Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse

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Bone morphogenetic protein-4 (BMP-4) is a member of the TGF-β superfamily of polypeptide signaling molecules, closely related to BMP-2 and to Drosophila decapentaplegic (DPP). To elucidate the role of BMP-4 in mouse development the gene has been inactivated by homologous recombination in ES cells. Homozygous mutant Bmp-4−/− embryos die between 6.5 and 9.5 days p.c., with a variable phenotype. Most Bmp-4−/− embryos do not proceed beyond the egg cylinder stage, do not express the mesodermal marker T(Brachyury), and show little or no mesodermal differentiation. Some homozygous mutants develop to the head fold or beating heart/early somite stage or beyond. However, they are developmentally retarded and have truncated or disorganized posterior structures and a reduction in extraembryonic mesoderm, including blood islands. These results provide direct genetic evidence that BMP-4 is essential for several different processes in early mouse development, beginning with gastrulation and mesoderm formation. Moreover, in the presumed absence of zygotic ligand, it appears that homozygous mutants can be rescued partially by related proteins or by maternal BMP-4.

[Key Words: BMP-4; mouse embryo; targeted mutation; lethal embryonic phenotype]

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Bone morphogenetic proteins-2 and -4 (BMP-2 and BMP-4) are two closely related members of the transforming growth factor (TGF)-β superfamily of secreted polypeptide signaling molecules (for review, see Kingsley 1994; Hogan 1995). The carboxy-terminal mature regions of the two proteins are 92% identical at the amino acid level. Moreover, they have been highly conserved during evolution, in Drosophila a single gene, decapentaplegic (dpp), encodes a protein with ~75% sequence identity to BMP-2 and BMP-4 in the carboxy-terminal mature region. At the functional level, human BMP-4 can rescue the dorsal-ventral pattern defects of dpp null mutants, and Drosophila DPP protein can induce ectopic bone in mice (Padgett et al. 1993; Sampath et al. 1993). Genetic analysis has shown that DPP is required at several different stages of Drosophila development. It acts as a dorsalizing morphogen in the dorsal–ventral patterning of the blastoderm embryo, mediates an inductive interaction between mesoderm and endoderm in the larval midgut, and plays a role in the proximal–distal patterning of the leg and wing imaginal discs (for review, see Campbell et al. 1993; Diaz-Benjumea and Cohen 1993; Wall and Hogan 1994).

The striking evolutionary conservation of BMP-4 and DPP supports the idea that BMP-4 plays crucial roles in development. Studies on the temporal and spatial patterns of Bmp-4 expression in the mouse embryo are consistent with this hypothesis. Low levels of Bmp-4 RNA have been detected as early as 6.5 days postcoitum (p.c.), around the time of gastrulation (Jones et al. 1991; Johansson and Wiles 1995; Fig. 1A, B) and by 7.5 days p.c. transcripts can be localized in the allantois, amnion, and posterior primitive streak (Jones et al. 1991; Fig. 1A, B) and by 7.5 days p.c. transcripts can be localized in the allantois, amnion, and posterior primitive streak (Jones et al. 1991; Fig. 1C, D). Expression is maintained in these regions through 8.5 and 9.0 days p.c. and is also seen in the mesoderm around the hindgut and foregut and in the ventral lateral mesoderm of the posterior body wall and gut (splanchnopleure and somatopleure) (Jones et al. 1991; Fig. 1F). As development proceeds, transcripts become localized to specific regions of the myocardium of the heart, the branchial arches, otic vesicle, and diencephalon. In later embryos, Bmp-4 transcripts are found in regions where inductive interactions occur between mesenchymal tissues and adjacent epithelium, for example, in the developing whisker follicles and the tooth bud (Jones et al. 1991). In addition, Bmp-4 is expressed in a complex and dynamic pattern in the mesoderm and apical ectodermal ridge of the limb bud, at a time when pattern formation is taking place (Jones et al. 1991; Francis et al. 1994). Taken together, these studies suggest that BMP-4 is involved in mesoderm formation and patterning during and after gastrulation, and in mediating instructive in-
interactions between many different cell types during development (for review, see Jones et al. 1991; Hogan et al. 1994). This hypothesis is reinforced by some in vitro studies with purified protein. For example, BMP-4 enhances the differentiation of hematopoietic cells from murine embryonic stem (ES) cells in culture (Johansson and Wiles 1995) and in the tooth bud BMP-4 induces the expression of several genes in the dental mesenchyme, including the BMP-4 gene itself (autoinduction) [Vainio et al. 1993].

In addition to these studies with mouse embryos, there is strong experimental evidence that BMP-4 acts as a ventralizing factor in mesodermal patterning during Xenopus development. Overexpression of BMP-4 leads to the up-regulation of posterior mesoderm markers such as Xhox-3, to the differentiation of ventral mesenchyme and blood cells in isolated animal caps, and to the duplication of the posterior body axis in “einstein” experiments [Dale et al. 1992; Jones et al. 1992; Fainsod et al. 1994; Hogan et al. 1994]. In addition, overexpression of a dominant negative BMP-2/4 type I receptor leads to a hyperdorsalized phenotype and the down-regulation of globin expression [Graff et al. 1994; Maeno et al. 1994; Suzuki et al. 1994]. These results have led to a model in which BMP-4 expressed from maternal and zygotic transcripts before and during Xenopus gastrulation actively promotes the differentiation of ventral–posterior mesoderm and counteracts the opposing effect of dorsalizing factors such as activin [Jones et al. 1992; Fainsod et al. 1994; Graff et al. 1994; Harland 1994].

To define precisely the role of BMP-4 in mouse development a mutation was generated in the gene by homologous recombination in ES cells. Most homozygous Bmp-4<sup>tmL1bh</sup> mutants are arrested at the egg cylinder stage and have little or no mesoderm. Some, however, develop to later stages but are grossly retarded and have defects in posterior structures. These results provide the first direct genetic evidence that BMP-4 is required at several different stages of mouse development, beginning with gastrulation and mesoderm formation.

**Results**

**Expression of Bmp-2 and Bmp-4 in early mouse embryos**

RNA for both Bmp-2 and Bmp-4 can be detected by RT–PCR in embryos from 6.5 to 10.5 days p.c. [Fig. 1A]. Using whole mount in situ hybridization, Bmp-2 transcripts cannot be localized in 6.5-day p.c. embryos, but low levels of Bmp-4 RNA can be seen in the posterior primitive streak [Fig. 1B]. By 7.5 days p.c. some Bmp-2 RNA is detected in the anterior, just lateral to the neural folds and in presumed cardiac mesoderm (pcm) [note lower background staining, cf. with Cl. (F)] After turning, Bmp-4 RNA is seen at high levels in the branchial arches (ba), heart (ht), foregut (fg), and posterior ventral mesoderm (pvm). [G] Bmp-2 RNA is highest in the anterior neural fold (nf) and in the heart (ht), foregut (fg) and allantois (out of focus).

**Targeted disruption of the mouse Bmp-4 gene**

As shown in Figure 2, A and B, and elsewhere [Feng et al. 1994] the mouse Bmp-4 gene consists of two protein-coding exons; the first encodes the signal sequence and the second encodes the remainder of the pro region and all of the carboxy-terminal mature protein. In the targeted allele, here designated Bmp-4<sup>tm1bh</sup> [Davisson 1995], all of the coding sequence of the first exon after amino acid
was found was an empty decidua, an empty yolk sac, was variable, even within a litter. In some cases all that
approximately Theiler stage 12) to a few embryos that had
bryo. In most cases mutants were arrested at the egg
alysis of yolk sac DNA. (This PCR analysis is dependent
ncluded empty decidua) generated by interbreeding either
hybrid genetic background

Phenotype of homozygous mutant embryos on a
(C57BL/6×129) hybrid genetic background

Initially, heterozygous Bmp-4tm1blh/+ (C57BL/6×129)
F₁ hybrids were intercrossed to generate homozygous and heterozygous embryos. These were collected be-
tween 7.5 and 10.5 days p.c. and identified by PCR anal-
ysis of yolk sac DNA. (This PCR analysis is dependent
on the presence in the second protein coding exon of an
oligonucleotide encoding stop codon in all three reading
frames.) All embryos genotyped as homozygous Bmp-
4tm1blh were abnormal in phenotype, whereas all normal
embryos were homozygous wild type or heterozygous mutant. The overall percentage of abnormal embryos in-
cluding empty decidua) generated by interbreeding either
(C57BL/6×129) hybrids or hybrids between the first and
third generation of backcrossing to C57BL/6 was 26%
(Table 1).

At each stage, the phenotype of Bmp-4tm1blh embryos was variable, even within a litter. In some cases all that
was found was an empty deciduum, an empty yolk sac, or a very small and completely disorganized or dead em-
bryo. In most cases mutants were arrested at the egg
cylinder stage. More advanced mutant embryos ranged in
phenotype from the neural fold/early somite stage [ap-
proximately Theiler stage 12] to a few embryos that had
undergone turning and had a beating heart [Theiler stage
14–15] (Fig. 3). All mutants were smaller and grossly re-
tarded compared with their littermates and had disorga-
nized or truncated posterior structures. In addition, when compared with wild-type littermates the mutant
visceral yolk sac often had a “blebby” appearance attrib-
utable to the paucity of extraneous mesoderm and blood
sacs underlying the endoderm layer [arrows, Fig. 3F, H].

Phenotype of homozygous mutant embryos during backcrossing onto a C57BL/6 inbred genetic background

To investigate the possible effect of genetic background on the homozygous mutant phenotype, hybrid (C57BL/
6×129) heterozygous mutant females were backcrossed
to C57BL/6 inbred males. From the fourth to the ninth
backcross generation (representing mice with between a
93.8% and 99.8% C57BL/6 genotype) embryos were col-
clected between 3.5 and 10 days p.c. Taking empty de-
cidua and abnormal embryos together, the overall per-
centage of putative homozygous mutants was 27% (Ta-
ble 2). In addition, in all cases where genotyping could be
done [by PCR analysis of yolk sac DNA], homozygous
Bmp-4tm1blh embryos were abnormal compared with their heterozygous and +/+ littermates.

The phenotype of Bmp-4tm1blh embryos still remained variable within a litter, and most failed to advance be-
yond the egg cylinder stage. Although the number of
embryos is still small, at 9.5 days p.c. no homozygous mutant embryos beyond the neural fold/early somite
stage have been recovered, raising the possibility that the
mutant phenotype is somewhat more severe on the
C57BL/6 background. Histological analysis of one of the
most advanced abnormal embryos in this backcross
group is shown in Figure 4A,C. One striking feature is
the relative paucity of blood islands containing red blood
cells in the mesoderm of the visceral yolk sac compared
with a normal littermate [Fig. 4B,D]. As noted above, this
was a feature of all Bmp-4tm1blh embryos that reached
this stage of development and beyond. In addition, there
were few red blood cells in the heart, dorsal aorta, and
vessels of the embryo [Fig. 4A,B, arrowheads].

Most of the abnormal embryos were arrested at the egg
cylinder stage. Sections of three such embryos analyzed
at 7.5 days p.c. are shown in Fig. 4 E–G, together with a
normal littermate [H]. In all abnormal embryos a small
amount of extraembryonic mesoderm can be distin-
guished [Fig. 4E–G, arrows] but an organized primitive
streak is not present.

Figure 2. Targeted inactivation of the Bmp-4 gene in ES cells and mice. [A] Genomic organization of the wild type and Bmp-4tm1blh
alleles and structure of the replacement vector. Exons are represented by solid rectangles. In the replacement vector a 1.6-kb genic
fragment containing the first protein-coding exon from amino acid position 7 and part of the following intron was replaced by the
neomycin resistance cassette from pMC1neoA+ . In addition, an oligonucleotide generating translational stop codons in all three
reading frames was inserted into the single SfiI site in the second coding exon [open box], thereby destroying this site. The
replacement vector has herpes virus thymidine kinase cassettes flanking the regions of homology. The open rectangles denote the
500-bp BamHI–BsmI fragment used as an external S’ probe and the 800-bp XbaI–BamHI fragment used as an internal S’ probe. [B]
Details of the mutated second coding exon. The position of the SfiI site and the inserted oligonucleotide is shown with respect to the
BMP-4 amino acid sequence. The positions of the primers used in the PCR–SfiI restriction analysis are denoted, together with the sizes
of the PCR products derived from the wild-type and mutant second exon before and after SfiI digestion. (Inset) PCR–SfiI restriction
analysis of yolk sac DNAs of three 9.5-day p.c. embryos derived from matings of heterozygous Bmp-4tm1blh/+ mice. The 452-bp band
indicates the presence of the mutated second coding exon, the 210- and 230-bp fragments indicate the presence of the wild-type second
coding exon. Accordingly, the genotypes of the embryos are definitively +/+ , +/−, and −/−. [B] BamHI, [B] BsmI, [C] ClaI, [E]
EcoRI; [S] SfiI; [Sp] SspI; [X] XbaI; [Xh] XhoI. Restriction enzyme recognition sites in brackets were destroyed in the cloning process.

Some of the founder chimeras were crossed with 129 females, and a colony of heterozygous Bmp-4tm1blh/+ mice.
inbred 129 mice was established. However, these have low fertility and most females with a copulation plug have failed to yield any embryos or decidual swellings. The four $Bmp-4^{tm1bhh}$ homozygous mutant embryos recovered to date have been small and abnormal, resembling those shown in Figure 4, E–G, and Figure 5, B and C. Some of the inbred 129 $Bmp-4^{tm1bhh/+}$ males were crossed with outbred ICR females to generate (ICR × 129)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Figure 2. (See previous page for legend.)}
\end{figure}
Table 1. Phenotypes observed during gestation

| Age of embryos [d.p.c.] | Total no. of decidua | Normal [+/+ and +/-] | Abnormal [-/-] | Empty decidua [no.] |
|-------------------------|---------------------|---------------------|----------------|---------------------|
|                         | de                  | ec                  | hf             | t                  | N.D.   |
| 7.5                     | 36                  | 25                  | 2              | 6                  | 0      | 0      | 3 |
| 8.5                     | 83                  | 54                  | 0              | 0                  | 0      | 17     | 12 |
| 9.5                     | 159                 | 133                 | 5              | 10                 | 1      | 6      | 0  |
| 10.5                    | 39                  | 25                  | 3              | 2                  | 0      | 1      | 8  |
| Totals [%]               | 317                 | 237 (74%)           | 53 (17%)       | 27 (9%)            |

(de) Dead embryo; (ec) egg cylinder; (hf) head-fold; (t) "turned;" [N.D.] not determined.

hybrids that were then interbred. The phenotype of embryos on this genetic background is summarized in Table 3. Taken together, empty decidua and abnormal embryos make up 24% of the total. Again, the phenotype of Bmp-4<sup>tm1lh</sup> embryos is variable and at 9.5 and 11.5 days p.c. some have been recovered that are grossly retarded but have undergone turning, have a beating heart and forelimb buds, and resemble the embryo shown in Figure 3A.

Abnormalities are not seen in homozygous mutants before ~6.0 days p.c.

To determine the earliest stage at which the mutant phenotype could be detected, embryos were collected as blastocysts at 3.5 days p.c. and as egg cylinder stage embryos at 5.5 days p.c. soon after implantation, and genotyped by PCR. At both stages, all the homozygous Bmp-

Figure 3. Phenotype of Bmp-4<sup>tm1lh</sup> and wild-type embryos at 9.5 days p.c. (A,B,E,F) Homozygous mutant embryos as judged by PCR analysis of yolk sac DNA; (C) a wild-type +/+ littermate of F. They were obtained on the C57BL/6×129 hybrid background. All the homozygotes are smaller than normal [see scale bars] and retarded in development. (D,G) Sections through the embryo shown in A at the levels indicated. These and other sections show that embryo A has optic (op) and otic vesicles, branchial arches (ba) 1, 2, and 3, somites, forelimb bud (fl) and heart (ht). The posterior of the embryo is abnormal and shows only a small hind gut diverticulum and neural plate [inset * in D]. Sections of the heart at the level of the atrioventricular cushion reveal an absence of mesenchymal cells in the region of the prospective cushions (+) between the myocardium and the endocardium. Very few red blood cells are present in the heart and blood vessels. The embryo in B has optic (op) and otic (ot) vesicles and a heart that was beating at the time of dissection. The posterior of the embryo is truncated. In E the embryo has prominent optic vesicles and a heart, but the posterior is truncated and disorganized. In F the neural folds are still open in the head region and the yolk sac has an abnormal blebbly appearance [arrows]. Viewed ventrally, the margin between the lateral body wall and the yolk sac has not begun to close around the ventral midgut region. (H) Section through the visceral yolk sac from a 9.5-day homozygous mutant. Contact between the endoderm [arrowheads] and extraembryonic mesoderm [arrows] is very limited and few red blood cells are present. (I) Yolk sac from a 9.5-day wild-type embryo showing close apposition of endoderm and mesoderm and red blood cells in the numerous blood islands [arrows]. [A–F] Bars, 0.25 mm; [H,I] bar, 50 μm.
Table 2. Phenotype observed during gestation

| Age of embryos [d.p.c.] | Total no. of decidua | Normal (+/ + or + /- ) | Abnormal (-/-) | Empty decidua [no. ] |
|-------------------------|----------------------|------------------------|---------------|---------------------|
|                         |                      | de ec hf t N.D.        |               |                     |
| 6.5                     | 15                   | 11 0 4 0 0 0           |               |                     |
| 7.5                     | 33                   | 23 1 8 0 0 1           |               |                     |
| 8.5                     | 24                   | 18 1 3 1 0 0 1        |               |                     |
| 9.5                     | 20                   | 15 0 1 2 0 0 2        |               |                     |
| Totals (%)              | 92                   | 67 (73%) 21 (23%) 4 (4%)|               |                     |

See Table 1 for abbreviations.

4tm11b embryos appeared morphologically normal. This was the case with embryos either on a predominantly C57BL/6 background or on an (ICR×129) hybrid background (Table 4).

Expression of molecular markers in homozygous mutant embryos

To investigate mesoderm formation in mutants at the molecular level, Bmp-4 tm11b genotyped embryos arrested at the egg cylinder stage at 7.25 days (as judged by morphology under the dissecting microscope) were hybridized with a probe for T [Brachyury]. Normally this gene is expressed first in the mesoderm of the early primitive streak from ~6.5 days p.c. [Herrmann 1991]. In the three homozygous mutant embryos examined in this way, no T expression was detected. In contrast, both the normal 7.25-day p.c. littermates and wild-type embryos collected at 6.5 days p.c., at the onset of gastrulation, showed characteristic T expression [Fig. 5A]. This finding supports the morphological evidence [see Fig. 4] that those mutant embryos, which are arrested at the egg cylinder stage, generate little or no primitive streak mesoderm.

Abnormal embryos that had developed to the neural fold stage were analyzed for the expression of the caudal-like homeodomain protein, Cdx-4 [Gamer and Wright 1993]. Normally this is expressed first in the allantois and tip of the posterior primitive streak in 7.0- to 7.5-day p.c. embryos and later extends rostrally in the ectoderm and mesoderm of the posterior primitive streak. To maintain the relationship between the embryo and its extraembryonic membranes, immunohistochemistry was carried out on sections without dissecting the embryo from the decidua, so that genotyping was not carried out and homozygous mutants were identified solely on the basis of abnormal morphology. No Cdx-4 expres-
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sion could be detected in the posterior of presumed Bmp-4 tmblh embryos. This is illustrated in Figure 5, B–E, which compares a normal embryo at 8.5 days p.c. with a littermate with an abnormal posterior region that does not show Cdx-4 expression.

Finally, embryos that had developed to the neural fold stage were examined for the expression of HNF-3β. This gene encodes a winged-helix protein that is normally expressed in the anterior primitive streak, node, and notochord during gastrulation (Sasaki and Hogan 1993). Studies in other laboratories have shown that embryos homozygous for a null mutation in the fibroblast growth factor (FGF) receptor gene, fgr-1, have a deficiency in posterior mesoderm and overexpress HNF-3β in the midline notochord region. This is thought to reflect the role of an FGF in promoting the proliferation and differentiation of posterior and paraxial mesoderm (Deng et al. 1995; Yamaguchi et al. 1995). In Bmp-4 tmblh embryos

Table 3. Phenotypes observed during gestation

| Age of embryos (d.p.c.) | Total no. of decidua | +/+ | +/− | −/− | Empty decidua* |
|---|---|---|---|---|---|
| 6.5 | 44 | 10 | 19 | 1 | 10 (11%) |
| 7.5 | 74 | 18 | 33 | 2 | 18 (21%) |
| 8.5 | 58 | 18 | 34 | 2 | 6 (7%) |
| 9.5 | 115 | 33 | 62 | 4 | 10 (11%) |
| 10.5 | 39 | 10 | 14 | 1 | 0 (%) |
| 11.5 | 4 | 1 | 2 | 0 | 0 (%) |
| Total | 334 | 90 (27%) | 164 (49%) | 36 (11%) | 44 (13%) |

See Table 1 for abbreviations.

*For wild-type ICR mice the percentage of empty decidua was 3% (7/238).
the pattern of expression of HNF-3β is essentially normal in the notochord and node (Fig. 5F,G).

Discussion

The results reported here provide the first direct genetic evidence that BMP-4 is essential for early mouse development. Although not all embryos were genotyped, the percentage of abnormal embryos and empty decidua together made up ~25% of the total collected from heterozygous Bmp-4tmIblh/+ crosses on all genetic backgrounds. Moreover, all homozygous Bmp-4tmIblh embryos genotyped were abnormal and all normal embryos genotyped were either wild type or heterozygous mutant (Tables 1–3; data not shown). Therefore, Bmp-4 is the second member of the TGF-β gene superfamily shown by genetic evidence to be required for mesoderm formation and gastrulation in the mouse, the other being nodal (Zhou et al. 1993; Conlon et al. 1994). In contrast, homozygous null Bmp-5 and Gdf-5 mice are viable and fertile, although the adults do show defects in the skeletal system (Kingsley et al. 1992; Storm et al. 1994). Homozygous null activinβA and activinβB mice have relatively minor defects, for example, in ectodermal differentiation and craniofacial development, but show no abnormalities in gastrulation and mesoderm formation (Vassalli et al. 1994; Matzuk et al. 1995). Therefore, the most important questions that need to be addressed are (1) the nature of the specific processes in early development that require BMP-4, and (2) the reason for the variability in the phenotype of homozygous mutants.

The simplest hypothesis, summarized in Figure 6, and discussed in more detail below, is that BMP-4 is first required both for the proliferation and survival of epiblast cells just before gastrulation and for mesoderm formation. Beyond this stage, development of homozygous mutant embryos depends on the supply of exogenous BMP-4 from the maternal environment or on the ability of other signaling molecules to compensate for the absence of BMP-4. In the presence of these factors, development can proceed, albeit at a slower rate than normal, until the next step at which BMP-4 is required, for example, the differentiation and proliferation of posterior mesoderm fated to give rise to extraembryonic mesoderm of the amnion, allantois, and yolk sac and to ventral–lateral mesoderm in the embryo [Lawson and Pedersen 1992]. Homozygous mutants may be arrested at this stage or proceed to the next stage at which a BMP-4-dependent process is essential for embryo survival in the uterus [Copp 1995]. For example, this might be the production of an adequate blood supply and circulatory system, fusion of the allantois with the chorion, or closure of the ventral body wall. According to this model, the variability of the phenotype of homozygous mutants is dependent in part on the stochastic nature of the processes involved and the availability of rescuing factors. In addition, it is possible that on some genetic backgrounds modifier genes influence the supply of rescuing factors or the dependence of complex processes on BMP-4.

BMP-4 is essential for normal gastrulation and mesoderm formation

We have found that homozygous mutant 3.5-day p.c. blastocysts and 5.5-day p.c. egg cylinder stage embryos appear normal, at least under the dissecting microscope. In contrast, abnormal embryos and empty decidua are first observed ~6.5 days p.c., the time when gastrulation begins [Tables 1–3] and when very low levels of Bmp-4 transcripts have been detected in the embryo (Jones et al. 1991; Johansson and Wiles 1995; Fig. 1A). Abnormal Bmp-4tmIblh embryos arrested at this stage do not express T[Brachyury], a marker for early primitive streak mesoderm [Herrmann 1991], although expression is seen in their heterozygous littermates (Fig. 5A). One possible explanation for these results is that BMP-4 is required for the survival and proliferation of epiblast cells at the time when they become competent to respond to mesoderm-inducing factors. An alternative hypothesis is that BMP-4 is required for the survival of newly differentiated mesoderm cells; in the absence of ligand the mesoderm dies and more epiblast cells differentiate to compensate, ultimately leading to the epiblast being depleted. A third hypothesis is that BMP-4 is acting solely as a mesoderm-inducing factor. However, if this were the case it might be expected that the epiblast would continue to proliferate without generating any mesoderm, giving rise to embryos with the phenotype seen in homozygous nodal or msd mutants [Conlon et al. 1994; Holdener et al. 1994]. Further studies are required to distinguish between these possibilities. For example, it would be informative to culture blastocysts in vitro and test whether Bmp-4tmIblh inner cell mass [ICM] cells continue to proliferate in vitro at the same rate as normal, and give rise to ES cell lines that generate mesoderm. A similar approach has been used to demonstrate a requirement for FGF-4 in the proliferation of ICM and embryonic ectoderm cells [Feldman et al. 1995].

It is possible that the hypotheses outlined above are not mutually exclusive and that BMP-4 is required for both the proliferation of epiblast cells in the egg cylinder

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### Table 4. Genotyping of pre- and peri-implantation embryos

| Background | Age of embryos [d.p.c.] | Total | +/- | +/− | −/− | Empty decidua [no.] |
|------------|-------------------------|-------|-----|-----|-----|---------------------|
| C57BL/6    | 3.5                     | 10    | 2   | 6   | 2   | N.A.                |
| ICR        | 3.5                     | 22    | 6   | 13  | 4   | N.A.                |
| ICR        | 5.5                     | 45    | 14  | 20  | 8   | 3                   |
| Total [%]  | 77                      | 22 [29%] | 39 [49%] | 14 [18%] | 3 [4%] |
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**Figure 6.** Model for the multiple requirements for BMP-4 in early mouse development. (A) Homozygous *Bmp-4 tmlblh* embryos appear normal at the blastocyst stage and at the early egg cylinder stage immediately after implantation [Table 4]. (B) *Bmp-4* transcripts can be detected at very low levels at 6.5 days p.c. [Jones et al. 1991; Johansson and Wiles 1995] and many homozygous *Bmp-4 tmlblh* embryos do not develop beyond the egg cylinder stage and do not express T(Brachyury). Therefore, we propose that BMP-4 is required for the proliferation of epiblast cells (red) and for their differentiation into mesoderm. (C) Some *Bmp-4 tmlblh* homozygous mutants can be rescued by maternal BMP-4 or by BMP-2 expressed in the embryo and develop slowly to the primitive streak/head fold stage. However, there is a deficiency in posterior embryonic mesoderm and extra-embryonic mesoderm in the allantois and visceral yolk sac, suggesting that BMP-4 is required for their proliferation or differentiation (red). *Bmp-4* transcripts can be detected in these regions by whole mount in situ hybridization (data not shown). (D) Some *Bmp-4 tmlblh* mutants can be rescued to the early somite/beat heart stage before or after turning. These embryos probably cease development because of a failure in the development of extraembryonic structures such as the blood islands and allantois, tissues in which BMP-4 is expressed (red). There may also be a failure in the growth of the lateral plate mesoderm so that the margins between the yolk sac and the amnion, and the splanchnopleure and somatopleure do not move ventrally [arrows] to close along the ventral midline of embryo during and after turning.

and for their differentiation into mesoderm. In *Xenopus* and *Drosophila* embryos, BMP-4 and DPP appear to function by specifying the fate of cells in the marginal zone and blastoderm, respectively. In the mouse this function of regulating cell fate may be conserved but linked to the control of cell proliferation. A somewhat similar conclusion has been reached by two groups studying the role of FGF in early mouse development. In the absence of a signal from the FGF receptor-1 (FGFR-1), cells in the primitive streak appear to proliferate more slowly than normal and to differentiate preferentially into axial rather than paraxial mesoderm [Deng et al. 1995; Yamaguchi et al. 1995]. However, a marked expansion of *HNF-3β*-expressing axial mesoderm compared with normal was not seen in *Bmp-4 tmlblh* homozygous embryos [Fig. 5F,G].

Our hypothesis that BMP-4 is essential for gastrulation is strongly supported by the phenotype of embryos homozygous for a likely null mutation in the *Bmpr* gene, which encodes a BMP-2/4 type I serine–threonine kinase trans-membrane receptor [Mishina et al. 1995]. Homozygous mutant embryos die at ~6.5 days, are small in size, and do not form mesoderm, as judged by the absence of expression of a number of early mesoderm markers, including T(Brachyury) [Y. Mishina and R.R. Behringer, pers. comm.].

**Development of Bmp-4 tmlblh embryos beyond gastrulation**

Although BMP-4 appears to be first required for gastrulation some homozygous *Bmp-4 tmlblh* mutants develop beyond this stage [Tables 1–3]. One possibility is that these embryos are rescued by maternal BMP-4 diffusing in from decidual cells, which express *Bmp-4* RNA at 6.5 and 7.5 days [Jones et al. 1991]. Alternatively, embryonic BMP-2, which is 92% identical to BMP-4 in the mature region, may compensate for the absence of BMP-4, particularly if its expression is up-regulated in the homozygous mutant. *Bmp-2* is expressed in the embryos from 6.5 days p.c. and in some tissues (amnion, allantois, heart) its localization overlaps with that of *Bmp-4* (Fig. 1A,B; Lyons et al. 1995). Transcripts for two other members of the BMP superfamily, *Vgr-2* (*Gdf-3*) and BMP-7, have been detected in the 6.5- and 7.5-day p.c. embryo [Jones et al. 1992; Lyons et al. 1995] but they encode proteins with only 50%–60% sequence identity to BMP-4, and might not be expected to signal through the same or closely related receptors. The hypothesis that some homozygous *Bmp-4 tmlblh* embryos are being rescued by BMP-2 is supported by the fact that no homozygous *Bmpr* mutants, which lack a type 1 receptor are capable of binding both BMP-2 and BMP-4, develop beyond the egg cylinder stage (Y. Mishina and R.R. Behringer, pers. comm.).

Assuming that some rescue of *Bmp-4 tmlblh* mutants is possible, then gastrulation could occur and development proceed, albeit more slowly than normal, to the next stage when a BMP-4-dependent process becomes rate-limiting. This appears to be the proliferation and differentiation of the mesoderm of the posterior primitive streak, which is fated to give rise to the extraembryonic mesoderm of the yolk sac and allantois and to the ventral–lateral mesoderm of the posterior body wall and gut. This conclusion is based on several observations. First, in all *Bmp-4 tmlblh* embryos that develop beyond the neural fold/early somite stage there is a deficiency of mesoderm cells and blood islands in the visceral yolk sac, often leading to a blebbly appearance of the yolk sac [Fig. 3F,H]. Also, in sections of embryos that develop a heart and vascular system, the number of red blood cells seen inside these structures is greatly reduced compared with normal [Figs. 3D,G and 4A]. In addition, the allantois is often relatively small [see Fig. 3F] and the posterior of the...
embryo truncated and disorganized [Figs. 3A, B and 5B, C]. This is manifested at the molecular level by the absence of Cdx-4 staining the posterior of abnormal embryos [Fig. 5B–E]. Finally, in some embryos that reach the stage of "turning," the ventral aspect of the body wall appears more "open" than normal (for example, see Fig. 3E, F). The process of turning involves a major realignment of the embryo and its extraembryonic membranes. In particular, the margins between the lateral body wall and the yolk sac, and the body wall and the amnion, have to shift ventrally and close down to a narrow ventral umbilical ring [Kaufman 1992] [see Fig. 6D]. Although very little is known about the mechanisms underlying this process of ventral body wall closure, it probably requires extensive proliferation of the ventral–lateral mesoderm of the somatopleure and splanchnopleure, tissues in which Bmp-4 transcripts are expressed at high levels [Jones et al. 1991].

According to our model, in the early mouse embryo Bmp-4 is required for the differentiation of mesoderm derived from the posterior primitive streak and for the development of the ventral–lateral mesoderm of the body wall and gut (somatopleure and splanchnopleure). This conclusion is compatible with studies on both the expression of Bmp-4 in Xenopus embryos [Fainsod et al. 1994] and its function as a ventralizing factor [for review, see Harland 1994]. For example, we and others have shown that in Xenopus BMP-4 promotes the differentiation of posterior–ventral mesoderm, including hematopoietic tissue, and these cell types are deficient in Bmp-4embryos that develop beyond gastrulation. In addition, our findings support the speculation that the dorsal–ventral axis of invertebrate and vertebrate embryos has been inverted during evolution [Arendt and Nubler-Jung 1994]. This is based in part on the finding that DPP is a dorsalizing factor in Drosophila, whereas the closely related ligand Bmp-4 behaves as a ventralizing factor in both Xenopus and early mouse embryos.

Expression studies in the later mouse embryo have suggested that Bmp-4 is required for the development of a number of different structures, including the heart, limb, skin, diencephalon, and teeth [Jones et al. 1991]. Although limb buds and heart did develop in some Bmp-4embryos, they appeared less well developed than those of normal littermates [Fig. 3D, G]. However, it is not possible to determine whether this is attributable to a specific requirement for Bmp-4 in these organs or to a secondary delay in overall development attributable to defects in other systems [e.g., the hematopoietic system]. In the future, techniques such as tissue-specific gene targeting could be used to investigate more directly the role of Bmp-4 in organ development in older embryos.

The effect of genetic background on the phenotype of Bmp-4embryos

If our model outlined above and in Figure 6 is correct, there are several ways in which the genetic background of the embryo or the mother could affect the phenotype of homozygous Bmp-4embryos. For example, there could be genetic differences in the synthesis and accumulation of rescuing factors such as maternal BMP-4 or embryonic BMP-2 or in the relative affinity of trans-membrane signaling receptors for BMP-4 and BMP-2. In addition, there could be genetic differences in the extent to which specific developmental processes such as posterior mesoderm proliferation and differentiation are dependent on one class of signaling molecules rather than another, for example, on FGFs compared with BMPs. One way to address these possibilities is to generate mice carrying various combinations of mutations in genes encoding different ligands and receptors, and such experiments are under way.

Materials and methods

Construction of the targeting vector

Genomic clones were isolated from a 129/Sv mouse genomic library [Stratagene] using a mBmp-4 cDNA probe. Four overlapping λ-clones [λG, λH, λL, λM] were isolated and partial sequencing and Southern blot analysis revealed the structure of the mBmp-4 gene as shown in Figure 2A. A replacement vector was constructed in a pBRA32 derivative [Blessing et al. 1993] using as the 5' homology region a 3.1-kb fragment spanning from the BsmI site in the first coding exon [amino acid position 7 in the leader sequence] to an upstream BamHI site. The entire insert of clone λM, spanning from a Sau3A site in the intron between the two coding exons to a Sau3A site 18.4 kb downstream, was used as the 3' homology region. This was modified by inserting a short DNA fragment consisting of the two partially complementary oligonucleotides 5'-CTAGTAACTGTA-3' and 5'-GTAACTAGTCA-3' into the single SfiI restriction site located in the second coding exon just upstream of the DNA sequence coding for the mature region of BMP-4. This insertion destroys the SfiI restriction site and introduces stop codons in all reading frames [Fig. 2B]. To facilitate positive/negative selection, thymidine kinase cassettes derived from the constructs pMC1TKA+ and pPGKTKA+ [Rudnicki et al. 1992] were attached to the ends of the homology regions [Fig. 2]. The neomycin/G418 resistance cassette derived from the construct pMC1neo'A+ replaced 1.6 kb of genomic DNA consisting of the first coding exon from codon position 7 in the leader peptide and a part of the following intron. According to standard nomenclature [Davison 1995] the mutant allele is designated here as Bmp-4tmblh.

Electroporation and selection of ES cells

ES cells [5×10⁴] of line D3 [kindly provided by Dr. Tom Doetzman, University of Cincinnati, OH] at passage 15 were electroporated with 200 μg of NorI-digested replacement vector DNA in a total volume of 800 μl of PBS using a single pulse from a gene pulsar [Bio-Rad] at 800 V and 3 μF. The cells were then placed onto irradiated male primary mouse embryo fibroblasts in Dulbecco's modified Eagle medium [DMEM] without sodium pyruvate [Specialty Media] containing 15% fetal bovine serum [HyClone] and supplemented with nonessential amino acids [0.01 mM], l-glutamine [0.1 mM], 50 μg/ml of gentamycin sulfate, 0.0006% β-mercaptoethanol [Sigma], and 1000 U/ml of ESGRO. Unless otherwise stated, all supplements were from Gibco-BRL. Selection was initiated after 24 hr by adding geneticin [Gibco-BRL] at a final concentration of 300 μg/ml, and, 48 hr later, gancyclovir [Syntex] at a final concentration of 2×10⁻⁶ M. Cells were kept under selection for 7-10 days with a daily change of medium. Colonies were picked and replated.
without selection into duplicate 96-well cell culture dishes, one of which contained a feeder layer of irradiated embryonic fibroblasts. After 2 days, the cells growing on feeders were frozen in 10% DMSO, 30% serum and stored at −70°C and the duplicate plate was used to prepare genomic DNA for Southern blotting and PCR analysis. Of 288 double-resistant colonies screened, 48 were found to have a correctly targeted allele, giving an overall frequency of 1 in 6. Southern blot analysis with a Neo probe showed only a single insert [data not shown].

Southern blotting and PCR–SfiI restriction analysis

For DNA extraction, ES cells, tail biopsies, yolk sac fragments, and embryos were lysed in [100 mm Tris-HCl (pH 7.5), 50 mm EDTA, 0.5% SDS, 0.1 mg/ml of proteinase K] and digested over-night at 56°C. DNA was prepared by phenol chloroform extraction and ethanol precipitation and redissolved in TE (10 mm Tris-HCl at pH 7.5, 0.1 mm EDTA). Southern blots were performed essentially as described [Church and Gilbert 1984]. The 5' probe [Fig. 2] is a 500-bp BamHI–BsmI fragment. The expected sizes for DNA digested with Sphel are 6.5 kb for the wild-type allele and 6.0 kb for the BMP-4 targeted allele. The internal 3' probe is a 800-bp XbaI–BamHI fragment located at the end of the 3' homology arm. This hybridized with a 12-kb fragment in both wild-type and targeted alleles after digestion of genomic DNA with BamHI.

PCR–SfiI restriction analysis was performed using one primer identical to a sequence (5'-CCAGACTAGTCCATCACAATG-3') 230 bp upstream of the SfiI restriction site in the second coding exon and the other identical to a sequence (5'-TTGAGTGATCAGCCAGTGGA-3') on the reverse strand 210 bp downstream of the same SfiI restriction site [Fig. 2B]. After amplification using Vent DNA polymerase (New England Biolabs), according to the manufacturer's instructions, the PCR products were subjected to SfiI restriction enzyme digestion and analyzed on a 1.5% agarose gel. The product derived from the wild-type second exon will be cleaved by SfiI and yield 210- and 230-bp fragments, whereas the product from the mutated second exon will remain as a 452-bp fragment [Fig. 2B].

Generation of chimeras and colonies of mice heterozygous for the Bmp-4 targeted allele

ES cells from four targeted clones were injected in C57BL/6 host blastocysts that were implanted into pseudopregnant females as described [Hogan et al. 1995]. The resulting chimeras were bred to C57BL/6 females and agouti offspring analyzed for the presence of the Bmp-4 targeted allele by Southern blotting. Thirteen chimeras from one cell line, B286, transmitted the mutation through the germ line. Heterozygous offspring were either interbred or backcrossed with C57BL/6 inbred males. This colony is currently at the ninth backcross generation. The chimeras were also bred to 129/Sv females, generating a colony of heterozygous Bmp-4 targeted + 129/Sv inbred mice. These were also crossed to outbred ICR mice [Harlan Sprague-Dawley] to generate [129 x ICR] F1 hybrids.

For collecting embryos, heterozygous males and females were mated and noon on the day of plug taken as 0.5 days p.c. The embryos were genotyped using PCR–SfiI restriction analysis on DNA obtained from a part of the yolk sac.

RT–PCR

RT–PCR was performed as described [Johansson and Wiles 1995]. Briefly, total RNA was isolated from ES cells and embryos between 6.5 and 10.5 days p.c. by an adaptation of the LiCl/urea method of Auffrey et al. [1980]. The ES cells [line TL1] were cultured for two passages in the absence of mouse embryo fibroblasts, and embryos of one age were pooled. Three independent cDNA syntheses were carried out, using three different RNA samples from each stage examined. cDNA was synthesized using oligo(dT) and the PCR was quantified using hypoxanthine phosphoribosyltransferase (HPRT) as an internal standard. The Bmp-2 and Bmp-4 primers were designed as described by Johansson and Wiles [1995]. The HPRT primer sequences are 5' primer [5' to 3'] TTGTTGGAATTCAATTC-CAGACAAG and 3' primer GCATTAAAAGGAACTGTA-TGCAACAG. The expected length of the cDNA amplification product is 648 bp, whereas the genomic DNA length is 1.3 kb.

Whole-mount in situ hybridization

This was performed essentially as described [Sasaki and Hogan 1993] with minor modifications. For example, 2% bovine mannheim blocking reagent was used in addition to 10% heat-inactivated serum to block nonspecific sites and bovine manhein purple-precipitating reagent was used instead of NBT-BCIP as the coloring reagent.

Immunohistochemistry

This was performed as described [Gamer and Wright 1993] using a 1:100 dilution of Cdx4 antibody.

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