PITX2 Insufficiency Leads to Atrial Electrical and Structural Remodeling Linked to Arrhythmogenesis

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Background—*Pitx2* is a homeobox transcription factor that plays a pivotal role in early left/right determination during embryonic development. *Pitx2* loss-of-function mouse mutants display early embryonic lethality with severe cardiac malformations, demonstrating the importance of *Pitx2* during cardiogenesis. Recently, independent genome-wide association studies have provided new evidence for a putative role of *PITX2* in the adult heart. These studies have independently reported several risk variants close to the *PITX2* locus on chromosome 4q25 that are strongly associated with atrial fibrillation in humans.

Methods and Results—We show for the first time that *PITX2C* expression is significantly decreased in human patients with sustained atrial fibrillation, thus providing a molecular link between *PITX2* loss of function and atrial fibrillation. In addition, morphological, molecular, and electrophysiological characterization of chamber-specific *Pitx2* conditional mouse mutants reveals that atrial but not ventricular chamber-specific deletion of *Pitx2* results in differences in the action potential amplitude and resting membrane potential in the adult heart as well as ECG characteristics of atrioventricular block. Lack of *Pitx2* in atrial myocardium impairs sodium channel and potassium channel expression, mediated in part by miRNA misexpression.

Conclusions—This study thus identifies *Pitx2* as an upstream transcriptional regulator of atrial electric function, the insufficiency of which results in cellular and molecular changes leading to atrial electric and structural remodeling linked to arrhythmogenesis. **(*Circ Cardiovasc Genet*. 2011;4:269-279.)**

Key Words: *Pitx2* ■ arrhythmia ■ atrial fibrillation ■ gene regulation ■ polymorphism ■ transcription factors

Pitx2 is a homeobox transcription factor that plays a pivotal role in early left/right determination during embryonic development, downstream of the *nodal/lefty* signaling pathway.[1] The expression of *Pitx2* is confined to the left side of the embryo within the lateral plate mesoderm. With further development, it continues to be mainly confined to the left side in different organs, such as the stomach and the heart.[2,3] *Pitx2* loss-of-function mouse mutants displayed early embryonic lethality with severe cardiac malformations,[4–7] demonstrating the importance of *Pitx2* during cardiogenesis.

Clinical Perspective on p 279

Recent genome-wide association studies have suggested new roles for *Pitx2* in the adult heart.[8–10] These authors have independently reported several risk variants on chromosome 4q25 that are strongly associated with atrial fibrillation (AF) in distinct human populations. AF-associated risk variants are adjacent to *PITX2,* and although these studies[8–10] do not provide any experimental evidence that links regulation of *PITX2* expression/activity to the risk variants, it is plausible that modulation of the expression and/or activity of *PITX2* in the adult heart have the potential to play a role in AF.

In the present study, we have confirmed the high prevalence of these genetic variants in a small cohort of AF patients and furthermore we demonstrate for the first time that *PITX2C* expression is significantly decreased in human patients with sustained AF, thus providing a molecular link between loss of function of *PITX2* and AF. In addition, we report herein morphological, molecular, and electrophysiological characterization of chamber-specific *Pitx2* conditional mouse mutants. Deletion of *Pitx2* in the atrial chambers results in viable offspring. Electrophysiological studies in *Pitx2* atrial chamber–specific adult hearts revealed differences in the resting membrane potential, action potential amplitude, and conductive disturbances as demonstrated by ECG measurements. Furthermore, lack of *Pitx2* in the adult

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atria alters sodium and potassium channel expression, corroborating the electrophysiological findings. Thus, we provide evidence that *Pitx2* is an upstream transcriptional regulator of distinct signaling pathways that provide cellular, molecular, and electrophysiological substrates linked to atrial arrhythmogenesis.

Methods

Human Tissue and DNA Samples

Atrial myocardial tissue samples were obtained from patients undergoing cardiac surgery. The atrial samples were classified as patients with (AF) and without (no AF) a recorded history of AF. Detailed information regarding tissue processing is provided in the online-only Data Supplement. The study conforms to the principles outlined in the *Declaration of Helsinki*.

Genomic DNA samples from 47 patients diagnosed of having AF and 100 healthy donors with no cardiac structural and/or functional diseases were obtained from the Spanish National DNA Bank (BNADN, Salamanca). Polymerase chain reaction (PCR) amplification of both single nucleotide polymorphisms (SNPs) (rs2200733 and rs13143308) was carried out using flanking oligonucleotides, as detailed in online-only Data Supplement Table 1, followed by direct sequencing. This study was approved by the Ethics Committees of the Spanish National DNA Bank (BNADN, Salamanca) and of the University of Jaén, and the investigation conforms to the principles outlined in the *Declaration of Helsinki*.

Transgenic Mouse Lines, Breeding Strategy, and Mouse Genotyping

The *Pitx2*floxed, NppaCre, and Mlc2vCre transgenic mouse lines have been previously described.[5,11,12] Generation of conditional atrial (NppaCre) and ventricular (Mlc2vCre) mutant mice was performed by intercrossing hemizygous Cre deletor mice with homozygous *Pitx2*floxed mice, which resulted in atrial-specific ($NppaCre^{+}Pitx2^{-/-}$) and ventricular ($Mlc2vCre^{+}Pitx2^{-/-}$) *Pitx2* mutant mice, respectively. DNA for PCR screening was extracted from adult ear and/or tail samples and from the yolk sac in embryos. Screening of Cre and *Pitx2*floxed alleles was routinely done using used specific primers, as detailed in online-only Data Supplement Table 1. Further details are provided as in the online-only Data Supplement. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

Quantitative Reverse Transcriptase–PCR Analyses

Tissue sample isolation and processing for RNA isolation were performed using standard procedures. Reverse transcriptase (RT)-PCR was performed in the Mx3005Tm QPCR System with an MxPro QPCR Software 3.00 (Stratagene) and SYBR Green detection system. Detailed information regarding mRNA and microRNA quantitative (q)RT-PCR analyses are provided in the online-only Data Supplement.

ECG Recordings and Electrophysiological Measurements

Mice were anesthetized with 2 mg/kg Ketamine (Parker-Davis) intraperitoneally. ECG recordings were registered and analyzed using a digital acquisition and analysis system (Power Laboratory/4SP; www.adinstrument.com). Transmembrane action potentials were recorded in isolated left and right atria of male control mice and atrial-specific *Pitx2* conditional mice (n=5 per group) and in thin papillary muscles from male control mice and ventricular-specific *Pitx2* conditional mice (n=5 per group) through glass microelectrodes filled with 3 mol/L KCl (tip resistance, 8 to 15 mol/LΩ) using procedures described previously.[13,14] Further details are provided in the online-only Data Supplement.

Cell Culture and Transfection Assays

HL-1 mouse immortalized atrial myocardial cells were used to assay microRNA-1 gain-of-function experiments as well as *Pitx2c* gain- and loss-of-function assays. Transfection experiments were performed using standard condition as detailed in the online-only Data Supplement.

Statistical Analyses

qRT-PCR data statistical analyses were performed using unpaired Student *t* test. Probability values <0.05 were considered statistically significant and are stated on each corresponding figure legend. Deviation from the Hardy-Weinberg equilibrium was tested by Fisher exact test. General linear models were carried out for testing the genotype dependence on the independent age and group variables, as detailed in the online-only Data Supplement Methods. Allele frequencies were estimated from genotype frequencies by gene counting. Further detail information regarding the statistical analyses is provided in the online-only Data Supplement.

Results

rs2200733 and rs13143308 Correlate With AF

We performed a direct resequencing approach to study the frequency of 2 SNPs previously associated with AF[8] in a small cohort of Caucasian patients with AF. A total 47 patients (25 men and 22 women) with paroxysmal or permanent AF were recruited for the study (online-only Data Supplement Table 2). Thirty patients presented isolated AF; 17 patients were also diagnosed with cardiomyopathy and/or valvulopathy. Ages ranged from 38 to 90 years (68±11 years); 100 patients (44 men and 56 women) without any cardiac structural or electrophysiological diagnosis were recruited as the control population. Control ages ranged from 43 to 69 years (52±6 years) (online-only Data Supplement Table 3). rs2200733 (C/T or T/T) was observed in 20 of 47 (42%) AF patients and 22 of 100 (22%) control patients (odds ratio [OR], 2.607; 95% confidence interval [CI, 1.158 to 5.908; Fisher exact test; $P<0.01$; Table 1). rs13143308 (T/T or T/G) was observed in 26 of 47 (55%) AF patients and was present in 10 of 100 (10%) control patients (OR, 10.900; 95% CI, 4.325 to 29.594; Fisher exact test; $P<0.001$; Table 1) (online-only Data Supplement Figure 1). Thus, the data demonstrate a highly significant prevalence of rs2200733 (C/T or T/T) and rs13143308 (T/T or T/G) in patients with AF compared with control subjects. No significant differences were obtained related to age for rs2200733 (C/T or T/T) or for rs13143308 (T/T or T/G), respectively, using general linear models. Furthermore, an increased frequency of rs2200733 (C/C or C/T) but not of rs13143308 is obtained if patients are subdivided into isolated AF (rs2200733, 16/30; 53%) and AF patients with valvulopathy and/or cardiomyopathy (rs2200733, 4/17; 23%), although none of them reached statistical significance.

PITX2c Expression Is Impaired in Patients With AF

To test if AF is linked to changes in the expression levels of *PITX2*, we analyzed the expression levels of *PITX2C*, the major PITX2 isoform expressed in the adult heart, in right and left atrial appendage biopsies of human patients diagnosed with AF compared with samples from patients without a history of AF (no AF) (online-only Data Supplement Table 4). Importantly, *PITX2C* expression, as revealed by qRT-PCR, is decreased in right atria of AF patients (n=5) as

Table 1. SNP Genotypes in AF Patients

	AF Type		Sex		
	Isolated	With CM/VM	Male	Female	Total
rs2200733					
AF patients					
C/C	14/30 (47%)	13/17 (76%)	17/25 (68%)	10/22 (45%)	27/47 (57%)
C/T	14/30 (47%)	3/17 (18%)	9/25 (36%)	8/22 (17%)	17/47 (36%)
T/T	2/30 (6%)	1/17 (6%)	1/25 (4%)	2/22 (9%)	3/47 (5%)
Control subjects					
C/C	NA	NA	35/44 (79%)	43/56 (77%)	88/100 (88%)
C/T	NA	NA	9/44 (21%)	13/56 (33%)	22/100 (22%)
T/T	NA	NA	0/44 (0%)	0/56 (0%)	0/100 (0%)
rs13143308					
AF patients					
G/G	13/30 (42%)	8/17 (50%)	11/25 (44%)	10/22 (45%)	21/47 (45%)
G/T	13/30 (42%)	6/17 (37%)	10/25 (40%)	9/22 (41%)	19/47 (40%)
T/T	5/30 (16%)	2/17 (13%)	4/25 (16%)	3/22 (13%)	7/47 (15%)
Control subjects					
G/G	NA	NA	42/44 (95%)	48/56 (86%)	90/100 (90%)
G/T	NA	NA	2/44 (5%)	8/56 (14%)	10/100 (10%)
T/T	NA	NA	0/44 (0%)	0/56 (0%)	0/100 (0%)

	Isolated AF	
	Paroxysmal	Permanent
rs2200733 AF patients C/T or T/T	16/27 (59%)	1/3 (33%)
rs13143308 AF patients G/T or T/T	17/27 (62%)	1/3 (33%)

	Age	
	51–70 y	71–90 y
rs2200733 AF patients C/T or T/T	11/16 (68%)	5/11 (45%)
rs13143308 AF patients G/T or T/T	13/16 (81%)	4/11 (36%)

CM indicates cardiomyopathy and VM, valvulopathy.

compared with control subjects (n=5; Figure 1). Similarly, *PITX2C* expression is also decreased in left atria of AF patients (n=4) compared with control subjects (n=4; Figure 1) (online-only Data Supplement Table 5). Importantly, ENPEP, gene coding for glutamyl aminopeptidase A, which is located in the vicinity of *PITX2* in chromosome 4q25, display randomized expression levels in AF patients (online-only Data Supplement Figure 2). Thus, these data provide for the first time evidence of an association between loss of function of *PITX2* and AF in human patients.

Atrial and Ventricular Chamber-Specific *Pitx2* Deletion Leads to Chamber-Specific Defects

To obtain a suitable model of *Pitx2* loss of function, we have generated conditional tissue-specific *Pitx2* mutant mice by intercrossing a *Pitx2* floxed mouse line[5] with 2 distinct Cre deletor mouse lines, which rendered atrial-specific (NppaCre)[11] and ventricular-specific (Mlc2vCre)[12] *Pitx2* mutant models, respectively. Deletion of *Pitx2* within the atrial chambers using NppaCre and deletion of *Pitx2* in the ventricular chambers using Mlc2vCre resulted in viable chamber-specific homozygous *Pitx2*-deleted mice. qRT-PCR analyses of *Pitx2* expression in the atrial chambers and the ventricular chambers revealed that *Pitx2b* and *Pitx2c* transcript levels were reduced approximately ≈60% (online-only Data Supplement Figure 3), respectively, whereas *Pitx2a* expression was undetectable. Thus, these conditional *Pitx2* mice represent *Pitx2* loss-of-function deficiency models within the atrial and ventricular chambers. Within the present study, we have centered our attention on the atrial-specific *Pitx2* mouse mutant.

Adult NppaCre^{+}*Pitx2*$^{-/-}$ mutant mice display moderate enlargement of the atrial chambers with myocardial wall thinning, whereas the ventricular chambers display an overt increase in size and volume (Figure 2A through 2C), which is also characterized by a mild increase in the interventricular septum and left ventricular free wall thickness (Figure 2D through 2G). Increased fibrous tissue deposition is detectable within the ventricular but not the atrial chambers (Figure 2H through 2M), in line with procollagen qRT-PCR analyses (Figure 2N through 2O).

To investigate whether such morphological defects were present in atrial-specific *Pitx2* conditional mouse mutants during

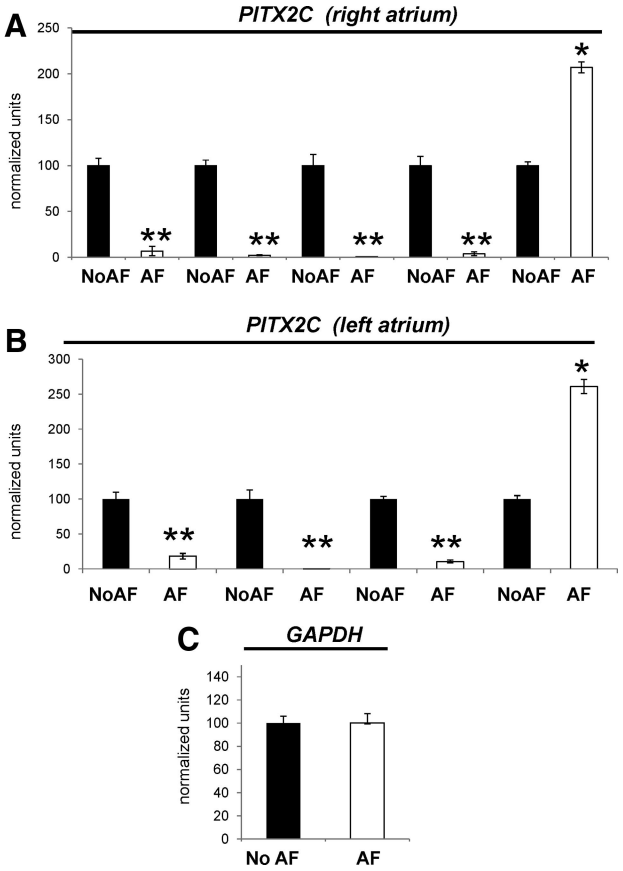

Figure 1. *PITX2C* qRT-PCR analysis in AF patients. **A**, *PITX2C* expression in right atrial biopsies from AF patients and no-AF patients. In 4 of 5 comparisons (80%), *PITX2C* expression is decreased approximately 80% to 90% in AF patients as compared with no-AF patients. **B**, *PITX2C* expression in left atrial biopsies from AF patients and no-AF patients. In 3 of 4 (75%) comparisons, *PITX2C* expression is similarly decreased (approximately 80% to 90%) in AF patients as compared with no-AF patients. **C**, GAPDH normalization against PPIA, which display no differences between AF and no-AF patients in left atria (n=4), serving as internal control. Similar results were obtained for right atrial (n=5) samples. *$P<0.05$, **$P<0.01$.

embryonic development, control ($NppaCre^{-}Pitx2^{flox/flox}$) and mutant ($NppaCre^{+}Pitx2^{-/-}$) mouse embryos were generated, collected at distinct developmental stages, and morphologically analyzed. Lack of *Pitx2* in the developing atrial myocardium partially impaired cardiac development because a subset (7/22; ≈30%) of E13.5 $NppaCre^{+}Pitx2^{-/-}$ mouse embryos displayed enlarged and thinner atrial chambers, as illustrated in Figure 3A through 3H), but no other morphogenetic defects were observed. Atrial length but not width was significantly larger in $NppaCre^{+}Pitx2^{-/-}$ as compared with $NppaCre^{-}Pitx2^{flox/flox}$ embryos at this stage, as reflected in Figure 3I. Enlarged atrial chambers, in both the right and left atria, become more patent at fetal (5/10 at E15.5; ≈50% and 12/15 at E17.5; ≈80%) stages and was characterized by a thinner myocardial wall as compared with wild-type age-matched control mice (Figure 3E through 3H). No ventricular defects are observed in $NppaCre^{+}Pitx2^{-/-}$ embryos during development and thus ventricular defects are likely to be secondary to atrial chamber dysfunction.

Because *Pitx2* expression in the developing atria is confined to the left atrial chamber, we explored the expression profile of several cardiac markers in the left atrial appendages of $NppaCre^{+}Pitx2^{-/-}$ mutant embryos as compared with age-matched control mice. In line with previous reports,[15] *Bmp10* expression was highly upregulated in the left atrial chambers. In addition, a significant increase on *Nkx2.5* expression was observed. On the contrary, *Gata6*, *Mef2c,* and *Nppa* transcript levels were downregulated, whereas *islet-1* and *Gata4* displayed no significant differences (Figure 3J).

Atrial-Specific *Pitx2*-Deficient Mice Display Electrophysiological Defects

To address potential electrophysiological changes in the mutant mice, we analyzed the ECG recordings and action potentials of adult *Pitx2* chamber-specific mutants. ECG recordings were similar between nontransgenic control adult mice (data not shown), $NppaCre^{+}$ (Figure 4A), and $NppaCre^{-}Pitx2^{flox/flox}$ (Figure 4C) adult mice, displaying in all cases rhythmic ECG recordings. However, 40% (4/10) of $NppaCre^{+}Pitx2^{-/-}$ mutants display impaired ECG recordings characteristic of an atrioventricular (AV) node block (Figure 4B). In addition, p waves are missing in most (5/6; ≈85%) of the remaining adult $NppaCre^{+}Pitx2^{-/-}$ mutant mice (Figure 4D). Morphological examination of the ventricular conduction system in $NppaCre^{+}Pitx2^{-/-}$ mutants demonstrates that the sinoatrial node (data not shown) and the ventricular conduction system is properly organized (Figure 4J through 4M), yet the AV node and bundle of His display reduced fibrous tissue insulation (Figure 4J through 4K).

In addition, we studied the electrophysiological properties of dissected right and left atrial samples corresponding to the NppaCre*Pitx2* background (control and mutants) and left ventricular samples of control and conditional mutants corresponding to the Mlc2vCre*Pitx2* background. The characteristics of action potentials were recorded on multicellular preparations, and the results are summarized in Table 2. Left atria from NppaCre*Pitx2*-deficient mice displayed a significantly more depolarized resting membrane potential (RMP) (-83.8 ± 4.2 versus -87.0 ± 2.7 mV) and a smaller action potential amplitude (109.8 ± 0.6 versus 114.1 ± 1.8 mV) than those from control littermate control mice ($P<0.05$). The depolarization of the RMP would suggest that the absence of *Pitx2* correlates with a decrease in the expression and/or function of the channels that generate the ionic currents involved in the control of the resting membrane potential, for instance, the inward rectifier current (I_{K1}). Furthermore, this depolarization may inactivate the Na^{+} channels responsible of the AP upstroke explaining the reduced action potential amplitude observed in *Pitx2*-deficient mice. It is interesting to note that the effects observed are chamber-specific because significant differences were apparent only in the left atria.

Molecular Determinants of the Electrophysiological Measurements in Atrial-Specific *Pitx2*-Deficient Mouse Mutants

To further investigate the molecular substrates underlying the decreased action potential amplitude and the depolarized RMP in the left atria of $NppaCre^{+}Pitx2^{-/-}$ adult hearts, we

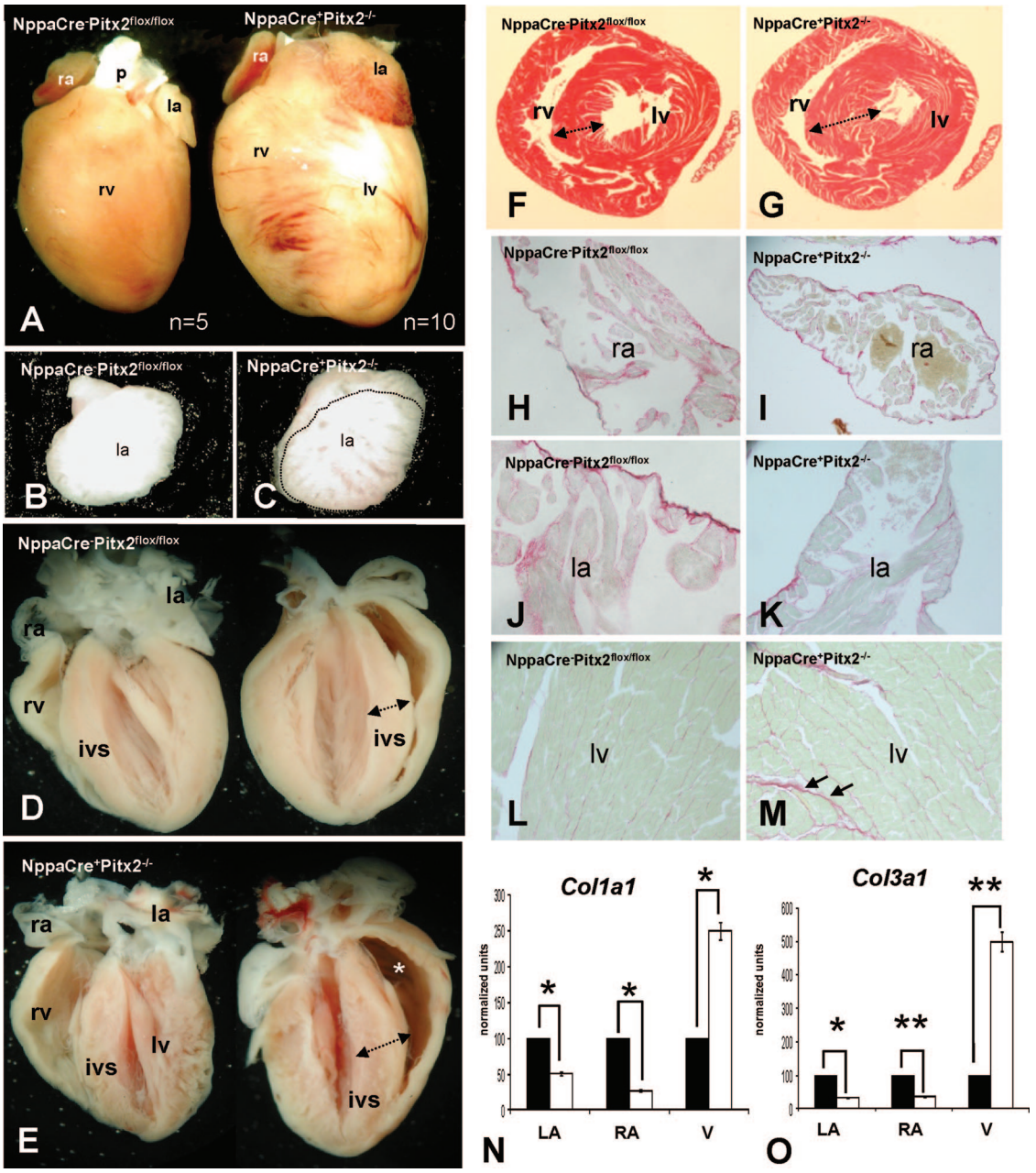

Figure 2. Morphological remodeling of adult atrial-specific *Pitx2* conditional mutants. Whole-mount ventral views (**A**) and isolated left atria (**B** and **C**) corresponding to adult NppaCre^{-}*Pitx2*$^{flox/flox}$ (**A** and **B**) and NppaCre^{+}*Pitx2*$^{-/-}$ (**A** and **C**) hearts, respectively. Observe the increased heart size in NppaCre*Pitx2*$^{-/-}$ compared with control NppaCre^{-}*Pitx2*$^{flox/flox}$ hearts. Left atria (la) size is significantly enlarged in NppaCre^{+}*Pitx2*$^{-/-}$ (**B**) compared with control NppaCre^{-}*Pitx2*$^{flox/flox}$ (**C**) hearts. Dashed lines in **C** represent the overlay of the left atria dimensions illustrated in **B**. Four-chambered views of adult NppaCre^{-}*Pitx2*$^{flox/flox}$ (**D**) and NppaCre^{+}*Pitx2*$^{-/-}$ (**E**) hearts are shown. Note that atrial-specific *Pitx2* mutants (**E**) display enlarged atrial and ventricular chambers and thickening of the interventricular septum (IVS) (double arrows) compared with control (**D**) and right ventricular (rv) lumen is significantly dilated (asterisk, **E**). Transversal histological sections of adult ventricular NppaCre^{-}*Pitx2*$^{floxed/floxed}$ (**F**) and NppaCre^{+}*Pitx2*$^{-/-}$ (**G**) chambers illustrate a significant IVS thickness (double arrows). Red sirius staining of atrial (**H** through **K**) and ventricular (**L** and **M**) histological sections of NppaCre^{-}*Pitx2*$^{flox/flox}$ (**H**, **J**, and **L**) and NppaCre^{+}*Pitx2*$^{-/-}$ (**I**, **K**, and **M**) adult hearts demonstrate increased fibrosis in the ventricular (arrows, **M**) but not the atrial chambers in atrial-specific *Pitx2* conditional mutants. qRT-PCR analyses of *Col1a1* (**K**) and *Col3a1* (**L**) expression in NppaCre^{-}*Pitx2*$^{flox/flox}$ (black bars) and NppaCre^{+}*Pitx2*$^{-/-}$ (white bars) adult hearts are shown. *$P<0.05$, **$P<0.01$.

compared the expression of the major determinants of sodium current (I_{Na}) and the inward rectifier current (I_{K1}) by qRT-PCR. As shown in Figure 4C and 4D, *Scn5a* and *Scn1b* expression was severely impaired in the both left and right atrial chambers, with milder or no changes in the ventricular chambers of NppaCre*Pitx2*$^{-/-}$ adult hearts. Similarly *Kcnj2*, *Kcnj12,* and *Kcnj4* expression was severely reduced in the left atrial myocardium but not ventricular chambers (Figure 4E through 4G). Consistent with these findings, Western blot analysis showed that Kir2.1 (*Kcnj2*) and Nav1.5 (*Scn5a*) channel expression is decreased in the atrial chambers of NppaCre^{+}*Pitx2*$^{-/-}$ mice (Figure 4L).

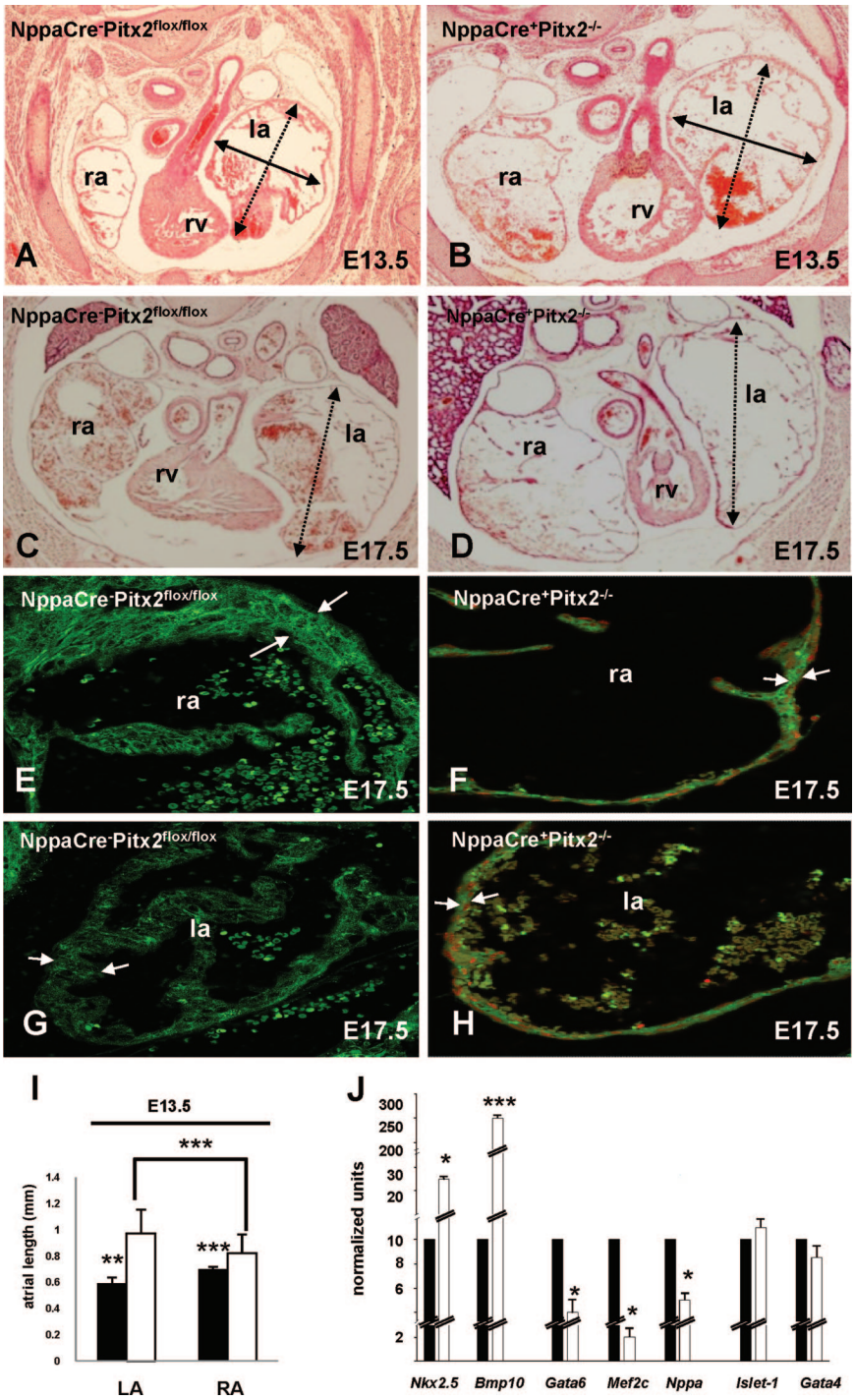

Figure 3. Morphological remodeling of embryonic atrial-specific *Pitx2* conditional mutants. Transversal histological sections of E13.5 (**A** and **B**) and E17.5 (**C** through **H**) embryonic hearts corresponding to NppaCre^{-}*Pitx2*$^{flox/flox}$ (**A**, **C**, **E**, and **G**) and NppaCre^{+}*Pitx2*$^{-/-}$ (**B**, **D**, **F**, and **H**) embryos illustrate a significant atrial chamber enlargement (**A** through **D**) and myocardial thinning (**E** through **H**). **F**, Mean dorso-ventral length (double arrows in **A** through **D**) of the right atrial (ra) and left atrial (la) appendages in multiple E13.5 transversal sections demonstrate a statistically significant increase in length in atrial-specific *Pitx2* conditional mutants (black bars) compared with control (white bars). **F**, Expression levels of *Nkx2.5*, *Bmp10*, *Gata6*, *Mef2c*, *Nppa*, *Islet-1,* and *Gata4* in E17.5 left atrial appendages corresponding to atrial-specific *Pitx2* conditional mutants (white bars) compared with control (black bars). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Pitx2 Modulates miR-1 Expression, Thereby Controlling I_{K1} but Not I_{Na} Components

To further understand the regulatory role of *Pitx2* on ion channel expression, we tested whether lack of *Pitx2* in the adult left atrial chambers impairs microRNA expression. miR-1 qRT-PCR analyses of adult NppaCre*Pitx2*$^{-/-}$ left atrial myocardium demonstrate a significant increase of miR-1 expression (Figure 5A). Thus, these results support a role for *Pitx2* in repressing miR-1 expression, which in turn, can regulate *Kcnj2* expression.[16] However, it is unknown whether *Scn5a* and *Scn1b* can also be modulated by miR-1. We therefore overexpressed miR-1 in HL-1 atrial cardiomyocytes, which resulted in decreased *Gja1* and *Kcnj2* transcripts levels (Figure 5B), in line with previous reports,[16] but did not modify *Scn5a* and/or *Scn1b* expression (Figure 5B). To further investigate if *Pitx2* directly regulates miR-1 expression and/or *Scn5a* expression, we transiently transfected HL-1 atrial adult cardiomyocytes with *Pitx2*c. Overexpression of *Pitx2*c resulted in decreased miR-1 and increased *Scn5a* and *Scn1b* expression (Figure 5C). Furthermore, *Pitx2* silencing decreased *Scn5a* and *Scn1b* expression in HL-1 cells (Figure 5D).

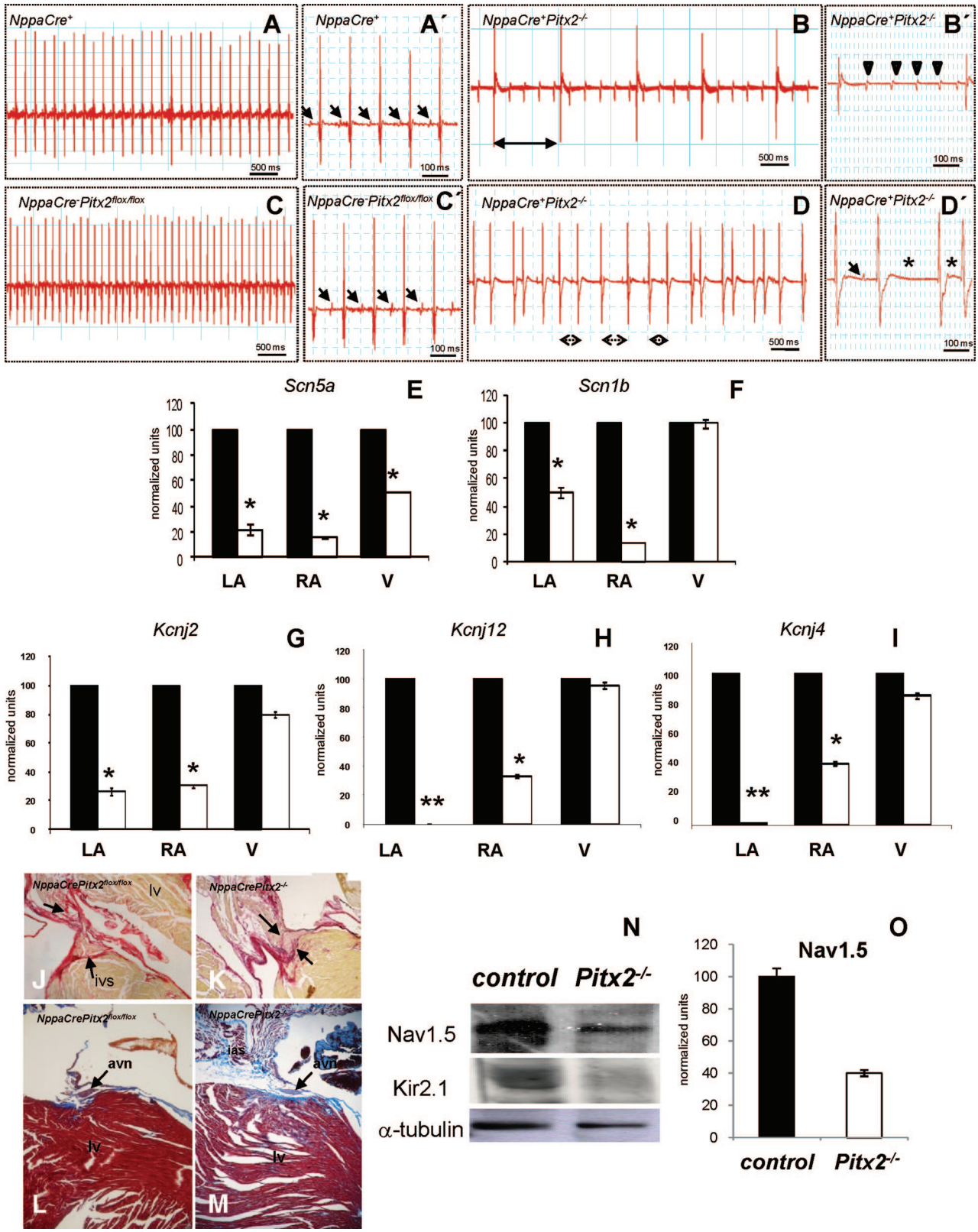

Figure 4. Electrophysiological and molecular remodeling of adult atrial-specific *Pitx2* conditional mutants. Shown are representative ECG recordings (**A** through **D**) of adult NppaCre^{+} (n=5) (**A**), NppaCre^{-}*Pitx2*$^{flox/flox}$ (n=10) (**C**), and NppaCre^{+}*Pitx2*$^{-/-}$ (**B** and **D**) hearts, respectively. Observe that in control mice (NppaCre^{+} and NppaCre^{-}*Pitx2*$^{flox/flox}$), conserved R-R intervals are recording, and in all cases a p wave can be distinguished (arrows, **A′** and **C′**). In 40% (4/10) of the atrial-specific conditional mutant mice (NppaCre^{+}*Pitx2*$^{-/-}$), an AV block ECG pattern can be observed, as delineated by arrowheads in **B** and **B′**, whereas in the remaining atrial-specific conditional mutant (6/10), in all but one, a p wave (arrow) was frequently missing (asterisks), as illustrated in **D** and **D′**, and irregular R-R intervals were also recorded (**D**, double arrows). **E** through **I** correspond to qRT-PCR expression analyses of *Scn5a* (**E**), *Scn1b* (**F**), *Kcnj2* (**J**),

Table 2. Electrophysiological Measurements in Atrial Chamber-Specific *Pitx2* Insufficient Mice

Preparation	*Pitx2* Conditional	RMP, mV	APA, mV	Vmax, V/s	APD_{20}, ms	APD_{50}, ms	APD_{90}, ms
Left atria (n=5)	NppaCre^{-}Pitx2$^{flox/flox}$	−87.0±2.7	114.1±1.8	174.0±8.7	7.3±1.4	23.3±3.5	72.2±8.9
	NppaCre^{+}Pitx2$^{-/-}$	−83.8±4.2*	109.8±0.6*	170.0±14.8	7.3±0.8	24.3±2.6	89.7±10.0
Right atria (n=5)	NppaCre^{-}Pitx2$^{flox/flox}$	−86.4±2.9	111.7±1.1	160.0±8.6	6.8±1.0	25.6±2.7	93.0±7.5
	NppaCre^{+}Pitx2$^{-/-}$	−85.4±1.7	111.5±2.0	164.0±13.4	9.1±2.2	31.7±5.6	102.3±8.8
Ventricular papillary muscle (n=5)	Mlc2vCre^{-}Pitx2$^{flox/flox}$	−85.3±2.1	116.4±1.7	190.0±14.9	8.8±1.7	37.0±5.8	145.1±14.6
	Mlc2vCre^{+}Pitx2$^{-/-}$	−86.2±3.0	114.0±2.5	186.0±8.7	14.6±2.5	40.2±3.8	124.8±7.8

APA indicates action potential amplitude; APD, action potential duration; and Vmax, maximum upstroke velocity.

Discussion

AF is the most common cause of arrhythmogenesis in the human population, yet, the genetic cause of AF remains elusive.[17–19] Point mutations in potassium or sodium channel genes have been associated with familial AF but account for only a small AF fraction.[19–23] Recent genome-wide association studies[8–10] have reported several risk variants on chromosome 4q25, adjacent to *PITX2* gene, which are associated with AF. Although these studies do not provide any experimental evidence that links regulation of *PITX2* expression/activity to the risk variants, it has been suggested that *PITX2* might be the causative link. In the present study, we demonstrate that 2 SNPs, rs2200733 and rs13143308, are highly prevalent in a cohort of Spanish patients with AF, supporting previous findings from other populations. In addition, rs2200733 is more prevalent in patients with isolated AF as compared with patients with AF and other cardiac structural defects, providing a potential role for SNP genotyping as a stratification tool as recently suggested.[24] The mechanisms by which these SNPs regulate *PITX2* function, however, remain unknown.

At the transcriptional level, we demonstrate for the first time in this study that *PITX2C* is significantly decreased in human patients with sustained AF, thus providing a molecular link between *PITX2* loss of function and AF. We have also generated chamber-specific conditional *Pitx2* mouse mutants, which display a ≈60% reduction of *Pitx2* expression in the chamber myocardium, thus providing an experimental model of *Pitx2* insufficiency. Such incomplete *Pitx2* deletion might be attributed to incomplete and/or patchy Cre recombination in the atrial chamber myocardium.[11] Importantly, Cre recombination (NppaCre) is mainly restricted to the atrial appendage myocardium, with some weak and patchy expression in the AV node (V. Christoffels, personal communication) but excluding the sinoatrial and pulmonary veins myocardium.[11] Lack of *Pitx2* expression in the atrial myocardium leads to a progressive enlargement of the atrial chambers, which is consistent with the increased proliferation rate in the left compared with the right atrium already observed from early developmental stages.[25] Furthermore, *Bmp10* is highly upregulated in the left atrial chambers,[15] supporting a role of *Pitx2* controlling atrial chamber dimensions, because overexpression of *Bmp10* plays a crucial role regulating physiological hypertrophy.[26] Thus, these findings support the hypothesis that selective upregulation of *Bmp10* in the atrial myocardium, mediated by *Pitx2*, leads to increase cell proliferation and thus larger atrial chambers. Critically, atrial dilatation has been widely reported as a putative mechanism triggering the onset and maintenance of arrhythmogenic processes, including AF, in the adult heart.[27]

At the functional level, atrial deletion of *Pitx2* leads to ion channel remodeling events, which have been previously linked to familiar cases of AF,[21,28] supporting the notion that *Pitx2* acts upstream of these AF-prone pathways. In context, Wang et al[29] have recently reported that *Pitx2* plays important role inhibiting sinoatrial pacemaker activity in the left atrium, thus providing susceptibility to atrial arrhythmias. Importantly, our atrial-specific deletion of *Pitx2* provides evidence of ion channel remodeling within the atrial chamber myocardium independent of altering sinoatrial node function. Specifically, we show that *Pitx2* loss of function leads to downregulation of *Scn5a* and *Scn1b*. Genetic studies have revealed that point mutations in SCN5A and SCN1B are associated with familiar cases of AF,[28] and *Scn5a* loss-of-function mouse mutants also display increased atrial susceptibility to atrial arrhythmogenesis.[30] Surprisingly, lack of *Pitx2* expression in atrial myocardium leads to downregulation *Kcnj2*, *Kcnj4,* and *Kcnj14* expression, in contrast to the proarrhythmogenic pattern of expression observed in humans,[31] although loss of the resting membrane potential has been also associated with atrial electric remodeling and AF.[32]

Atrial chamber-specific *Pitx2* conditional mutants also display conductive disturbances, such as an AV block. Importantly, P-wave recording is frequently missing in the NppaCRe-Pitx2flox/flox (control) and NppaCre^{+}*Pitx2*$^{-/-}$ atrial chamber-specific *Pitx2* conditional mutants, demonstrating too an atrial chamber dysfunction. Curiously, the morphological characteristics and anatomic location of the sinoatrial node and the ventricular conduction system of atrial-chamber specific conditional mutants is unaltered, yet

Figure 4 (Continued). *Kcnj12* (**H**), and *Kcnj4* (**I**) in right atrium (RA), left atrium (LA), and ventricular (V) chambers corresponding to atrial-specific adult *Pitx2* conditional hearts (white bars) as compared with control mice (black bars). Histological sections of the AV conduction system of adult NppaCre^{-}*Pitx2*$^{flox/flox}$ (**J** and **L**) and NppaCre^{+}*Pitx2*$^{-/-}$ (**K** and **N**) hearts are stained with picrosirius (**J** and **K**) and Mallory trichrome (**L** and **M**). Western blot analyses (**N**) of Nav1.5 and Kir2.1 expression correspond to adult NppaCre^{-}*Pitx2*$^{flox/flox}$ (wt) and NppaCre^{+}*Pitx2*$^{-/-}$ (*Pitx2*$^{-/-}$) hearts. α-Tubulin served as internal loading control. **O**, Semiquantitative illustration of Nav1.5 protein expression normalized to α-tubulin expression corresponding to control mice as compared with atrial-specific *Pitx2* mutants. *$P<0.05$, **$P<0.01$.

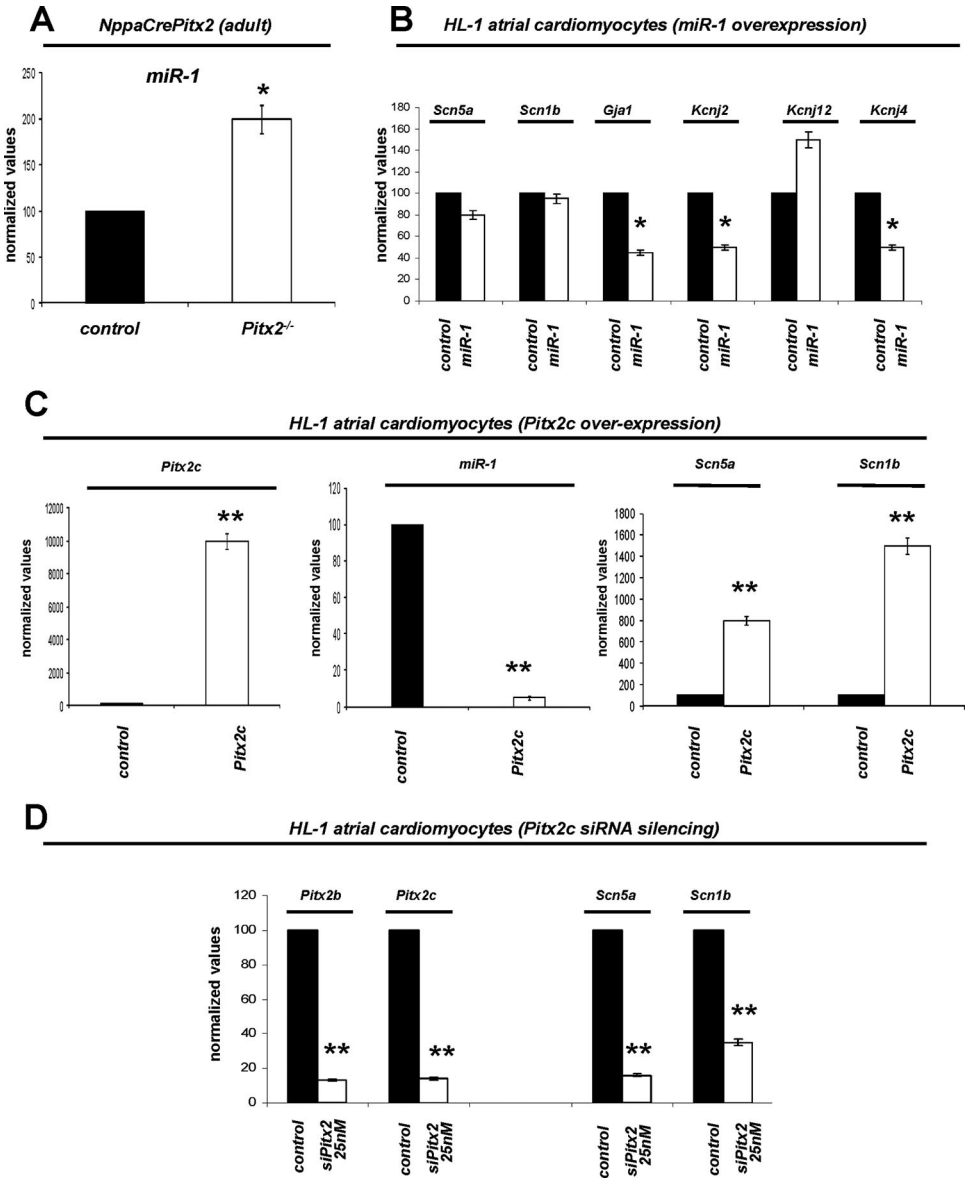

Figure 5. *Pitx2*-mediated microRNA molecular pathway. **A**, qRT-PCR analyses of microRNA miR-1 expression in adult NppaCre^{-}*Pitx2*$^{flox/flox}$ (control) and NppaCre^{+}*Pitx2*$^{-/-}$ (*Pitx2*$^{-/-}$) hearts. Observe that miR-1 expression is increased approximately 2-fold in atrial-specific mutant mice as compared with control mice. **B**, qRT-PCR analyses of *Scn5a*, *Scn1b*, *Gja1*, *Kcnj2*, *Kcnj12,* and *Kncj4* expression corresponds to miR-1 overexpression in HL-1 atrial cardiomyocytes. Overexpression of miR-1 leads to a significant decrease in the expression of *Gja1, Kcnj2,* and *Kcnj4*, in line with previous reports,[14] whereas *Scn5a, Scn1b,* and *Kcnj12* display no significant differences. **C**, qRT-PCR analyses of *Pitx2c*, *miR-1*, *Scn5a,* and *Scn1b* expression in *Pitx2c*-transfected HL-1 atrial cardiomyocytes. Overexpression of *Pitx2c* leads to downregulation of *miR-1* and enhanced expression of *Scn5a* and *Scn1b*. **D**, qRT-PCR analyses of *Pitx2b, Pitx2c, Scn5a,* and *Scn1b* expression in *Pitx2* siRNA-transfected HL-1 atrial cardiomyocytes. Silencing of *Pitx2c* leads to downregulation of *Scn5a* and *Scn1b*. *$P<0.05$, **$P<0.01$.

there is reduced insulation of the AV node and bundle of His.[33] Thus, it is plausible that such AV block might be caused by *Pitx2* deficiency in the AVN (Cre recombination), leading to sodium channel impairment, which in turn can underlie AV block, as previously proposed in mice and humans.[34] Importantly, AV block is an independent risk factor for AF in humans,[28,35] and the observation of AV block in our mouse mutant model further implicates *Pitx2* in the development of atrial proarrhythmogenic substrates.

Atrial dilation and ion channel remodeling are highly linked events. However, it is unclear whether *Pitx2* directly regulates these ion channels or whether this might be caused by remodeling due to atrial chamber dilation. Our results provide for the first time evidence that lack of *Pitx2* results in impaired expression of sodium (I_{Na}) and potassium (I_{K1}) channel expression in atrial but not the ventricular chambers, consistent with the altered electrophysiological properties recorded in the left but not right adult *Pitx2*-deficient atrial myocardium. Moreover, our *Pitx2* gain- and loss-of-function experiments in vitro provide direct evidence that ion channel remodeling triggered by *Pitx2* is independent of atrial chamber dilation. In addition, we demonstrate that *Pitx2* can also modulate I_{K1} channel expression indirectly, through regulation of miR-1,[36] whereas *Scn5a* regulation appears to be

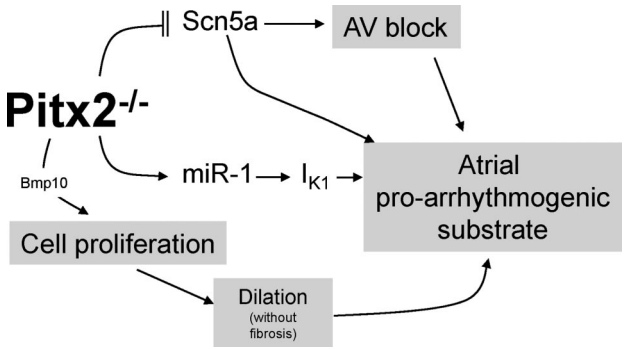

Figure 6. *Pitx2*-mediated signaling pathways in the developing and adult heart. Schematic representation of the *Pitx2*-mediated signaling pathways is revealed by the morphological, electrophysiological, and molecular analysis of atrial chamber-specific *Pitx2* conditional mutants.

directly exerted by *Pitx2*. Thus, together these data support the notion that impaired *Pitx2* expression leads to reduced ion channel expression and function, thereby providing cellular and molecular substrates for the onset of arrhythmogenic events. Unfortunately, the size limits of the murine heart makes this model unsuitable to decipher if ion channel remodeling caused by impaired *Pitx2* expression is sufficient to induce AF, and resolution of this issue will have to wait for the generation of *Pitx2* loss of function in larger animal models.

In conclusion, we provide the first direct evidence for a relationship between impaired *Pitx2* function and distinct cellular, molecular, and electrophysiological pathways (Figure 6) can provide increased susceptibility to induce and/or promote atrial arrhythmogenesis, supporting the notion that *Pitx2* acts hierarchically upstream of these AF-prone pathways.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Atrial fibrillation (AF) is the most frequent cardiac arrhythmia, leading to a high risk of mortality and morbidity. Though its prevalence is high, genetics of AF has remained rather elusive, with sporadic reports on point mutations in a wide variety of ion channel–encoding genes. Recently, genome-wide association studies have unraveled genetic variants (associated with AF risk) that are located close to the homeobox transcription factor *PITX2* in a large proportion of AF patients. In the present investigation, we corroborated these findings in a small cohort of AF patients. We also provided evidence that *PITX2* is downregulated in AF patients and experimentally demonstrated that *Pitx2* insufficiency results in cellular and molecular changes leading to atrial electrical and cellular remodeling linked to atrial arrythmogenesis. Thus, these findings provide insights into signaling pathways that are implicated in the pathogenesis of AF.