Alk3/Bmpr1a Receptor Is Required for Development of the Atrioventricular Canal Into Valves and Annulus Fibrosus


Abstract—Endocardial cushions are precursors of mature atrioventricular (AV) valves. Their formation is induced by signaling molecules originating from the AV myocardium, including bone morphogenetic proteins (BMPs). Here, we hypothesized that BMP signaling plays an important role in the AV myocardium during the maturation of AV valves from the cushions. To test our hypothesis, we used a unique Cre/lox system to target the deletion of a floxed Alk3 allele, the type IA receptor for BMPs, to cardiac myocytes of the AV canal (AVC). Lineage analysis indicated that cardiac myocytes of the AVC contributed to the tricuspid mural and posterior leaflets, the mitral septal leaflet, and the atrial border of the annulus fibrosus. When Alk3 was deleted in these cells, defects were seen in the same leaflets, ie, the tricuspid mural leaflet and mitral septal leaflet were longer, the tricuspid posterior leaflet was displaced and adherent to the ventricular wall, and the annulus fibrosus was disrupted resulting in ventricular preexcitation. The defects seen in mice with AVC-targeted deletion of Alk3 provide strong support for a role of Alk3 in human congenital heart diseases, such as Ebstein’s anomaly. In conclusion, our mouse model demonstrated critical roles for Alk3 signaling in the AV myocardium during the development of AV valves and the annulus fibrosus. (Circ Res. 2005;97:219-226.)

Key Words: bone morphogenetic protein signaling ■ heart development ■ atrioventricular canal ■ Cre–lox system

In humans, abnormalities in atrioventricular (AV) valves are among the most frequent congenital heart defects,1 which reflects the complexity of the mechanisms regulating the development of the valvular apparatus. In mouse embryos, the first visible manifestation of this process is the formation of endocardial cushions in the AV canal (AVC) around E9.5 (Figure 1A). This is a well-documented event that requires inducting signaling by the adjacent AV myocardium.2–6 Recent lineage studies showed that mature AV valves are formed almost entirely from these endocardial cushions.7,8 Interestingly, however, these cushions remain in contact with the AV myocardium throughout leaflet formation. Indeed, the mural leaflets of both AV valves are supported by AV myocardium at their ventricular side (Figure 1A). This myocardium remains as late as E17.5 when it delaminates.7 The septal leaflet of the tricuspid valve remains in contact with the septum (Figure 1A) until E17.5 when it delaminates.7 The mitral septal leaflet is never supported by myocardium at its ventricular side (Figure 1A), but is in contact with the AV myocardium-derived mitral gully at its anterior and posterior margins (anterior and posterior in reference to the heart correspond to cranial and caudal in respect to the body).7 The fact that the AV myocardium remains in contact with the developing leaflets suggests that it plays a role during valvular morphogenesis in addition to its original inductive role in cushion formation.

Signaling molecules originating from the AV myocardium during cushion formation include, among others, the bone morphogenetic proteins (BMPs).5,9,10 We recently showed that conditional ablation of Alk3, the type IA receptor for BMPs, in the entire myocardium from E10.5 onwards resulted in abnormal cardiac morphogenesis at E11.5, including defects in AV cushion formation.11 Embryos with cardiac-specific deletion of Alk3 died at mid-gestation, precluding...
further analysis of the role of Alk3 at later stages of development. Here, we hypothesized that Alk3 is required specifically in cardiac myocytes of the AVC for the development of AV valves from the cushions. To separate the multiple functions of Alk3 in the myocardium from its possible role in a subpopulation of myocytes underlying valve formation, we used a unique Cre/lox system driven by a heart-region–specific enhancer from the avian GATA6 gene (cGATA6) to selectively delete a floxed Alk3 allele in cardiac myocytes of the AVC.

Materials and Methods
Detailed Materials and Methods are available in the online data supplement available at http://circres.ahajournals.org.

Animals
Transgenic mice with cGATA6-Cre overexpression, a floxed Alk3 allele, a conventional null Alk3 allele, and the R26R transgenic mice have been described previously. The procedures for echocardiographic examination of mice and for surface ECG have been reported previously. For optical mapping studies, hearts were Langendorff perfused and stained with a bolus injection of the voltage sensitive dye, Di-4-ANEPPS. vECGs were recorded from each heart using Ag-AgCl electrodes placed 1 mm from the heart surface. Left atrial pressure measurement was performed under anesthesia using a 1.4F micromanometer catheter (Millar Instrument Inc).

Histology
Adult hearts and embryos were fixed in 4% paraformaldehyde/PBS and embedded in paraffin. Serial sections (6 μm) were stained with Picric Acid Sirius Red, Masson’s trichrome, or Van Gieson method. Immunohistochemistry was performed using the MF20 antibody against sarcomeric myofilaments (Developmental Studies Hybridoma Bank, University of Iowa), connexin 43 (Zymed Laboratories Inc), peristin, and -cardiac actinin (Sigma-Aldrich). Sections were also processed for radioactive in situ hybridization as described using a probe for peristin. To detect Cre-dependent LacZ expression, tissues were processed for whole-mount LacZ staining.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
Expression of atrial natriuretic factor (ANF) was measured by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) and normalized to 36B4 encoding a constitutive ribosomal phosphoprotein.

Statistics
Results obtained for cGATA6-Cre/Alk3 mice (Cre, Alk3null/flox) were compared with control littermates (Cre, Alk3null/flox, and Cre, Alk3wt/flox) using the unpaired 2-tailed t-test and a significance level of P = 0.05.

Results
Cardiac Myocytes of the Atrioventricular Canal Contribute to the Formation of Atrioventricular Valve and the Annulus Fibrosus
To study the fate of the cardiac myocytes in the AVC, we used transgenic mice expressing Cre under the control of a cGATA6 heart-specific enhancer and LacZ-reporter mice for Cre activity (R26R). Previous studies indicated that the activity of the cGATA6 enhancer can be detected in a subset of cardiac myocytes as early as E7.5 in mouse embryos. It becomes restricted to cardiac myocytes of the AVC at E9.5,
at the heart looping stage. Cells in the R26R mice that have undergone Cre-mediated recombination express LacZ irreversibly, allowing for the mapping of their fate. In E11.5 (Figure 1B), E14.5 (Figure 1C), and adult (not shown) cGATA6-Cre/R26R mice, consistent Cre-mediated DNA recombination was observed at the posterior right side of the AVC. Fewer LacZ-positive cardiac myocytes were seen on the left (Figure 1C) and anterior sides of the AVC. This pattern of Cre-mediated recombination in the AVC can also be appreciated on coronal and sagittal sections of E11.5 cGATA6-Cre/R26R embryos (Figure 1D through 1F; Figure 1AA through 1CC). Variability in the extent of Cre-mediated recombination was observed, mostly on the left side of the AVC (Figure 1C, compare right and left panels). This is likely to result from heterogeneity of expression of the cGATA6-Cre transgene inherent to conventional transgenic methods. Therefore, the cGATA6-Cre mice are a unique and powerful tool to target cardiac myocytes of the AVC, but variation in the penetrance of the phenotype of cGATA6-Cre/floxed mice can be expected. In addition, the left ventricle and right atrium were marked by patches of LacZ-positive cells (Figure 1B). These cells are clonal derivatives of a limited population of cardiac myocytes that expressed the cGATA6-Cre transgene outside of the AV boundaries during early cardiogenesis. A detailed analysis of the activity of the cGATA6-Cre heart-specific enhancer using cGATA6-LacZ embryos was shown previously.

Coronal and sagittal sections through the hearts of embryonic (E11.5, E14.5), neonatal (N7.0), and adult cGATA6-Cre/R26R mice were immunostained with the MF20 antibody to identify cardiac myocytes (Figure 1D through 1N; Figure 1AA through 1CC) or counterstained with Van Gieson method to stain the AV valves and annulus fibrosus (Figure 1O through 1Z; Figure 1DD through 1EE). LacZ-positive cardiac myocytes found at the right side of the AVC at E11.5 (Figure 1E) contributed to the myocardial layer present in the tricuspid mural leaflet (Figure 1H and 1L). They were also identified in the myocardium at the atrial border of the developing right annulus fibrosus, which is formed by fusion of epicardially-derived sulcus tissue (star, Figure 1H and 1L) with cushion tissue (yellow arrow, Figure 1L). In N7.0 and adult hearts, they remained present in the tricuspid mural leaflet (Figure 1Q and 1W) and at the border of, or sometimes embedded in, the right annulus fibrosus (Figure 1P and 1V). Similarly, LacZ-positive cardiac myocytes found at the posterior right side of the AVC at E11.5 (Figure 1CC) contributed to the tricuspid posterior leaflet and to the atrial border of the posterior anulus fibrosus (Figure 1EE). Finally, LacZ-positive cardiac myocytes found at the left side of the AVC at E11.5 (Figure 1F) contributed to the myocardium of the mitral gully (Figure 1M), which is in contact with the posterior side of the mitral septal leaflet (Figure 1I). In N7.0 and adult hearts, LacZ-positive cells remained present in the mitral septal leaflet (Figure 1S and 1Y), but only in the superior part that was in contact with the gully during development. During development and after birth, LacZ-positive cells were also found at the border of the left annulus fibrosus (Figure 1J, 1N, 1T, and 1Z), but to a lesser extent than on the right and posterior anulus (Figure 1P and 1EE). LacZ-positive cells were not identified in the tricuspid septal leaflet, in the mitral mural leaflet, or in the fibrous continuity between the septal leaflets (not shown). Thus, cells expressing the cGATA6-Cre transgene contributed to the formation of the AV valves, more specifically to the tricuspid mural and posterior leaflets, and to the mitral septal leaflet. In addition, their presence at the atrial border of the anulus fibrosus suggested that they also contribute to the development of the insulation between the atrial and ventricular chambers.

Deletion of Alk3 in the Atrioventricular Canal

To target the deletion of Alk3 in cardiac myocytes of the AVC, mice hemizygous for both cGATA6-Cre and the conventional null Alk3 allele were bred to mice homozygous for the floxed Alk3 allele. Mice with AVC-targeted deletion of Alk3 (cGATA6-Cre/Alk3) were found at weaning (detailed breeding strategy and numbers are available in the online data supplement) and no premature mortality was noticed over a 15-month period. Because cells expressing the cGATA6-Cre transgene contributed to the formation of the mitral septal leaflet and the tricuspid mural and posterior leaflets (Figure 1), we focused our analysis on these leaflets in 3-month-old mice. Histological analysis of coronal sections revealed defects of the mitral septal leaflet in 45% of 3-month-old cGATA6-Cre/Alk3 mice (n=11). Typically, the abnormal valves had longer leaflets than control valves (Figure 2B). To test if mitral valve elongation in cGATA6-Cre/Alk3 mice resulted in blood reflux, left atrial pressure was measured, followed by histological analysis of the heart. A significant 2-fold increase in left atrial pressure consistent with mitral insufficiency was found in cGATA6-Cre/Alk3 mice in which mitral valve defects were confirmed by histology (Control: 8±2 mm Hg; Mutant: 17±3 mm Hg; P<0.05), cGATA6-Cre/Alk3 mice with no detectable abnormal mitral valve were similar to control littermates (7±1 mm Hg).

In addition, histological analysis revealed defects of the tricuspid mural leaflet in 36% of cGATA6-Cre/Alk3 mice (n=11) (Figure 2D). Typically, the abnormal valves had longer mural leaflets than control valves. To analyze the tricuspid posterior leaflet, sagittal sections were obtained from 6 cGATA6-Cre/Alk3 mice (Figure 2E through 2H). The posterior annulus fibrosus was displaced downward into the right ventricular cavity in one of them (compare position of arrowheads, which indicate the annulus fibrosus, in Figure 2E and 2F). In addition, the tricuspid posterior leaflet adhered to the ventricular wall and contained nonfibrous tissue (star, Figure 2H).

Valvular Defects Originate During Development

To get some insights into the causes of the valvular defects seen in adult cGATA6-Cre/Alk3 mice, we examined the fate of the AV myocardium. We first examined the developing tricuspid mural leaflet in which cells expressing the cGATA6-Cre transgene contributed significantly (Figure 1H and 1L). AV myocardium is normally present at the ventricular side of this leaflet up to E17.5 when it is removed by apoptosis. Accordingly, sections through the heart of E14.5
and E15.5 control embryos stained with the MF20 antibody showed the presence of a homogeneous layer of cardiac myocytes in the tricuspid mural leaflet (Figure 3A and 3C). In cGATA6-Cre/Alk3 embryos, this layer was present at E14.5 (3 of 3 analyzed) but was no longer detectable at E15.5 (5 of 6 analyzed) (Figure 3B and 3D). Thus, in the absence of Alk3, premature disappearance of the myocardial layer in the tricuspid mural leaflet occurred. We also examined the myocardium in the mitral gully in contact with the mitral septal leaflet (Figure 1M). At E15.5, there was no detectable difference between control and cGATA6-Cre/Alk3 embryos (not shown).

Because BMP signaling in AV myocardium is necessary for AV cushion formation and development, we examined the expression of peristin, a robust marker of mesenchymal cells, in cGATA6-Cre/Alk3 mice. In E11.5 and E14.5 control embryos, strong peristin expression was detected by in situ hybridization in the cushions and AV valves, respectively (Figure 4A and 4C). In cGATA6-Cre/Alk3 embryos, peristin expression was unchanged at E11.5 (Figure 4B) but was dramatically decreased at E14.5 specifically in AV valves (Figure 4D), whereas sites outside the heart were unaffected (ie, ribs, Figure 4E and 4F).

Ventricular Preexcitation in cGATA6-Cre/Alk3 Mice

Because lineage analysis suggested that cardiac myocytes of the AVC contribute to the formation of the insulation between the atrial and ventricular chambers (Figure 1), we characterized the electric conduction properties of the cGATA6-Cre/Alk3 mice. Surface electrocardiogram (ECG) records of 6 of 33 mice, aged between 2 and 6 months, showed absence of the PR interval (Figure 5A and 5B). Volume-conducted electrocardiograms (vECGs) were recorded in isolated Langendorff perfused hearts and showed a delta wave in 4 of 4 mice with abnormal surface ECG (Figure 5C and 5D). These AV conduction abnormalities are compatible with ventricular preexcitation syndrome. To confirm the presence of an accessory pathway in cGATA6-Cre/Alk3 mice, high-resolution optical mapping with the voltage sensitive dye Di-4-ANEPPS were recorded in isolated Langendorff perfused hearts from the posterior side while the hearts were beating spontaneously in sinus rhythm (Figure 5E and 5F). This imaging system provides a detailed picture of electrical activation from the epicardial surface of the heart.
Control hearts (n=4) showed a mature apex-to-base ventricular activation pattern, with no evidence of conduction abnormalities (Figure 5E). In stark contrast, in 4 of 4 hearts with abnormal surface ECG, a base-to-apex activation pattern was observed, with evidence of a posterior paraseptal bypass tract (Figure 5F).

Bypass Tract Results From Disruption of the Annulus Fibrosus
To gain further insight on the nature of the accessory pathway in cGATA6-Cre/Alk3 mice, 3 hearts that showed evidence of a posterior paraseptal bypass tract were analyzed by histology. Serial sagital sections were stained with Masson’s trichrome (Figure 6A). In the posterior paraseptal region (boxed; Figure 6A), the annulus fibrosus isolated the left chambers in control hearts (Figure 6B). In 1 heart from a cGATA6-Cre/Alk3 mouse, the annulus fibrosus was disrupted, resulting in a direct myocytic connection between the atrium and ventricle (Figure 6C). Interestingly, the same heart also presented with the downward displacement of the posterior tricuspid leaflet described above (Figure 2F). Adjacent sections were immunostained with an antibody against periostin (Figure 6D and 6E). Periostin protein was localized at the border of the annulus fibrosus in control hearts (Figure 6D) but was not detectable in the region between the atrium and ventricle in the heart with the bypass tract (Figure 6E).

To conduct the electrical impulse, the cardiac myocytes in the bypass tract have to be connected by gap junctions formed by connexins (Cx). Cx43 is the major Cx isoform found in the ventricular myocardium. Based on our histological data showing a muscular accessory conduction pathway (Figure 6C), we examined the expression of Cx43 in that region. Immunofluorescence showed the presence of Cx43 in the cardiac myocytes of the bypass tract (Figure 6G) providing a substrate for conduction of the impulse between the atrium and the ventricle. In control heart, the annulus fibrosus interrupted Cx43 expression between the atrium and ventricle (Figure 6F).
Functional Repercussion of Atrioventricular Canal-Targeted Deletion of Alk3

At echocardiography (Figure 7A), 3-month-old cGATA6-Cre/Alk3 mice (n=17) had significantly decreased left ventricular (LV) ejection fraction (Figure 7B) and increased LV-end–systolic dimension (Figure 7C) and LV-end–diastolic dimension (Figure 7D) when compared with control mice. Heart rate, heart weight, and body weight were similar in both groups (not shown). LV dysfunction in 3-month-old cGATA6-Cre/Alk3 mice was accompanied by significant increase in ANF expression (Figure 7E). LV dysfunction probably results from the presence in the LV of clusters of cardiac myocytes in which cGATA6-Cre induced DNA recombination (Figure 1B).12 This suggests that Alk3 is necessary for normal cardiac contractile function or development. LV dysfunction could also be attributed, in part, to the conduction defects because the component of atrial systole is impaired in some of the cGATA6-Cre/Alk3 mice. Indeed, LV ejection fraction was less (P<0.05) in cGATA6-Cre/Alk3 mice with ventricular preexcitation (59.3±2.1%) as compared with cGATA6-Cre/Alk3 mice with normal surface ECG (64±1.3%).

Discussion

To test the hypothesis that AV myocardium contributes to the development of functional AV valves and that BMP signaling is required in that process, we used a unique Cre/lox system driven by a cGATA6 enhancer12 to selectively delete a floxed Alk3 allele in cardiac myocytes of the AVC.

Atrioventricular Valve Development

Lineage analysis indicated that cardiac myocytes expressing the cGATA6-Cre transgene were present in the AVC myocardium in contact with the developing tricuspid mural and posterior leaflets, and with the mitral septal leaflet. They were still present in the corresponding mature leaflets (Figure 1). This is in agreement with another recent observation in which LacZ- and SERCA-positive cells where found in the AV valves of αMHC-Cre/R26R mice.7 When Alk3 was deleted in these cells, defects were seen in the same leaflets, ie, the tricuspid mural leaflet and mitral septal leaflet were longer, and the tricuspid posterior leaflet was displaced and adherent to the ventricular wall (Figure 2). Therefore, even if these
leaflets are formed almost entirely from mesenchymal cells derived from the endocardial cushions, cardiac myocytes from the AVC are also necessary for their normal development. The defects in cGATA6-Cre/Alk3 mice also indicate a role for BMP signaling during AV valve maturation.

The defects in valve development in the absence of Alk3 take place after the formation of the endocardial cushion (Figure 4B), with the premature disappearance of the myocardial layer in the tricuspid leaflet at E15.5 (Figure 3D). This suggests that Alk3 is required for cardiac myocyte survival at E14.5, consistent with the antiapoptotic function of Alk3 in embryonic myocardium. Concomitantly, periostin expression was dramatically decreased in the AV valves at E14.5 (Figure 4D), which could result from the premature disappearance of the myocardial layer, at least in the tricuspid mural leaflet. The role of periostin in valve development is unknown. However, increased periostin expression is observed in several mouse models of hyperplastic valves suggesting that it is required for proper fibrogenic morphogenesis of the AV valves (S. Conway, personal communication, 2005). In other cell types, periostin interacts with integrins to promote cell adhesion. Taken together with our data, this suggests that periostin could play an important role in the formation of mature fibrous valve leaflets.

Annulus Fibrosus Development

Lineage analysis indicated that cardiac myocytes expressing the cGATA6-Cre transgene were present at the atrial border of the developing and mature annulus fibrosus (Figure 1). This is in agreement with the previous observation that the formation of the annulus fibrosus results in the incorporation of the AV myocardium in the myocardium of the atrium. The persistent presence of AV myocardium at the border of the annulus fibrosus suggested that it contributes to the development of the insulation between the atrial and ventricular chambers. When Alk3 was deleted in cardiac myocytes of the AVC, AV conduction defects compatible with ventricular preexcitation were observed (Figure 5). In one mouse (n=3), downward displacement (Figure 2F) and disruption (Figure 6C) of the posterior annulus fibrosus created a myocytic connection between the left atrium and ventricle. This bypass tract expressed Cx43 (Figure 6G). This is similar to the clinical finding in patients with Wolff-Parkinson-White (WPW) ventricular preexcitation syndrome that muscular connections outside of the specialized conduction system are almost always composed of myocardial fibers expressing Cx43. Therefore, BMP signaling plays an important role in AVC myocardium for the proper development of the insulation between the atrial and ventricular chambers. The exact mechanism involved remains to be defined.

Recently a mouse model of WPW syndrome was generated by overexpressing the AMP-activated protein kinase with a cardiac myocyte connection between the left atrium and ventricle. This bypass tract (not shown). Our mice are therefore a unique model of ventricular preexcitation, different from the previously described model of glycogen-storage disease.

Clinical Significance

The defects in AV valves and annulus fibrosus seen in cGATA6-Cre/Alk3 mice show some similarities with Ebstein’s anomaly. Ebstein’s anomaly of the tricuspid valve is a rare congenital heart defect that occurs with a probability of 1:20,000 and represents ~40% of congenital malformations of the tricuspid valve. It has a wide spectrum of clinical variations ranging from intrauterine death to asymptomatic survival to late adulthood. Ebstein’s anomaly is characterized by a downward displacement of the posterior tricuspid leaflet resulting in a smaller right ventricular chamber and the tricuspid posterior leaflet adheres frequently to the wall of the chamber. It can be associated with WPW ventricular preexcitation syndrome caused by discontinuity of the annulus fibrosus. The defects seen in cGATA6-Cre/Alk3 mice provide strong support for a role of Alk3 in human congenital heart diseases, such as Ebstein’s anomaly.

In conclusion, our mouse model demonstrates critical roles for Alk3 signaling in the AV myocardium during the development of AV valves and the annulus fibrosus.

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Material and Methods

Animals. Transgenic mice with cGATA6-Cre overexpression (FVB), a floxed Alk3 allele (129/B6), a conventional null Alk3 allele (C57), and the R26R transgenic mice (FVB) have been described previously. To genotype progeny by PCR, DNA was isolated from tail biopsies of weaned mice or yolk sacs of embryos. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

To target the deletion of Alk3 in cardiac myocytes of the AVC, mice hemizygous for both cGATA6-Cre and the conventional null Alk3 allele were bred to mice homozygous for the floxed Alk3 allele. This breeding strategy generates 25% of mice in which Cre is present together with a conventional null allele and a floxed allele for Alk3, following a previously established breeding strategy. In that genotype, Cre action will result in AVC-targeted deletion of Alk3 (cGATA6-Cre/Alk3). The advantage of this strategy is that only one allele of Alk3 must be recombined by Cre, the other being already a null allele, which greatly increases recombination efficiency. Because original lines only were used for breeding, the F1 offspring that were analyzed were always in the same mixed background. In embryonic hearts, DNA recombination was detected only in the presence of cGATA6-Cre in a Alk3null/flox background (not shown).

cGATA6-Cre/Alk3 mice were found at weaning (20% Cre+,Alk3null/flox versus control groups: 32% Cre−,Alk3wt/flox, 28% Cre−,Alk3null/flox, and 20% Cre+,Alk3wt/flox, n=465) and no premature mortality was noticed over a 15-month period. The variation to the
Mendelian ratio seen in mice harboring the cGATA6-Cre transgene, irrespective of Alk3 genotypes, might result from interference in the mix FVB/129/B6/C57 background with the transmission of the cGATA6-Cre transgene. Mendelian transmission of the transgene was seen in the original cGATA6-Cre mouse colony in a pure FVB background (50% Cre\(^-\), 50% Cre\(^+\), n=273).

**Echocardiogram and electrophysiological analysis.** Mice were anesthetized with ketamine (0.065 mg/g), acepromazine (0.002 mg/g), and xylazine (0.013 mg/g) injected intraperitoneally. The procedures for echocardiographic examination of mice and for surface ECG have been reported previously\(^6\). A total of 32 mice were examined by ECG at 3, 6, and 12 months of age.

**Left atrial pressure measurement.** Mice (3 months old) were anesthetized as described above and ventilated via intubation. A 1.4F micromanometer catheter (Millar Instrument Inc) was inserted in the right carotid artery and advanced into LV to record LV hemodynamics. Via a sternal thoracotomy, another catheter was used for the direct measurement of left atrial pressure. At the end of the experiment, the hearts were fixed and processed as described below for histological analysis.

**Heart Isolation and Optical Mapping Studies.** Hearts were isolated by performing a thoracotomy, cannulated and Langendorff perfused as previously described\(^7,8\). Briefly, hearts were stained with a bolus injection of the voltage sensitive dye, Di-4-ANEPPS and recordings were made in the bin mode, which allows for an array of 64x64 pixels to be acquired at 947 frames/sec with 12-bit resolution\(^7\). Ten to fifteen activation sequences were then averaged to improve the signal-to-noise ratio of the fluorescent
signal. vECGs were recorded from each heart using Ag-AgCl electrodes placed approximately 1 mm from the heart surface.

**Histology.** Adult hearts and embryos were fixed in 4% paraformaldehyde/PBS and embedded in paraffin. Serial sections (6 µm) were stained with hematoxylin and eosin, modified Masson’s trichrome, or Van Gieson stain for morphologic studies. The MF20 antibody against sarcomeric myofilaments was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Immunostaining was performed using streptavidin-biotin amplification system (Zymed Laboratories Inc.), followed by a chromogen/substrate system (Vector Laboratories). Immunofluorescence was performed using antibodies against connexin 43 (Zymed Laboratories Inc.), periostin, and α-cardiac actinin (Sigma-Aldrich). To detect Cre-dependent LacZ expression, tissues were processed for whole-mount staining.

**In situ hybridization.** Embryos were fixed in 4% paraformaldehyde/PBS and embedded in paraffin. Serial sections (6 µm) were processed for radioactive in situ hybridization as described. Serial sections were incubated with 35S-labeled riboprobes, processed for emulsion autoradiography, counterstained with Hoechst, and visualized by dark-field and epifluorescence microscopy. Tissue was visualized by Hoechst dye staining of nuclear DNA. Silver grains were visualized in the dark field and were represented in false color (pink) in figures. The probe for periostin was kindly provided by E. Delot, UCLA, Los Angeles.

**Real-time quantitative RT-PCR.** RNA was prepared using the TRI reagent (Sigma-Aldrich). Expression of ANF was measured by real-time quantitative RT-PCR and normalized to 36B4 encoding a constitutive ribosomal phosphoprotein.
Statistics. Results obtained for cGATA6-Cre/Alk3 mice (Cre\(^+\),Alk3\(^{\text{null/flox}}\)) were compared to control littermates (Cre\(^-\),Alk3\(^{\text{null/flox}}\) and Cre\(^+\),Alk3\(^{\text{wt/flox}}\)) using the unpaired two-tailed t test and a significance level of P<0.05.
References


