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Lim1 is required in both primitive streak-derived tissues and visceral endoderm for head formation in the mouse

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SUMMARY

***Lim1* is a homeobox gene expressed in the extraembryonic anterior visceral endoderm and in primitive streak-derived tissues of early mouse embryos. Mice homozygous for a targeted mutation of *Lim1* lack head structures anterior to rhombomere 3 in the hindbrain. To determine in which tissues *Lim1* is required for head formation and its mode of action, we have generated chimeric mouse embryos and performed tissue layer recombination explant assays. In chimeric embryos in which the visceral endoderm was composed of predominantly wild-type cells, we found that *Lim1*$^{-/-}$ cells were able to contribute to the anterior mesendoderm of embryonic day 7.5 chimeric embryos but that embryonic day 9.5 chimeric embryos displayed a range of head defects. In addition, early somite stage chimeras generated by injecting *Lim1*$^{-/-}$ embryonic stem cells into wild-type tetraploid blastocysts lacked forebrain and midbrain neural tissue. Furthermore, in explant recombination assays, anterior mesendoderm from *Lim1*$^{-/-}$ embryos was unable to maintain the expression of the anterior neural marker gene *Otx2* in wild-type ectoderm. In complementary experiments, embryonic day 9.5 chimeric embryos in which the visceral endoderm was composed of predominantly *Lim1*$^{-/-}$ cells and the embryo proper of largely wild-type cells, also phenocopied the *Lim1*$^{-/-}$ headless phenotype. These results indicate that *Lim1* is required in both primitive streak-derived tissues and visceral endoderm for head formation and that its inactivation in these tissues produces cell non-autonomous defects. We discuss a double assurance model in which *Lim1* regulates sequential signaling events required for head formation in the mouse.**

Key words: *Lim1*, Chimera, Primitive streak, Visceral endoderm, Head formation, Mouse

INTRODUCTION

The organizer experiment of Spemann and Mangold demonstrated that the dorsal lip region of an amphibian gastrula stage embryo could direct the formation of a secondary embryonic axis and induce neural tissue from ectoderm when transplanted to a host amphibian embryo (Spemann and Mangold, 1924). Later, Spemann also observed that the inductive properties of the dorsal lip region changed during the course of gastrulation. Transplantation of dorsal lip regions from early gastrula stage embryos resulted in the formation of secondary axes that contained head structures whereas transplantation of dorsal lip regions from late gastrula stage embryos resulted in the formation of secondary axes that contained trunk and tail structures. These findings led Spemann to propose the existence of a distinct head and trunk organizer in vertebrate embryos (Spemann, 1931).

In the mouse, the node is a morphologically distinct structure located at the rostral end of the primitive streak of embryonic day (E) 7.5 embryos. The node from late streak stage embryos is able to induce a secondary embryonic axis when transplanted to the lateral aspect of a similarly staged mouse host embryo; however, the induced secondary axis in these embryos lack any discernable anterior neural tissue (Beddington, 1994). One possible explanation for the failure of the node to induce anterior neural tissue is that at the late streak stage, the node may be equivalent to the amphibian late gastrula dorsal lip or trunk organizer. The inductive properties of the presumptive node have been tested at an earlier stage, when it may be analogous to the early dorsal lip, by transplanting tissue from the posterior epiblast region of early primitive streak stage embryos (Tam et al., 1997). Cells from this region express several node-specific genes and fate mapping studies indicate that they contribute descendants to node derivatives including the definitive endoderm and notochord (reviewed by Tam and Behringer, 1997). When transplanted to a late streak stage host embryo, the posterior epiblast tissue is able to induce a secondary embryonic axis but

none of the induced structures displayed any anterior characteristics (Tam et al., 1997). These experiments suggest that the mouse node, unlike the amphibian dorsal lip region, is not sufficient to induce ectoderm to become anterior neural tissue.

Recent experiments have implicated the visceral endoderm in anterior neural induction in mouse. The visceral endoderm is an extraembryonic tissue that surrounds the epiblast of the egg cylinder stage embryo (Rossant, 1986). During gastrulation the visceral endoderm is replaced by definitive endoderm that derives from the anterior portion of the primitive streak (Lawson and Pedersen, 1987). Although no morphological asymmetries are apparent in the visceral endoderm, molecular studies have shown that a distinct anterior-posterior pattern exists in the visceral endoderm prior to the formation of the primitive streak (Rosenquist and Martin, 1995; Hermesz et al., 1996; Thomas and Beddington, 1996; Thomas et al., 1998). These studies revealed that the VE-1 antigen and the homeobox genes *Hesx1*/*Rpx* and *Hex* are expressed in the anterior visceral endoderm that underlies the ectoderm fated to form the anterior portion of the neural plate. Ablation experiments have shown that, if the anterior visceral endoderm is removed at the early streak stage, expression of *Hesx1*/*Rpx* in the anterior neuroectoderm in late streak/headfold stage embryos is absent or greatly reduced (Thomas and Beddington, 1996).

Chimera experiments have provided genetic evidence that extraembryonic tissues play an important role in establishing anterior identity in the mouse embryo. Mice homozygous for a retroviral insertion in the *nodal* gene fail to gastrulate and die during embryogenesis (Conlon et al., 1991). In chimeric embryos in which the visceral endoderm was composed of primarily *nodal* mutant cells and the embryonic portion of primarily wild-type cells, the gastrulation defect was rescued but the resulting embryos lacked anterior head structures (Varlet et al., 1997). Similarly, *Otx2*, a homeobox gene expressed in the visceral endoderm and the ectoderm, has been shown to be required in the visceral endoderm and subsequently in the neuroectoderm for development of the forebrain and midbrain (Rhinn et al., 1998). Furthermore, the expression of the anterior neural marker *Bf1* and the midbrain/hindbrain neural marker *Engrailed1* (*En1*) can occur in the absence of embryonic mesendoderm as seen in embryos homozygous for a mutation in *Cripto*, which encodes a putative

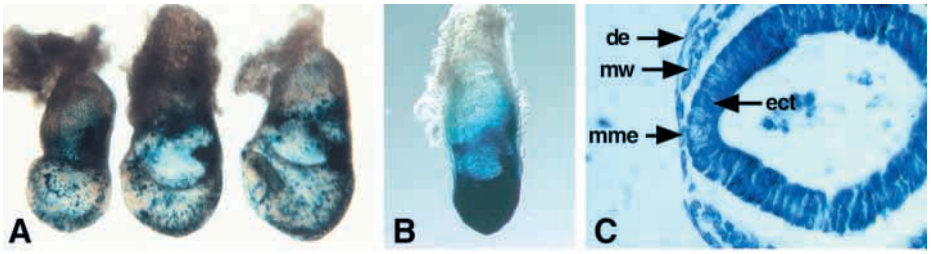

Fig. 1. E7.5 *Lim1*$^{-/-}$ ↔ +/+ chimeras. (A) Distribution of *Lim1*$^{-/-}$ cells (3-21) in *Lim1*$^{-/-}$ ↔ +/+ chimeras. *Lim1*$^{-/-}$ cells are randomly distributed in the embryo proper. (B) Phenotypic appearance of a E7.5 *Lim1*$^{-/-}$ ↔ +/+ chimera (3-21) with a contribution of *Lim1*$^{-/-}$ cells estimated to be greater than 95%. Unlike E7.5 *Lim1*$^{-/-}$ embryos, no constriction is present between the embryonic and extraembryonic region of *Lim1*$^{-/-}$ ↔ +/+ chimeric embryos. (C) Transverse section of a E7.5 *Lim1*$^{-/-}$ ↔ +/+ chimera (3-21) at the level of the presumptive forebrain region. The contribution of *Lim1*$^{-/-}$ cells (blue) is estimated to be greater than 95%. *Lim1*$^{-/-}$ cells are present in the definitive endoderm (de), the mesodermal wings (mw), the midline mesendoderm (mme) and the ectoderm (ect).

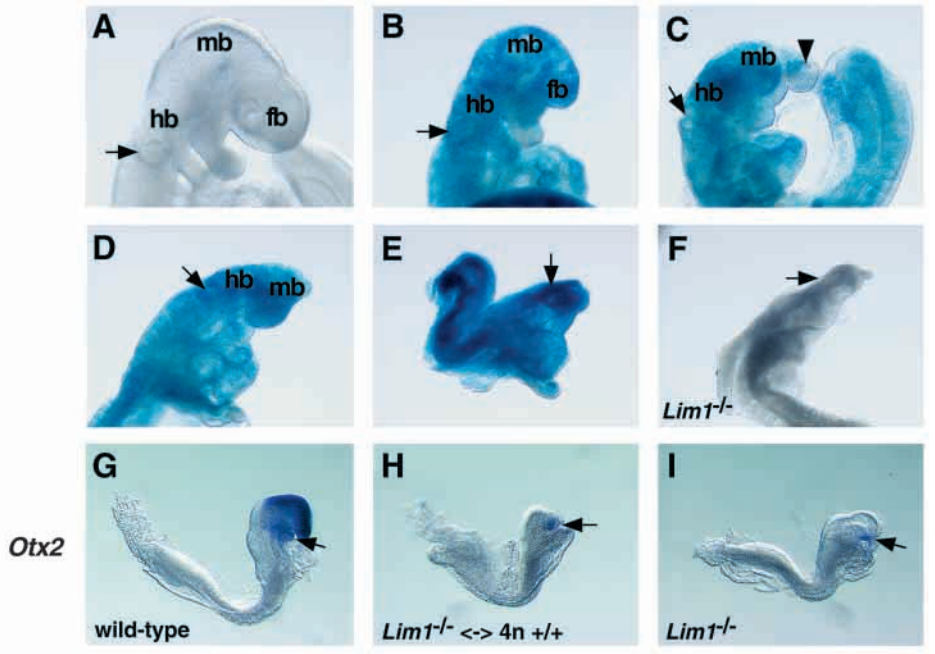

Fig. 2. Head phenotype of E9.5 *Lim1*$^{-/-}$ ↔ +/+ chimeras. (A) Non-chimeric embryo with normal anterior neural development displaying forebrain (fb), midbrain (mb) and hindbrain (hb). (B) Moderate percentage chimera in which the forebrain region is reduced. An optic placode was not present. (C) Moderate percentage chimera having a small anterior projection bearing a single optic placode (arrowhead). (D) A moderate to high percentage chimera in which the forebrain region is absent. (E) A very high percentage chimera that lacks head structures just anterior to the otic vesicle. (F) *Lim1*$^{-/-}$ embryo displaying a loss of head structures just anterior to the otic vesicle. All chimeras shown here were generated using *Lim1*$^{-/-}$ ES cell line 3-21. The arrows in A-F mark the otic vesicle. (G) Expression of *Otx2* in an E8.0 wild-type embryo. (H) Expression of *Otx2* in a E8.0 *Lim1*$^{-/-}$ ↔ tetraploid (4n) +/+ chimera. (I) Expression of *Otx2* in an E8.0 *Lim1*$^{-/-}$ embryo. The arrows in G-I mark the first branchial arch.

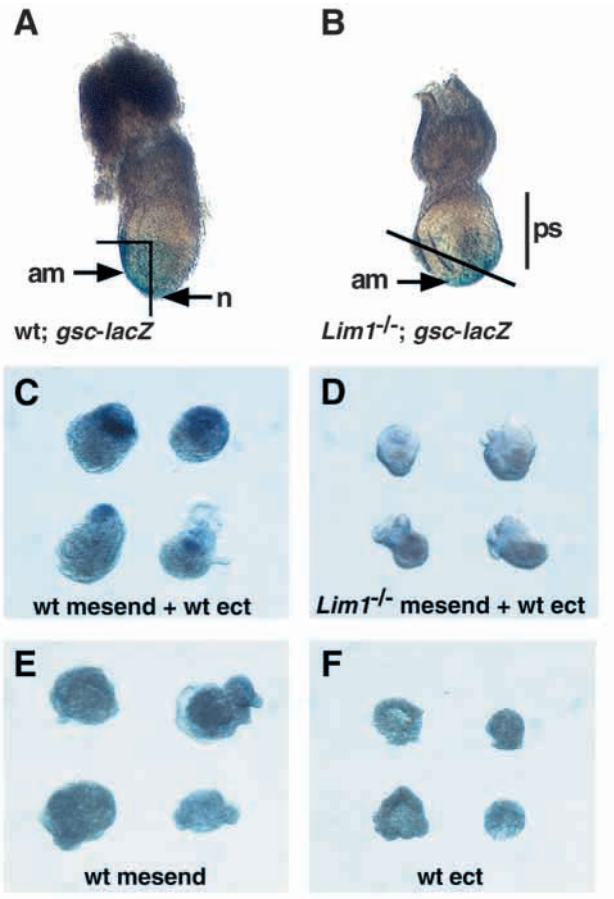

Fig. 3. Expression of *Otx2* in tissue layer recombination explants. (A) *lacZ* expression in a E7.5 wild-type embryo that carries a *lacZ* transgene integrated into the *gsc* locus. The *lacZ* transgene is expressed in the anterior mesendoderm and the node. (B) *lacZ* expression in a E7.5 *Lim1*$^{-/-}$ embryo that carries a *lacZ* transgene targeted to the *gsc* locus. The *lacZ* transgene is expressed in the distal portion of embryo in the rostral portion of the primitive streak and in the anterior mesendoderm region. The lines through the embryos in A and B indicate the cuts made to isolate anterior mesendoderm. The line outside the embryo in B indicates the extent of the primitive streak. am, anterior mesendoderm; n, node; ps, primitive streak. (C) Expression of *Otx2* in control E7.5 wild-type anterior mesendoderm + E6.5 wild-type ectoderm explants. (D) Lack of *Otx2* expression above background in E7.5 *Lim1*$^{-/-}$ anterior mesendoderm + E6.5 wild-type ectoderm explants. (E) Absence of *Otx2* expression in E7.5 wild-type mesendoderm explants cultured alone. (F) Absence of *Otx2* expression in E6.5 wild-type ectoderm explants cultured alone.

signaling molecule (Deng et al., 1998). These results suggest that the anterior visceral endoderm is necessary for mammalian head formation (Bouwmeester and Leyns, 1997).

The mouse *Lim1* homeobox gene (also known as *Lhx1*) is expressed in both the anterior visceral endoderm and in primitive streak-derived tissues including the node, the mesodermal wings and the anterior mesendoderm that underlies the presumptive anterior neural plate (Barnes et al., 1994; Shawlot and Behringer, 1995; Belo et al., 1997, Shimono and Behringer, 1999). *Lim1* expression in the anterior primitive streak-derived tissues is transient and is downregulated after embryonic day (E) 7.5 (Barnes et al., 1994; W. S., unpublished data). To study the function of *Lim1* during mouse embryogenesis, we previously generated a mutation in *Lim1* by homologous recombination in embryonic stem (ES) cells (Shawlot and Behringer, 1995). Homozygous mutant embryos have defects in node and axial mesendoderm development and do not form head structures anterior to rhombomere 3 in the hindbrain (Shawlot and Behringer, 1995). These studies however, have not resolved the cellular site of *Lim1* action. To determine in which tissues *Lim1* is required for head formation and its mode of action, we have performed chimera studies and tissue layer recombination assays. Surprisingly, we found that *Lim1* is required in both primitive streak-derived tissues and extraembryonic tissues for head formation and that its inactivation in these tissues produces cell non-autonomous defects. Thus the anterior visceral endoderm is necessary for head formation but this process also requires streak-derived tissues. We discuss a double assurance model in which *Lim1* regulates sequential signaling events from the anterior visceral endoderm and the anterior definitive mesendoderm that are necessary for head formation in the mouse.

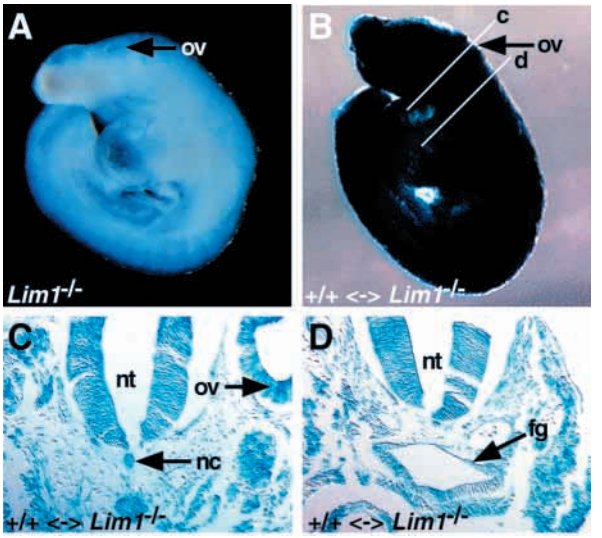

Fig. 4. Analysis of +/+ ↔ *Lim1*$^{-/-}$ chimeras at E9.5. (A) Phenotype of a E9.5 *Lim1*$^{-/-}$ embryo showing the absence of head structures just anterior to the otic vesicle (ov). Taken from Shawlot and Behringer (1995). (B) Phenotype of a E9.5 +/+ ↔ *Lim1*$^{-/-}$ chimera. The embryo proper contains a high percentage of wild-type cells (blue) but the embryo lacks head structures anterior to the otic vesicle. The lines through the embryo indicate the level of the transverse section in C and D. (C) Transverse section of a +/+ ↔ *Lim1*$^{-/-}$ chimera at the level of the otic vesicle. (D) Transverse section of same embryo at the level of the heart. In both sections, wild-type cells (blue) have contributed extensively to the neural tube (nt), notochord (nc) and foregut endoderm (fg) but do not rescue head development.

MATERIALS AND METHODS

Generation of ES cell lines

ES cell lines were isolated from blastocysts obtained from *Lim1*$^{+/-}$ females crossed with *Lim1*$^{+/-}$, ROSA26 males using standard methods (Robertson, 1987). Twelve independent ES cell lines were derived and

genotyped by Southern hybridization using a 5′ *Lim1* probe (Shawlot and Behringer, 1995).

Generation and X-gal staining of chimeras

Chimeras were generated by blastocyst injection (Bradley, 1987). To assess the role of *Lim1* in epiblast tissues, ES cells were injected into blastocysts obtained from Swiss Webster mice (Taconic, Germantown, NY). To assess the role of *Lim1* in the visceral endoderm, $Lim1^{+/+}$ ES cells (ES31) were injected into blastocysts obtained by crossing $Lim1^{+/-}$ mice. Injected blastocysts were transferred to pseudopregnant Swiss Webster females and recovered at E7.5 and E9.5. Embryos were fixed in 0.2% glutaraldehyde and processed for X-gal staining. Embryos were postfixed in 4% paraformaldehyde and paraffin embedded (Hogan et al., 1994).

$Lim1^{-/-} \leftrightarrow$tetraploid +/+ chimeras were generated by injecting $Lim1^{-/-}$ ES cells into wild-type tetraploid blastocysts (Swiss Webster strain). 2-cell stage wild-type embryos were isolated and electrofused using a CF-150 Cell Fusion Instrument (Biochemical Laboratory Services, Budapest, Hungary). Embryos were fused in M2 medium with 3 pulses of 90 volts and 94 microseconds. Using these parameters, 80-90% of the embryos fused within 1 hour. The fused embryos were cultured to the blastocyst stage in M16 media. Blastocyst injections were performed on the morning of E4. Approximately one third of the injected tetraploid embryos were recovered at E8.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed by standard methods (Wilkinson, 1992). The *Otx2* probe used was pBSotd 9 (Ang et al., 1994). Hybridization and washes were performed at 65°C.

Tissue layer recombination explant cultures

Ectoderm from the distal tip of the egg cylinder was isolated from E6.5 early streak stage embryos using pulled glass capillary needles. Anterior mesendoderm from E7.5 embryos was isolated using tungsten needles after incubating the embryos in 0.5% trypsin, 0.25% pancreatin in PBS at 4°C for 10 minutes (Ang and Rossant, 1993). Tissue fragments were cultured for 2 days in drops of Dulbecco's modified Eagles (DME) media containing 15% fetal calf serum, 2 mM glutamine and 0.1 mM 2-mercaptoethanol.

Generation of *Lim1-τ-lacZ* mice

The 5′ 1.2 kb *Hin*dIII-*Hin*dIII DNA fragment and the 3′ 4.3 kb *Bam*HI-*Bam*HI DNA fragment from the *Lim1* locus (Shawlot and Behringer, 1995) were inserted into the IRES-τ-*lacZ*-loxPGKneo-lox vector of Mombaerts et al. (1996). Correct targeting results in the replacement of the entire *Lim1*-coding region with the *lacZ*-neo cassette, thereby creating a null allele. The targeting vector was electroporated into W95 129/Sv ES cells and 96 G418-resistant clones were isolated. The G418-resisitant ES cell clones were analyzed by Southern hybridization using the 5′ and 3′ DNA probes described by Shawlot and Behringer (1995). One correctly targeted ES cell clone was identified that produced a 5 kb wild-type band and a 1.8 kb targeted band in genomic DNA digested with *Kpn*I and hybridized with the 5′ probe. Correct targeting was confirmed by digestion of the genomic DNA with *Eco*RI and hybridization with the 3′ probe, which produced a 12 kb wild-type band and a 10 kb targeted band. Blastocyst injections were performed as described previously (Mombaerts et al., 1996).

RESULTS

Function of *Lim1* in primitive streak-derived tissues

Previous studies have demonstrated that ES cells injected into blastocysts do not contribute extensively to the extraembryonic endoderm (Beddington and Robertson, 1989). We have used this developmental bias of ES cells to generate highly polarized *Lim1* chimeras in which the embryo proper is predominantly of one genotype and the extraembryonic endoderm is of another.

Twelve independent *Lim1* ES lines were generated from blastocysts obtained from $Lim1^{+/-}$ females mated to $Lim1^{+/-}$ males homozygous for the ROSA26 *lacZ* transgene (data not shown) which is widely expressed during development (Friedrich and Soriano, 1991). To determine if $Lim1^{-/-}$ cells can contribute normally to primitive streak-derived tissues, we injected separately two $Lim1^{-/-}$ ES cell lines, 3-21 and ES27, into wild-type blastocysts. These chimeric embryos are referred to as $Lim1^{-/-} \leftrightarrow$+/+ chimeras. As controls, we also injected two $Lim1^{+/+}$ ES cell lines, ES10 and ES31, into wild-type blastocysts. These chimeric embryos are referred to as $Lim1^{+/+} \leftrightarrow$+/+ chimeras. The injected blastocysts were transferred to pseudopregnant recipients and potential chimeric embryos were isolated at E7.5 and stained with X-gal to determine the location of the ES-derived cells.

Approximately 150 E7.5 $Lim1^{-/-} \leftrightarrow$+/+ chimeras were recovered. The chimeric embryos resulting from the injection of $Lim1^{-/-}$ ES cell lines 3-21 and ES27 were similar. When compared with $Lim1^{+/+} \leftrightarrow$+/+ control chimeras (data not shown), the *lacZ*-marked $Lim1^{-/-}$ cells were randomly distributed in $Lim1^{-/-} \leftrightarrow$+/+ chimeras (Fig. 1A). $Lim1^{-/-}$ cells did not appear to be excluded from or concentrate in any region of the embryo proper. Unlike E7.5 $Lim1^{-/-}$ mutant embryos, very high percentage $Lim1^{-/-} \leftrightarrow$+/+ chimeras (greater than 95% $Lim1^{-/-}$ cells) did not have a constriction between the embryonic and extraembryonic region (Fig. 1B). As the extraembryonic tissues in these chimeric embryos are composed of predominantly wild-type cells, the constriction present in $Lim1^{-/-}$ embryos is likely to be caused by a defect in extraembryonic tissues.

To analyze the contribution of $Lim1^{-/-}$ cells to the germ layers, transverse sections of E7.5 neural plate stage $Lim1^{-/-} \leftrightarrow$+/+ chimeras were made. In moderate and high percentage $Lim1^{-/-} \leftrightarrow$+/+ chimeras, *lacZ*-tagged $Lim1^{-/-}$ cells were able to contribute to the anterior midline mesendoderm, the mesodermal wings and the definitive anterior endoderm even in chimeric embryos in which the contribution of mutant cells was estimated to be greater than 95% (Fig. 1C). Thus it is unlikely that the mutant cells are rescued by the presence of wild-type cells. These results indicate that $Lim1^{-/-}$ cells can contribute normally to the primitive streak-derived tissues of E7.5 $Lim1^{-/-} \leftrightarrow$+/+ chimeras.

To determine whether *Lim1* is required in primitive streak-derived cells for the development of anterior structures, we analyzed the head phenotype of E9.5 $Lim1^{-/-} \leftrightarrow$+/+ chimeric embryos. The visceral endoderm of these chimeric embryos is predominantly composed of wild-type cells. The chimeric embryos generated with $Lim1^{-/-}$ ES cell lines 3-21 and ES27 were phenotypically similar and the results from these two lines were combined. Non-chimeric and low percentage chimeric embryos had normal anterior head development (Fig. 2A). In contrast, 16 of 23 chimeric embryos with an estimated contribution of $Lim1^{-/-}$ cells greater than 50% had defects in head development. Moderate percentage chimeric embryos (50-75% $Lim1^{-/-}$ cells) were microcephalic with the forebrain region either reduced or absent (Fig. 2B-D). Very high

percentage chimeric embryos (>95% *Lim1*$^{-/-}$ cells) lacked head structures just anterior to the otic vesicle and were identical to E9.5 *Lim1*$^{-/-}$ embryos (Fig. 2E, F). *Lim1*$^{+/+}$↔+/+ control chimeras (*n*=20) that were generated using ES cell lines ES10 and ES31 had normal head development (data not shown). As *Lim1* is not expressed in the central nervous system until E10 (Barnes et al., 1994; Fujii et al., 1994), it is unlikely that the anterior defects observed in E9.5 *Lim1*$^{-/-}$↔+/+ chimeras are due to a requirement for *Lim1* in the anterior neural tissue itself. Despite the absence of a constriction between the embryonic and extraembryonic region of E7.5 chimeric embryos with a very high contribution of mutant cells, a headless phenotype identical to that seen in *Lim1*$^{-/-}$ embryos was observed in E9.5 chimeric embryos with a very high contribution of mutant cells. This suggests that the constriction and any defects in cell movement caused by the constriction are not the primary cause of the headless phenotype.

To analyze anterior neural development at an earlier stage, we next examined *Otx2* expression in early somite stage E8.0 *Lim1*$^{-/-}$↔+/+ tetraploid chimeras. When ES cells are introduced into tetraploid blastocysts, the embryo proper is almost completely ES cell-derived while the extraembryonic endoderm is composed of mainly tetraploid cells (Nagy et al., 1990). To generate these chimeras, we injected *Lim1*$^{-/-}$ ES cells (3-21) into wild-type tetraploid blastocysts. In E8.0 wild-type embryos, *Otx2* is expressed in the forebrain and midbrain and in the first branchial arch (Fig. 2G) (Ang et al., 1994). In E8.0 *Lim1*$^{-/-}$↔+/+ tetraploid chimeras (*n*=2) and E8.0 *Lim1*$^{-/-}$ embryos (*n*=2), *Otx2* was expressed only in the first branchial arch indicating that anterior neural tissue was not present (Fig. 2H,I). These results indicate that *Lim1* is required in primitive streak-derived tissues for the development of anterior head structures.

Lim1 tissue layer recombination explants

Previous studies have shown that, in explant cultures, E7.5 anterior mesendoderm can maintain the expression of *Otx2* in E6.5 ectoderm (Ang et al., 1994). As *Lim1*$^{-/-}$ cells were able to contribute normally to the anterior mesendoderm of high percentage E7.5 *Lim1*$^{-/-}$↔+/+ chimeras but E8.0 *Lim1*$^{-/-}$↔+/+ tetraploid chimeras lacked anterior neural structures, we hypothesized that *Lim1* regulated the production of a factor that acted cell non-autonomously. To test this hypothesis, we performed tissue layer explant recombination experiments using anterior mesendoderm from E7.5 *Lim1*$^{-/-}$ embryos. To confirm that anterior mesendoderm is present in E7.5 *Lim1*$^{-/-}$ embryos, we examined the expression of the *goosecoid* (*gsc*) gene, an anterior mesendoderm marker expressed between E7.0 and E7.75 (Faust et al., 1995). To visualize *gsc* expression, we used a mouse strain containing a *lacZ* insertion in the *gsc* locus. E7.5 embryos heterozygous for this insertion expressed *lacZ* in the node and the anterior mesendoderm (M. W. and R. B., unpublished data) (Fig. 3A). In E7.5 *Lim1*$^{-/-}$ embryos carrying the *gsc-lacZ* marker, *lacZ* expression was observed in the distal portion of the embryo corresponding to the anterior portion of the primitive streak and the anterior mesendoderm (Fig. 3B). The location of the anterior aspect of the primitive streak in *Lim1*$^{-/-}$ embryos, as revealed by the *gsc-lacZ* marker, is in agreement with previous molecular marker studies that examined the expression of the primitive streak and node markers *Brachyury*, *nodal* and *HNF3β* (Shawlot and Behringer, 1995). These *gsc-lacZ* marker results indicate that anterior type mesendoderm is present in *Lim1*$^{-/-}$ embryos and that it is located in the distal portion of the embryo.

In control explant experiments, wild-type E7.5 (late streak-neural plate stage) anterior mesendoderm was recombined with E6.5 (early streak stage) distal tip ectoderm, cultured for 2 days and then assayed for *Otx2* expression by RNA whole-mount in situ hybridization. 11 of 13 wild-type recombinant explants expressed *Otx2* (Fig. 3C). When cultured alone, 0/7 mesendoderm explants expressed *Otx2* (Fig. 3E). Two of 12 ectoderm explants expressed *Otx2* when cultured alone (Fig. 3F). The two ectoderm explants that expressed *Otx2* may have contained some mesendoderm tissue, or alternatively, the ectoderm may have already received inductive signals from the mesendoderm prior to its isolation. When E7.5 *Lim1*$^{-/-}$ anterior mesendoderm from the distal tip region, which excludes the posterior region of the embryo, was recombined with E6.5 wild-type ectoderm, 0/11 explants expressed *Otx2* above background (Fig. 3D). The results of these explant recombination experiments indicate that *Lim1*$^{-/-}$ anterior mesendoderm is defective in anterior neural signaling.

Function of *Lim1* in extraembryonic tissues

To determine if *Lim1* is required in extraembryonic tissues for anterior neural development, we injected *Lim1*$^{+/+}$ ES cells (ES31) into blastocysts obtained from *Lim1*$^{+/-}$ intercrosses. Approximately one quarter of the injected blastocysts should be of the genotype *Lim1*$^{-/-}$ and give rise to chimeras in which the extraembryonic tissues are composed almost exclusively of *Lim1*$^{-/-}$ cells. These embryos are referred to as +/+↔*Lim1*$^{-/-}$ chimeras. Of the thirty embryos recovered at E9.5, eight were phenotypically indistinguishable from *Lim1*$^{-/-}$ embryos, which lack head structures just anterior to the otic vesicle (Fig. 4A). We did not observe any chimeric embryos with microcephaly. After staining with X-gal, four of the headless embryos were estimated to contain greater than 50% wild-type cells with one chimeric embryo containing greater than 95% wild-type cells in the embryo proper (Fig. 4B). Histological analysis of this chimeric embryo demonstrated that the wild-type cells had contributed extensively to the anterior portion of the embryo including the neural tube, notochord and foregut tissues (Fig. 4C,D). A similar distribution of wild-type cells was seen in the three moderate percentage +/+↔*Lim1*$^{-/-}$ headless chimeras (data not shown). We were not able to genotype the extraembryonic tissues from the chimeric embryos by PCR because the yolk sac endoderm layer that we isolated for genotyping was presumably contaminated with mesoderm cells that derive from the embryo proper. Because of this, we cannot exclude the possibility that the headless phenotype was rescued in some chimeric embryos. However, the observation that four chimeric embryos with a substantial contribution of wild-type cells had a complete headless phenotype indicates that *Lim1* is required in extraembryonic tissues for head formation.

Lim1 is not required in the visceral endoderm for *Lim1* expression in primitive streak-derived tissues

Next, we determined if the lack of anterior neural development in +/+↔*Lim1*$^{-/-}$ chimeras was due to an inability of the mutant visceral endoderm to induce *Lim1* expression in the primitive

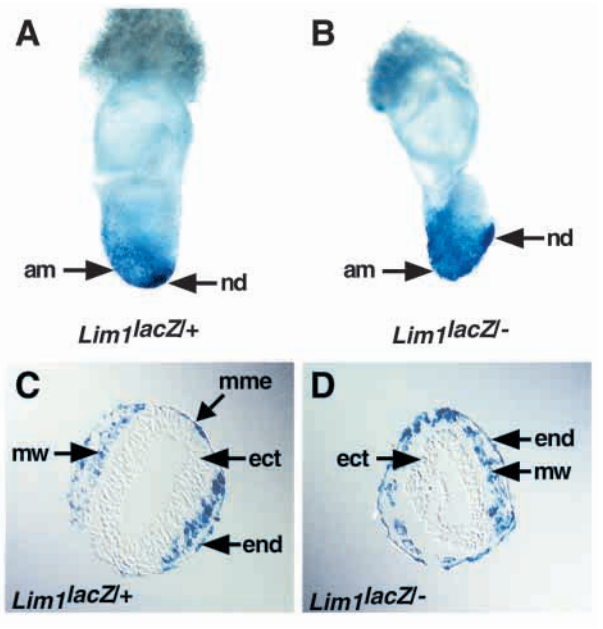

Fig. 5. Analysis of *Lim1* expression in primitive streak-derived tissues. (A) Expression of *lacZ* in an E7.5 $Lim1^{lacZ/+}$ embryo. (B) Expression of *lacZ* in an E7.5 $Lim1^{lacZ/-}$ mutant embryo. Expression of *lacZ* is present in the node region and anterior mesendoderm in both the wild-type and mutant embryos. (C) Transverse section of an E7.5 $Lim1^{lacZ/+}$ embryo. (D) Transverse section of $Lim1^{lacZ/-}$ mutant embryo. In both embryos, *lacZ* is expressed in the mesodermal wings and the endoderm. ect, ectoderm; end, endoderm; mme, midline mesendoderm; mw, mesodermal wing.

streak-derived tissues. We analyzed expression directed by the endogenous *Lim1* promoter in *Lim1* mutant embryos that carry a *τ-lacZ* transgene targeted to the *Lim1* locus (A. K. and T. J., unpublished data). This $Lim1^{lacZ}$ allele is functionally equivalent to our original *Lim1*-null allele. The details of these mice will be published elsewhere. $Lim1^{lacZ/+}$ males were mated with $Lim1^{+/-}$ females and embryos were collected at E7.5 (neural plate stage) and stained with X-gal. In $Lim1^{lacZ/+}$ embryos, *lacZ* expression was observed in the node and the anterior mesendoderm, which is identical to the endogenous *Lim1* expression pattern (Fig. 5A). Analysis of transverse histological sections showed that *lacZ* was expressed in the mesodermal wings, the endoderm and the anterior midline mesendoderm tissue (Fig. 5C). In $Lim1^{lacZ/-}$ mutant embryos, *lacZ* expression was also observed in the node region and in the anterior mesendoderm (Fig. 5B). Analysis of transverse sections showed that *lacZ* was expressed in the mesodermal wings and the endoderm of *Lim1* mutant embryos (Fig. 5D). These results indicate that *Lim1* is not required in the visceral endoderm to induce *Lim1* expression in the primitive streak-derived tissues. Thus, the $+/+ \leftrightarrow Lim1^{-/-}$ chimera results indicate that *Lim1* is required in extraembryonic tissues to directly induce anterior neural tissue.

DISCUSSION

Our results demonstrate that *Lim1* is required in both primitive streak-derived tissues and extraembryonic tissues for head formation and that its inactivation in these tissues leads to cell non-autonomous defects. Anterior visceral endoderm therefore is necessary for head formation but additional signals from the streak-derived definitive anterior mesendoderm are also essential for head development. These findings are consistent with previous recombination explant experiments in mice showing that mid- to late streak stage anterior mesendoderm can induce anterior neural fates in naïve epiblast (Ang and Rossant, 1993). We have also demonstrated that *Lim1* is not required in the anterior visceral endoderm to initiate or maintain *Lim1* expression in primitive streak-derived tissues. Furthermore, *Lim1* is not required in the primitive streak-derived tissues to maintain *Lim1* expression in the anterior visceral endoderm because *Lim1* expression is observed in the anterior visceral endoderm of E7 *Wnt3* mutant embryos, which lack a primitive streak and associated tissues (Liu et al., 1999). Based on our chimera findings we hypothesize that *Lim1* regulates sequential signaling events, first in the anterior visceral endoderm and then subsequently in the anterior definitive mesendoderm that underlies the future anterior neural plate.

A double assurance model for head development

Spemann (1927) described a two-step induction or double assurance model for neural plate determination in amphibian embryos. Spemann hypothesized that the first step occurred by forward spreading of a neuralizing agent in the surface layer from the dorsal blastopore lip to the adjacent ectoderm. The second step occurred when the involuting dorsal mesoderm came to underlie the presumptive neural plate. Spemann wrote, “It would be entirely conceivable that [its] induction which occurs after its exposure to the subjacent mesoderm is merely the continuation of another induction which was initiated when the [prospective mesodermal and ectodermal] materials were still lying side by side on the surface” (Spemann, 1927; Hamburger, 1988).

A two-step model for anterior neural induction in the mouse has recently been proposed (Thomas and Beddington, 1996). This model suggests that the visceral endoderm is responsible for inducing anterior identity in the embryo and that this identity is subsequently reinforced and maintained by primitive streak-derived anterior mesendoderm. Our results provide genetic support for this model. We hypothesize that *Lim1* regulates the production of a secreted factor or factors from the anterior visceral endoderm at the pre- to early streak stage that causes the overlying ectoderm to differentiate as anterior neural tissue. This anterior neural fate is labile, however, and a second *Lim1*-regulated signal from the primitive streak-derived definitive mesendoderm, which replaces the visceral endoderm beginning at the mid- to late streak stage, is required to maintain or complete the differentiation of the anterior ectoderm as anterior neural tissue.

We interpret our chimera results with respect to this two-step induction model (Fig. 6). In $Lim1^{-/-} \leftrightarrow +/+$ chimeras, the first signaling event occurs normally because the visceral endoderm is composed of wild-type cells. The second signaling event, however, is altered because the definitive mesendoderm is a mixture of both wild-type cells and $Lim1^{-/-}$ cells. Consequently, there is a reduction in the amount and or the distribution of the second *Lim1*-regulated signal, which results in the development of chimeric embryos with microcephaly.

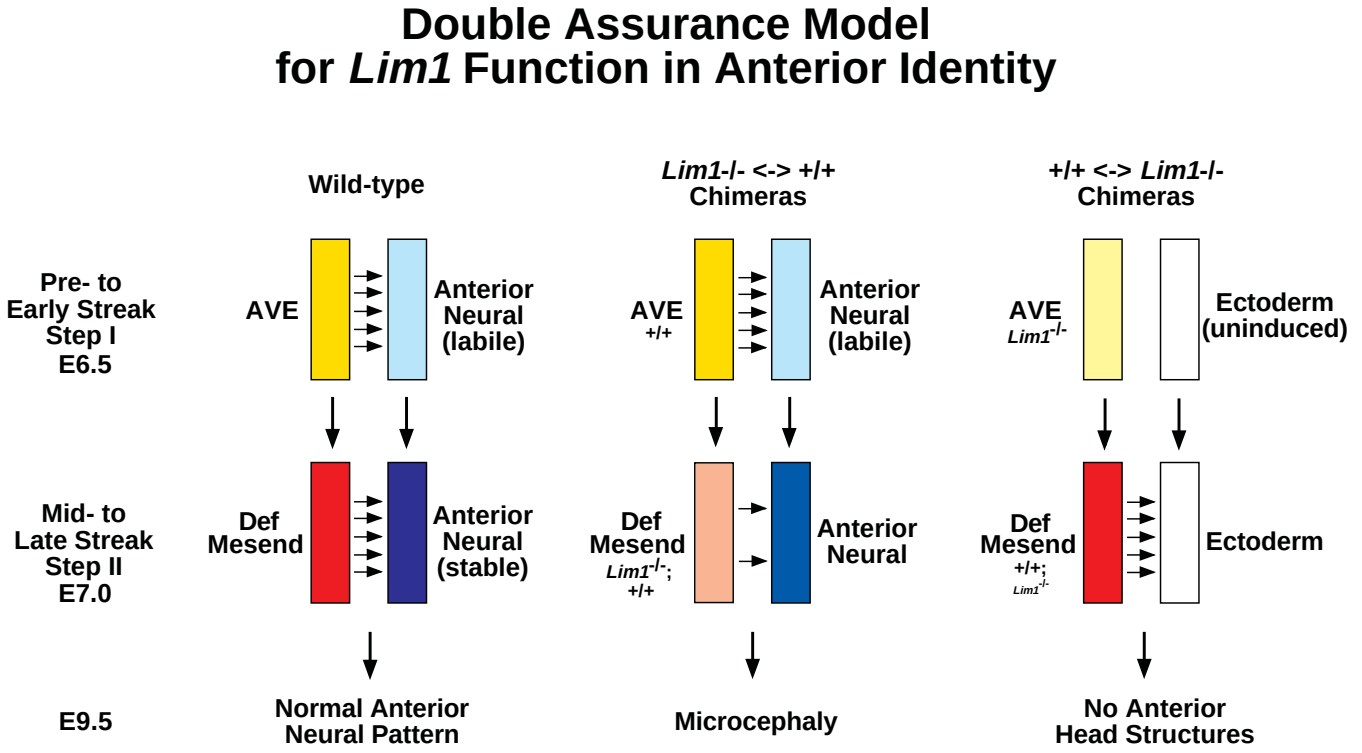

Fig. 6. Model for *Lim1* function in establishing anterior identity. *Lim1* is expressed in the anterior visceral endoderm (AVE) in pre- and early streak stage embryos. *Lim1* is also expressed in the primitive streak-derived definitive mesendoderm (Def Mesend) that replaces the visceral endoderm at the mid- to late primitive streak stage. *Lim1* chimera experiments indicate that two sequential signaling events are required for head development. The first *Lim1*-regulated signaling event from the anterior visceral endoderm induces the anterior ectoderm towards an anterior neural fate. This anterior neural fate is labile and is stabilized by a second *Lim1*-regulated signal from the definitive mesendoderm. In $Lim1^{-/-} \leftrightarrow +/+$ chimeras, the first signaling event occurs normally but the second signaling event is quantitatively reduced because fewer wild-type cells are present in the definitive mesendoderm. This results in chimeras with microcephaly. In $+/+ \leftrightarrow Lim1^{-/-}$ chimeras, the first signaling event from the anterior visceral endoderm does not occur and no subsequent anterior development takes place despite the presence of a high percentage of wild-type cells in the definitive mesendoderm. The size of the genotype labels next to the mesendoderm boxes approximates the contribution of wild-type and mutant cells in the mesendoderm.

In $+/+ \leftrightarrow Lim1^{-/-}$ chimeras, the visceral endoderm is predominantly composed of $Lim1^{-/-}$ cells so the first signaling event does not occur and the ectoderm remains uninduced. In the absence of the first signal, the second signal from the wild-type cells in the definitive mesendoderm is unable to direct anterior neural development despite the expression of *Lim1* by the definitive anterior mesendoderm. Consequently no microcephalic embryos are present and the chimeras completely lack anterior head structures.

Our *Lim1* chimera studies are consistent with this two-step model for anterior neural induction. However, the phenotype of *Cripto* mutant mice does not appear to fit with this model (Deng et al., 1998). *Cripto* mutant mice form primitive streak-derived extraembryonic mesoderm but not other streak-derived tissues. Still, *Bf1* and *En1*, forebrain and midbrain/hindbrain marker genes respectively, are expressed in E8.5 *Cripto* mutant embryos. Interestingly, anterior visceral endoderm markers are expressed but are located in the distal region of the mutant embryo. These findings suggest that anterior neural fates can be expressed in the absence of embryonic streak-derived tissues. One possible explanation to reconcile the *Cripto* findings with the results reported here and by others (Liu et al., 1999) is that Cripto functions to render the initial signal from the visceral endoderm labile (Thomas and Beddington, 1996). Thus, in the absence of Cripto, the initial visceral endoderm-derived signal is stable and can cause the expression of anterior neural fates in the epiblast in the absence of embryonic streak-derived tissues.

One candidate *Lim1* downstream gene is the *cerberus*-related gene, *Cerr1*. In *Xenopus*, *cerberus* encodes a secreted molecule that can induce ectopic head structures when its mRNA is injected into *Xenopus* embryos (Bouwmeester et al., 1996). In mouse, *Cerr1* is expressed in both the anterior visceral endoderm and the primitive streak-derived anterior mesendoderm (Belo et al., 1997; Thomas et al., 1997; Biben et al., 1998; Shawlot et al., 1998) and its expression is downregulated in $Lim1^{-/-}$ embryos (Shawlot et al., 1998). The partitioning of the same signaling molecule to both the visceral endoderm and the anterior definitive mesendoderm would allow the anterior ectoderm to be continuously in contact with the inducing signal(s) for an extended period of time despite the visceral endoderm being displaced during the course of gastrulation. Surprisingly, *Cerr1*-null mice created by gene targeting have normal head development, indicating that additional or alternative factors are involved in mouse head formation (W. S. and R. B., unpublished data).

In summary, we found that *Lim1* is required in both primitive streak-derived tissues and extraembryonic tissues for head formation and that its inactivation in these tissues produces cell non-autonomous defects. Our results imply that sequential *Lim1*-regulated signals from distinct and separate tissue layers are required for the establishment of anterior identity in the mouse. The results of our study will aid in the identification of *Lim1* downstream genes involved in vertebrate head formation.

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