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Failure of ventral body wall closure in mouse embryos lacking a procollagen C-proteinase encoded by *Bmp1*, a mammalian gene related to *Drosophila tolloid*

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SUMMARY

The mouse *bone morphogenetic protein1 (Bmp1)* gene encodes a secreted astacin metalloprotease that cleaves the COOH-propeptide of procollagen I, II and III. BMP-1 is also related to the product of the *Drosophila* patterning gene, *tolloid (tld)*, which enhances the activity of the TGFβ-related growth factor Decapentaplegic and promotes development of the dorsalmost amnioserosa. We have disrupted the mouse *Bmp1* gene by deleting DNA sequences encoding the active site of the astacin-like protease domain common to all splice variants. Homozygous mutant embryos appear to have a normal skeleton, apart from reduced ossification of certain skull bones. However, they have a persistent herniation of the gut in the umbilical region and do not survive beyond birth. Analysis of the amnion of homozygous mutant embryos reveals the absence of the fold that normally tightly encloses the physiological hernia of the gut. At the electron microscopic level, the extracellular matrix of the amnion contains collagen fibrils with an abnormal morphology, consistent with the incorporation of partially processed procollagen molecules. Metabolical labelling and immunofluorescence studies also reveal abnormal processing and deposition of procollagen by homozygous mutant fibroblasts in culture.

Key words: BMP-1, procollagen C-proteinase, null mutation, mouse embryo, amnion, collagen fibrils.

INTRODUCTION

Bone Morphogenetic Protein-1 (BMP-1) was initially identified as a component of a bone extract able to induce ectopic cartilage and bone when implanted in rats (Wozney et al., 1988). It belongs to an evolutionarily conserved family of secreted metalloproteinases with a characteristic structure; an NH2-terminal activation region and astacin-like protease domain, followed by EGF-like motifs and CUB protein-protein interaction domains (Wozney et al., 1988; Fukagawa et al., 1994; Takahara et al., 1994, 1996; Hwang et al., 1994). In both humans and mice, the gene encoding BMP-1 (*Bmp1*; also known as mammalian *Tld)* gives rise to at least three alternatively spliced transcripts, which encode three different proteins, varying in the number of EGF-like and CUB domain repeats, and in the presence or absence of a histidine-rich domain (Takahara et al., 1994). Recently, a mammalian gene closely related to *Bmp1*, known as *Tolloid-like*, *(Tll)*, has been identified, with overlapping but distinct expression patterns during embryonic development (Takahara et al., 1996). Alternative splicing has not been reported for mouse *Tll*.

Genes encoding secreted astacin metalloproteinases related to BMP-1 have been isolated from *Xenopus* (xBMP-1) (Maeno et al., 1993), sea urchin (BP10, SpAN, suBMP-1) (Lepage et al., 1992; Reynolds et al., 1992; Hwang et al., 1994), *C. elegans* (quoted in Finelli et al., 1994), hydra (HMP1) (Yan et al., 1995) and *Drosophila* (TOLLOID (TLD) and TLD-related-1 (TLR-1), also known as TOLKIN (TOK)) (Shimell et al., 1991; Ngyugen et al., 1994; Finelli et al., 1994, 1995).

Genetic analysis in *Drosophila* has shown that *tld* plays a role in dorsal-ventral patterning of the blastoderm embryo. *Tld* is expressed dorsally and, in null mutants on a wild-type background, the dorsalmost tissue, the amnioserosa, is transformed to a more ventral cell fate with an altered rate of cell proliferation (Shimell et al., 1991; Arora and Nusslein-Volhard, 1992). Genetic manipulations have also shown that TLD enhances the dorsalizing activity of Decapentaplegic (DPP), a TGFβ superfamily extracellular signalling protein which is the *Drosophila* homolog of mammalian BMP-2 and BMP-4. Thus, increasing the dose of wild-type *dpp* genes suppresses the ventralized phenotype of weak *tld* mutations, and partially suppresses that of null *tld* mutants (Ferguson and Anderson, 1992). Although *tlr-1/tok* expression partially overlaps with that of *tld* at the blastoderm stage, it also has unique sites in later embryos, and

null *tlr-1* mutants show larval and pupal defects. Moreover, *tlr-1* cannot rescue *tld* mutants (Nguyen et al., 1994; Finelli et al., 1995). Based on the genetic interaction between *dpp* and *tld*, several functions have been proposed for TLD protein. For example, it has been argued that the protease releases active DPP from an inactive protein-protein complex (Ferguson and Anderson, 1992; Childs and O'Connor, 1994; Finelli et al., 1994). TLR-1/TOK may perform the same function for other DPP-related signalling molecules, for example 60A (Nguyen et al., 1994; Finelli et al., 1995).

Recent experiments have clearly established that mammalian and chick BMP-1 is identical with an enzyme known as procollagen C-proteinase, that cleaves the C-terminal peptide from procollagen I, II and III (Kessler et al., 1996; Li et al., 1996). Other studies have provided evidence that BMP-1 activates lysl oxidase, an enzyme essential for the formation of covalent cross-links which stabilize the fibrous forms of both collagen and elastin (Panchenko et al., 1996) and that it cleaves the terminal peptides from the α2 and γ2 chains of laminin 5, a component of hemidesmosomes (Robert Burgeson, Harvard Medical School, personal communication). These results raise the possibility that mouse embryos lacking BMP-1 activity would have major defects in extracellular matrix assembly and function, and in osteogenesis. Moreover, if BMP-1 has the additional function of enhancing the activity or availability of TGFβ-related growth and differentiation factors, one might expect homozygous mutants to have defects in some of the many organs and tissues where these factors are required for normal development (Hogan, 1996). We report here that, unexpectedly, most homozygous *Bmp1* mutant mouse embryos develop until late gestation, with relatively few mophological defects. However, they show a persistent herniation of the gut, probably due to a defect in the amnion and ventral body wall, and die at birth. Analysis of the extracellular matrix of the amnion shows that it contains abnormal collagen fibrils, and metabolic labelling and immunofluorescence studies also reveal abnormal processing and deposition of procollagen by homozygous mutant fibroblasts in culture.

MATERIALS AND METHODS

Construction of the targeting vector

Genomic clones were isolated from a 129/Sv mouse λ-Fix II genomic library (Stratagene) using the *Bmp1* cDNA, F8 (Fukagawa et al., 1994). An approximately 2.6 kb *Xba*I-*Xba*I fragment containing at least two coding exons was subcloned into the *Xba*I site of pBluescriptIIKS+ (Stratagene) and a 1.1 kb region encoding amino acids 150-249 (Fukagawa et al., 1994), which includes the putative active-site zinc-binding motif (Bode et al., 1992), was deleted using an *Exo*III/Mung Bean deletion kit (Stratagene). The resulting 1.3 kb *Eco*RI-*Pvu*II fragment, of which 218 bp at the 3′ end was from the *Pvu*II-*Kpn*I fragment (759-976 bp) of pBluescriptIIKS+, was used as the 5′ homology region. A 4.5 kb *Xba*I-*Xba*I fragment located 3′ of the 2.6 kb *Xba*I-*Xba*I fragment was used as the 3′ homology region. The *Bam*HI site located at the 3′ end of the pMC1neo^{r}A+ cassette was recreated by ligation between a *Pvu*II site from the 5′ homology region and the blunt-ended *Bam*HI site from the neo^r cassette. To facilitate negative selection, HSV thymidine kinase cassettes derived from the constructs pMC1TKA+ and pPGKTKA+ (Rudnicki et al., 1992) were attached to the ends of the homology regions (Fig. 1). According to standard nomenclature (Davisson, 1995) the mutant allele is here designated *Bmp-1*^{tm1blh} or *Bmp1*^{tm1}, for short.

Electroporation and selection of ES cells

5×10^7 ES cells of line R1 (kindly provided by Dr A. Nagy) at passage 12 were electroporated with 150 μg of *Not*I-digested replacement vector DNA in a total volume of 800 μl phosphate-buffered saline (PBS) as described (Winnier et al., 1995). The cells were then plated

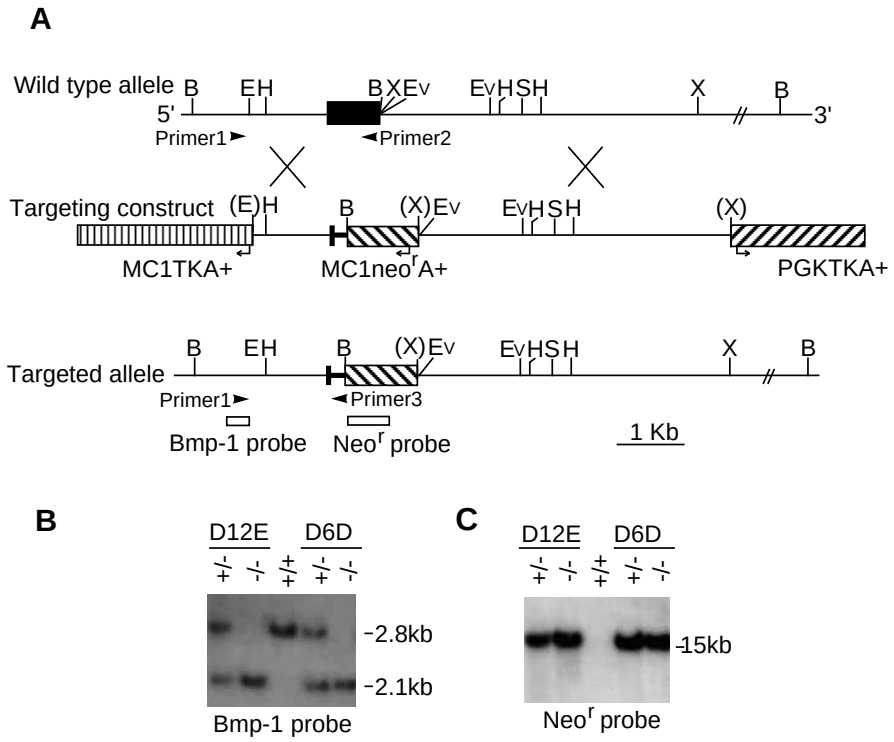

Fig. 1. Generation of *Bmp1*-deficient mice by homologous recombination. (A) Restriction maps showing the endogenous 129/Sv *Bmp1* gene, the structure of the targeting vector and the targeted *Bmp1*^{tm1} allele. The black box denotes the region encoding amino acids 111-249. The precise exon-intron organization was not confirmed within this region but if the genomic organization of *Bmp1* is conserved between human and mouse (Takahara et al., 1995), then the deletion would encompass part of one exon and all of two others. The 5′ and 3′ ends of the targeting vector contain the herpes simplex virus thymidine kinase (TK) gene driven by the MC1 and PGK promoters, respectively. The MC1 neo^r cassette and vector sequences (thick horizontal line) delete most of the 5′ exon, the intervening intron, the 3′ exon and 43 nucleotides of the following intron. (B) Southern blot hybridization using the 5′ probe of *Bam*HI-digested DNA from offspring of two lines. The wild-type allele gives a 2.8 kb fragment while the targeted allele gives a 2.1 kb fragment. (C) Southern blot hybridization of *Bam*HI digested genomic DNA using a neo^r probe. This shows there has been no rearrangement at the 3′ end of the targeted allele. Abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Sac*I; EV, *Eco*RV; X, *Xho*I. TBASE Accession number is TG-000-03-915.

onto irradiated neo^r primary mouse embryonic fibroblasts and cultured as described (Winnier et al., 1995). Selection was initiated 24 hours after plating by adding geneticin (GibcoBRL) at a final concentration of 300 μg/ml and, 48 hours later, gancyclovir (kindly provided by Syntex) at a final concentration of 2 μM. Of 600 double resistant colonies screened by Southern blotting of genomic DNA, 25 were found to have a correctly targeted allele, giving an overall frequency of 1/24.

DNA analysis and genotyping

For DNA extraction, 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 proteinase K) and digested overnight at 56°C. DNA was prepared by phenol chloroform extraction and ethanol precipitation and redissolved in TE (1 mM Tris/HCl pH 7.5; 0.1 mM EDTA). Using a 0.4 kb *Eco*RI/*Sau*3A fragment as probe (Fig. 1), DNA digested with *Bam*HI gave rise to 2.8 kb and 2.1 kb bands for the wild-type allele and the *Bmp1^{tm1}*mutated allele, respectively. Genotyping was also performed using PCR with the primers shown in Fig. 1. The primers are 5′ TGGAGCTTCAGTGTCATGTG 3′ (primer1) 5′ TGGCTG-TATGTTCTTGCGTA 3′ (primer 2) and 5′ GCTATGACCATGAT-TACGCC 3′ (primer 3). The wild-type allele gives a product of 2.1 kb and the mutant allele a product of 1.4 kb.

Generation of chimeras and mice heterozygous for *Bmp1^{tm1}*

ES cells from three targeted clones, D12E, D6D and D6A, were injected into C57BL/6 host blastocysts which were implanted into pseudopregnant ICR females as described (Hogan et al., 1994). The resulting male chimeras were mated with Black Swiss females and agouti offspring genotyped by Southern blot analysis. All three cell lines generated *Bmp1^{tm1}*heterozygous offspring. The offspring from two of the three lines, D12E and D6D, were used for phenotype analysis.

Histological analysis and in situ hybridization

All the procedures for histological analysis at the light microscope level were essentially as described (Hogan et al., 1994). Briefly, embryos with placenta, yolk sac and amnion intact were fixed in 4% paraformaldehyde in PBS, dehydrated through increasing concentrations of ethanol, 70%, 90%, 95% and 100% two times, transferred into xylene and embedded in paraffin. Sections (7 μm) were stained with hematoxylin and eosin or used for non-radioactive in situ hybridization as described (Sasaki and Hogan, 1994). Riboprobe template DNAs were as follows; a 1.5 kb cDNA *Bmp1*A1 for m*Bmp1* (Fukagawa et al., 1994), c21 for m*HNF-3β* (Sasaki and Hogan, 1994), rat cDNA fragment corresponding to nucleotides 1-1542 for *Isl-1* (Karlson et al., 1990; Thor et al., 1991), and 500 bp *Hin*dIII-*Pst*I fragment for m*Pax-3* (Goulding et al., 1991) The *Bmp1* probe hybridizes with all three alternatively spliced transcripts.

Electron microscopy

Tissue for electron microscopy was placed in 2% glutaraldehyde in phosphate buffer, postfixed in osmium tetroxide, stained en bloc with uranyl acetate, processed by standard dehydration in graded ethanols before infiltration and embedding in Spurr's embedding medium (EM Sciences, Fort Washington, PA). Thin sections were stained with uranyl acetate-lead citrate before viewing in a Philips 300 electron microscope.

Metabolic labelling of mouse embryo fibroblasts

Confluent mouse embryo fibroblasts prepared as described (Hogan et al., 1994) were incubated with 75 μg/ml ascorbate for 20 hours, depleted in labeling medium (Dulbecco's modified Eagle's medium with 5% dialyzed fetal bovine serum, 75 μg/ml ascorbate) without isotope for 2 hours and radiolabeled with 20 μCi/ml L-(2,3-^{3}H)proline (Du Pont-New England Nuclear) for 20 hours. Preincubation and labeling media were buffered to pH 7.0, and soybean trypsin inhibitor (Sigma) added to 100 μg/ml to reduce cell layer-associated proteolytic activity capable of cleaving procollagen telopeptides (Lee et al., 1990; Bateman et al., 1987). Radiolabeled collagen forms were isolated from the culture medium of the same number of cells, prepared for SDS-PAGE, separated with or without reduction on 5% SDS-polyacrylamide gels and analyzed by autofluorography as described (Lee et al., 1990).

Immunofluorescent staining of collagen

Mouse embryo fibroblasts were grown on sterile coverslips in the presence of ascorbic acid (50 mg/ml), fixed with acetone at –20°C for 20 minutes and labeled with a 1:100 dilution of a primary antibody specific for the α1(I) collagen carboxyl-telopeptide retained on mature collagen (Bernstein et al., 1996), as described (Dzamba et al., 1993). The secondary antibody was rhodamine-conjugated donkey anti-rabbit at a dilution of 1:100.

Skeletal preparation

Skeletal preparations of embryos were performed as described (Hogan et al., 1994).

RESULTS

Homozygous *Bmp1* mutant embryos exhibit a persistent herniation of the gut

As shown in Fig. 1, the mouse *Bmp1* gene was disrupted by replacing DNA sequences, including an exon(s) encoding amino acids 150-249 and that part of an upstream exon corresponding to amino acids 111-149, with a neomycin resistance cassette. This deletion was designed to include the Zn^{2+}-binding active site of the astacin-like protease domain that is common to all splice variants of BMP-1protein (Dumermuth et al., 1991; Bode et al., 1992; Takahara et al., 1994). Screening of a cDNA library generated from homozygous *Bmp1^{tm1}* mouse embryo fibroblasts (Takahara et al., 1996) yielded several cDNAs potentially encoding a truncated BMP1 protein

Table 1. Genotype and phenotype of embryos obtained by mating *Bmp1^{tm1}* heterozygotes

	Genotype		
Age	+/+	+/–	–/–
Postnatal:			
2 days	6	25	1*
Percent of total	19%	78%	3%
3 weeks	45	88	0
Percent of total	34%	66%	0%
Prenatal:			
16.5 d.p.c.	5	15	6†
Percent of total	19%	58%	23%
Herniated gut	0	0	4
17.5 d.p.c.	21	52	30†‡
Percent of total	20%	50%	30%
Herniated gut	0	0	25

The phenotype of progeny resulting from intercrosses between heterozygotes on a (129 × Black Swiss) background was scored at the indicated ages.

*This pup showed abdominal bleeding.

†One of the –/– embryos showed severe growth retardation without herniation of the gut.

‡2 of these were dead and degenerating. Gut herniation could not be determined.

with an in frame deletion of amino acids 96-249. This confirms that the protease domain of BMP1 was deleted in the mutant allele. It also suggests that, by comparison with the organization of the human *Bmp1* gene (Takahara et al., 1995), the deletion was extended to include amino acids 96-249 through splicing out from the mutant transcript of the remainder of the partially deleted exon. It should be noted that a *tolloid* mutation producing an in-frame deletion in the protease domain generates inactive and non-interacting TLD protein (Finelli et al., 1994).

Three ES cell lines with a correctly targeted *Bmp1*^{tm1} allele gave rise to founder mouse lines; the phenotypes of two, D12E and D6D, are reported here and were identical. Heterozygotes were normal, suggesting that any mutant protein made from the targeted gene was not antimorphic and did not interfere with normal development. However, following heterozygous matings, only one homozygous mutant pup was recovered a few days after birth, and this was dying with abdominal hemorrhage (Table 1). When embryos were examined at 16.5 and 17.5 days p.c. their size and external features, including skin, appeared grossly normal. However, 24% of them had a herniated gut (Fig. 2B) and all of these were genotyped as homozygous mutant (Table 1). Although it has not been examined directly, we assume that pups born with this defect die and are cannibalized shortly after birth. A small number of homozygous mutants have been found dead or severely growth retarded before 17.5 days p.c (Table 1) but the phenotype of these embryos or their precise time of death has not been examined. Homozygous mutant embryos examined at 9.5 days p.c. showed no obvious morphological defects.

Homozygous mutant embryos lack the membrane normally present around the physiological hernia of the gut

From about 11.0 days p.c., loops of the midgut normally herniate through the ventral body wall, returning to the peritoneal cavity by about 16 days p.c. followed by closing of the umbilical ring around the umbilical vessels (Kaufman, 1992). The herniated gut is normally surrounded by a thin, bilayered membrane consisting of ectodermal and mesodermal cells separated by extracellular matrix material. This membrane is continuous with both the ventral body wall and the amnion (Figs 2D, 3, 7). The mesodermal layer gives rise to the connective tissue and extracellular material known collectively as Wharton's jelly around the umbilical vessels. Careful dissection of 11.0, 13.5 and 17.5 days p.c. embryos showed that, in homozygous mutants, the amnion is intact but the part of the membrane normally covering the herniated gut is absent (Fig. 2A,D). To eliminate the possibility that this defect was due to damage during dissection, embryos were fixed and serially sectioned without disturbing the placenta, visceral yolk sac and amnion. As shown in Fig. 2D,E both the amnion and Wharton's

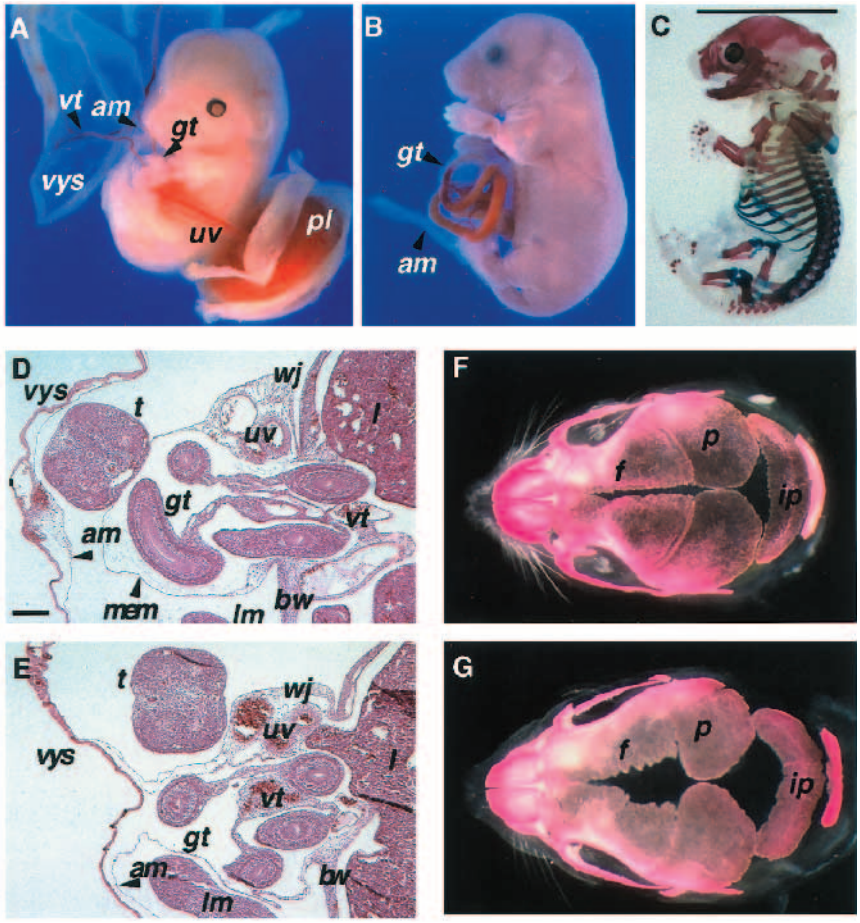

Fig. 2. Phenotype of BMP-1-deficient embryos. (A) Homozygous mutant at 13.5 days p.c. The visceral yolk sac (vys) and placenta (pl) were separated to show the integrity of the vitelline (vt) and umbilical (uv) blood vessels. The embryo is surrounded by the amnion (am), but no membrane covers the herniated gut (gt). (B) Homozygous mutant at 17.5 days p.c. showing the persistent gut herniation. Most of the amnion has been removed during dissection. (C) Skeleton of a homozygous mutant at 17.5 days p.c. stained with alizarin red (bone) and alcian blue (cartilage). No obvious abnormalities were seen in the axial or limb elements. The bar shows the limits used to measure skull length in Table 1b. (D) Section through the umbilical region of a 13.5 day p.c. wild-type embryo stained with haematoxylin and eosin. The herniated gut (gt) is surrounded by a membrane (mem) consisting of an ectodermal and mesodermal layer. The mesodermal layer is continuous with the somatopleural mesoderm of the ventral body wall (bw), and the connective tissue and Wharton's jelly (wj) around the umbilical vessels (uv). (E) Section through the umbilical region of a 13.5 day p.c. homozygous mutant. The membrane around the gut is absent. The mesodermal layer of the amnion (am) is continuous with the somatopleural mesoderm and Wharton's jelly. Three embryos, which were serially sectioned, showed this phenotype. (F) Dorsal membranous skull bones from a wild-type embryo at 17.5 days p.c. For clarity these were dissected from the rest of the skull. (G) Dorsal skull bones of a homozygous mutant at 17.5 days p.c. Note the smaller size of the frontal (f), parietal (p) and interparietal (ip) bones. The rest of the skull was normal. Bar corresponds to 250 μm in d and e. Abbreviations: l, liver; lm, hind limb; t, tail; vt, vitelline vessels in the midgut mesentery.

jelly are present in homozygous mutant embryos, but the fold of membrane tightly enclosing the gut is absent. No cellular remnants were seen, as might be expected if a membrane had been formed initially but was ruptured during development.

One possible explanation for the persistent herniation in *Bmp1^{tm1}* embryos is that the gut is malformed or longer than normal. However, no significant difference was seen in the length of the intestines dissected from +/+, +/– and –/– embryos at 17.5 days p.c. (Table 2) and no abnormalities were seen in gut morphology, apart from some necrosis in a few specimens. Another possibility is that the peritoneal cavity of homozygous mutants is smaller than normal, so that the gut cannot be enclosed. Although the cavity volume was not measured directly, no difference was observed in either the wet weight of +/+, +/– and –/– embryos at 17.5 days p.c., or their crown-to-rump length (Table 2). Moreover, no distortion was seen in the thoracic skeleton of homozygous mutant embryos (see Fig. 2C), that might compress the peritoneal cavity.

Bmp1 is expressed in the umbilical region of the midgestation mouse embryo

Bmp1 is expressed in the mouse embryo from around 6.5 days p.c. and transcripts can be detected throughout the paraxial and lateral mesoderm (Fukagawa et al., 1994). Since there was a defect in the umbilical region of homozygous mutant embryos, we asked whether the gene is, in fact, expressed in this region. Sections of embryos at 11.0 days p.c. were hybridized with antisense digoxigenin-labelled riboprobe. As shown in Fig. 3, *Bmp1* RNA is present in the splanchnopleure of the gut, the somatopleure of the ventral body wall and in the mesoderm (Wharton's jelly) around the umbilical vessels. The mesodermal cells are only loosely packed in these regions, so that the level of expression may be underestimated relative to other regions where the cells are more densely packed. High levels of *Bmp1* signal were also noted in the floorplate of the spinal cord and in the endocardial cushion of the heart (Fig. 3A, and data not shown).

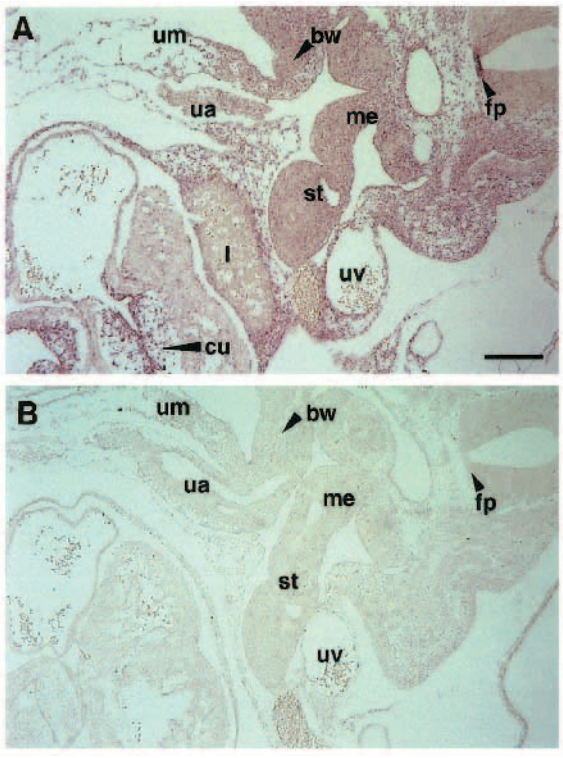

Fig. 3. *Bmp1* expression in a wild-type embryo. Sections through the trunk of an 11.0 days p.c. embryo were hybridized with digoxigenin-labeled riboprobe as described in Materials and Methods. (A) *Bmp-1* transcripts are present in many tissues. Note the intense signal in the floorplate (fp) and lower levels in the somatopleure mesoderm of both the ventral body wall (bw), the umbilical region (um) and the umbilical artery and vein (ua and uv). (B) A near section hybridized with a *Bmp1* sense strand riboprobe. Additional abbreviations: cu, endocardial cushion of the heart; l, liver; st, stomach; me, mesentry. Scale bar, 200 mm.

Homozgous *Bmp1^{tm1}* mutants have defects in skull development but not in dorsal-ventral patterning of the neural tube

Since BMP1 has procollagen C-proteinase activity (Kessler et al., 1996; Li et al., 1996), we examined homozygous mutants for skeletal defects. At 17.5 days p.c., homozygous *Bmp1^{tm1}* embryos had no obvious axial and limb skeleton abnormalities (Fig. 2C and data not shown). However, 25 of 28 embryos showed a reduction in the size of the frontal, parietal and interparietal membrane bones of the skull, although the overall antero-posterior length of the skull was not significantly different from that of heterozygous or wild-type embryos (Table 1; Fig. 2F,G).

High levels of *Bmp1* expression are also normally seen in the floorplate of the neural tube (Fukagawa et al., 1994; Sasaki and Hogan, 1994; Takahara et al., 1994) and Fig. 3A), suggesting a role in D-V patterning. However, no difference was seen in the spatial organization of the neural tube of 11.0 day p.c. homozygous mutant embryos, as judged by the expression patterns of *HNF3β*, *Isl1* and *Pax3*, which are markers for the floorplate, motor neurons and dorsal neural tube, respectively (Karlsson et al., 1990; Goulding et al., 1991; Thor et al., 1991; Sasaki and Hogan, 1994) (data not shown).

Abnormal collagen processing and assembly in homozygous *Bmp1^{tm1}* mutants

Although no gross defects were seen in the skeleton, skin and

Table 2. Dimensions of 17.5 days p.c. embryos

Dimensions	Genotype +/+	+/–	–/–	
weight (g)	0.87±0.16 (*n*=7)	0.97±0.15 (*n*=25)	0.94±0.10 (*n*=8)	*P*=0.199
crown-rump length (cm)	1.8±0.4 (*n*=6)	2.0±0.2 (*n*=28)	2.1±0.2 (*n*=12)	*P*=0.147
gut length* (cm)	4.5±0.9 (*n*=3)	4.8±0.8 (*n*=9)	4.9±1.3 (*n*=11)	*P*=0.762
skull length† (mm)	9.4±0.8 (*n*=8)	9.3±0.7 (*n*=25)	9.0±1.0 (*n*=15)	*P*=0.742

Means and standard deviations are shown for each group of embryos. The Kruskal-Wallis Test was used to calculate significance of difference (*P* values) in the last column. There is no significant difference in any dimension between +/+, +/– and –/– embryos.

*The total length of the duodenum, jejunum and ileum was measured as the gut length.

†The bar shown in Fig. 2C denotes the skull length.

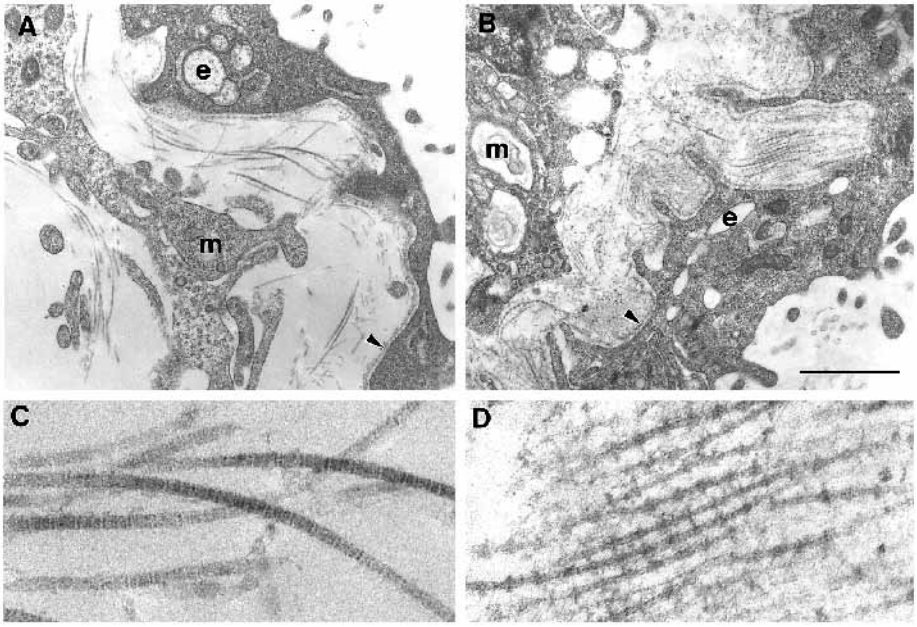

Fig. 4. Abnormal collagen fibrils in the amnion of homozygous mutants. Amnions from 13.5 day p.c. normal (A,C) and homozygous mutant (B,D) embryos were examined by transmission electron microscopy. In both cases, the epithelial ectodermal (e) cells are underlaid by a basal lamina (arrow) and separated from the mesodermal cells (m) by extracellular material. However, in the homozygous mutants, the collagen fibrils are thinner and have a 'barbed wire' appearance. The periodicity of the 'barbs' is approximately 65 nm, the same as the periodicity of normal collagen fibrils, suggesting that they represent attached C-terminal procollagen peptides extending from surface of the fibrils. The extracellular space in the mutant also contains large amounts of amorphous material, which may represent procollagen molecules not incorporated into fibrils. Scale bar, 1 μm in A,C and 0.2 μm in C,D.

connective tissue of homozygous mutants, we examined collagen processing and assembly in detail, by transmission electron microscopy of the 13.5 days p.c. amnion and ventral body wall, and by metabolic labelling and immunofluorescence of embryonic fibroblasts in culture.

As shown in Fig. 4, at the electron microscope level there are clear differences in the collagen fibrils deposited in the amnion of wild-type and homozygous mutant embryos. In both cases, the fibrils have a periodicity of approximately 65 nm, but the fibrils of the mutant are thinner than those of the wild type and have a 'barbed wire' appearance. In addition, they are surrounded by large amounts of amorphous material. The basal lamina underlying the ectodermal layer of the mutant amnion appears normal (arrow in Fig. 4A,B).

Mouse embryo fibroblasts were grown on coverslips and then stained with antibody specific for the carboxyl-telopeptide of α1(I) collagen. Wild-type fibroblasts showed an extensive network of clearly defined type I collagen fibrils (Fig. 5A,B). By contrast, the signal from the homozygous mutant cells was lower, more diffuse, and less organized into clearly defined fibrils than in the wild-type cells (Fig. 5C,D).

Finally, wild-type, heterozygous and homozygous mutant mouse embryo fibroblasts in culture were labelled for 20 hours with [^{3}H]proline and collagen forms secreted into the medium analyzed by SDS-PAGE under reducing and non-reducing conditions. As shown in Fig. 6A,B, cells of all genotypes processed collagen to mature α1(I) and α2(I) chains. However, there were relatively fewer mature α chains in the medium of homozygous mutant cells, compared with wild-type and heterozygous cultures. By contrast, in homozygous mutant cultures there were more unprocessed collagen forms that migrated near the top of the gel under non-reducing conditions (Fig. 6B, compare the amounts of radioactive material in this position in the three lanes). This behaviour under non-reducing conditions is characteristic of unprocessed procollagen and partially processed

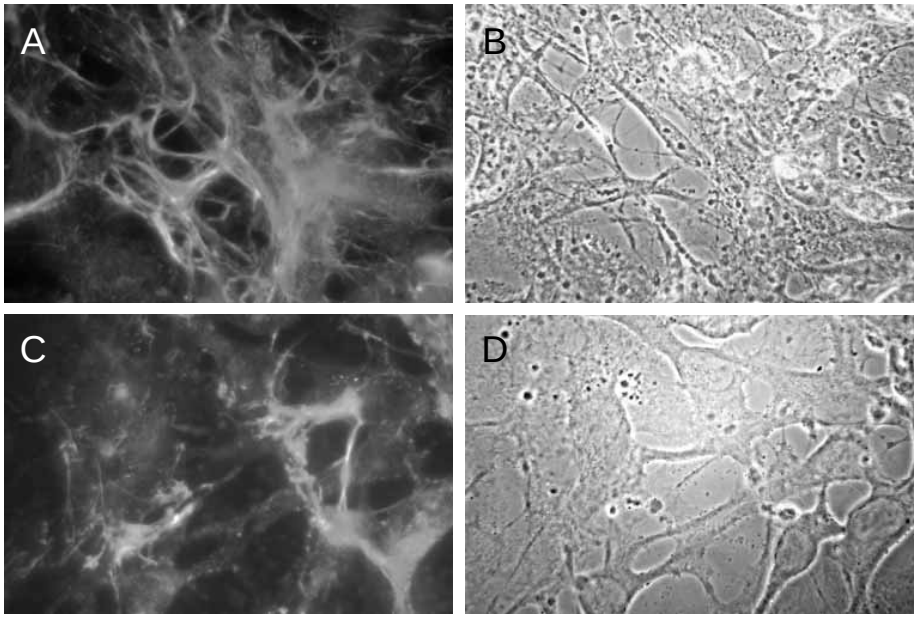

Fig. 5. Immunofluorescent staining of the collagen matrix assembled by mutant and wild-type fibroblasts. Mouse embyo fibroblasts were grown on coverslips as described and stained with antibody specific for the α1(I) collagen carboxyl-telopeptide. Samples were viewed by immunofluorescence (A,C) or phase-contrast (B,D) microscopy. Wild-type cells show an extensive, well-defined matrix of collagen fibrils while homozygous mutant cells assemble less matrix and the collagen fibrils are less well defined.

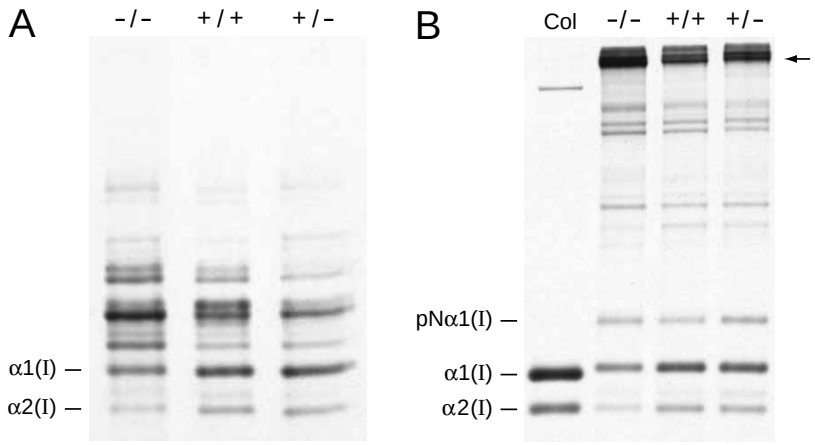

Fig. 6. Processing of procollagen in cultured mouse embryo fibroblasts. Mouse embryo fibroblasts from homozygous (–/–) and heterozygous (+/–) mutant and wild-type (+/+) embryos were grown to confluency and labeled for 20 hours with [^{3}H]proline. Collagen forms were analyzed by SDS-PAGE with (A) or without (B) reduction. Pepsin-digested human type I collagen was used as a size marker in the lane marked 'Col' in B. In cultures of all three genotypes, processing of collagen to mature α1(I) and α2(I) chains occurs. However, in the homozygous mutants, these chains were less abundant. Procollagens and collagen processing intermediates that retain the C-propeptide, form intermolecular disulfide bonds and therefore migrate near the top of the gel in unreduced conditions (B), while mature collagen chains and intermediate such as pNα1(I), which retain the N-propeptide but not the C-propeptide, do not.
Comparison of A and B shows that homozygous mutant fibroblasts generate more procollagen and incompletely processed forms retaining the C-propeptides that migrate near the top of the gel under nonreducing conditions than do heterozygous and wild-type cultures.

forms that retain the C-terminal propeptide and can form intermolecular disulfide bonds.

DISCUSSION

We have shown here that the mouse *Bmp1* gene is required for normal embryonic development. This was achieved by generating embryos homozygous for a mutation that causes an in-frame deletion of the protease domain, confirmed by sequencing cDNAs from homozygous mutant fibroblasts. The fact that heterozygous animals are normal and that a deletion within the protease domain of *Drosophila* TLD produces an inactive and non-interacting protein, make it very likely that the *Bmp1^{tm1}* mutation is a null. The majority of *Bmp1^{tm1}* homozygous mutants have persistent herniation of the gut combined with failure of ventral body wall closure in the umbilical region and do not survive beyond birth. In addition, homozygous mutants have defects in skull bone development. The etiology and significance of these abnormalities are discussed below.

Homozygous *Bmp1^{tm1}* mutants have defects in collagen processing but these are compatible with skeletogenesis

Given that BMP1 has been shown to have procollagen C-proteinase activity (Kessler et al., 1996; Li et al., 1996) and that procollagens I, II and III were thought to require processing of C-propeptides in order to assemble into fibrils (Prockop and Hulmes, 1994), it was somewhat unexpected that homozygous *Bmp1^{tm1}* embryos show no obvious abnormalities in the axial and appendicular skeleton (Fig. 2C). However, the frontal, parietal and interparietal bones of the skull are smaller than normal when examined at 17.5 days p.c. (Fig. 2F,G), suggesting that membrane bone formation is delayed. It is also possible that subtle differences in the endochondral bones of homozygous mutants might be observed at the ultrastructural level, but this was not explored. As it is, comparison at the electron microscope level of the collagen fibrils in the amnion of homozygous mutant and wild-type embryos does reveal interesting defects (Fig. 4). The homozygous mutant fibrils (probably of type I collagen) have the same 65 nm periodicity as normal fibrils, but are thinner and have a 'barbed wire' appearance. This is compatible with some procollagen molecules with attached C-terminal propeptide extensions being incorporated into the fibrils (David Birk, Tufts University, personal communication), but with lower efficiency than fully processed collagen. The presence of amorphous extracellular matrix material around the collagen fibrils in vivo suggests that at least some of the unprocessed collagen accumulates and is not immediately degraded. Immunofluorescence staining of the extracellular matrix laid down by homozygous mutant embryonic fibroblasts also shows fewer, less well organized collagen fibrils than normal (Fig. 5). Finally, metabolic labelling studies on the biosynthesis and processing of collagen by these cells demonstrates that a substantial amount of fully processed type I collagen is produced in the absence of BMP1, but that more unprocessed or partially processed collagen is present (Fig. 6). One explanation for these findings with cultured cells, and for the relatively minor defects in connective tissue, cartilage and bone in the homozygous mutant embryos, is that other proteins are compensating for the absence of BMP1, both in vivo and in vitro. The most likely candidate is the product of the mammalian *Tolloid-like (Tll)* gene recently described by Takahara et al. (1996). Although it is not yet known whether TLL protein has procollagen C-proteinase activity, the gene is expressed in many of the same embryonic tissues as *Bmp1*, including the developing skeletal system. In the long term, the idea that *Tll* is compensating for the absence of *Bmp1*, at least in some tissues and for some activities, can be tested by studying the phenotype of embryos homozygous for null mutations in both genes.

Homozygous *Bmp1^{tm1}* embryos are defective in ventral body wall/amnion development

The most striking defect in homozygous mutant embryos is the persistent herniation of the gut (Fig. 2B). Very little is known about the normal mechanisms by which the gut is returned to the peritoneal cavity by 16.0 days p.c., allowing the ventral body wall to close around the umbilical vessels. However, it is likely that an important role is played by the so-called 'wall of the physiological umbilical hernia' (Kaufman, 1992), a thin, bilayered membrane continuous with the amnion and body

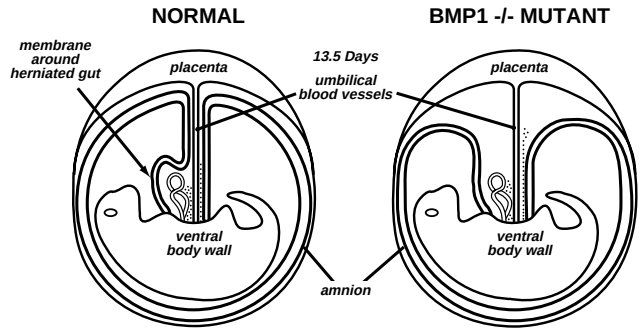

Fig. 7. Schematic representation of the development of the ventral body wall and amnion of wild-type and mutant embryos. (Left) The midgestation mouse embryo is normally surrounded by the amnion, which consists of an ectodermal and mesodermal layer and is continuous with the ventral body wall. A fold of the amnion tightly encloses the herniated gut and mesodermal cells give rise to Wharton's jelly (dots) around the umbilical blood vessels. In homozygous *Bmp-1^{tm1}* embryos, a defect in the adhesiveness of the amnion results in a failure to form a fold or membrane around the herniated gut.

wall which surrounds the loops of the gut and holds them in a compact configuration until they are internalized (see Fig. 7 for schematic diagram). In the homozygous *Bmp1^{tm1}* mutants, the amnion is present but there is no fold covering the loops of the gut and, as a result, they lie free in the extraembryonic coelomic cavity. The most likely explanation for this phenotype is that the defects in the collagen fibrils in the amnion interfere with the ability of the mesodermal cells in the region of the umbilicus to form a cohesive extracellular matrix able to withstand the pressure generated by the expansion of the herniated gut. However, we cannot rule out the possibility that proliferation and/or survival of cells in the amnion of homozygous mutants is reduced compared with wild type so the membrane cannot grow fast enough to completely surround the herniating gut. In addition, the mesodermal cells of the ventral body wall/amnion may have altered cell-cell or cell-matrix adhesion, so that they do not attach to the umbilical vessels efficiently. Further experiments will be required to distinguish between these possibilities.

It should be noted here that the phenotype of *Bmp-1^{tm1}* mice resembles that of human neonates with gastroschisis, a condition in which the gut is herniated adjacent to the umbilicus without a surrounding membrane. While gastroschisis is rare, a genetic component has been reported for some cases (Salinas et al., 1979).

Have the functions of *Bmp1* and *tolloid* in embryonic development been conserved during evolution?

As outlined in the Introduction, *Bmp1* is a member of the same conserved gene family as *Drosophila tld* and *trl-1/tok.* Genetic analysis suggests that TOLLOID functions by enhancing the activity of DPP, possibly by proteolytically cleaving a protein that associates with DPP, forming an inactive complex (Ferguson and Anderson, 1992; Childs and O'Connor, 1994; Finelli et al., 1994). Biochemical analysis, on the other hand, has shown that mammalian and chick *Bmp1* encodes a procollagen C-proteinase (Kessler et al 1996; Li et al., 1996), a function supported by our analysis of the homozygous null mutant embryos and cells reported here. How may these rather disparate biological activities be reconciled with the fact that the *Bmp1/tolloid* gene family appears to be ancient (members are present in hydra and sea urchin) and conserved during evolution. Two possibilities can be considered. First, family members may fall into two subclasses. Some members, such as mammalian *Bmp1* and *Tll*, *Xenopus Bmp1* and sea urchin *Bmp1* may be involved in the processing of components of the extracellular matrix such as fibrillar collagen and laminin 5. By contrast, other members, such as sea urchin *BP10* and *SpAN*, and *Drosophila tld* and *tlr-1/tok*, may be involved in the cleavage of extracellular proteins that specifically bind and sequester growth factors of the TGFβ/BMP superfamily and are therefore involved in embryonic patterning, rather than matrix assembly. This idea has been proposed previously (Hwang et al., 1994) and has some evidence in its support. For example, unlike *Bmp1*, which is widely expressed, su *BP10* and *SpAN* and *Drosophila tld*, have restricted temporal and spatial patterns of expression, and are transcribed only in the dorsal region of the early embryo; in the case of *tld*, the expression pattern is very similar to that of *dpp*. This hypothesis would predict that genes encoding products closely related related to su BP10 and SpAN may be present and awaiting discovery in mammals, and may be involved in modulating the activity of TGFβ/BMP signalling molecules.

An alternative (or additional) possibility is that mammalian, Xenopus and sea urchin BMP1/TLL proteins have a wider range of substrates than the structural extracellular matrix molecules so far identified, and do indeed act on TGFβ/BMP-associated proteins in addition to collagen and laminin 5. As a corollary to this, these putative growth-factor-associated proteins may have domain structures that include motifs present in extracellular matrix proteins. In support of this hypothesis, it should be noted that the proteins encoded by *Drosophila shortened gastrulation (sog)* and vertebrate *chordin*, which are thought to interact with and inactivate DPP and BMP4, respectively, are large, complex proteins with motifs related the N-terminal peptide of procollagen (Francois and Bier, 1995; Holley et al., 1995; De Robertis and Sasai, 1995). Further analysis, in a range of different organisms, should help to distinguish between these alternatives.

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