# *Pitx2* prevents susceptibility to atrial arrhythmias by inhibiting left-sided pacemaker specification

Jun Wang[a], Elzbieta Klysik[a], Subeena Sood[b], Randy L. Johnson[c], Xander H. T. Wehrens[b,d], and James F. Martin[a,1]

[a]Institute of Biosciences and Technology, Texas A&M System Health Science Center, Houston, TX 77030; Departments of [b]Molecular Physiology and Biophysics and [d]Medicine (in Cardiology), Baylor College of Medicine, Houston, TX 77030; and [c]Department of Biochemistry and Molecular Biology, MD Anderson Cancer Center, Houston, TX 77030

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**Atrial fibrillation (AF), the most prevalent sustained cardiac arrhythmia, often coexists with the related arrhythmia atrial flutter (AFL). Limitations in effectiveness and safety of current therapies make an understanding of the molecular mechanism underlying AF more urgent. Genome-wide association studies implicated a region of human chromosome 4q25 in familial AF and AFL, ≈150 kb distal to the *Pitx2* homeobox gene, a developmental left–right asymmetry (LRA) gene. To investigate the significance of the 4q25 variants, we used mouse models to investigate *Pitx2* in atrial arrhythmogenesis directly. When challenged by programmed stimulation, $Pitx2^{null+/-}$ adult mice had atrial arrhythmias, including AFL and atrial tachycardia, indicating that *Pitx2* haploinsufficiency predisposes to atrial arrhythmias. Microarray and in situ studies indicated that *Pitx2* suppresses sinoatrial node (SAN)-specific gene expression, including *Shox2*, in the left atrium of embryos and young adults. In vivo ChIP and transfection experiments indicated that Pitx2 directly bound *Shox2* in vivo, supporting the notion that Pitx2 directly inhibits the SAN-specific genetic program in left atrium. Our findings implicate *Pitx2* and *Pitx2*-mediated LRA-signaling pathways in prevention of atrial arrhythmias.**

atrial flutter | atrial tachycardia | cardiac conduction | left–right asymmetry | homeobox

Atrial fibrillation (AF), the most common adult arrhythmia, increases in prevalence with age, eventually afflicting 5% of the population over age 65 years and 10% of those over age 80 years. Moreover, patients with AF have a significantly increased risk of stroke, heart failure, and dementia (1–3). Electrical impulses critical for a normal heartbeat are initiated in the sinoatrial node (SAN) or pacemaker region. In AF, rapid and irregular atrial activity overrides normal SAN function, often resulting in irregular impulse conduction to the ventricles. In many cases, ectopic electrical activity originates in the pulmonary veins and may serve to trigger and maintain AF (1, 4). The related arrhythmia, atrial flutter (AFL), displays more regular and organized electrical activity than does AF (5). Significantly, current treatments for AF are suboptimal because of incomplete effectiveness and deleterious side effects. It also has been recognized that untreated AF results in pathologic remodeling that makes AF more likely to recur (6). Thus, it is critically important to uncover the genetic mechanisms underlying AF to aid in patient management and to develop more safe and effective therapies.

The pituitary homeobox (*Pitx*) family of homeobox genes containing three genes, *Pitx1*, *Pitx2*, and *Pitx3*, is a subgroup within the larger *Paired*-related superfamily of homeobox genes (7, 8). *Pitx2* was identified as the gene mutated in Rieger syndrome I, a haploinsufficient disorder that includes ocular, tooth, and anterior body wall defects as primary characteristics (9). Importantly, the *Pitx2* gene encodes three isoforms: *Pitx2a*, *Pitx2b*, and *Pitx2c*. The *Pitx2c* isoform plays a critical role as a late effector in left–right asymmetry (LRA), a fundamental component of organ morphogenesis in vertebrates. The signaling pathways regulating LRA are initiated in the presomite-stage embryo and are mediated in large part through Nodal signaling. *Pitx2c* is the major downstream effector of the *Nodal* pathway (10).

Recent genome-wide association studies identified sequence variants on chromosome 4q25 that were associated with increased risk for AF in multiple human populations (11–13). Moreover, the 4q25 variants were strongly associated with AF cases diagnosed at an earlier age (<60 years) and with recurrence after ablation therapy (14). In a small Icelandic cohort, the sequence variants also were strongly associated with AFL (11). These correlative sequence variants were found in proximity to *Pitx2*, suggesting that *Pitx2* may be the AF locus in this region. We show that *Pitx2c* is expressed in the immediate postnatal period in left atrium and pulmonary vein. Moreover, $Pitx2^{null+/-}$ mice that are heterozygous for a *Pitx2*-null allele that removes all isoform function (15) are prone to atrial arrhythmias. In vivo ChIP assays reveal that *Pitx2c* binds directly to *Shox2*, suggesting that Pitx2c directly represses the SAN genetic program in the left atrium. Together, our findings support *Pitx2* as a bona fide susceptibility gene for atrial arrhythmias.

## Results

***Pitx2c* Is Expressed in the Postnatal Left Atrium and Pulmonary Vein.** To determine if *Pitx2* is expressed in the postnatal heart, we generated a *Pitx2* LacZ knockin allele, $Pitx2^{LacZ}$, to follow *Pitx2* expression efficiently (*Methods*). LacZ staining in postnatal day 3 (P3) pups indicated that *Pitx2* was expressed in the left atrium and pulmonary veins (Fig. 1 *A–C*). Sections confirmed that $Pitx2^{LacZ}$ directed LacZ activity in the left atrium and pulmonary veins (Fig. 1 *D–G*). *Pitx2* expression also was detected in the right ventricle, although this expression was weak (Fig. 1*B*). By P42, *Pitx2* expression was detected in the left atrial myocardium, although at diminished levels (Fig. 1 *H–J*). At 1 year, *Pitx2* expression was found only in rare left atrial myocardial cells (Fig. 1 *K–N*). To gain insight into postnatal *Pitx2* isoform expression, we investigated LacZ expression in a transgenic line, $Pitx2c^{tg3K}$, in which LacZ is directed by *Pitx2c* regulatory elements (16). Because this *Pitx2c* transgene is influenced by copy number and position effect, this experiment provided only qualitative information about *Pitx2* isoform expression in left atrium. In 3-month-old $Pitx2c^{tg3K}$ transgenic mice, we found LacZ activity in right ventricle and left atrium, supporting the notion that *Pitx2c* was expressed in the postnatal left atrium and right ventricle (Fig. S1 *A–D*). RT-PCR experiments also indicated that *Pitx2c* was expressed in left atrium (Fig. 1*O*). Quantitative RT-PCR indicated that *Pitx2c* expression levels in a 3-month-old left atrium were ≈25% of that found in the embryo 13.5 days postconception (dpc) (Fig. 1*P*). Together, these

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[1]To whom correspondence should be addressed. E-mail: jmartin@ibt.tamhsc.edu.

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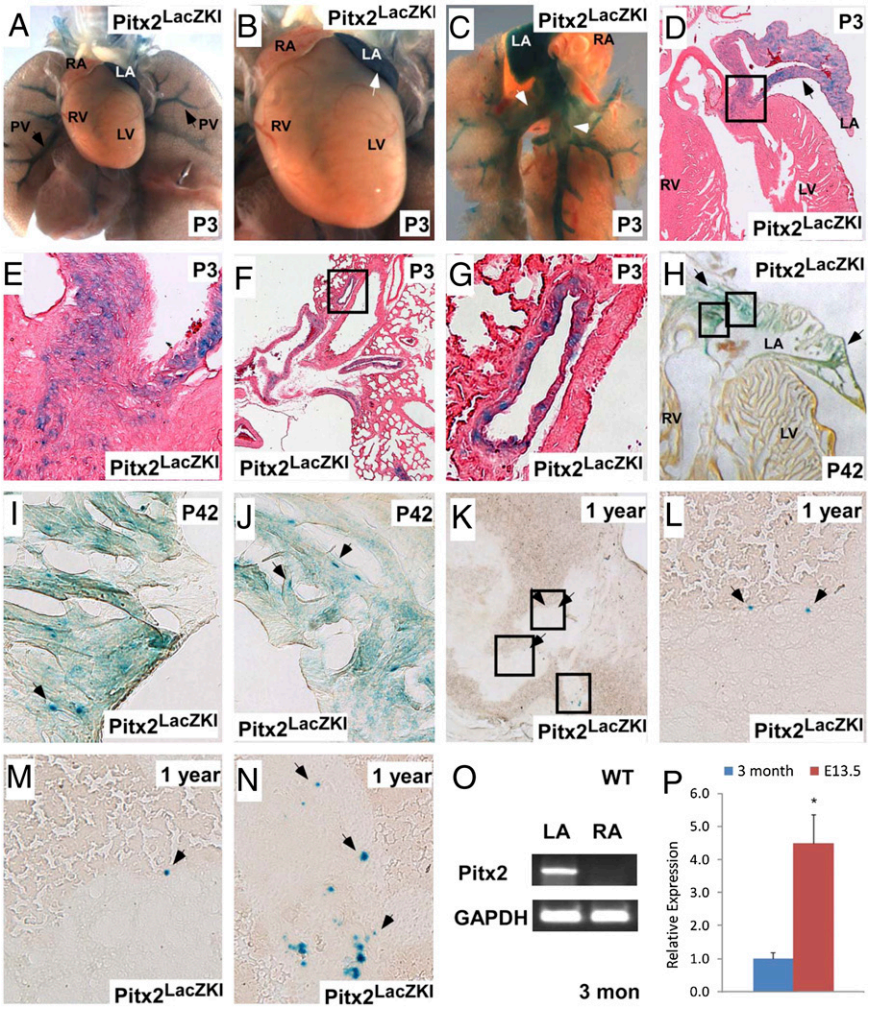


**Fig. 1.** *Pitx2* is expressed in the left atrium and pulmonary vein of postnatal mice. (*A–G*) Whole-mount LacZ staining and sagittal sections of *Pitx2* $^{LacZ}$ allele at P3. *Pitx2* is expressed in the left atrium, right ventricle, and pulmonary vein (arrows). Boxed areas in *D* and *F* are shown at higher magnification in *E* and *G*, respectively. (*H–J*) LacZ staining (arrows) on sagittal sections of *Pitx2* $^{LacZ}$ allele on P42 shows LacZ activity in the left atrium. Boxed areas in *H* are shown at higher magnification in *I* and *J*. (*K–N*) LacZ staining (arrows) on sagittal sections of 1-year-old *Pitx2* $^{LacZ}$ allele. *Pitx2* is expressed in rare left atrial myocardial cells in left atrium. Boxed areas in *K* are shown at higher magnification in *L*, *M*, and *N*. (Magnification in E, G, I, J and L-N: ×200.) (*O*) RT-PCR of *Pitx2c* in WT left atrium and right atrium shows *Pitx2c* is expressed in left atrium. (*P*) Quantitative RT-PCR analysis of *Pitx2c* comparing embryonic and adult stages. Arrows indicate Lac staining. *, statistically significant difference ($P < 0.05$). LA, left atrium; LV, left ventricle; PV, pulmonary vein; RA, right atrium; RV, right ventricle.

findings indicate that *Pitx2c* is expressed in postnatal left atrium, pulmonary vein, and right ventricle and that *Pitx2c* expression in left atrium is silenced during aging.

**Baseline Electrophysiological Features of *Pitx2*$^{null+/-}$ Mice.** To test the hypothesis that *Pitx2c* is a susceptibility gene for atrial arrhythmias such as AF and AFL, we first used conventional six-lead surface ECG and four-lead bipolar intracardiac electrograms to record simultaneously in WT and *Pitx2*$^{null+/-}$ mice (Fig. S2). Under baseline conditions, there were no differences between *Pitx2*$^{null+/-}$ and WT mice in heart rate, interval between two consecutive R wave peaks (RR), interval from beginning of the P wave to the peak of the R wave (PR), or in the time elapsing from the beginning of the QRS complex to the end of the T wave (corrected Q-T interval) on surface ECGs (Table 1), with the exception of a small but significant prolongation of the QRS interval in *Pitx2*$^{null+/-}$ mice. These findings were corroborated using bipolar intracardiac electrogram recordings. Endocardial programmed electrical stimulation was used to determine atrial effective refractory period, atrioventricular nodal effective refractory period, and sinus node recovery time at a basic cycle length of 100 ms. There were no significant differences between *Pitx2*$^{null+/-}$ and WT mice (Table 1). During these electrophysiological studies, no episodes of spontaneous AF were observed in any of the mice studied.

**Pacing-Induced Atrial Arrhythmias in *Pitx2*$^{null+/-}$ Mice.** Although *Pitx2*$^{null+/-}$ mice were in normal sinus rhythm at baseline, we wanted to test the notion that *Pitx2c* haploinsufficiency predisposed mice to atrial arrhythmias. There is precedent for this idea, because a mouse model for a gain-of-function mutation in ryanodine receptor type II (RyR2) provided a substrate for AF that was insufficient to produce spontaneous arrhythmias. RyR2 mutants required a second arrhythmogenic triggering event, in this case activation of $Ca^{2+}$/calmodulin-dependent protein kinase II, which was uncovered by increased heart rate (17).

Inducibility of atrial arrhythmias, determined twice in each mouse, was measured with surface leads and an intraatrial catheter using the stimulation protocol previously described (17, 18). Episodes of AFL/tachycardia, defined as rapid but regular atrial rhythm lasting >1,000 ms (19), were observed frequently in *Pitx2*$^{null+/-}$ mice (Fig. 2 *A–C*). Based on a-wave morphology and rate on the atrial electrogram, episodes of pacing-induced atrial arrhythmias were observed more frequently in *Pitx2*$^{null+/-}$ (100%, six of six mice) than in WT mice (one of four mice, Fisher's exact test, $P = 0.033$). One of the *Pitx2*$^{null+/-}$ mice showed an atrial arrhythmia only once with an episode lasting for at least 16 s, whereas five of six *Pitx2*$^{null+/-}$ mice developed arrhythmias following both burst-pacing stimuli. Mean duration of atrial arrhythmias in *Pitx2*$^{null+/-}$ mice, confirmed by rapid and regular a-wave on atrial electrograms, was 164 ± 131 s. In addition, we observed an episode of AFL/tachycardia in one of the WT mice (although this episode only lasted 6.8 s). Taken together, our findings indicate that *Pitx2c* haploinsufficiency predisposes mice to atrial arrhythmias, including AFL/tachycardia.

**Table 1. Baseline cardiac electrophysiological parameters in WT and *Pitx2*$^{null+/-}$ mice**

| ECG intervals (ms) | WT ($n$ = 4) | Pitx2$^{null+/-}$ ($n$ = 6) | Statistics |
|---|---|---|---|
| HR | 470.5 ± 36.3 | 487.8 ± 8.6 | NS |
| RR | 129.7 ± 9.4 | 123.2 ± 2.2 | NS |
| PR | 44.9 ± 1.3 | 46.1 ± 0.74 | NS |
| QRS | 9.1 ± 0.2 | 10.7 ± 0.4 | $P$ = 0.05 |
| QTc | 23.5 ± 1.2 | 26.8 ± 1.1 | NS |
| SNRT | 179.8 ± 31.0 | 176 ± 13.5 | NS |
| AVERP | 52.0 ± 2.2 | 56 ± 1.3 | NS |
| AERP | 44.5 ± 0.3 | 47.7 ± 1.4 | NS |

All values expressed mean ± SEM. AERP, atrial effective refractory period; AVERP, atrioventricular nodal effective refractory period; HR, heart rate; NS, nonsignificant; PR, interval from beginning of P waves to the peak of R wave; QRS, duration of interval between beginning of Q wave to peak of S wave; QTc, duration of Q-T interval corrected for heart rate; RR, interval between two consecutive R wave peaks; SNRT, sinus node recovery time.

***Pitx2c* Inhibits the Pacemaker Gene Program in Left Atrium.** To gain insight into the mechanisms underlying predisposition to AFL/tachycardia, we performed microarray analysis on *Pitx2*$^{null}$-mutant and control hearts (*Methods* and Fig. S3). Interestingly the microarray study revealed that the SAN genes *Shox2* and *Tbx3*, as well as a number of channel genes, were up-regulated in the *Pitx2*$^{null}$-mutant embryos when compared with controls (Fig. 3*A*). Among the up-regulated channel genes was *Kcnq1*, a potassium-channel gene that has been implicated in familial AF through a gain-of-function mutation (20).

We next used quantitative RT-PCR and whole-mount in situ hybridization to gain further insight into the mechanisms underlying predisposition to AFL/tachycardia in *Pitx2c* mutants. We examined expression of *Hcn4,* a hyperpolarization-activated, cyclic nucleotide-gated (HCN) channel that has a critical role in SAN automaticity (21, 22). In 12.5-dpc control embryos, *Hcn4* was expressed in the large caval veins posterior to the heart, as well as in the forming SAN region (Fig. 3*Ca* and Fig. S4 *A* and *B*). In *Pitx2*$^{null+/-}$ mutants, *Hcn4* appeared to be modestly up-regulated in the left superior caval vein that also expresses *Pitx2c* (Fig. 3*Cb* and Fig. S4 *C* and *D*) (23). In *Pitx2*$^{null-/-}$ embryos, the anatomy of the junction between the large veins and the atrium was disrupted (24, 25), and *Hcn4* was expressed continuously in the abnormal vein–atrial junction in *Pitx2*$^{null-/-}$-mutant embryos and in the posterior wall of the mutant left atrium (Fig. 3*Cc* and Fig. S4 *E* and *F*). Quantitative RT-PCR indicated that *Hcn4* was quantitatively up-regulated in both *Pitx2*$^{null+/-}$ and *Pitx2*$^{null-/-}$ mutant embryos (Fig. 3*B*).

*Tbx3* is a T-box gene that is expressed in SAN progenitors and is required for correct separation of the SAN from working atrial myocardium (26). In *Pitx2*$^{null+/-}$ mutants, *Tbx3* expression was more prominent in the left atrioventricular canal, whereas in *Pitx2*$^{null-/-}$ mutants *Tbx3* was expanded and up-regulated in the left atrioventricular canal and in the midline cells at the base of the common venous sinus (Fig. 3 *B* and C *d–f*).

*Shox2*, a homeodomain containing transcription factor expressed in the developing SAN, has been shown recently to be required for SAN development (27, 28). At both 12.5 and 13.5 dpc in *Pitx2*$^{null+/-}$ embryos, *Shox2* was expressed similarly to control SAN progenitors, although expression was up-regulated in left superior caval vein and interatrial septum of *Pitx2*$^{null+/-}$ embryos (Fig. 3*C g*, *h*, *j*, and *k* and Fig. S4 *G–J* and *M–P*). *Shox2* up-regulation was confirmed using quantitative RT-PCR (Fig. 3*B*). In *Pitx2*$^{null-/-}$ embryos, *Shox2* was expanded in the posterior wall of the left atrium and left atrial myocardium (Fig. 3 *C i* and *l* and Fig. S4 *K*, *L*, *Q*, and *R*). Quantitative RT-PCR also indicated that *Shox2* was up-regulated in *Pitx2*$^{null-/-}$ embryos (Fig. 3*B*).

In addition to genes identified in the microarray, we also investigated candidate genes in the *Pitx2* mutants. *Nppa,* encoding the atrial natriuretic peptide hormone that regulates intravascular volume, has been reported to be a *Pitx2* target and has been implicated in familial AF (29, 30). Whole-mount in situ hybridization and quantitative RT-PCR indicated that *Nppa* expression was up-regulated in *Pitx2*$^{null+/-}$ and *Pitx2*$^{null-/-}$-mutant embryos (Fig. 3 *B* and *C m–o*).

We next evaluated gene expression in the left atrium of P42 *Pitx2*$^{null+/-}$ mice that were prone to atrial arrhythmias as shown in Fig. 2. Microarray analysis followed by quantitative RT-PCR verification revealed that genes expanded in the adult heart were similar to those up-regulated in *Pitx2*$^{null}$-mutant embryos. A number of potassium-channel genes, including *Kcnq1*, were up-regulated in the *Pitx2*$^{null+/-}$ P42 left atrium, as well as the SAN genes *Shox2* and *Hcn4* (Fig. 3 *D* and *E*).

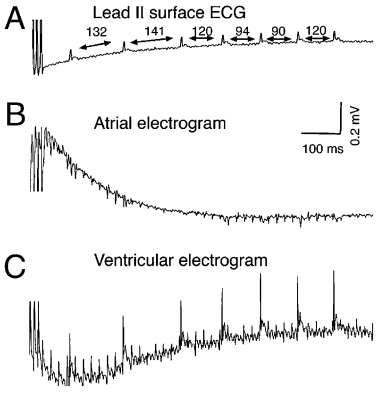


**Fig. 2.** Surface ECG (*A*) and intracardiac electrograms (*B* and *C*) revealing an episode of pacing-induced atrial tachycardia in a *Pitx2*$^{null+/-}$ mouse. (*A*) Surface ECG showing the last three paced beats of the burst protocol. The tracing reveals the absence of P waves and irregular RR intervals, suggestive of atrial arrhythmia. (*B*) Atrial electrogram shows the presence of rapid and regular a waves, suggestive of AFL/tachycardia. (*C*) Ventricular electrograms reveals irregular intervals between v waves.

***Pitx2c* Directly Represses *Shox2*.** To understand the underlying molecular mechanisms in the predisposition to atrial arrhythmia in *Pitx2*$^{null+/-}$ mice, we generated an allele of *Pitx2*, *Pitx2*$^{Flag}$ (Fig. 4 *A–C*). We found that *Pitx2*$^{Flag}$ directed *Pitx2c* expression in embryoid bodies cultured from 7–10 days (Fig. 4*D*). In the P0 heart, we observed strong expression of Pitx2c protein in mice that were heterozygous for the *Pitx2*$^{Flag}$ allele (Fig. 4*D*).

Bioinformatics analysis revealed a *Pitx2c* recognition element in the *Shox2* gene that was conserved in the mouse, human, dog, and cow genomes (Fig. 4*E* and *Methods*). Moreover, in vivo ChIP indicated that the *Pitx2c* recognition element, located in *Shox2* intron 2, was bound by Pitx2c in both embryoid bodies and the adult left atrium (Fig. 4*F*). Transfection experiments using a 2-kb *Shox2* genomic fragment that included the Pitx2c binding site indicated that Pitx2c repressed *Shox2* expression in P19 cells and that this repression was lost when the *Pitx2c* binding site was mutated (Fig. 4*G*). Together, our findings support the hypothesis that *Shox2* is a direct target for Pitx2c repression in the embryonic and adult left atrium.

## Discussion

Atrial arrhythmias such as AF and AFL are common human arrhythmias with potentially devastating clinical consequences. Our findings, taken together with previously reported data, indicate that *Pitx2c* is an important susceptibility gene for atrial arrhythmias that normally represses the pacemaker gene program in the left atrium. We identify *Shox2*, a critical regulator of the SAN gene program, as a *Pitx2c* target gene. The observation

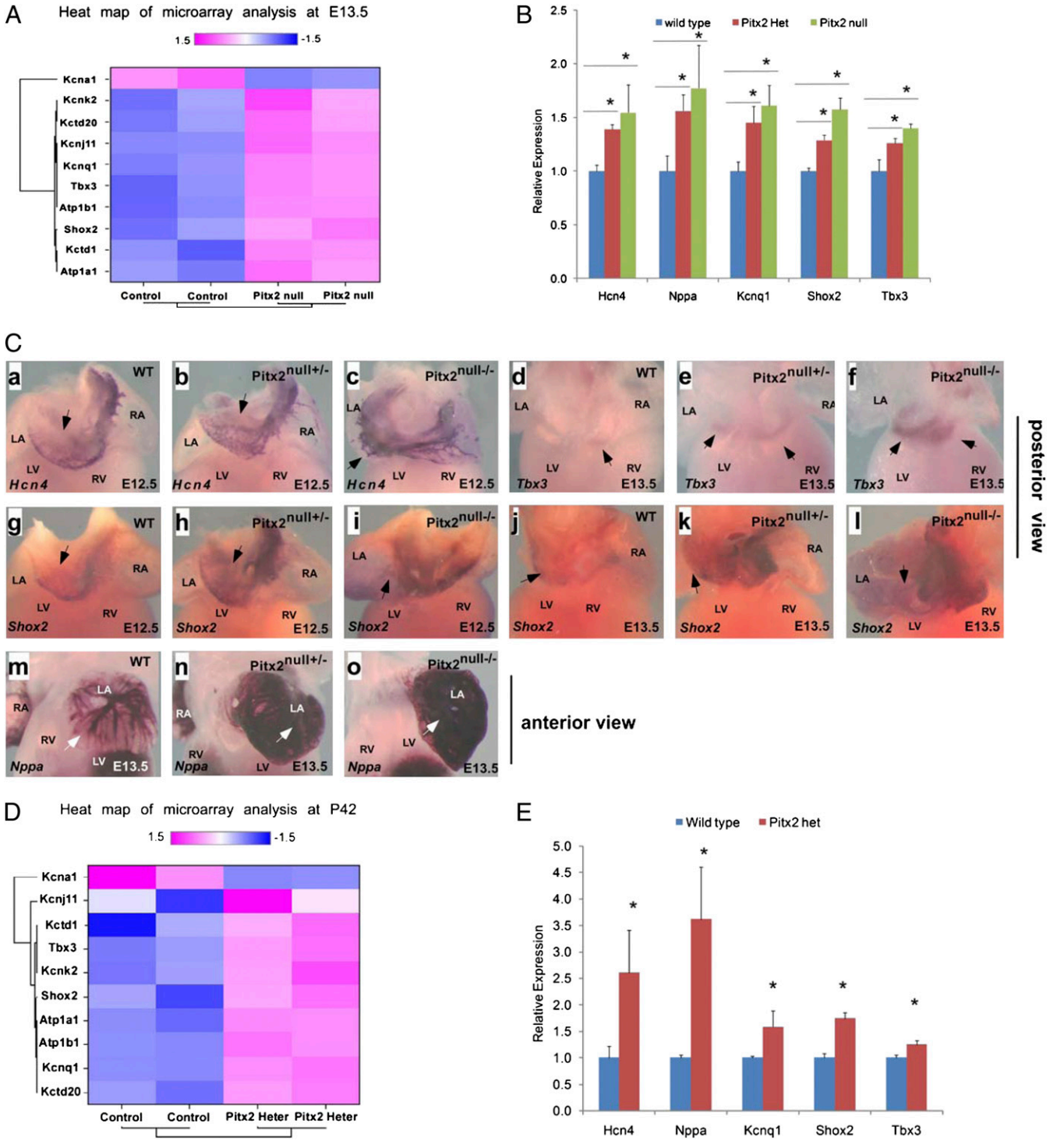


**Fig. 3.** *Pitx2* inhibits the SAN program. (*A*). Heat map of microarray data from *Pitx2*$^{null-/-}$-mutant embryo hearts compared with WT hearts at 13.5 dpc. All pacemaker genes and ion-channel genes designated to be present at significant levels were identified in the normalized data, followed by log2 transformation, subjected to hierarchical clustering and heat-map generating. (*B*) Quantitative RT-PCR validation of microarray analysis of *Pitx2*-mutant hearts compared with WT control hearts at 13.5 dpc. The quantitative real-time RT-PCR analysis showed that *Kcnq1, ANF, Hcn4, Shox2*, and *Tbx3* were up-regulated in *Pitx2*-mutant hearts. (*C*). Whole-mount in situ analysis at 12.5 dpc and 13.5 dpc with indicated probes. Genotypes and probes are labeled. Arrows designate areas of relevant gene expression in control and mutant embryos. (*D*) Heat map of microarray data and (*E*) quantitative RT-PCR validation of microarray on WT and *Pitx2*$^{null+/-}$ mouse hearts at P42. Potassium-channel genes including *Kcnq1*, as well as the SAN genes *Shox2* and *Hcn4*, were up-regulated in the *Pitx2*$^{null+/-}$-mutant hearts. In *B* and *E*, * indicates statistically significant differences ($P < 0.05$).

that *Pitx2c* is most highly expressed during embryogenesis and in the early postnatal heart suggests that the arrhythmogenic substrate is established relatively early in life.

***Pitx2c* Directly Inhibits Pacemaker Specification.** Our in vivo ChIP data and transfection experiments indicate that *Pitx2c* normally silences *Shox2* in the left atrium. Previous work has shown that

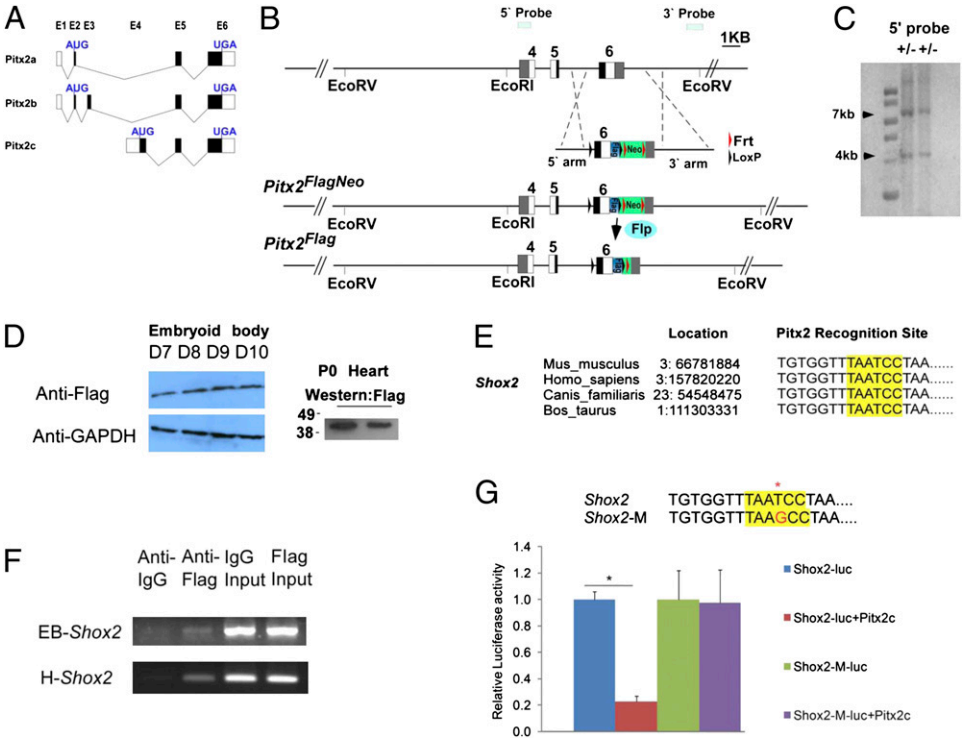


**Fig. 4.** *Pitx2* directly represses *Shox2*. (*A*) Summary of exon usage by *Pitx2* isoforms. AUG and UGA: start and stop codons; E1->E6, exons 1->6. (*B*) Targeting strategy of generating the $Pitx2^{Flag}$ allele. (*C*) Southern blots for the $Pitx2^{Flag}$ allele showing the WT 7-kb and mutant 4-kb bands. (*D*) Western blot showed $Pitx2^{Flag}$-directed Pitx2 expression in embryoid bodies and P0 heart. D7-D10, culture day 7–10. (*E*) Alignment of *Pitx2* recognition elements in *Shox2*. (*F*) In vivo ChIP indicated that *Shox2* was bound by Pitx2 in embryoid bodies (EB) and adult left atrium (H). (*G*) Luciferase activity assay experiments indicated that Pitx2 repressed *Shox2* in P19 cells and that this repression was lost when the *Pitx2* binding site was mutated. At top, highlight in red indicates point mutation inserted to disrupt Pitx2 binding. * indicates statistically significant differences ($P < 0.05$).

*Shox2*-mutant mice have defective SAN development with reduced expression of *Hcn4* and *Tbx3*, indicating that *Shox2* positively regulates the SAN genetic program. *Shox2* functions, at least in part, by inhibiting *Nkx2.5* expression in SAN progenitors (Fig. S5) (27, 28). *Nkx2.5* promotes the atrial working myocardial program and represses the SAN genetic pathway (31, 32). Transfection experiments revealed that Shox2 directly represses the *Nkx2.5* 5′ flanking region and that this repression was independent of Shox2 DNA binding activity (28). Although this genetic program is intact on the right side of the embryo, on the left side *Pitx2c* disrupts the negative cascade by inhibiting *Shox2* expression (Fig. S5).

*Tbx3* also promotes the SAN phenotype while inhibiting the working atrial myocardial phenotype through both direct and indirect mechanisms. There is strong evidence that Tbx3 binds to and inhibits atrial genes, such as *Cx43*. Tbx3 also promotes the expression of SAN genes, most likely through a poorly understood indirect mechanism (26). Moreover, recent findings indicate that *Tbx3* is genetically downstream of *Shox2* (28). Together, our data support a model whereby Pitx2c directly represses *Shox2* and inhibits the SAN genetic program in left atrium.

Our findings support the notion that *Pitx2c* loss of function promotes ectopic automaticity in the left atrium. Under normal conditions, the left atrium should be protected from impulse generation. In $Pitx2^{null+/-}$ mutants, our data reveal that left atrial myocardium expresses genes that are characteristic of the SAN such as *Hcn4*. We suggest that the $Pitx2^{null+/-}$ null mutant left atrium provides an arrhythmogenic substrate that would enhance other pathologic triggers for atrial arrhythmias.

**Atrial Fibrillation and Left–Right Asymmetry: A Direct Molecular Connection.** Our findings indicate that the *Pitx2c*-mediated LRA signaling cascade inhibits SAN specification by disrupting a negative regulatory transcriptional cascade. Previous work showed that Pitx2c directs formation of left and right anatomic characteristics that separate the systemic and pulmonic circulation that is vital for normal cardiac function. Morphologic abnormalities in $Pitx2^{null}$ homozygous mutants include right atrial isomerism with bilateral venous valves, deficiency of the primary interatrial septum, and anomalous pulmonary venous drainage (31, 33). In $Pitx2c^{-/-}$-mutant embryos, caval and pulmonary veins drain into an abnormal medial venous sinus. Moreover, there was a deficiency in contribution of the *Pitx2*-expressing lineage to pulmonary veins in *Pitx2c* homozygous mutant embryos (25) because of a defect in pulmonary vein precursors (24).

Mommersteeg and colleagues (31) reported that *Pitx2c* homozygous mutants had a bilateral SAN that expressed SAN markers such as *Hcn4*. Our findings suggest that Pitx2c regulates the precise location of the SAN by directly inhibiting *Shox2*, a transcriptional regulator of SAN gene program. Moreover, we show that *Pitx2* haploinsufficiency promotes the occurrence of atrial tachyarrhythmias.

Our data uncover a remarkably close connection between LRA and the SAN genetic program. *Pitx2c* is a direct target of the TGFβ superfamily member *Nodal* that is expressed transiently at early stages of development (10). Left-sided *Pitx2c* expression persists in organ primordia well after *Nodal* has been silenced (34, 35). Furthermore, the identification of *Pitx2c* as a susceptibility gene for atrial arrhythmias provides deeper insight into the genetic pathways that predispose to AF and AFL. Importantly, the *Nodal-Pitx2c* signaling cassette is evolutionarily conserved, raising the possibility that *Nodal* also is implicated in predisposition to atrial arrhythmias. Indeed, mutations in *Nodal* have been identified in human patients with LRA defects but have not been reported in familial AF (36).

**Other Mechanisms Contributing to *Pitx2c* Predisposition to Atrial Arrhythmia.** In addition to the *Shox2*-mediated mechanism, we found other gene expression changes in *Pitx2c* mutants that suggest other contributory mechanisms for predisposition to atrial arrhythmias. Atrial natriuretic peptide, encoded by *Nppa*, regulates multiple ion channels in atrial cardiomyocytes. An atrial natriuretic peptide gain-of-function mutation that may promote arrhythmias within atrial myocardium has been identified in human patients (29). Our finding that *Nppa* was up-regulated in

*Pitx2c* mutants suggests that similar mechanisms may contribute to the predisposition to atrial arrhythmia in *Pitx2c* mutants.

Previous work indicated that a gain-of-function substitution in a conserved serine of *Kcnq1* (S140G) results in familial AF (20). Moreover, separate mutations in *Kcnq1*—a serine-to-proline (S209P) substitution and a valine-to-methionine (V141M) substitution—also were reported in familial AF (37, 38). In vitro experiments indicate that the gain-of-function mutations in *Kcnq1* may be caused by defects in channel inactivation (39). Our data indicate that *Kcnq1* is up-regulated in *Pitx2c* mutants. Together these findings suggest that *Pitx2c* likely has multiple targets that are relevant to the predisposition to atrial arrhythmia.

## Methods

**Mouse Alleles and Transgenic Lines.** The $Pitx2^{null}$ allele, which removes function of all *Pitx2* isoforms, and the $Pitx2^{tg3k}$ transgenic line have been described previously (15, 16).

**Real-Time Quantitative PCR.** Total RNA was isolated from embryonic hearts and adult hearts using RNeasy Micro Kit (QIAGEN), followed by RT-PCR using Super Script™ II reverse transcriptase (Invitrogen). Real-time thermal cycling was performed using Mx3000P thermal cycler (Stratagene) with SYBR Green JumpStart™ Taq ReadyMix™ (Sigma). Sequences of primers are available upon request.

**Cardiac Electrophysiology.** Cardiac electrophysiology was performed as described (40).

**Microarray Studies.** Total RNA was extracted from embryonic hearts or P42 hearts and purified using either RNeasy Micro Kit (QIAGEN) for embryonic hearts or TRIzol Reagent (Invitrogen) for postnatal hearts. DNA microarray analysis was performed using the OneArray Mouse Whole Genome Array that interrogated 17,455 unique genes using 29,972 probes (Phalanx Biotech Group). Graphical presentations of the array data are shown in Fig S3.

**ChIP.** ChIP analysis was performed using a ChIP assay kit (Upstate).

**Generation of Reporter Constructs.** Using rVista2.0 (41) and Ensembl genomic alignments (http://www.ensembl.org/), we analyzed *Shox2* genomic sequence and the genomic sequence5 kb upstream of *Shox2* to identify conserved *Pitx2* binding sites. This analysis was followed by the ChIP analysis, and the *Pitx2* biding site "TAATCC" in intron between exon 2 and exon3 of *Shox2* was validated.

**Site-Directed Mutagenesis.** Site-directed mutagenesis of the Pitx2 binding sites in the *Shox2* intron was achieved by using the QuikChange II site-directed mutagenesis kit (Stratagene).

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