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Cardiomyopathy in *Irx4*-Deficient Mice Is Preceded by Abnormal Ventricular Gene Expression

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To define the role of Irx4, a member of the *Iroquois* family of homeobox transcription factors in mammalian heart development and function, we disrupted the murine *Irx4* gene. Cardiac morphology in Irx4-deficient mice (designated $Irx4^{\Delta ex2/\Delta ex2}$) was normal during embryogenesis and in early postnatal life. Adult $Irx4^{\Delta ex2/\Delta ex2}$ mice developed a cardiomyopathy characterized by cardiac hypertrophy and impaired contractile function. Prior to the development of cardiomyopathy, $Irx4^{\Delta ex2/\Delta ex2}$ hearts had abnormal ventricular gene expression: Irx4-deficient embryos exhibited reduced ventricular expression of the basic helix-loop-helix transcription factor *eHand* (*Hand1*), increased *Irx2* expression, and ventricular induction of an atrial chamber-specific transgene. In neonatal hearts, ventricular expression of *atrial natriuretic factor* and α-*skeletal actin* was markedly increased. Several weeks subsequent to these changes in embryonic and neonatal gene expression, increased expression of hypertrophic markers *BNP* and β-*myosin heavy chain* accompanied adult-onset cardiac hypertrophy. Cardiac expression of *Irx1, Irx2*, and *Irx5* may partially compensate for loss of *Irx4* function. We conclude that Irx4 is not sufficient for ventricular chamber formation but is required for the establishment of some components of a ventricle-specific gene expression program. In the absence of genes under the control of Irx4, ventricular function deteriorates and cardiomyopathy ensues.

The atrial and ventricular chambers of the mammalian heart are exquisitely tailored for their precise roles in circulating blood. Unique properties of atrial and ventricular cells, conserved throughout vertebrate evolution, enable the specialized roles that each chamber plays in cardiac function (2, 17, 22, 23, 31, 37, 41). Structurally, atrial myocytes have poorly developed sarcoplasmic reticulums and disorganized sarcomeres compared to ventricular myocytes and contain dense-core secretory granules that are absent in the ventricles. Atrial myocytes display shorter times of contraction and relaxation than their ventricular counterparts, and misexpression of chamber-specific contractile proteins results in abnormal myocardial function (20, 41, 47). Presumably such differences have evolved to accommodate the specific hemodynamic load of each chamber; these differences may also be important for myocardial adaptation to diseases such as hypertension and hypertrophy (12, 13, 17, 28).

The anatomical and functional differences between atrial and ventricular myocardium reflect the expression of specific genes in each chamber. Experiments with chickens suggest that external positional information acts on the precardiac cells in the earliest stages of differentiation, but soon after cardiac differentiation the plasticity of the myocytes is lost, and cardiac cells are irreversibly programmed as atrial or ventricular (40, 55, 56). Subsequently, however, the establishment of chamber-specific gene expression occurs as a gradual and dynamic process throughout embryogenesis. Prior to heart tube formation, expression of ventricle-specific gene *myosin light chain 2v* (*MLC2v*) is already regionalized, presumably in the ventricular precursors (34). During heart tube formation and subsequent morphogenetic remodeling to form the mature heart, regionalization of most other transcripts is evident, so that by the time the heart has two atria and ventricles the majority of chamber-specific genes are expressed in their final anatomical compartments (10, 29, 33, 35, 38, 53, 56). Some genes exhibit delayed regionalization; for example, the *atrial natriuretic factor* (*ANF*) gene is expressed in both embryonic atria and ventricles but at birth ventricular expression is down-regulated (17, 57). Despite progress in determining the patterns of chamber-specific gene expression during mammalian development, the factors that control the assignment of one gene to its predominant site of expression are not known.

We have recently identified in chickens, mice, and humans a new member of the *Iroquois* gene family, *Irx4*, whose cardiac expression is restricted to the ventricles of the developing heart

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(1, 9). *Irx4* is the earliest marker of the ventricular precursors and is expressed in ventricular myocardium during all stages of cardiac development, including during adulthood. Transient misexpression of mouse Irx4 or of a dominant-negative Irx4 molecule in chicken embryos disrupted the chamber-specific expression of cardiac *myosin heavy chain* genes (1). By virtue of its homology to *Iroquois* patterning genes and its ventricle-specific expression pattern, Irx4 is a good candidate for a molecule involved in regulating ventricular specification in the developing heart. To fully elucidate its role in heart development and function, we disrupted the murine *Irx4* gene. Irx4-deficent mice develop adult-onset cardiac hypertrophy that is preceded by abnormal ventricular gene expression.

MATERIALS AND METHODS

Generation of *Irx4*-targeted and transgenic mice. A genomic clone comprising the *Irx4* gene was isolated by screening a 129/SvJ mouse genomic phage library with the *Irx4* cDNA. A replacement targeting construct was constructed (see Fig. 1); this resulted in the deletion of a 650-bp fragment that includes the 3′ end of exon 2 (including the translation initiation codon), intron 2, and part of exon 3. The *thymidine kinase* gene driven by the *PGK* promoter was inserted at the end of the 3′ region of homology. The targeting construct was electroporated into 3×10^7 C1 embryonic stem cells (30). Three separate embryonic stem cell lines were injected into mouse blastocysts; one chimeric mouse transmitted the targeted allele through the germ line. $Irx4^{\Delta ex2/\Delta ex2}$ and $Irx4^{\Delta ex2/+}$ mice were maintained on a mixed (SvJ × BlackSwiss) background. Genotyping was performed by PCR using three primers designed to amplify the wild-type and mutant alleles; primer sequences are available upon request. Reverse transcription-PCR (RT-PCR) was performed using primer pairs i (GCGGGCCGGCTCTTTCCTG) and iv (AGTTCTAGCTCCTTGTCGTCTTTG) or ii (CCCGGCATGTCCTACCC GCAGTTT) and iii (GCAGGCCCGGAATCAGCCAGTGTG). SMyHC3-HAP transgenic mice were generated as previously described (54) and were crossed with $Irx4^{\Delta ex2/\Delta ex2}$ mice.

Physiological measurements. Echocardiography of adult mice was performed as previously described (36) using a Sonos 5500 (Hewlett-Packard) with a 12-MHz transducer. Conscious systolic blood pressure was measured by tail cuff using a Visitech BP2000. Mice were acclimatized to the instrument twice a day for 5 days; sequential measurements were acquired twice a day for 3 days. In vivo left ventricle (LV) physiological measurements and electrophysiological analysis were performed as previously described (4, 24). All physiological analyses were done blinded to the genotypes of the animals.

Analysis of gene expression. In situ hybridization of whole embryos was performed as previously described (44). In situ hybridization on paraffin sections was done using a modification of the whole-mount protocol. Northern blots were prepared and hybridized according to standard protocols, using cDNA or oligonucleotide probes. Blots were quantitated using a phosphorimager (Molecular Dynamics) and normalized to the signal from a *GAPDH* probe. Fold increases are reported as means of three to five individual samples and are significantly different from control values at P values of <0.05. Oligonucleotide probes corresponding to α-*skeletal actin*, α-*myosin heavy chain* (α*MHC*), β-*myosin heavy chain* (β*MHC*), *phospholamban, serca2, MLC1a, MLC1v, MLC2a*, and *MLC2v* were synthesized according to previously published sequences (36). cDNA probes are listed below. Alkaline phosphatase (AP) staining of embryos was done as previously described (54).

cDNA probes. The cDNA probes used were α*MHC* (35), β*MHC* (35), *Chisel* (R. P. Harvey, unpublished data), *COUP-TFII* (42), *dHand* (49), *eHand* (49), *FOG-2* (51), *Hermes* (25), *Irx4* (9), *Irx2* (referred to as *Irx6* in reference 15) (7), *MLC1a* (35), *MLC1v* (35), *MLC2a* (33), *MLC2v* (38), *MLC3f*(32), *Msg1* (19), and *Tbx5* (10). *ANF, BNP, BMP10*, and *FGF12* cDNAs were cloned by PCR amplification of reverse-transcribed heart RNA using primers based on the published mouse sequences (GenBank accession no. K02781, D16497, AF101033, and AF020738, respectively). Mouse *Irx1* and *Irx5* cDNAs were obtained from a mouse embryonic heart cDNA library (Stratagene) that was screened with the *Irx4* cDNA. Mouse *Irx3* cDNAs were identified initially in a library screen; the probe used here was obtained as an expressed sequence tag (GenBank accession no. AI154095).

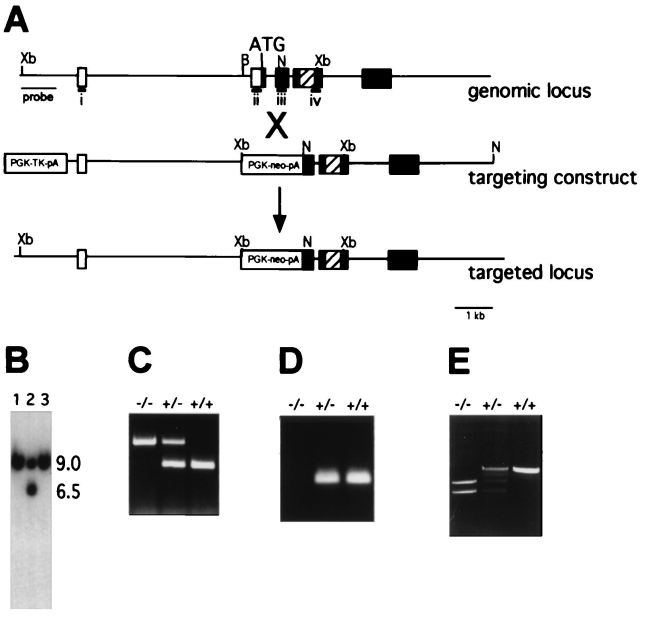

FIG. 1. Targeted disruption of *Irx4*. (A) Diagram of the *Irx4* genomic locus, targeting construct, and targeted locus. Open boxes, untranslated sequences; solid boxes, coding sequences; hatched boxes, homeodomain-coding sequences. Only relevant restriction enzyme sites are shown. (B) Southern blot of *Xba*I-digested embryonic stem cell DNA using a probe external to the targeting construct, showing a targeting event (lane 2) as evidenced by two bands representing the endogenous 9-kb allele and the 6-kb targeted allele. (C) PCR identification of wild-type and targeted alleles in $Irx4^{+/+}$ (+/+), $Irx4^{\Delta ex2/+}$ (+/−) and $Irx4^{\Delta ex2/\Delta ex2}$ (−/−) mice. (D) RT-PCR of mouse heart RNA for all three genotypes using primers internal to the deletion (ii and iii in panel A). (E) RT-PCR of mouse heart RNA for all three genotypes using primers external to the deletion (i and iv in panel A). Xb, *Xba*I; B, *Bam*HI; N, *Not*I.

RESULTS

Mice with a targeted disruption of *Irx4* were generated by homologous recombination in embryonic stem cells (Fig. 1). The first coding exon and part of the second coding exon of *Irx4* were eliminated, resulting in targeted allele designated $Irx4^{\Delta ex2}$ (Fig. 1A to D). One-fourth of the offspring of $Irx4^{\Delta ex2/+}$ animals were $Irx4^{\Delta ex2/\Delta ex2}$ animals, thereby indicating that Irx4 is not essential for viability. There was no increase in mortality in $Irx4^{\Delta ex2/\Delta ex2}$ animals compared to that of wild-type mice. Northern blot and RT-PCR analyses revealed that two transcripts are still transcribed at normal levels from the $Irx4^{\Delta ex2}$ allele. The sequencing of RT-PCR products generated using oligonucleotide primers outside the deleted region (Fig. 1E; see Materials and Methods) defined the structure of the *Irx4* transcripts produced from the $Irx4^{\Delta ex2}$ allele. Two transcripts that contained *Irx4* sequences were identified. The 5′ ends of these transcripts contained exon 1 of the *Irx4* gene, the 3′ end of the *PGKneo* gene, and sequences from *Irx4* exon 3; all of *Irx4* exon 2 and part of exon 3 were deleted. All of these transcripts lacked a ribosome binding site and initiation codon (data not shown). We concluded that the $Irx4^{\Delta ex2}$ allele does not encode a functional Irx4 protein.

We assessed cardiac gene expression in the hearts of $Irx4^{\Delta ex2/\Delta ex2}$ embryos and 10-day-, 6-week-, and 24-week-old animals by Northern blot analysis or in situ hybridization. At all time points examined, $Irx4^{\Delta ex2/\Delta ex2}$ and wild-type LVs con-

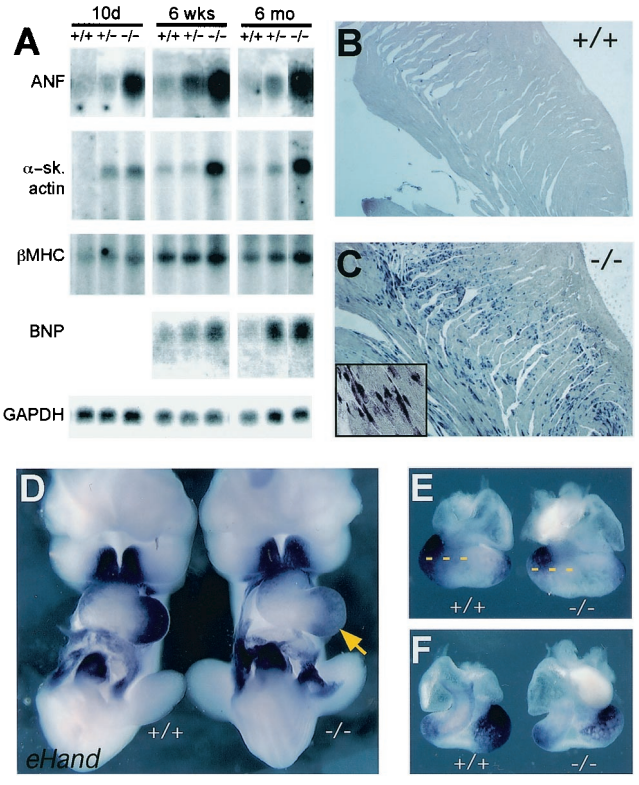

FIG. 2. Gene expression in wild-type (+/+) and *Irx4*$^{\Delta ex2/\Delta ex2}$ (−/−) mice. (A) Northern blot analysis of gene expression of ventricular RNA for wild-type, heterozygous (+/−), and *Irx4*$^{\Delta ex2/\Delta ex2}$ mice aged 10 days, 6 weeks, or 6 months. Representative signals for *ANF,* α-*skeletal actin,* β*MHC, BNP*, and *GAPDH* (as a loading control) are shown. (B and C) Expression of *ANF* by in situ hybridization on longitudinal sections of wild-type (B) and *Irx4*$^{\Delta ex2/\Delta ex2}$ (C) hearts at 6 months of age showing increased *ANF* transcript levels in *Irx4*$^{\Delta ex2/\Delta ex2}$ ventricles. (D to F) *eHand* expression in E10.5 embryos viewed ventrally (D) or in hearts dissected from E10.5 embryos viewed from the back (E) or the front (F). The arrow indicates lower *eHand* mRNA levels in *Irx4*$^{\Delta ex2/\Delta ex2}$ embryonic hearts. Dashed lines provide a comparison of the domains of *eHand* expression.

tained equal amounts of α-*cardiac myosin heavy chain* (*MHC*), *phospholamban, serca2, MLC1a, MLC1v, MLC2a, MLC2v*, and *Tbx5*. In contrast, at 10 days postbirth *ANF* and α-*skeletal actin* mRNA levels were higher by factors of 5.2 ± 0.6 and 5.8 ± 1.3 in *Irx4*$^{\Delta ex2/\Delta ex2}$ hearts than in wild-type hearts, respectively (Fig. 2A). At 6 weeks, β*MHC* mRNA levels were also increased (by a factor of 2.1 ± 0.2 versus the wild-type level). By 24 weeks, *Irx4*$^{\Delta ex2/\Delta ex2}$ hearts contained increased levels of *ANF* (factor of 6.7 ± 1.3), *BNP* (factor of 4.3 ± 0.6), α-*skeletal actin* (factor of 5.4 ± 0.8), and β*MHC* (factor of 2.7 ± 0.15) mRNAs compared to mRNA levels in wild-type hearts (Fig. 2A). Heterozygous *Irx4*$^{\Delta ex2/+}$ animals exhibited intermediate increases of these mRNAs, suggesting an inverse dose relationship between Irx4 levels and RNA expression. In situ hybridization of an *ANF*-specific probe to sections of adult *Irx4*$^{\Delta ex2/\Delta ex2}$ myocardium demonstrated an uneven distribution of *ANF*, with *ANF* mRNA localized mainly in the trabecular zone, an area of normal *ANF* expression in fetal, but not postnatal, life (Fig. 2B and C). No difference in *ANF* expression between wild-type and *Irx4*$^{\Delta ex2/\Delta ex2}$ mice was observed in embryonic day 13.5 (E13.5) fetal hearts (data not shown).

To determine if Irx4 regulates the developmental expression of chamber-specific transcription factors implicated in cardiac development or of other chamber-specific genes, in situ hybridization of whole-mount *Irx4*$^{\Delta ex2/\Delta ex2}$ embryos (E10.5 to E11.5) was performed with cDNA probes. The expression patterns of *Chisel, COUP-TFII, dHand, FGF12, FOG-2, Hermes, Msg1, BMP10, Tbx5, MLC1a, MLC1v, MLC2a, MLC2v,* α*MHC*, and β*MHC* in wild-type and *Irx4*$^{\Delta ex2/\Delta ex2}$ embryonic hearts were comparable (data not shown). However, expression of the basic helix-loop-helix (bHLH) transcription factor *eHand*, which at E10.5 to E11.5 predominates in the LV and part of the right ventricle (RV) (5, 49), was altered (Fig. 2D to F). *eHand* expression in *Irx4*$^{\Delta ex2/\Delta ex2}$ embryos was diminished in the anterior and ventral regions of the developing LV (n = 4).

To further explore the role of Irx4 in directing chamber-specific gene expression, we mated *Irx4*$^{\Delta ex2/+}$ mice to transgenic mice expressing human AP under the control of the *slow myosin heavy chain 3* (*SMyHC3*) promoter. The *SMyHC3* gene is the quail homolog of the chicken *atrial myosin heavy chain* gene, and the transgenic mice express AP robustly in developing atria but not in ventricles (54). Heterozygous *SMyHC3-HAP/Irx4*$^{\Delta ex2/+}$ mice were mated to *Irx4*$^{\Delta ex2/+}$ mice to generate *SMyHC3-HAP/Irx4*$^{\Delta ex2/\Delta ex2}$ embryos. At E9.5, the atrial cham-

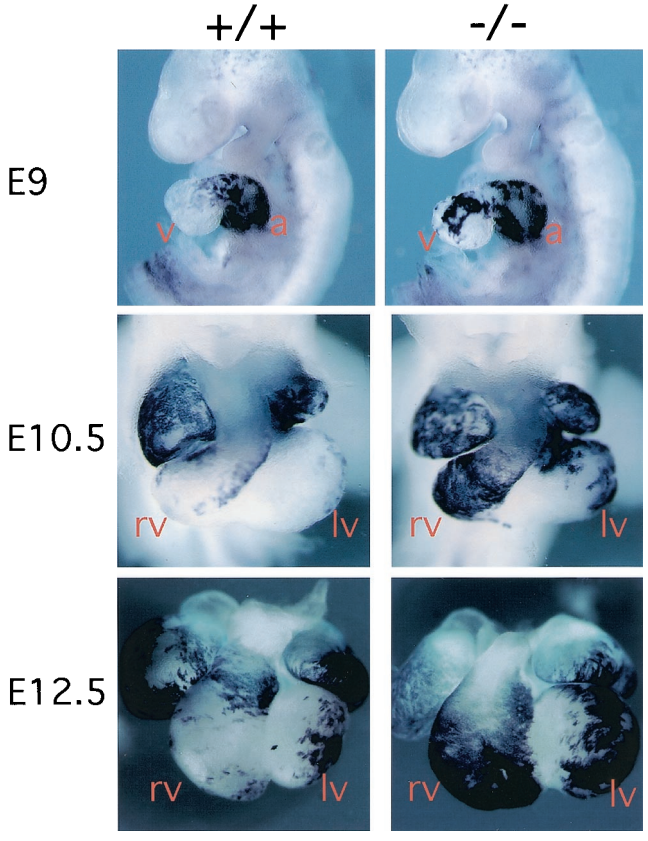

FIG. 3. SMyHC3-HAP transgene expression in wild-type (+/+) and *Irx4*$^{\Delta ex2/\Delta ex2}$ (−/−) embryos at E9, E10.5, and E12.5. Hearts were removed from E12.5 embryos for better visualization. a, atrium; v, ventricle; lv, LV; rv, RV.

ber-specific transgene was expressed in the presumptive LV as well as the atria of *SMyHC3-HAP*/*Irx4*$^{\Delta ex2/\Delta ex2}$ embryos (Fig. 3), showing derepression of the *SMyHC3-HAP* transgene in a portion of the ventricles. At E10.5 and E12.5, marked AP staining was detected in both the LV and RV (Fig. 3). However, AP expression was nonuniform and restricted to the LV and RV free walls at E10.5; by E12.5 the entire RV expressed the reporter gene, but transgene expression was excluded from the region to the left of the interventricular septum. These data imply a role for Irx4 and other factors in regulating chamber-specific gene expression in the early embryo; Irx4 can function to repress atrial gene expression within developing ventricular chambers.

Members of the *Iroquois* gene family in *Xenopus laevis* and *Drosophila melanogaster* are functionally interchangeable and partially redundant (3, 11, 18, 26, 27). To determine whether other Iroquois family members compensated for the lack of Irx4 in mutant mice, we attempted to identify additional Iroquois genes exhibiting cardiac expression. An E10 embryonic heart cDNA library was screened with the *Irx4* cDNA, and three additional Iroquois genes were identified: *Irx1, Irx2*, and *Irx5*. Expression of *Irx1*, *-2*, *-3*, and *-5* was assessed in wild-type and *Irx4*$^{\Delta ex2/\Delta ex2}$ hearts. Only *Irx1, Irx2*, and *Irx5* were expressed in the heart (Fig. 4). *Irx1* and *Irx2* were detectable in a subset of cells near the interventricular groove (Fig. 4C, D, F, and G). *Irx5* was present in both atria and ventricles but was excluded from the atrioventricular junction (Fig. 4A, B, and D). Although the levels of *Irx1* and *Irx5* in the mutant hearts were not significantly altered (data not shown), increased *Irx2* expression was observed in *Irx4*$^{\Delta ex2/\Delta ex2}$ hearts (Fig. 4F and G; $n = 3$).

To determine the consequences of Irx4 deficiency on postnatal cardiac structure and function, histopathologic and hemodynamic studies were performed. The hearts from 10-day-old *Irx4*$^{\Delta ex2/\Delta ex2}$ and *Irx4*$^{\Delta ex2/+}$ pups were indistinguishable from those of wild-type pups (assessed by morphology, heart weight-to-body weight ratios, and histology; data not shown). Chamber-specific analyses demonstrated right atrium (RA) enlargement in *Irx4*$^{\Delta ex2/\Delta ex2}$ mice at 6 weeks of age, with an average increase in RA weight/body weight ratio of 51% compared to that for their wild-type or heterozygous littermates ($n = 6$; $P < 0.02$), a finding suggestive of RV dysfunction. Hearts from mature *Irx4*$^{\Delta ex2/\Delta ex2}$ mice, age 24 weeks, exhibited significant increases in the ratios of each chamber weight to body weight compared to those from wild-type or heterozygous mice (left atrium, +44%; RA, +57%; LV, +18%; RV, +32%; $n = 7$; $P < 0.05$ for each). In vivo assessments confirmed LV hypertrophy (Table 1) in adult mutant mice; wall thickness was greatest in homozygous *Irx4*$^{\Delta ex2/\Delta ex2}$ mice, but LV hypertrophy was evident in heterozygous *Irx4*$^{\Delta ex2/+}$ mice compared to wild-type mice. Despite increased wall thickness, light microscopy revealed normal myocardial histology without fibrosis in adult heterozygous and homozygous mice. Immunohistochemistry and electron microscopy revealed no pro-ANF secretory granules (data not shown), an ultrastructure unique to atrial cardiocytes (17).

Cardiac echocardiography demonstrated abnormal ventricular function in *Irx4*$^{\Delta ex2/\Delta ex2}$ mice including increased end-systolic dimensions, reduced fractional shortening, and diminished velocity of fiber shortening compared to wild-type mice (Table 1 and Fig. 5). In vivo physiological measurements confirmed echocardiographic findings and showed increased end-systolic volumes and decreased ejection fractions (data not shown) in *Irx4*$^{\Delta ex2/\Delta ex2}$ mice at 10 and 24 weeks of age. Blood pressure and electrical parameters were normal in *Irx4*$^{\Delta ex2/\Delta ex2}$ mice (data not shown). We conclude that Irx4 deficiency adversely effects ventricular function and causes a cardiomyopathy characterized by myocardial hypertrophy, chamber dilation, and systolic dysfunction.

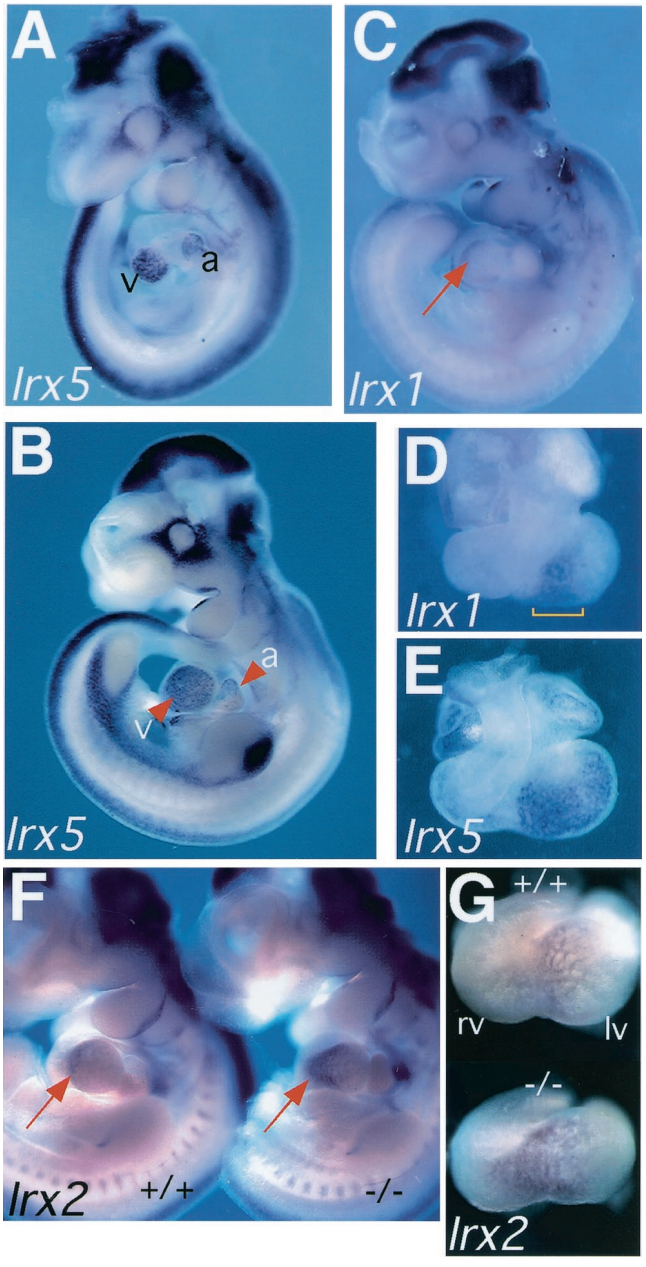

FIG. 4. Cardiac expression of Irx family genes. *Irx5* is robustly expressed in both atria and ventricles of E9.5 (A) and E10.5 (B and E) embryos. Expression of *Irx1* was detected in a subset of ventricular cardiocytes at E10.5 (arrow in panel C, bracket in panel D). *Irx2* is also expressed in a pattern that overlaps *Irx1* (F and G; red arrows in panel F); *Irx2* expression is increased in E10.5 *Irx4*$^{\Delta ex2/\Delta ex2}$ embryonic hearts (F and G) a, atrium; v, ventricle; rv, RV; lv, LV.

TABLE 1. Echocardiographic parameters for wild-type, heterozygous $Irx4^{\Delta ex2/+}$ mice, and homozygous $Irx4^{\Delta ex2/\Delta ex2}$ mice[a]

Mouse age (wks)	Genotype (*n*)	HR (min^{-1})	LVAW (mm)	LVPW (mm)	LVEDD (mm)	LVESD (mm)	LVFS (%)	VcF (circumferences/s)
6	Wild type (6)	559 ± 41	0.78 ± 0.02	0.78 ± 0.02	3.20 ± 0.06	1.51 ± 0.12	53 ± 3	ND
	$Irx4^{\Delta ex2/+}$ (4)	551 ± 17	0.87 ± 0.01	0.86 ± 0.01	3.00 ± 0.11	1.33 ± 0.05	55 ± 2	ND
	$Irx4^{\Delta ex2/\Delta ex2}$ (6)	566 ± 22	0.90 ± 0.06	0.90 ± 0.06	3.22 ± 0.09	1.65 ± 0.15	48 ± 3	ND
24–30	Wild type (6)	547 ± 41	0.85 ± 0.04	0.85 ± 0.04	3.37 ± 0.17	1.51 ± 0.20	55 ± 5	10.7 ± 1.2
	$Irx4^{\Delta ex2/+}$ (5)	525 ± 27	1.00 ± 0.04*	1.00 ± 0.04*	3.20 ± 0.20	1.46 ± 0.14	55 ± 2	9.4 ± 0.4
	$Irx4^{\Delta ex2/\Delta ex2}$ (7)	453 ± 24	1.06 ± 0.03*	1.06 ± 0.03*	3.65 ± 0.15	2.22 ± 0.22*#	40 ± 4*#	7.1 ± 0.6*

[a] *, significant difference ($P < 0.05$) compared with wild-type mice; #, significant difference ($P < 0.05$) compared with $Irx4^{\Delta ex2/+}$ mice. HR, heart rate; LVAW, LV anterior wall thickness; LVEDD, LV end-diastolic diameter; LVFS, LV fractional shortening; LVPW, LV posterior wall thickness; LVESD, LV systolic diameter; VcF, velocity of fiber shortening; ND, not determined.

DISCUSSION

We have shown that mice with a targeted disruption of the ventricle-specific homeodomain gene *Irx4* exhibit aberrant ventricular gene expression and maturity onset cardiomyopathy. Decreased ventricular *eHand* expression and derepression of an atrial chamber-specific transgene in *Irx4*-targeted embryos indicate a role for Irx4 in some, but not all, aspects of ventricle-specific gene expression and patterning during heart development. Inappropriate postnatal ventricular expression of *ANF,* α*-skeletal actin*, and β*MHC* in $Irx4^{\Delta ex2/\Delta ex2}$ mice suggests that Irx4 participates in lifelong maintenance of the ventricular phenotype. While not essential for ventricular chamber formation, Irx4 is required for normal ventricular function.

The response of *eHand* and of the *SMyHC3-HAP* transgene in $Irx4^{\Delta ex2/\Delta ex2}$ embryos indicates that Irx4 controls specific aspects of ventricle-specific gene expression in the developing mouse heart. Aberrant *eHand* expression in the $Irx4^{\Delta ex2/\Delta ex2}$ embryo may indicate that Irx4 functions in a manner analogous to Iroquois proteins in *Drosophila* and *Xenopus* that establish boundaries of proneural bHLH expression (3, 26, 27) or simply reflects a role for Irx4 in maintaining increased *eHand* expression levels. The regulation of *eHand* gene expression in heart development has not been well defined; however it has been shown that *eHand* expression is decreased in mice lacking *Nkx2-5* or *FOG-2* (5, 50, 52). *Irx4* expression is reduced in mice lacking Nkx2-5 (9) but not in FOG-2-deficient mice (52), suggesting that parallel pathways regulate cardiac *eHand* expression. This is the case in *Drosophila*, where parallel pathways involving the *Drosophila* homologs of *Irx4* (*Iroquois* genes) and *FOG-2* (*u-shaped*) are responsible for the regulation of proneural bHLH genes (16, 26). The role of eHand in cardiomyocytes is unclear and appears not to be related to normal cardiac differentiation, but instead is likely to be related to growth and the looping of the myocardium (21, 45, 46).

Our observation that lack of Irx4 results in the derepression of *ANF* in the ventricles shortly after birth indicates that Irx4 is a key negative modulator of *ANF*. *Irx4* is expressed in the postnatal ventricular myocardium (9), supporting a role in regulating gene expression after birth as well as in the embryo. The regulation of the *ANF* gene in cardiac development is complex (17, 48, 57). *ANF* is initially expressed in the RV precursors, after which its expression appears in the atrial precursors. Subsequent to chamber formation, *ANF* expression remains strong in the atria and the trabecular region of the ventricles. After birth, ventricular *ANF* expression decreases to less than 1% of atrial levels. Irx4 is therefore likely to be involved in repressing *ANF* expression in the ventricular myocardium after birth. We believe that the increased *ANF* expression in Irx4-deficient mice is independent of the development of cardiomyopathy in these animals, which only becomes physiologically apparent at 6 weeks of age and which is functionally and morphologically measurable at 6 months of age. Despite aberrant *ANF* expression, we note that ventricular myocytes lacking Irx4 do not express *ANF* granules as do atrial cells; presumably these ventricular cells lack molecular factors and/or cellular machinery required to produce these secretory granules.

The *SMyHC3* gene is the quail ortholog of chicken *AMHC1*, which previously we have shown to be repressed by Irx4 (1). *SMyHC3* elements promote atrial chamber-specific transgenic expression in mice (54); although there is no mammalian ortholog of *SMyHC3*, the mechanisms for transcriptional regulation of chamber-specific expression appear to have been conserved during myosin gene evolution. It is not known if Irx4 directly binds to *SMyHC3* regulatory elements. The DNA-binding site of Irx4 has not been defined; however a bipartite AT-rich binding site in the *achaete-scute* regulatory sequence has been defined for *Drosophila* Iroquois protein araucan (26). Since the *SMyHC3* promoter region does not contain such a sequence, we suggest either that Irx4 has different DNA-binding specificity than its *Drosophila* counterparts or that Irx4 acts via protein-protein interactions as do other three-amino-acid length extended class homeodomain proteins (39, 43).

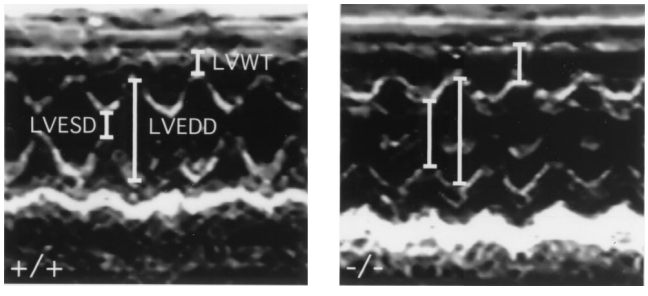

FIG. 5. Altered ventricular dimensions and function in 6-month-old wild-type and $Irx4^{\Delta ex2/\Delta ex2}$ mice. M-mode echocardiography shows increased LV wall thickness (LVWT) and LV end-systolic diameter (LVESD) but normal LV end-diastolic diameter (LVEDD) in $Irx4^{\Delta ex2/\Delta ex2}$ mice (right) compared to those for wild-type animals (left).

Our data implicate other molecules in specification of the ventricular phenotype. Some are likely to be *Iroquois* gene family members that incompletely compensate for Irx4 deficiency in $Irx4^{\Delta ex2/\Delta ex2}$ mice. In *Drosophila* and *Xenopus*, *Iroquois* genes are known to be redundant and functionally interchangeable (3, 11, 18, 26, 27). A deletion of at least two of the *Drosophila Iroquois* genes *araucan*, *caupolican*, and *mirror* is required to cause a morphological defect, and deletion of all three results in more-profound abnormalities (11, 18, 26). We have shown that besides *Irx4* three additional *Iroquois* genes are expressed in the developing mouse heart. Two of these, *Irx1* and *Irx2*, are expressed in an overlapping pattern in a subset of ventricular cells on the left of the interventricular groove. Additionally, embryonic ventricular *Irx2* expression is increased in $Irx4^{\Delta ex2/\Delta ex2}$ mice. It is intriguing that the sites of expression of *Irx1* and *Irx2* colocalize with regions where SMyHC3 transgene induction does not occur in $Irx4^{\Delta ex2/\Delta ex2}$ embryos. In addition we and others (6, 14, 15) have identified a novel *Iroquois* gene, *Irx5*. Although widely expressed in both ventricles and atria, *Irx5* is excluded from the atrioventricular junction and the outflow tract. Collectively these observations suggest that combinatorial interactions between several Iroquois transcription factors refine the spatial regulation of cardiac gene expression. It is noteworthy in this regard that Irx3 has been shown to play a role in a combinatorial process of neuronal precursor definition in concert with other homeodomain proteins (8).

Our previous studies in which Irx4 function was disrupted with a putative dominant-negative Irx4 molecule in fact indicated a role for Irx4 in ventricle-specific gene expression but not in ventricular morphogenesis (1). While these experiments clearly demonstrated that a dominant-negative molecule could modulate chamber-specific gene expression in a vertebrate heart, they were greatly limited due to multiple technical issues. The chicken cardiac myosin heavy-chain genes are the only chamber-specific genes identified in chicken hearts to date and do not have mammalian orthologs; thus it is difficult to anticipate the response of mammalian chamber-specific genes to similar experimental manipulations. Furthermore, the dominant-negative molecule used in these experiments is predicted to interfere with the actions of multiple Irx family proteins. In addition, the chicken embryos did not survive and are not well suited for physiological measurements of cardiac function; therefore we were not able to address the functional consequences of the manipulation. Also, the timing of viral misexpression is only adequate to disrupt Irx4 function much later than the initiation of *Irx4* expression in the developing heart. Thus, using gene targeting, we have been able to address the functional consequences of Irx4 deficiency and now have a useful tool to elucidate the molecular pathways regulated by Irx4.

Previously described etiologies of cardiomyopathy in mice and humans have involved contractile proteins, cytoskeletal proteins, or signaling molecules. The development of cardiomyopathy in $Irx4^{\Delta ex2/\Delta ex2}$ mice reveals a novel transcriptional pathway in the regulation of ventricular function. We speculate that this cardiomyopathy indicates that Irx4-deficient myocytes lack normal functional properties, thus leading to decompensation when subjected to ventricular load. While it is recognized that cardiac pathologies, in particularly cardiomyopathies, cause ventricular expression of *ANF, BNP,* β*MHC*, and α*-skeletal actin* (12, 13, 17), the expression of *ANF* and α*-skeletal actin* in postnatal ventricular $Irx4^{\Delta ex2/\Delta ex2}$ myocytes is far in advance of cardiac dysfunction, indicating that Irx4-mediated repression of these (and presumably other) genes is important for physiological ventricular function. Identification of other genes regulated by Irx4 and other Iroquois family members should offer further insights into the differences between atrial and ventricular myocytes.

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