are required during different but overlapping phases of HVM development. Wg acts early in both the hindgut ectoderm and mesoderm to establish the HVM primordium and promote its development. Wg also regulates the expression of htl, which is required later to promote the specification and differentiation of the HVM by activating the mitogen-activated protein kinase (MAPK) cascade. Our work indicates that during the development of the HVM, the hindgut ectoderm is required as a template and acts as a source of signalling by Wg and a presently unknown FGF signal.

MATERIALS AND METHODS

Fly stocks

The following flies were used: Oregon R, a null allele of twist, twist1996 (Simpson, 1983), a null allele of wingless, wgCM (Baker, 1987), a temperature sensitive allele of wg, wgE114 (Gonzalez et al., 1991), a hypomorphic allele of heartless, htl28 (Shishido et al., 1997), a loss-of-function allele of string, stg9M3 (Hoch et al., 1994) and an amorphic allele of fork head, fkh1 (Weigel et al., 1989). We also used the following GAL4 and UAS lines: 455.2GAL4 (Hinz et al., 1994), twistGAL4 (Bayles and Bate, 1996), 24BGAL4 (Brand and Perrimon, 1993), UASCAD2 (Dunin Borkowski and Brown, 1995), UASSweeper (John and Brand, 1997, from W. Gehring), UASArmS10C (an activated form of arm, Pai et al., 1997). The embryos were fixed and stained with anti-Wg antibody before calculating the stage of the earliest embryo.

Temperature shift experiments

wgE114 embryos produce viable larvae at 18°C whereas at 25°C they exhibit a wg null phenotype (Baker, 1988; Bejsovec and Martinez-Arias, 1991). The null phenotype at 25°C is due to the retention of Wg in the secretory apparatus (Gonzalez et al., 1991). Thus loss of functional Wg at the restrictive temperature depends on the half-life of the protein, which has been estimated to be approximately 20 minutes (Skaer and Martinez-Arias, 1992).

We kept the wgE114 stock in large cages at 18°C. Prior to collection of embryos, the apple juice agar plates were changed several times to ensure that the female flies did not retain fertilised embryos. wgE114 embryos from hourly collections at 18°C were transferred to small Eppendorf tubes and placed in a PCR machine where they were kept at 18°C until the desired stage, before shifting to 29°C. The embryos were then left to develop to stage 14 before being fixed. As a control for timing embryos at different temperatures, Oregon R embryos from 1-hour lags at 18°C or 29°C were transferred to a PCR machine and left to develop for a number of hours at 18°C or 29°C, respectively. The embryos were fixed and stained with anti-Wg antibody before calculating the stage of the earliest embryo.

Histochemistry

Whole-mount in situ hybridisation of a digoxigenin-labelled bagpipe probe (bag; Azpiazu and Frasch, 1993; Boehringer Mannheim) was performed according to the method of Tautz and Pfeifle (Tautz and Pfeifle, 1989) as modified by Ruiz-Gómez and Ghysem (Ruiz-Gómez and Ghysem, 1993). Immunological staining of whole-mount embryos was performed essentially as in Rushton et al. (Rushton et al., 1995) using the Vectastain Elite ABC kit (Vector Laboratories) with the slight modification that we simultaneously incubated both primary antibodies in double-labelling experiments. To double label for anti-Wg and bag we first immunologically stained the embryos, as described above, developing the stain without salts. This was followed by in situ hybridisation (see above), changing from Triton-X to Tween as the detergent in PBT.

The following primary antibodies were used: anti-muscle Myosin (Kiehart and Feghali, 1986), anti-Wg (available from Developmental Studies Hybridoma Bank, from S. Cohen), anti-β-galactosidase (Cappel and Promega), anti-CD2, (Dunin Borkowski and Brown, 1995), anti-Connectin (Meadows et al., 1994), anti-Twist (Roth et al., 1995), anti-Heartless (Shishido et al., 1997), anti-Dichaete (Sanchez-Soriano and Russell, 1998) and anti-diphospho-ERK (Gabay et al., 1997).

Embryos were analysed using a Zeiss Axioplan microscope. Any negatives taken were scanned with a Nikon Coolscan. Other images were captured using a JVC analogue camera linked to a PC. Images and figures were processed in Adobe Photoshop 5.0.

RESULTS

The origin and development of the hindgut ectoderm and visceral mesoderm

The caudal mesoderm gives rise to two populations of visceral mesoderm, the HVM and the LVM (Georgias et al., 1997; Broihier et al., 1998; Kusch and Reuter, 1999; B. San Martin, PhD thesis, University of Cambridge, 1999). The HVM primordium is distinguishable at stage 10 both by the expression of bagpipe (bag, Fig. 1A) and by virtue of its relatively higher levels of Twist (Fig. 1B). It includes all mesodermal cells caudal to the 15th Wg stripe (Fig. 1C), which, unlike mesoderm cells in the trunk (Dunin Borkowski et al., 1995), are organised in...
multiple layers soon after gastrulation (not shown). In contrast, the LVM primordium arises from cells adjacent and egg-posterior to the HVM (Broihier et al., 1998; B. San Martin, PhD thesis, University of Cambridge, 1999).

The hindgut ectoderm is derived from a ring of cells that lies just anterior to the posterior midgut primordium at the cellular blastoderm stage (Hartenstein et al., 1985, see reviews by Skaer, 1993; Campos-Ortega and Hartenstein, 1997). At stage 7, these cells invaginate into the embryo and, by stage 10, form a hollow tube that extends by cell division and rearrangement. HVM cells become closely associated with the invaginated hindgut ectoderm at stage 11 (Fig. 1D and see Fig. 6). Later in this stage, all HVM cells begin to express Connectin (Fig. 2G, Gould and White, 1992; Nose et al., 1992) and this together with Twist expression, can be used to follow the cells as they move over the hindgut ectoderm tube.

As the germ band retracts, the hindgut tube undergoes considerable morphological rearrangements. By stage 13, it lies longitudinally (arrow in Fig. 2F) from the anus and bends at right angles to join the posterior midgut. Over time, the bend flattens laterally and the hindgut lengths, revealing morphological subdivisions (Hoch and Pankratz, 1996; B. S. M. and M. B., unpublished observations). HVM cells continue to cover the ectodermal tube during these stages and as they mature, they begin to express Myosin (Fig. 2M).

**The hindgut ectoderm develops independently of mesoderm**

To determine whether hindgut ectoderm requires interactions with mesoderm to form normally, we followed its development in twist mutant embryos that lack mesoderm (Simpson, 1983; Leptin and Grunewald, 1990; Leptin, 1991). In these embryos, cells of the hindgut ectoderm can form a tube that bends anteriorly, lengthens partially at stage 13 and expresses Wg and Dichaete in restricted domains, similar to wild-type embryos (Fig. 2B,D, Hoch and Pankratz, 1996; Sanchez-Soriano and Russell, 2000). Thus, characteristic features of ectodermal gut development occur in the complete absence of surrounding visceral mesoderm.

**Hindgut ectoderm is required for the development of the surrounding visceral mesoderm**

To test whether hindgut ectoderm is needed for the proper development of the HVM, hindgut ectoderm cells were selectively killed early in their embryogenesis using the GAL4/UAS system (Brand and Perrimon, 1993). We used 455.2GAL4 (Hinz et al., 1994), which is expressed in the primordium of the hindgut ectoderm from stage 9 onwards but not in the caudal mesoderm (Fig. 2E,F), to drive expression of reaper, a gene whose protein product promotes death of those cells in which it is expressed (White et al., 1994; Nordstrom et al., 1996; White et al., 1996).

As in wild-type embryos, Twist is expressed strongly in the prospective HVM at early stage 11 in embryos carrying 455.2GAL4;UASreaper constructs (compare Fig. 1D with 2I). However, even though the morphology of the hindgut ectoderm appears normal at this stage (not shown), the bulk of the HVM is not closely associated with the hindgut ectoderm (Fig. 2I). By late stage 11, the number of HVM cells expressing Connectin (Fig. 2H) or Twist (not shown) is clearly reduced. This is concomitant with the severe disruption in the morphology of the hindgut ectoderm. During stage 13, many HVM cells die (Fig. 2K). The surviving cells are those that attach to any remaining hindgut ectoderm (Fig. 2L); these cells persist, differentiating to form a variable amount of visceral muscle (Fig. 2N,O). Thus, we conclude that the hindgut ectoderm acts as a template to promote the development and differentiation of the HVM.

**Wg signalling is essential for HVM development**

The hindgut ectoderm might be required simply because it is an essential substrate for the development of the HVM. To test whether it is also the source of essential signals, we analysed the formation of the HVM in embryos with mutations in dpp, hh and wg, which are known to be expressed in the hindgut ectoderm during embryogenesis (Hoch and Pankratz, 1996). Of these, only Wg is essential for the differentiation of the HVM (Fig. 3B and Fig. 6, not shown for Dpp and Hh). In embryos mutant for wg, bap expression is reduced (Fig. 3A) and Twist expression fails to be enhanced in the HVM at stage 10 (not shown). By stage 11, even fewer cells express Connectin and Twist (not shown). By mid stage 12, some embryos completely lack Connectin and Twist expression in the caudal mesoderm (not shown), while in other embryos only a few cells maintain their expression and these cells are unable to cover the whole of the hindgut ectoderm (shown for Connectin in Fig. 3D). The few remaining cells may eventually express Myosin (Fig. 3B), but this is difficult to assess because some syncitial somatic muscle cells appear to attach ectopically to the hindgut ectoderm in these embryos.

In wg embryos, proctodeal cells fail to divide after gastrulation (Skaer and Martinez-Arias, 1992). Thus, the hindgut ectoderm is very small and this reduction in the size could indirectly cause the defects observed in the development of surrounding visceral mesoderm. To test this, we compared the development of the HVM in string (stg) embryos, where all cells fail to divide after the cellular blastoderm stage (cycle 13, Edgar and O’Farrell, 1989). As in wg embryos, a very small ectodermal hindgut develops, but many more visceral mesoderm cells maintain...
Fig. 2. (A-D) Hindgut ectoderm development in the absence of visceral mesoderm. (A,B) Wg expression in the hindgut ectoderm of wild-type and *twi*ΔID96 embryos, respectively (stage 13, lateral views). Asterisks mark the lumen of the gut. Arrows and arrowheads point to the posterior and anterior hindgut Wg domains, respectively. (C,D) Dichaete expression in the hindgut ectoderm of wild-type and *Df(2R)S60/twi*ΔID96 embryos, respectively (stage 15, dorsal view). Though the hindgut ectoderm fails to elongate properly in *Df(2R)S60/twi*ΔID96 embryos, Dichaete expression is still restricted to the central portion of the hindgut (arrows mark the extent of this expression and asterisks mark the lumen of the gut). *Df(2R)S60/twi*ΔID96 embryos fail to retract their germ band fully and this may lead indirectly to defects in the morphology of the hindgut ectoderm. (E,F) 455.2GAL4 drives expression in the hindgut ectoderm. CD2 expression in 455.2GAL4; UASCD2 embryos at stage 10 (E) and stage 13 (F) (lateral views). The CD2 expression that is initiated in the primordium of the hindgut ectoderm (arrow in E) is maintained throughout embryogenesis (arrow in F). By stage 13 (F), CD2 expression has spread to the posterior midgut (arrowhead) and some cells of the ventral midline (asterisk). 455.2GAL4 does not drive expression in the caudal mesoderm (asterisk in E). (G-O) The hindgut ectoderm is needed for the proper specification and differentiation of the visceral mesoderm. (G,H) Connectin expression in wild-type and 455.2GAL4; UAS*Reaper* embryos at late stage 11 (lateral-caudal views). Fewer HVM cells express Connectin in 455.2GAL4; UAS*Reaper* embryos (compare arrows in G and H). (I) Twist expression in 455.2GAL4; UAS*Reaper* embryos at early stage 11 (ventral-caudal view). Twist is expressed strongly in the prospective HVM (arrow in I), but the majority of cells are not in close association with the hindgut ectoderm. (J-L) Connectin expression in wild type (J) and 455.2GAL4; UAS*Reaper* (K,L) embryos at stage 13. (J,K) Lateral views; (L) dorsal view. Arrow in K indicates a Connectin-expressing cell that is no longer attached to the hindgut ectoderm and, by its condensed morphology, appears to be dying (compare with wild-type cell, arrow in J). (L) Visceral mesoderm cells maintain Connectin expression when attached to hindgut ectoderm (arrow) even if only a small remnant remains (arrowhead). Asterisk marks the lumen of the remaining tube. (M-O) Myosin expression in the HVM at stage 15 (dorsal views, asterisks mark the hindgut lumen). (M) Wild-type embryo, arrow indicates the visceral muscle. (N,O) 455.2GAL4; UAS*Reaper* embryos raised at 25°C, arrows point to the visceral muscle that surrounds the remnant of hindgut ectoderm. Anterior is towards the left.

Connectin expression (compare Fig. 3D with 3E). These cells eventually envelop the whole of the ectodermal tube and differentiate to express Myosin (not shown). Thus, the defects in *wg* embryos cannot solely be explained by the reduced size of the hindgut ectoderm, suggesting that Wg signalling is required directly for the normal development of the HVM. **Wg signalling activity is required within the mesoderm**

If Wg signals to the surrounding mesoderm and promotes the development of visceral mesoderm, then the Wg signalling pathway should be activated in the caudal mesoderm. Consequently, in *wg* embryos, the development of the HVM might be rescued if the Wg signalling pathway was activated autonomously throughout the mesoderm. One of the components needed to transduce the Wg/Wnt signalling pathway is Armadillo (Arm; McGrea et al., 1990; Peifer and Wieschaus, 1990). We used a constitutively active form of *arm* (*arm*Δ10C) under the control of the UAS promoter (Pai et al., 1997) and two mesodermal GAL4 drivers, *twist*GAL4 and 24BGAL4 (Brand and Perrimon, 1993; Baylies and Bate, 1996), to activate the Wg pathway in the mesoderm. *twist*GAL4 activates expression throughout the mesoderm from stage 6 onwards and is maintained in the HVM throughout embryogenesis (B. S. M. and M. B., unpublished observations). In contrast, 24BGAL4 drives weak and patchy
expression at stage 9 (B. S. M. and M. B., unpublished observations), which is reinforced by late stage 10, and spreads to most mesoderm cells, including those of the HVM.

Expression of \textit{arm}^{S10C} under the control of \textit{twist}GAL4, but not 24BGAL4, partly rescues the development of the HVM in \textit{wg}^{-} embryos as seen by an increase in the expression of Connectin in \textit{wg}^{-} mutant embryos (D), more cells express Connectin in \textit{stg}^{-} (E) and \textit{fkh}^{1} (F) embryos. There is some rescue of Connectin expression in the HVM of \textit{wg}^{-} embryos that express \textit{arm}^{S10C} under the control of \textit{twist}GAL4 (G) or 455.2GAL4 (I), but not 24BGAL4 (H).

(J-L) Lateral views of Connectin expression at stage 14 in wild-type (J) and \textit{wg}^{IL114} (K,L) embryos; arrows indicate the HVM and arrowheads mark the extent of the hindgut ectoderm. (K) Only a few mesoderm cells maintain Connectin expression around the hindgut when \textit{wg}^{IL114} embryos are raised at the restrictive temperature throughout embryogenesis. (L) When Wg function is removed at mid stage 11 by shifting \textit{wg}^{IL114} embryos to 29ºC early in stage 11, the morphology of the hindgut ectoderm is improved and visceral mesoderm cells envelop the hindgut tube. Anterior is towards the left.

In \textit{wg}^{-} embryos, both hindgut ectoderm and mesoderm respond to the activation of the Wg signalling pathway and promote the development of the HVM.

Non-hindgut ectodermal sources of Wg can promote the development of the HVM

Although \textit{wg} is expressed in the primordium of the hindgut ectoderm, it is also expressed in epidermal stripes and transiently in the mesoderm (Baker, 1987; Baker, 1988; van den Heuvel et al., 1989; Gonzalez et al., 1991; Ingham and Hidalgo, 1993; Lawrence et al., 1994). Any of these sources of Wg might promote the development of the HVM. Indeed, in \textit{fork head} (\textit{fkh}) embryos, which lack Wg expression in the hindgut ectoderm (Hoch and Pankratz, 1996; B. S. M. and M. B., unpublished observations) many more caudal mesoderm cells begin to express Connectin (Fig. 3F) than in \textit{wg}^{-} embryos (Fig.3D). By stage 12 however, Connectin expression begins to diminish quite rapidly in \textit{fkh} embryos, but this is most likely due to the loss of hindgut ectoderm at later stages (Wu and Lengyel, 1998; B. S. M. and M. B., unpublished observations). This suggests that at least for the early stages of HVM development, the source of Wg need not be the hindgut ectoderm alone.

Loss of Wg signalling after stage 11 does not affect HVM development

Ablation of the hindgut ectoderm through directed expression
of reaper reveals that the HVM needs to be in contact with the hindgut ectoderm to differentiate (Fig. 2). Wg signalling might mediate this requirement for the hindgut ectoderm. To test this, we used the temperature-sensitive wgIL114 to remove Wg function after the HVM has begun to migrate over the hindgut ectoderm (mid stage 11, see Materials and Methods).

As expected, more cells develop as hindgut ectoderm when Wg function is first removed at mid stage 11 (Fig. 3L), compared with wgIL114 embryos raised at the restrictive temperature throughout (Fig. 3K). In addition, Connectin expressing visceral mesoderm cells envelop the hindgut ectoderm (Fig. 3L). Thus, loss of Wg after the initial HVM migration over the hindgut ectoderm does not prevent HVM cells from developing further.

Htl is essential for the development of the HVM

Since Wg is not required after mid stage 11, the requirement for the hindgut ectoderm could reflect the need for a second signalling pathway in the interactive process that underlies HVM differentiation. Indeed we find that htl, which encodes the FGF receptor tyrosine kinase DFR1 (Shishido et al., 1993; Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997), is essential for HVM development (Fig. 4 and see Fig. 6). Although the development of the hindgut ectoderm appears relatively normal in htl embryos, hindgut visceral muscle fails to differentiate altogether (Fig. 4C). The early stages of HVM development appear relatively normal, thus at stage 10, bap expression is established normally in the caudal visceral mesoderm (Fig. 4A). Defects in HVM development first appear at stage 11, once the HVM has begun to move over the hindgut ectoderm. Initial Connectin expression is reduced both in intensity and in cell number (Fig. 4B), while Twist expression is rapidly lost from HVM cells late in stage 11 (not shown). Expression of both markers is lost completely between stages 12 and 13 (not shown). Thus, Htl is necessary to promote the development of the HVM as it migrates over the hindgut ectoderm and to allow its further differentiation.

Htl functions through the activation of the MAPK signalling cascade

Htl is initially expressed throughout the mesoderm, though its expression is particularly strong in the primordium of the HVM (Fig. 4D) where it is maintained throughout embryogenesis (not shown). This is consistent with the possibility that the visceral mesoderm responds to an FGF signal from the hindgut ectoderm. If so, we would also expect activation of signalling cascades downstream of Htl in HVM cells.

Receptor tyrosine kinase activation can trigger a number of signalling cascades (Ullrich and Schlessinger, 1990), though these kinases generally activate the MAPK signalling pathway that uses the MAPK kinase (MEK) and extracellular signal-regulated kinase (ERK) isoforms (see reviews by Cobbs and Goldsmith, 1995; Seger and Krebs, 1995). Activation of this cascade can be followed by staining embryos against the double phosphorylated form of ERK (diphospho-ERK, Gabay et al., 1997). We find that diphospho-ERK is expressed weakly in HVM cells between stage 10-12 (Fig. 4E) and that this expression is reduced in htl embryos (Fig. 4F).

That Htl functions through the activation of the MAPK pathway in the development of the HVM is suggested by two additional observations. First, the loss of Connectin and Myosin expression in the HVM of htl embryos can be partially rescued when an activated form of Ras (a component of the MAPK signalling pathway) is expressed throughout the mesoderm (Fig. 4G, not shown for Myosin). Secondly, targeted mesodermal expression of a dominant negative form of Htl (DNHtl, Beiman et al., 1996) or of a
dominant negative form of Raf (also a component of the MAPK signalling cascade), both lead to a reduction in the number of Connectin-expressing HVM cells (Fig. 4H,I, cf. Fig. 3C).

Wg and Htl act over sequential and overlapping phases of HVM development

Our analysis shows that the establishment of the HVM primordium at stage 10 relies on Wg but not on Htl. Consequently, there is an early Htl-independent role of Wg. However, in this first phase of HVM development, Wg is also required for the normal development of Htl expression in the HVM and in wg embryos, expression of Htl in the cells that normally give rise to the HVM does not become enhanced at stage 9-10 (Fig. 5A).

At stage 11 and 12, Wg and Htl partly function independently of each other in controlling Connectin expression in the HVM. Thus, the loss of Connectin expression in the HVM is more severe in embryos mutant for both wg and htl than in either wg or htl mutant embryos. All wg;htl double mutant embryos lose expression of Connectin in the HVM by stage 12 (Fig. 5B), whereas at the same stage, some wg embryos maintain Connectin expression in a few cells (Fig. 3D) and there is weak expression of Connectin in htl embryos (Fig. 5C). If Wg can act in parallel with Htl to promote Connectin expression in the HVM, this explains why misexpression of Wg throughout the hindgut ectoderm of a htl mutant embryo directs strong expression of Connectin in HVM cells at stage 12 (Fig. 5D).

Differentiation of the HVM requires Htl (Fig. 4C). Wg is not required past stage 11 for the continued development of the HVM (Fig. 3L), and the HVM fails to differentiate and express Myosin when Wg is misexpressed in the hindgut ectoderm of a htl mutant embryo (not shown).

DISCUSSION

Distinct mechanisms govern the development of the visceral mesoderm in the midgut and hindgut

The gut in Drosophila consists of two cell layers, an inner epithelial layer and an external muscle layer. Our work shows that interactions between the inner ectodermal layer of the hindgut and the surrounding mesoderm are essential for the normal specification and differentiation of the HVM. This is very different from the development of the CVM, which, in the absence of midgut endoderm (e.g. in serpent mutant embryos; Reuter, 1994), can develop normally until at least late stage 12 (B. San Martin, PhD thesis, University of Cambridge, 1999).

The differences between the development of the CVM and the HVM reflect the way in which they form. The CVM arises from metamerically repeated clusters of cells, from parasegment 2-12 (Tremml and Bienz, 1989; Azpiazu and Frasch, 1993), which on allocation may already have acquired information as to their relative position in the A/P axis. These cells form a continuous template in the A/P axis over which the midgut endoderm spreads before coalescing as a tube. The HVM, by contrast, develops as a single cluster of cells that employ the ectoderm of the hindgut as a template over which to spread and differentiate.

The development of the midgut endoderm and hindgut ectoderm

Whereas the hindgut ectoderm can develop fairly normally in the absence of visceral mesoderm, the development of the midgut endoderm requires the presence of the CVM. This may reflect distinct morphogenetic events in the development of the hindgut and midgut. Anterior and posterior midgut endoderm
originate as clusters of cells and migrate over a considerable distance before they meet in the centre of the embryo (Skaer, 1993; Campos-Ortega and Hartenstein, 1997). Thus the endoderm is probably not intrinsically patterned, at least in the A/P axis (Bienz, 1994). The circular midgut visceral mesoderm, which arises locally in a metamerically repeated fashion, not only acts as a scaffold for the migration of the endoderm but also provides A/P patterning cues (Reuter et al., 1993; Bienz, 1994; Bienz, 1996; Tepass and Hartenstein, 1994). In contrast to the midgut endoderm, the hindgut ectoderm maintains its tubular structure throughout embryogenesis and is intrinsically patterned, as seen by the restricted expression of a number of segmentation genes (Skaer, 1993; Bienz, 1994, B. S. M. and M. B., unpublished observations). Consequently, the hindgut ectoderm can develop independently of surrounding visceral mesoderm.

The control of HVM development

The control of HVM development by Wg and Htl is sequential and interdependent (Fig. 6). At stage 10, Wg, secreted from multiple sources, promotes the normal formation of the HVM primordium and enhances Htl expression in this tissue. However Htl itself is not needed until stage 11 for the normal development of the HVM.

As in the embryonic somatic mesoderm, Wg may regulate Htl expression indirectly by first enhancing Twist expression. At stage 10, Twist is expressed at high and low levels in adjacent metamerically repeated domains (Dunin Borkowski et al., 1995). In the trunk mesoderm, modulation of this pattern of Twist expression depends on wg function (Bate and Rushton, 1993; Riechmann et al., 1997) and is thought to lead to the differential expression of Htl (Shishido et al., 1997). Twist expression is also modulated in the caudal mesoderm with the HVM cells expressing Twist most strongly (B. San Martin, PhD thesis, University of Cambridge, 1999). This strong Twist expression is lost in wg mutants as is the strong expression of Htl (B. San Martin, PhD thesis, University of Cambridge, 1999).

Although Htl expression in the HVM depends on Wg, both Wg and Htl also function independently of each other in promoting the development of the HVM. Thus wg;htl double mutants exhibit a more severe phenotype than wg or htl mutants and loss of htl can be partly rescued by misexpression of Wg throughout the hindgut ectoderm. Later, Htl promotes the differentiation of the HVM whereas Wg is not required in this last phase of HVM development and is unable to substitute for Htl.

The subdivision of the caudal mesoderm

Although the primordia of the HVM and LVM arise next to each other, Twist is expressed at relatively high levels in the HVM but at relatively low levels in the LVM. As in the trunk of the embryo, this modulation of Twist expression is necessary for the correct allocation of mesodermal cell fates (Baylies and Bate, 1996; B. San Martin, PhD thesis, University of Cambridge, 1999). Thus, reduced Twist function appears to lead to a reduction in the HVM and overexpression of twist leads to a reduced LVM primordium (B. San Martin, PhD thesis, University of Cambridge, 1999).

Wg is required for the correct allocation of alternative cell fates in the trunk mesoderm. If Wg were to play a similar role in the caudal mesoderm we would expect an expansion in the primordium of the LVM at the expense of the HVM in wg mutant embryos. This is not the case however, and the LVM primordium is specified normally in wg mutant embryos (B. San Martin, PhD thesis, University of Cambridge, 1999).

Wg also appears to play distinct roles in the trunk and caudal mesoderm. In htl embryos, the uncoordinated migration of the trunk mesoderm at stage 10 leads to a reduction in dorsal mesodermal cell fates (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997). In contrast however, the primordia of the HVM and LVM are specified normally in htl mutant embryos (B. San Martin, PhD thesis, University of Cambridge, 1999).

Extrinsic factors in the development of the mesoderm

The patterning of the trunk mesoderm depends on the integration of a combination of factors that are intrinsic and extrinsic to the mesoderm (Baylies et al., 1998; Frasch, 1999). Cells that are ectodermal in origin are major sources of inductive signals. First, the ectoderm is an essential source of inductive signals that reinforce A/P patterning and subdivide the mesoderm along the dorsoventral axis (Bate and Rushton, 1993; Staehling-Hampton et al., 1994; Baylies et al., 1995; Frasch, 1995; Bate and Baylies, 1996; Riechmann et al., 1998). Second, the specification of the ventral mesodermal glial cell fate requires inducing activity from midline cells of the central nervous system, and these cells are also ectodermal in origin (Lüer et al., 1997).

Our work indicates that the initial development of the HVM depends on the ectoderm of the developing hindgut. It is probably the case that the entire gut ectoderm functions as a source of signals necessary for the development of overlying visceral mesoderm. Indeed, the FVM fails to form in the absence of foregut ectoderm (B. San Martin, PhD thesis, University of Cambridge, 1999). Furthermore, since both Wg and Htl are required for the normal specification of FVM and HVM, it may well be that similar mechanisms operate in the development of both these tissues. Later in development, we find that the HVM is subdivided by localised patterns of gene expression that may represent different functional domains of the gut (B. S. M., unpublished observations). It will be important to show whether, like the early development of the HVM, these later refinements in the patterning process depend on signals derived from the ectoderm of the gut.

We thank Helen Skaer and Matthias Landgraf for their helpful comments on the manuscript, as well as Miriam Gomez, Alfonso Martinez Arias, Fernando Roch and Keith Brennan for useful discussions. We would also like to thank the following people who generously provided us with reagents and fly stocks: Alfonso Martinez Arias, Keith Brennan, Mary Baylies, Jordi Casanova, Kaoru Saigo, Helen Skaer, Tahila Volk, Andrea Brand, Enrique Martin Blanco, Thomas Klein, Claire Ainsworth, Rob White, Dan Keihart, Siegfried Roth, Uwe Hinz and Steve Russell. This work was supported by a Wellcome Trust Prize studentship and fellowship to Beatriz San Martin.


