Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning

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Summary

Cardiac cushion development provides a valuable system to investigate epithelial to mesenchymal transition (EMT), a fundamental process in development and tumor progression. In the atrioventricular (AV) canal, endocardial cells lining the heart respond to a myocardial-derived signal, undergo EMT, and contribute to cushion mesenchyme. Here, we inactivated bone morphogenetic protein 2 (*Bmp2*) in the AV myocardium of mice. We show that *Bmp2* has three functions in the AV canal: to enhance formation of the cardiac jelly, to induce endocardial EMT and to pattern the AV myocardium. *Bmp2* is required for myocardial expression of *Has2*, a crucial component of the cardiac jelly matrix. During EMT, Bmp2 promotes expression of the basic helix-loop-helix factor *Twist1*, previously implicated in EMT in cancer metastases, and the homeobox genes *Msx1* and *Msx2*. Deletion of the Bmp type 1A receptor, *Bmpr1a*, in endocardium also resulted in failed cushion formation, indicating that *Bmp2* signals directly to cushion-forming endocardium to induce EMT. Lastly, we show that *Bmp2* mutants failed to specify the AV myocardium with loss of *Tbx2* expression uncovering a myocardial, planar signaling function for *Bmp2*. Our data indicate that *Bmp2* has a crucial role in coordinating multiple aspects of AV canal morphogenesis.

Key words: Bone morphogenetic protein, Epithelial-mesenchymal transition, Cardiac morphogenesis, Mouse

Introduction

Abnormal valve development is a common human congenital anomaly, affecting approximately 1% of all live births (Armstrong and Bischoff, 2004; Barnett and Desgrosellier, 2003). Because of its prevalence, it is important to understand the mechanisms underlying cushion and valve development. During early heart development, a subset of endocardial cells break homotypic cell adhesive contacts, undergo cytoskeletal changes and invade the matrix-rich cardiac jelly to form endocardial cushions (Armstrong and Bischoff, 2004; Eisenberg and Markwald, 1995). The localized nature of the epithelial to mesenchymal transition (EMT) suggests a mechanism to molecularly distinguish the myocardium and/or endocardium of the atrioventricular (AV) canal from the rest of the non-cushion-forming heart. The signals that induce EMT have been investigated in chick embryos using a three-dimensional collagen gel invasion assay (Runyan and Markwald, 1983). Data from these experiments revealed that Tgfβ2 and Tgfβ3 perform crucial and sequential functions in cushion morphogenesis. In chick embryos, Tgfβ2 is thought to mediate the activation of endocardium, while Tgfβ3 functions in mesenchymal invasion (Camenisch et al., 2002).

In mouse embryos, the signals that induce EMT are less well understood. Data from the in-vitro collagen gel assays suggested that Tgfβ2 is the signal required for EMT; however, mice homozygous for a null allele of Tgfβ2 still make cushions, indicating that other signals are required in vivo to induce EMT in mammals (Bartram et al., 2001; Camenisch et al., 2002; Sanford et al., 1997). Notch signaling, through regulation of the *Snai1* (previously *Snail*) repressor, has recently been shown to be required for cushion formation through downregulation of vascular/endothelial-cadherin (VE-cadherin) in cushion endocardium (Timmerman et al., 2004). In zebrafish, Wnt signaling has been implicated in the EMT required for valve formation (Hurlstone et al., 2003). However, mice with an endocardial-specific deletion of β-catenin have cushions, although defective, suggesting that Wnt signaling is not essential for EMT in mammals (Liebner et al., 2004).

In addition to providing a signal to the endocardium for AV cushion formation, the AV myocardium is phenotypically distinct from the chamber myocardium. Chamber myocardium is coupled intercellularly, has a fast contraction pattern and expresses the chamber-specific genes *Cx40* (*Gja5* – Mouse Genome Informatics), *Anf* (*Nppa* – Mouse Genome Informatics) and chisel (*Smpx* – Mouse Genome Informatics). By contrast, the smooth-walled myocardium of the AV canal retains an embryonic phenotype and fails to express genes that are found in the chamber myocardium (Habets et al., 2002).

T-box (Tbx) genes have been implicated in regional patterning of the myocardium (Plageman and Yutzey, 2005). *Tbx2* has been shown to repress expression of chamber-specific genes in the AV myocardium. Mice with a *Tbx2* loss-of-function have expansion of chamber-specific genes into the AV myocardium (Harrelson et al., 2004). Moreover, *Tbx2* has been

shown to bind to an element in the *Anf* gene that represses *Anf* expression in AV myocardium through competition with *Tbx5* (Habets et al., 2002). In other experiments, *Tbx20* has been shown to directly repress *Tbx2*, indicating that a *Tbx* gene regulatory network functions to regionally pattern the myocardium (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005).

Previous data revealed that *Bmp2* is expressed in the AV myocardium at 9.5 and 10.5 days post coitum (dpc) and in the cushion mesenchyme at later stages (Lyons et al., 1990; Sugi et al., 2004). Moreover, gene inactivation studies in mice revealed that *Bmp2* was necessary for early myocardial development (Zhang and Bradley, 1996). *Bmp2* has been suggested to have a role in valve morphogenesis in mouse embryos, based on data from the collagen gel invasion assay (Sugi et al., 2004). In that work, Bmp2 could substitute for myocardium to induce endocardial EMT. Moreover, noggin treatment of explants efficiently inhibited EMT. However, other data indicated that Bmp2 was insufficient to induce EMT on its own but could enhance Tgfβ transformation activity (Yamagishi et al., 1999).

Here, we provide strong genetic evidence that *Bmp2* induces AV cushion EMT in mammals. We inactivated *Bmp2* specifically in the AV myocardium and found that *Bmp2* mutant embryos failed to form AV cushions. Expression of *Has2* was decreased in *Bmp2* mutant embryos with reduced formation of the cardiac jelly. Furthermore, inactivation of the Bmp type 1A receptor, *Bmpr1a*, in endocardium also resulted in loss of AV cushion formation, indicating that Bmp2 signaled directly to the endocardial cushions. Our findings also indicate that the basic helix-loop-helix (bHLH) transcription factor *Twist1* is a downstream effector of Bmp2 in EMT. We also found that *Bmp2* has a planar signaling function to regulate patterning of the AV myocardium through regulation of *Tbx2* expression. Taken together, our data indicate that *Bmp2* has a crucial function in coordinating cushion morphogenesis with AV myocardial patterning.

Materials and methods

Generation of the *Bmp2^{floxneo}* and *Bmp2^{null}* alleles

The *Bmp2^{floxneo}* allele has been described (Ma and Martin, 2005). The *Bmp2^{floxneo}* allele contains LoxP sites flanking Bmp2 exon 3 that encodes the mature peptide. Deletion of this exon was shown to result in a null *Bmp2* allele. To generate the *Bmp2^{null}* allele, we used the *NestinCre* transgene, which directs efficient cre activity in the germline (Trumpp et al., 1999). For genotyping, DNA was extracted from yolk sacks or tails of embryos and adult mice, respectively. PCR was used to determine the genotype of the *Bmp2^{null}* allele with the primers (5′-AAG TCT CCT CCT TCA TCA GTA TAC GCT CG-3′) and (5′-GAT ATC GAA TTC GAT ATC AAG CTG AT-3′) located in the neomycin cassette. The amplified product is 100 bp.

Whole-mount in-situ hybridization

Whole-mount and section in-situ hybridization was performed as previously described (Lu et al., 1999). Details about probes will be provided upon request. In all in-situ experiments, at least three mutants and three control embryos were analyzed for each experiment.

lacZ staining and histology

For histology, embryos were fixed overnight in Bouin's fixative or buffered formalin, dehydrated through graded ethanol and embedded in paraffin. Sections were cut at 7-10 μm and stained with H&E. Staining for *lacZ* was as previously described (Lu et al., 1999).

Immunohistochemistry

Tissue sections were deparaffinized, rehydrated and boiled for 5 minutes in 10 mmol/l sodium citrate for antigen retrieval. After washing in PBS, the nuclear factor of activated T-cells (NFAT) c1 (Cat. No.: 56602; BD Pharmingen, San Jose, CA 95131, USA) primary antibody (1:250 dilution), was applied and incubated overnight at 4°C. The sections were washed and incubated consecutively with biotin-labeled secondary antibody (sc-2017; Santa Cruz, CA 95060 USA). The secondary antibodies were detected using the avidin-biotin method. To detect Phospho-Smad1/Smad5/Smad8, sections were blocked in PBS/0.05% BSA and primary antibody (#9511′; Cell Signaling, MA 01915, USA) was applied with 1:200 dilution for overnight at 4°C. Sections were washed and incubated with secondary antibody (Cat. No.: DPVR-15DAB). Color was developed with diaminobenzidine (DAB) provided by the kit of secondary antibody and the sections were counterstained with hematoxylin. For control staining, preimmune serum was used instead of the primary antibody.

Results

Bmp2 has a dynamic expression pattern in the heart

Bmp2 expression was examined by whole-mount in-situ hybridization with a *Bmp2* exon3 probe (Fig. 1). *Bmp2* was detected in the cardiac crescent at 7.5 dpc, consistent with its role in early cardiac morphogenesis (Fig. 1A) (Schultheiss et al., 1997; Zhang and Bradley, 1996). At 8.5 dpc, *Bmp2* was expressed bilaterally in the sinus venosus (Fig. 1B,C), while at 9.5 and 10.5 dpc, *Bmp2* was abundantly expressed in the AV myocardium and at lower levels in the outflow tract (OFT; Fig. 1D,E,F,G). In the 11.5 dpc embryo, *Bmp2* mRNA expression in the AV canal was extinguished, although low-level expression was still detected in the OFT and ventricular myocardium (Fig. 1H). By 12.5 dpc, *Bmp2* expression was detected at low levels in the OFT and left ventricular myocardium (Fig. 1I). Taken together, these data indicate that, in addition to an early role in cardiac induction, *Bmp2* also probably functions in the development of the inflow and AV canal. *Bmp2* also may have a role in OFT and ventricular myocardial development.

Inactivation of *Bmp2* in the AV myocardium

To directly investigate *Bmp2* function in cardiac development, we constructed a *Bmp2* conditional null allele, the *Bmp2^{floxneo}* allele, which contained *LoxP* sites surrounding exon 3 encoding the mature *Bmp2* peptide. Deletion of exon 3 has been shown to result in a *Bmp2* null allele (Ma and Martin, 2005). To inactivate *Bmp2* in the heart, we used the *Nkx2.5^{cre}* knock-in allele, which directs cre activity to the anterior splanchnic mesoderm of the primary heart field and within the mature heart (Fig. 2A,B) (Liu et al., 2004; Moses et al., 2001).

To generate *Bmp2* cardiac-specific mutant embryos, we crossed the *Nkx2.5$^{cre+/–}$* mice to the *Bmp2$^{floxneo+/–}$* mice to obtain *Nkx2.5^{cre}; Bmp2^{floxneo}* compound heterozygotes. Intercrosses between these mice and the *Bmp2^{floxneo}* homozygous mice resulted in recovery of 25% of embryos that were *Nkx2.5^{cre}; Bmp2$^{floxneo/floxneo(fff)}$*, hereafter referred to as *Bmp2* CKO. This crossing strategy required that both *Bmp2^{floxneo}* alleles undergo cre-mediated recombination to generate a *Bmp2* null cell. Because there is a delay in the timing

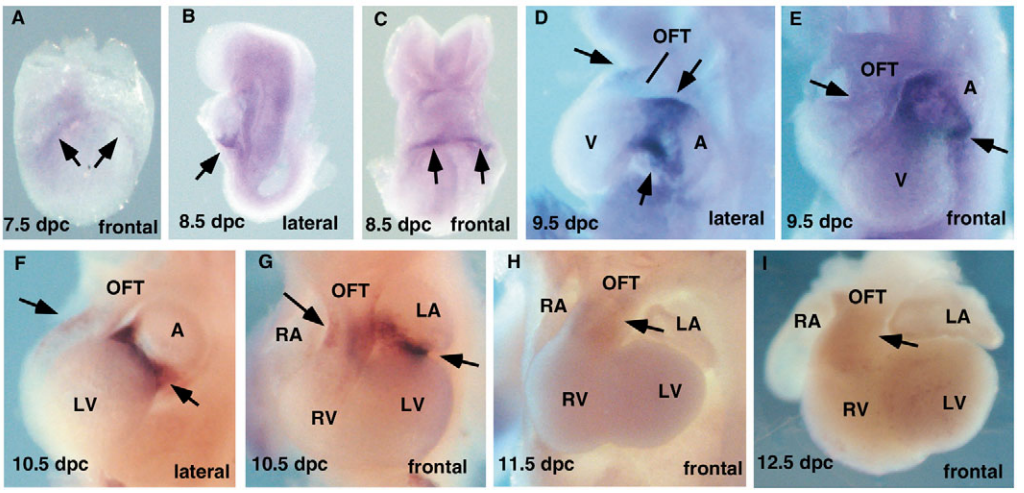

Fig. 1. *Bmp2* expression pattern during cardiogenesis. Whole-mount in-situ hybridization with *Bmp2* exon3 probe shows *Bmp2* expression pattern (A-I). At 7.5 dpc, *Bmp2* expression can be visualized bilaterally in the primitive heart tube (A, indicated by arrows). Side view (B) and frontal view (C), indicating that at 8.5 dpc *Bmp2* is expressed in the sinus venosus. At 9.5 dpc, both lateral view (D) and frontal view (E) showed that *Bmp2* is highly expressed in the myocardium of the AV canal, as well as lower level of expression in the outflow tract (indicated by arrows). (F,G) Side view and frontal view, showing that at 10.5 dpc *Bmp2* expression is maintained in the myocardium of the AV cushion region and in the outflow tract (arrows). At 11.5 and 12.5 dpc, *Bmp2* expression decreased significantly, as denoted by arrows (H,I). A, atrium; LA, left atrium; LV, left ventricle; OFT, outflow tract; RA, right atrium; RV, right ventricle; V, ventricle.

of cre-mediated recombination as intracellular cre protein accumulates, we predicted that the two-allele recombination strategy would result in a slight delay in *Bmp2* cardiac inactivation (Nagy, 2000). This would provide a stable genetic system to study *Bmp2* in either the sinus venosus or the AV canal.

To determine the timing and extent of the *Bmp2* cardiac-specific deletion, we performed whole-mount analysis with the *Bmp2* exon 3 probe that is deleted in the *Bmp2* CKO mutant embryos. In wild-type and *Bmp2* CKO mutant embryos, expression of *Bmp2* exon 3 was readily detectable bilaterally in the sinous venosus at 8.5 dpc (Fig. 2C,D). However, by 9.0 dpc *Bmp2* was undetectable in AV myocardium of *Bmp2* CKO mutant embryos (Fig. 2E,F). At 9.5 dpc, *Bmp2* exon 3 was absent in the AV myocardium and formation of the AV cushions was disrupted in the *Bmp2* CKO embryos (Fig. 2G-

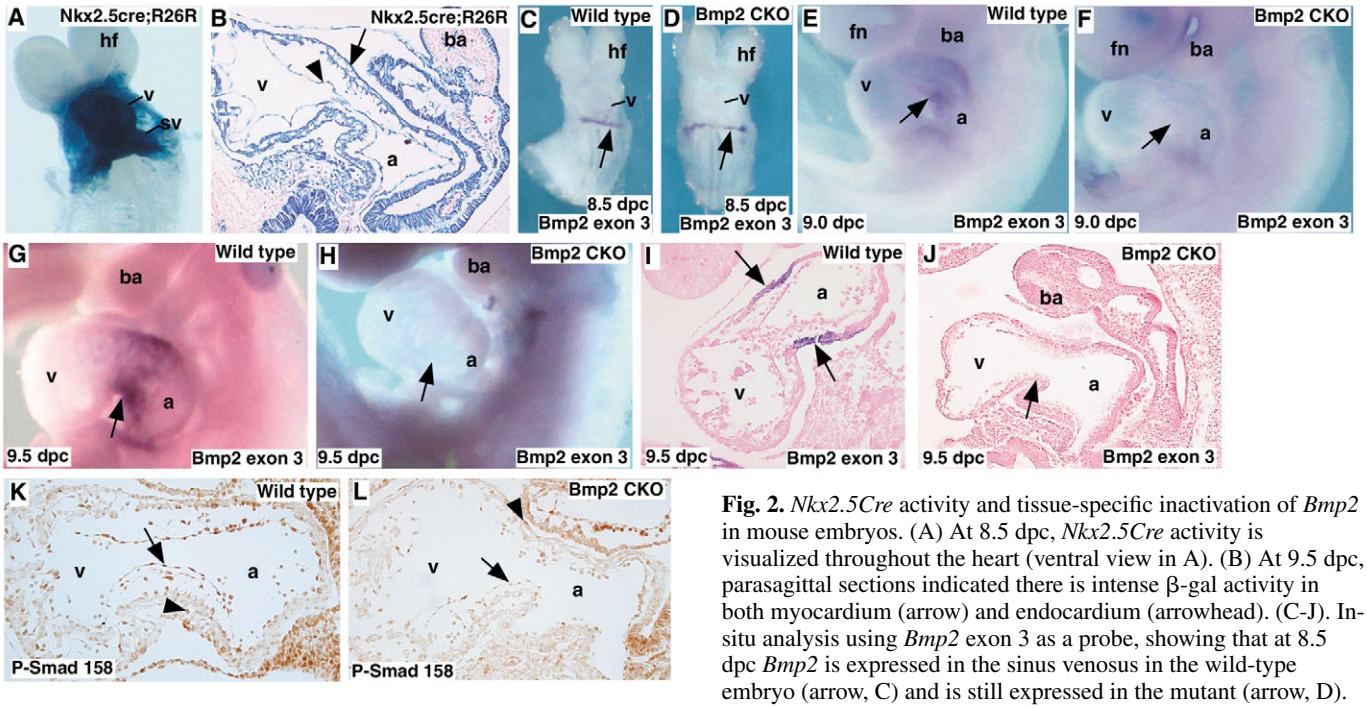

Fig. 2. *Nkx2.5Cre* activity and tissue-specific inactivation of *Bmp2* in mouse embryos. (A) At 8.5 dpc, *Nkx2.5Cre* activity is visualized throughout the heart (ventral view in A). (B) At 9.5 dpc, parasagittal sections indicated there is intense β-gal activity in both myocardium (arrow) and endocardium (arrowhead). (C-J). In-situ analysis using *Bmp2* exon 3 as a probe, showing that at 8.5 dpc *Bmp2* is expressed in the sinus venosus in the wild-type embryo (arrow, C) and is still expressed in the mutant (arrow, D). At 9.0 dpc (E,F) and 9.5 dpc (G,H,I,J), *Bmp2* is highly expressed in the myocardium of the control AV canal (arrow, E,G,I) but is absent in the *Bmp2* CKO mutant (arrow, F,H,J). I and J are parasagittal sections of embryos in G and H. (K,L) Immunohistochemistry of phospho-Smad1/5/8, effectors of Bmp signaling, indicating Bmp-responding cells in the endocardium (denoted by arrow) and myocardial cells (arrowhead) of the AV canal at 9.5 dpc (K). In the *Bmp2* CKO mutant, the phospho-Smad 1/5/8 signal is reduced in both cell populations (L). a, atrium; ba, branchial arch; fn, fronto-nasal process; hf, head fold; sv, sinus venosus; v, ventricle.

Table 1. Embryo recovery: *Nkx2.5*^{Cre};*Bmp2f/+* × *Bmp2f/f* intercrosses

Stage (dpc)	*Nkx2.5*^{Cre};*Bmp2f/+*	*Bmp2f/+*	*Bmp2f/f*	*Nkx2.5*^{Cre};*Bmp2f/f*	Mutant
8.5-9.0	21	23	18	31	33%
9.5	144	136	171	170	27%
10.5	6	11	2	12	38%
12.5-birth	18	17	10	0	0%

J). These data indicate that the early expression of *Bmp2* is intact in the *Bmp2* CKO embryos but by 9.0 dpc, *Bmp2* expression in the AV myocardium is absent, allowing us to focus on *Bmp2* function in the AV canal.

Bmp2 signals directly to the cushion endocardium

To establish functionally that Bmp signaling had been disrupted in the *Bmp2* CKO mutant embryos, we performed immunostaining with an antibody that recognizes P-Smad 1/5/8. Smad 1, Smad 5 and Smad 8, the receptor regulated (R-Smad) effector molecules for Bmp signaling, are phosphorylated by a ligand-bound receptor complex. Phosphorylation of the Bmp-dependent R-Smad is an indication that cells are actively receiving a Bmp signal. In the 9.5 dpc wild-type embryos, approximately 80% of endocardial cells in the AV canal were stained for P- Smad1/5/8 (Fig. 2K). We also noted that approximately 10-15% of the AV myocardium was positive for Smad 1/5/8 immunostaining. By contrast, in the *Bmp2* CKO mutant embryos, Smad 1/5/8-positive cells were drastically reduced in the endocardium, and only a few scattered cells in the AV myocardium were immunoreactive (Fig. 2L). From these data, we conclude that Bmp signaling to the AV cushion-forming endocardium is severely disrupted in the *Bmp2* CKO embryos at 9.5 dpc, while Bmp signaling in the AV myocardium is significantly reduced.

Embryonic lethality and AV cushion defects in the absence of *Bmp2* in the AV myocardium

Analysis of litters at embryonic time points revealed that *Bmp2* CKO mutant embryos were indistinguishable from wild-type littermates at 8.5 and 9.0 dpc. At 9.5 dpc, *Bmp2* CKO mutant embryos had abnormal morphology of the AV canal constriction but were still recovered at Mendelian frequencies (Table 1). By 10.5 dpc, all *Bmp2* CKO mutants exhibited pericardial effusion and growth retardation, indicating that these embryos suffered from heart failure at this stage.

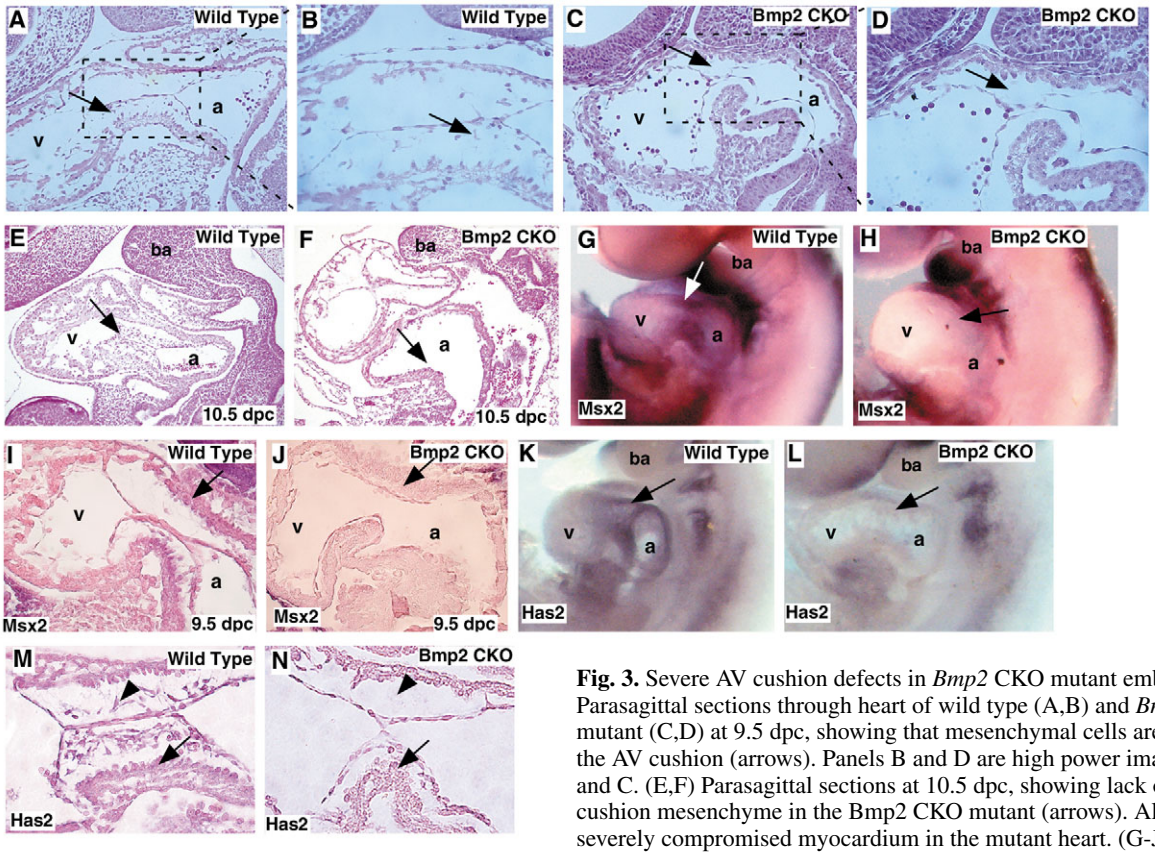

Fig. 3. Severe AV cushion defects in *Bmp2* CKO mutant embryos. Parasagittal sections through heart of wild type (A,B) and *Bmp2* CKO mutant (C,D) at 9.5 dpc, showing that mesenchymal cells are absent in the AV cushion (arrows). Panels B and D are high power images of A and C. (E,F) Parasagittal sections at 10.5 dpc, showing lack of AV cushion mesenchyme in the Bmp2 CKO mutant (arrows). Also note the severely compromised myocardium in the mutant heart. (G-J) In-situ hybridization indicated that *Msx2* is expressed in the AV myocardium and migrating mesenchymal cells (arrow, G), but its expression is diminished in the mutant (arrow, H). Panels I and J are parasagittal sections of embryos in G and H, showing lack of *Msx2* expression in the mutant (arrows). (K-N) *Has2*, encoding an extracellular matrix protein, is present in the AV myocardium (arrow) and migrating mesenchyme (arrowhead) in the 9.5 dpc wild-type embryos (K,M) but is reduced in the *Bmp2* CKO myocardium (L,N). M and N are parasagittal sections of K and L. a, atrium; ba, branchial arch; v, ventricle.

Histological analysis revealed there was no discernible abnormality in cardiac structure at 8.5 dpc in the *Bmp2* CKO mutant embryos (data not shown, see Fig. 5A,B). However at 9.5 dpc, all *Bmp2* CKO mutant embryos had severe defects in AV cushion morphogenesis (Fig. 3A-D). In the wild-type embryos, there was expansion of the space between myocardium and endocardium as the cardiac jelly was laid down by the AV myocardium (Krug et al., 1985). However, in the *Bmp2* CKO mutant embryos the space between the myocardium and the endocardium failed to expand in 43% (10 of 23) of the 9.5 dpc *Bmp2* CKO mutant embryos, indicating a defect in cardiac jelly formation (Fig. 3A-F,I-J). We noted that the expression of *Has2*, a crucial component of the cardiac jelly, was reduced in the *Bmp2* CKO mutant myocardium (see below).

In wild-type embryos, migrating mesenchymal cells were observed in the forming AV cushions at 9.5 dpc (Fig. 3A,B). Parasagittal sections through *Bmp2* CKO mutant embryos indicated that the invasive mesenchyme failed to form (Fig. 3C,D). The failed EMT phenotype was observed in all *Bmp2* CKO mutant embryos, including embryos with cardiac jelly deposition, suggesting that the EMT defect was probably not secondary to cardiac jelly loss (Fig. 3C,D). Analysis of *Has2* and *Msx2,* markers of the transformed, migrating mesenchyme, revealed that the endocardium had not been induced to undergo EMT (Fig. 3G-N). Taken together, these data indicate that Bmp2 is required to both promote cardiac jelly formation and induce AV endocardial EMT.

Inactivation of *Bmpr1a* in the endocardium disrupts endocardial cushion formation

In order to establish more firmly that Bmp2 signaled directly to the underlying endocardium, we generated mouse embryos that were deficient in the competence to receive Bmp signals within the endocardium. We inactivated the type 1A Bmp receptor, *Bmpr1a*, in the endocardium using the *Tie2 cre* transgenic line (Kisanuki et al., 2001). *Bmpr1a* is a major type 1 receptor for *Bmp2* and *Bmp4* (von Bubnoff and Cho, 2001). Because germline mutation of *Bmpr1a* is early embryonic lethal, we used the *Bmpr1a* conditional null ($Bmpr1a^{flox}$) allele (Mishina et al., 2002; Mishina et al., 1995). We generated embryos that carried $Tie2^{cre}$ and the $Bmpr1a^{flox}$ and $Bmpr1a^{null}$ ($Bmpr1a^{n/f}$) alleles. We examined $Tie2^{cre}$; $Bmpr1a^{n/f}$ mutant embryos at 9.5 dpc and found that embryos deficient for *Bmpr1a* in the cushion endocardium failed to form cushions (Fig. 4A,B). It is notable that one-third of $Tie2^{cre}$; $Bmpr1a^{n/f}$ mutants made cushions. This probably results from variability of cre activity; however, it is possible that other type 1 receptors also have a function in the cushion endocardium.

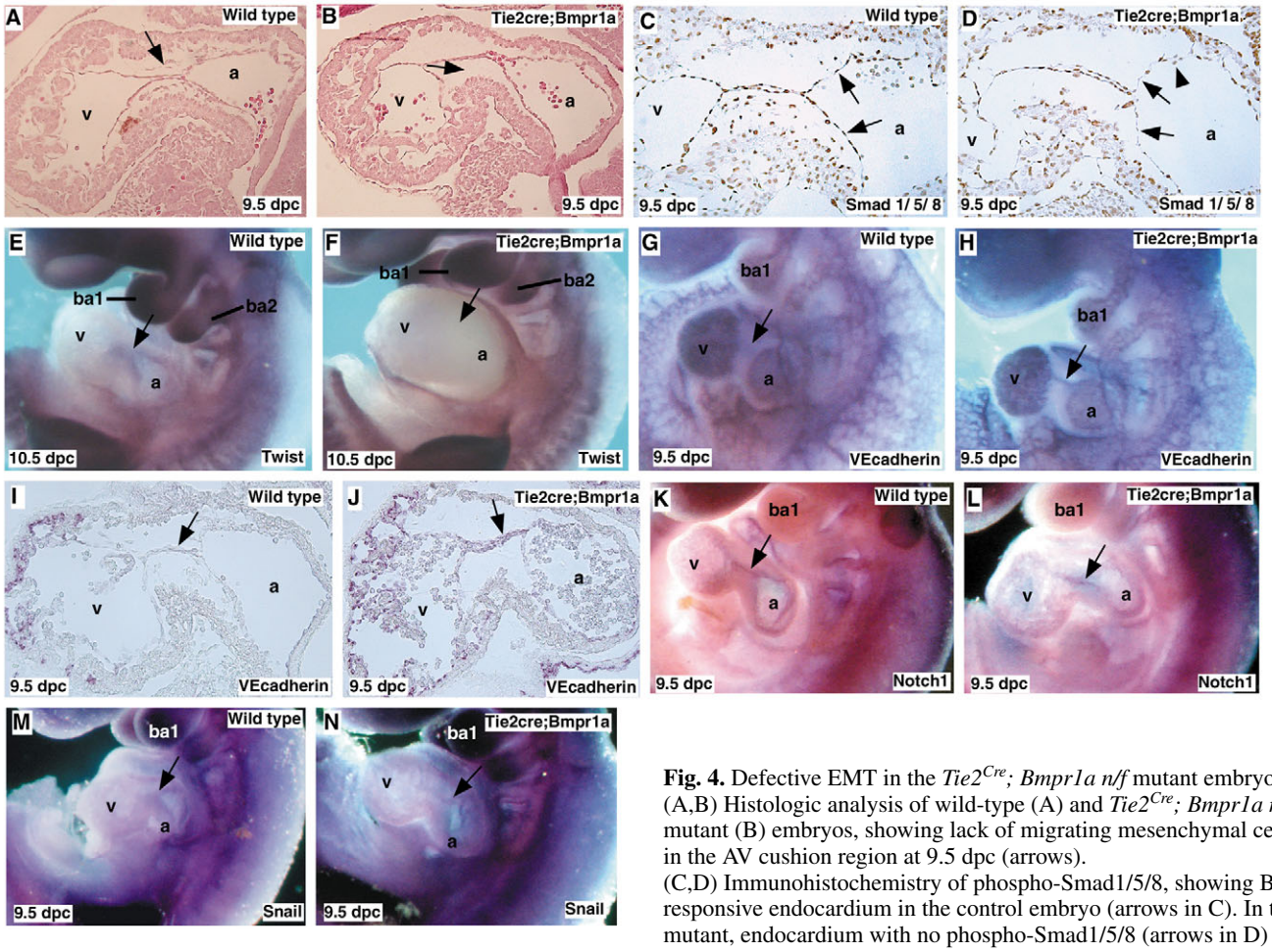

Fig. 4. Defective EMT in the $Tie2^{Cre}$; *Bmpr1a n/f* mutant embryos. (A,B) Histologic analysis of wild-type (A) and $Tie2^{Cre}$; *Bmpr1a n/f* mutant (B) embryos, showing lack of migrating mesenchymal cells in the AV cushion region at 9.5 dpc (arrows). (C,D) Immunohistochemistry of phospho-Smad1/5/8, showing Bmp-responsive endocardium in the control embryo (arrows in C). In the mutant, endocardium with no phospho-Smad1/5/8 (arrows in D) or reduced phospho-Smad1/5/8 is shown (D, arrowhead). (E-N) Whole-mount in-situ hybridization analysis with *Twist1* (E,F), VE-cadherin (G-J), *Notch1* (K,L) and *Snai1* (M,N). Genotypes are shown, and arrows denote hybridization signal. a, atrium; ba1, first branchial arch; ba2, second branchial arch; v, ventricle.

To determine the extent of Bmpr1a inactivation in the *Tie2^{cre}; Bmpr1a$^{n/f}$* mutants, we examined Smad 1/5/8 immunostaining in the AV endocardium of wild-type and *Bmpr1a* mutant embryos. In the wild type, most endocardial cells stained positive for P-Smad 1/5/8 (Fig. 4C), while in the *Tie2^{cre}; Bmpr1a* mutant embryos approximately 50% fewer strong positive cells were detected (Fig. 4D). We noted that the main reduction in P-Smad 1/5/8 immunoreactivity in *Tie2^{cre}; Bmpr1a* mutants was in endocardium located toward the atrium (Fig. 4D, *n*=3). The significance of this observation is presently unclear. These data indicate that while endocardial inactivation of *Bmpr1a* results in incomplete loss of endocardial Bmp responsiveness, the AV cushion phenotype is strong, revealing that AV cushion EMT is very sensitive to small decreases in Bmp signaling. Moreover, our findings support the hypothesis that other type 1 receptors may have overlapping function in the AV canal endocardium.

We studied expression of *Twist1*, encoding a bHLH transcription factor that is expressed in AV endocardium and has been implicated in EMT in cancer metastasis (Yang et al., 2004). In wild-type embryos, we found that *Twist1* was expressed in both AV endocardium and cushion mesenchyme (Fig. 4E and see Fig. 5E,G). In the *Tie2^{cre}; Bmpr1a$^{n/f}$* mutant AV canal, we found that *Twist1* expression was reduced at 9.5 dpc and absent by 10.5 dpc (Fig. 4F).

Twist has been shown to negatively regulate expression of E-cadherin in breast cancer cells, prompting us to examine expression of *VE-cadherin* in the AV canal endocardium of *Tie2^{cre}; Bmpr1a$^{n/f}$* mutant embryos (Kang and Massague, 2004). During EMT, *VE-cadherin* is normally downregulated (Fig. 4G,I); however, in the *Tie2^{cre}; Bmpr1a$^{n/f}$* mutant endocardium VE-cadherin expression persisted when compared with the wild-type embryo (Fig. 4H,J). Notch signaling has been shown to be required for EMT in the AV canal (Timmerman et al., 2004). In the *Tie2^{cre}; Bmpr1a* mutants, we found that expression of *Notch1* was similar to

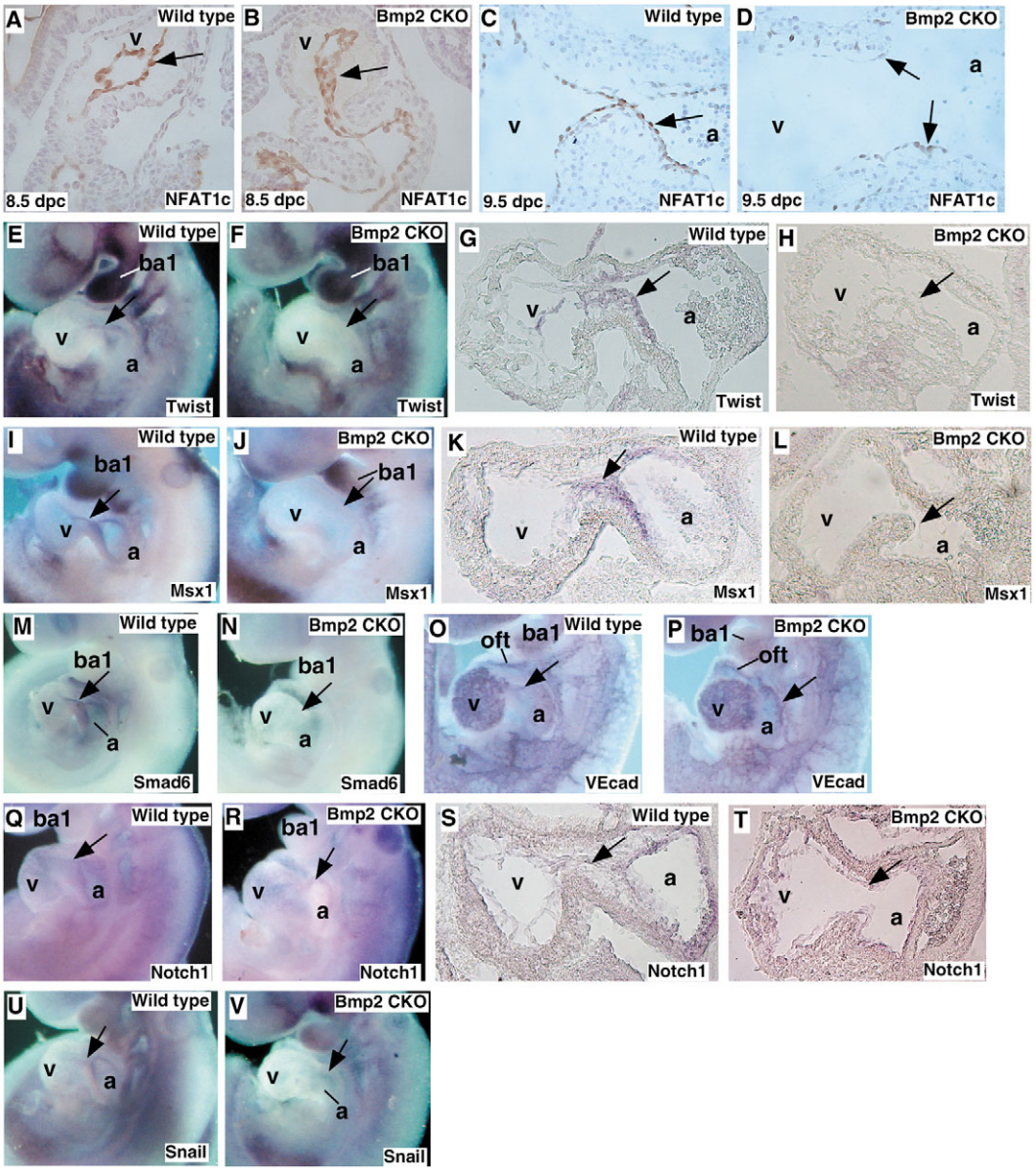

Fig. 5. Defective endocardium in *Bmp2* CKO mutant embryos. Immunohistochemistry of NFATc1 in wild-type (A) and *Bmp2* CKO mutant (B) embryos at 8.5 dpc. At 9.5 dpc, there is upregulation of NFATc1 expression in the AV cushion endocardium (C) in wild-type embryos but in the Bmp2 mutant many of the endocardial cells are negative and there is no regional specificity of the signal (D). Arrows denote positively stained cells. Whole-mount in-situ hybridization followed by sectioning for some probes with *Twist1* (E-H), *Msx1* (I-L), *Smad6* (M,N), VE-cadherin (O,P), *Notch1* (Q-T) and *Snai1* (U,V) probes at 9.5 dpc in wild-type and Bmp2 CKO mutant embryos. Arrows denote positive hybridization signal or areas where signal is up- or downregulated in the mutant embryo. Probes and genotypes are labeled. a, atrium; ba1, first branchial arch; v, ventricle.

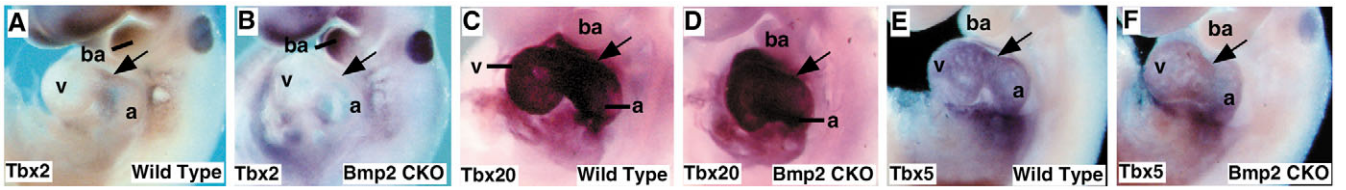

Fig. 6. *Tbx* expression in *Bmp2* CKO mutant embryos at 9.5 dpc. *Tbx2* mRNA expression is located in the myocardium of the AV canal in wild-type embryos (A) but is not present in the mutant (B, indicated by arrow). Other *Tbx* genes, including *Tbx5* and *Tbx20*, are expressed in the myocardium of the atrium and ventricle in wild-type embryos (C,E) and also in the *Bmp2* CKO mutant embryos (D,F). Arrows denote hybridization signal. a, atrium; ba, branchial arch; v, ventricle.

wild-type embryos (Fig. 4K,L). We also examined expression of *Snai1*, a zinc finger transcription factor that has been implicated in EMT and has been shown to be a target of Notch signaling in the AV endocardium (Timmerman et al., 2004). By contrast to *Twist1*, *Snai1* expression was unaffected by loss of *Bmpr1a* in AV endocardium (Fig. 4M,N). Taken together, these data indicate that Bmp2 signals directly to the cushion endocardium through the type 1 receptor, *Bmpr1a*.

Bmp2 regulates multiple genes in the AV endocardium

NFATc1 is regulated by a calcium-calcineurin pathway and can translocate to the nucleus upon activation (Crabtree and Olson, 2002). Moreover, *NFATc1* expression is upregulated in activated endothelial cells within the AV canal. At 8.5 dpc, *NFATc1* was present in the endocardium of both wild-type and *Bmp2* CKO mutant embryos (Fig. 5A,B). By 9.5 dpc, *NFATc1* was upregulated and nuclear localized in the wild-type AV cushion endocardium (Fig. 5C), while expression in the *Bmp2* CKO mutant embryo AV canal failed to be upregulated (Fig. 5D).

As for the $Tie2^{cre}$; $Bmpr1a^{n/f}$ mutant embryos, expression of *Twist1*, normally detected in the AV cushion endocardium and mesenchyme of wild-type embryos was not detected in the *Bmp2* CKO mutant endocardium (Fig. 5E-H). Bmp signaling regulates the *Msx1* homeobox gene in other developmental fields (Liu et al., 2005; Vainio et al., 1993). Expression of *Msx1* was detected in the AV endocardium of wild-type embryos but was absent in the *Bmp2* CKO mutant embryos (Fig. 5I-L). The inhibitory Smad, *Smad6*, is a negative regulator of Bmp signaling that is also transcriptionally regulated by Bmp signaling. Furthermore, *Smad6* has been shown to be a negative regulator of valve development (Desgrosellier et al., 2005; Galvin et al., 2000; Ishida et al., 2000). In wild-type embryos, *Smad6* was expressed in the AV endocardium, while in the *Bmp2* CKO mutants, *Smad6* expression was absent (Fig. 5M,N). This finding uncovers a negative feedback loop that functions to limit the extent of endocardial EMT.

During activation of the AV endocardium, VE-cadherin expression is downregulated in wild-type embryos, but in the *Bmp2* CKO mutant VE-cadherin persists (Fig. 5O,P). A Notch-Snai1 signaling pathway has been implicated in VE-cadherin downregulation in AV endocardium (Timmerman et al., 2004). In the *Bmp2* CKO mutants, *Notch 1* and *Snai1* expression was reduced compared with wild-type embryos, indicating that the Notch and Bmp2 signaling pathways cooperate in AV cushion morphogenesis (Fig. 5Q-V). Taken together, our data indicate that Bmp2 induces EMT-promoting genes, such as *Twist1* and *Snai1*, and other genes that restrain EMT such as *Smad6*. Moreover, these data indicate that *Bmp2* CKO mutants, in contrast to *Bmpr1a*-deficient embryos, have defects in Notch-Snai1 signaling in the AV cushion endocardium (see Discussion).

Bmp2 regulates *Tbx2* in the AV myocardium to control chamber-specific gene expression

Previous studies in chick embryos had suggested that Bmp2 regulated *Tbx2* expression in the developing heart, prompting us to examine *Tbx2* expression in *Bmp2* CKO mutants (Yamada et al., 2000). In wild-type embryos, *Tbx2* was expressed specifically in the AV myocardium, while in the *Bmp2* CKO mutant embryos *Tbx2* expression was undetectable (Fig. 6A,B). By contrast, expression of *Tbx5* and *Tbx20* were unaffected by *Bmp2* deletion (Fig. 6C-F). These data indicate that Bmp2 promotes expression of *Tbx2* and so is important to maintain the correct ratio of *Tbx* genes in the AV myocardium.

In the AV canal, *Tbx2* is known to repress chamber-specific gene expression. Consistent with this, expression of the chamber-specific genes *Anf*, chisel, and *connexin 40* was limited to the ventricular and atrial myocardium in wild-type embryos (Fig. 7A,C,E,G,I,K). By contrast, in the *Bmp2* CKO mutant embryos, expression of all three chamber-specific genes was expanded into the AV myocardium (Fig. 7B,D,F,H,J,L). Markers of the AV myocardium, such as Tgfβ2 and *Lef1*, were lost in the *Bmp2* CKO mutant embryos (Fig. 7M-P). From these findings, we conclude that Bmp2 signaling directs regionalized myocardial patterning.

Discussion

In this work, we investigated *Bmp2* function in cardiac development by deleting *Bmp2* specifically in the AV myocardium. Our findings provide strong genetic evidence that *Bmp2* is a crucial signal for AV cushion formation in mammals. We found that *Bmp2* promotes cardiac jelly formation and signals directly to the AV endocardium through the type 1A receptor, *Bmpr1a*, to induce EMT. Moreover, our data reveal that *Twist1* is one target of *Bmp2* in the AV canal endocardium, suggesting a novel signaling pathway regulating EMT in valve formation. In addition, our findings indicate that *Bmp2* concurrently regulates myocardial patterning through regulation of *Tbx2* expression. Taken together, our data provide insights into the mechanisms coordinately regulating cushion development and myocardial patterning in mammals.

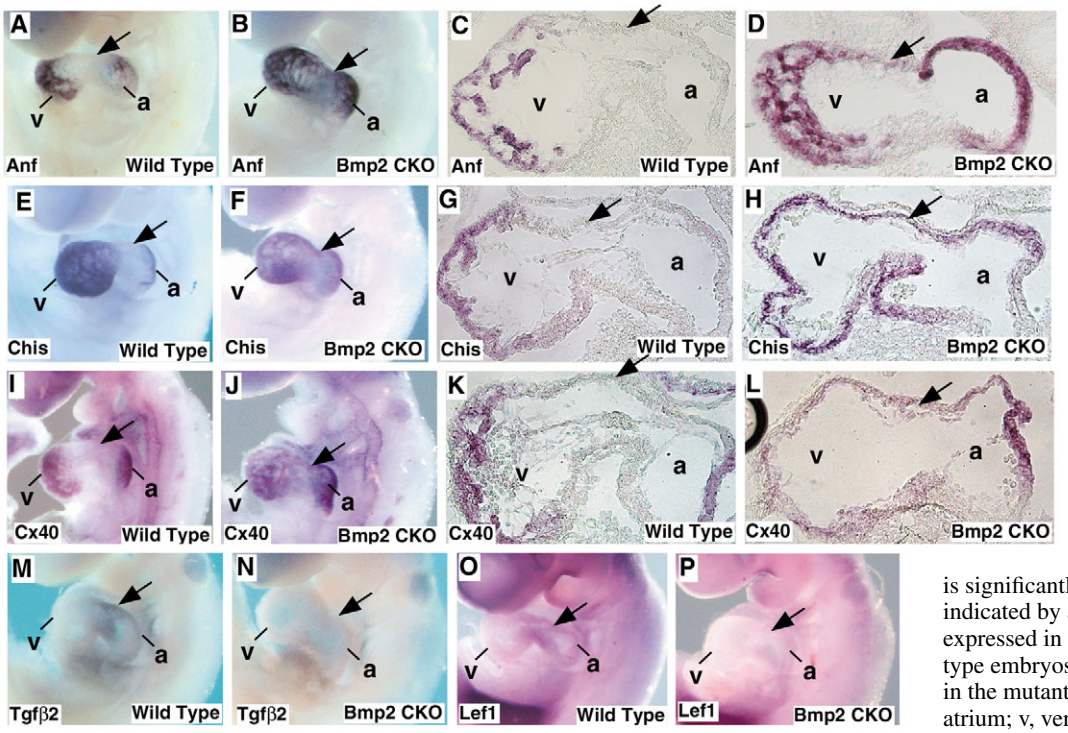

Fig. 7. AV myocardium defect in the *Bmp2* CKO mutants at 9.5 dpc. In wild-type embryos, chamber-specific genes, including *Anf* (A,C), chisel (E,G) and connexin 40 (I,K), are expressed in the myocardium of the atrium and ventricle but not in the AV myocardium (denoted by arrows). In the *Bmp2* CKO mutant, the expression of *Anf* (B,D), chisel (F,H) and connexin 40 (J,L) is expanded into the AV canal myocardium (denoted by arrows). Tgfβ2 is expressed in the AV myocardium in wild-type embryos (M) but is significantly reduced in the mutant, as indicated by arrows (N). *Lef1* is also expressed in the AV myocardium in wild-type embryos (O) but is significantly reduced in the mutant (P, indicated by arrow). a, atrium; v, ventricle.

Bmp2 is a crucial inducer of EMT in the AV cushion

The early lethality of the germline *Bmp2* null mice has hampered definitive insight into the role of *Bmp2* in AV cushion development (Zhang and Bradley, 1996). Recently reported experiments, using the collagen gel invasion assay, suggested that Bmp2 could substitute for myocardium to induce EMT in mouse embryos (Sugi et al., 2004). However, other experiments, also using the collagen gel invasion assay, showed that Bmp2 functioned only in combination with Tgfβ3 to promote EMT in chick embryos (Yamagishi et al., 1999). Furthermore, chick embryos treated with a retrovirus expressing Noggin caused defects in the OFT but did not affect the AV canal (Allen et al., 2001). Our data indicate that Bmp2 is the myocardial-derived signal that induces EMT in mammals. One likely explanation for the different results obtained from these studies is species-specific differences between mice and chicks (Camenisch et al., 2002).

Our findings indicate that *Bmpr1a* is a crucial type 1 receptor in the AV endocardium to transduce the Bmp signal required for EMT. Inactivation of *Bmpr1a* using the *Tie2^{cre}* transgenic line disrupted EMT in most mutant embryos examined. The *Tie2^{cre}; Bmpr1a n/f* mutant cushion phenotype indicates a direct requirement for a Bmp signal to induce EMT in the endocardium. Furthermore, our findings indicate that the *Tie2^{cre}; Bmpr1a n/f* mutants have normal cardiac jelly deposition, as *Has2* is expressed and cardiac jelly is deposited normally. Taken together, these findings provide strong evidence that the cushion defect in the *Bmp2* CKO mutants is not secondary to failure of cardiac jelly deposition or disruption of an indirect signal relay mechanism.

It is important to note that *Alk2* has also been shown to be required for EMT in the in-vitro explant system using chick embryos (Desgrosellier et al., 2005). A constitutively active *Alk2* was able to induce EMT, while Alk2 antisera could effectively inhibit EMT. Furthermore, mouse embryos deficient for *Alk2* in cushion endocardium had a severe defect in AV cushion morphogenesis (Wang et al., 2005). Our data indicate that the Notch-Snai1 pathway was intact in the *Tie2^{cre}; Bmpr1a n/f* mutant embryos, while this pathway was disrupted in the *Alk2* and *Bmp2* mutant embryos (this work) (Wang et al., 2005). This indicates that *Bmpr1a* and *Alk2* may activate distinct pathways in cushion endocardium. However, other genes such as *Msx1*, were reduced in both the *Bmpr1a* and *Alk2* mutants. Taken together, these findings indicate that signaling through *Alk2* and *Bmpr1a* activates distinct but overlapping target pathways. Alternatively, as some (approximately 33%) *Tie2^{cre}; Bmpr1a n/f* mutants made AV cushions, it is possible that there is partial redundancy between type 1 receptors in AV endocardium, similar to that observed in chondrocytes (Yoon et al., 2005). Future studies will be required to investigate these questions in more detail.

Previous work investigating Bmp ligands in cardiac development uncovered considerable functional redundancy. For example, *Bmp7* mutants have no phenotype in the heart as a result of redundancy with other Bmp ligands (Luo et al., 1995). *Bmp6; Bmp7* double mutants have delayed formation of the OFT cushions (Kim et al., 2001), while the *Bmp5; Bmp7* double mutants have severe defects in cushion formation (Solloway and Robertson, 1999). Moreover, we found evidence for overlapping function between *Bmp4* and *Bmp7* in OFT development (Liu et al., 2004). By contrast, we have found that *Bmp2* function is essential in the AV canal. It will be important in the future to investigate the functional relationship of the distinct classes of Bmp ligands in cushion morphogenesis.

Tgfβ and Bmp signaling in AV cushion morphogenesis

Tgfβ signaling is a crucial signal in avians for EMT in the AV cushions, as determined by the collagen invasion assay

(Barnett and Desgrosellier, 2003). However, although Tgfβ2 is clearly important for normal cushion development in mice, EMT can still occur in the Tgfβ2 null mice (Sanford et al., 1997). Our data, and the work of Sugi et al. (Sugi et al., 2004), indicate that much of the function of Tgfβ has been co-opted by Bmp signaling in mammals. In mice, current evidence suggests that Tgfβ2 expression is regulated by Bmp signaling. Inactivation of *Bmpr1a* in myocardium using an MHC cre transgene resulted in downregulation of Tgfβ2 expression in the AV canal (Gaussin et al., 2002). This suggested a cell-autonomous requirement for Bmp signaling in myocardium to maintain Tgfβ2 expression, which was important for the later aspects of AV cushion development. Furthermore, Bmp2 induces Tgfβ2 in explants cultured in collagen gel (Sugi et al., 2004), and Tgfβ2 expression is off or reduced in the AV cushions of *Bmp2* CKO mutant embryos (this work). Future experiments will be required to firmly establish the genetic relationship between Bmp signaling and Tgfβ2 transcriptional regulation.

The role of *Twist1* in AV cushion EMT

Twist1, first identified in *Drosophila* as a crucial regulator of gastrulation and mesoderm formation, has previously been recognized as an important developmental control gene in higher vertebrates. *Twist1* has been shown to be the gene mutated in Saethre-Chotzen syndrome, a haploinsufficient disorder that primarily affects cranial and limb development (Howard et al., 1997). In mouse embryos, *Twist1* is expressed in presomitic mesoderm, neural crest-derived mesenchyme of the head and branchial arches and in limb bud mesenchyme (Gitelman, 1997). *Twist1* null mutant embryos had failure of rostral neural tube closure, most likely as a result of a head mesenchyme deficiency, but defects in cardiac morphogenesis were not reported (Chen and Behringer, 1995).

Particularly relevant to the present study is the recent observation that *Twist1* promotes EMT in cancer cells and facilitates tumor metastasis. Twist1 regulates EMT through a mechanism involving repression of *E-cadherin* transcription (Kang and Massague, 2004; Yang et al., 2004). Our data indicate that *Twist1* expression was lost in both the *Bmp2* CKO and *Tie2^{cre}; Bmpr1a n/f* mutant embryos. Moreover, we observed a modest defect in VE-cadherin downregulation in the *Bmp2* and *Bmpr1a* mutant AV canals. These findings suggest that *Twist1* is an effector of the Bmp-signaling pathway in the AV canal that promotes endocardial EMT.

We observed only mild defect in VE-cadherin downregulation, despite loss of *Twist1* in the *Bmp2* CKO mutant AV canal. This is probably a result of compensatory function of the closely related *Twist2* gene. There are two *Twist* genes in mammals, *Twist1* and *Twist2*, which have redundant functions in cytokine regulation (Bialek et al., 2004; Li et al., 1995; Sosic et al., 2003). Further experiments will be required to investigate this possibility.

It is notable that the *Snai1* transcription factor has recently been shown to be required for AV canal EMT as part of the *Notch* signaling pathway (Timmerman et al., 2004). In *Drosophila*, *Twist* and *Snai1* are both required for correct dorsoventral patterning and mesoderm induction as part of the *Dorsal/Twist/Snai1* pathway (Stathopoulos and Levine, 2002). In vertebrates, both *Snai1* and *Twist1* have been shown to promote EMT through inhibition of *E-cadherin* expression. Our findings suggest that Bmp and Notch signaling cooperate to influence *VE-cadherin* expression. It will be interesting to investigate the functional relationship of Notch and Bmp signaling in AV canal EMT in future experiments.

Bmp2 regulates myocardial differentiation

We provide evidence that *Bmp2* is upstream of *Tbx2* in the AV myocardium. Our observation that myocardial patterning in the *Tie2^{cre}; Bmpr1a* mutants is normal argues against the hypothesis that an endocardial-derived signal regulates myocardial patterning. Further experiments will be required to investigate whether Bmp2 directly regulates *Tbx2* expression.

Our findings can be interpreted in the context of recent *Tbx20* inactivation studies that led to conflicting interpretations of the *Bmp2-Tbx2* regulatory relationship. *Tbx20* mutant embryos had expanded *Tbx2* expression throughout the myocardium despite diminished *Bmp2* expression, suggesting that *Tbx2* regulation is *Bmp2*-independent (Cai et al., 2005; Stennard et al., 2005). Another, independently generated, *Tbx20* null allele resulted in ectopic Bmp2 with resulting expanded *Tbx2* (Singh et al., 2005). Our data support the conclusion that Bmp2 promotes *Tbx2* expression in the AV myocardium.

It is interesting to note that *Tbx20* and *Tbx2* are co-expressed in the AV myocardium, indicating an AV-canal-specific mechanism that allows *Tbx2* to escape *Tbx20*-imposed repression. Our data suggest that Bmp2-signaling counters the repressive activity of Tbx20 in the AV myocardium. Because *Tbx20* is expressed normally in the *Bmp2* CKO mutant embryos, Bmp2 may regulate Tbx20 post-transcriptionally. Bmp2 may also induce genes that functionally inhibit Tbx20 activity. The precise mechanism underlying this inhibition of Tbx20 is presently unclear and awaits future experiments.

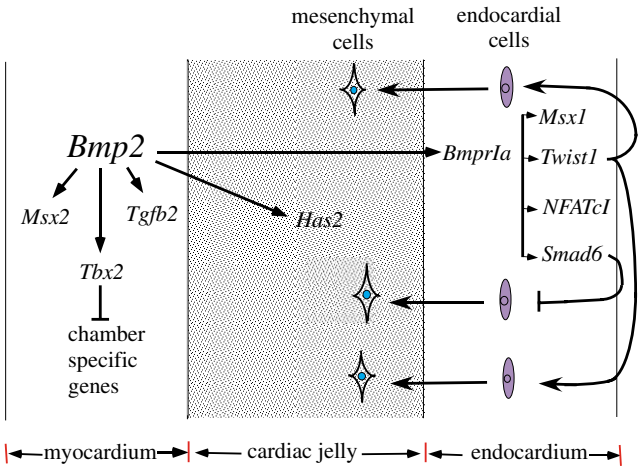

Fig. 8. Summary of *Bmp2* function in the AV canal. Bmp2 signals to the cushion endocardium through Bmpr1a to promote expression of genes that support EMT, such as *Twist* and *NFATc1*. Bmp2 also induces expression of *Smad6*, an inhibitory Smad, to limit the extent of EMT in the forming AV cushion. An additional function of Bmp2 is to induce expression of *Has2*, thereby accentuating cardiac jelly deposition. Finally, within the AV myocardium, Bmp2 signaling is required for *Tbx2* expression, which normally represses expression of chamber-specific genes in the AV canal. Arrows denote genetic, not necessarily direct, relationships.

In conclusion, our findings reveal that *Bmp2* is required for EMT in the mammalian AV canal (see Fig. 8). We have uncovered a genetic pathway involving the type 1 Bmp receptor, *Bmpr1a*, which functions in EMT. Our data also revealed that Bmp2 signaling is required for normal deposition of the cardiac jelly. Within the myocardium, Bmp2 functions upstream of *Tbx2* to control regionalized myocardial patterning.

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