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Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development

Hongbing Zhang[1,*] and Allan Bradley[1,2]

[1]Department of Molecular and Human Genetics, and [2]Howard Hughes Medical Institute, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA

*Current address: Carciovascular Research Institute, University of California, San Francisco, Parnassus Avenue, San Francisco, CA 94143, USA

SUMMARY

To address the function of bone morphogenetic protein-2 (BMP2) in mammalian development, mice with a targeted deletion of the *Bmp2* mature region were generated using embryonic stem cell technology. This mutation caused embryonic lethality when homozygous. Mutant embryos failed to close the proamniotic canal, which caused the malformation of the amnion/chorion. BMP2-deficient embryos also exhibited a defect in cardiac development, manifested by the abnormal development of the heart in the exocoelomic cavity. These defects are consistent with the expression of *Bmp2* in the extraembryonic mesoderm cells and promyocardium. Thus BMP2 is a critical factor for both extraembryonic and embryonic development.

Key words: BMP2, Gene targeting, ES cell, Cardiac development

INTRODUCTION

The bone morphogenetic proteins (BMPs) are a group of secreted signaling proteins that were originally identified by their ability to induce ectopic bone formation when implanted into rodents (Urist, 1965). Molecular cloning of the BMPs revealed that they belong to the transforming growth factor-β (TGF-β) superfamily (Wozney et al., 1988). The TGF-β family includes, but is not limited to, various TGF-βs, bone morphogenetic proteins (BMPs), mammalian inhibin and activins, Mullerian inhibiting substance (MIS), growth and differentiation factors (GDFs), the *Drosophila decapentaplegic* (*dpp*) and *60A* gene products, and the *Xenopus Vg1* gene product. At present, the known group of BMPs consist of at least seven members, BMP2 through BMP8 (reviewed by Kingsley, 1994).

BMPs are synthesized as large prepro protein precursors, which are cleaved to release the dimeric C-terminal mature regions. The mature region, which defines assignment as a TGF-β family member, demonstrates the bone morphogenetic activity (reviewed by Hogan et al., 1994). BMP2 has gained attention because it is the predominant form in the natural bone morphogenetic protein extract (Wozney et al., 1988; Sampath et al., 1990) and it is widely expressed during mouse development (see below). The sequence of bone induction events initiated by BMP2 closely resembles endochondral bone formation during embryogenesis, which strongly suggests that BMP2 is a morphogenetic factor controlling osteogenesis (Rosen and Thies, 1992; Wang et al., 1990; Reddi, 1981). This view is supported by the localization of *Bmp2* transcripts in the condensing precartilagenous mesenchyme cells of the 12.5 day mouse embryo and in the hypertrophic cartilage of the long bones and digits of the 15.5 day mouse embryo, the sites involved in bone formation (Lyons et al., 1989, 1990).

Accumulating evidence suggests that the *Bmp2* gene product is involved in morphogenetic processes other than the developing skeletal system. *Bmp2* and *Bmp4* are the mammalian homologues of a *Drosophila* gene, *dpp*, with BMP2 showing about 75% amino acid identity to DPP protein. BMP2, BMP4 and DPP protein seem to be functionally interchangeable in certain experimental systems (Padgett et al., 1993; Sampath et al., 1993; Vainio et al., 1993). Powerful genetic analysis has shown that DPP is responsible for body pattern formation at several distinct stages of *Drosophila* development (Ferguson and Anderson, 1992; Capovilla et al., 1994). This strongly indicates that BMP2 might be involved in early mammalian development. Other evidence in support of this hypothesis is derived from expression studies. Murine *Bmp2* cDNA was cloned from an E8.5 mouse embryonic cDNA library (Dickinson et al., 1990) while chondrification and ossification centers appear later than day 11 of gestation (Rugh, 1991). Furthermore, *Bmp2* is highly expressed in the myocardial layer of the mouse heart at E9.5 which suggests that it might have a role in cardiac development (Lyons et al., 1989, 1990). *Bmp-2* is also expressed at a variety of other embryonic stages (Lyons et al., 1995).

However, the role of BMP2 in murine embryonic development cannot be determined solely from its expression pattern. To study the function of BMP2 in vivo, embryonic stem cell (ES) technology (Bradley, 1991) has been utilized to generate a deletion of the *Bmp2* mature region in the mouse genome, and the expression pattern of *Bmp2* between day 7.5 and day 9.5 of gestation has been defined. Here, we show that BMP2 is essential for early embryonic development. A homozygous null mutation of *Bmp2* causes the failure of amnion/chorion formation and abnormal cardiac development. The expression of *Bmp2* mRNA in both the extraembryonic mesoderm and

embryonic mesoderm where development of amnion/chorion and heart initiates, respectively, is consistent with the phenotype of the mutants. These data support the role of BMP2 in both extraembryonic and embryonic development.

MATERIALS AND METHODS

Targeting vector BMP2-14

To generate the targeting vector for positive-negative selection, a 129SvEv genomic library (Matzuk et al., 1992) was screened, using a 1.124 kb *Bmp2* cDNA probe (Lyons et al., 1989). Overlapping phages covering the entire *Bmp2* coding region were isolated and mapped. The map was consistent with a previous publication (Feng et al., 1994). The 5′ homologous arm of the targeting vector was a 5.0 kb *Sal*I-*Xho*I fragment which contains part of the BMP2 propeptide. The 3′ homologous arm was a 2.0 kb *Bgl*II-*Not*I (*Not*I is from the polylinker in the phage) fragment which is downstream of the BMP-2 mature region. The PGK-*hprt* expression cassette was cloned between the two homologous arms and a MC1-*tk* (thymidine kinase) expression cassette was cloned into the polylinker site of the plasmid backbone. The vector was designed such that the entire mature region and about two-thirds of the *Bmp2* propeptide coding region would be deleted by correct targeting. The 5′ flanking probe was an 800 bp *Spe*I-*Sal*I fragment upstream of the 5′ homologous arm and the 3′ flanking probe was a 1 kb *Xba*I-*Bgl*II fragment downstream of the 3′ homologous region. The targeting experiment yielded 7 targeted clones from 248 $HAT^r/FIAU^r$ clones screened. The enrichment (the ratio of HAT^r clones to $HAT^r/FIAU^r$ clones) was about 15-fold.

Transfection of ES cells and generation of germline chimeras

The transfection and culture of ES cells was performed as described previously (Ramirez-Solis et al., 1993). Briefly, AB2.1 ES cells were transfected with 25 μg *Xho*I-linerized BMP2-14 per 10^7 cells using a Bio-Rad gene pulser and grown under double selection as described. The targeted ES cells were injected into C57BL/6 blastocysts (McMahon and Bradley, 1990), which were then transferred into the uteri of pseudopregnant females. Resulting male chimeras were mated to C57BL/6 females, and heterozygous offspring were then interbred.

Southern blot analysis

DNA isolation and Southern blot analysis were performed as described previously (Matzuk et al., 1992). Genomic DNA was digested with either *Spe*I or *Xho*I and electrophoresed in a 0.7% agarose gel. The screening strategy for targeted clones is illustrated in Fig. 1A. The high stringency wash using the 3′ flanking probe was at 75°C (1× SSC, 0.1% SDS).

Histological analysis of embryos

Embryos between day 7.0 and day 8.0 of gestation were processed without dissecting away the maternal decidua. Embryos after day 8.0 were dissected free of the uterine muscle and decidua, and the yolk sacs were saved for DNA analysis. Histological procedures were performed as described by Kaufman (1992b). The embryos were fixed overnight in Bouin's solution and transferred to 70% ethanol. The fixed embryos were dehydrated through increasing concentrations of ethanol, cleared in xylene and embedded in paraplast. Embryos were sectioned at 6-9 μm using a Reichert-Jung microtome and stained with hematoxylin and eosin.

In situ hybridization

The probe used to survey the expression of *Bmp2* was a 400 bp *Eco*RI-*Hin*dIII fragment which contains the *Bmp2* propeptide. The probe fragment was excised from the 1.124 kb *Bmp2* cDNA fragment (Lyons et al., 1989) and subcloned into the *Eco*RI-*Hin*dIII sites of pSK^-. Antisense riboprobe was transcribed from the T7 promoter and sense control riboprobe was transcribed from the T3 promoter.

The probe used for the molecular marker of the cardiac lineage was a 400 bp fragment from *Csx* (cardiac-specific homeobox gene) cDNA.

Section in situ hybridization procedures were performed as described (Sundin et al., 1990) with the following modification. Sections of 5-9 μm were mounted onto poly-Lysine-treated slides. The hybridization and post hybridization washes were performed as described (Wilkinson, 1992). Briefly, the sections were hybridized overnight at 50°C. The stringency washes were at 64°C. Exposure times were 10 to 16 days. Autoradiography, Hoechst 33258 staining and photography were performed as described by Sundin et al. (1990).

Whole mount in situ hybridization procedures were as described (Takada et al., 1994) with the following modification. The hybridization and high stringency washes were performed at 70°C. The incubation time required to see the *Csx* signal was 1-2 hours.

RESULTS

Deletion of the *Bmp2* and generation of BMP2-deficient mice

To disrupt the *Bmp2* gene in mice, a replacement targeting vector, BMP2-14, was generated which contained a total of 7.2 kb of homology with the *Bmp2* gene. A 4.0 kb region of the *Bmp2* gene was replaced with a PGK-*hprt* mini-gene cassette (Fig. 1A). The entire *Bmp2* mature region, and about two-thirds of the propeptide should be deleted by the predicted homologous recombination event (Fig. 1A). The targeting

Table 1. Analysis of embryos and adult mice from heterozygotes matings

	Genotype of normal embryos and neonates			Genotype of abnormal embryos			Fraction of abnormal embryos	
Day	+/+	+/–	–/–	+/+	+/–	–/–	Genotyped	Untyped
E7.25-7.75	7*	16*	9*	–	–	–	0/32 (0%)*	13/48 (27%)†
E8.5	12	21	0	0	0	12	12/45 (27%)	36/128 (28.1%)
E9.5	21	33	0	0	0	11	11/65 (17%)	29/134 (21.6%)
E10.5	14	23	0	0	0	9	9/46 (20%)	14/74 (18.9%)
E11.5	1	2	0	0	0	2	9/46 (20%)	
Postnatal	38 (35%)	70 (65%)	0					

Many resorbed embryos were found at E10.5 and later, only some of these could be genotyped. All of the embryos judged to be abnormal proved to be homozygous for the mutant allele.

*Embryos at E7.25. At this stage homozygous mutant embryos could not be distinguished from their littermates.

†Embryos at E7.5. About one quarter of these embryos could be distinguished by morphology from their littermates, these embryos were processed for histology and were not genotyped.

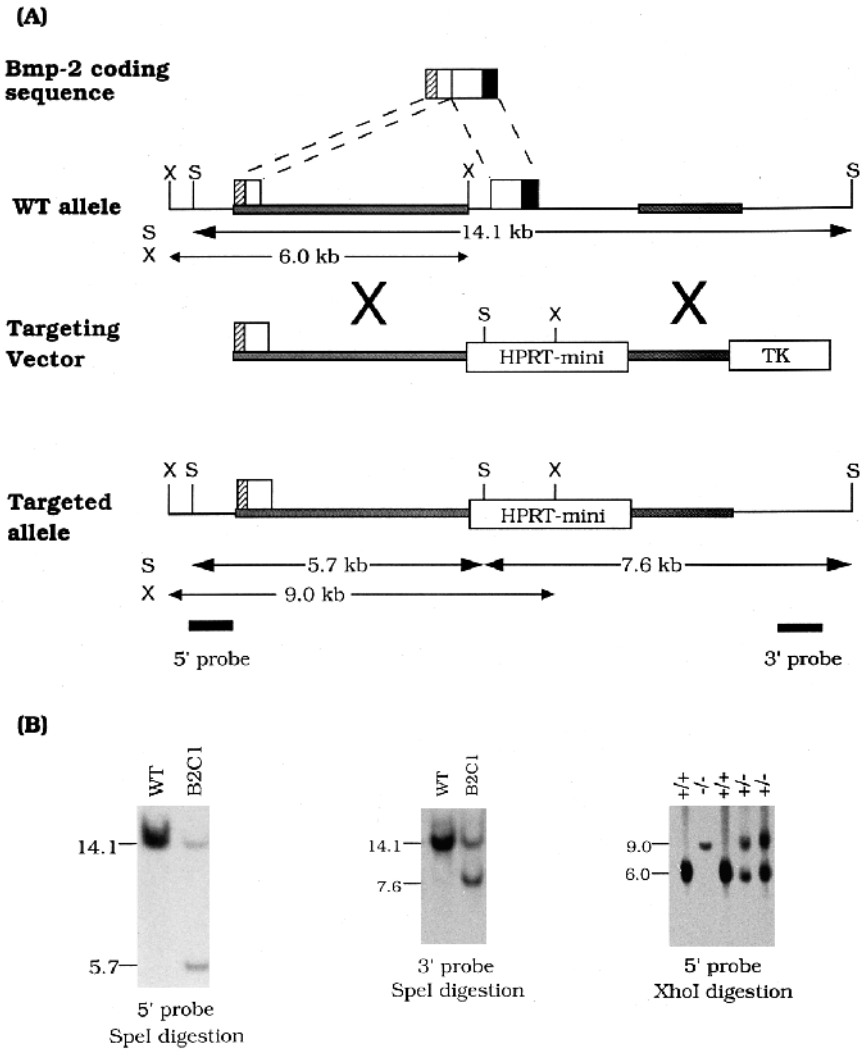

Fig. 1. Targeted deletion of the *Bmp2* mature region in ES cells and in mice. (A) Strategy for targeted mutation of the *Bmp2* gene. The mouse *Bmp2* coding sequence is distributed into two exons. The second exon encodes the C-terminal mature region (black box) that has the bone morphogenetic activity. Homologous recombination (thick crossover) between the targeting vector and the endogenous *Bmp2* gene in the mouse ES cell results in the deletion of the whole *Bmp2* mature region (black box) and about two-thirds of the propeptide (blank box). The striped box represents the location of the signal peptide. The targeting vector contains 5.2 kb of isogenic DNA homologous to the 5′ sequence of the mouse *Bmp2* gene (including the exon which has the start codon), 2.0 kb of sequence homologous to the 3′ end of the mouse *Bmp2* gene, a PGK-*hprt* expression cassette, and a MC1-*tk* (thymidine kinase) expression cassette. The recombinant allele can be distinguished from the wild type by restriction of genomic DNA with *Spe*I or *Xho*I and Southern blot analysis using 5′ and 3′ external probes, as indicated. Restriction sites: S, *Spe*I; X, *Xho*I. (B) Southern blot analysis of DNA isolated from either targeted ES clones or embryos from heterozygous matings. Symbols: +/+, wild type; +/–, heterozygous; –/–, mutant. (Left) DNA from targeted ES clones digested with *Spe*I and hybridized with the 5′ flanking probe. Shown are 14.1 kb wild-type and 5.7 kb mutant fragments. (Center) The same filter as left striped and rehybridized with the 3′ flanking probe. Shown is the 7.6 kb mutant fragment. (Right) DNA isolated from embryos of heterozygotes matings digested with *Xho*I and hybridized with the 5′ flanking probe. Shown are 6.0 kb wild-type and 9.0 kb mutant fragments.

vector was electroporated into AB 2.1 ES cells and Southern blot analysis identified clones with the deleted *Bmp2* allele (Fig. 1B). This deletion allele (*bmp2*^{m1}) will ensure that no *Bmp2* mRNA is transcribed and therefore no BMP2 protein should be produced in the homozygous mutant animals. Targeted clones were micro-injected into C57BL/6 blastocysts and the resulting chimaeric mice were crossed with C57BL/6 mice. Chimeras generated from two independently derived targeted clones (B2C1 and B2C5) transmitted the mutation through the germline. The analysis of the BMP2 deficient embryos in this study are derived from mice generated from both targeted ES cell clones on the mixed (129SvEv × C57BL/6) genetic background.

Heterozygous mutant (*bmp2*^{m1}/+) mice were phenotypically normal, and these mice were intercrossed to determine whether homozygous mutant mice (*bmp2*^{m1}/*bmp2*^{m1}) were viable. Three-week-old offspring from this cross were genotyped and a 2:1:0 ratio of heterozygous to wild-type to homozygous mutant mice was observed (Table 1). To determine the reason for the lack of homozygous progeny, embryos from heterozygous intercross matings were analyzed at successive stages of development. Homozygous mutant embryos were observed but these died between day 7.0 and 10.5 of gestation (Table 1). The mutant phenotype was evident as early as E7.75 (Fig. 2 and Table 2). Southern blot (Fig. 1B) or PCR analysis (data not shown) confirmed that about one quarter of the embryos were homozygous for the mutant allele at E7.75 (Table 1). At E8.5, *bmp2*^{m1}/*bmp2*^{m1} embryos could be distinguished from their littermates because they either

Table 2. Number of homozygous mutant embryos with defects in amnion/chorion development and cardiac development

		Cardiac defects		Other defects	
Gestational age	Proamniotic canal open	Heart in exocoelomic cavity	No heart	Delayed allantois	Not turned
E7.75	3/4	N/A	N/A	N/A	N/A
E8.5	20/29	10/12	2/12	12/12	N/A
E9.5	10/14	9/12	2/12	9/9	9/9
E10.5	2/2				

Each entry shows the number of embryos with a particular defect out of the total number of mutant embryos that were analyzed for that defect. At E7.75, the abnormal embryos were scored as homozygous mutants based on their morphology. N/A, not applicable.

retained an open proamniotic canal or exhibited abnormal development of the heart (see later). At E9.5 homozygous mutant embryos could be identified because they had not finished turning (Table 2). All of the embryos which were scored as abnormal at 8.5 and 9.5 days were confirmed to be homozygous mutants when genotyped.

Amnion/chorion defects caused by an open proamniotic canal

In normal mouse embryos the proamniotic canal is a transient structure. Fig. 3A illustrates how this structure is formed during the development of a normal mouse embryo. The mesoderm starts to form at the most posterior end of the embryo at E6.75. Mesoderm cells (both the extraembryonic and embryonic components), proliferate and migrate towards the anterior part of the embryo. The amnion and the chorion, which are extraembryonic tissues of the embryo, start to form at the time of mesoderm migration and are well differentiated tissues at about E7.5. The proamniotic canal is transiently formed as the two amniotic folds expand and coalesce into a single cavity. Each amniotic fold consists of two types of cells, the ectoderm and the extraembryonic mesoderm. The ectodermal cells of the amniotic fold are of both embryonic and extraembryonic origins. The formation and separation of the amnion and chorion normally concludes with the fusion of the two amniotic folds which closes the proamniotic canal (Fig. 2A,C,F). The proximal part of the canal becomes part of the chorion and the distal part is included in the amnion (reviewed by Beddington, 1992; Kaufman, 1992a).

The earliest discernable defect in *bmp2*^{m1}/*bmp2*^{m1}

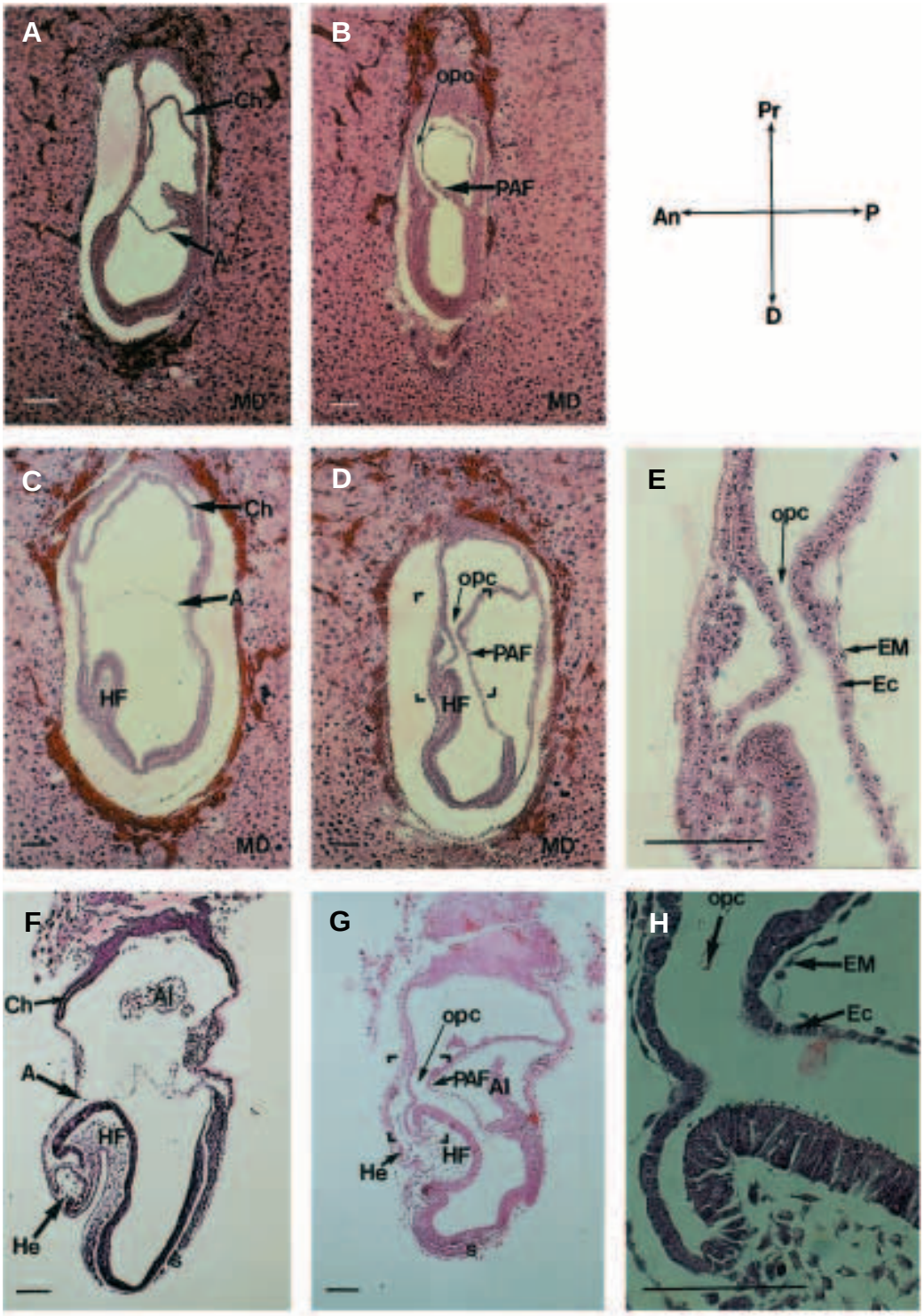

Fig. 2. Formation of amnion/chorion in homozygous mutant mice and normal mice at various gestational stages. (A,B) Sagittal sections of wild-type (A) and mutant (B) embryos at E7.5 + 2 hours. (C,D,E) Sagittal sections of wild-type (C) and mutant (D,E) embryos at E7.5+5 hours. The bracketed region in D is shown at higher magnification in E. (F,G,H) Sagittal sections of wild-type (F) and mutant (G,H) embryos at E8.0. The bracketed region in G is shown at higher magnification in H. (E,H) Higher magnification of the proamniotic canal region of the embryo bracketed in D and G. Notice the presence of two cell layers. The defect in the formation of amnion/chorion, the open proamniotic canal (OPC), is shown in B,D,E,G and H. The amnion (A), chorion (Ch), posterior amniotic fold (PAF), headfold (HF), maternal decidua (MD), heart (He), somite (S), allantois (Al), extraembryonic mesoderm (EM) and ectoderm (Ec) are labeled. The ectodermal cells of the proamniotic canal are of either embryonic or extraembryonic origin. The orientation of the embryos is indicated by anterior (An), posterior (P), proximal (Pr) and distal (D). Bars, 100 μm.

embryos was the persistence of the proamniotic canal (Fig. 2B,D,E). About 69% of the homozygous mutant embryos had this defect at E8.5 (Table 2), which became more obvious at later stages (Fig. 2G,H, 4C, and Table 2). At E9.5, the proamniotic canal became a tubular structure which linked the headfold to the placenta (Fig. 4C). The proamniotic canal in the E8.5 and E9.5 mutant embryos consisted of two cell layers (Fig. 2E), which resembled the ectodermal and extraembryonic mesodermal cells that form the proamniotic canal in wild-type embryos at E7.0 (Kaufman, 1992a).

The cardiac defect in BMP2-deficient mice

The amnion/chorion was formed appropriately in about 30% of the homozygous mutant embryos, yet none of these developed to term (Tables 1 and 2). This suggested that BMP2 may have another critical developmental function in addition to its involvement in closure of the proamniotic canal. The abundant expression of *Bmp2* in the myocardial layer of the heart suggested that BMP2 might have a role in heart morphogenesis (Lyons et al., 1990).

During normal mouse embryogenesis, the heart is the first organ to develop (for a detailed description see DeRuiter et al., 1992; Kaufman and Navaratnam, 1981). Presomite mouse embryos have both a left and a right mesodermal layer between the endoderm and ectoderm. The two layers of mesodermal cells migrate from the most posterior part of the embryo and meet at the anterior part of the embryo (Fig. 3A). At this stage coelomic cavities appear in the mesodermal layer, which initiates cardiac development. The intraembryonic part of the splanchnic mesoderm, which is situated ventral to the coelomic cavity, differentiates into promyocardium. This event occurs in the mesoderm between the most proximal part of the headfold and the origin of the amnion. At the 1-2 somite stage, the endocardial elements which are inside the myocardial layer aggregate to form a pair of blind-ended tubes separated in the ventral midline. At the 3-4 somite stage, the heart tubes become more prominent and after the 4 somite stage, the bilateral heart tubes fuse at the midline. Subsequently, extensive cardiac morphogenesis takes place, resulting in the formation of the septa and valves by localized cellular differentiation and migration.

In the normal embryo at E8.5 (about 7 somites), there is a relatively large heart tube (Fig. 4B) which consists of two cell layers, the myocardium and the endocardium (Fig. 4F). At the equivalent stage in homozygous mutant embryos, heart development was delayed and occurred at an abnormal site. About 75% of the homozygous mutant embryos had this defect (Table 2). As shown in Fig. 4A and E, the heart of the mutant embryo was disorganized and formed in the putative exocoelomic cavity, while in normal embryos the heart was formed in its proper place inside the amniotic cavity (Fig. 4F). The morphological changes of the heart in mutant embryos was more obvious at later stages. At E9.5, only a single heart tube was present (Fig. 4C and G) while in wild-type embryos at this stage there was a relatively more differentiated heart that consisted of a well-formed atrioventricular canal which separated the future atrium and ventricle (Fig.

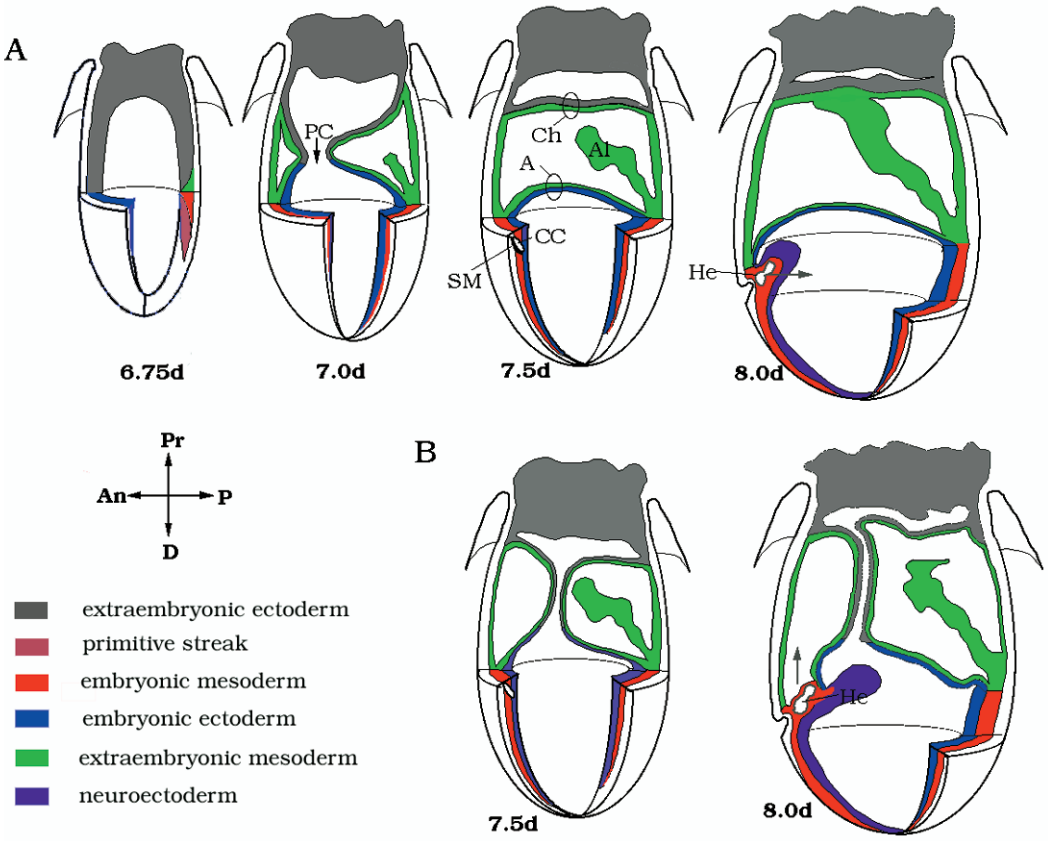

Fig. 3. A simplified diagram for the function of BMP-2 in mouse. (A) A diagram depicting the development of the amnion/chorion and the heart in a wild-type embryo between stages E6.75 and E8.5. (B) A diagram showing the defects in a homozygous mutant embryo. The proamniotic canal closes in normal embryos (A) which leads to the proper formation of the amnion/chorion but does not close in mutant embryos (B). Also, the interaction between the cardiac progenitor cells and the ectodermal cells or endodermal cells will determine whether the primitive heart will migrate dorsally, resulting in the formation of the heart in the amniotic cavity (A). In mutant embryos, these interactions have been lost, which leads to the formation of the heart in the exocoelomic cavity (B). The arrow shows the direction of the migration of the cardiac progenitor cells. Amnion (A), heart (He), allantois (Al), splanchnic mesoderm (SM), coelomic cavity (CC), chorion (Ch) and proamniotic canal (PC) are labeled. Different cell types (except the outside endodermal cells) are represented by various colors. The foregut invagination site is marked by an indentation.

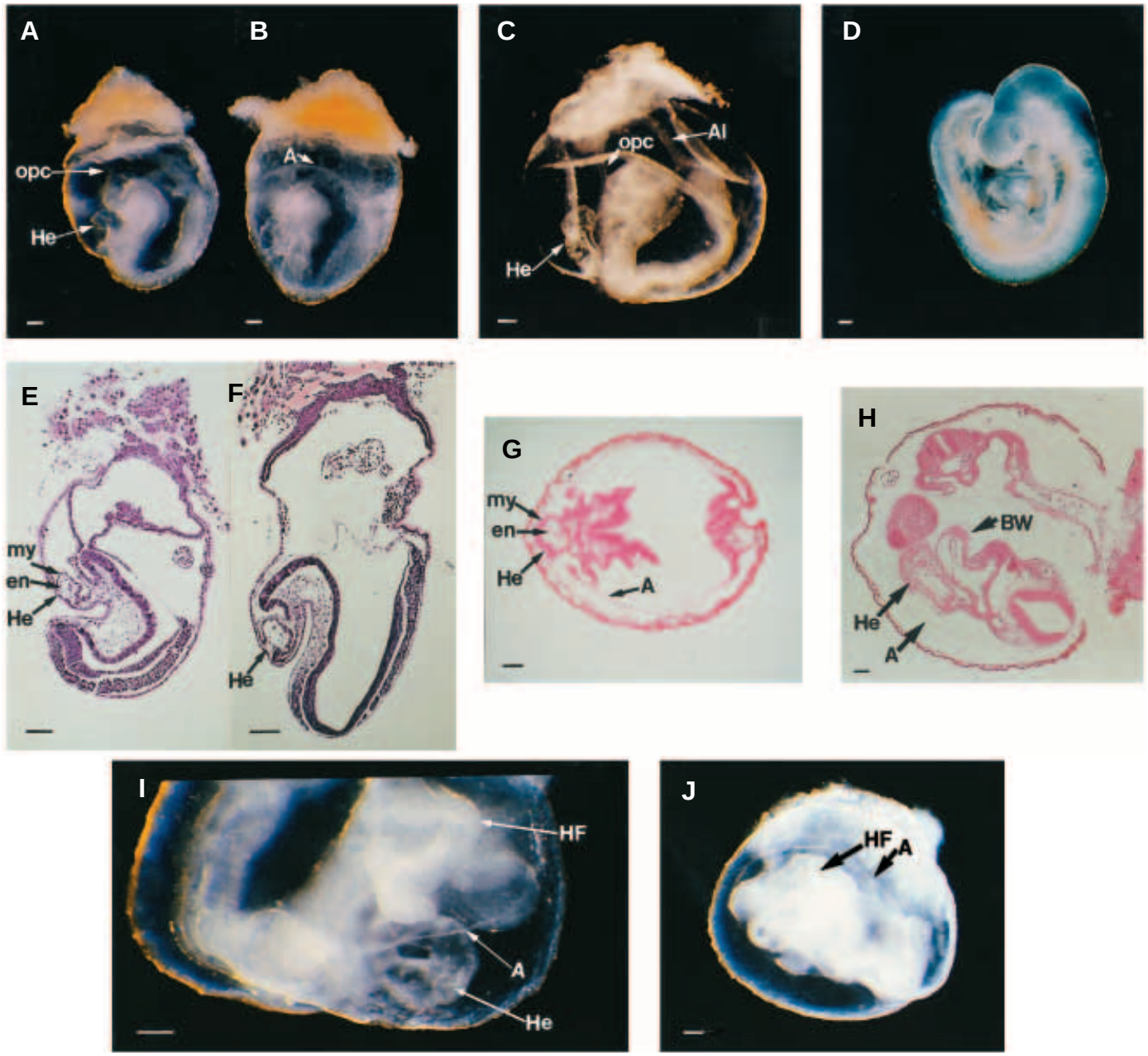

Fig. 4. Cardiac development in mutant and normal embryos. (A,B) Morphology of mutant (A) and wild-type (B) embryos at E8.5. (C,D) Morphology of mutant (C) and wild-type (D) embryos at E9.5. (E,F) Sagittal sections of mutant (E) and wild-type (F) embryos at E8.5. (G,H) Transverse sections of mutant (G) and wild-type (H) embryos at E9.5. (I,J) Morphology of a mutant embryo at E9.5 which exhibited the cardiac defect (I) but did not have the amnion/chorion defect (J). The orientation of the embryo was chosen such that the amnion and heart could be shown within the intact embryo. In normal embryos (B,F,H), the heart (He) is formed in the amniotic cavity (caudal to the origin of the amnion), while in homozygous mutant embryos (A,C,E,G,I), it is formed in the presumptive exocoelomic cavity. Amnion (A), heart (He), body wall of the heart (BW), allantois (Al), headfold (HF), myocardium (my) and endocardium (en) are labeled. Bars, 100 μm.

4H and D). The heart tube normally forms within the body-wall at a position which is caudal to the origin of the amnion and inside the amniotic cavity (Fig. 4F). However, in the homozygous mutant embryos, the heart tube was formed inside the exocoelomic cavity instead of the putative amniotic cavity (Fig. 4E). In embryos with the amnion/chorion defect, the amniotic cavity was still continuous with the ectoplacental cavity (Fig. 4C). In some homozygous mutant embryos, the heart was formed in the exocoelomic cavity even though the amnion of these embryos had developed appropriately (Fig. 4I and J).

To confirm that the structures which had developed in abnormal positions in the mutant embryos were heart tubes, a cardiac specific molecular marker, *Csx* (cardiac-specific homeobox, also named *Nkx-2.5*), was used as a probe in whole mount in situ analysis on both +/+ and $bmp2^{m1}/bmp2^{m1}$ embryos (Fig. 5). *Csx* expression was restricted to the myocardial cells (Komuro and Izumo, 1993; Lints et al., 1993). The

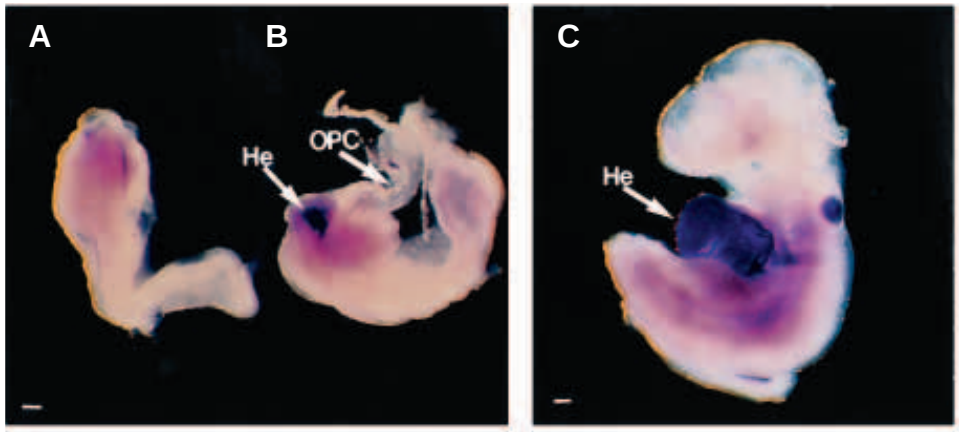

Fig. 5. Expression of *Csx* in mutant and normal embryos. *Csx* expression is displayed by whole mount in situ hybridization analysis on mutant (A,B) and normal (C) embryos at E9.5. The *Csx* expression in heart tissue (He) is labeled. In the mutant embryo (B), *Csx* is expressed, but the position of the heart is anterior to the remnant of the open proamniotic canal (OPC). In one of the mutant embryos (A), the heart tissue appeared to be absent based on the lack of *Csx* staining. Bars, 100 μm.

expression of *Csx* confirmed that the tissue which had developed in an abnormal position was from the cardiac lineage (Fig. 5B).

Among the subset of homozygous mutants in which the amnion had formed at E8.5 and E9.5, some had no sign of heart development. This was confirmed by the absence of expression

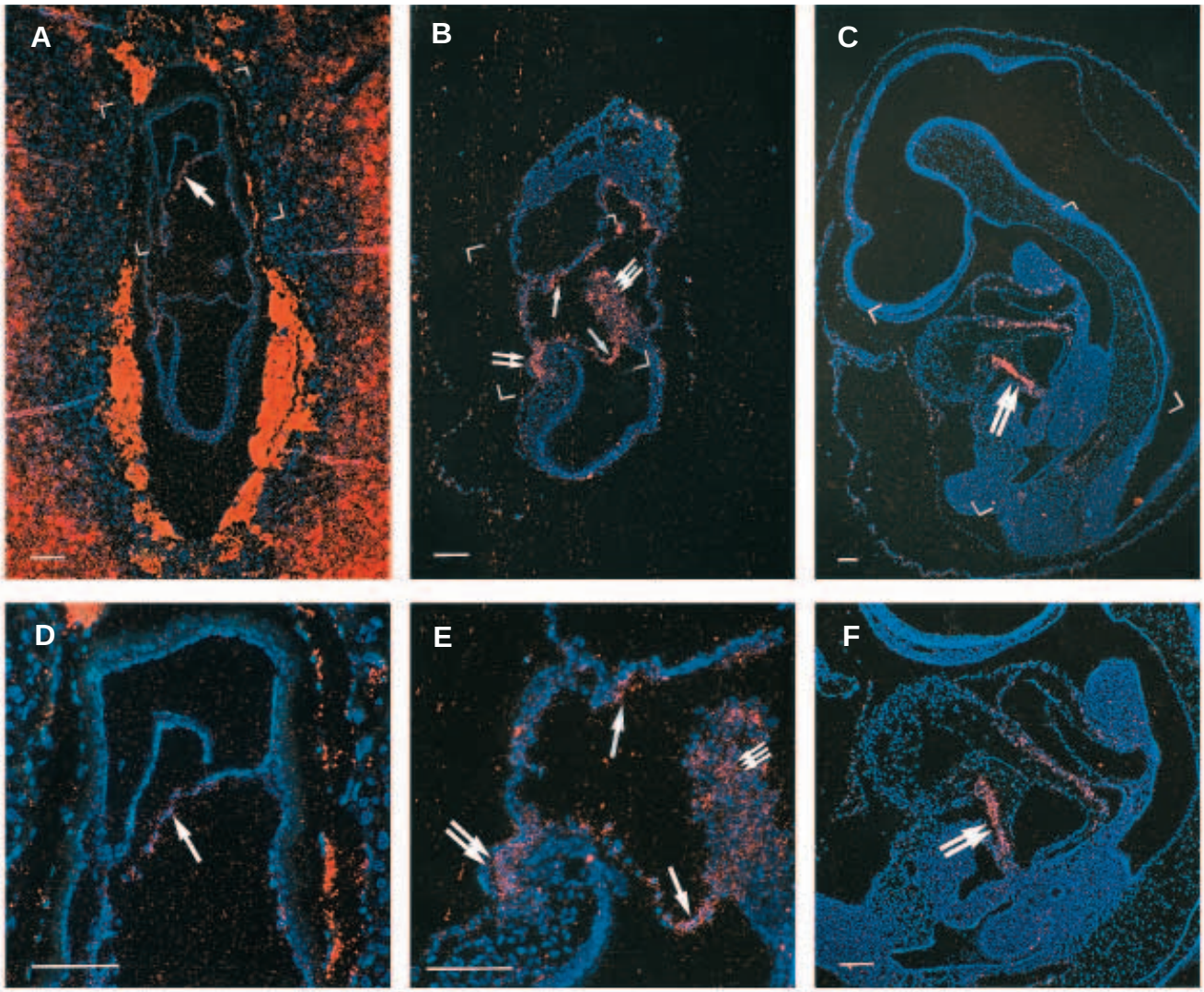

Fig. 6. Expression pattern of *Bmp2* in normal mouse development. *Bmp2* expression revealed by RNA section in situ analysis at E7.5 (A,D), E8.0 (B,E) and E9.5 (C,F). The single arrow shows the expression of *Bmp2* in mesodermal cells of chorion and amnion (A,B,D,E). The double arrow shows *Bmp2* expression in the promyocardium and surrounding mesodermal cells (B,E) and the atrioventricular canal of the heart (C,F). The triple arrow shows *Bmp2* expression in the allantois (B and E), which consists of extraembryonic mesoderm. Bars, 100 μm.

of *Csx* (Fig. 5A). Thus the heart did not form in a few homozygous mutant embryos.

Expression pattern of *Bmp2* between E7.5 and E9.5

A detailed survey of *Bmp2* expression later than E9.5 has been reported (Lyons et al., 1989, 1990). The expression of *Bmp2* correlates with some functions of this gene in certain in vitro assay systems (Vainio et al., 1993; Niswander and Martin, 1993). Since the developmental defects in $bmp2^{m1/}bmp2^{m1}$ mice appeared earlier than E9.0, the expression of *Bmp2* was examined during normal mouse embryogenesis before E9.0.

The earliest detectable expression of *Bmp2* was at E7.5 just before the closure of the proamniotic canal. At this stage *Bmp2* was expressed in the mesodermal cells of the proamniotic canal although the message level was low (data not shown). After the formation of the amnion and chorion, *Bmp2* was expressed in the extraembryonic mesodermal cells lining the chorion and amnion (Fig. 6A,D). Additionally, *Bmp2* was strongly expressed in the maternal decidua. At about E8.0, *Bmp2* was expressed throughout the extraembryonic mesoderm which includes the mesodermal cells of the amnion and chorion, and the allantois (Fig. 6B,E). When differentiation of the promyocardium had started, a high level of *Bmp2* expression was detected in the mesodermal cells rostral to the foregut invagination site (Fig. 6B,E). These *Bmp2* expressing cells are in the promyocardium and the adjacent mesoderm (Fig. 6B,E). *Bmp2* expression was also detected at E9.5 in the outer myocardial cells of the atrioventricular canal (Fig. 6C,F), which is consistent with previous reports (Lyons et al., 1990).

Defects in allantois and other tissues

Allantois is a tissue composed of extraembryonic mesoderm cells. The allantois will become part of the umbilical cord which provides a direct nutrient exchange between the embryo and the placenta. The allantois, although present in all $bmp2^{m1/}bmp2^{m1}$ homozygotes, was delayed in development (Fig. 4C and Table 2). In some cases, the allantois did not reach the ectoplacental cone (data not shown). Since *Bmp2* is highly expressed in the allantois (Fig. 6B,E), it is likely that the delay in allantoic development is a primary consequence of BMP2 deficiency.

At E9.5, a number of other defects were observed which are interpreted as secondary consequences of heart defects. The homozygotes have an open neural tube and blood cells can be found in the headfold (data not shown). Somites were present in these embryos but the mutant embryos were smaller than their normal littermates (Fig. 4C,D).

DISCUSSION

In this study, we have investigated the role of *Bmp2* in murine development by generating BMP2-deficient mice using ES cell technology. The null mutation in the *Bmp2* gene leads to embryonic lethality between E7.5 and E9.0. Specifically, the proamniotic canal in BMP2-deficient embryos failed to close and the heart in mutant embryos either formed in the exocoelomic cavity or did not develop.

BMPs, synthesized as secreted molecules, are thought to be involved in various developmental processes indicated by their restricted and highly regulated expression patterns and the phenotypic consequences of mutations in some of the family members (Kingsley, 1994). For instance, experiments have shown that BMP2 might be one of the signals between the apical ectodermal ridge and the underlying mesenchyme cells that can effect the outgrowth of the limb bud (Niswander and Martin, 1993). The data described here indicate that BMP2 is involved in the interaction between the ectodermal cells and mesodermal cells during several different developmental processes.

The role of BMP-2 in amnion/chorion development

The formation of amnion/chorion starts by the protrusion of the amniotic folds at E7.0 and ends with the closure of the proamniotic canal at E7.5 (Kaufman, 1992a). The process by which the proamniotic canal closes is unknown except for the histological evidence which suggests that the canal is a very transient structure. It is not understood how the tubular structure, which is composed of ectodermal and extraembryonic mesodermal cells, fuses. The relatively strong expression of *Bmp2* in the extraembryonic mesoderm component of the chorion and amnion (Fig. 6A), which is the same cell lineage as the mesodermal cells lining the proamniotic canal, suggests that BMP2 is involved in this process. In the homozygous mutant embryos, the canal usually did not close (Fig. 2). The formation of amnion/chorion involves the proliferation or differentiation of the ectodermal/mesodermal cells. A detailed analysis of the open proamniotic canal in the mutant embryos revealed that the mesodermal cells were still present, suggesting that it is not the death or ablation of certain cell types that causes the defect. We hypothesize that the rate of proliferation of either the mesodermal or the ectodermal cells is reduced in the mutant embryos, leading to a delay in the closure of the canal. As other developmental processes proceed (such as the headfold's expansion into amniotic cavity), the canal can no longer close (Fig. 3B). An alternative possibility is that the fate of the mesodermal or ectodermal cells has changed due to lack of BMP2, resulting in the failure of the closure of the proamniotic canal. In vitro experiments suggest that BMP2 and BMP4 can have a similar function, for instance during early tooth development (Vainio et al., 1993). However, BMP4 cannot fully rescue the defects in a BMP2 deficient embryo, despite the high conservation between these two proteins (BMP4 shares 92% homology with BMP2 at the amino acid level) and the expression of *Bmp4* in the amnion and chorion at E7.5 (Jones et al., 1991).

The role of BMP-2 in cardiac development

In this report, we have shown that *Bmp2* is expressed in the promyocardium and adjacent mesodermal cells (Fig. 6B,E) and that the heart is formed in the exocoelomic cavity in homozygous mutant embryos (Fig. 4). The identity of the heart was confirmed by a molecular marker of the cardiac cell lineage, *Csx* (Fig. 5).

As illustrated in Fig. 3A, cardiac differentiation starts after the mesodermal cells, originating from the posterior part of the embryo, reach the anterior end of the embryo (for a detailed description see DeRuiter et al., 1992; Kaufman and Navaratnam, 1981). The splitting of the mesoderm in the presumptive pericardial region, which precedes the formation of the two pericardial coelomic cavities, begins at about the same time as the initiation of the invagination of the foregut (Kaufman and

Navaratnam, 1981). The promyocardium will appear right between the most proximal part of the headfold and the origin of the amnion in this region (Kaufman and Navaratnam, 1981). At this stage, the interaction between the mesodermal cells and ectodermal/endodermal cells defines two critical processes that might be related to each other: first, the boundary between embryonic and extraembryonic tissues is determined and maintained; second, primitive cardiac tubes migrate dorsally which results in the formation of the developing heart in the amniotic cavity. The cardiac phenotype of $bmp2^{m1}/bmp2^{m1}$ embryos and the expression of *Bmp2* throughout the mesoderm cells at the site of cardiac differentiation suggest that BMP2 is involved in these cellular interactions. In the absence of BMP2, the heart starts to form in the exocoelomic cavity (Figs 3B and 4A,C,E,G). The dorsal migration of the primitive cardiac tube is thought to be the result of the extensive growth of the head fold (DeRuiter et al., 1992). However, the fact that the heart of the homozygous mutant embryos which had extensive head fold growth formed in the exocoelomic cavity suggests that head fold growth is not the sole determinant of the dorsal migration (Fig. 4C and I). The cardiac defect appears to be independent of the amnion/chorion defect since some of the embryos that had a properly closed proamniotic canal and an overtly normal amnion/chorion did have the cardiac defect (Fig. 4I and J).

A recent study has shown that *Csx* mutants have myogenic and morphogenetic defects in the heart tubes (Lyons et al., 1995). It will be interesting to see if BMP2 acts on cells expressing *Csx*. However, *Bmp2* is not expressed in the pharyngeal endoderm where *Csx* is expressed (data not shown). Expression studies on BMP2 receptor(s) will shed light on this issue, if the receptor(s) is specifically expressed. This issue is complicated by the fact that there are multiple receptors which bind BMP2/BMP4 (Koenig et al., 1994; Yamaji et al., 1994).

In $bmp2^{m1}/bmp2^{m1}$ embryos, the allantois was underdeveloped (Table 2). Since BMP2 is highly expressed in allantois (Fig. 6B,E), this raises a possibility that BMP2 acts as an autocrine factor in this particular tissue. An alternative explanation is that the allantois is a differentiated tissue consisting of different cell types where BMP2 has a paracrine role.

An unaddressed question that confronts all studies of the mutation of diffusible factors is that some BMP2 activity from the heterozygous mother might alter the severity of the homozygotes phenotype. This question cannot be addressed without the availability of viable homozygous animals. Circumstantial evidence from transgenic overexpression studies of *Bmp2* (Blessing et al., 1993) suggests that *Bmp2* has to be regulated very precisely during normal mouse development. If BMP2 was passed to the zygotic environment from the maternal decidua, it would have to pass through various cellular barriers to reach the correct locations in the embryo. The correlation of the mutant phenotype with the expression of *Bmp2* strongly suggests that the zygotic gene functions locally.

The role which BMP2 plays during development is complex. Defining the function of BMP2 will be aided by studies on its receptor(s) and downstream signalling molecules. Some in vitro studies suggest that BMP2 is involved in many other signaling processes regulating cell growth and differentiation (Vainio et al., 1993; Niswander and Martin, 1993). Although our studies cannot supplement the evidence for function of BMP2 in these processes, the application of tissue-specific knockouts (Gu et al., 1994) will enhance our understanding of BMP2 function both in embryogenesis and during adult development.

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