**HNF3β and Lim1 interact in the visceral endoderm to regulate primitive streak formation and anterior-posterior polarity in the mouse embryo**

Aitana Perea-Gómez1, William Shawlot2,*, Hiroshi Sasaki3, Richard R. Behringer2 and Siew-Lan Ang1,‡

1Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, BP163, 67404 Illkirch cedex, CU de Strasbourg, France
2Department of Molecular Genetics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA
3Laboratory of Developmental Biology, Institute for Molecular and Cellular Biology, Osaka University, Osaka 565, Japan

*Present address: Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis 55455, USA
‡Author for correspondence (e-mail: siew-lan@igbmc.u-strasbg.fr)

**SUMMARY**

Recent embryological and genetic experiments have suggested that the anterior visceral endoderm and the anterior primitive streak of the early mouse gastrula function as head- and trunk-organising centers, respectively. Here, we report that HNF3β and Lim1 are coexpressed in both organising centers suggesting synergistic roles of these genes in regulating organiser functions and hence axis development in the mouse embryo. To investigate this possibility, we generated compound HNF3β--/--;Lim1--/- mutant embryos. An enlarged primitive streak and a lack of axis formation were observed in HNF3β--/--;Lim1--/-, but not in single homozygous mutant embryos. Chimera experiments indicate that the primary defect in these double homozygous mutants is due to loss of activity of HNF3β and Lim1 in the visceral endoderm. Altogether, these data provide evidence that these genes function synergistically to regulate organiser activity of the anterior visceral endoderm. Moreover, HNF3β+/-;Lim1+/- mutant embryos also exhibit defects in mesoderm patterning that are likely due to lack of specification of anterior primitive streak cells.

Key words: HNF3β, Lim1, Anteroposterior axis, Mesoderm, Patterning, Visceral endoderm

**INTRODUCTION**

One of the major questions that arises when studying development of the mouse embryo is the understanding of the mechanisms that allow generation of anteroposterior (A-P) polarity. Indeed after implantation, the mouse embryo is a symmetrical cup-shaped structure consisting of an outer layer of visceral endoderm (VE) and an inner layer of ectoderm. The first morphological sign of asymmetry is the thickening of the embryonic ectoderm, or epiblast, at the junction with the extraembryonic ectoderm in the future posterior region of the embryo. This feature marks the appearance of the primitive streak, where mesoderm is produced by delamination of epiblast cells (Tam and Behringer, 1997, for a review).

Embryological manipulations have shown that the anterior region of the primitive streak and its later derivative, the node, can induce the formation of an anteriorly truncated secondary axis when grafted ectopically in lateral regions of the embryo (Beddington, 1994). Thus, the anterior primitive streak was thought to be the mouse equivalent of the vertebrate gastrula organiser, first defined by Spemann and Mangold in the newt embryo, and subsequently found in all vertebrate species examined (reviewed by Lemaire and Kodjabachian, 1996). Recent genetic and embryological data suggest, however, that an extraembryonic tissue of the mouse gastrula, the anterior visceral endoderm (AVE), displays organising properties as it is necessary to induce and pattern the anterior regions of the mouse embryo (reviewed in Beddington and Robertson, 1998). Indeed ablation experiments have demonstrated a requirement of the AVE for the correct expression of forebrain markers (Thomas and Beddington, 1996). Moreover, chimera analysis has shown that the VE expression of several genes coding for secreted molecules (Nodal) or transcription factors (Otx2 and HNF3β), is essential for head development (Varlet et al., 1997; Rhinn et al., 1998; Dufort et al., 1998). These findings have led to the proposal that the AVE of the mouse embryo displays head organising properties whereas the anterior primitive streak can induce trunk structures. Remarkably, several genes conserved through evolution and expressed in the organiser of different vertebrate embryos are found both in the AVE and in the anterior primitive streak of the mouse gastrula, further confirming that both tissues contribute to organiser function. These genes code for secreted molecules such as Nodal (Varlet et al., 1997) or for transcription factors like the homeodomain proteins Otx2 and Goosecoid (Gsc), the winged helix factor HNF3β and the LIM homeodomain protein Lim1 (Ang and Rossant, 1994; Filosa et al., 1997; Barnes et al., 1994, Shawlot and Behringer, 1995). In addition, Gsc, Lim1, the
homeodomain protein Hex (Thomas et al., 1998) and the secreted molecule Cerberus-like (Cer-l) (Belo et al., 1997) are specifically expressed in the AVE before the appearance of the primitive streak in the posterior side of the embryo, suggesting that the head organiser arises independently of the trunk organiser and that anterior regions are defined before posterior regions of the mouse embryo.

Among the genes expressed in both the AVE and the anterior primitive streak of the mouse embryo, those that code for transcription factors are likely to play a role in specifying the organising properties of these tissues. In vivo function of these genes has been addressed by gene targeting experiments. Otx2 has been shown to function in the VE for the induction of forebrain and midbrain (Rhinn et al., 1998, reviewed in Simeone, 1998). Although Gsc null mutants are not affected during early development (Rivera-Perez et al., 1995; Yamada et al., 1995), analysis of Gsc−/−;HNF3B−/− compound mutant embryos has demonstrated that Gsc functions synergistically with HNF3β to pattern the neural tube. The expression patterns of these two genes in early mouse embryos suggest that the interaction could take place during gastrulation either in the anterior primitive streak or in the AVE (Filosa et al., 1997). HNF3β mutant embryos show severe defects in dorsoventral (D-V) patterning of the neural tube due to the loss of an organised node and axial mesoderm structures, while the A-P axis is correctly specified in most cases (Ang and Rossant, 1994; Weinstein et al., 1994). However, a role for HNF3β in the VE for the development of anterior neural tissue has been inferred from chimera analysis (Dufort et al., 1998). Lim1 homozygous mutant embryos show a truncation of the neural tube rostral to rhombomere 3, which is due to a defect in AVE (Shawlot and Behringer, 1995; W. S. and R. R. B., unpublished results). Taken together, these results suggest that the mutation of only one of the transcription factors expressed in the organising centers of the mouse gastrula does not completely impair axis development. These factors might therefore act redundantly or synergistically in the anterior primitive streak and the AVE, as already demonstrated in the case of Gsc and HNF3β (Filosa et al., 1997).

In this paper, we have investigated the possible interactions and/or redundant functions of HNF3β and Lim1. A comparative analysis of the expression domains of the two genes has shown that they are coexpressed in the VE layer surrounding the epiblast before primitive streak formation. During gastrulation, a subpopulation of cells in the anterior primitive streak coexpresses the two genes. In the VE, Lim1 expression is maintained at strong levels only in anterior regions, where it is coexpressed with HNF3β. Their early coexpression in organising centers of the mouse gastrula prompted us to generate compound mutant embryos. HNF3β−/−;Lim1−/− mutant embryos show severe germ layer disorganisation and a lack of embryonic ectoderm derivatives. Epiblast development is impaired as early as 6.5 days postcoitum (d.p.c.) as we observe an expansion of the primitive streak territory and a loss of anterior and distal regions. Chimera analysis shows that the primary defect in HNF3β−/−;Lim1−/− mutant embryos lies in the VE. Based on this result and on the analysis of molecular markers of the VE in double mutant embryos, we propose a model for the function of HNF3β and Lim1 in the VE in restricting primitive streak formation to posterior regions of the epiblast, thereby establishing A-P polarity in the mouse embryo. In addition, double mutant embryos also show D-V patterning defects in mesoderm formation that are likely due to an additional function of the two genes in the specification of anterior primitive streak cells.

**MATERIALS AND METHODS**

**Generation and genotyping of wild-type and mutant mice**

Lim1 heterozygous mutant mice on a 129/Sv x C57B1/6 background (Shawlot and Behringer, 1995) were crossed with HNF3β heterozygous mutant mice on a 129/Sv x C57B1/6 background (Ang and Rossant, 1994) to generate double heterozygous animals. For the genotyping of pups, DNA was extracted from tail tips as described in Laird et al. (1991). For genotyping of 7.5 d.p.c. or older embryos, yolk sac DNA extraction was performed according to Moens et al. (1992). For 6.5 d.p.c. embryos, DNA was extracted from eutopic abortion cone cultures as described (Ang and Rossant, 1994). PCR analysis of the Lim1 locus was performed using the same primers as in Shawlot and Behringer (1995). The DNA was amplified for 35 cycles (94°C/1 minute, 55°C/30 seconds, 72°C/1 minute) in 25 µl volume containing 67 mM Tris-HCl, pH 8.8, 6.7 mM MgCl2, 170 µg/ml BSA, 16.6 mM (NH4)2SO4, 1.5 mM dNTPs, 10% DMSO and 150 ng of each oligonucleotide. For the HNF3β locus, PCR analysis was performed using primers and conditions described in Filosa et al. (1997). Staging of mutant and wild-type embryos was described in Downs and Davies (1993).

**In situ hybridisation, immunohistochemistry and histology**

Whole-mount in situ hybridisation was performed as described previously (Conlon and Herrmann, 1993). The number of specimens used for in situ hybridisation of each gene is indicated in the figure legends as n.X. For section in situ hybridisation, deccuades were fixed for 2 hours in 4% paraformaldehyde in PBS, equilibrated in 20% sucrose and embedded in OCT (Tissue-Tek, Miles). In situ hybridisation on sections was performed as described in Gradwohl et al. (1991). Staging of mutant and wild-type embryos was according to Down and Davies (1993).
endoderm isolated from visceral mesoderm after digestion in pancreatic and trypsin as described (Hogan et al., 1994). DNA samples prepared from the endodermal fraction were genotyped for the \(HNF3\beta\) and \(HNF3\beta\) loci by PCR.

RESULTS

Expression of \(Lim1\) and \(HNF3\beta\) in early mouse embryos

Earlier studies have shown that \(Lim1\) and \(HNF3\beta\) are expressed in the anterior primitive streak as well as in the AVE (Barnes et al., 1994; Shawlot et al., 1995; Filosa et al., 1997). To more precisely compare the expression domains of \(HNF3\beta\) and \(Lim1\), we analysed the expression of these two genes from gastrulation to the headfold stage (5.5-7.75 d.p.c.), by radioactive in situ hybridisation on adjacent sections and by double-labelling analyses of embryos in whole mounts. Sagittal sections of 5.5 d.p.c. embryos show expression of both \(Lim1\) and \(HNF3\beta\) in the VE (Fig. 1A-C). \(HNF3\beta\) is expressed in the VE lining both the embryonic and extraembryonic regions, whereas \(Lim1\) expression is found exclusively in the VE in contact with the epiblast. At the onset of gastrulation (6.5 d.p.c.), both transcripts are detected in the newly formed primitive streak at the posterior proximal region of the embryo, and \(HNF3\beta\) is still expressed in the whole endoderm germ layer (Fig. 1D,F). In contrast, \(Lim1\) transcripts can no longer be detected in the region of the VE overlying the primitive streak (arrow in Fig. 1E). As gastrulation proceeds and the primitive streak elongates, \(Lim1\) transcripts are progressively restricted to the AVE and are also observed in the mesoderm wings (Fig. 1E and data not shown).

Double-labelling by in situ hybridisation with a \(Lim1\) RNA probe and immunocytochemistry with a \(HNF3\beta\) antiserum on whole embryos at 6.5 d.p.c. demonstrated that \(HNF3\beta\) and \(Lim1\) are coexpressed in the AVE and in the anterior primitive streak (Fig. 1G,H,J,K). However, in the anterior primitive streak, the majority of the cells and specifically the more distal ones express only \(HNF3\beta\) (Fig. 1H). Thus as we had previously shown for Gsc (Filosa et al., 1997), \(Lim1\) expression is encompassed by the domain of expression of \(HNF3\beta\) in the anterior primitive streak. The mesoderm cells exiting the primitive streak express \(Lim1\) and \(HNF3\beta\), whereas the endoderm cells overlying the anterior primitive streak express \(HNF3\beta\) alone (data not shown). In the anterior mesoderm wings only \(Lim1\) is expressed (Fig. 1K). By the early headfold stage (7.5 d.p.c.) when the primitive streak has reached the distal tip of the embryo and the node and head process are formed, both \(HNF3\beta\) and \(Lim1\) are expressed in these structures (Fig. 1I). Flatmounts of the distal region of the embryo show a large number of cells coexpressing \(HNF3\beta\) and \(Lim1\) in the node and in the head process (Fig. 1L). However, \(Lim1\) expression in the head process seems to be transient as in late head fold stage embryos (7.75 d.p.c.) only \(HNF3\beta\) protein is detected in anterior midline tissues (data not shown).

In summary, our results demonstrate that \(Lim1\) and \(HNF3\beta\) are coexpressed in the VE surrounding the epiblast before gastrulation and in a population of cells in the anterior primitive streak and its derivatives (the node and axial mesoderm cells) during gastrulation.

Table 1. Frequencies of genotypes among embryos with mutant phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.5 d.p.c. (n=70)</td>
</tr>
<tr>
<td>(HNF3\beta) (-/-) (Lim1) (+/+)</td>
<td>6.25</td>
</tr>
<tr>
<td>(-/-) (+/-)</td>
<td>12.50</td>
</tr>
<tr>
<td>(+/+) (+/-)</td>
<td>6.25</td>
</tr>
<tr>
<td>(+/-) (-/-)</td>
<td>12.50</td>
</tr>
<tr>
<td>(-/-) (-/-)</td>
<td>6.25</td>
</tr>
</tbody>
</table>

\(n\), Number of embryos analysed.

The fit to mendelian expectations was tested with a \(\chi^2\) goodness-of-fit test:

- 6.5 d.p.c.: \(\chi^2=3.49, \text{df}=5, 0.9>\text{P}>0.5\)
- 7.5 d.p.c.: \(\chi^2=3.25, \text{df}=5, 0.9>\text{P}>0.5\)

Early lethality of \(HNF3\beta\(-/-\);\(Lim1\)-/- double mutants

The identification of domains of coexpression of \(HNF3\beta\) and \(Lim1\) raised the possibility that these genes interact in cells of the VE and of the anterior primitive streak. To study genetic interactions between these genes during development, we have generated double heterozygous animals. These mice appeared normal and fertile and when intercrossed gave rise to mutant embryos of five different genotypes at the expected Mendelian frequencies at 6.5 d.p.c. and 7.5 d.p.c. (Table 1). However, from 8.75 d.p.c. onwards, embryos of the \(HNF3\beta\)-/-;\(Lim1\)-/- genotype were not recovered, suggesting that these embryos die between 7.5 and 8.75 d.p.c. Thus, \(HNF3\beta\)-/-;\(Lim1\)-/- double mutant embryos (referred to as \(HNF3\beta\);\(Lim1\) embryos) die 2 days earlier than single \(HNF3\beta\)-/- or \(Lim1\)-/- mutants (Ang and Rossant, 1994; Shawlot et al., 1995; Weinstein et al., 1994). In contrast, embryos of the genotypes \(HNF3\beta\)-/-;\(Lim1\)+/- and \(HNF3\beta\)+/-;\(Lim1\)-/- were found at the expected Mendelian ratios at 10.5-11.5 d.p.c., indicating that these embryos do not die earlier than single mutants (data not shown).

\(HNF3\beta\),\(Lim1\) embryos show severe germ layer disorganisation

The earlier lethality of \(HNF3\beta\);\(Lim1\) embryos suggested that these mutants are more severely affected than the single homozygous mutants. This result was confirmed by morphological and histological analysis. \(HNF3\beta\);\(Lim1\) embryos are easily recognizable morphologically from their littermates as early as 6.75 d.p.c. by the compact appearance of their embryonic region (Fig. 2A-D and data not shown). In wild-type embryos, the cavitation process is over at this stage and has produced a central lumen called the amniotic cavity, resulting in their characteristic cup-shaped appearance (Fig. 2A). In contrast, the amniotic cavity is absent or very small in \(HNF3\beta\);\(Lim1\) embryos (Figs 2D, 3B). Single mutant embryos show a central cavity despite their abnormal shape and characteristic constriction at the embryonic-extraembryonic junction (Fig. 2B,C).

Sagittal sections through a \(HNF3\beta\);\(Lim1\) embryo at 6.75 d.p.c. show that the epiblast (which normally gives rise to the embryonic tissues upon gastrulation) is disorganised (Fig. 3B) and lacks the typical columnar aspect observed in a wild-type littermate (Fig. 3A). The extraembryonic ectoderm is prematurely detached from the embryonic ectoderm (asterisk
in Fig. 3B) but other extraembryonic structures, including proximal and distal VE, look normal. The space between embryonic and extraembryonic ectoderm is filled with cells of a mesenchymal appearance. It is to note that none of the embryos of the other genotypes showed such phenotypic features (data not shown).

At 7.75 d.p.c., HNF3β,Lim1 embryos have a very small embryonic region (Fig. 2H) when compared to wild-type (Fig. 2E) or single mutant embryos (Fig. 2F,G), whereas the extraembryonic region looks normal in size. Unlike in the wild-type embryos at this stage (Fig. 3C), we could not distinguish the three germ layers in the embryonic region of HNF3β,Lim1 embryos. Neither a continuous mesoderm layer nor neur ectodermal folds are visible. Instead, the embryonic region of these embryos is severely disorganised and presents multiple cavities (Fig. 3D), whereas the extraembryonic region and the outer VE layer seem to be correctly formed. In these embryos, it is also possible to identify some extraembryonic mesoderm lining the chorion and the proximal VE (arrowhead in Fig. 3D).

Thus, HNF3β,Lim1 embryos show a new and more severe phenotype than the single HNF3β−/− and Lim1−/− mutant embryos. The organisation of the germ layers is strongly impaired in the embryonic region, presumably as a consequence of the disorganisation of the epiblast at earlier stages. However, gastrulation has occurred as mesoderm is produced in these embryos.

**Absence of embryonic ectoderm derivatives in HNF3β,Lim1 mutants**

At 7.75 d.p.c., the ectoderm of a wild-type embryo consists of an epithelial layer that was never observed in HNF3β,Lim1 embryos (Fig. 3C,D). To further characterise the defects in the ectodermal layer of HNF3β,Lim1 embryos, we analysed the expression of Oct4 and Sox1 in these embryos. Oct4 belongs to the family of POU domain transcription factors and is expressed at 7.5 d.p.c. in definitive ectoderm in the posterior half as well as in the primitive streak (Fig. 4A; Rosner et al., 1990). However in HNF3β,Lim1

---

**Fig. 1.** Lim1 and HNF3β are coexpressed in the VE, anterior primitive streak and its derivatives in early mouse embryos. (A-F) Radioactive in situ hybridisation on adjacent sagittal sections. (A,D) Bright-field images of sections shown in B and E, respectively. Arrowheads in A point to the embryonic-extraembryonic junction, while the arrow in D points to the VE overlying primitive streak. (B,C) At 5.5 d.p.c., Lim1 transcripts are found in the VE lining the epiblast with apparently stronger levels in the distal region, while HNF3β is expressed in the VE lining the embryonic and extraembryonic regions. (E) At 6.5 d.p.c., Lim1 is still expressed in the VE except in the region overlying the primitive streak (arrow). Lim1 transcripts are also found in the anterior primitive streak (arrowhead) and in the mesoderm wings. (F) HNF3β is still expressed in the whole VE as well as in the anterior primitive streak (arrowhead). (G-L) Whole-mount in situ hybridisation using Lim1 RNA probe (purple) and immunohistochemistry with anti-HNF3β antibody (brown). The level of the transverse section depicted in J and K is indicated by horizontal black lines in G and H, respectively. (G,J) At 6.25 d.p.c., Lim1 and HNF3β are coexpressed in the AVE (arrow in J) and in the anterior primitive streak (arrow in G). (H,K) A 6.5 d.p.c. embryo showing that the most anterior cells of the primitive streak express only HNF3β (arrowhead in H), whereas more posteriorly both HNF3β and Lim1 are expressed (arrowhead in K). There is also coexpression of HNF3β and Lim1 in the AVE (arrow in K). In the anterior mesoderm wings, only Lim1 is expressed (short arrow). (L) At 7.5 d.p.c., Lim1 and HNF3β are expressed in the node and in the head process. (L) Flatmount of the distal region of the embryo depicted in I shows cells coexpressing Lim1 and HNF3β (arrowhead in L). Anterior is to the left in A-I, and to the top in J-L. Abbreviations: a, anterior; p, posterior. Scale bar, 50 μm.
Role of HNF3β and Lim1 in mouse axis development

mutants, Oct4 transcripts were absent, suggesting that definitive ectoderm cells are missing in these embryos (Fig. 4A'). Sox1 is an SRY-related factor expressed in newly formed neural tissue (Fig. 4B and Collignon et al., 1996). In HNF3βLim1 embryos at 7.75 d.p.c., Sox1 expression was not detected (Fig. 4B'), confirming the lack of neurectoderm suggested by the histological analysis. Altogether, these results show that HNF3βLim1 embryos lack embryonic ectoderm cells and their derivatives at late gastrulating stages. In contrast, the extraembryonic ectoderm of the chorion seems to be correctly formed (asterisk in Fig. 3B,D).

To provide insights into the mechanisms leading to the loss of ectoderm cells, we analysed expression of two epiblast markers, Otx2 and Oct4, in HNF3βLim1 embryos at 6.5 d.p.c. The homeobox gene Otx2 is normally expressed in epiblast cells of wild-type embryos at this stage, except those at the posterior proximal end (Fig. 4C; Ang et al., 1994). In contrast, Otx2 expression was not detected in HNF3βLim1 embryos (Fig. 4C'), suggesting abnormal specification of these cells. Oct4 expression, however, was still observed in HNF3βLim1 embryos (Fig. 4D') at 6.75 d.p.c., albeit at reduced levels compared to wild-type embryos (Fig. 4D). This apparent lower level of expression is reminiscent of the situation in the primitive streak of normal embryos, where Oct4 appears to be progressively downregulated as Brachyury (T) expression is turned on in cells of the primitive streak (Fig. 4D and data not shown). These results suggest that HNF3βLim1 embryos present defects in patterning of epiblast cells along the A-P axis, although these data alone do not exclude the possibility that an earlier block in epiblast differentiation occurs in HNF3βLim1 embryos.
An enlarged primitive streak territory leads to ectopic mesoderm formation in HNF3β,Lim1 embryos

We next examined whether the primitive streak develops normally in HNF3β,Lim1 embryos, by analysing expression of T in these embryos. At 6.5 d.p.c., T expression is restricted to primitive streak cells in the posterior side of wild-type embryos (Fig. 5A; Wilkinson et al., 1990). In contrast, we observed that T transcripts are not restricted to the posterior side in HNF3β,Lim1 embryos at the same stage (Fig. 5A'), but are present in the whole embryonic region. This result, together with the absence of Otx2 expression and the low levels of Oct4 transcripts, strongly suggests that the epiblast of HNF3β,Lim1 embryos has adopted characteristics of primitive streak cells.

This hypothesis is further supported by the expression of Fgf8 in HNF3β,Lim1 embryos. During gastrulation, Fgf8 is expressed in the primitive streak (Fig. 5B; Cossley and Martin, 1995). In HNF3β,Lim1 embryos, Fgf8 expression was detected in the entire embryonic region (Fig. 5B'), thus confirming the expansion of the primitive streak territory in these mutants.

The expanded primitive streak domain in HNF3β,Lim1 embryos suggest that mesoderm might be ectopically produced in these embryos. To examine the extent of mesoderm formation in HNF3β,Lim1 embryos, we analysed expression of two early markers of mesodermal precursors, MesP1 and Lefty2. The basic helix-loop-helix gene, MesP1, is expressed in mesoderm cells as well as in the primitive streak from the middle part of the primitive streak of a wild-type embryo and in mesoderm cells emerging from it. (D') Widespread expression of Lefty2 in the whole embryonic region of a HNF3β,Lim1 embryo (n=1). (E) A wild-type embryo showing Gsc expression in anterior primitive streak as well as in VE cells. (E') Gsc transcripts are not detected in a HNF3β,Lim1 embryo (n=4). Anterior is to the left. Magnification is the same for all panels. Scale bar in A, 100 μm.

Fig. 4. Absence of embryonic ectoderm derivatives in HNF3β,Lim1 embryos. Whole-mount in situ hybridisation on wild-type embryos (A-D) and HNF3β,Lim1 embryos (A'-D'). (A) Oct4 is expressed in ectoderm cells in the posterior half of wild-type embryos. (A') Absence of Oct4 transcripts in a 7.75 d.p.c. HNF3β,Lim1 embryo (n=2). (B) Frontal view of a wild-type embryo showing Sox1 expression in the neural plate. (B') Absence of Sox1 expression in a HNF3β,Lim1 embryo (n=2). (C) Otx2 is expressed in the anterior two thirds of the epiblast of a wild-type embryo at 6.75 d.p.c. (C') Otx2 expression is not detected in a HNF3β,Lim1 embryo at 6.75 d.p.c. (n=2). (D) Oct4 expression in the epiblast of a wild-type embryo at 6.75 d.p.c. (D') Reduced levels of Oct4 expression in the abnormal epiblast of a HNF3β,Lim1 embryo at 6.75 d.p.c. (n=4). Anterior is to the left except in B. Magnification is the same for A,B,A',B' and C,D,C',D', respectively. Scale bar in A and C, 100 μm.

Fig. 5. Enlarged primitive streak region in HNF3β,Lim1 embryos. Whole-mount in situ hybridisation on 6.5 d.p.c. (A,A',E,E') and 6.75 d.p.c. (B-D,B'-D') wild-type (A-E) and HNF3β,Lim1 embryos (A'-E'). (A) T transcripts are restricted to the posterior region in a wild-type embryo. (A') HNF3β,Lim1 embryo showing ectopic T expression in the whole embryonic region (n=4). (B) Fgf8 is expressed in the primitive streak of wild-type embryos. (B') HNF3β,Lim1 embryo showing Fgf8 expression in the whole embryonic region (n=3). (C) MesP1 expression in the primitive streak and in the nascent mesoderm cells of a wild-type embryo. (C') Abundant distal and lateral expression of MesP1 in a HNF3β,Lim1 embryo (n=1). (D) Lefty2 is expressed in all panels. Scale bar in A, 100 μm.
onset of gastrulation to the late streak stage (Fig. 5C and Saga et al., 1996). In HNF3\(\beta\),Lim1 embryos, MesP1 expression was detected in the distal region as well as in the cells surrounding the small central amniotic cavity of the embryo at 6.75 d.p.c. (Fig. 5C'). Likewise, the TGF\(\beta\)-family member Lefty2, which marks the middle part of the primitive streak as well as the mesoderm cells exiting from it (Fig. 5D; Meno et al., 1996), is expressed in a larger domain including the whole distal embryonic region of HNF3\(\beta\),Lim1 embryos (Fig. 5D'). These observations suggest that in HNF3\(\beta\),Lim1 embryos mesoderm formation is not confined to posterior regions as in wild-type embryos, but occurs throughout the embryonic region.

Anterior primitive streak cells are missing in HNF3\(\beta\),Lim1 embryos

As HNF3\(\beta\) and Lim1 are coexpressed in the anterior primitive streak, we analysed in more detail the status of this structure using the homeobox gene Gsc. Gsc is expressed in the anterior primitive streak (Fig. 5E; Blum et al., 1992), and its expression has been associated to the presence of organiser cells in several vertebrate species (reviewed by Tam and Behringer, 1997). In single Lim1 or HNF3\(\beta\) mutant embryos, Gsc is still expressed but ectopically located in a proximal region of the embryo, presumably due to the lack of elongation of the primitive streak (Shawlot and Behringer, 1995; Ang and Rossant, 1994). At 6.5 d.p.c., Gsc expression was
not detected in HNF3βLim1 embryos, suggesting that the anterior primitive streak and the organiser cells are missing in these mutants (Fig. 5E'). As the anterior primitive streak is the region where the precursors of the axial mesoderm are located, the absence of this structure suggests that there could also be defects in mesoderm patterning in HNF3βLim1 embryos.

Abnormal VE patterning in HNF3βLim1 embryos

Recent findings have revealed a key role of the VE layer in the patterning of the mouse embryo before and during gastrulation (Beddington and Robertson, 1998, 1999). The early disruption in germ layer formation and organisation in HNF3βLim1 embryos, as well as the coexpression of HNF3β and Lim1 in the VE before the onset of gastrulation, prompted us to examine the status of the VE in HNF3βLim1 embryos.

Three genes that are asymmetrically expressed in the VE are Cer-l, Hex and Lefty1. Cer-l, a gene that shares sequence homology with Xenopus Cerberus (X-cer), encodes a secreted molecule which starts to be expressed in the AVE before the onset of gastrulation and is subsequently found in the definitive endoderm, midline mesoderm and newly formed somites (Fig. 6A and Belo et al., 1997). Hex is a homeobox gene expressed first in the distal VE and then asymmetrically in the AVE before primitive streak formation (Fig. 6B; Thomas et al., 1998). Lefty1 is a TGFβ-related molecule, which is expressed specifically in AVE at 6.5 d.p.c. (Oulad-Abdelghani et al., 1998). In HNF3βLim1 embryos, Cer-l, Hex and Lefty1 transcripts are severely reduced or absent in the VE. (Fig. 6A',B',C', respectively). Moreover, Otx2 and Gsc, which normally mark also the AVE, are not expressed in the VE of HNF3βLim1 embryos (Figs 4C', 5E', respectively). In contrast, a general marker of VE, Pem (Lin et al., 1994), is expressed in HNF3βLim1 embryos at 7.5 d.p.c.; however, expression persists distally in these mutants in contrast to the wild-type situation where Pem-positive VE cells have been displaced to proximal regions (Fig. 6D,D'). Thus, although VE is formed in HNF3βLim1 embryos, its molecular patterning seems to be severely impaired, which might be the primary cause of the germ-layer defect in these mutants.

Synergistic functions of HNF3β and Lim1 in the VE

In order to examine specifically the role of HNF3β and Lim1 in the VE, we have generated chimeric embryos by injecting wild-type embryonic stem (ES) cells carrying a ubiquitously expressed lacZ transgene into morulae obtained from crosses between double heterozygous mice. In these experiments, one in 16 chimeric embryos would be expected to be derived from a double homozygous mutant morula. This approach takes advantage of the observation that ES cells colonize predominantly the epiblast and the embryonic derivatives in ES cell-morula chimeras. Thus, in a highly chimeric embryo, the extraembryonic ectoderm and the VE are of host origin whereas the epiblast is completely derived from the ES cells (Fig. 7A).

Out of 33 embryos obtained from ES cell-morula
aggregation experiments and analysed at 7.5 d.p.c., 24 were chimeric as revealed by β-galactosidase staining. Of these, two chimeras were derived from injection into HNF3β+/–;Lim1+/– morulae, as determined by PCR-genotyping of yolk-sac endoderm, and looked very similar to HNF3β,Lim1 embryos. These embryos show an embryonic region that is highly disorganised, formed of cells with a round morphology that do not resemble ectoderm or neuroectoderm tissue (Fig. 7B). In contrast, their extraembryonic region is well formed. In these chimeric embryos, only the VE and the extraembryonic ectoderm were lacZ-negative (pink cells) and therefore derived from the double mutant morula, whereas the rest of the embryonic and extraembryonic tissues were derived from wild-type ES cells expressing lacZ (blue cells). The lack of rescue of the double mutant phenotype despite the high colonisation of the epiblast by wild-type cells, demonstrates that the primary defect in HNF3β,Lim1 embryos is in VE cells.

It is noteworthy that some lacZ+ cells, with a VE-like morphology, are found in the endoderm of the double mutant-derived chimeric embryos (arrow in Fig. 7B), suggesting that the exclusion of the ES cells from the extraembryonic tissues of the chimeras is not complete. One possible explanation for the incomplete exclusion of ES cells from the VE is that the mutant embryo-derived cells in the chimeras are somewhat compromised in their ability to contribute to the VE layer. Alternatively, the lacZ+ cells in the VE could correspond to gut endoderm cells whose morphology is abnormal as a consequence of defects in the epiblast. Even if the first hypothesis turns out to be the correct one, the presence of a few wild-type cells in the VE of the chimeras is not sufficient to rescue the mutant phenotype, further supporting important roles of HNF3β and Lim1 in the VE.

**Mesoderm patterning is affected in HNF3β,Lim1 embryos**

Despite abnormal epiblast and VE development, mesoderm is formed in HNF3β,Lim1 embryos as shown by the expression of Mes1 and Lefty2. To examine whether different D-V types of mesoderm are produced in double mutant embryos, we determined expression of molecular markers expressed specifically in these different cell types at 7.5–7.75 d.p.c. Using whole-mount in situ hybridisation, we analysed the expression of T, a marker of primitive streak and axial mesoderm in wild-type embryos at 7.5 d.p.c. (Fig. 8A; Wilkinson et al., 1990). In HNF3β,Lim1 embryos, T expression was not observed, indicating that axial mesoderm is not produced in the mutants (Fig. 8A’). This result is not surprising as HNF3β+/– single mutants lack the notochord precursors (Ang and Rossant, 1994; Weinstein et al., 1994) and Lim1+/– mutants are devoid of prechordal mesoderm (Shawlot and Behringer, 1995). However, the absence of T expression suggests also that the primitive streak has been lost in HNF3β,Lim1 embryos at 7.5 d.p.c. Thus, mesoderm production is completed by this stage in the double mutants, whereas in the single mutants of the same stage, the primitive streak is still present.

To determine if other types of mesoderm are produced in HNF3β,Lim1 embryos, we analysed the expression of the bHLH gene Twist, a marker of paraxial and lateral ventral mesoderm at 7.5 d.p.c. (Fig. 8B; Wolf et al., 1991). In HNF3β,Lim1 embryos, Twist is widely expressed in the embryonic region, indicating that non-axial mesoderm derivatives are produced in these embryos (Fig. 8B’). To identify specifically paraxial mesoderm cells, a dorsal type of mesoderm that gives rise to the somites later in development, we used two molecular markers specific for this population of mesoderm cells, the Tbox-related gene Tbx6 and the Notch ligand Delta1 (Fig. 8C; Chapman et al., 1996; Bettenhausen et al., 1995). At 7.75 d.p.c., neither of these two paraxial mesoderm markers was detected in HNF3β,Lim1 embryos (Fig. 8C’ and data not shown), whereas the single mutants show expression of both genes (data not shown). Taken together, these results indicate that dorsal mesoderm derivatives (axial and paraxial) are absent in HNF3β,Lim1 embryos.

In order to analyse the formation of ventral embryonic mesoderm in HNF3β,Lim1 embryos, we characterised the expression of Bmp4 and Flik1. The TGFβ-related molecule BMP4 is expressed at 7.5 d.p.c. in the ventral mesoderm of the proximal region of the embryo, in the base of the allantois and in the chorion (Fig. 8D; Winnier et al., 1995; Waldrip et al., 1998). In HNF3β,Lim1 embryos, Bmp4 transcripts were found in a band at the embryonic-extraembryonic junction indicating the presence of ventral embryonic mesoderm cells in these mutant embryos (Fig. 8D’). In addition, the expression of Bmp4 in the chorion of HNF3β,Lim1 embryos confirmed the histological observation that this structure is correctly formed in these mutants. Flik1 is a tyrosine kinase receptor expressed in early endodermal precursors (Fig. 8E; Yamaguchi et al., 1993). In HNF3β,Lim1 embryos, the expression of Flik1 was detected not only in the proximal region of the embryo but also in the more distal region (Fig. 8E’). This distal expression suggest that in HNF3β,Lim1 mutants embryonic ventral mesoderm cells are ectopically located. Altogether these results indicate that ventral embryonic mesoderm cells are formed in HNF3β,Lim1 embryos.

Finally, we investigated the formation of the most ventral mesoderm derivative, the extraembryonic mesoderm, in HNF3β,Lim1 embryos. To determine the presence of visceral yolk sac extraembryonic mesoderm, we analysed the expression of the growth factor Fgf3 (Fig. 8F; Wilkinson et al., 1989). In HNF3β,Lim1 embryos as well as in the wild-type littermates, Fgf3 transcripts were found in the extraembryonic region demonstrating that the round cells observed in the extraocoelomic cavity of the mutants in histological sections are indeed extraembryonic mesoderm cells (Fig. 8F’). In summary, only ventral mesoderm (embryonic and extraembryonic) is produced in HNF3β,Lim1 embryos. The dorsal mesoderm derivatives, paraxial and axial mesoderm, are missing indicating that mesoderm patterning is severely impaired in HNF3β,Lim1 embryos.

**DISCUSSION**

**Abnormal A-P patterning of the epiblast in HNF3β,Lim1 embryos**

The first morphological sign of A-P pattern in the epiblast of the mouse embryo is the site of formation of the primitive streak at the posterior end of the embryo. The genetic pathway that initiates primitive streak formation remains to be elucidated, but expression of T on one side of the epiblast at the onset of gastrulation marks posterior primitive streak cells. In HNF3β,Lim1 embryos, T expression in the epiblast is no
longer restricted posteriorly, but is instead expressed throughout the epiblast by the mid-streak stage. Thus, A-P polarity of the epiblast is abnormal in HNF3β,Lim1 embryos and widespread expression of T strongly suggests that mutant epiblast cells are transformed into primitive streak cells. The loss of epiblast cells is confirmed by the absence and reduction of expression of Otx2 and Oct4, respectively. In addition, mid-streak-stage embryos also show ectopic mesoderm formation as demonstrated by the expression of MesP1 and Lefty2. As a consequence of these early patterning defects, ectoderm and neuruectoderm cells that are derived from distal and anterior epiblast cells (Lawson et al., 1991) are missing in these embryos at 7.5-7.75 d.p.c. These epiblast defects are not observed in single homozygous HNF3β and Lim1 mutants. Altogether, these data demonstrate that HNF3β and Lim1 function synergistically to establish A-P patterning of the epiblast and to restrict primitive streak formation to the posterior side of mouse embryos.

**HNF3β and Lim1 regulate the patterning function of the VE**

A detailed analysis of the expression patterns of HNF3β and Lim1 in situ hybridization on sections and double-labelling experiments, demonstrates two distinct sites of coexpression. At 5.5 d.p.c., HNF3β and Lim1 are coexpressed in the distal two thirds of the VE. 1 day later, these genes are coexpressed in the AVE and in the anterior primitive streak of mouse embryos. Since the whole epiblast is affected in HNF3β, Lim1 embryos, it is unlikely that loss of activity of HNF3β and Lim1 in anterior primitive streak cells is the primary cause of the epiblast defects. Rather, the earlier and widespread coexpression of HNF3β and Lim1 in the VE suggests that these two genes are required in this tissue. VE has been shown to regulate growth and cell survival as well as A-P patterning of the early mouse embryo (Beddington and Robertson, 1998). The phenotype of HNF3β, Lim1 embryos strongly suggests that the patterning function rather than the trophic function of the VE is affected in these mutant embryos. In particular, the finding that Otx2, Gsc, Cer-l, Hex and Lefty1 expression are lost in the VE of HNF3β, Lim1 embryos indicates that the patterning of VE cells is severely affected in these embryos. In contrast, a general VE marker Pem is normally expressed in HNF3β, Lim1 embryos. Therefore, some aspects of VE development occur normally in these embryos. Furthermore, histological sections, BrdU incorporation studies and TUNEL analysis of cell death performed on HNF3β, Lim1 embryos during gastrulation do not show any evidence of massive cell death or proliferation defects in the epiblast (Fig. 3B,D; data not shown), which would be expected if the trophic function of the VE was affected in these embryos (Tam and Behringer, 1997). Altogether, these results demonstrate that the epiblast defects observed in HNF3β, Lim1 embryos are due to loss of the patterning function of the VE.

To confirm that the patterning defects in HNF3β, Lim1 embryos are due to the function of HNF3β and Lim1 in the VE, we have generated chimeric embryos containing double mutant cells in the VE layer and a completely wild-type embryonic region. These chimeric embryos show no rescue of the HNF3β, Lim1 phenotype, demonstrating that the abnormal patterning of the epiblast and the expanded primitive streak region observed in HNF3β, Lim1 embryos are due to a requirement of HNF3β and Lim1 functions in the VE. A direct role for these genes in extraembryonic ectoderm of chimeric embryos, which is also made of double mutant HNF3β, Lim1 cells, can be excluded since the two genes are not expressed in this tissue. Thus, HNF3β and Lim1 are required in the VE for its patterning function.

Interestingly, HNF3β, Lim1 embryos show defects that are similar to those observed in mutant embryos for the Smad2 gene, a transducer of TGFβ activin signalling (Waldrip et al., 1998). Chimeric studies have also shown that the requirement for Smad2 is in extraembryonic tissues, VE cells and/or extraembryonic ectoderm cells. Lim1 and HNF3β are not expressed in the VE of Smad2Rohml embryos, suggesting that the Smad2Rohml phenotype is in part due to the lack of VE expression of HNF3β and Lim1, and that Smad2 functions upstream of these genes. This hypothesis is tempting in light of the data obtained in Xenopus and zebrafish embryos, demonstrating that the homologs of these two genes are inducible by the TGFβ superfamily molecule Nodal (Toyama et al., 1995; Rebert and Dawid, 1997; Feldman et al., 1998; Howell and Hill, 1997).

**VE-derived signals pattern the epiblast of the mouse embryo**

A requirement of HNF3β and Lim1 in the VE for correct A-P patterning of the epiblast implies that extrinsic signals derived from the VE and dependent on HNF3β and Lim1 are required to maintain the pluripotentiality of epiblast cells and/or to prevent them from adopting a primitive streak fate. Our analysis of gene expression in the VE of HNF3β, Lim1 embryos suggests that Cer-l and Lefty1 are candidate molecules for signalling between VE and epiblast cells.

Gain-of-function as well as biochemical experiments performed in Xenopus embryos have demonstrated that Cerberus (X-cer) triggers head induction and suppresses trunk-tail mesoderm formation by inhibiting the signalling activities of Nodal, BMP4 and Wnt through direct binding to these molecules (Bouwmeester et al., 1996, Hsu et al., 1998; Piccolo et al., 1999). Although Cer-l is not able to mimic the head-inducing activity of X-Cer when injected ventrally in Xenopus embryos, it is able to inhibit BMP activity in Xenopus and mammalian assays (Belo et al., 1997; Biben et al., 1998; Pearce et al., 1999), as well as Nodal and X-Wnt8 activities in Xenopus assays (J. A. Belo and E. M. De Robertis, personal communication).

Another interesting candidate signalling molecule expressed in VE and whose expression is lost in HNF3β, Lim1 embryos is Lefty1. In zebrafish, a novel gene called antivin, belonging to a new subclass of the TGFβ superfamily that also contains the mouse Lefty1 and Lefty2 genes, has been shown to function as an antagonist of TGFβ signalling (Thisse and Thisse, 1999). Moreover, Lefty1 has been shown to have anti-BMP activity in Xenopus animal cap assays (Meno et al., 1997). In addition, a null mutation of Lefty1 in mice demonstrates that this gene regulates the expression of Lefty2 and Nodal in the lateral plate mesoderm, yet the Lefty1 mutant embryos gastrulate normally (Meno et al., 1998). Thus, we propose that HNF3β and Lim1 function in the VE to control directly or indirectly the expression of Cer-l, Lefty1 and possibly other unidentified secreted molecules. These molecules would then act in combination to block mesoderm formation in the anterior...
regions of the epiblast, thus permitting the development of anterior neural structures. Consistent with a role for the anterior visceral endoderm to block mesoderm formation in the anterior epiblast, explants of the anterior epiblast isolated from the VE of wild-type embryos at 6.5 d.p.c. and cultured for 2 days show widespread expression of T (A. P.-G. and S.-L. A., unpublished results).

**HNF3β and Lim1 may be required in the anterior primitive streak to specify dorsal mesoderm cell fates**

The function proposed above for Lim1 and HNF3β in the VE would explain the abnormal patterning of the epiblast as well as the absence of ectoderm derivatives observed in HNF3β;Lim1 embryos. However, it does not explain the absence of dorsal mesoderm and the exclusive production of extraembryonic and ventral mesoderm populations. The molecular mechanisms involved in the development of different D-V populations of mesoderm are not well understood in the mouse embryo. Analyses of Fgfr1 and BMP4 mutants have provided some evidence for the existence of dorsalising and ventralising signals involved in patterning mesoderm cells as they ingress through the different segments of the primitive streak (Yamaguchi et al., 1994; Deng et al., 1994; Winnier et al., 1995). It is generally thought that ventral signals such as BMP4 would be produced by extraembryonic ectoderm and later by ventral mesoderm, whereas dorsalising signals would be produced by the anterior primitive streak expressing BMP antagonists such as chordin, noggin and follistatin. Indeed ectopic graft of the node of a late streak embryo into the posterior proximal region of the epiblast induces the formation of a truncated secondary axis containing somites derived from host tissue (Beddington, 1994). Thus, a likely explanation for the absence of dorsal mesoderm derivatives in HNF3β;Lim1 embryos is the lack of anterior primitive streak shown by the absence of Gsc expression. Loss of axial mesoderm would be a direct consequence of the absence of precursors normally located in the anterior primitive streak, whereas the absence of paraxial mesoderm would be due to the lack of dorsalising signals. The absence of anterior primitive streak cells itself could be a direct effect of the lack of expression of HNF3β and Lim1 in these cells, but it could also be a consequence of the defect in VE discussed above. In Smad2β/β/embryos, the anterior primitive streak also fails to form, suggesting that VE signals patterning the epiblast could also be required for the induction of the anterior primitive streak. To address synergistic function of these two genes in anterior primitive streak cells, we will derive HNF3ββ−/−;Lim1−/− ES cells in the future to generate the reverse type of chimeric embryos, whereby the VE and the embryonic region are made up entirely of wild-type and HNF3ββ/lim1 mutant cells, respectively. In addition, synergistic functions of the two genes in notochordal precursors in the anterior primitive streak are clearly required for notochord development, as HNF3ββ−/−;Lim1−/− embryos lack a notochord and show fusion of somites in the midline, in contrast to normal development of these two structures in HNF3ββ−/− and Lim1−/− embryos (data not shown).

**How do HNF3β and Lim1 interact in the visceral endoderm?**

The present study demonstrates that HNF3β and Lim1 interact in the VE to pattern the epiblast. The coexpression of the two genes in VE cells strongly suggests that the two proteins act in the same cell. An interaction at the cellular level could involve the direct regulation of a common target (e.g. Cer-I) or the involvement in parallel pathways leading to the same signalling event. In any case, loss of one of the two genes is not sufficient to abolish patterning of the epiblast. A role of HNF3β in the VE to ensure the proper elongation of the primitive streak has been previously demonstrated with chimeric embryos (Dufort et al., 1998). Although Lim1 function in the VE has not yet been reported, Lim1 appears to be required in the VE and the prechordal mesoderm to induce head development in chimeric embryos (W. S. and R. R. B, unpublished results). Thus these two genes have separate functions in the VE, but the double mutant analysis reveals that they also share a new and earlier function of patterning the epiblast.

In summary, we have shown that HNF3β and Lim1 function synergistically in the VE before and during gastrulation to establish the A-P polarity of the embryo, by restricting the extent of epiblast territory that contributes to the primitive streak. The model that we propose invokes a role for these transcription factors in regulating the expression of secreted molecules from the VE that act on the epiblast by preventing mesoderm formation in the anterior regions of the embryo (Fig. 9).

The loss of posterior neural tissues in double mutant embryos is most likely due to additional functions of the two genes in establishment of anterior primitive streak cells that are required for trunk development. Future experiments will be aimed at testing different steps of the model proposed in Fig. 9 by coculturing epiblast isolated from wild-type embryos with cells expressing the different candidate signalling molecules.

---

We are grateful to Drs R. Beddington, M. Blum, P. Chambon, H. Hamada, R. Harvey, D. Henrique, B. Herrmann, B. L. Hogan, M. Kuehn, G. Martin, V. E. Papaloannou, Y. Saga, H. Scholer, F. Perrin-
REFERENCES


Role of HNF3β and Lim1 in mouse axis development


