

ARTICLE

Bmp2 and *Bmp4* Genetically Interact to Support Multiple Aspects of Mouse Development Including Functional Heart Development

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Summary: Bone morphogenetic proteins (BMPs) have multiple roles during embryogenesis. Current data indicate that the dosage of BMPs is tightly regulated for normal development in mice. Since *Bmp2* or *Bmp4* homozygous mutant mice show early embryonic lethality, we generated compound heterozygous mice for *Bmp2* and *Bmp4* to explore the impact of lowered dosage of these BMP ligands. Genotyping pups bred between *Bmp2* and *Bmp4* heterozygous mice revealed that the ratio of adult compound heterozygous mice for *Bmp2* and *Bmp4* is much lower than expected. During embryogenesis, the compound heterozygous embryos showed several abnormalities, including defects in eye formation, body wall closure defects, and ventricular septal defects (VSD) in the heart. However, the ratio of the compound heterozygous embryos was the same as expected. Caesarean sections at E18.5 revealed that half of the compound heterozygotes died soon after birth, and the majority of the dead individuals exhibited VSD. Survivors were able to grow to adults, but their body weight was significantly lower than control littermates. They demonstrated progressive abnormalities in the heart, eventually showing a branched leaflet in atrioventricular valves. These results suggest that the dosage of both BMP2 and 4 is critical for functional heart formation during embryogenesis and after birth. *genesis* 47:374–384, 2009. © 2009 Wiley-Liss, Inc.

Key words: haploid insufficiency; ventricular septum defect; mitral valve; tricuspid valve; microphthalmia

INTRODUCTION

Bone morphogenetic proteins (BMPs) are the members of the TGF- β superfamily and were found by their osteogenic activity when placed in ectopic locations (de Caestecker, 2004; Kishigami and Mishina, 2005). However, recent studies of several organisms demonstrated that BMPs have other roles during embryogenesis, including the dorsoventral and/or anterior-posterior axis formation

and processes of organogenesis, such as cardiac and lung development (Mishina, 2003; Zhao, 2003). BMP2 and BMP4 are the first discovered members of the BMP family and are rediscovered to have these alternate roles. Homozygous mutant embryos for *Bmp2* show developmental abnormalities in fusion of the preamniotic canal at E7.5 and defects in cardiac development and embryonic turning, and die by E9.5 (Zhang and Bradley, 1996). BMP2 is necessary for migration but not for induction of neural crest cells (Correia *et al.*, 2007). It is also necessary for PGC formation (Ying and Zhao, 2001). Homozygous mutant embryos for *Bmp4* show abnormal development in the posterior half of the embryo and defects in germ cell development causing death by E10.5 (Lawson *et al.*, 1999; Winnier *et al.*, 1995; Ying *et al.*, 2001). Severity of the phenotype of *Bmp4* homozygous mutant embryos varies, depending partly on genetic background. For example, no mesoderm induction is observed in a C57BL6 background but the mutant embryos show a much milder phenotype in an outbred background and develop an apparent anterior-posterior axis (Furuta and Hogan, 1998; Winnier *et al.*, 1995). Recent studies using a conditional null allele revealed important roles of BMP2 and BMP4 in later stages of embryogenesis. BMP4 is required for the endocardial epithelial-to-mesenchymal transformation (EMT) in the atrioventricular canal (AVC) (Jiao *et al.*, 2003) and outflow

Additional Supporting Information may be found in the online version of this article.

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tract septation (Liu *et al.*, 2004). BMP4 also plays critical roles in morphogenesis of lung epithelial cells (Eblaghie *et al.*, 2006). BMP2 is critical for the formation of the endocardial cushions in the AVC and subsequent endocardial EMT (Ma *et al.*, 2005; Rivera-Feliciano and Tabin, 2006).

BMP ligands bind to both Type I and Type II receptors to form heteromultimers for their signal transduction. Three Type I receptors (BMPRIA, BMPRIB, and ACVRD), and three Type II receptors (BMPRII, ActRIIA, and ActRIIB) behave as receptors for BMPs. Homozygous mutants for *Bmpr1a*, which encodes a Type I receptor for BMP2 and BMP4, develop no mesoderm and die by E8.5 (Mishina *et al.*, 1995). Homozygous mutants for *Bmpr2* also show a phenotype identical to homozygous mutants for *Bmpr1a* (Beppu *et al.*, 2000). Homozygous mutant embryos for *Acrv1*, which encodes a Type I receptor for BMP2 and BMP7, start to gastrulate, but stop developing at the late streak stage of gastrulation (Gu *et al.*, 1999; Komatsu *et al.*, 2007; Mishina *et al.*, 1999). The fact that homozygous mutants for BMP receptors show more severe phenotypes than their ligands prompted us to hypothesize that loss of *Bmp2* may be compensated, in part, by BMP4, and loss of *Bmp4* may be compensated by BMP2. Both genes are highly conserved with over 90% similarity in the mature proteins, thus it is reasonable to hypothesize that *Bmp2* and *Bmp4* may play synergistic roles for proper embryonic development, and generation of compound mutant embryos may further reveal functions of these genes.

The amount of BMP signaling is regulated at different levels. Recent findings demonstrated that extracellular antagonists including noggin and chordin tightly regulate the amount of signaling (Balemans and Van Hul, 2002; Yanagita, 2005). Indeed, compound mutant embryos of *noggin* and *chordin* display a wide range of severity in their phenotype depending on the numbers of functional copies of BMP antagonists (Anderson *et al.*, 2002; Bachiller *et al.*, 2000), suggesting that tight regulation of the amount of BMP signaling is critical for normal development. It is reported that a small portion of heterozygous mutant mice for *Bmp4* in a C57BL6 background demonstrate some phenotypes including polydactyl, a shorter head, and circling behavior (Dunn *et al.*, 1997), but their survivability was not affected. A hypomorphic allele for *Bmp4* was recently reported (Goldman *et al.*, 2006). It was generated by introduction of a mutation in the cleavage site of the BMP4 precursor. Analyses of mice homozygous for this mutation revealed that the proper amount of BMP4 is critical for placental vasculature formation and ventral body wall closure (Goldman *et al.*, 2006). No heterozygous phenotype was reported originally for *Bmp2* (Zhang and Bradley, 1996), however, we recently found that some heterozygous embryos for *Bmp2* exhibited closure defects in the cephalic region of the neural tube (Castranio and Mishina, 2008). Also, we recently generated a floxed allele for *Bmp2* and the presence of a neo selection cassette in the locus dramatically reduced levels of *Bmp2* (Singh *et al.*, 2008). This leads to closure defects in cephalic region of the neural tube as well as in the ven-

tral body wall (Singh *et al.*, 2008). Therefore, it would be a reasonable hypothesis that mice that are compound heterozygous mutant for *Bmp2* and *Bmp4* would develop additional phenotypes during development.

Here, we found that compound heterozygous mutant mice for *Bmp2* and *Bmp4* were underrepresented at the time of weaning. Approximately half of the compound heterozygous fetuses showed defects in formation of the interventricular septum (ventricular septal defects, VSD) during development and died soon after birth. A small portion of the compound heterozygous fetuses showed a variety of phenotypes including closure defects in the ventral abdominal body wall, suggesting that those organs are sensitive for a decreased dose of BMPs during development. Interestingly, the survivors progressively developed abnormal valve structure in the heart by 2 months after birth. These results suggest that the dosage of both of BMP2 and 4 is critical for functional heart formation during embryogenesis and after birth, and that lowered BMP signaling is a potential cause of congenital and postnatal heart abnormalities such as VSD.

RESULTS

Bmp2/Bmp4 Compound Heterozygotes Show Disadvantage for Survival

First, we set up matings between *Bmp2* heterozygous and *Bmp4* heterozygous mice, which had a mixed genetic background of 129SvEv and C57BL6. In this combination, 25% of pups should be compound heterozygotes for *Bmp2* and *Bmp4* (*Bmp2*^{+/-};*Bmp4*^{+/-}). However, genotyping after the weaning stage revealed that only 10.6% of pups out of 415 mice were compound heterozygotes, whereas the other genotypes were in reasonable numbers (36.3% for wild type, 29.2% for *Bmp4* single het, 23.9% for *Bmp2* single het). These survived compound heterozygotes were smaller than littermates in both sexes at 3 weeks (Fig. 1, total 89 males and 94 females). The relative body weight of the survived compound heterozygous males and females to wild-type males and females were 68% and 59%, respectively (see Fig. 1). Swapping the genotypes of parents did not cause any overt changes in the ratio and body weight of compound heterozygotes (data not shown). Since both *Bmp4* null and *Bmp2* null embryos die in early stages of development, we speculated that compound heterozygotes might demonstrate an embryonic phenotype either similar to these nulls or something unique. Results of timed mating revealed that we recovered expected ratios of compound heterozygotes during mid to late gestation, suggesting that compound heterozygotes were able to survive much longer than homozygous null embryos for each gene. Some of the compound heterozygotes developed interesting phenotypes depicted in Figure 2. They showed severe edema as early as E13.5 (2 out of 36 at E13.5, Fig. 2c,k,l) suggesting defects in heart function. Some others showed eye defects (7 out of 73 at E13.5-E15.5, Fig. 2d,k) or body wall closure

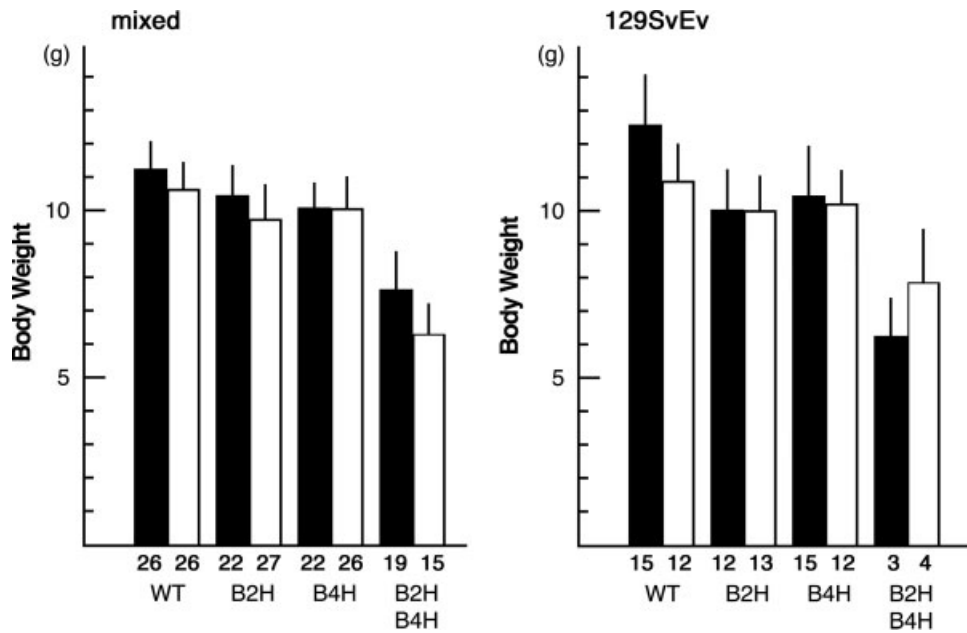


FIG. 1. Body weight of 3-week-old mice. Mixed background (left) and 129SvEv background (right). Black bar, male; white bar, female; WT; wild type, B2H, *Bmp2* heterozygous mice, B4H, *Bmp4* heterozygous mice, B2HB4H, compound heterozygous mice for *Bmp2* and *Bmp4*. Numbers of animals measured are shown underneath of each bar. B2HB4H mice are smaller in their body weight than other genotype in both sexes ($P < 0.0001$).

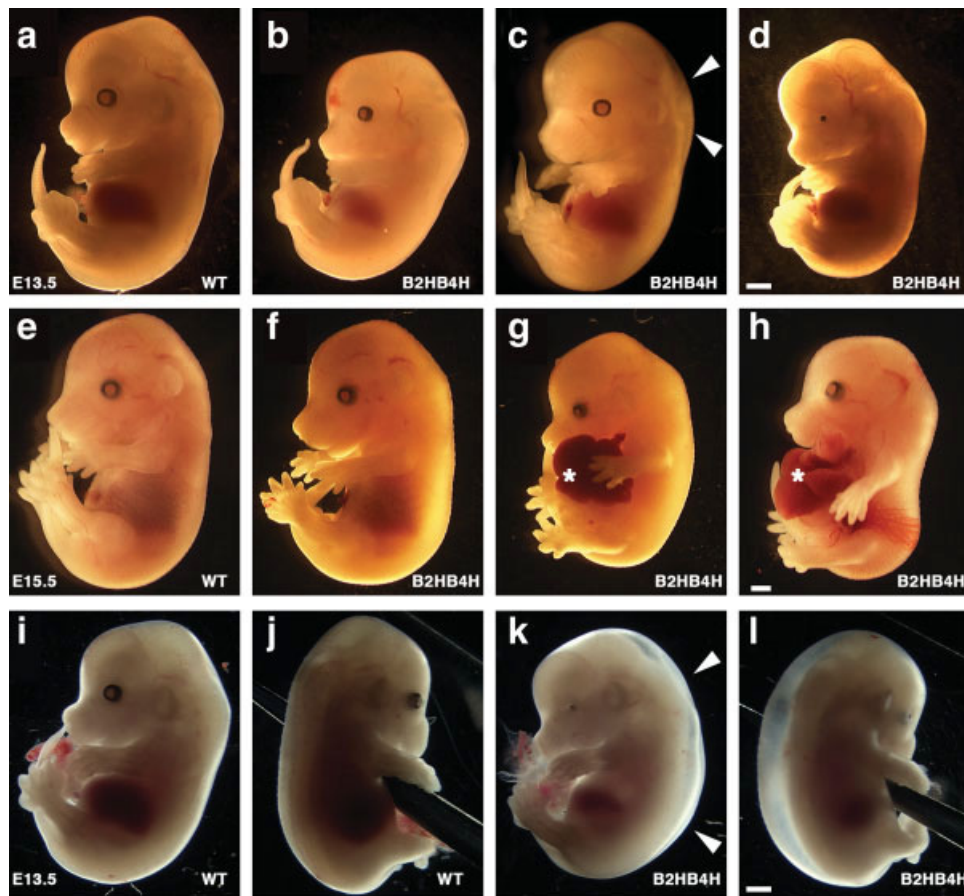


FIG. 2. Abnormalities developed in the compound heterozygous embryos. Whole view of mouse embryos at E13.5 (a–d and i–l), E15.5 (e–h), wild type (a, e, i, and j), and compound heterozygous (b–d, f–h, and k, l) embryos. Compound heterozygous embryos showed no significant abnormality (b and f), edema (c and k, l, white arrowheads), small eye (d and k, l), and hernia (g and h, asterisk). i and j, and k and l are the same embryo, respectively. Scale bar, 1 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 1
Ratio of Genotyping of Offspring From the Breeding Between *Bmp2* het and *Bmp4* het

Stage	Wild type (%)	<i>Bmp2</i> het (%)	<i>Bmp4</i> het (%)	Compound het (%)	Total	Resorption site
E13.5	18 (15.4)	37 (31.6)	25 (21.4)	36 (30.8)	117	6
E14.5	9 (20.5)	9 (20.5)	12 (27.3)	14 (31.8)	44	21
E15.5	26 (25.0)	27 (26.0)	28 (26.9)	23 (22.1)	104	25
E18.5	23 (28.4)	18 (22.2)	19 (23.4)	21 (35.9)	81	9
3 wk	129 (39.8)	80 (24.7)	86 (26.5)	29 (9.0)	324	n/a

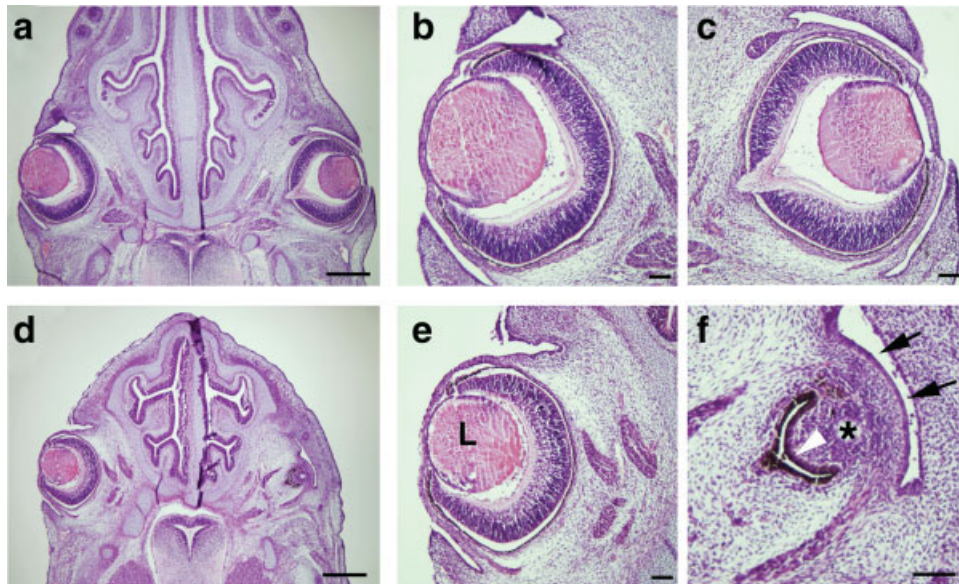


FIG. 3. Histological observation of eye abnormalities. H&E staining of head sections at E13.5. Wild type (a–c) and compound heterozygous (d–f) embryos are shown. The compound heterozygous mutant showed mild (e) and severe (f) forms of microphthalmia. L, lens; asterisk, remnant of lens; arrows, presumptive corneal-conjunctival epithelium; white arrowhead, degenerated retina replaced by pigment. Scale bar, 500 μ m (a, d), 100 μ m (b, c, e, f).

defects that lead to hernia (7 out of 73 at E13.5–E15.5, Fig. 2g,h, asterisk). However, the majority of compound heterozygotes in these stages were grossly normal (55 out of 73 at E13.5–E15.5, depicted in Fig. 2b,f), as well as all of the wild type or single heterozygous mutant embryos for each gene (except small portion of *Bmp2* heterozygous embryos, see below). These results indicated that severe edema or body wall closure defects were not the only cause of the shortage of compound heterozygotes by the time of weaning. Ratios of compound heterozygotes were relatively normal in mid/late gestation (Table 1), indicating that the majority of compound heterozygotes can survive to late gestation, which excludes the possibility that the majority of them die earlier. Since previous studies have indicated the importance of BMP signaling in heart development, and a small number of the embryos showed edema as shown in Figure 2, we hypothesized that abnormalities that develop in the heart of compound heterozygous embryos would compromise their survivability during the perinatal period.

Histological observation revealed that some of the compound heterozygous mutant embryos developed microphthalmia (see Fig. 3). The particular compound embryo shown in Figure 3 had smaller a left eye than that of wild

type (mild form of microphthalmia), whereas the right eye showed severe microphthalmia characterized by lens remnant, corneal-conjunctival epithelium, and double-layered retinal pigment epithelium. These results suggest that the retina started to degenerate soon after its formation and was subsequently replaced by the retinal pigment (inner layer of pigmented epithelium) (Fig. 3f).

We previously noticed that a small portion of heterozygous embryos for *Bmp2* develop neural tube closure defects especially in the cephalic region (Castranio and Mishina, 2008). The frequency of the neural tube defects was not increased by addition of *Bmp4* heterozygosity to the *Bmp2* heterozygous null background (6 out of 41 embryos for *Bmp2* heterozygous embryos and 4 out of 42 embryos for *Bmp2/Bmp4* compound heterozygous embryos) suggesting that dosage of BMP4 has less of an impact on developing the neural closure phenotype than that of BMP2.

Compound Heterozygotes Show Developmental Abnormalities in Heart

Histological observation of the E13.5 heart revealed that wild-type embryos are about to fuse the interven-

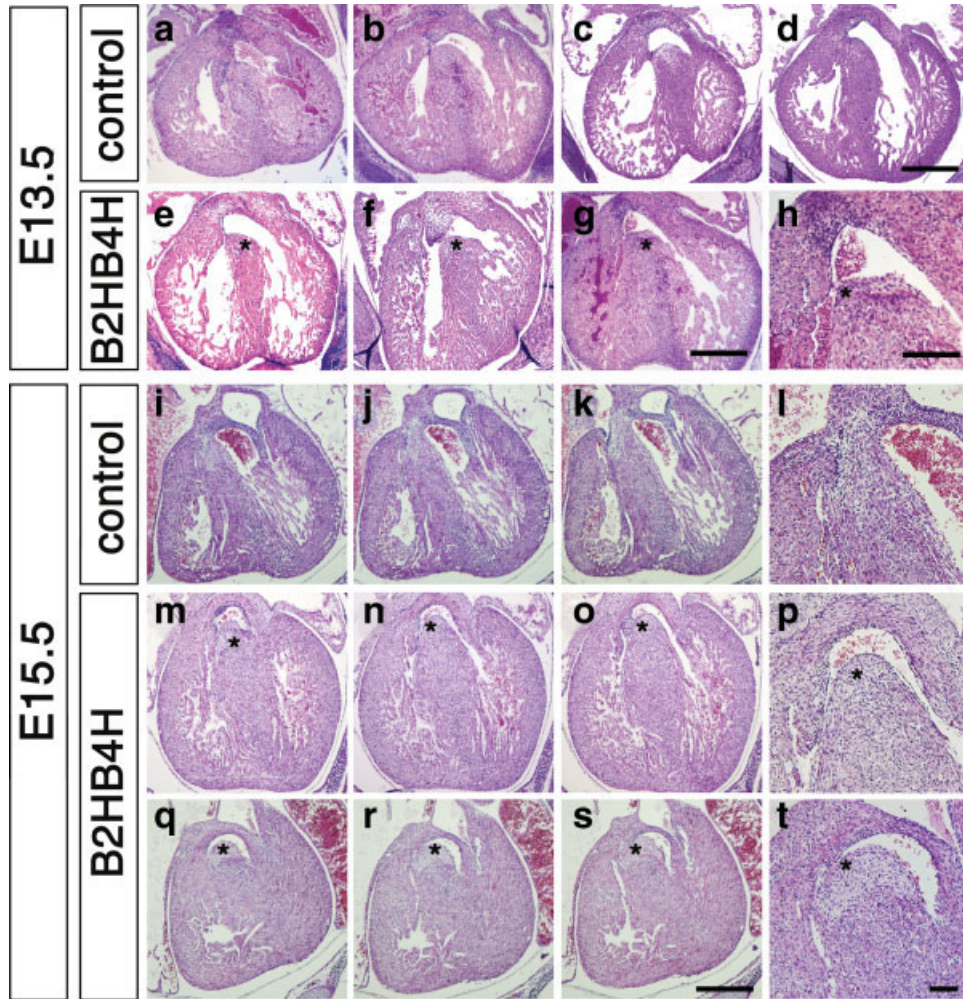


FIG. 4. Heart abnormalities developed in compound heterozygous embryos. H&E staining of heart sections at E13.5 (a–h) and E15.5 (i–t). At E13.5, ventricular septum started to close in wild type (a and b), *Bmp2* heterozygous (c), *Bmp4* heterozygous (d) embryos, but in the compound heterozygous embryos, all of ventricular septum have not closed yet (e–h, asterisks). Two examples for the WT and three examples for the compound heterozygotes are shown. At E15.5, all ventricular septum are completely closed in wild type (i–k; sequential with 35 μ m interval, l; magnified image of j). In the compound heterozygous embryos the ventricular septum has not closed (asterisks, m–o; sequential images with 35 μ m interval, p; magnified image of a region in n, q–s; sequential images with 35 μ m interval, t; magnified image of a region in r). Two examples are shown. Scale bar, 500 μ m (a–g); 200 μ m (h); 250 μ m (j–k, m–o, q–r); 100 μ m (l, p, and t).

Table 2
Results of C-Section

Genotype	Total	Infrequent breath (%)	Dead (%)	Body weight (mg) average (STD)
Wild type	23	2 (8.7)	0 (0)	1,143.0 (120.5)
<i>Bmp2</i> Het	18	1 (5.6)	1 (5.6)	1,162.5 (87.7)
<i>Bmp4</i> Het	19	1 (5.3)	1 (5.3)	1,137.2 (191.4)
Compound Het	21	12 (57.1)**	11 (52.3)**	993.1 (111.0)*
Total	81	16 (19.8)	15 (18.5)	1,107.1 (147.1)

* $P < 0.001$, Student *t*-test.

** $P < 0.001$ to *Bmp2* Het or *Bmp4* Het, Fisher's exact test.

tricular septum to the top portion of cardiac wall (2/4 are fused, 2/4 are not, Fig. 4a–d). In the case of compound heterozygous mice, 6 out of 6 showed ventricular septal defects (VSD) (Fig. 4e–h). To distinguish if this as a persistent defect or a delay of fusion, we next made

histological observations at E15.5. At this stage, the septum in nearly all of wild type (13/14) and in all of both single heterozygous embryos examined were fused, whereas the majority of the compound heterozygous embryos still showed VSD (12/16) (Fig. 4m–t).

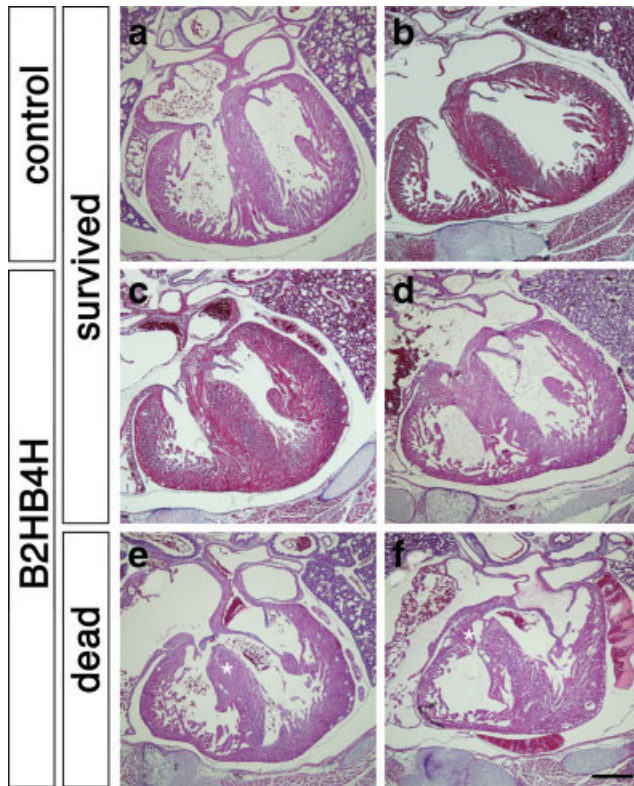


FIG. 5. Defects in heart septation in the compound heterozygous mutants at E18.5. Fetuses were recovered at E18.5 by C-section and breathing was initiated by mild stimuli. Histological sections of heart were stained with H&E. Wild type (**a** and **b**) and majority of the compound heterozygous pups that revived after C-section (**c** and **d**, four of five) showed no evidence of VSD. Compound heterozygous mutants that did not revive showed VSD (**e** and **f**, asterisks, six of eight). Two examples for each case are shown. Scale bar, 500 μ m.

Since the ratio of the compound heterozygotes is relatively normal in mid/late gestation (Table 1), and we observed little death of pups during lactation period, we speculated that some of the compound heterozygotes die immediately after birth. E18.5 embryos were recovered from pregnant females by C-section and were evaluated for their ability to survive. All pups started to breathe, however, about half of compound heterozygotes showed respiratory difficulties, and died within 30 minutes (Table 2). Compound heterozygotes were smaller than other types of pups ($P < 0.001$). Among compound heterozygotes, the ones that died had lower body weights than survivors in body weight ($949 \text{ mg} \pm 10.6$ vs. $1042 \text{ mg} \pm 9.6$ mg, respectively, $P < 0.005$). Examination of heart histology revealed a very clear contrast. The majority of compound heterozygotes that survived after C-section showed a relatively normal heart histology (4/5), including complete formation of the interventricular septum (Fig. 5c,d). Interestingly, all of the compound heterozygotes that died within 30 minutes after C-section showed VSD (Fig. 5e,f). This result suggests that perinatal death is probably due to

heart malfunction caused by VSD because we did not see any overt changes in the lung (see below). Other types of abnormalities including atrial septal defects (ASD), double outlet right ventricle (DORV), and persistent truncus arteriosus (PTA) were not observed in the compound heterozygous mutant pups (Supporting Information Fig. 1 and data not shown).

We examined levels of several markers including MSX2, a known downstream target of BMP signals (Brugger *et al.*, 2004) and SOX9, a marker for neural crest derivatives that populate atrioventricular cushions (Nie *et al.*, 2008), but no overt changes were observed between wild type and the compound heterozygous heart (Supporting Information Fig. 2).

To understand the effects of lowered BMP signaling on heart development after birth, we made histological observations on 3-week-old hearts. The heart wall seemed normal (Fig. 6a,d,g), but leaflets in the tricuspid valve and mitral valve in hearts of compound heterozygotes showed abnormal morphology (Fig. 6e,h, and Fig. 6f,i, respectively). They were prolonged and more prominent in particular when body weight was low. These defects were not obvious in C-sectioned samples at E18.5 (Fig. 5 and data not shown) suggesting that these defects are developed postnatally. Histological observation of 2-month and 4-month-old hearts showed more dramatic changes in valve form. They were elongated and branched (Fig. 6o,q,r, $n = 4$ for control and compound heterozygous mutants). No abnormalities were found in single heterozygous mutant mice for either for *Bmp2* or *Bmp4*.

No Overt Abnormalities are Found in Lung

Since half of compound heterozygotes fail to initiate continuous breathing, we also examined the histology of the lungs. At E15.5, there were no overt morphological changes (Supporting Information Fig. 3a,b). However, at 3 weeks, the color of lungs in compound heterozygotes was pale compared with that in control (Supporting Information Fig. 3c,d). The histology of lungs at the time of C-section revealed less alveolar spaces in compound heterozygotes (Supporting Information Fig. 3e-l), however, the changes were minimal. Three-week-old samples showed the similar tendency, lower numbers of alveolar vesicles (Supporting Information Fig. 3m-p); however, the thickness of the epithelial layer of bronchioles was comparable. SP-C and CC10, markers for alveoli epithelium, were produced in both control and compound heterozygous mutant lung in comparable manners (Supporting Information Fig. 4).

Genetic Background Affects Severity of the Phenotypes

Because the genetic background of the mice used were a mixture of 129SvEv and C57BL6, variability of the phenotypes might be explained by variability of genetic background. In fact, it is reported that phenotypes of *Bmp4* null embryos change in different

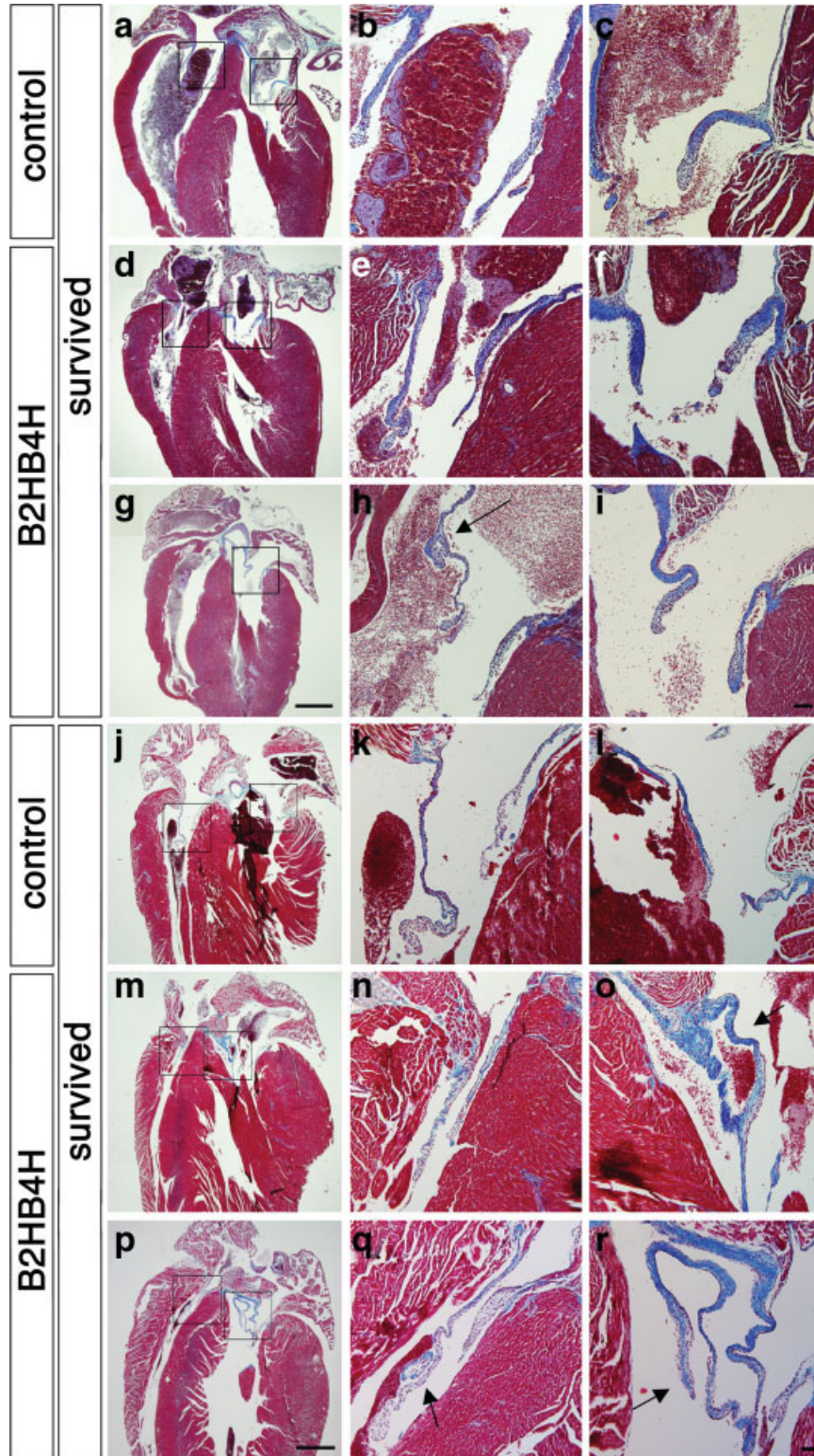


FIG. 6. Valve abnormalities in the compound heterozygous mutant pups. Heart sections including atrioventricular valves from 3-week-old pups (a–i) and from adults (4 months for j–o and 2 months for p–r) were stained with Masson's trichrome. Wild type (a–c, j–k) and two examples of compound heterozygous pups (d–f, g–i, m–o, p–r) are shown for each stage. Magnified images of right (tricuspid) and left (mitral) atrioventricular valves are shown in b, e, h, k, n and q and c, f, i, l, o, and r, respectively. h is a magnified image from an adjacent of g. Blue staining indicates atrioventricular valves. An arrow indicates branched atrio-ventricular valve (h, o, q, r). Scale bar, 1 mm (a, d, g, j, m, and p); 100 μ m (b, c, e, f, h, i, k, l, n, o, q, and r).

Table 3
Ratio of Genotyping of Offspring in Different Genetic Background

Strain	Stage	Wild type (%)	<i>Bmp2</i> het (%)	<i>Bmp4</i> het (%)	Compound het (%)	Total
Mixture	E15.5	26 (25.0)	27 (26.0)	28 (26.9)	23 (22.1)	104
129SvEv	E15–16	12 (25.1)	16 (28.5)	14 (25.0)	14 (25.0)	56
C57BL6	E15–16	24 (40.0)	13 (21.7)	13 (21.7)	10 (16.7)	60
Mixture	3 wk	129 (39.8)	80 (24.7)	86 (26.5)	29 (9.0)	324
129SvEv	3 wk	98 (31.9)	92 (30.0)	99 (32.2)	18 (5.9)	307
C57BL6	3 wk	36 (42.4)	25 (29.4)	24 (28.2)	0 (0.0)	85

Results from E15.5 were shown for mixed background. Results from E15.5 and E16.5 were combined for 129SvEv and C57BL6.

backgrounds. In an outbred background, homozygous null embryos can survive longer and reach the organogenesis stages whereas in a C57BL6 background, their phenotype resembles *Bmpr1a* or *Bmpr2* null mutant embryos (Furuta and Hogan, 1998; Winnier *et al.*, 1995). Phenotypes of *Bmp4* heterozygous mutants are also affected by genetic background. In a C57BL6 background, small proportions of heterozygous mutant mice show minor abnormalities including an extra digit and circling behavior (Dunn *et al.*, 1997). We previously noticed that *Bmp2* heterozygous mutants in a C57BL6 background showed closure defects in the cephalic neural tube more frequently than heterozygous mutants in a mixed background of 129SvEv and C57BL6 (Castranio and Mishina, 2008). These facts suggest that the genetic background influences the severity of the phenotypes. To address this issue, we generated compound heterozygous mutants in a 129SvEv background and in a C57BL6 congenic background. For the latter case, we backcrossed heterozygous mice to C57BL6 more than seven times (expected ratio of C57 is over 99%). In mid-to-late gestation, a ratio of the compound heterozygotes was the same as expected (approximately 25%) (Table 3). A total of 307 pups in a 129SvEv background were genotyped at 3 weeks after birth and an under representation of compound heterozygotes were observed (Table 3). Like in mixed background, compound heterozygotes were smaller than wild type or heterozygotes for each gene [Fig. 1, the relative body weight of the survived compound heterozygotes to wild type was 53% (male) or 62% (female)]. In contrast, at 3 weeks none of the over 80 pups in a C57BL6 background were compound heterozygotes, suggesting that they died during the perinatal period due to severe defects caused by the genetic background (Table 3). These results also may suggest that genetic modifiers in the C57BL6 background may account, at least in part, for the variable phenotypes of compound heterozygous mutants found in the mixed genetic background.

DISCUSSION

Dosage of BMP Signaling and Related Phenotype

We used a genetic approach to partially reduce the levels of BMP signaling and found that a proper dosage of BMP2 and BMP4 is critical for several aspects of normal development including functional heart formation.

Most of the compound heterozygous individuals that died during the perinatal period showed ventricular septal defects, which might affect the initiation and continuation of breathing. Several reports using conditional and hypomorphic alleles of BMP ligands, receptors, and signaling molecules have demonstrated that BMPs play critical roles during heart development, including septation in the outflow tracts, proliferation of the heart wall, and EMT in the atrial-ventricular cushion (Delot *et al.*, 2003; Gaussin *et al.*, 2002, 2005; Jiao *et al.*, 2003; Kaartinen *et al.*, 2004; Kim *et al.*, 2001; Liu *et al.*, 2004; Ma *et al.*, 2005; Nie *et al.*, 2008; Park *et al.*, 2006; Rivera-Feliciano and Tabin, 2006; Stottmann *et al.*, 2004; Stroud *et al.*, 2007; Wang *et al.*, 2005). Histological observations of the compound heterozygous mutants described here suggest that proper formation of the interventricular septum may be the most sensitive process affected by the reduced levels of BMP signaling. *Bmp2* is highly expressed in the atrioventricular canal myocardium between E11.5 and E14.5, and also expressed in endocardial cushion from E12.5 (Abdelwahid *et al.*, 2001). *Bmp4* is highly expressed in outflow tract myocardium and endocardial cushion (Abdelwahid *et al.*, 2001). *Bmpr1a* is expressed nearly ubiquitously in the developing stage of the heart whereas *Bmpr1b* expression in the heart is very low (Dewulf *et al.*, 1995). These expression patterns suggest that BMP2 and BMP4 produced in endocardial cushion signals through BMPRIA to play a role for fusion of interventricular septum to endocardial cushion. These facts also suggest that BMP2 and BMP4 play roles for morphological integrity of the tricuspid and mitral valves that are developed from atrioventricular canal.

A closure defect in the posterior ventral body wall is another dosage sensitive process. It has a nearly identical phenotype of that found in the conditional mutant embryos of *Bmpr1a* induced by *Dermo1-Cre* (Sun *et al.*, 2007). Double homozygous mutations of *Msx1* and *Msx2* show a similar omphalecele phenotype (Ogi *et al.*, 2005) suggesting that during mid-gestation, both BMP2 and BMP4 work together to maintain a sufficient level of BMP signaling through BMPRIA to regulate levels of *Msx1* and *Msx2*. Some of the compound heterozygous embryos for *Bmp2* and *Bmp4* developed hypomorphic eyes, which would be a similar phenotype to that caused by a retina-specific disruption of *Bmpr1a* in *Bmpr1b* null background (Murali *et al.*, 2005). In this case, proliferation of the cells in the retinal neuroectoderm is

drastically reduced at E11.5. Interestingly, no overt phenotype is observed if *Bmpr1b* allele is wild type (Murali *et al.*, 2005). These suggest that BMP2 and BMP4 work together to send signaling through both BMPRIA and BMPRII for proper retinal development during mid-gestation.

Some of the *Bmp2* heterozygous mutant embryos develop closure defects in the cephalic neural tube leading to perinatal death (Castranio and Mishina, 2008). Interestingly, the ratio of heterozygous mutant embryos demonstrating the neural tube defects is altered depending on their genetic background. We recently generated a hypomorphic allele for *Bmp2* by knocking in a *polII-neo* cassette, of which expression level was 10% compared with a wild-type allele, and embryos having the hypomorphic allele over the null allele showed higher frequency of the neural tube defects (Singh *et al.*, 2008). However, as described in the Result section, the frequency of the neural tube defects in the compound heterozygous embryos for *Bmp2* and *Bmp4* was not increased compared with that in *Bmp2* heterozygous embryos. Although small proportions of *Bmp4* heterozygous mutant mice show abnormalities, no perinatal death is reported in both inbred (congenic) and mixed background (Dunn *et al.*, 1997) (unpublished observation). We have dissected over 500 heterozygous embryos for *Bmp4* in various genetic backgrounds but never found neural tube closure defects in this genotype (data not shown). Together, the data suggest that dosage of BMP2, but not BMP4, would be of primary importance for development of neural tube closure defects in the cephalic region. Generation of a hypomorphic allele for *Bmp4*, by introducing a mutation in the cleavage site for maturation is previously reported (Goldman *et al.*, 2006). It would be an interesting future experiment to further reduce the amount of BMP2 and BMP4 by combining these hypomorphic alleles to uncover relatively less sensitive biological processes, which may include limb bud formation and subsequent digit specification.

Potential Interactions of BMP Ligands for Their Functions

Several reports demonstrate that compound homozygous mutants reveal overlapping functions of BMP ligands. For example, double homozygous null embryos for *Bmp5* and *Bmp7* die by E10.5 even though the impact of the loss of one of these genes for proper embryogenesis is negligible (Solloway and Robertson, 1999). Double homozygous null embryos for *Bmp6* and *Bmp7* show similar defects in interventricular septation as we have shown in this article (Kim *et al.*, 2001). These results suggest that some of the BMPs share redundant functions especially in the tissues where they are co-expressed. Compound heterozygous mutant mice for *Bmp4* and *Bmp7* showed an abnormal skeletal phenotype, which also suggests that dosage of BMP signaling may play a role in the morphology of bones (Katagiri *et al.*, 1998).

It is reported that heterodimer molecules for BMPs may have functions distinct from their homodimers. In frog, purified BMP4/7 heterodimer has a potent mesoderm inductive ability compared with a mixture of BMP4 homodimer and BMP7 homodimer (Suzuki *et al.*, 1997). In cell culture and in vivo bone defect models, BMP2/7 heterodimer shows a more potent ability for induction of alkaline phosphatase and differentiation toward osteoblast lineages (Koh *et al.*, 2008; Tsuji *et al.*, 1998; Zhu *et al.*, 2006). In *Drosophila*, Screw-Dpp heterodimers shows potent activity for specification of dorsal tissues (Shimmi *et al.*, 2005). Although these are the examples of heterodimers between the Dpp subgroup (BMP2, BMP4) and the 60A subgroup (BMP5, BMP6, BMP7), these facts suggest BMP2/4 heterodimer may also have unique functions. It is an alternative possibility that an alteration of the amount of heterodimers for BMP2 and BMP4 may be a cause of the abnormalities reported here. A knock-in experiment of *Bmp2* gene into the *Bmp4* locus (or vice versa) may shed light on this possibility.

BMP Dose and Heart Functions

Most of the surviving compound heterozygous mutants can live for over a year, but the majority are smaller in body size (weight and stature). One of the interesting possibilities for smaller body size would be insufficient circulation. Since wall thickness of heart chambers in the compound heterozygous mutants are seemingly thinner than that of control at neonatal stage (see Fig. 5), it is possible to speculate that ejection volume in the mutants could be smaller. An alternative possibility is that insufficient circulation is caused by the abnormal morphology of valve leaflets, as they age (see Fig. 6). Atrioventricular cushion-specific disruption of *Bmpr1a* causes defects in leaflet formation in both mitral and tricuspid valves, which in turn leads to higher blood pressure in the atrium (Gaussin *et al.*, 2005; Stroud *et al.*, 2007). Thus, we speculate that reduction of BMP2 and/or BMP4 signaling through BMPRIA in the valves would be one of the causes of morphological abnormalities found in human cases. It is reported that cell proliferation in heart valves stops 1 week after birth (Kruithof *et al.*, 2007). There is no overt difference in proliferative ability of cells in valves at E18.5 and 1 week after birth (data not shown), suggesting BMP signaling may be important for morphological changes of the cells in the valves during postnatal development. It will be of interest in the future to assess functionality of the heart of survived compound heterozygotes because it is expected that abnormal valve structure may interfere with blood flow from atriums to ventricles.

MATERIALS AND METHODS

Mouse

Generation and characterization of mutant mouse strains were described elsewhere (Winnier *et al.*, 1995;

Zhang and Bradley, 1996). All mouse experiments were performed in accordance with NIEHS/NIH guidelines covering the humane care and use of animals in research.

PCR Genotyping

Mouse genotypes were initially determined by Southern blot described elsewhere (Winnier *et al.*, 1995; Zhang and Bradley, 1996). Subsequently, each allele was identified by PCR using the following primers. For *Bmp2*, primer 1, 5'-AGCATGAACCCTCATGTGTTGG-3'; primer 2, 5'-GTGACATTAGGCTGCTGTAGCA-3'; primer 3, 5'-GAGACTAGTGAGACGTGCTACT-3'. For *Bmp4*, primer 4, 5'-TTGATCTTTCGACCTGTTTIA-3'; primer 5, 5'-ACGACCATCAGCATTCCGG-3'; primer 6, 5'-GAATTCGC CAATGACAAGAC-3'.

Histology

Embryos were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with Hematoxylin and Eosin (H&E). Some sections from adult heart were stained with Masson's trichrome to visualize collagen fibers.

Immunohistochemistry

Immunohistochemistry was performed by standard procedures. Briefly, 6 μ m paraffin sections were subjected to antigen retrieval (10 mM citrate buffer, pH 6.0 with microwave treatment for 10 minutes) and subsequently incubated with anti-SOX9 (H-90, Santa Cruz), anti-MSX2 (M1069, Sigma), anti-SP-C (AB3428, Millipore), or anti-CC10 (T-18, Santa Cruz) with dilution of 1/10-1/100 at 4°C for overnight. Either HRP conjugated or Alexa fluor-488 labeled secondary antibodies were used for detection.

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