**nodal** expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation

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**SUMMARY**

Mouse nodal, a member of the TGFβ family of secreted growth factors is essential for gastrulation. We recently generated a nodal\textsuperscript{lacZ} reporter allele by homologous recombination in ES cells. In the present study, β-galactosidase staining in the perigastroplation-stage embryo has demonstrated the site of highest nodal expression is localised to the prospective posterior region of the epiblast marking the site of primitive streak formation. We also documented transient nodal\textsuperscript{lacZ} expression in the visceral endoderm prior to and during early streak formation. A mosaic analysis using wild-type ES cells to rescue nodal-deficient embryos allowed us to document functionally distinct nodal activities in the embryonic ectodermal and primitive endodermal cell lineages. nodal signaling in the ectoderm is necessary for primitive streak formation as the gastrulation defect of nodal-deficient embryos can be rescued by the inclusion of small numbers of wild-type cells. In addition, we show that chimeric embryos composed of nodal-deficient primitive endoderm fail to develop rostral neural structures. Thus we conclude that the action of nodal, a TGFβ-related growth factor expressed in the primitive endoderm, is critical for patterning of the anterior aspects of the A-P axis.

Key words: nodal, mouse gastrulation, primitive endoderm, chimeras

**INTRODUCTION**

Nodal, a member of the TGFβ family of secreted growth factors, was originally identified due to its close linkage with the 413.d proviral integration site (Zhou et al., 1993; Conlon et al., 1994). Homozygous 413.d mutant embryos lacking nodal expression cannot initiate primitive streak formation and are arrested at the gastrulation stage of development (Conlon et al., 1991, 1994). The recent identification of conserved nodal homologs in Xenopus and chick (Smith et al., 1995; Jones et al., 1995) has underscored the key role played by the nodal signaling pathway during vertebrate development. Comparisons of the carboxyl-terminal signaling domains of Xenopus, chick and mouse nodal sequences indicate these molecules to share between 52 and 78% amino acid identity. Interestingly individual Xenopus nodal homologs seem to have distinct functional activities. For example, both Xnr-1 and Xnr-2 act as potent inducers of dorsal mesoderm cell types in manipulated frog embryos, and can also completely rescue UV-ventralized embryos (Jones et al., 1995). By contrast, Xnr-3 lacks mesoderm-inducing activities per se, but rather appears to be required for cell migration and movement (Smith et al., 1995). On the other hand, several aspects of the nodal signaling pathway are highly conserved between higher and lower vertebrates. Recent studies demonstrate that the mouse nodal gene is transiently expressed in a discrete population of lateral plate mesoderm on the left side of the early somite-stage embryo, where its expression appears to be functionally correlated with the direction of axial rotation and the establishment of the definitive left-right (L/R) axis (Collignon et al., 1996; Lowe et al., 1996). Similarly both the chick Cnr-1 (Levin et al., 1995) and Xenopus Xnr-1 (Lowe et al., 1996) genes are asymmetrically expressed in left lateral mesoderm, suggesting a conserved molecular pathway determines the vertebrate L/R body axis.

nodal gene expression has been detected at the onset of gastrulation both by RT-PCR (Zhou et al., 1993) and in situ hybridization (Conlon et al., 1994). We recently used gene targeting techniques to introduce a lacZ reporter gene under control of the endogenous cis-regulatory elements into the mouse nodal locus (Collignon et al., 1996). The increased resolution afforded by β-gal staining has allowed us to precisely describe highly dynamic and transient nodal expression at early stages of mouse development. In the present study, we demonstrate that nodal is expressed throughout the epiblast tissue prior to overt streak formation, but becomes rapidly localized within the embryonic ectoderm to mark the future caudal region where the streak will form. Intriguingly, we find that nodal is also transiently expressed in the primitive endoderm cells overlying the embryonic ectoderm when gastrulation is initiated. Although the precise developmental role of the primitive endoderm in mammals has yet to be established, the equivalent cell lineage in chick, the hypoblast, is known to provide inductive signals influencing the position and
orientation of the primitive streak (Waddington, 1933; Azar and Eyal-Giladi, 1981).

Here we also analyse nodal signaling in the primitive endoderm during gastrulation and establishment of the anterior-posterior (A-P) axis. We have exploited the pronounced developmental bias of ES cells, which colonize almost exclusively derivatives of the embryonic ectoderm following re-introduction to blastocyst-stage embryos (Beddington and Robertson, 1989), to distinguish nodal activities in the embryonic ectoderm and the primitive endoderm lineages of the embryo. Our previous experiments demonstrated that 413.d mutant ES cells injected into wild-type host blastocysts were capable of contributing to a wide variety of normal embryonic and adult tissue types in chimeras (Conlon et al., 1991). The present experiments show that very few wild-type epiblast cells are required to efficiently rescue the gastrulation defect of 413.d mutant embryos, endorsing an essential role for the nodal signaling pathway in primitive streak initiation. However, we observe profound developmental defects in mosaic embryos in which the primitive endoderm lineage is composed exclusively of nodal-deficient cells. The rostral-most regions of the developing central nervous system (CNS) were consistently lacking in these embryos. These findings demonstrate an essential role for the primitive endoderm lineage in the establishment of anterior pattern during mouse gastrulation.

MATERIALS AND METHODS

Mouse strains and ES cells

The nodallacZ allele was maintained on a 129/Sv background and animals genotyped as described (Collignon et al., 1996). Mice carrying the 413.d retroviral integration were maintained on a 129/Sv background and genotyped by PCR as described (Conlon et al., 1994). Animals carrying the ROSA26 gene trap integration (Friedrich and Soriano, 1991) were obtained from the Jackson Laboratories (Tg(ROSA26)RSor strain, Jackson Laboratories, Bar Harbor). A similar gene trap line designated BT-5 (V. Episkopou and E.J.R., unpublished) was maintained on a 129xC57BL/6 hybrid background. The single independent copy of a lacZ reporter gene derived from the ROSA retroviral vector (Friedrich and Soriano, 1991) carried by both strains is ubiquitously expressed throughout embryogenesis (Friedrich and Soriano, 1991; V. Episkopou and E.J.R., unpublished). Heterozygous ROSA26 and BT-5 animals were identified by X-gal staining of ear tissue biopsies.

For analysis of nodal-lacZ expression during development, embryos were collected from matings between heterozygous nodallacZ males and 129/Sv or CD-1 (Charles River, Wilmington, MA) females and stained for β-galactosidase activity as described (Hogan et al., 1994), using either X-gal or Salmon-gal (Biosynth) as the substrate. Embryos were postfixed in 4% paraformaldehyde, embedded in paraffin wax and sectioned at 9 μm. Sections were dewaxed using standard procedures, mounted in either 80% glycerol or Permount and photographed under Normarski optics.

ES cell lines were isolated from blastocysts obtained from 413.d/+ females crossed to either 413.d/-, ROSA26+/- or 413.d/-, B7T+/- males, as described (Robertson, 1987). Individual ES cell lines were genotyped with respect to the 413.d locus by Southern blot (Conlon et al., 1991). Genotyping for the ROSA26 or BT-5 allele was assessed by staining individual ES cell lines for β-galactosidase activity (Hogan et al., 1994). The experiments presented here were carried out using two independent lacZ+ wild-type ES cell lines (R26.1 and BT5.7) and two lacZ+ 413.d homozygous nodal-deficient ES cell lines (ER.1 and ER.4).

Generation and analysis of chimeras

Chimeras were generated by blastocyst injection as described (Bradley, 1987). Blastocysts were collected from matings between 413.d heterozygous animals, or from outbred CD-1 strain animals (Charles River, Wilmington, MA). For production of chimeric embryos with differing ES cell contributions, the number of ES cells injected into the blastocoele cavity was varied from 2-4 up to a maximum of 12-14 cells. Following transfer into pseudopregnant foster females, the manipulated embryos were recovered either at day 7.5 or 10.5 of development. Embryos were fixed and processed either for β-galactosidase staining or in situ hybridization. In experiments using embryos from 413.d intercrosses, the genotype of the host blastocyst was determined retrospectively from a sample of extraembryonic tissue. Briefly, individual visceral yolk sacs were dissected from 10.5 day conceptuses, washed in PBS, and the endoderm layer isolated manually following digestion in pancreatic-trypsin as described (Hogan et al., 1994). DNA samples prepared from the endodermal fraction were genotyped with respect to the 413.d locus by PCR as described (Conlon et al., 1994).

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization using digoxigenin-labelled RNA probes was performed as described (Wilkinson, 1992). Additionally, a fluorescein-labelled RNA probe detected with Magenta-Phos was used for double labelling as described (Levin et al., 1995). The Shh En-1 and Krox-20 probes have been described previously (Echelard et al., 1993; Davis and Joyner, 1988; Wilkinson et al., 1989). The Wnt-8b probe was kindly provided by Scott Lee and Andrew McMahon (S. Lee and A. McMahon, personal communication).

RESULTS

nodal expression domains in early mouse embryos

nodal transcripts are present in ES cells (Conlon et al., 1994). Consistent with this, correctly targeted ES cell clones carrying the nodallacZ reporter allele stained uniformly for β-gal activity in the undifferentiated state (data not shown). To determine the onset of nodal expression in vivo, nodallacZ heterozygous embryos were examined for β-gal activity from 3.5 day post coitum (d.p.c.) onwards. No detectable activity was observed at the blastocyst stage (data not shown). However, shortly after implantation (5.5 d.p.c.) low levels of staining were detected throughout the embryonic ectoderm and associated overlying primitive endoderm (Fig. 1A). No lacZ expression was detected in the more proximally located extraembryonic endoderm population (data not shown).

By approximately 6.0 d.p.c., just prior to overt streak formation, β-gal activity was detected in the embryonic epiblast and primitive endoderm (Fig. 1B). Low but uniform lacZ expression was seen throughout the visceral endoderm cell population (Fig. 1B-E) and was most noticeable in a morphologically distinct region of anterior endoderm lying approximately 10-15 μm distal to the junction of the embryonic and extraembryonic tissues, which retains a more thickened character. However, the more obvious staining seen in this population likely reflects increased cell density since analysis of numerous independent embryos revealed β-gal-positive cells in all regions of the visceral endoderm, including the proximal region overlying the extraembryonic ectoderm. By contrast, within the embryonic ectodermal nodal activity was highest in
the posterior proximal quadrant suggesting gene activity is downregulated on the prospective anterior side of the embryo. Interestingly expression remained radially symmetrical at levels coincident with or just distal to the region where the embryonic and extraembryonic cell populations abut (Fig. 1C). However, 10-15 μm distal to this region corresponding to the level at which a distinct thickening of the anterior visceral endoderm is visible, expression is clearly strongest on the prospective posterior side of the egg cylinder (Fig. 1D). Similarly towards the distal tip of the egg cylinder, lacZ expression was markedly higher on the posterior side (Fig. 1E). These results demonstrate that the primitive streak forms at the site of highest nodal activity within the ectoderm.

The pattern of nodal expression changes rapidly during the next few hours of development. By early streak stages, nodal activity was completely absent in the anterior ectoderm, although high levels persist on the posterior side of the embryo (Fig. 1F). Mesoderm cells exiting the streak proximally and laterally briefly retain lacZ expression (Fig. 1G,H). At this stage, we failed to detect lacZ expression in the overlying visceral endoderm. Within the ectoderm, nodal expression was rapidly lost as the streak elongates distally and, by late streak stages, only a few cells, strictly confined to the posterior side of the embryo, show weak β-gal staining (Collignon et al., 1996; data not shown). In sum, nodal is expressed prior to primitive streak formation. Expression continues during the initial stages of streak induction and is then rapidly downregulated as the streak elongates. Subsequently nodal expression is detected in a small subset of node progenitors, and following the formation of the morphologically distinct node becomes restricted to the edges of the notochordal plate (Collignon et al., 1996).

The block to gastrulation in 413.d nodal mutant embryos is rescued by wild-type ES cells

Based on the results above, the gastrulation defect displayed by nodal-deficient embryos could potentially reflect an essential role for nodal signaling in the primitive endoderm, the embryonic ectoderm or both cell lineages. To distinguish these possibilities, we undertook a mosaic analysis using wild-type ES cells...
and *nodal*-deficient ES cells. It is known that ES cells display a marked developmental bias when introduced into recipient blastocysts, almost exclusively colonizing the embryonic epiblast (Beddington and Robertson, 1989). Thus mosaic embryos contain ES cell derivatives largely confined to the embryonic portion, while the primitive endoderm and its derivatives are of host origin. In the present experiments, we used ES cell lines carrying ubiquitously expressed *lacZ* transgenes (Friedrich and Soriano, 1991) to simultaneously mark and follow the fates of the injected ES cells. It was first important to confirm that ES cell derivatives preferentially colonise the epiblast lineage in chimeras obtained from blastocyst injection. A panel of chimeric embryos (*n* = 12), obtained by injecting wild-type *lacZ*-marked ES cells, were retrieved at early gastrulation stages, stained for β-gal activity and serially sectioned. As shown in Fig. 2, we found no evidence for the presence of *lacZ*-expressing cells in either the extraembryonic ectoderm or primitive endoderm lineage in embryos in which the epiblast was strongly colonised by ES cell derivatives.

To test the role of nodal activity in the primitive endoderm and epiblast, we used the strategy outlined schematically in Fig. 3. Thus wild-type ES cells were injected into 413.3d mutant blastocysts to generate chimeric conceptuses in which the primitive endoderm was composed exclusively of *nodal*-deficient cells (class I chimeras). Alternatively, in the reciprocal experiment, *nodal*-deficient ES cells injected into wild-type blastocysts gave chimeras in which the primitive endoderm was genetically wild type (class II chimeras). In control experiments, wild-type ES cells were injected into wild-type blastocysts (class III chimeras).

![Fig. 2](image)

**Fig. 2.** ES cell derivatives fail to colonize the extraembryonic and primitive endoderm lineages in chimeric embryos. Chimeric embryos generated by injecting wild-type *lacZ*-expressing ES cells into wild-type embryos were recovered at early gastrulation stages, stained in Salmon-gal and serially sectioned. (A-C, D-F) Three transverse sections from two independent embryos, respectively. (A,D) Sections correspond to a level slightly above the extraembryonic junction, (B,E) sections correspond to a region midway down the embryonic regions and (C,F) sections correspond to regions close to the distal tip of the egg cylinder. *lacZ*-expressing cells are confined to the embryonic ectoderm and nascent mesoderm. The anterior side of the embryos is to the left.

![Fig. 3](image)

**Fig. 3.** Experimental strategy for evaluating nodal activities in the ectoderm and primitive endoderm cell lineages. Injection of *lacZ*+ wild-type ES cells (blue) into 413.3d *nodal*-deficient blastocysts leads to the generation of mosaic embryos in which the primitive endoderm (green) is exclusively *nodal*-deficient in origin (class I chimeras). Alternatively, injection of *lacZ*+ *nodal*-deficient ES cells (yellow) into wild-type blastocysts results in mosaic embryos in which the primitive endoderm (orange) is wild type (class II chimeras). As a control, wild-type ES cells were injected into wild-type blastocysts (class III).
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However, we observed pronounced abnormalities affecting the

turning. In the majority of extensive chimeras, the trunk and

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tinctive somites, derivatives of paraxial mesoderm, were occa-

sionally seen in these embryos. This, together with the point of insertion of

of the VYS, allowed the identification of the posterior end of the

axis. Derivatives of the lacZ+ wild-type ES cells were scattered

randomly along the extent of the rudimentary axis (Fig. 4A),

reflecting substantial mixing with host epiblast cells prior to

gastrulation (Lawson et al., 1991; Lawson and Pedersen,


More strongly colonized embryos (10-30% wild-type con-

tribution) displayed a more robust A-P axis (Fig. 4B), with

morphologically distinct anterior and posterior structures. A

well-developed neural tube was frequently visible in this class

of chimera, although it was often kinked in appearance. Dis-

tinctive somites, derivatives of paraxial mesoderm, were occa-

sionally observed, but were abnormally shaped and fused

across the midline (Fig. 4B). By contrast, extensively colonized

chimeras (wild-type contribution 30% or higher) were rela-

tively well developed. These frequently possessed a grossly

normal heart (Fig. 4C,E), which in all cases was visibly beating

at the time of dissection. Additionally, as shown in Fig. 4E, an

orderly array of paired somites was visible in more posterior

regions of the axis and some of these embryos had undergone

turning. In the majority of extensive chimeras, the trunk and
caudalmost structures appeared grossly normal (Fig. 4C,E).

However, we observed pronounced abnormalities affecting the
development of anterior neural structures. For example, as
shown in Fig. 4C,E, the neural structures rostral to the otic
vesicle are clearly defective. In the example shown in Fig. 4E,
the region of the forebrain and midbrain appears to be largely
missing and a single, narrow, fused head structure is present
anterior to the otic vesicle.

Defects in the formation of the anterior neural axis have been
described in embryos carrying loss-of-function mutations in the
HNF-3β (Ang and Rossant, 1994; Weinstein et al., 1994),
Lim-1 (Shawlot and Behringer, 1995) and Otx-2 (Acampora et
al., 1995; Matsuo et al., 1995; Ang et al., 1996) genes. These
three genes are expressed in both the visceral endoderm and in
derivatives of the embryonic ectoderm (Monaghan et al., 1993;
Acampora et al., 1995; I. V., J. C. and E. J. R., unpublished
data). Strikingly, all three mutations result in a similar
phenotype during gastrulation. Thus, homozygous mutant
embryos exhibit a characteristic narrowing or constriction at the
junction of the extraembryonic and embryonic ectoderm at
early to mid-streak stages. In all cases, this defect is associated
with a greatly reduced primitive streak and impairment of the
normal cell movements associated with gastrulation.

We wanted to know if the anterior defects described above in
class I chimeras were also associated with similar morpho-

tological abnormalities during gastrulation. To test this, blasto-
cysts from 413.d+/+ heterozygous intercrosses were injected
with wild-type lacZ+ ES cells and the resulting chimeric
embryos examined at 7.5 days of gestation. As shown in Fig.
5, approximately 25% of chimeras (3/13 analyzed) were aber-

rantly shaped and showed a pronounced constriction at the
embryonic-extraembryonic region, even in cases where the
embryonic ectoderm was extensively colonized by lacZ+ cells
and thus largely wild type in character (Fig. 5C,D). Only a
quarter of the embryos exhibited this abnormality suggesting
they probably correspond to chimeras derived from 413.d
homozygous mutant blastocysts.

Molecular characterization of the anterior defects in

chimeras

To more clearly delineate the nature of the anterior truncations
described above, day 10.5 chimeras were further analyzed by
assessing the expression of Krox-20, En-1 and Wnt-8b mRNAs,
subpopulations, respectively (Table 2). At 9.5 days of development, Krox-20 is known to be expressed in rhombomeres 3 and 5 and by day 10 is confined to rhombomere 5 (Wilkinson et al., 1989). Wnt-8b marks the dorsal region of the telencephalon and expression extends rostrally into the diencephalon at 10.5 days of development (S. Lee and A. McMahon, personal communication). In double labeling in situ hybridization experiments control chimeric embryos (class III) showed the expected patterns of Krox-20 and Wnt-8b expression (Fig. 6A). In contrast, extensively rescued chimeras generated from nodal mutant blastocysts express Krox-20 in the region of the otic vesicle, but there was no evidence for the expression of Wnt-8b (Fig. 6A,B). To further characterise the anterior neural tissue populations, additional chimeras were analysed for the expression of En-1. At 10.5 d.p.c., En-1 is strongly expressed throughout a ring of neural tissue at the hindbrain-midbrain junction (Davis and Joyner, 1988; Fig. 6C). In keeping with the Krox-20 results, none of the poorly rescued chimeras analysed showed evidence for expression of En-1 mRNA in the developing CNS, although a specific hybridization signal was present in the developing somites and limb buds. However, in two extensively rescued chimeras, the hindbrain-midbrain region, or isthmus, had clearly been induced as evidenced by the presence of a characteristic stripe of En-1 expression (Fig. 6D,E). Collectively these gene marker studies show that, while posterior regions of the midbrain encompassing the hindbrain-midbrain boundary do form, the rostral-most neural structures fail to form in this class of chimeras. In particular, we note that none of the strongly rescued chimeras show evidence for the formation of forebrain tissue.

The anterior truncations seen in mutant embryos lacking either Lim-1 or Otx-2 (Shawlot and Behringer, 1995; Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996) suggest these molecules are essential for specification of the prechordal plate, node and notochord during gastrulation. These axial mesoderm populations derived from the anterior streak are known to participate in the rostrocaudal patterning of the CNS (reviewed Shimamura et al., 1995; Placzek et al., 1993). To assess whether similar defects in ventral midline structures contribute to the anterior axis abnormalities described above, chimeras derived from nodal mutant blastocysts were examined for expression of Shh mRNA. At 10.5 d.p.c., Shh mRNA is normally present in the notochord and ventral floor plate along the length of the spinal cord and hindbrain. In the mid and forebrain regions, expression expands to occupy more dorsal regions and extends anteriorly to the rostral-most region of prosencephalon (Echelard et al., 1993; Shimamura et al., 1995). All five of the class I chimeras analysed at 10.5 d.p.c. expressed Shh mRNA in midline structures including the notochord, floor plate and gut structures (Fig. 7A–E). However, we observed intermittent expression caudal to the hindbrain region (recognized by the presence of the otic vesicle), suggesting these embryos fail to form a continuous notochord. Considering the node gives rise to the notochord (Beddington, 1994; Sulik et al., 1994), these defects probably reflect functional abnormalities of the node at early stages of development. Sections through the most anterior regions of these chimeras (Fig. 7D) showed no discernable notochord. Shh-expressing cells were confined to a narrow stripe of ventrally placed neuroectodermal cells, a pattern similar to that seen in the normal hindbrain-midbrain region of wild-type embryos (Shimamura et al., 1995). Together these marker gene studies, summarised in Table 2, demonstrate that the forebrain and possibly anterior portions of the midbrain...
nodal function in primitive endoderm

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nodal function in primitive endoderm

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structures fail to form in chimeric conceptuses derived from homozygous 413.d blastocysts.

A requirement for nodal signaling in the primitive endoderm

Results above clearly indicate that nodal-deficient endoderm can support gastrulation, showing that nodal signaling in the primitive endoderm is not required for primitive streak formation within the epiblast. However, even when wild-type cells extensively colonize the founder epiblast population, we observe incomplete formation of the most rostral aspects of the neural axis. The prechordal plate is responsible for induction of the midbrain and forebrain (Placzek et al., 1993). However, the morphogenesis and progenitor cell populations of this tissue remain poorly defined (Sulik et al., 1994). Thus either the lack of nodal signaling in the primitive endoderm lineage, or reduced nodal signaling in the embryonic ectoderm during early gastrulation could potentially disturb the formation of anterior midline populations.

To distinguish these possibilities, we first examined whether formation of the prechordal plate was affected in chimeras extensively colonized by nodal-deficient ES cells. We compared the development and colonization patterns of nodal-deficient and wild-type ES cells injected into wild-type blastocysts (class II and class III chimeras, respectively; Fig. 3). Chimeric embryos analyzed at early headfold stages (7.5 d.p.c.) were found to be grossly morphologically normal irrespective of the genotype of the injected ES cells. To examine the possibility that nodal mutant cells might be selectively excluded from specific cell populations such as the prechordal

Fig. 5. Abnormal gastrulation generated from 413.d mutant blastocysts (class I chimeras). (A,C) Two examples of 7.5 d.p.c. chimeras exhibiting a constriction (blue arrowheads) at the embryonic/extraembryonic boundary. (B,D) Same embryos as in A and C, stained with salmon-gal to show the distribution of wild-type lacZ+ ES cell derivatives. Abbreviations: hf, headfold; ps, primitive streak.

Fig. 6. Anterior neural defects found in class I chimeras at 10.5 d.p.c. (A) Whole-mount in situ hybridization analysis of Wnt-8b (purple stain) and Krox-20 (light pink stain, arrowhead) expression in wild type (left) and two class I chimeras (right). Wnt-8b expression is absent in both rescued chimeras. Krox-20 expression in r5 is detected in the chimera exhibiting the most extensive rescue (shown at higher magnification in B). (C-E) Whole-mount in situ hybridization analysis of En-1 expression in wild type (C) and two extensively rescued class I chimeras (D,E) at day 10.5 p.c. (C) High magnification view of the anterior region of a wild-type embryo. The En-1 expression domain includes the isthmus, with expression extending both anteriorly and posteriorly into the midbrain and hindbrain, respectively. A pronounced D-V band of hybridization demarcates the hindbrain-midbrain boundary. (D,E) High magnification view of two chimeras showing expression of En-1; the hindbrain-midbrain region has clearly formed in both embryos (arrowheads) in a region of the developing brain rostral to the otic vesicle. The more posterior regions of the trunk were well developed in both embryos and closely resembled the chimera shown in B. Note that the apparent signal in the forebrain region of the control embryo is due to background: in this experiment, the embryos were incubated in substrate for a longer period of time to maximize the staining in the chimeric embryos. Three additional less well rescued chimeras showed no evidence for staining in the anterior regions, although the somites and limb buds showed the predicted pattern of En-1 expression (data not shown).
plate or midline, chimeras were serially sectioned. As shown in Fig. 8, nodal-deficient cells exhibit a perfectly normal colonization pattern. Thus derivatives of nodal mutant ES cells extensively contributed to dorsal and ventral regions of the node, and anterior mesoderm populations (Fig. 8D,E, data not shown). As expected (Beddington and Robertson, 1989; see Fig. 2), ES cell derivatives fail to colonize the primitive endoderm lineage. Thus lacZ cells were not detected in the visceral yolk sac endoderm population. Interestingly, in sections of headfold-stage chimeras, numerous β-gal ES cell derivatives were seen in the outermost layer of cells overlying the mesoderm in the embryonic region (Fig. 8D,E). β-gal staining thus identifies derivatives of the midline that contribute to the definitive endoderm population. These data confirm previous studies indicating that the definitive embryonic endoderm rapidly displaces the primitive endoderm during gastrulation (Lawson and Pedersen, 1987; Tam and Beddington, 1996). Additionally, we noted several highly chimeric embryos that displayed a marked absence of lacZ-expressing cells in the notochordal plate and midline (Fig. 8A,B). Conversely, in weakly colonized chimeric embryos, ES cell derivatives were occasionally confined to the midline (Fig. 8C). Together these observations suggest that a small number of founder epiblast cells contribute to the formation of the node and its derivatives and that little cellular mixing occurs following allocation of this population.

Interestingly, none of the large number (n=30) of class II chimeras examined at mid streak to early head fold stages, in which the majority of the epiblast derivatives were nodal-deficient, showed overt abnormalities. Thus, the pronounced morphological constriction seen at gastrulation in class I chimeric embryos resulting from the injection of 413.d nodal-deficient blastocysts (Fig. 5), can be specifically attributed to the absence of nodal expression in the primitive endoderm lineage. Finally, to exclude the possibility that chimeras containing a large component of nodal mutant cells develop anterior patterning defects at later stages, the embryonic development of class I and class II chimeras was compared at 10.5 d.p.c. Clear anterior defects were present in extensively chimeric embryos obtained from nodal mutant blastocysts (Fig. 9C,F). In marked contrast, all of the class II chimeras examined (n=20) in which lacZ nodal-deficient ES cell derivatives constituted greater than 80% of the embryo showed correct patterning of the anterior CNS structures (Fig. 9A,B). In both classes of chimera, the lacZ-marked wild-type and nodal-deficient cells respectively were uniformly distributed along the entire extent of the A-P and dorsal-ventral axis. Collectively these experiments show that the anterior defects documented in chimeras generated from nodal mutant blastocysts results from loss of nodal signaling in the primitive endoderm during early gastrulation.

DISCUSSION

Elegant single cell marking studies in the mouse have shown that the cells of the early epiblast are regionalized with respect to their fate prior to the initiation of gastrulation (Lawson et al., 1991; Lawson and Pedersen, 1992). However, relatively little is known about the molecular signaling pathways that

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<th>Probe</th>
<th>No. chimeras hybridized</th>
<th>No. showing CNS expression</th>
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<tr>
<td>Shh</td>
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<td>Wnt-8b</td>
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* As a positive control for the hybridization conditions, chimeric embryos were processed together with wild-type embryos.
† For the Krox-20 and En-1 probes, a positive hybridization signal was only seen in those chimeras showing extensive rescue of the normal body axis.
‡ To ensure that low levels of En-1 expression were detected, embryos were incubated in substrate for longer than optimal time periods. A low level of background staining in the surface ectoderm was present in both the control and chimeric embryos.

Fig. 7. Expression of Shh mRNA in mid-gestation embryos. (A) Shh expression in wild type (left) and class I chimeras (right) at 10.5 days. (B,C) Lateral and frontal views, respectively, of a second class I chimera. In addition to the obvious defect in the anterior expression pattern, both chimeras show discontinuous staining of the notochord and/or gut. (A,B) Transverse sections of the chimera in B and C taken at anterior and posterior levels, respectively, to show Shh expression in the ventral part of the neural tube (D) and in notochord and gut posteriorly (E). Abbreviations: di, diencephalon; fp, floorplate; g, gut; hb, hindbrain; h, heart; mb, midbrain; nt, neural tube; op, optic vesicle; ov, otic vesicle; nc, notochord; te, telencephalon.
position the primitive streak within the radially symmetrical epiblast or the activities required to initiate the process of streak formation. A number of genes initially expressed uniformly in the epiblast become asymmetrically localized prior to overt streak formation. For example, both Fgf-8 (Crossley and Martin, 1995) and nodal appear to be expressed in a gradient, with highest levels of activity confined to the prospective caudal region of the future axis. Thus a strong argument can be made that the epiblast acquires molecular asymmetry prior to overt streak induction.

The finding that nodal-deficient embryos fail to gastrulate (Conlon et al., 1994) has been taken as evidence that, as for other TGFβ members acting in lower vertebrates, nodal normally induces the formation of nascent mesoderm (Conlon and Beddington, 1995). However, there is also evidence that both mouse nodal and Xnr-3 may regulate the migratory properties of cells (Conlon et al., 1994; Smith et al., 1995). Consistent with this, the present analysis of nodal.lacZ expression indicates that nodal is only transiently detected during early streak formation. Thus its activity is not required continuously during gastrulation as might be expected if it acts primarily as a mesoderm-inducing activity, or is required to maintain the structure of the streak region.

The present chimera experiments indicate that very low levels of nodal activity provided by a minor population of wild-type cells are sufficient to alleviate the block to gastrulation in nodal-deficient epiblast tissue. This is illustrated in Fig. 4, where the introduction of a small number of lacZ+ nodal-deficient ES cells displays severe anterior abnormalities. Histological sections at anterior (D), future thoracic (E) and more posterior regions (F) of this chimera show extensive colonization by wild-type ES cell derivatives and the absence of anterior structures. Abbreviations: ht, heart; g, gut; lb, limb bud; nc, notochord; nt, neural tube; V, ventral side; D, dorsal side of the embryo.
tulation. In our experiments analyzing a large panel of chimeric embryos (n=138) at 10.5 d.p.c. only 15%, as opposed to the expected 25%, were derived from nodal mutant blastocytes. The inability to rescue a proportion of mutant embryos likely stems from failure of nodal-expressing wild-type cells to occupy the prospective caudal region of the epiblast. These results suggest that localized nodal signaling within the embryonic ectoderm is necessary to promote the formation of the primitive streak.

At 10.5 days of development, many of the partially rescued chimeras resemble HNF-3β mutants. HNF-3β mutant mice fail to form a node or notochord, although they gastrulate to form a well-patterned A-P axis (Ang and Rossant, 1994; Weinstein et al., 1994). Numerous weakly colonized class I chimeras exhibit a similar phenotype suggesting that node morphogenesis is highly abnormal. Since these embryos are largely composed of nodal-deficient cells, the probability that significant numbers of wild-type cells become incorporated into the node progenitor population is very low. Thus it seems likely that nodal expression in the cells at the distal tip of the streak is normally required for node morphogenesis. Additionally, strongly colonized embryos analyzed at 10.5 d.p.c., exhibit discrete gaps in the A-P Shh expression domain. Thus the orderly production of the notochord is disturbed. It seems likely that nodal expression in the ventral lateral edges of the mature notochordal plate is important in maintaining the integrity of this structure and the orderly generation of the notochord and midline derivatives. Interestingly, the nodal.lacZ domain in the distal region of the streak is induced at the most caudal limit of the Shh expression domain. Thus nodal expression may be activated by short-range, possibly contact-mediated, Shh signaling. Consistent with this suggestion, ectopically supplied Shh protein has been shown to induce the expression of the nodal homolog Cnr-1 in manipulated chick embryos (Levin et al., 1995). It will be interesting to learn whether nodal expression is disturbed in Shh mutant embryos.

Shortly after implantation nodal is transiently expressed in the layer of primitive endoderm cells that invests the epiblast tissue. Cell marking and mosaic experiments have shown that the primitive endoderm lineage contributes exclusively to the extraembryonic tissues (reviewed Tam and Beddington, 1992). Beyond possible roles in supporting the growth of the underlying ectoderm, the primary function of the visceral endoderm remains poorly understood. Because nodal is expressed in both the endoderm and ectoderm lineages, we devised an experimental strategy that allowed us to test whether nodal expression was essential for any aspects of endoderm function. These studies show that the transient expression of nodal in the visceral endoderm is not required for the initiation of gastrulation.

Strikingly nodal signaling in this cell layer appears essential to confer correct anterior patterning of the neural plate at later stages of development. Thus mosaic embryos comprised largely of wild-type cells developing in combination with mutant primitive endoderm lack the most rostral aspects of the axis. Conversely, mosaic embryos composed largely of nodal mutant ectoderm derivatives developing in conjunction with wild-type primitive endoderm form a normal A-P axis. We found that the forebrain, and possibly anterior midbrain, structures fail to form in the absence of nodal-expressing primitive endoderm. As induction of the forebrain, and possibly regions of the midbrain, in the vertebrate CNS is contingent on signals provided by the prechordal plate tissue (reviewed Shimamura et al., 1995), it seems likely that formation of this tissue is adversely affected in these chimeras. A number of morphological studies have shown that the prechordal plate forming at the rostral midline of the embryo is composed of a complex population of closely associated endoderm and anterior mesoderm cells (Poelmann, 1981; Sulik et al., 1994). While the embryonic origins of the endoderm cells that contribute to the prechordal plate have yet to be established, our experiments demonstrate that nodal expression in the primitive visceral endoderm prior to gastrulation is required for the correct morphogenesis of this population of cells, and hence the establishment of correct anterior positional identity in the developing neural tissue.

Recently evidence has been provided for important inductive interactions between the primitive endoderm and underlying ectoderm during early embryogenesis. Thus expression of the homeobox gene Hesx1/Rpx (Thomas and Beddington, 1996), first detected at the start of gastrulation, is confined to a small anterior domain of primitive endoderm, but a few hours later becomes apparent in the underlying anterior ectoderm. This second domain of ectodermal Hesx1 expression is in part dependent on signals from the endoderm, since expression in the ectoderm is lost or severely depleted by physical removal of the anterior endoderm at earlier stages (Thomas and Beddington, 1996). A similar finding has been reported for Otx-2. Thus a lacZ reporter allele of Otx-2 is initially activated in the visceral endoderm layer of Otx-2 mutant embryos, but then fails to be expressed in the underlying anterior ectoderm (Acampora et al., 1995), suggesting that expression of Otx-2 itself in the anterior endoderm is normally required for its subsequent induction in the ectoderm. Moreover, loss of Lim-1, normally expressed at early head fold stages in anterior mesoderm (Barnes et al., 1994; Shawlot and Behringer, 1995) and also in the anterior visceral endoderm (I. V., J. C. and E. J. R., unpublished data) results in a loss of the anterior neuroectodermal Otx-2 expression domain (Shawlot and Behringer, 1995). In keeping with these findings, a series of elegant tissue recombination experiments have endorsed an important role for anterior mesendoderm populations in patterning the developing neural plate (Ang and Rossant, 1993; Ang et al., 1994).

Mosaic embryos developing within a nodal-deficient visceral endoderm exhibit a distinctive physical constriction between the embryonic and extraembryonic regions, similar to that seen in HNF-3β-, Otx-2- and Lim-1-deficient mutants. Since these mutants also exhibit defects in the formation of anterior regions of the neural axis, it is tempting to speculate that these molecules participate in a common pathway that is initiated prior to gastrulation by signals provided from the primitive endoderm. Evidence is now accumulating that the primitive endoderm is patterned with respect to the prospective A-P axis at early stages of postimplantation mouse development. Thus, at gastrulation, expression of Hesx1/Rpx (Hermesz et al., 1996; Thomas and Beddington, 1996), and the antigenic epitope VE-1 (Rosenquist and Martin, 1995) is localized to a distinct patch of prospective anterior visceral endoderm lying slightly distal to the junction with the extraembryonic ectoderm. This molecular and physical asymmetry may reflect...
a role for the visceral endoderm in establishing anterior identity within the cells of the underlying ectoderm (Thomas and Beddington, 1996). It will be interesting to examine whether nodal, expressed throughout the visceral endoderm of the pregastrulation-stage embryo, participates in setting up this marked regional identity within the endoderm. Most recently a novel secreted molecule, cerberus, normally expressed in the anterior endoderm of Spemann’s organizer has been shown to induce ectopic head structures in manipulated Xenopus embryos (Bouwmeester et al., 1996). Collectively these findings, in conjunction with the experiments in mouse discussed above, serve to underscore an important involvement of the anterior endoderm in the induction of anterior regions of the vertebrate body axis.

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