

# Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3

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**Receptors for bone morphogenetic proteins (BMPs), members of the transforming growth factor- $\beta$  (TGF $\beta$ ) superfamily, are persistently expressed during cardiac development, yet mice lacking type II or type IA BMP receptors die at gastrulation and cannot be used to assess potential later roles in creation of the heart. Here, we used a Cre/lox system for cardiac myocyte-specific deletion of the type IA BMP receptor, ALK3. ALK3 was specifically required at mid-gestation for normal development of the trabeculae, compact myocardium, interventricular septum, and endocardial cushion. Cardiac muscle lacking ALK3 was specifically deficient in expressing TGF $\beta$ 2, an established paracrine mediator of cushion morphogenesis. Hence, ALK3 is essential, beyond just the egg cylinder stage, for myocyte-dependent functions and signals in cardiac organogenesis.**

**B**one morphogenetic proteins (BMPs), named for their first-discovered role in bone differentiation, mediate a diverse spectrum of developmental events, such as dorsal-ventral patterning, mesoderm specification, and the spacing of embryo implantation (1, 2). BMPs are postulated to mediate cardiac development in mammals, by extrapolation from the role of decapentaplegic, a related factor in *Drosophila* (3), their function in cardiac looping in fish (4) or frogs (5), and, especially, studies of cardiac myogenesis in avians (6, 7). Avian explant studies also implicate BMPs as an essential paracrine signal from myocardium adjacent to the endocardial cushion during the epithelial-mesenchymal transformation and later events that ultimately give rise to the atrio-ventricular (AV) valves (8). This process is known to involve TGF $\beta$ 2 and TGF $\beta$ 3 as well (9–12), but the molecular connection between BMPs and TGF $\beta$  is uncertain.

Despite inferences from the distribution of BMPs during heart formation (13), and BMP effects on cardiac differentiation in teratocarcinoma cells (14), evidence analogous to that for other species is lacking in mammals themselves. One barrier to using conventional germline deletions to resolve these issues in mice is the greater potential for redundancy among BMP family members than in flies. In the mid-gestation heart, BMP2 and -4 are found, respectively, in myocardial layers of the AV canal and in the AV cushion itself, BMP5, -6, and -7 are initially homogeneous throughout the myocardium, and BMP10, which is heart-specific, is found exclusively in trabeculae (14, 15). This diversity, with overlapping and nonoverlapping programs of expression, suggests the utility of addressing the question via BMP receptors, instead, which are smaller in number.

BMPs bind two serine/threonine kinase receptors, type II (BMPRII) and type I (ALK3/BMPRI-IA and ALK6/BMPRI-IB), which form a heteromeric signaling complex acting in series, as for other TGF $\beta$  family receptors (16). In the presence of ligand, the type II receptor phosphorylates the type I receptors, which activate signaling by intracellular effectors including Smad transcription factors (16). ALK3 is ubiquitous throughout de-

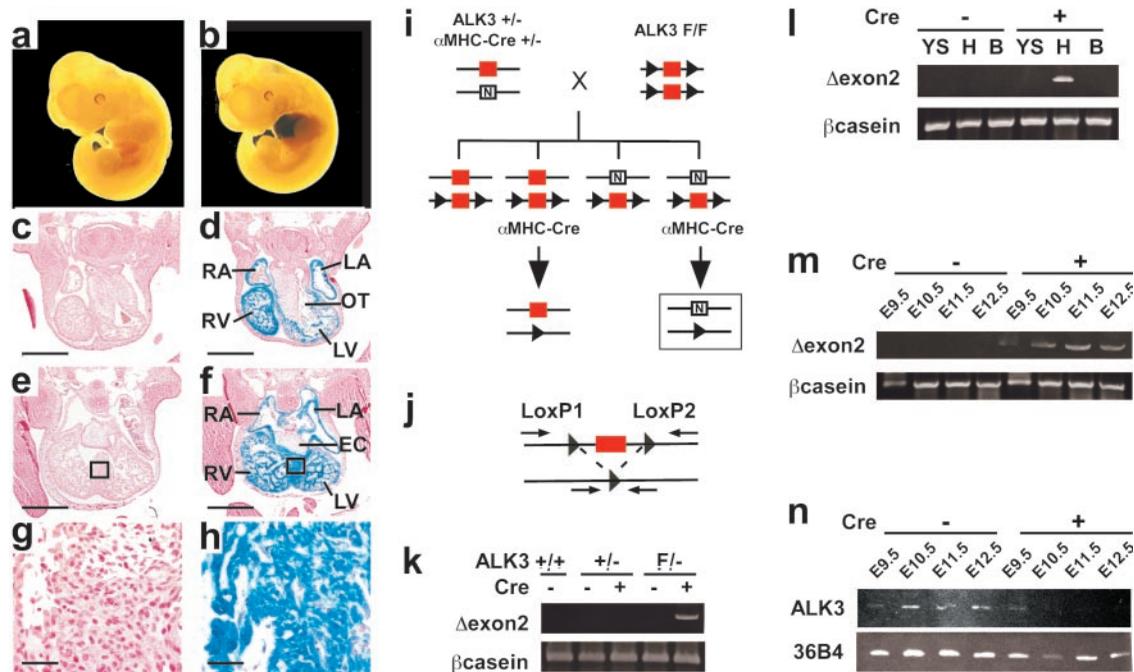
velopment, whereas ALK6 is absent from the heart at mid-gestation (17). The developing heart also expresses ALK2/ActRIA (5, 18), which can function as a type I BMP receptor with preference for BMP6 and -7 (19). ALK3, ALK2, and BMPRI-II are each essential for gastrulation and mesoderm formation (18, 20, 21); mice lacking just BMP4 also fail to progress, typically, beyond the egg cylinder stage (22). In each instance, lethality before mesoderm is formed precludes detecting a role in cardiac development *per se*. This gap in predicting gene function is highlighted by the existence of human mutations in receptors for BMPs and TGF $\beta$  as a cause of clinical cardiovascular disorders including pulmonary hypertension and hereditary hemorrhagic telangiectasia (23, 24).

Even where survival in mice to later stages occurs, extrinsic defects might confound the interpretation of cardiac phenotypes, such as abnormalities of the amnion/chorion, observed with deletion of *BMP2* (25). Analogously, deletion of *BMP5* plus *BMP7* results in death between E9.5 and E10.5, ascribed to cardiac defects (whereas their deletion singly has no apparent effect on the heart), but severe malformations also occurred in neural crest, branchial arches, and, notably, the allantois (26). Hence, the defects or delays in heart morphogenesis might plausibly result, at least in part, from abnormal development external to the heart, including the developing placenta.

Conditional deletion of ALK3, by targeted expression of Cre recombinase (27), provides a means to circumvent early lethality, confounding abnormalities, and, potentially, the greater redundancy among BMP family members than among their receptors. Mechanistically, deleting ALK3—unlike secreted proteins—also would establish unambiguously which cell type in the heart is the *direct* target for all resulting abnormalities. By conditional deletion of ALK3, we demonstrate an obligatory myocyte-dependent role for ALK3 in AV cushion and septal morphogenesis, functions that cannot be deduced or extrapolated from the conventional null phenotype (which is lethal before cardiac fate determination). Our study provides conclusive evidence that even single mutations in the BMP/ALK3 pathway can disrupt cardiac morphogenesis in mammals, proving that other BMP receptors do not substitute for ALK3 here, and potentially

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Abbreviations: AV, atrio-ventricular; BMP, bone morphogenetic proteins; MHC, myosin heavy chain; TGF $\beta$ , transforming growth factor- $\beta$ ; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; RT, reverse transcription.



**Fig. 1.** Cardiac-restricted recombination of the floxed *ALK3* allele. (a–h) The prevalence and specificity of early recombination using  $\alpha$ MHC-Cre was tested at E11.5 in *ROSA26* Cre-reporter embryos, by whole-mount  $\beta$ -galactosidase staining (a and b) and by coronal sections at the level of the outflow tract (c and d) and endocardial cushion (e and f). The box in e and f denotes the region of interventricular septum shown at higher magnification in g and h. (a, c, e, and g) *ROSA<sup>F/+</sup>*; (b, d, f, and h) *ROSA<sup>F/+</sup>; αMHC-Cre<sup>+/-</sup>*. Scale bar, 500  $\mu$ m (c–f) or 30  $\mu$ m (g and h); RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; OT, outflow tract; EC, endocardial cushion. (i–n) Cardiac-specific disruption of *ALK3*. (i) Homozygous floxed *ALK3* mice (F/F) were bred with  $\alpha$ MHC-Cre mice that are hemizygous for *ALK3* (+/-), to produce a cardiac-specific deletion of exon 2 (boxed). red, wild-type exon 2; arrowhead, LoxP site; N, null allele (*PGK-neo* cassette). (j) Deletion of the 2.2-kb DNA fragment containing exon 2 is detected by PCR, using primers LoxP1 and LoxP2, which amplify a 170-bp product. (k–m) PCR analysis of *ALK3* recombination ( $\Delta$ exon2) and a control gene ( $\beta$ casein) at E11.5. (k) Recombination in myocardium was contingent on co-inheritance of  $\alpha$ MHC-Cre and the floxed *ALK3* allele. (l) Recombination was detected in heart (H) of *ALK3<sup>F/+</sup>; αMHC-Cre<sup>+/-</sup>* embryos, but not the yolk sac (YS) or body (B). (m) Recombination increased progressively in myocardium of *ALK3<sup>F/+</sup>; αMHC-Cre<sup>+/-</sup>* embryos from E9.5–E11.5. (n) RT-PCR analysis of *ALK3* and a control gene (*36B4*) expression in myocardium of *ALK3<sup>F/+</sup>* embryos, in the presence (+) or absence (–) of  $\alpha$ MHC-Cre. Note the absence of *ALK3* elicited by Cre from E10.5 and onward.

implicates *ALK3* signaling in endocardial cushion defects or other congenital heart disorders.

## Materials and Methods

**Generation of the Conditional *ALK3* Allele.** A neomycin cassette driven by the PGK promoter and flanked by two loxP sites was placed in an *EcoRI* site in intron 2 of the 129/SvEv *ALK3* gene (20) by homologous recombination. The third loxP sequence was placed in one of the two *HindIII* sites in intron 1, introducing a new *NheI* site for later diagnosis. The left and right homologous arms were 5.4 and 5.5 kb, respectively. For negative selection, an MC1 herpes simplex virus thymidine kinase cassette was placed at the 5' end. The vector was linearized for stable transfection by using *XhoI* and introduced into AB-1 ES cells by electroporation. Correctly targeted clones were injected into blastocysts for chimera production. Heterozygous mice were mated with *CMV-Cre* transgenic mice (28) for site-specific recombination between the loxP motifs. Progeny that executed recombination between the second and third loxP motifs, resulting in an expressed but loxP-tagged allele, were identified by Southern blot (*ALK3<sup>F/+</sup>*). The mating strategy is detailed in Fig. 1.

**PCR and Reverse Transcription (RT)-PCR.** To genotype progeny by PCR, DNA was isolated from yolk sacs of embryos or tail biopsies of weaned mice. The specificity and timing of DNA recombination were tested using primers 5' and 3' to the first and second loxP motifs. RNA was prepared from five embryonic hearts by using TRIzol (GIBCO). Expression of *MEF2A*, *MEF2C*, *ANF*,  $\alpha$ MHC, and  $\beta$ MHC was measured by real-time quantitative RT-PCR (29), and corrected for *36B4*, encoding a

constitutive ribosomal phosphoprotein. For other genes studied, amplification was performed under conditions of linearity. Primer sequences are available on request.

**Histology.** Embryos were fixed in 10% neutral formalin and embedded in paraffin. Serial sections (6  $\mu$ m) were stained with hematoxylin and eosin for morphology studies. Apoptosis was monitored using a terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay and fluorescein-conjugated sheep polyclonal antibody against digoxigenin (Intergen, Purchase, NY). Sections were counterstained for sarcomeric MHC by using MF20 (Hybridoma Bank, University of Iowa, Iowa City, IA), Texas Red-conjugated goat IgG against mouse IgG (Molecular Probes), and Hoechst 33258. To detect Cre-dependent  $\beta$ -galactosidase expression, embryos were harvested at E11.5, processed for whole-mount staining, photographed, and sectioned (30).

**In Situ Hybridization.** Embryos were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections (6  $\mu$ m) were incubated with <sup>35</sup>S-labeled riboprobes, processed for emulsion autoradiography, counterstained with Hoechst, and visualized by dark-field and epifluorescence microscopy (31). Probes were kindly provided by M. C. Jones, Institute of Cancer Research, London (BMP2); B. Hogan, Vanderbilt University Medical Center, Nashville, TN (BMP4); V. Rosen, Genetics Institute, Cambridge, MA (BMP10); R. J. Schwartz, Baylor College of Medicine, Houston (Nkx2.5); M. A. van Rooijen, Hubrecht Laboratory NIOB, Utrecht, The Netherlands (Smad6); and

H. L. Moses, Vanderbilt–Ingram Cancer Center, Nashville, TN (TGF $\beta$ 3).

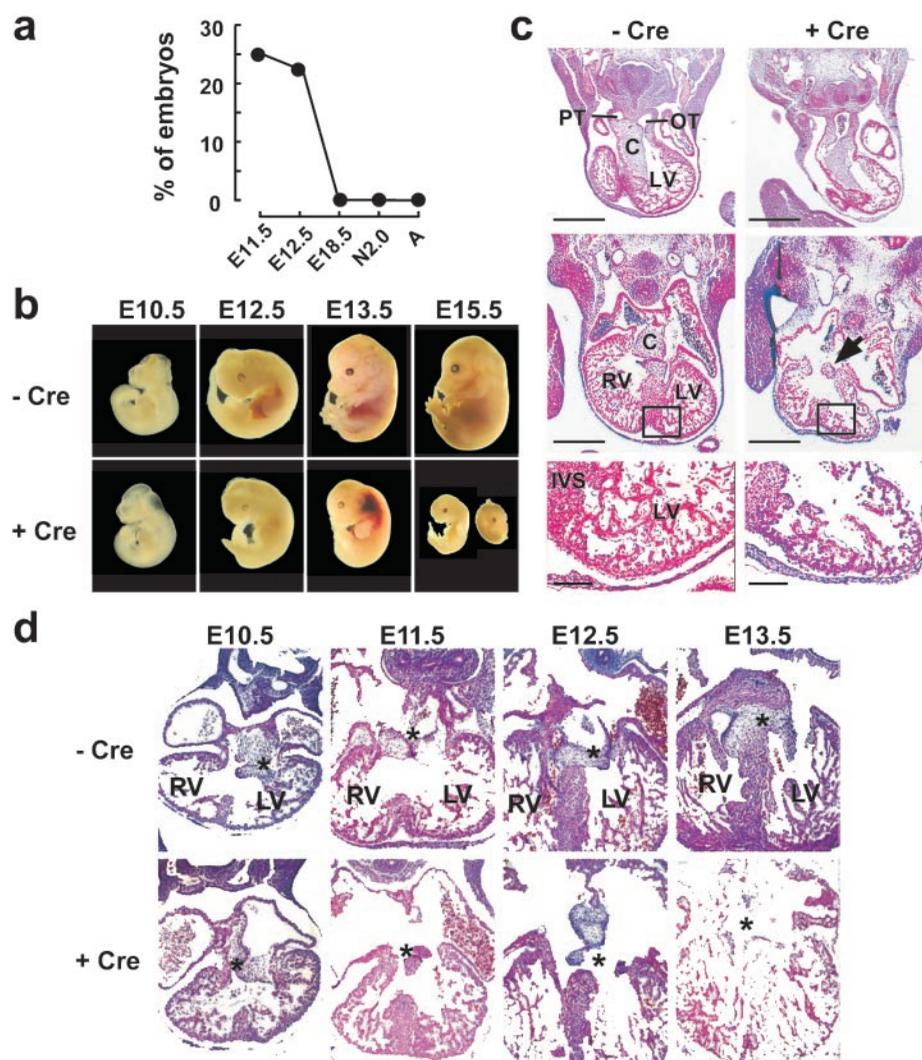
**Statistics.** TUNEL assays were compared by analysis of variance and the unpaired two-tailed *t* test, using a significance level of  $P < 0.05$ . RT-PCR results were compared by analysis of variance and Scheffé's test, using a significance level of  $P < 0.05$ .

## Results

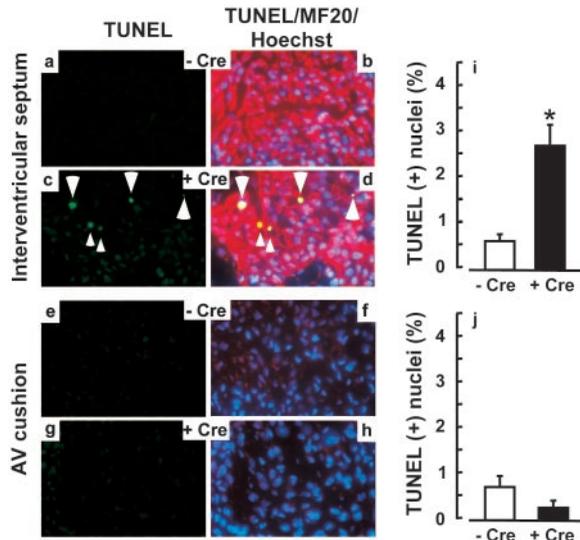
**Conditional, Cardiac-Specific Deletion of ALK3.** We previously reported transgenic mice expressing Cre under the control of the cardiac  $\alpha$ MHC promoter, which elicit efficient DNA recombination in myocardium (30). To establish the extent of recombination during cardiac organogenesis, we bred the  $\alpha$ MHC-Cre mice to a Cre-dependent *lacZ* knock-in at the ubiquitously transcribed locus (32), and harvested embryos at E11.5. In agreement with the transient expression of endogenous  $\alpha$ MHC

throughout myocardium at that stage (33), whole-mount X-Gal staining revealed localized induction of *lacZ* in the heart, contingent on the presence of Cre (Fig. 1 *a* and *b*). Recombination in atrial and ventricular myocytes was virtually homogeneous, in coronal sections at the level of the outflow tract (Fig. 1 *c* and *d*) and AV cushion (Fig. 1 *e–h*). No promiscuous recombination was seen elsewhere or in nonmyocyte components of the heart, and there was no effect on cardiac development from the Cre transgene alone.

To produce the conditional mutation of *ALK3*, exon 2 (encoding 35% of the extracellular ligand-binding domain) was flanked by loxP sites, using homologous recombination. Deletion of exon 2 was sufficient to inactivate *ALK3* function completely (Y.M. and R.R.B., unpublished results). Homozygous floxed *ALK3* mice (F/F) were bred to  $\alpha$ MHC-Cre mice that were hemizygous for *ALK3* (+/–) (Fig. 1*i*). DNA recombination was detected as a 170-bp PCR product that results from



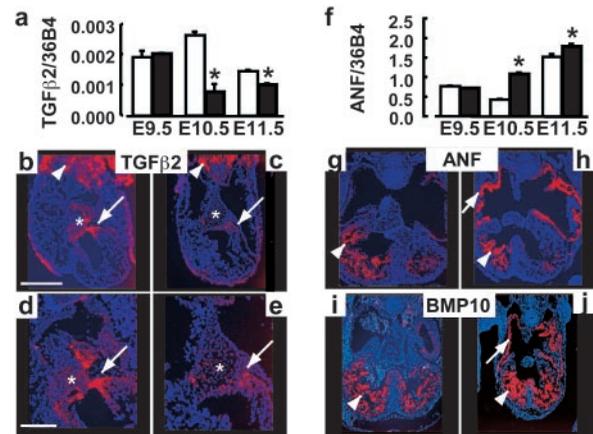
**Fig. 2.** *ALK3* is essential for cardiac septation and AV cushion morphogenesis. (a) A myocyte-dependent function of *ALK3* is required for survival beyond mid-gestation. Prevalence of the  $ALK3^{F/F};\alpha MHC-Cre^{+/-}$  genotype in litters from the breeding strategy in Fig. 1*i*, at E10.5–E18.5, 2 days after birth (N2.0) or after weaning (A). (b) Morphology of  $ALK3^{F/F}$  embryos during development, in the presence (Lower) or absence (Upper) of  $\alpha$ MHC-Cre. (c) Hematoxylin- and eosin-stained coronal sections through the heart of E11.5  $ALK3^{F/F}$  embryos, in the presence (Right) or absence (Left) of  $\alpha$ MHC-Cre. Top, at the level of the outflow tract; Middle, at the level of the AV cushion; Bottom, higher magnification of trabeculae in the left ventricle. Note the defects in the endocardial cushion (arrow), interventricular septum, and trabeculae of *ALK3* mutant embryos. PT, pulmonary trunk; OT, outflow tract; C, endocardial cushion; LV, left ventricle; RV, right ventricle; IVS, interventricular septum. [Scale bar, 500  $\mu$ m (Top and Middle) or 50  $\mu$ m (Bottom).] (d) Time course of AV cushion development in  $ALK3^{F/F}$  embryos in the absence or presence of  $\alpha$ MHC-Cre. Asterisks denote progressive fusion of the two AV cushions if *ALK3* is intact, and the defect in fusion if deleted in cardiac myocytes.



**Fig. 3.** *ALK3* is essential for cardiac myocyte survival. (a–h) Apoptosis was quantified by TUNEL analysis at E11.5 in the interventricular septum (a–d) and AV cushion (e–h). Green, TUNEL reaction; red, MF20 antibody to sarcomeric MHC (Texas red); blue, Hoechst dye 33258. (i and j) Mean  $\pm$  SE for the interventricular septum (i) and endocardial cushion (j). – Cre, control embryos (*ALK3<sup>F/-</sup>*); + Cre, mutant embryos (*ALK3<sup>F/-</sup>;  $\alpha$ MHC-Cre<sup>+/-</sup>*); \*,  $P < 0.05$ .

deletion of the 2.2-kb fragment containing exon 2 (Fig. 1j). Recombination occurred in the heart of E11.5 embryos that were *ALK3<sup>F/-</sup>;  $\alpha$ MHC-Cre<sup>+/-</sup>*, contingent on co-inheritance of Cre and the floxed *ALK3* allele (Fig. 1k). Recombination was restricted to the heart, and was not detected in the yolk sac or body (Fig. 1l). Recombination of the floxed *ALK3* allele was faintly detectable at E9.5 and maximal by E11.5 (Fig. 1m). RT-PCR confirmed the Cre-dependent loss of *ALK3* expression in myocardium from E10.5 onwards (Fig. 1n). *ALK6*, the type IB BMP receptor, is not expressed in the heart at this stage (17).

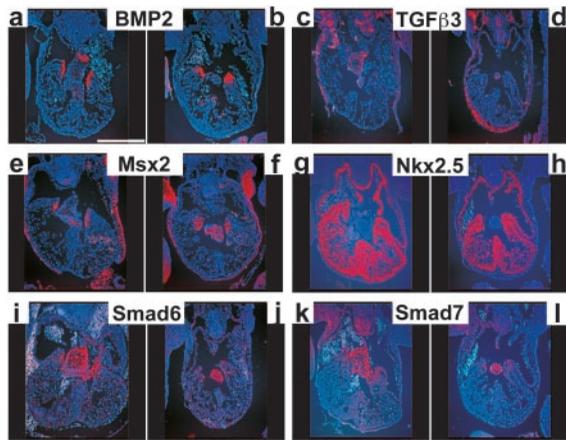
**ALK3 Is Required for Cardiac Septation and Normal Cushion Morphogenesis.** No newborn or weaned pups were found with the *ALK3<sup>F/-</sup>;  $\alpha$ MHC-Cre<sup>+/-</sup>* genotype, of 15 litters examined (Fig. 2a). All three other potential genotypes were present in a Mendelian ratio (not shown). Myocyte-restricted deletion of *ALK3* (*ALK3<sup>F/-</sup>;  $\alpha$ MHC-Cre<sup>+/-</sup>*) was invariably lethal before E18.5, with marked resorption at E15.5 and growth retardation or internal hemorrhages at E12.5–13.5 (Fig. 2b). To seek potentially direct effects of the null mutation, and not secondary or tertiary consequences, we analyzed E11.5 embryos: at that stage DNA recombination is maximal, yet no early lethality or change in gross morphology is seen. In control embryos (*ALK3<sup>F/-</sup>*), endocardial cushions have developed in the outflow tract and AV canal, and the ventricles show a typical pattern of trabeculation (Fig. 2c Left; see also Fig. 1). Cardiac-specific deletion of *ALK3* (*ALK3<sup>F/-</sup>;  $\alpha$ MHC-Cre<sup>+/-</sup>*) invariably produced heart defects, involving the interventricular septum, trabeculae, and AV cushion (Fig. 2c Right). No defects were seen in the outflow tract. All mutant embryos showed the same phenotype, with some variation in severity. We tested whether this ensemble of defects might result from enhanced apoptosis in ventricular myocytes, AV cushion, or both. At E11.5 a 5-fold increase was seen in TUNEL-positive myocytes in the interventricular septum of *ALK3<sup>F/-</sup>;  $\alpha$ MHC-Cre<sup>+/-</sup>* mice (Fig. 3 a–d and i), but no change in the AV cushion (Fig. 3 e–h and j). Thus, an *ALK3*-mediated pathway is required for cardiac myocyte survival here, consistent with the antiapoptotic function of BMPs in some settings (26).



**Fig. 4.** *ALK3* regulates *TGFβ2*, *ANF*, and *BMP10* in myocardium. (a and f) Quantitative RT-PCR analysis (mean  $\pm$  SE) for *TGFβ2* (a) and *ANF* (f). Expression was unaffected until E10.5. White, control (*ALK3<sup>F/-</sup>*); black, mutant (*ALK3<sup>F/-</sup>;  $\alpha$ MHC-Cre<sup>+/-</sup>*); \*,  $P < 0.05$ . (b–e and g–j) *In situ* hybridization for *TGFβ2* (b–e), *ANF* (g and h), and *BMP10* (i and j). Hearts were sectioned at E11.5, at the level of the AV cushion. b, d, g, and i, Control (*ALK3<sup>F/-</sup>*); c, e, h, and j, mutant (*ALK3<sup>F/-</sup>;  $\alpha$ MHC-Cre<sup>+/-</sup>*). (d and e) Higher magnification of AV canal corresponding to b and c, respectively. Expression patterns with the hemizygous floxed allele did not differ from those reported for these genes in a wild-type background. *TGFβ2* expression was defective in the ventricle surrounding the AV canal (arrows) but normal in noncardiac tissue (arrowheads). Asterisks denote the adjacent AV cushions. *ANF* and *BMP10* were unchanged in the ventricle (arrowheads), but expressed precociously in the atria (arrow). [Scale bar, 500  $\mu$ m (b, c, and g–j) or 50  $\mu$ m (d and e).]

**ALK3 Is Required for Cardiac Expression of *TGFβ2*.** Defects in organization of the AV cushion, where  *$\alpha$ MHC-Cre* was not expressed, indicate that *ALK3*-dependent crosstalk from cardiac myocytes to the cushion is essential for normal AV valve formation in mammals, as is known to be true for avians (9–12). To establish more precisely the earliest step(s) in cushion morphogenesis affected by disruption of *ALK3* in the myocytes, cushion development was examined at E10.5–13.5 (Fig. 2d). In normal mice, the two AV cushions formed by epithelial-to-mesenchymal transformation (E10.5) fuse at E11.5–12.5 to form the AV valves (E13.5). Despite conditional disruption of *ALK3*, the AV cushions appeared normal at E10.5 in all mutant embryos, suggesting that the initial epithelial-to-mesenchymal transformation occurs normally. By E11.5–12.5, however, the two cushions were reduced in size in more than 60% of embryos, often misaligned (not seen in the same short-axis plane of section), and unfused even when in contiguity. Fusion of the cushions and leaflet formation were deficient even at E13.5, when myocardial necrosis precludes further analyses. Thus, continued expression of *ALK3* in cardiac myocytes is indispensable for subsequent development of the cushion and, hence, the AV valves.

Because *TGFβ2* and  $\beta$ 3 are both essential paracrine factors for cushion development in avians (9–11), we tested whether *ALK3* was required for their expression in mice. By quantitative RT-PCR (Fig. 4a), *TGFβ2* expression was normal at E9.5, when disruption of *ALK3* was incipient, but decreased 75% at E10.5, thus preceding the first detected changes in cushion morphogenesis. Myocyte-restricted deletion of *ALK3* decreased expression of *TGFβ2* specifically in myocardium adjacent to the AV canal, whereas sites outside the heart were unaffected (Fig. 4 b–e). As measured by quantitative RT-PCR, or *in situ* hybridization, genes for other *TGFβ*/*BMP* signaling proteins that may participate in heart valve formation were unchanged: *TGFβ3*, *BMP2*, *BMP4*, *BMPII*, the Hox gene *Msx2*, and the inhibitory Smads, *Smad6* and  $\beta$ 7 (Figs. 4 d and e and 5, and unpublished



**Fig. 5.** Cardiac gene expression after conditional, myocyte-restricted deletion of *ALK3*. (a–l) *In situ* hybridization analysis of E11.5 hearts sectioned at the level of the AV cushion. a, c, e, g, i, and k, Control embryos (*ALK3<sup>f/f</sup>*); b, d, f, h, j, and l, mutant embryos (*ALK3<sup>f/f</sup>; αMHC-Cre<sup>+/+</sup>*). a and b, *BMP2*; c and d, *TGFβ3*; e and f, *Msx2*; g and h, *Nkx2.5*; i and j, *Smad6*; k and l, *Smad7*. (Scale bar, 500 μm.)

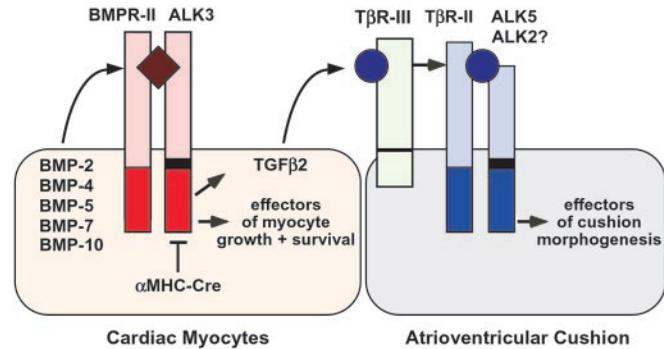
results). Time-points later than E11.5 were not analyzed; older mutant embryos were typically friable.

Conversely, by quantitative RT-PCR, expression of *ANF* in the mutant embryos increased 3-fold at E10.5 (Fig. 4f). By *in situ* hybridization, baseline *ANF* expression was greatest in the ventricular trabeculae at this stage, whereas the increase localized to the atria (Fig. 4g and h). We therefore tested a second trabeculae-restricted gene, *BMP10* (13). In mutant embryos, trabecular expression of *BMP10* was preserved, but with precocious expression in the atria, where it would normally be found only at later time points (ref. 13; Fig. 4i and j). Hence, *ALK3* inhibits or delays the appearance of *ANF* and *BMP-10* in the atria: these findings argue against a generalized maturation arrest as the basis for cardiac phenotypes resulting from the absence of *ALK3*.

The cardiogenic transcription factors *Nkx2.5*, *eHAND*, *Tbx2*, and *Pitx2* each may require endogenous BMPs for their induction in avian embryos (6, 7, 31, 34). However, expression of these four transcription factors was not affected by heart-specific deletion of *ALK3* (Fig. 5g and h and unpublished results). Hence, either the initial expression of *ALK3* suffices, or an alternative signal. However, necrosis and friability would interfere with assessing *ALK3*-dependent genes even 48 h after heart-specific disruption of the gene. Small (10–30%) yet consistent defects occurred in expression of *MEF2A*, *MEF2C*, *MLC<sub>2v</sub>*, and *βMHC*, and other markers of cardiac differentiation—i.e., *αMHC*, *dHAND*, *GATA4*, and *FOG2*—were unchanged (not shown).

## Discussion

The mechanistic focus of this study was to determine—by conditional, lineage-restricted deletion of *ALK3*—if an essential role is served by the continued expression of this BMP receptor in cardiac myocytes subsequent to onset of the cardiac “fate.” Disruption of *ALK3* in differentiated myocytes unmasked an essential requirement for *ALK3* in heart morphogenesis, beyond myocyte determination (6, 7, 14, 35), involving the ventricular septum, trabeculae, compact myocardium, and endocardial cushion. Second, although BMP signaling via *ALK3* may be required to maintain (as well as induce) *Nkx2.5* in *Xenopus* (35), *ALK3* appears dispensable for its expression in mice, and for other transcription factors tested, once the cardiac fate is established. Third, given the prevalence of AV cushion defects and proven cross-talk in avians between myocardium and the devel-



**Fig. 6.** *ALK3* is essential for cardiac myocyte expression of *TGFβ2* and AV cushion morphogenesis. Genetic interactions between BMPs and *TGFβ* signaling are shown schematically. In mid-gestation, cardiac myocytes express *BMP2* and *BMP4* adjacent to the AV cushion, *BMP5* and *BMP7* more homogeneously, and *BMP10* in trabeculae (in addition to BMP production by other sources). Deletion of *ALK3* in cardiac muscle cells, using *αMHC-Cre*, results in defective AV cushion morphogenesis, associated with the lack of *TGFβ2* produced by cardiac myocytes, a proven paracrine signal for this process in avians (10). The type III *TGFβ* receptor (*TβR-III*) is needed for *TGFβ2* binding to *TβR-II* (39) and, in avian explants, AV cushion morphogenesis (11). In avians, *ALK2* also mediates cushion formation, not the type I *TGFβ* receptor *ALK5* (36), but given early lethality for both null alleles, it is unknown whether this is true in mice (18, 40). Myocyte-restricted deletion of *ALK3* disinhibits the expression of *ANF* and *BMP10* in the atria, but causes defects in the ventricular septum, trabeculae, and compact myocardium.

oping cushion, we tested whether disruption of *ALK3* in myocytes caused defective expression of established paracrine signals for cushion morphogenesis. *ALK3* was essential for cardiac myocyte expression of *TGFβ2*, suggesting a myocyte-dependent pathway in forming the AV cushion and the resulting cardiac valves (Fig. 6).

Germline mutations of *ALK3* and *BMPR-II* lead to death before cushion formation or even cardiac specification. For this reason, the conventional null alleles cannot be used readily to test whether, where, and how BMP signals function during cardiac morphogenesis. By contrast, the conditional loss of *ALK3* in mid-gestation myocardium quickly leads to multiple cardiac defects, which coincide with the distribution of BMPs in the heart: *BMP2* and *BMP4* adjacent to the AV cushion, *BMP5* and *BMP7* more homogeneously, and *BMP10* in the trabeculae. Deletion of *BMP5* and *-7* in tandem results in cardiac defects that involve the AV cushion, septum, free wall, and trabeculae—resembling heart-specific deletion of *ALK3*—but with extensive abnormalities of the allantois, neural crest, and vasculature that make a role for BMPs within the heart less straightforward to assert (26). Myocyte-restricted deletion of *ALK3* enabled us, with one mutation, to overcome the confounding problems of redundancy among multiple BMP ligands, systemic and local confounding effects, and the ambiguous identity of the key target cell.

These functions of *ALK3* in cardiac morphogenesis are essential and not supplanted by other type I receptors. By contrast, cardiac-lethal effects of deleting *BMP7* plus *BMP6* preferentially involve the outflow tract (15), a region unaffected by the loss of *ALK3*. Although *ALK6* is not expressed in the mid-gestation heart and thus is extraneous here, *ALK2/ActRIA* is expressed in the mouse heart at this stage and binds a subset of BMP family members (18). Notably, for *Xenopus* and avians, *ALK2* has reported functions during cardiac morphogenesis, in laterality and AV cushion transformation, respectively (5, 36). Mice lacking *ALK2* die in early gastrulation, before even the earliest steps in cardiac specification, and a requirement for *ALK2* in extraembryonic tissues complicates even the use of chimeric

mice to address its possible function in the mammalian heart (18). Notwithstanding possible redundancy between *ALK3* and *ALK2* in other circumstances, *ALK2* does not overcome the cardiac-lethal phenotype unmasked by conditional deletion of *ALK3*, as with the essential role for each in gastrulation. Whether this, in turn, reflects distinctions in ligand preference or downstream effectors is presently conjectural.

Dependence of the AV cushion on ALK3—despite the absence of Cre from this site—indicates conclusively that crosstalk from myocytes to the cushion, in some form, is obligatory for AV valve formation in mice. Down-regulation of TGF $\beta$ 2 by the disruption of ALK3 in cardiac muscle cells suggests that a paracrine circuit, as in avian explants (9–12), might also apply to mammalian cushion morphogenesis (Fig. 6). Although TGF $\beta$ 2 is one of many potential downstream candidate genes that might contribute to developmental defects in ALK3-deficient hearts, it is noteworthy that mice lacking TGF $\beta$ 2 die soon after birth with cardiac defects including malformations of the ventricular septum and AV valves (37).

In humans, congenital heart defects occur with a prevalence of at least 1% in newborns, and are even more common in death

before term (38). Most frequent are defects in septation and the cardiac valves, and few single-gene etiologies are known. The invariable defects in myocardium and AV cushion resulting from conditional deletion of *ALK3* provide strong support for its assessment as a candidate gene in human congenital heart disease.

**Note Added in Proof.** Construction of the floxed ALK3 mice and their use to show the requirement for ALK3 in limb development have been described since the submission of our report (41, 42).

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