The somatic-visceral subdivision of the embryonic mesoderm is initiated by dorsal gradient thresholds in *Drosophila*

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SUMMARY

The maternal dorsal regulatory gradient initiates the differentiation of the mesoderm, neuroectoderm and dorsal ectoderm in the early *Drosophila* embryo. Two primary dorsal target genes, *snail* (*sna*) and *decapentaplegic* (*dpp*), define the limits of the presumptive mesoderm and dorsal ectoderm, respectively. Normally, the sna expression pattern encompasses 18-20 cells in ventral and ventrolateral regions. Here we show that narrowing the sna pattern results in fewer invaginated cells. As a result, the mesoderm fails to extend into lateral regions so that fewer cells come in contact with dpp-expressing regions of the dorsal ectoderm. This leads to a substantial reduction in visceral and cardiac tissues, consistent with recent studies suggesting that dpp induces lateral mesoderm. These results also suggest that the dorsal regulatory gradient defines the limits of inductive interactions between germ layers after gastrulation. We discuss the parallels between the subdivision of the mesoderm and dorsal ectoderm.

Key words: *Drosophila*, mesoderm, induction, dorsal, *decapentaplegic*, gradient

INTRODUCTION

The dorsal (dl) regulatory gradient initiates the differentiation of the embryonic mesoderm, neuroectoderm and dorsal ectoderm (Govind and Steward, 1991; Jiang and Levine, 1993). There are peak levels of dl protein along the ventral surface of precellular embryos and progressively lower levels in lateral and dorsal regions (Rushlow et al., 1989; Steward, 1989; Roth et al., 1989). High concentrations of dl initiate the expression of mesoderm genes in ventral regions (Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991; Ip et al., 1992a), while low levels trigger the expression of neuroectodermal regulatory genes in lateral regions (Ip et al., 1992b), dl also functions as a repressor that restricts the expression of certain genes to dorsal regions where they are important for the differentiation of the dorsal ectoderm (Jiang et al., 1992, 1993; Huang et al., 1993; Kirov et al., 1993, 1994; summarized in Fig. 1).

In an effort to determine how the dl regulatory gradient initiates these three territories of tissue differentiation, previous studies have characterized target genes that are directly regulated by different concentrations of nuclear dl protein. These studies suggest that there are two classes of dl target promoters. Type 1 promoters contain low affinity dl-binding sites, so that expression is restricted to ventral regions where there are high concentrations of dl (Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991; Ip et al., 1992a). Type 2 promoters contain high affinity dl-binding sites and, consequently, they can be activated in both ventral and lateral regions, the presumptive mesoderm and neuroectoderm, in response to both high and low levels of dl (Ip et al., 1992b; Jiang and Levine, 1993).

Transcriptional repression is essential for converting these two thresholds into three territories of tissue differentiation (summarized in Fig. 1). The dl target gene, *snail* (*sna*), contains a type 1 promoter and, consequently, its expression is restricted to the ventral mesoderm (Leptin, 1991; Kosman et al., 1991; Alberga et al., 1991; Ip et al., 1992a). The sna protein functions as a sequence-specific repressor, and type 2 promoters that contain sna repressor sites are excluded from the ventral mesoderm and restricted to the lateral neuroectoderm (Ip et al., 1992b; Gray et al., 1994; summarized in Fig. 1). The third tissue territory, the dorsal ectoderm, is established by dl-mediated repression. dl activates target genes in the presumptive mesoderm and neuroectoderm, and also works as a repressor that restricts the expression of genes such as *zerknüllt* (*zen*; Jiang et al., 1992, 1993; Kirov et al., 1993; Lehming et al., 1994) and *decapentaplegic* (*dpp*; Huang et al., 1993) to the dorsal ectoderm. In principle, these latter genes can be activated throughout the early embryo, but they are excluded from the ventral mesoderm and lateral neuroectoderm by dl. The *zen* and *dpp* promoters contain dl-binding sites and closely linked `corepressor` sites; the corepressors mediate long-range repression, or silencing (Doyle et al., 1989; Ip et al., 1991; Jiang et al., 1992, 1993; Kirov et al., 1993; Lehming et al., 1994).

After the three tissue territories are established prior to cellularization, they are subsequently subdivided into multiple
cell types during gastrulation and germ band elongation. For example, the dorsal ectoderm gives rise to dorsal epidermis and the amnioserosa (Ray et al., 1991; Rushlow and Arora, 1990). In the present study, we investigate the role of the dl regulatory gradient in the subdivision of the embryonic mesoderm. After formation of the ventral furrow and invagination of the ventralmost cells, the internal mesodermal layer appears to be ‘naïve’ and the cells are capable of forming any particular mesodermal lineage. Normally, the ventralmost mesoderm, which is in contact with the overlying neuroectoderm, forms the somatic mesoderm (Dohrmann, 1990; Leptin et al., 1992; Azpiazu and Frasch, 1993; Bate and Rushton, 1993). In contrast, the lateral mesoderm, which abuts the dorsal ectoderm, gives rise to the gut musculature and heart tissues (Bodmer et al., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). Recent studies suggest that the initial subdivision of the mesoderm into ventral and lateral lineages involves inductive interactions with the dorsal ectoderm (Staehling-Hampton et al., 1994; Frasch, 1995). In particular, lateral mesoderm that comes into contact with dpp-expressing cells in the dorsal ectoderm is ‘induced’ to form the gut muscles and heart.

Here we show that a subtle alteration in the threshold response of sna to the dl regulatory gradient can disrupt the subdivision of the mesoderm into somatic, visceral and cardiac lineages. The type I sna promoter contains low affinity dl-binding sites and unlinked E boxes, which are binding sites for the twist (twi) protein and other helix-loop-helix (HLH) activators. The two proteins work synergistically to activate sna within the limits of the presumptive mesoderm in ventral and ventrolateral regions of precellular embryos (Ip et al., 1992a). The present study exploits a previously described fusion gene (‘Psnag’) that contains the sna-coding sequence attached to a heterologous twist (twi) promoter sequence (Ip et al., 1994). The twi promoter includes two regulatory regions, the distal element (DE) and proximal element (PE). The 260 bp PE sequence contains low affinity dl-binding sites, but lacks E box sequences. As a result, the PE can be activated only by peak levels of dl protein in ventral regions (Jiang and Levine, 1993). The chimeric PE-sna fusion gene is expressed in just a subdomain of the normal mesoderm anlagen, spanning the ventralmost 12-16 cells (rather than 18-20 cells). Evidence is presented that these narrow limits of sna expression cause a reduction in both the ventral furrow and the number of invaginated cells. Consequently, the mesoderm fails to extend into lateral regions, and fewer cells come into contact with the dpp-expressing regions of the dorsal ectoderm. This causes a severe reduction of lateral mesoderm derivatives, including both the gut muscles and heart. In contrast, the somatic musculature is still formed. We discuss these results in the context of dl thresholds and draw parallels between the subdivision of the mesoderm and dorsal ectoderm.

**MATERIALS AND METHODS**

**Embryo collections and fly strains**

The sna;2xPE-sna mutant strain was generated by crossing the 2xPE-sna P-transposon into a sna+ genetic background. The snaihG05 allele was used for this purpose since previous studies have shown that it probably corresponds to a null allele (Grau et al., 1984), despite the...
The 2xPE-sna fusion gene was expressed in transgenic embryos via P-transformation. It was subsequently crossed into a sna− genetic background, so that the only source of sna protein corresponds to the 2xPE-sna fusion gene (Fig. 2C). The distribution of sna mRNAs was visualized by in situ hybridization using a digoxigenin-labeled sna antisense RNA probe. As predicted from previous analyses of the PE promoter sequence (Jiang et al., 1991; Jiang and Levine, 1993), the synthetic sna fusion gene is expressed within narrower limits as compared with the wild-type endogenous gene (compare Fig. 2B and C).

In the remainder of this study, we describe the consequences of the reduced sna expression pattern with regard to mesoderm differentiation.

Narrowed limits of sna expression reduce the number of invaginating cells

Previous studies suggest a correlation between the limits of the sna expression pattern and the extent of the ventral furrow (Ip et al., 1994). To obtain more rigorous evidence that the narrowed sna pattern results in a reduction in mesoderm invagination, embryos were double stained to reveal the distribution of twi protein and dpp RNA; cross sections of these embryos are presented in Fig. 3. Transgenic sna− embryos carrying the 2xPE-sna fusion gene (sna−; 2xPE-sna) exhibit narrowed limits of twi expression in ventral regions (Fig. 3B; compare with A). Thus, narrowing the limits of the sna pattern could be a corresponding narrowing of the twi pattern. This observation is consistent with the finding that twi expression is initially normal in sna− mutants, but the pattern prematurely disappears (Leptin, 1991; Ray et al., 1991). Altered patterns of sna and twi expression do not influence the dorsal limits of the dpp pattern (Fig. 3 A and B).

Reduced limits of sna and twi expression result in a narrowing of the ventral furrow (data not shown), and the invagination of fewer cells (Fig. 3, compare C and D). The ventral furrow encompasses about 18 to 20 cells in wild-type embryos (Leptin and Grunewald, 1990). All of these cells are fully invaginated during the rapid phase of germ band elongation (Fig. 3C); note that invaginated, twi-expressing cells can be seen in both bottom and top portions of this section due to germ band elongation. The original lateral limits of the dpp expression pattern are maintained in both the wild-type and mutant embryos (Fig. 3C,D; blue RNA staining).

More definitive evidence that fewer cells invaginate in mutant embryos as compared with wild-type was obtained by analyzing slightly older embryos. After one round of mitotic divisions, the invaginated cells migrate into lateral regions immediately after the completion of the rapid phase of germ band elongation (Leptin and Grunewald, 1990). This migration converts the presumptive mesoderm into a single internal layer of cells that is in tight contact with the overlying ectoderm (Fig. 3E,F). At this time, the width of the mesoderm spans about 40 cells in wild-type embryos, but only about 24-30 cells in the mutant embryos. Normally, the mesodermal cells migrate dorsally until they reach the dorsal ectodermal cells that contact the amnioserosa. In contrast, in mutant embryos, the mesodermal cells fail to reach the dorsalmost ectoderm due to the reduced number of invaginated cells. Although individual mesodermal cells undergo more extensive spreading in order to compensate for the reduced numbers, the mesodermal layer
remains intact and narrower than normal. It should be noted that the dorsoventral limits of the 2xPE-sna expression pattern, and ventral furrow, are somewhat variable along the antero-posterior axis (e.g., Fig. 2C); there is also variation among different mutant embryos.

In wild-type embryos, the mesodermal layer extends virtually throughout the region of the dorsal ectoderm containing dpp-expressing cells (Frasch, 1995; Fig. 3E). In contrast, in mutant embryos, the mesoderm usually extends just 1 or 2 cells beyond the ventral limit of the dpp expression pattern in the dorsal ectoderm (Fig. 3F); in some instances, they fail to reach the dpp-expressing cells at all (the exact limits of lateral migration depend on the plane of sectioning and is variable among different embryos).

**Narrowing the limits of the presumptive mesoderm results in loss of visceral and heart lineages**

Recent studies suggest that dpp-expressing cells in the dorsal ectoderm induce the lateral mesoderm to form the visceral mesoderm and heart lineages. *dpp*− embryos form only somatic
derivatives (M. F., unpublished observation). Moreover, ectopic expression of dpp transforms the presumptive somatic regions so that it forms visceral derivatives (Frasch, 1995). To determine whether reducing the mesodermal layer disrupts this subdivision of the mesoderm into somatic, visceral and cardiac tissues, we examined the expression of tinman (tin) (Bodmer et al., 1990; Bodmer, 1993; Azpiazu and Frasch, 1993) and bagpipe (bap) (Azpiazu and Frasch, 1993) in wild-type and mutant embryos. Previous studies have shown that tin is initially expressed throughout the entire presumptive mesoderm, beginning with the formation of the ventral furrow. After invagination and lateral migration, tin expression is lost in ventral regions of the mesoderm (presumptive somatic regions), but maintained in lateral regions that will form the visceral and heart lineages. This maintenance of the late tin pattern depends on induction by dpp-expressing cells (Frasch, 1995).

As shown previously, tin expression persists throughout the entire mesoderm after germ band elongation and lateral
migration (Bodmer, 1993; Azpiazu and Frasch, 1993; Fig. 4A). In slightly older embryos, tin expression is maintained only in lateral regions (Fig. 4C), corresponding to the cells that contact the dorsal ectoderm. Mutant embryos (sna−; 2xPE-sna) also exhibit early tin expression immediately after lateral migration of the mesoderm (Fig. 4B), although the staining is less extensive due to restricted lateral migration. In older embryos, tin expression is virtually lost, although staining is sometimes seen to be retained in just one or two of the lateralmost cells (Fig. 4D; compare with C). This reduction in the late tin pattern correlates with the general failure of the mesoderm to reach dpp-expressing regions of the dorsal ectoderm (see Fig. 3 and Discussion). There is a correlation between the extent of migration and proportion of cells that express tin (Fig. 4D).

Previous studies suggest that the late tin expression pattern is important for the activation of the homeobox gene, bap,
which subsequently controls the differentiation of the visceral mesoderm (Azpiazu and Frasch, 1993). In wild-type embryos, the dorsoventral limits of bap expression appear to coincide with those of the late tin pattern in the lateral mesoderm (Fig. 4E). This embryo was double stained to show bap RNAs in the normal lateral mesoderm (blue), as well as the expression of an ectodermal gene, Cf1a (brown). The Cf1a pattern serves as a marker for the extent of mesoderm lateral expansion. In both wild-type and mutant embryos, the ventral limits of the bap expression pattern are positioned below the center of the Cf1a domains. However, mutant embryos exhibit a reduced bap pattern that correlates with the reduction in tin expression (Fig. 4F). These results suggest that reducing the invaginated mesoderm restricts lateral migration, so that fewer cells come into contact with the dorsal ectoderm and receive the inductive signal from dpp. Consequently, there is a selective reduction in the expression of regulatory genes required for differentiation of the heart and gut musculature (see below; Discussion).

**Reduced heart in mutant embryos**

The consequences of restricting mesoderm-dorsal ectoderm interactions were investigated by analyzing the expression of a number of marker genes in advanced-staged mutant embryos. These studies suggest that tissues arising from the lateralmost regions of the mesoderm are the most severely disrupted, while those arising from ventral regions are less affected.

The heart, or dorsal vessel, is derived from the lateralmost regions of the mesoderm (Bate and Martinez-Arias, 1993; Bodmer, 1993; Azpiazu and Frasch, 1993). The expression of two ‘marker genes’, tin and even-skipped (eve), suggests that mutant embryos possess severely reduced hearts. Fig. 5 shows dorsal views of wild-type and mutant embryos undergoing dorsal closure, just after germ band shortening. tin and eve are expressed in different tissues of the heart, corresponding to cardioblasts and pericardial cells, respectively (Fig. 5A,C). Normally, the developing heart extends from the labium/prothorax through the seventh abdominal segment. Mutant embryos exhibit a severe reduction in heart tissues, particularly in abdominal regions (Fig. 5B,D).

Additional marker genes were analyzed in order to assess the differentiation of other mesodermal derivatives, including the visceral mesoderm and somatic musculature. A fasciclin III probe was used to examine the early differentiation of the visceral mesoderm (Strong et al., 1994; Fig. 6). Wild-type and mutant embryos were double stained to visualize fas III protein and eve RNA. Both expression patterns are reduced in mutant embryos (Fig. 6B; compare with A), indicating a loss of both visceral and heart tissues. Analysis of markers for the somatic musculature, such as the myosin heavy chain (MHC) gene, suggests that derivatives of the ventral mesoderm differentiate and are not as severely disrupted (data not shown). In addition, the use of a nautilus (Michelson et al., 1990) hybridization probe suggests that the mutant embryos possess essentially a normal number of founder somatic myoblasts. These results suggest that limiting the scope of the dpp-mesoderm interaction causes a selective loss in the derivatives of the lateral mesoderm, including the heart and gut musculature.

**DISCUSSION**

We have presented evidence that differential thresholds established by the dl gradient are directly responsible for the subdivision of the embryonic mesoderm into visceral and somatic lineages. Classical ablation experiments in short germ band insects demonstrated that the neurogenic ectoderm and the
defines the lateral limit of asymmetry is a direct consequence of the dl gradient, which
Haget, 1953). In
dorsal ectoderm possess distinctive properties with regard to
mesoderm induction (Bock, 1939, 1941; Seidel et al., 1940; 
Haget, 1953). In Drosophila, it would appear that this
asymmetry is a direct consequence of the dl gradient, which
defines the lateral limit of dpp expression in the dorsal
ectoderm. Recent studies suggest that dpp-expressing cells
induce the lateralmost mesoderm to form both heart and visceral
derivatives (Staehling-Hampton et al., 1994; Frasch, 1995). The
mechanisms underlying the subsequent subdivision of the
lateral mesoderm into these two distinct lineages are currently
unknown. This study provides evidence that the subdivision of
the mesoderm is surprisingly nonplastic. A slight reduction in
the limits of the presumptive mesoderm leads to a severe loss
of specific mesodermal lineages, particularly those arising from
the lateral mesoderm such as the heart and gut muscles.

dl thresholds and the subdivision of embryonic
tissues
Gastrulation is essential for the juxtapositioning of diverse
embryonic tissues, which subsequently interact to define cell
fate. It has become increasingly clear in a variety of embryonic
systems that the interacting tissues are not naive, but instead
possess an intrinsic developmental bias. In insects, the type of
mesodermal derivatives that are obtained depend on the source of
the ectoderm. Dorsal ectoderm induces the differentiation of
‘lateral’ mesoderm, including both visceral and cardiac
mesoderm derivatives (Seidel et al., 1940). In contrast, neuro-
genic ectoderm (or neuroectoderm) appears to be required for
the differentiation of somatic derivatives (Bock, 1939, 1941; 
Seidel et al., 1940; Haget, 1953). This study provides evidence
that the parameters of mesoderm-ectoderm inductive interac-
tions are stringently set by differential threshold responses to
the dl gradient, as summarized in Fig. 7.

sna is activated precisely within the limits of the presum-
tive mesoderm through synergistic interactions between dl and
and one of its target genes, twi (Kosman et al., 1991; Leptin, 1991;
Ip et al., 1992a). Previous studies suggested a link between the
sna expression pattern and the limits of the ventral furrow and
invaginates mesoderm. Mutant embryos with an altered sna
pattern show a corresponding disruption in mesoderm differ-
entiation. For example, there are gaps in the sna expression
pattern in dl/+; twi/+ double heterozygotes, and these regions fail
to invaginate (Kosman et al., 1991). There is reason to
believe that the bHLH twi activator must form a heterodimer
with one or more ubiquitously expressed bHLH proteins, such
as daughterless (da), which is maternally expressed (González-
Crespo and Levine, 1993). sna expression is virtually elimi-
nated in dal+; dl/+; twi/+ triple heterozygotes and, conse-
quently, no ventral furrow forms and there is a severe loss of
mesodermal derivatives. It was not possible to establish a
causal link between the altered sna pattern and invagination,
since these mutants impair dl activity and, as a result, a number
of target genes are disrupted in addition to sna.

The present study provides evidence for a direct link between
the sna expression pattern and mesoderm invagination. The use
of the PE heterologous promoter introduces a rather subtle per-
turbation in essentially normal embryos. The only discernible
difference between wild-type embryos and the mutant embryo
(sna−; 2xPE-sna) is the narrowing of the sna expression pattern,
from approximately 18-20 cells to about 12-16 cells (see Fig.
2). The dl gradient and its interacting partners such as bHLH
activators and corepressors, are not altered. The initial
expression of primary dl target genes, including dpp, zen, rho
and twi appear normal (data not shown).

Drosophila dorsoventral patterning is inflexible
It would appear that there are no compensatory cell divisions
to re-establish the normal number of invaginated cells in sna−;
2xPE-sna mutants. In principle, an additional division cycle
would be sufficient to permit expansion of the mesodermal layer into proximity with the dorsal ectoderm. The conse-
quences of a slightly narrowed sna expression pattern provide
perhaps the most striking example of the inflexibility of the
dorsoventral (DV) patterning process. Other examples include
the dorsoventral patterning defects observed in dl-bHLH double
heterozygotes (González-Crespo and Levine, 1993). Moreover,
dl/+ heterozygotes, containing just a 2-fold reduction in the
normal levels of dl protein, exhibit a high incidence of lethality
(Simpson, 1983). dl/+ heterozygotes (more accurately, embryos
derived from heterozygous females) show a slight narrowing of
the sna expression pattern, so it is conceivable that this lethality
stems, at least in part, from disruptions in mesoderm-dorsal
ectoderm inductive interactions.

The inflexibility of dorsoventral patterning is also observed
for the subdivision of the dorsal ectoderm. An apparent dl
target gene, short gastrulation (sog), is expressed in lateral
regions of precellular embryos (François et al., 1994). It has
been proposed that lateral stripes of sog expression correspond
to a ‘sink’ that helps establish a dpp activity gradient in the
dorsal half of gastrulating embryos (François et al., 1994). 
Peak dpp activity is restricted to the dorsalmost regions, while
lower levels extend into dorsolateral and lateral regions. This
dpp activity gradient is thought to be responsible for subdivi-
sing the dorsal ectoderm into the amnioserosa and dorsal

Fig. 6. Reduction of visceral mesoderm in mutant embryos. Lateral
views of late extended embryos that were double stained to visualize
the fasciclin III protein (brown) and eve protein (blue). Wild-type
(A) and mutant (B) embryos. At this stage, fas III stains all of the
cells of the presumptive visceral musculature, which can be seen as a
continuous lateral band of brown stain. The presumptive pericardial
cells (blue) are located just dorsally of the visceral mesoderm.
Mutant embryos show a severe reduction in both the visceral and
cardiac lineages. vm, visceral mesoderm; hp, heart progenitors.
epidermis (Ferguson and Anderson, 1992; François et al., 1994). Peak levels of dpp work synergistically with another TGF-β homologue, screw (scw), to initiate the differentiation of amnioserosa (Arora et al., 1994). dpp and scw are required for the maintenance and refinement of the zen expression pattern. Initially, zen, like dpp, is expressed in a broad dorsal on/ventral off pattern due to repression by the dl gradient. During the completion of cellularization, dpp and scw maintain the zen pattern only in the dorsalmost 4-5 cells (the presumptive amnioserosa) where dpp is at peak activity. This refinement of the zen pattern appears similar to the restriction of the late tin pattern within the lateral mesoderm. Both processes require ‘induction’ by peak levels of dpp activity. Previous studies have shown that dpp/+ heterozygotes fail to maintain and refine the zen expression pattern and, consequently, there is a loss of the amnioserosa resulting in embryonic lethality.

These examples of dosage-sensitive embryonic lethality (dll+ and dpp+/) contrast with the high degree of plasticity seen for tissue differentiation in other embryonic systems. For example, in sea urchins, the primary mesenchyme arises through the ingestion of the micromeres (Cameron et al., 1991). Loss of micromeres through ablation causes the growing tip of the archenteron (the future foregut) to undergo additional, ‘unscheduled’ divisions. The resulting cells form the mesenchyme in response to the loss of the micromeres.

The inflexibility of dorsoventral patterning also contrasts with the plasticity of anteroposterior (AP) patterning in Drosophila. Embryos containing between 1 and 6 copies of the bicoid (bcd) gene develop properly and ultimately give rise to normal adults (Struhl et al., 1989; Driever and Nüsslein-Volhard, 1988). bcd is essential for head differentiation and the initiation of the segmentation cascade. Normal embryos containing two copies of bcd show 7 pair-rule stripes of eve and fushi tarazu (ftz) expression, which extend from the cephalic furrow to subterminal regions at the posterior pole, at about 10% egg length. Heterozygotes containing one-half the normal levels of bcd show eve and ftz stripes that extend over a broader region of the AP axis. Remarkably, all 7 pair-rule stripes are compressed within a narrow, central region of embryos containing 6 copies of bcd, but they develop normally presumably due to changes in programmed cell death and division cycles.

In summary, evidence was presented that the maternal dl gradient sets the limits of inductive interactions between germ layers. Altering the threshold response of just one primary target gene, sna, resulted in the narrowing of the presumptive mesoderm. Consequently, there is a general failure of the invaginated mesoderm to expand into lateral regions and come into contact with dpp-expressing cells in the dorsal ectoderm. These cells are ‘induced’ to form lateral derivatives, including both the visceral and cardiac lineages. In mutant embryos (sna++; 2XPE-sna), there is a reduction in the ventrolateral limits of the sna pattern, but the dpp pattern is unchanged. Consequently, lateral migration is less extensive and fewer cells come into contact with the dorsal ectoderm. This results in a substantial reduction in both visceral and cardiac derivatives.

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