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Elisabeth M. Zeisberg, … , Seigo Izumo, William T. Pu

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Research Article **Cardiology**

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Morphogenesis of the right ventricle requires myocardial expression of *Gata4*

Elisabeth M. Zeisberg,[1,2] Qing Ma,[3] Amy L. Juraszek,[3,4] Kelvin Moses,[5] Robert J. Schwartz,[5] Seigo Izumo,[1] and William T. Pu[3,6]

[1]Cardiovascular Division and [2]Center for Matrix Biology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, USA. [3]Department of Cardiology, Children's Hospital Boston, Boston, Massachusetts, USA. [4]Department of Pathology, Harvard Medical School, Boston, Massachusetts, USA. [5]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, USA. [6]Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA.

Mutations in developmental regulatory genes have been found to be responsible for some cases of congenital heart defects. One such regulatory gene is *Gata4*, a zinc finger transcription factor. In order to circumvent the early embryonic lethality of *Gata4*-null embryos and to investigate the role of myocardial *Gata4* expression in cardiac development, we used Cre/loxP technology to conditionally delete *Gata4* in the myocardium of mice at an early and a late time point in cardiac morphogenesis. Early deletion of *Gata4* by *Nkx2-5*^{Cre} resulted in hearts with striking myocardial thinning, absence of mesenchymal cells within the endocardial cushions, and selective hypoplasia of the RV. RV hypoplasia was associated with downregulation of *Hand2*, a transcription factor previously shown to regulate formation of the RV. Cardiomyocyte proliferation was reduced, with a greater degree of reduction in the RV than in the LV. Late deletion of *Gata4* by Cre recombinase driven by the α *myosin heavy chain* promoter did not selectively affect RV development or generation of endocardial cushion mesenchyme but did result in marked myocardial thinning with decreased cardiomyocyte proliferation, as well as double-outlet RV. Our results demonstrate a general role of myocardial *Gata4* in regulating cardiomyocyte proliferation and a specific, stage-dependent role in regulating the morphogenesis of the RV and the atrioventricular canal.

Introduction

Congenital heart defects are the most common developmental anomaly in newborns (1). Formation of the normal heart involves the transformation of a linear heart tube into the mature 4-chambered organ. The linear heart tube grows rapidly by the proliferation of cardiomyocytes and by the addition of new cardiomyocytes to the arterial pole of the heart. These new cardiomyocytes, derived from splanchnic mesoderm adjoining the arterial pole of the heart (termed the secondary heart field), give rise to the outflow tract (OFT) and also contribute to the development of the RV (2–4). As the heart tube lengthens, it loops to the right, and the resulting structure is septated by growth and maturation of the endocardial cushions, structures derived from the endothelial lining of the heart by an epithelial-to-mesenchymal transition (EMT) (5).

Genetic dissection of heart development has shown that a hierarchy of transcription factors regulates these morphogenic events (6). Relatively independent genetic programs control the development of each heart chamber, which is consistent with the frequent occurrence of selective chamber hypoplasia in severe forms of congenital heart disease (6). For example, during mouse heart development, the transcription factor *Hand2* (*dHAND*) is predominantly expressed in the RV, while the related transcription factor *Hand1* (*eHAND*) is predominantly expressed in the LV (7). Deletion of Hand2 results in severe hypoplasia of the RV segment (8).

It has been found that some cases of congenital heart defects were caused by mutations in developmental regulatory genes. Mutation of the cardiac transcription factor *Nkx2-5* is associated with familial congenital heart disease and has been associated with up to 4% of cases of tetralogy of Fallot and secundum atrial septal defect (9, 10). Haploinsufficiency for the transcription factor *Tbx1* in patients with 22q11 chromosomal microdeletions causes OFT anomalies (11, 12), while mutation of the transcription factor *Tbx5* is associated with congenital heart disease in Holt-Oram syndrome and in nonsyndromic congenital heart disease (13).

Recently, mutation of the transcription factor *Gata4* was linked to congenital heart disease characterized by atrial or ventricular septal defects (14, 15). In the heart, *Gata4* is expressed in cardiomyocytes and their mesodermal precursors, as well as in the endocardium and the epicardium (Figure 1, E, F, I, and J, and Figure 5, A, C, and E). Gata4 binds to and regulates expression of a number of myocardium-expressed genes (16, 17). Embryos lacking *Gata4* arrested in development and died due to a defect in the visceral endoderm that caused aberrant ventral morphogenesis and cardiac bifida (18–20). Using alleles of *Gata4* that cause partial loss of function, we and others have previously demonstrated that *Gata4* is required for later heart development (21, 22). However, these experiments were not able to address the tissue-restricted requirements for *Gata4*, particularly during early stages of heart development. To evaluate the function of *Gata4* within the myocardial compartment, we crossed floxed *Gata4* mice with mouse lines in which expression of Cre recombinase is initiated within the myocardium at an early and a late time point in cardiac development. We found that myocardial expression of *Gata4* was required in a stage-dependent manner for proliferation of cardiomyocytes, formation of the endocardial cushions, development of the RV, and septation of the OFT.

Nonstandard abbreviations used: *ANT*, *atrial natriuretic factor*; *BNP*, *brain natriuretic peptide*; EMT, epithelial-to-mesenchymal transition; $G4^{MC}$, $Gata4^{flox/flox}$;MHCαCre^{+}; $G4^{NK}$, $Gata4^{flox/flox}$;$Nkx2\text{-}5^{WT/Cre}$; MHC, myosin heavy chain; MHCαCre; α MHC promoter driving Cre recombinase expression; OFT, outflow tract; qRT-PCR, quantitative RT-PCR; *TNC*, *tenascin C*.

Conflict of interest: The authors have declared that no conflict of interest exists.

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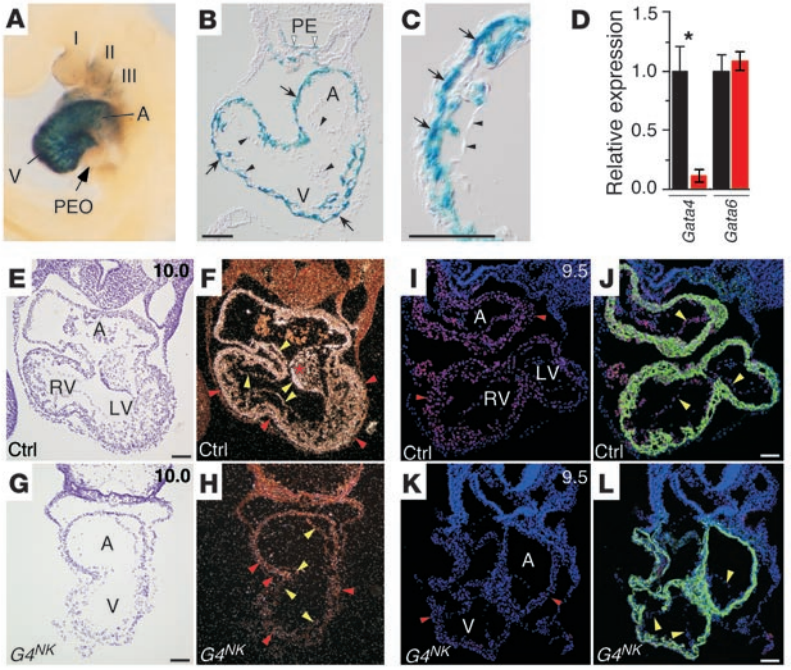

Figure 1
Early cardiomyocyte-restricted inactivation of *Gata4* by *Nkx2-5*^{Cre}. (**A**–**C**) The spatial pattern of recombination driven by *Nkx2-5*^{Cre} was determined using the R26Rstop*lacZ* reporter. X-gal–stained E9.5 embryos were examined in whole-mount (**A**) or in transverse sections (**B** and **C**). Strong staining was detected in cardiomyocytes (arrows, **B** and **C**) of the atrium (A) and ventricle (V). Weaker activity was detected in the branchial arch (I–III), epithelium, and pharyngeal endoderm (PE). No Cre activity was detected in the proepicardial organ (PEO) or endocardium (arrowheads). (**D**) Efficient inactivation of *Gata4* by *Nkx2-5*^{Cre}. *Gata4* and *Gata6* expression was measured by qRT-PCR in RNA isolated from hearts of E9.5 control (*Gata4*$^{flox/flox}$, black bars) or *G4*^{NK} embryos (red bars). *Gata4* expression was downregulated in *G4*^{NK} embryos compared with that of controls ($n = 3$; $^*P < 0.05$). (**E**–**H**) In situ hybridization for *Gata4*. *Gata4* expression was robust in myocardium (red arrowheads), endocardium (yellow arrowheads), and endocardial cushions (asterisk) of control embryos (**E** and **F**) but was not detectable in *G4*^{NK} embryos (**G** and **H**). Shown are brightfield (**E** and **G**) and darkfield views (**F** and **H**). (**I**–**L**) Immunofluorescent detection of *Gata4* in control (**I** and **J**) and *G4*^{NK} embryos (**K** and **L**). *Gata4* staining was strongly reduced in cardiomyocytes (red arrowheads) and endocardial cells (yellow arrowheads) of *G4*^{NK} embryos. **J** and **L** show the same field as **I** and **K**, with the addition of green labeling of cardiomyocytes (desmin). Red staining indicates *Gata4*; blue staining denotes nuclei; purple staining indicates colocalization of *Gata4* in the nucleus. Original magnification, ×40 (**A**). Scale bars: 100 μm (**B**, **C**, and **E**–**L**).

Results

Myocardial-restricted deletion of Gata4 early in cardiac development. Previously, in order to study the temporal and spatial requirements for *Gata4* in heart development, we generated a floxed *Gata4* allele (*Gata4*^{flox}) in which exon 2, containing the start codon and 46% of the coding sequence, is flanked by loxP sites (22). Expression of Cre recombinase resulted in deletion of exon 2, yielding a null allele (22). In order to investigate the cardiomyocyte-restricted function of *Gata4* in cardiac development, we used Cre/loxP technology to conditionally ablate *Gata4* in cardiomyocytes at 2 distinct time points in cardiac development. We achieved early cardiomyocyte-restricted deletion of *Gata4* using *Nkx2-5*^{Cre}, in which Cre recombinase expression is driven by the endogenous *Nkx2-5* locus (23). We previously reported that *Nkx2-5*^{Cre} initiated Cre expression around E7.5 in the cardiac progenitor pool and that robust Cre-mediated recombination occurred in cardiomyocytes by E9.5 (23). In order to further delineate the temporal and spatial pattern of Cre activity, we crossed *Nkx2-5*^{Cre} mice with R26Rstop*lacZ* mice, in which expression of *lacZ* requires Cre-mediated excision of a stop cassette (24). Consistent with our prior results (23), *Nkx2-5*^{Cre} activated *lacZ* expression in most cardiomyocytes of E9.5 embryos (arrows, Figure 1, B and C). Cre-mediated expression of *lacZ* did not occur in the endocardium (arrowheads, Figure 1, B and C) or in the proepicardial organ (Figure 1A). Outside the heart, *lacZ* activity was detected in the pharyngeal endoderm and in a patchy distribution in branchial arch epithelium (Figure 1A and Figure 4, A–C).

Next, we obtained *Gata4*$^{flox/flox}$;*Nkx2-5*$^{WT/Cre}$ (*G4*^{NK}) embryos from timed matings and we confirmed loss of the *Gata4* transcript in *G4*^{NK} hearts by quantitative RT-PCR (qRT-PCR) and in situ hybridization. *Gata4* expression was reduced 90% in *G4*^{NK} hearts compared with that in controls ($P < 0.05$; $n = 3$; Figure 1D), while the expression of the closely related gene *Gata6* was unaffected (Figure 1D). In situ hybridization showed that *Gata4* expression in the myocardium was not detectable above background by E9.5 in *G4*^{NK} embryos (compare red arrowheads in Figure 1H and Figure 1F). Surprisingly, we found that *Gata4* expression in the endocardium was also reduced to background levels in *G4*^{NK} embryos (compare yellow arrowheads in Figure 1H and Figure 1F). *Gata5* and *Gata6* expression was not altered in *G4*^{NK} embryos (Supplemental Figure 3; supplemental material available online with this article; doi:10.1172/JCI23769DS1).

To further confirm the loss of myocardial and endocardial *Gata4* expression, we examined Gata4 protein expression in *G4*^{NK} hearts by immunofluorescence staining (Figure 1, I–L). In control embryos, robust Gata4 staining (red stain) was localized to the nucleus (blue stain) of cardiomyocytes and endocardial cells (Figure 1, I and J). However, in *G4*^{NK} embryos, Gata4 signal was strongly decreased in both cardiomyocytes and endocardial cells (Figure 1, K and L). As the antibody used recognizes a peptide in the C terminus of Gata4, loss of immunoreactivity in *G4*^{NK} hearts suggests that excision of the floxed region of *Gata4* (exon 2, encoding the N-terminal portion of *Gata4* including the start codon) resulted in loss of Gata4 protein. Loss of endocardial *Gata4* expression was not due to a generalized defect in endothelial differentiation into endocardium, as the endocardial-specific marker Nfatc1 was expressed normally in *G4*^{NK} hearts (Supplemental Figure 1). Because Cre activity was absent in the endocardium of *Nkx2-5*^{Cre} hearts (Figure 1, B and C), loss of endocardial *Gata4* expression in *G4*^{NK} hearts suggests that a paracrine signal derived from the myocardium regulates endocardial *Gata4* expression.

Early cardiomyocyte-restricted ablation of *Gata4* in *G4*^{NK} embryos resulted in lethality by E11.5 (Figure 2A). However, overall development of *G4*^{NK} embryos at E9.5, as assessed by the number of somites, was not impaired (control, 20.8 ± 2.8 somites, $n = 15$; mutant, 21.7 ± 0.5 somites, $n = 7$). By E10.5, *G4*^{NK} embryos were

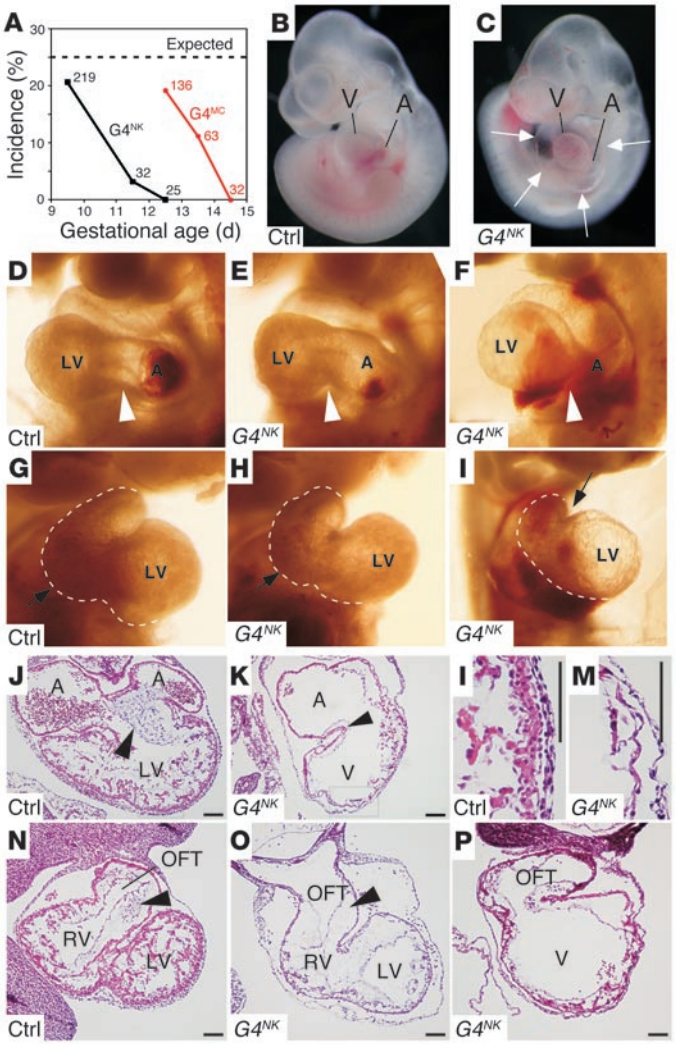

Figure 2
Phenotypic characterization of mutants after early cardiomyocyte-restricted *Gata4* deletion. (**A**) Survival of mutant embryos after myocyte-restricted deletion of *Gata4*. Numbers indicate total number of embryos genotyped at each gestational age. Expected Mendelian incidence was 25%. (**B** and **C**) Unstained whole-mount control and $G4^{NK}$ embryos at E10.5, showing pericardial effusion (white arrows) of the mutant embryo. (**D**–**I**) Control and mutant hearts, viewed from the left lateral (**D**–**F**) and right anterior oblique (**G**–**I**) positions. In control (**G**) and in 6 of 21 mutant (**H**) embryos systematically examined, the RV and OFT (arrows) connected normally to the right lateral aspect of the LV. In the remaining 15 of 21 mutant embryos, the RV was very hypoplastic or not apparent, and the OFT connected to the rostral aspect of the LV (**I**). The groove at the atrioventricular junction (arrowhead) was preserved in mutant embryos. (**J**–**P**). H&E-stained transverse sections at the level of the atrioventricular canal (**J** and **K**) and the OFT (**N**–**P**). (**L** and **M**) Higher-magnification views of boxed regions in **J** and **K**, respectively. Mutant embryos had severe myocardial hypoplasia with reduced trabeculation. Arrowheads indicate the endocardial cushions, which were small and markedly hypocellular in mutant embryos (**K** and **O**) compared with those of controls (**J** and **N**). In some mutant embryos, the RV and OFT were thin walled but normally positioned (**O**), while in other mutant embryos the RV was not apparent, and the OFT arose in an abnormal position from the ventricular chamber (**P**). Embryos in **D**–**P** had 31–32 somites. Original magnification, ×20 (**B** and **C**), ×60 (**D**–**I**). Scale bars: 100 μm (**J**–**P**).

mildly delayed in overall development (control, 31.6 ± 2.2 somites, $n = 19$; mutant, 28.5 ± 2.2 somites, $n = 6$; $P < 0.05$) and had large pericardial effusions, a sign of heart failure (compare Figure 2B and Figure 2C).

At E9.5, most $G4^{NK}$ embryos could be recognized on whole-mount examination by characteristic cardiac malformations. $G4^{NK}$ embryos had normally positioned atria and intact atrioventricular grooves (arrowheads, Figure 2, D–F). Fifteen of 21 $G4^{NK}$ embryos systematically examined in whole mount displayed a single predominant ventricular chamber that connected to an OFT located toward the rostral side of the chamber (compare arrows in Figure 2G and Figure 2I). The remaining 6 of 21 $G4^{NK}$ embryos had normal- to mildly hypoplastic–appearing RVs that connected to normally positioned OFTs (Figure 2, H and O). On histological sections, all $G4^{NK}$ embryos displayed marked myocardial hypoplasia, affecting both the compact and trabecular myocardium (Figure 2, J–M). The atrioventricular and OFT endocardial cushions were small and contained few mesenchymal cells (arrowheads, Figure 2, J, K, N, and O), which indicates a defect in the EMT of endocardial cells that normally generates cushion mesenchyme. This defect was due at least in part to the aforementioned downregulation of *Gata4* in the endocardium, as we have found that expression of *Gata4* in the endocardium is required for EMT (J. Rivera-Feliciano et al., manuscript submitted for publication).

Myocardial Gata4 regulates formation of the RV. Early myocyte-restricted deletion of *Gata4* in $G4^{NK}$ embryos resulted in the formation of a single predominant ventricular chamber in the majority of embryos (Figure 2, I and P). In order to determine the molecular identity of the chamber, we examined the expression of chamber-specific markers. In normal embryos, at E9.5 the atrial chamber expressed *myosin light chain 2a* (*MLC2a*) but not *MLC2v*, while the ventricular chambers expressed both *MLC2a* and *MLC2v*. This expression pattern was maintained in $G4^{NK}$ hearts (Supplemental Figure 2), which indicates that specification of atrial and ventricular chambers occurs normally in $G4^{NK}$ hearts. The genes *Hand1* and *Tbx5* are normally expressed at higher levels in the LV than in the RV (8, 25). In $G4^{NK}$ hearts, the expression pattern of *Hand1* and *Tbx5* was not altered, with the predominant ventricular chamber preferentially expressing these markers (Figure 3, I and J, and data not shown). These data indicate that the predominant ventricular chamber in $G4^{NK}$ hearts is the LV. The data also demonstrate that *Hand1* is not directly regulated by *Gata4*.

To determine whether a hypoplastic RV was incorporated into the tubular structure connecting the predominant ventricular chamber

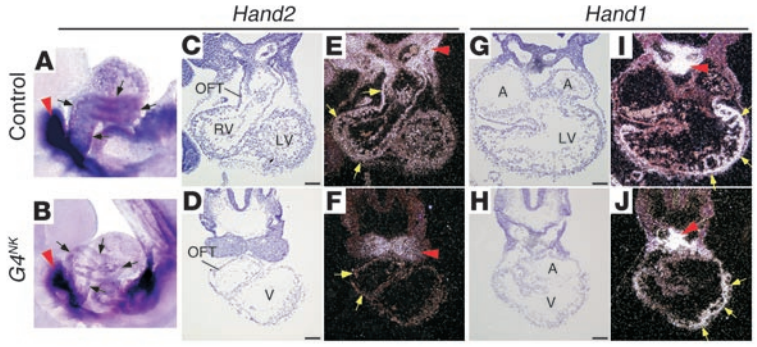

Figure 3
Expression of *Hand* genes after early myocyte-restricted *Gata4* deletion. (**A**–**F**) Whole-mount (**A** and **B**) and section (**C**–**F**) in situ hybridization for *Hand2*. In the hearts of control embryos (**A**, **C**, and **E**), *Hand2* was predominantly expressed in the RV and OFT (arrows), although its expression was also detected in the LV. In $G4^{NK}$ embryos (**B**, **D**, and **F**), *Hand2* expression was significantly decreased in the hypoplastic RV and OFT (arrows). Expression of *Hand2* was detected in the branchial arches of both control and $G4^{NK}$ embryos (arrowheads). (**G**–**J**) Section in situ hybridization for *Hand1*. *Hand1* expression was unchanged in mutant embryos compared with that in control embryos, localizing predominantly to the outer curvature of the LV (arrows), as well as to the branchial arches (arrowheads). Adjacent sections from the same mutant embryo were used in **F** and **J**. Brightfield (**C**, **D**, **G**, and **H**) and darkfield views (**E**, **F**, **I**, and **J**) are shown. Original magnification, ×32 (**A** and **B**). Scale bars: 100 μm (**C**–**J**).

to the aortic sac in mutant embryos, we examined the expression of the OFT marker *tenascin C* (*TNC*) by in situ hybridization. In control embryos, the entire length of the OFT from the morphological RV to the aortic sac expressed *TNC* (Supplemental Figure 2) (26). In contrast, in $G4^{NK}$ embryos with a single predominant chamber, *TNC* was expressed in the distal end of the OFT. However, the proximal end of the outflow tube near its junction with the predominant ventricular chamber was *TNC* negative (Supplemental Figure 2) and likely represented a severely hypoplastic RV.

The RV hypoplasia found in $G4^{NK}$ hearts was highly reminiscent of the phenotype of *Hand2*-null embryos, which failed to form the RV (8). *Hand2* is expressed in the limb buds, the branchial arches, and the myocardium of the OFT and the RV (8). By both whole-mount and section in situ hybridization, we found that *Hand2* transcript levels were decreased in $G4^{NK}$ hearts compared with those in controls (Figure 3, A–F). The decrease in *Hand2* transcript level was not due to nonspecific RNA degradation, as adjoining sections from the same embryo yielded robust signals to other probes (e.g., *Hand1*, Figure 3J). Decreased *Hand2* levels were also not simply a consequence of hypoplasia of the RV, as *Hand2* expression in the OFT was also strongly downregulated (arrows, Figure 3, E and F). *Hand2* remained robustly expressed in the branchial arches of mutant embryos (arrowheads, Figure 3, A–F). These data demonstrate that normal expression of *Hand2* requires *Gata4* and suggest that abnormal regulation of *Hand2* in $G4^{NK}$ mutants contributes at least in part to the selective requirement of *Gata4* for RV development.

The RV and OFT are derived at least in part from cardiomyocytes generated in a secondary heart field located at the junction of the splanchnic mesoderm with the caudal surface of the OFT (2–4). In chicken embryos, this secondary heart field contains progenitor cells that express both *Nkx2-5* and *Gata4* (2). One hypothesis to account for the RV hypoplasia seen in $G4^{NK}$ mutants is that *Gata4* inactivation in these progenitors impairs formation or migration of cardiomyocytes destined to contribute to the RV. To address this hypothesis, we assessed the timing and location within the secondary heart field of *Gata4*, *-5*, and *-6* expression and of Cre activity driven by $Nkx2\text{-}5^{Cre}$. By E9.5, when RV hypoplasia became apparent, few cells within this region had undergone recombination to express *lacZ* (Figure 4, A and B). At E10.5, we found that $Nkx2\text{-}5^{Cre}$ activation of the R26Rstop*lacZ* reporter within the splanchnic mesoderm was more robust (Figure 4C), as has been previously reported (27). More importantly, we found that *Gata4* was not expressed in splanchnic mesoderm at its junction with the OFT in wild-type embryos (arrowheads, Figure 4, E and I), while *Gata5* and *Gata6* were expressed in this region (arrowheads, Figure 4, F, G, J, and K). These data indicate that *Gata4* inactivation by $Nkx2\text{-}5^{Cre}$ in the secondary heart field is not responsible for hypoplasia of the RV.

Myocardial-restricted deletion of Gata4 late in cardiac development. In order to study the function of myocardial *Gata4* expression at a late time in heart development, we used transgenic mice in which Cre recombinase expression was driven by the α myosin heavy chain (MHC) promoter (MHCαCre). Prior characterization of this transgene demonstrated that MHCαCre catalyzed recombination only in the myocardium, starting at E9.5 and occurring in nearly all cardiomyocytes by E11.5 (28). We isolated $Gata4^{flox/flox}$;MHCαCre^{+} ($G4^{MC}$) embryos at E12.5 and examined *Gata4* expression by in situ hybridization and immunohistochemistry. In control embryos, *Gata4* expression was readily detected in myocardium (white arrowheads, Figure 5, A, C, and E), endocardium (yellow arrowheads, Figure 5, A and E), endocardial cushions (asterisk, Figure 5A), and epicardium (blue arrowheads, Figure 5, C and E). In $G4^{MC}$ embryos, myocardial *Gata4* expression was strongly reduced in both compact and trabecular myocardium (compare Figure 5, D and F with Figure 5, C and E), while *Gata4* continued to be expressed in epicardium and endocardium (blue and yellow arrowheads, Figure 5, B, D, and F). Expression of *Gata5* and *Gata6* were not altered in $G4^{MC}$ embryos (Supplemental Figure 4).

Late cardiomyocyte-restricted deletion of *Gata4* by MHCαCre resulted in lethality by E14.5 (Figure 2A). Mutant embryos had marked myocardial hypoplasia and double-outlet RV (Figure 5, G–L). While early excision of *Gata4* by $Nkx2\text{-}5^{Cre}$ resulted in the formation of a single predominant ventricular chamber in the majority of embryos, 2 well-formed ventricles were present after late *Gata4* inactivation by MHCαCre. In $G4^{MC}$ hearts, as in control hearts, the atrioventricular cushion divided the atrioventricular canal into separate RV and LV inflow tracts (asterisk, Figure 5, G and J). This result indicates that, unlike early myocardial *Gata4* deletion, late deletion does not impair initial steps of atrioventricular valve morphogenesis, including generation of cushion mesenchyme and growth and fusion of the atrioventricular endocardial cushions.

Myocardial Gata4 regulates cardiomyocyte proliferation. Hypoplasia of the myocardium was a prominent feature after both early and late myocyte-restricted *Gata4* deletion. This phenotype could have been due to increased cardiomyocyte apoptosis or decreased cardiomyocyte proliferation. TUNEL staining of $G4^{NK}$ and $G4^{MC}$ embryos at E9.0 and E12.5 showed that cardiomyocyte apoptosis did not increase compared with that of control littermates (data not shown). To determine whether myocardial *Gata4* inactivation

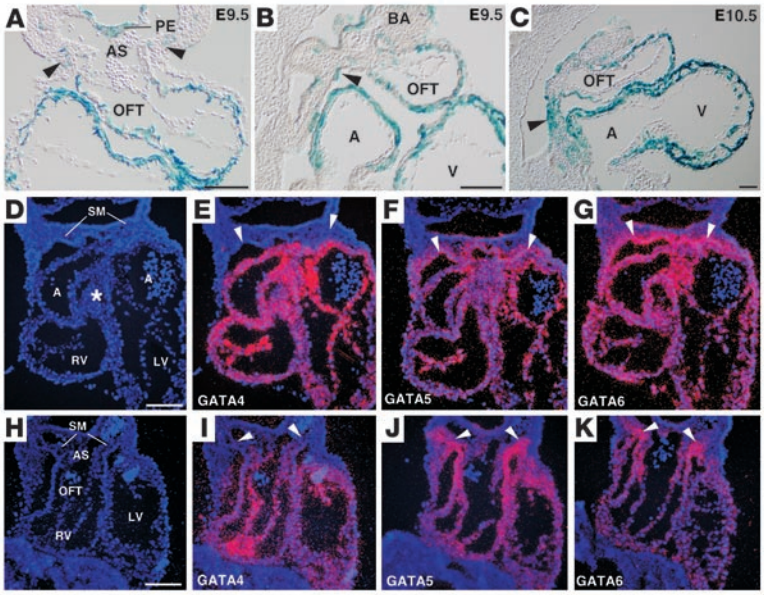

Figure 4
Pattern of *Gata4*, *Gata5*, and *Gata6* expression and *Nkx2-5*^{Cre} activity in the secondary heart field. (**A**–**C**) *Nkx2-5*^{Cre} activation of the R26Rstop*lacZ* reporter in the splanchnic mesoderm at its junction with the OFT (secondary heart field; arrowheads) at E9.5 (transverse section, **A**; sagittal section, **B**) and at E10.5 (sagittal section, **C**). Recombination in splanchnic mesoderm was detected in rare cells at E9.5 but was robust by E10.5. AS, aortic sac; BA, branchial arch. (**D**–**K**) Adjacent transverse sections of the same E9.5 control embryo (22 somites) were hybridized to probes for *Gata4*, *Gata5*, and *Gata6* (in situ signal displayed as red pseudocolor). *Gata5* and *Gata6* were detected in the splanchnic mesoderm (SM; arrowheads) and pharyngeal endoderm (data not shown). *Gata4* was not expressed in the splanchnic mesoderm, but was detected in the myocardium and endocardium. Sections in **D**–**G** were at the level of the caudal surface of the OFT (asterisk), while the plane of section in **H**–**K** was slightly more cranial, sectioned at the junction of the OFT with the aortic sac. Scale bars: 100 μm.

decreases cardiomyocyte proliferation, we measured cardiomyocyte proliferation by BrdU labeling. After both early and late *Gata4* deletion, the percentage of myocytes labeled by BrdU was significantly decreased ($P < 0.05$; Figure 6, A–H, and Table 1). Similar results were obtained in *G4*^{NK} embryos when cardiomyocyte proliferation was measured by staining for the M phase–specific marker phosphohistone H3 (data not shown). Early *Gata4* inactivation resulted in selective RV hypoplasia (Figure 2), while late *Gata4* inactivation did not (Figure 5). Consistent with these phenotypes, cardiomyocyte proliferation was more severely reduced in the RV than in the LV after early *Gata4* inactivation (Figure 6, A–F), but the reductions did not differ between RV and LV after late *Gata4* inactivation (Figure 6, G and H). These data suggest that *Gata4* is required in a cell-autonomous manner for normal proliferation of cardiomyocytes and that reduced cardiomyocyte proliferation might contribute to the selective RV hypoplasia seen in *G4*^{NK} hearts.

In order to identify transcriptional targets of *Gata4* that may mediate the effect of *Gata4* on cardiomyocyte proliferation, we asked whether expression of myocardial genes known to be important for myocardial growth was perturbed by *Gata4* deletion. *cyclin D1–D3* regulate the core cell-cycle machinery in response to mitogenic stimulation (29), and in other systems *Gata4* regulates proliferation by activating *cyclin D2* expression (30). *Bmp10* and *Hop* are both required for normal myocardial growth, and expression of both is regulated by *Nkx2-5*, which can coordinate with *Gata4* to regulate gene transcription (31, 32). qRT-PCR analysis of RNA isolated from *G4*^{MC} and *Gata4*$^{flox/flox}$ control hearts showed no difference in the expression of *Bmp10* (Figure 6I). Expression of *Hop* tended to be reduced in *G4*^{MC} hearts, but this did not reach statistical significance (Figure 6I). Unexpectedly, *cyclin D1–D3* were not downregulated in *G4*^{MC} hearts; rather, each was subtly but significantly upregulated ($P < 0.05$; Figure 6I). This indicates that *Gata4* does not directly regulate cardiomyocyte proliferation through *cyclin D1–D3*. Instead, *cyclin D1–D3* upregulation may be a compensatory response to altered expression of other components of the cell-cycle machinery following *Gata4* deletion.

Myocardial gene expression downstream of Gata4. *Gata4* has been implicated in the expression of a large number of myocardial genes, including the transcription factors *Mef2c*, *Carp*, and *Nkx2-5*, each of which is essential for normal cardiac morphogenesis (33–37). To determine whether abnormalities of cardiac morphogenesis seen in *G4*^{NK} or *G4*^{MC} mutants could be attributed to altered transcription of these genes in vivo, we measured transcript levels by qRT-PCR on RNA isolated from control and mutant hearts (Figure 7A). We found that expression of *Mef2c* and *Carp* were not significantly altered in mutant hearts after early or late myocardial *Gata4* deletion (Figure 7A). *Nkx2-5* expression was reduced by 50% in *G4*^{NK} hearts, but this decrease was expected due to haploinsufficiency for *Nkx2-5*. After late myocyte-restricted *Gata4* deletion, *Nkx2-5* expression was upregulated 1.7-fold in *G4*^{NK} hearts compared with that of controls ($P < 0.05$; Figure 7A). We previously found a similar degree of *Nkx2-5* upregulation in E12.5 hearts with a germline mutation that reduced *Gata4* expression by 70% (22). This might reflect upregulation secondary to heart failure, since *Nkx2-5* has previously been shown to be upregulated as a result of increased wall stress (38).

Gata4 has also been implicated in the expression of a number of cardiomyocyte structural genes. We measured the expression of 4 well-characterized *Gata4* targets in *G4*^{MC} hearts compared with that in controls. Surprisingly, we found that levels of *atrial natriuretic factor* (*ANF*), *brain natriuretic peptide* (*BNP*), and *MHC*β expression were unaltered in *G4*^{MC} hearts (Figure 7B). *MHC*α expression was downregulated 2-fold after late myocyte-restricted *Gata4* deletion ($P < 0.05$; Figure 7B), which is consistent with the prior finding that GATA binding sites in the proximal *MHC*α promoter are required for promoter activity in vitro (39).

Analysis of the *ANF* promoter in transgenic *Xenopus* embryos suggests that GATA binding sites in the *ANF* promoter are crucial for spatially restricting *ANF* expression from the OFT, rather than for determining the level of expression (40). To determine whether *Gata4* is required to similarly restrict the spatial distribution of *ANF* expression in mouse embryos, we used in situ hybridization to examine *ANF* expression after cardiomyocyte-restricted inactivation of *Gata4*. *ANF* is normally expressed in atrial and ventricular chamber myocardium, but not in the atrioventricular canal or OFT myocardium (41). In *G4*^{NK} hearts, *ANF* was expressed at comparable levels in atrial and ventricular chamber myocardium. However, in 2 out of 6 *G4*^{NK} embryos, *ANF* expression extended ectopically,

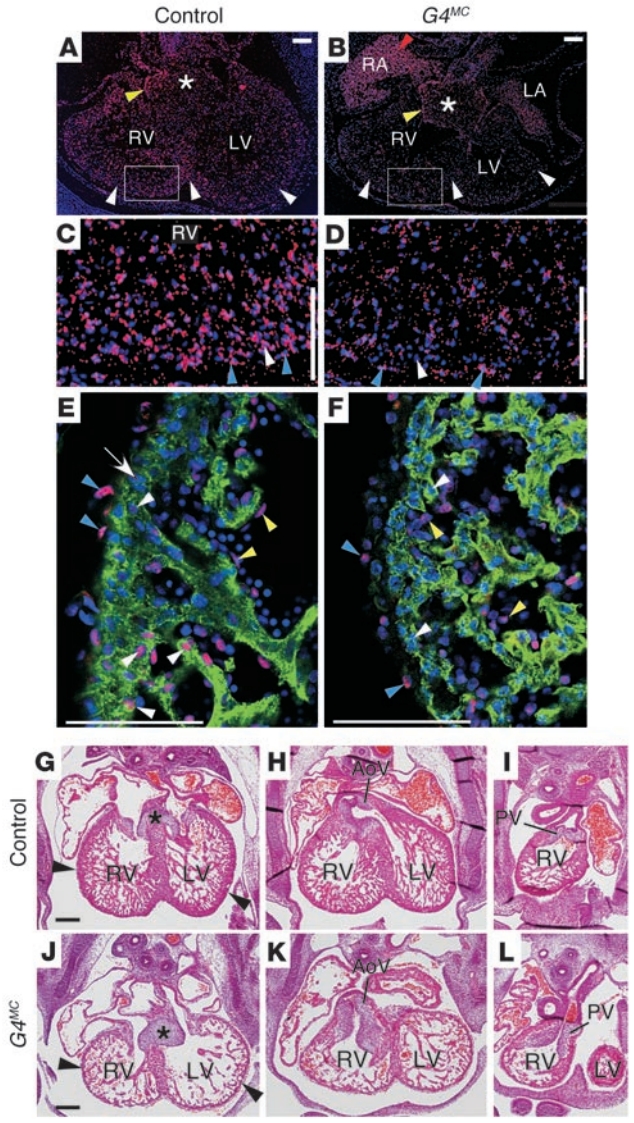

Figure 5
Late cardiomyocyte-restricted *Gata4* deletion by MHCαCre. (**A**–**D**) *Gata4* in situ hybridization (red pseudocolor) on sections from E12.5 $G4^{MC}$ and control embryos, showing inactivation of *Gata4* in the myocardium after late cardiomyocyte-restricted *Gata4* deletion (white arrowheads, compare **A** and **C** with **B** and **D**). Expression of *Gata4* in endocardium (yellow arrowheads) and endocardial cushions (asterisks) was unchanged in $G4^{MC}$ hearts compared with controls. Red blood cells gave a nonspecific signal in darkfield (red arrowhead, **B**). Blue staining indicates nuclei. Blue arrowheads indicate epicardium. (**E** and **F**) Reduced myocardial expression of Gata4 in E12.5 $G4^{MC}$ embryos compared with littermate controls. In the control ventricle (**E**), Gata4 staining was detected in myocardium (white arrowheads), epicardium (blue arrowheads), and endocardium (yellow arrowheads). Some myocyte nuclei in control embryos appeared Gata4 negative (white arrow). In the mutant ventricle (**F**), Gata4 was present in epicardium and endocardium but not in myocardium. Red staining indicates *Gata4*; green staining indicates desmin; blue staining indicates nuclei (TOPRO3). (**G**–**L**) Cardiac malformations after late cardiomyocyte-restricted *Gata4* deletion. Transverse sections of E13.5 embryos were stained with H&E. $G4^{MC}$ embryos (**J**–**L**) had marked hypoplasia of the compact myocardium (arrowheads) and reduced trabecular myocardium compared with that of control embryos (**G**–**I**). The aortic valve (AoV; **H** and **K**) and pulmonary valve (PV; **I** and **L**) arose from the RV in mutant embryos, which indicates the presence of a double-outlet RV. The atrioventricular canal was properly septated into right and left inflow channels by the endocardial cushions (asterisks), which were normally populated by mesenchymal cells (**G** and **J**). Scale bars: 100 μm (**A**–**D**), 50 μm (**E** and **F**), 200 μm (**G**–**L**).

well into the myocardium connecting the predominant ventricular chamber to the OFT (Figure 7C). Thus, in vivo *Gata4* is necessary to consistently restrict the *ANF* expression domain in mice, which is consistent with the requirement for GATA sites in the *ANF* promoter to restrict the *ANF* expression domain in *Xenopus* (40).

Discussion

To determine the myocyte-restricted function of *Gata4* in heart development, we characterized embryos in which *Gata4* was inactivated at an early and a late time point in heart development. Our results demonstrate distinct temporal requirements for myocardial expression of *Gata4* in the regulation of cardiac morphogenesis. Early myocardial expression of *Gata4* was necessary for normal cardiac expression of *Hand2* and for normal development of the RV. Early expression of *Gata4* in myocardium was also necessary to support endocardial *Gata4* expression and endocardial EMT. Late loss of myocardial *Gata4* expression did not selectively affect RV development but did result in abnormal OFT septation, manifested as double-outlet RV. Both early and late in cardiac development, normal cardiomyocyte proliferation required expression of *Gata4*.

Recently, tetraploid aggregation of *Gata4*-null embryonic stem cells was used to bypass the early developmental arrest of *Gata4*-null embryos (42). As in our embryos with cardiomyocyte-restricted *Gata4* deletion, tetraploid-rescued null embryos formed a thin, poorly trabeculated heart tube. However, early cardiomyocyte-restricted *Gata4* inactivation resulted in a milder phenotype than in tetraploid-rescued *Gata4*-null embryos, since the latter had incomplete segmentation and looping of the heart tube. This indicates that they arrested earlier in heart development than did our embryos with early myocyte-restricted *Gata4* inactivation ($G4^{NK}$). Tetraploid-rescued *Gata4*-null embryos lacked a proepicardial organ. This, in conjunction with the relatively unperturbed expression of myocardial genes, led the authors to conclude that disruption of myocardial development is non–cell autonomous and perhaps due to loss of signals from the proepicardial organ (42). However, our data directly demonstrate an important cardiomyocyte-autonomous function for *Gata4* in cardiomyocyte proliferation and RV formation. The $G4^{NK}$ phenotype is milder than the tetraploid-rescued *Gata4*-null phenotype, which supports additional roles for *Gata4* expression in non-myocyte lineages to regulate cardiac morphogenesis. Alternatively, the difference in phenotype may reflect a later deletion of *Gata4* in the $G4^{NK}$ hearts.

Because $G4^{NK}$ embryos are haploinsufficient for *Nkx2-5*, it is possible that reduced *Nkx2-5* dosage also contributed to the phenotype observed in $G4^{NK}$ embryos. However, this is unlikely for several reasons. First, *Nkx2-5* is not a direct regulator of RV specification or *Hand2* expression (43). Second, tetraploid-rescued *Gata4*-null hearts (42) had abnormalities similar to, but more severe than, $G4^{NK}$ hearts, which indicates that these phenotypes also occur in *Gata4*-null embryos that have a normal dose of *Nkx2-5*. Third, while *Nkx2-5* haploinsufficiency is associated with atrial septal defects and con-

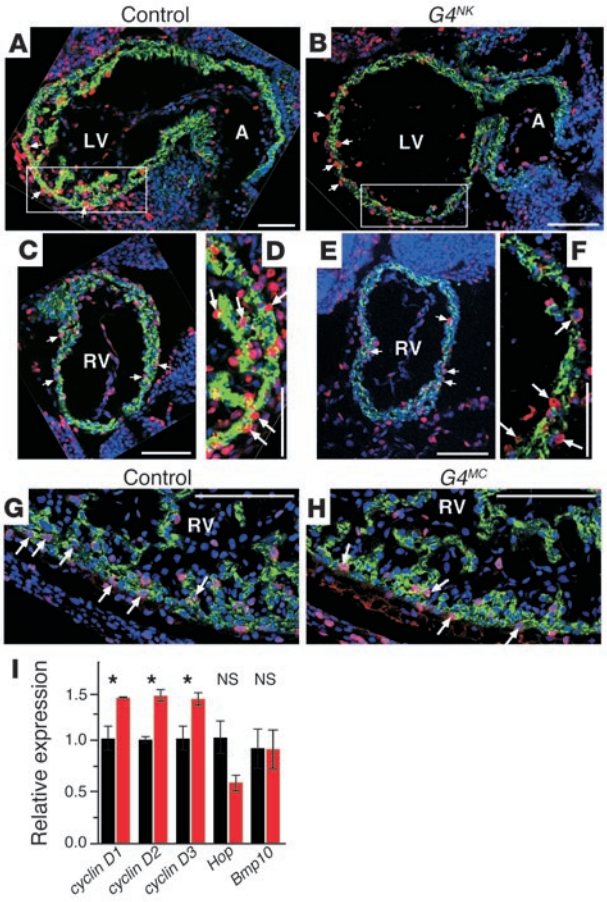

Figure 6
Decreased myocyte proliferation in mutants lacking myocardial *Gata4* expression. (**A**–**H**) Cardiomyocyte staining was assessed by BrdU labeling (red stain). Nuclei were counterstained with TOPRO3 (blue stain), and cardiomyocytes were identified by desmin staining (green stain). (**A**–**F**) Decreased cardiomyocyte proliferation in early myocyte-restricted *Gata4*-ablated ($G4^{NK}$) hearts at E9.5 (control, 21.4 ± 1.2 somites; mutant, 20.8 ± 0.7 somites). Arrows indicate representative BrdU-positive myocyte nuclei. Sagittal sections from the same control (**A**, **C**, and **D**) or $G4^{NK}$ (**B**, **E**, and **F**) embryo are shown. (**D** and **F**) Higher magnification views of the boxed regions in **A** and **B**. (**G** and **H**) Decreased cardiomyocyte proliferation in late myocyte-restricted *Gata4* ablated ($G4^{MC}$) hearts at E12.5. Arrows indicate representative BrdU-positive myocyte nuclei. (**I**) Expression of candidate *Gata4* target genes that control cardiomyocyte proliferation. RNA was isolated from E12.5 $G4^{MC}$ (red bars) and control hearts (black bars). *$P < 0.05$. Scale bars: 100 μm.

duction system abnormalities (44, 45), it has not been reported to result in an observable phenotype at this early stage of heart development. Finally, the myocardial hypoplasia of $G4^{NK}$ embryos was also seen in $G4^{MC}$ embryos, which expressed slightly elevated levels of *Nkx2-5*. Nevertheless, we cannot exclude the possibility that *Nkx2-5* haploinsufficiency contributed to the $G4^{NK}$ phenotype and to the differences between the phenotype of $G4^{NK}$ and $G4^{MC}$ hearts.

Gata4 regulates RV development. Severe hypoplasia of the RV occurred in most mutant embryos after early cardiomyocyte-restricted deletion, which indicates that *Gata4* plays an essential role in RV morphogenesis. Results of previous work have suggested that the OFT and at least a portion of the RV are derived from progenitors located within the secondary heart field (2–4). In chicks, cells within the secondary heart field express *Gata4* and *Nkx2-5* (2), and in mice, a secondary heart field enhancer has been shown to be dependent upon *Gata* activity (33). However, we found that in mice, *Gata4* was not expressed within the secondary heart field, whereas *Gata5* and *Gata6* were robustly expressed in this region, which suggests that the *Gata* isoforms active in the splanchnic mesoderm are species specific. In addition, the $Nkx2\text{-}5^{Cre}$–mediated excision in the secondary heart field occurred in a minority of cells at E9.5 (Figure 4, A and B), when hypoplasia of the RV was already apparent. These data indicate that hypoplasia of the RV in $G4^{NK}$ mutant embryos was not due to *Gata4* inactivation in RV precursors within the secondary heart field. Inactivation of *Gata4* within cells originating from the secondary heart field might occur after they have migrated into the heart tube and differentiated into cardiomyocytes; thus, our data are consistent with an important role of the secondary heart field in the development of the RV.

We found that RV hypoplasia after early myocyte-restricted *Gata4* inactivation was associated with a decrease in cardiomyocyte proliferation that was more severe in the RV than in the LV. At E9.5, RV cardiomyocytes may be more dependent upon *Gata4* for normal rates of proliferation than are LV cardiomyocytes. Alternatively, *Gata4* inactivation in RV cardiomyocyte precursors originating from the secondary heart field might impair their migration, expansion, or differentiation, which ultimately might be manifested as a decrease in cardiomyocyte proliferation. Distinguishing these models will require mapping the fate of RV progenitors in the $G4^{NK}$ mutant background.

The failure of RV development after early cardiomyocyte-restricted inactivation of *Gata4* was associated with downregulation of *Hand2* in the RV and the OFT. *Hand2* itself has been shown to be necessary for RV formation (8), which suggests that altered regulation of *Hand2* in $G4^{NK}$ embryos may contribute at least in part to the abnormal RV morphogenesis seen in these mutant embryos. Previous work has demonstrated that cardiac expression of *Hand2* is regulated by a GATA-dependent enhancer and that ablation of GATA sites within this enhancer dramatically reduces RV expression driven by this promoter in transgenic mice (46). These findings suggest that *Hand2* downregulation in $G4^{NK}$ mice might be a direct result of decreased *Gata4* binding to *Hand2* regulatory ele-

Table 1
Quantitative analysis of cellular proliferation in $G4^{NK}$ and $G4^{MC}$ hearts

	Control	Mutant	*P*
$G4^{NK}$			
Atria (%)	19 ± 3	8 ± 3	< 0.05
LV (%)	33 ± 3	18 ± 2	< 0.01
RV (%)	28 ± 2	13 ± 2	< 0.01
OFT (%)	13 ± 2	10 ± 3	NS
RV/LV (%)	88 ± 6	62 ± 4	< 0.05
$G4^{MC}$			
LV (%)	31 ± 4	21 ± 6	< 0.05
RV (%)	32 ± 3	17 ± 3	<0.05
RV/LV (%)	104 ± 10	87 ± 8	NS

Each group contained 3 to 5 embryos, each sectioned in 2 to 4 planes at least 40 μm apart.

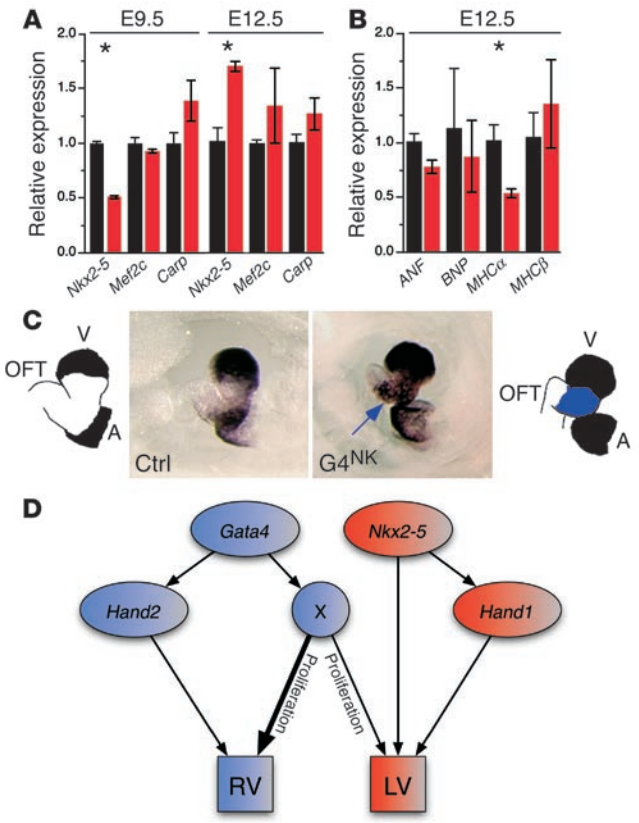

Figure 7

Gata4 regulation of cardiac gene expression. (**A**) Relative expression of transcription factors previously suggested to be *Gata4* targets. RNA was isolated from embryo hearts at E9.5 (red bars, $G4^{NK}$; black bars, $Gata4^{flox/flox}$;$Nkx2\text{-}5^{WT/WT}$ controls) or at E12.5 (red bars, $G4^{MC}$; black bars, $Gata4^{flox/flox}$;MHCαCre^{-} controls). (**B**) Relative expression of structural genes putatively identified as *Gata4* targets, in E12.5 hearts (red bars, $G4^{MC}$; black bars, $Gata4^{flox/flox}$;MHCαCre^{-} controls). Gene expression was measured by qRT-PCR and normalized to 18S ribosomal RNA. *$P < 0.05$. (**C**) Ectopic ANF expression in the OFT (blue arrow and shading), seen in 2 of 6 $G4^{NK}$ embryos examined at E9.5 by whole-mount in situ hybridization for ANF. Original magnification, ×32. (**D**) Model for the role of *Gata4* and *Nkx2-5* in the transcriptional regulation of ventricular formation. Distinct transcriptional programs control the formation of the LV and RV. *Gata4* regulates RV morphogenesis, in part through *Hand2*, whereas *Nkx2-5* regulates LV development in collaboration with *Hand1*. In addition to the specific role of *Gata4* in RV formation, *Gata4* regulates cardiomyocyte proliferation in both cardiac chambers via as-yet-unknown factors (X), which may be more dependent upon *Gata4* in the RV than in the LV.

ments. Interestingly, the GATA-dependent *Hand2* enhancer retains expression in *Gata4*-null mice, which may be due to functional substitution by *Gata5* and *Gata6* (46). The difference in normal *Hand2* enhancer activity in *Gata4*-null embryos versus decreased *Hand2* expression in $G4^{NK}$ embryos might be due to the failure of *Gata4*-null embryos to develop to the stage at which the *Hand2* enhancer requires *Gata4* function, as they arrest in development before E8.5.

Our results indicate that a *Gata4* transcriptional program is essential for RV formation and suggest that *Hand2* likely functions downstream of *Gata4* as a component of this program. Intriguingly, ablation of *Hand* genes was found to result in myocardial hypoplasia but was not associated with a change in cardiomyocyte proliferation (47). These results suggest that while *Hand2* likely contributes to the RV hypoplasia seen in $G4^{NK}$ embryos, other genes that influence cardiomyocyte proliferation must also be downstream of *Gata4* and contribute to the phenotype. The transcription factor *Nkx2-5* was previously reported to regulate expression of *Hand1* but not *Hand2* (43). *Hand1* is predominantly expressed in the LV and is an important regulator of myocardial growth and heart tube looping (48, 49); these findings led to the suggestion that *Nkx2-5* and *Hand1* selectively regulate LV formation (50). Thus, we propose a model in which *Gata4* acts through *Hand2* and other genetic pathways to regulate RV development, while *Nkx2-5* acts in conjunction with *Hand1* to regulate LV development (Figure 7D).

Severe hypoplasia of the RV was present in most, but not all, $G4^{NK}$ embryos. The incomplete penetrance of this phenotype was likely due to several factors. First, it is likely that subtle interembryo variability in the timing and extent of Cre-mediated *Gata4* inactivation occurred, which might result in different developmental outcomes. Second, the experiments were performed in a mixed genetic background, and genetic background has a strong influence on RV morphogenesis in the setting of decreased *Gata4* expression, as indicated by our finding that some *Gata4* heterozygous mice had RV hypoplasia in a pure C57BL/6 background but not in a mixed genetic background (data not shown).

Downstream targets of Gata4. Previous in vitro and in vivo promoter analysis has implicated *Gata4* in the regulation of a number of genes that play important roles in cellular proliferation, heart development, and myocardial contraction (16). Using qRT-PCR, we measured the expression level of 8 genes putatively activated by *Gata4* (*cyclin D2*, *Nkx2-5*, *Mef2c*, *Carp*, *MHCα*, *MHCβ*, *ANF*, and *BNP*) and found that only *MHCα* expression was downregulated in a manner suggestive of a required role for *Gata4* in activation of gene transcription. This might have been due to functional redundancy with other Gata factors. *Gata6* was expressed in myocardium at both E9.5 and E12.5, and its expression was not altered by *Gata4* deletion (Figure 1D and Supplemental Figures 3 and 4). At E9.5, *Gata5* was also expressed in myocardium and was unaffected by *Gata4* inactivation (Supplemental Figure 3). Thus, *Gata5* might have similarly mitigated the effect of *Gata4* inactivation on target gene expression. In addition, *Gata4* might regulate the regional expression of target genes; abnormalities of spatial distribution of a target gene might not be reflected in its overall expression level as measured by qRT-PCR. Indeed, while overall *ANF* expression was not altered in $G4^{NK}$ embryos, *ANF* was ectopically expressed in some of these hearts (Figure 7C). Altered expression of *ANF* might reflect a defect in patterning of the RV and OFT of $G4^{NK}$ hearts.

One of the most studied *Gata4* target genes is *Nkx2-5*. Several GATA-dependent enhancers control the expression of *Nkx2-5* (35–37). However, we found that after *Gata4* inactivation by $Nkx2\text{-}5^{Cre}$, *Nkx2-5* expression was downregulated by only 50% at E9.5, and this degree of downregulation was expected as a result of *Nkx2-5* haploinsufficiency. Late *Gata4* deletion resulted in a slight upregulation of *Nkx2-5*, similar to the *Nkx2-5* upregulation we previously observed in embryos with a germline mutation that reduced *Gata4* expression by 70% (22). Thus, our present data indicate that *Nkx2-5* expression after E9.5 is not dependent upon *Gata4*. Recently, BMP induction of *Nkx2-5* expression at the onset of cardiogenesis (E7.5 in mouse gestation) was shown to require

Gata4 binding to a composite GATA/Smad enhancer, which suggests that *Gata4* may be important for the induction rather than the maintenance of *Nkx2-5* expression (37). Alternatively, *Gata5* or *Gata6* may functionally substitute for *Gata4* to induce and/or maintain *Nkx2-5* expression.

Gata4 regulation of cardiomyocyte proliferation. We have found that myocardial inactivation of *Gata4* both early and late in heart development (in $G4^{NK}$ and $G4^{MC}$ mouse embryos, respectively) resulted in decreased cardiomyocyte proliferation. Myocardial hypoplasia in *Nkx2-5* knockout (43) and in *Hand* knockout mice (47) was not associated with altered cardiomyocyte proliferation or apoptosis, which suggests a defect in recruitment of cardiomyocyte precursors. Thus, the mechanism of myocardial hypoplasia due to *Gata4* inactivation is distinct and highlights the important role of *Gata4* in promoting the proliferation of cardiomyocytes.

In pulmonary artery smooth muscle, *Gata4* was found to regulate cellular proliferation, possibly through regulation of *cyclin D2* (30). In other systems, Gata factors both positively and negatively regulate cellular proliferation in a manner that is dependent on the cellular environment. For instance, *Gata1* inhibits proliferation and promotes terminal differentiation of erythroblasts (51), while it blocks interleukin-6–induced proliferation arrest in myeloid cells by sustaining *cyclin D2* expression (52). Gene expression array analysis identified multiple cell-cycle regulators downstream of *Gata1* that might mediate these effects (53). Using a focused candidate gene approach, we did not find significant downregulation of *cyclin D1–D3*, *Hop*, or *Bmp10* after myocardial *Gata4* inactivation. Unbiased array-based approaches will need to be taken in order to identify genes downstream of *Gata4* that regulate cardiomyocyte proliferation. Identification of these genes will be important for therapeutic strategies based upon expansion of cardiomyocyte populations.

Conclusion. Using Cre/loxP technology we have selectively inactivated *Gata4* within the myocardium at early and late stages of heart development. At both time points, we demonstrated that *Gata4* was an essential regulator of cardiomyocyte proliferation. Early in heart formation, myocardial inactivation of *Gata4* resulted in hypoplasia of the RV, which was associated with a downregulation of *Hand2* expression and a decrease in cardiomyocyte proliferation that was more severe in the RV than in the LV. These data have important implications for our understanding of the pathogenesis of congenital heart disease and for the development of therapeutic strategies that require modulation of cardiomyocyte proliferation.

Methods

Mice. $Gata4^{flox/flox}$ mice were generated by gene targeting followed by Flp-mediated removal of a Kan-Neo resistance cassette, as described previously (22). In these mice, exon 2, containing the start codon and 46% of the coding sequence, is deleted upon expression of Cre recombinase. $Nkx2\text{-}5^{Cre}$, MHCαCre, and R26Rstop*lacZ* alleles were described previously (23, 24, 28, 43). To obtain $G4^{NK}$ and $G4^{MC}$ embryos, timed matings were set up between $Gata4^{WT/flox}$, Cre-positive and $Gata4^{flox/flox}$, Cre-null mice. Noon of the day of the plug was defined as E0.5. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and Boston Children's Hospital.

Histology and gene expression. Embryos were dehydrated through an ethanol series and paraffin embedded. The *Gata4* probe was constructed by PCR amplification of exon 2, the exon deleted by Cre-mediated recombination. The *ANF* probe was a gift from C. Seidman (Harvard Medical School, Boston, Massachusetts, USA) (54). The full-length *TNC* probe was from an expressed sequence tag (dbEST ID 6838718). All other probes were developed as described previously (43). Section in situ hybridization was performed using ^{35}S-labeled probes as previously described (43). Darkfield signal was colored in red and superimposed on brightfield H&E- or DAPI-stained sections. Whole-mount in situ hybridization was performed using digoxigenin-labeled probes following previously established protocols (55).

Whole-mount X-gal staining for β-galactosidase was performed as previously described (56). Immunofluorescent staining for Nfatc1 or Gata4 was performed using monoclonal antibodies (Santa Cruz Biotechnology Inc.) and the ABC method (MOM kit; Vector Laboratories) with tyramide-Cy3 as the HRP substrate. Desmin counterstaining was performed using a Desmin rabbit antibody (BioMeda Corp.) and anti-rabbit Alexa Fluor 488 (Invitrogen Corp.). Nuclei were stained with TOPRO3 (Invitrogen Corp.). Phosphohistone H3 was detected with a polyclonal antibody (Upstate) using the ABC method and tyramide-Cy3. BrdU labeling was achieved by injecting pregnant mice with 2 mg of BrdU intraperitoneally. After 2 hours, embryos were harvested, and BrdU was detected with a monoclonal antibody (Sigma-Aldrich) using the ABC method and tyramide-Cy3. Detection was facilitated by antigen retrieval with HCl and trypsin digestion per instructions of the manufacturer (Sigma-Aldrich). TUNEL staining was performed on paraffin sections using the TMR Red In Situ Death Detection kit (Roche Diagnostics).

qRT-PCR. Hearts from E9.5 ($G4^{NK}$) and E12.5 ($G4^{MC}$) embryos were collected and frozen in liquid nitrogen. Hearts of each genotype ($G4^{NK}$, $n = 6$; $G4^{MC}$, $n = 3$) were pooled into a single sample, and RNA was prepared using the RNeasy kit with on-column DNase digestion (QIAGEN). Three samples were used per genotype. RNA integrity was verified and concentration determined using an Agilent Bioanalyzer (model 2100; Agilent Technologies). Real-time qRT-PCR was performed on an ABI 7700 Sequence Detector (Applied Biosystems) using Taqman probes (*Gata4*, *Gata6*, *Nkx2-5*, *Carp*, *ANF*, *cyclin D1*, *cyclin D2*, *cyclin D3*, *Hop*, and *Bmp10*, all from GenScript; and *18S* from Applied Biosystems). For primer and probe sequences, see Supplemental Table 1.

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Address correspondence to: William T. Pu, Department of Cardiology, Children's Hospital Boston, Enders 13, 300 Longwood Avenue, Boston, Massachusetts 02115, USA. Phone: (617) 919-2019; Fax: (617) 730-0140; E-mail: wpu@enders.tch.harvard.edu.

Seigo Izumo's present address is: Novartis Institute for Biomedical Research, Cambridge, Massachusetts, USA.

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