Pitx2c increases in atrial myocytes from chronic atrial fibrillation patients enhancing $I_{Ks}$ and decreasing $I_{Ca,L}$

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Aims

Atrial fibrillation (AF) produces rapid changes in the electrical properties of the atria (electrical remodelling) that promote its own recurrence. In chronic AF (CAF) patients, up-regulation of the slow delayed rectifier $K^+$ current ($I_{Ks}$) and down-regulation of the voltage-gated $Ca^{2+}$ current ($I_{Ca,L}$) are hallmarks of electrical remodelling and critically contribute to the abbreviation of action potential duration and atrial refractory period. Recent evidences suggested that Pitx2c, a bicoid-related homeodomain transcription factor involved in directing cardiac asymmetric morphogenesis, could play a role in atrial remodelling. However, its effects on $I_{Ks}$ and $I_{Ca,L}$ are unknown.

Methods and results

Real-time quantitative polymerase chain reaction analysis showed that Pitx2c mRNA expression was significantly higher in human atrial myocytes from CAF patients than those from sinus rhythm patients. The expression of Pitx2c was positively and negatively correlated with $I_{Ks}$ and $I_{Ca,L}$ densities, respectively. Expression of Pitx2c in HL-1 cells increased $I_{Ks}$ density and reduced $I_{Ca,L}$ density. Luciferase assays demonstrated that Pitx2c increased transcriptional activity of $KCNQ1$ and $KCNE1$ genes. Conversely, its effects on $I_{Ca,L}$ could be mediated by the atrial natriuretic peptide.

Conclusion

Our results demonstrated for the first time that CAF increases Pitx2c expression in isolated human atrial myocytes and suggested that this transcription factor could contribute to the CAF-induced $I_{Ks}$ increase and $I_{Ca,L}$ reduction observed in humans.

Keywords

Pitx2c • Chronic atrial fibrillation • Human atrial myocytes • $I_{Ks}$ • $I_{Ca,L}$

1. Introduction

Atrial fibrillation (AF) is the most frequent arrhythmia and the main risk factor associated with myocardial-related cerebrovascular events. Nowadays, treatment of AF is clearly suboptimal, mainly due to rapid changes in the electrical properties of the atria (electrical remodelling) induced by the arrhythmia itself. This electrical remodelling promotes the maintenance and recurrence of AF and is characterized by a marked shortening of the atrial action potential duration (APD) and refractoriness as a consequence of alterations in the expression and function of L-type $Ca^{2+}$ and inward rectifier and voltage-dependent $K^+$ channels. In chronic AF (CAF) patients, the L-type $Ca^{2+}$ current ($I_{Ca,L}$) decrease4 and the slow delayed rectifier $K^+$ current ($I_{Ks}$) increase5 critically contribute to the APD shortening.

Pitx2 is a transcription factor that plays a critical role in directing cardiac asymmetric morphogenesis. In humans, the PITX2 gene generates three main isoforms (Pitx2a, Pitx2b, and Pitx2c), the latter being the predominant isoform in the heart. Increasing evidence pointed to a role of Pitx2c in the pathophysiology of AF. Indeed, it has been described that single nucleotide variants on human chromosome 4q25, $\approx 170$ kb distal to the PITX2 gene, are associated with an increased risk of familial AF. Pitx2c expression can be increased10 or decreased11 in atrial appendage samples obtained from AF patients, compared with those from patients in sinus rhythm (SR). However, the putative role of

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Pitx2c in electrical remodelling in humans has not been explored as data available thus far have been obtained in mouse models. Therefore, this study was undertaken to measure Pitx2c expression in human atrial myocytes from SR and CAF patients and to determine the effects of Pitx2c on $I_{Ks}$ and $I_{Ca,L}$. Importantly, the results obtained demonstrated that Pitx2c expression is augmented in atrial myocytes from CAF patients. Furthermore, Pitx2c increases $I_{Ks}$ by enhancing expression of Ks channel encoding genes, whereas it decreases $I_{Ca,L}$ by a post-translational effect.

2. Methods

This study was approved by the Investigation Committee of the Hospital Universitario Gregorio Marañón (CNIC-13) and conforms to the principles outlined in the Declaration of Helsinki. Each patient gave written informed consent. Clinical data of the patients are included in Supplementary material online, Table S1.

2.1 Analysis of the mRNA expression in human atrial myocytes

Real-time quantitative polymerase chain reaction (qPCR) was performed using TaqMan gene expression assays (Life Technologies, USA). The cycle to threshold (Ct) values were normalized to 18S rRNA. To compare CAF vs. SR expression differences, the respective data were transformed from Delta Ct values to equivalent fold differences using the following equation: Fold Difference (mean ΔCtSR – mean ΔCtCAF) = 2(ΔCtSR-ΔCtCAF);7,16

2.2 Patch clamping

Outward $K^+$ currents and $I_{Ca,L}$ were recorded in human atrial myocytes and HL-1 cells cultured in the whole-cell patch-clamp technique (micropipette resistance < 3.5 MΩ). Human atrial myocytes were isolated from right atrial appendages obtained from SR ($n=10$) and CAF (SR $n=10$) patients as described previously,16,18 and transfected with CMV-Pitx2c (2 μg) by using Lipofectamine 2000. In HL-1 cells, $I_{Ca,L}$ was recorded by using Ba$^{2+}$ as charge carrier to increase current density and to eliminate Ca$^{2+}$-dependent inactivation.16,18,19 Outward $K^+$ currents ($I_{Ks}$) and $I_{Ca,L}$ were recorded at -5 mV and HMR-1556 (10 μM) and used to inhibit the transient outward ($I_{to}$) and the ultrarapid component of the delayed rectifier ($I_{Ks}$) $K^+$ currents. Series resistance was compensated manually and usually ≥90% compensation was achieved. Thus, no significant voltage errors (<5 mV) due to series resistance were expected with the micropipettes used.

2.3 Luciferase gene expression reporter assays

Luciferase activity assays were conducted in CHO cells transfected with the corresponding promoters20 cotransfected with an empty vector or with Pitx2c (100 ng). Luciferase activity was measured 48 h after transfection and normalized to sample protein concentration. All reporter assays were performed in triplicate.

2.4 Western blot analysis

Western blot analysis17 was conducted to measure Kv7.1, mink, and Cav1.2 protein expressions in HL-1 cells transfected or not with Pitx2c by using anti-Kv7.1 (1:250; Sigma, USA), anti-mink (1:250; Abcam, UK), and anti-Cav1.2 (1:1000; clone LS7/46; Neuromab, USA) antibodies.

2.5 Statistical analysis

Results are expressed as mean ± SEM. Unpaired t-test or one-way analysis of variance (ANOVA) followed by Newman–Keuls test was used where appropriate. In small-sized samples ($n<15$), statistical significance was confirmed by using non-parametric tests. Comparisons between categorical variables were performed using Z-test. To take into account repeated sample assessments, data were analysed with multilevel mixed-effects models. A value of $P<0.05$ was considered significant. Additional details are presented in Supplementary material.

3. Results

3.1 Pitx2c expression increases in atrial myocytes from CAF patients

The expression of Pitx2c was measured by qPCR in myocytes enzymatically isolated from atrial appendages obtained from SR ($n=10$) and CAF ($n=10$) patients. Comparison of ΔCt values demonstrated that Pitx2c expression was significantly larger in CAF myocytes (Figure 1A). Indeed, transformation of ΔCt values to fold differences demonstrated that Pitx2c expression was approximately two times larger in CAF than in SR myocytes (Figure 1B).

3.2 Pitx2c expression levels correlate with $I_{Ks}$ and $I_{Ca,L}$ densities in SR and CAF myocytes

An increase in $I_{Ks}$ and a decrease in $I_{Ca,L}$ densities are hallmarks of CAF-induced electrical remodelling.4,5,7,16 Therefore, we analysed a putative correlation between Pitx2c levels and $I_{Ks}$ and $I_{Ca,L}$ densities. In these experiments, myocytes enzymatically isolated from each atrial appendage were separated in two fractions: one for Pitx2c expression assay and another for $I_{Ca,L}$ or $I_{Ks}$ recordings. Interestingly, association studies demonstrated significant correlations of Pitx2c expression with $I_{Ks}$ density at +60 mV (Figure 1C) and $I_{Ca,L}$ density at +10 mV (Figure 1D), showing that the larger the Pitx2c expression the higher $I_{Ks}$ and the lower $I_{Ca,L}$ densities. To test whether these correlations were driven by differences between SR and CAF groups, a partial correlation was calculated. Pitx2c expression and $I_{Ks}$ or $I_{Ca,L}$ densities are correlated in the absence of the influence of the group effect (Figure 1E and F). Moreover, a positive correlation was observed between Pitx2c mRNA levels and the expression of KCNQ1 and KCNE1 mRNA. However, Pitx2c mRNA expression was not correlated with CACNA1C mRNA levels (Supplementary material online, Figure S1).

The existence of such correlations suggested that Pitx2c may modulate the expression of the channels that generate these currents. To test this hypothesis, we first analysed the properties of $I_{Ks}$ and $I_{Ca,L}$ recorded in SR and CAF myocytes. As demonstrated previously,5,7,16 cell capacitance of CAF myocytes was greater than that of SR myocytes ($96.9±0.2$ vs. $56.7±0.2$ pF, $P<0.0001$; $n=194$). In the presence of 4-aminopyridine (2 mM), used to inhibit the transient outward ($I_{to}$) and the ultrarapid component of the delayed rectifier ($I_{Ks}$) $K^+$ currents, a current whose time- and voltage-dependent properties are consistent with those of $I_{Ks}$ was recorded.5,7,16 Figure 2A and B shows current traces and current density–voltage curves for $I_{Ks}$ recorded in SR and CAF myocytes. $I_{Ks}$ was measured as the difference between the amplitudes at the end and beginning of 4 s pulses and normalized by cell capacitance to obtain current density. Confirming previous results,5,7,16 in myocytes obtained from CAF patients, $I_{Ks}$ density increased approximately two-fold compared with myocytes from SR patients ($2.3±0.2$ vs. $1.2±0.2$ pA/pF at $+60$ mV, $P<0.01$; $n=40$ and 86, respectively) and accordingly, KCNQ1 and KCNE1 mRNA expressions were also increased (Supplementary material online, Figure S1). Moreover, the time course of current activation was significantly faster, and the conductance curve was shifted to more hyperpolarized potentials in CAF myocytes relative to SR myocytes (Table 1). $I_{Ca,L}$ was
measured as the difference between the peak amplitude and the amplitude at the end of the pulses and normalized by cell capacitance to obtain the current density. As expected, CACNA1C mRNA expression (Supplementary material online, Figure S1) and $I_{\text{Ca,L}}$ density in CAF myocytes (2.0 ± 0.3 vs. 2.3 ± 0.3 pA/pF at +10 mV, P < 0.01; n = 27 and 41, respectively) (Figure 2C and D) were significantly smaller than in SR myocytes. The midpoint of the $I_{\text{Ca,L}}$ inactivation curve in CAF was significantly shifted to more positive potentials compared with SR myocytes (Table 1). However, no differences were observed in the activation ($t_{\text{act}} = 1.2 ± 0.1$ vs. $1.1 ± 0.1$ ms at +10 mV) and inactivation kinetics and voltage dependence of activation (Table 1).

### 3.3 Pitx2c increases $I_{\text{Ks}}$ density in HL-1 cells

The results suggested that Pitx2c could be involved in the CAF-induced $I_{\text{Ks}}$ increase and $I_{\text{Ca,L}}$ decrease. Next, we analysed the molecular mechanisms underlying the putative Pitx2c-induced $I_{\text{Ks}}$ increase and $I_{\text{Ca,L}}$ decrease in the mouse atrial-derived HL-1 cardiomyocyte-like cell line. Cells non-transfected with Pitx2c randomly patched (n = 32) could be separated into three groups, depending on the main outward K⁺ current recorded by applying 4 s pulses from −80 mV to potentials ranging from −80 to +40 mV. About 36% of the cells (Figure 3A) exhibited an outward current with time- and voltage-dependent properties concordant with those of $I_{\text{Ks}}$ and sensitive to dofetilide (1 μM)

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/109/3/431/2462557)

**Figure 1** Pitx2c increases in human atrial myocytes from CAF patients. (A) ΔCt values of Pitx2c mRNA measured by qPCR in isolated right atrial myocytes obtained from SR (n = 10) and CAF (n = 10) patients. Each bar represents mean ± SEM. (B) Relative expression levels of Pitx2c in SR and CAF samples. Correlation between Pitx2c expression (represented as 1/ΔCt) and $I_{\text{Ks}}$ density at +60 mV (C) and $I_{\text{Ca,L}}$ density at +10 mV (D) recorded in right atrial myocytes from SR (black circles) and CAF (white circles) patients. In (C) and (D), each point corresponds to the mean values of 1/ΔCt and current density obtained for each patient. (E and F) Partial correlation calculated to suppress the influence of the group effect. The corresponding residuals (labelled with ') were calculated after regressing $I_{\text{Ks}}$ or $I_{\text{Ca,L}}$ density ($Y$) and Pitx2c expression ($X_1$) to variable $X_2$ (to be in the SR or CAF group). Solid lines represent the linear regression to the data, and dashed lines represent the SEM of the fit. In (A) and (B), *P < 0.05 vs. SR. Unpaired t-test.
The results demonstrated that Pitx2c transfection did not significantly modify $I_{Kr}$ density, the voltage dependence of activation, or the time dependence of current activation or deactivation (Table 2 and Supplementary material online, Figure S2). In cells with $I_{Kr}$-predominant and $I_{Kr} + I_{Ks}$ patterns perfused with dofetilide, $I_{Ks}$ was recorded by applying 4 s pulses from −80 mV to potentials ranging between −80 and +60 mV, followed by repolarizing pulses to −30 mV to record the tail currents (Figure 3C and D). Under these conditions, the time-dependent current was completely inhibited by HMR-1556 (1 μM) (inset of Figure 3D). Importantly, Pitx2c significantly increased the current density (from 2.6 ± 0.4 to 6.1 ± 1.1 pA/pF at +60 mV; $P < 0.05$) (Figure 3D and E) and accelerated the time course of activation (Table 2). It also significantly increased the tail current density (Figure 3F) and shifted the activation curves to more hyperpolarized potentials (Table 2). Densitometric analysis from western blots conducted in HL-1 cells (Figure 4A) demonstrated that Pitx2c increased by approximately two-fold Kv7.1 and minK protein expression.
Figure 3  Pitx2c increases $I_{Ks}$ in HL-1 cells. Percentage of HL-1 cells with $I_{Kr}$-predominant, $I_{Ks}$-predominant, and $I_{Kr} + I_{Ks}$ patterns in non-transfected cells (A) or in cells transfected with Pitx2c (B). Outward $K^+$ current traces recorded in two $I_{Ks}$-predominant cells transfected (D) or not (C) with Pitx2c. The inset shows an outward $K^+$ current recorded at +60 mV and tail current recorded at −30 mV in the absence and presence of HMR-1556 (1 μM). Current density–voltage (E) and tail current density–voltage (F) relationships for dofetilide-resistant current (Dofe-insensitive) recorded in cells trans- fected or not with Pitx2c. In (F), continuous lines represent the fit of a Boltzmann function to the data. In (E) and (F), each point represents mean ± SEM of more than 15 cells from at least 4 independent batches in each group. In (B), (E), and (F), *$P$, 0.05 vs. cells non-transfected with Pitx2c.

Table 2  Electrophysiological properties of $I_{Kr}$ and $I_{Ks}$ recorded in HL-1 cells in the absence and presence of Pitx2c

|        | $I_{Kr}$ | $I_{Ks}$ |
|--------|----------|----------|
|        | $\tau_{act}$ (ms) | $\tau_{deactf}$ (ms) | $\tau_{deacts}$ (ms) | $V_{hact}$ (mV) | $k_{act}$ | $\tau_{act}$ (ms) | $\tau_{deactf}$ (ms) | $\tau_{deacts}$ (ms) | $V_{hact}$ (mV) |
| Pitx2c (−) | 866 ± 197 | 140 ± 25.2 | 905 ± 206 | −7.7 ± 2.0 | 8.0 ± 0.6 | 2800 ± 824 | 341 ± 74 | 2582 ± 595 | 36.5 ± 4.8 | 12.0 ± 2.5 |
| Pitx2c (+) | 780 ± 216 | 211 ± 54.5 | 1037 ± 197 | −8.6 ± 3.2 | 9.3 ± 2.2 | 856 ± 164* | 117 ± 23* | 1522 ± 359* | 25.6 ± 2.6* | 13.1 ± 2.0 |

Each value represents mean ± SEM of more than 15 cells from at least 4 independent batches in each group. $\tau_{act}$ time constants of $I_{Kr}$ and $I_{Ks}$ activation measured at 0 and +60 mV, respectively; $\tau_{deactf}$ and $\tau_{deacts}$, fast and slow time constants of $I_{Kr}$ and $I_{Ks}$ deactivation measured in the tail currents recorded at −60 and −30 mV, respectively, after pulses to +60 mV; $V_{hact}$ and $k_{act}$, midpoint and slope values of the activation curves for $I_{Kr}$ and $I_{Ks}$. *$P$, < 0.05 vs. cells non-transfected with Pitx2c.
It has been previously demonstrated that Pitx2c binds the DNA sequence TAATCC by means of a lysine residue located in position 9 of helix 3 in its homeodomain. Therefore, the consensus sequence TAATCC was searched near the transcription start site (TSS) of KCNQ1 and KCNE1 genes. In KCNQ1, a single consensus sequence was found at a distance of ~2 kb upstream of the TSS (Figure 5A). In KCNE1, two consensus sequences were found at ~1.4 and ~1.2 kb upstream of the TSS (Figure 5C). To determine whether Pitx2c may increase KCNQ1 and KCNE1 transcription, luciferase activity assays were conducted in CHO cells. A construction carrying the KCNQ1 proximal promoter and including the Pitx2c-binding site (~2186/0) was cloned in the pLightSwitch_Prom luciferase expression reporter vector (KCNQ1_WT) and transiently transfected. The same construction but with the Pitx2c-binding site mutated to AAAAAA (KCNQ1_mut) was also tested (Figure 5A). Cotransfection of Pitx2c significantly increased luciferase activity in cells transfected with KCNQ1_WT (Figure 5B), indicating that Pitx2c increased KCNQ1 transcription. However, Pitx2c did not increase luciferase activity in cells transfected with KCNQ1_mut, revealing that the presence of the consensus sequence TAATCC was critical for the Pitx2c effect on KCNQ1 transcription (Figure 5A). To determine the putative interaction of Pitx2c with KCNE1, a construction carrying the KCNE1 proximal promoter and including both Pitx2c-binding sites (~1429/+16) was cloned in the PGL3-Basic luciferase expression reporter vector (F2) and transfected in CHO cells. A shorter construction (F7) without the Pitx2c-binding sites (~311/+16) was also tested (Figure 5C). Pitx2c produced a 1.8-fold increase in luciferase activity in cells transfected with F2, demonstrating that Pitx2c was able to increase KCNE1 transcription.

Conversely, it did not increase luciferase activity in cells transfected with F7, indicating that the presence of the TAATCC sequence is critical for the Pitx2c effects on KCNE1 transcription. It is known that atrial natriuretic peptide (ANP) encoded by the NPPA gene is one of the main targets of Pitx2c and is frequently used to evaluate transcriptional activity of Pitx2c as the gold standard. Pitx2c increased NPPA transcription by interacting with several Pitx2c-binding sites within its promoter region. For this reason, CHO cells expressing the pLightSwitch_prom luciferase expression reporter vector carrying NPPA minimal promoter were used as a positive control. As expected, in both groups of experiments, transfection of Pitx2c significantly increased luciferase activity generated by the NPPA promoter (Figure 5B and D).

### 3.4 Pitx2c decreases \( I_{Ca,L} \) density in HL-1 cells

In HL-1 cells, both \( I_{Ca,L} \) and \( I_{Ca,T} \) can be recorded. To determine the effects of Pitx2c on \( I_{Ca,L} \), currents were recorded using Ba as the charge carrier (\( I_{ba} \)) and the holding potential was fixed to ~30 mV to fully inactivate \( I_{Ca,T} \). Under these conditions, ~80% of the cells exhibited a measurable \( I_{ba} \) that reached its maximum value at +20 mV when 500 ms pulses to potentials ranging between ~40 and ~50 mV were applied (\( n = 49 \) (Figure 6A)). Transfection of Pitx2c decreased the proportion of cells with measurable current to ~57% \(( P < 0.01, n = 53)\), without modifying cell capacitance \((40.7 \pm 8.3 \text{ pF}; P > 0.05)\). Pitx2c markedly decreased \( I_{ba} \) density at potentials between 0 and +50 mV \(( n = 23, P < 0.05)\) (Figure 6A and B). Moreover, it shifted the midpoint of the inactivation curve to more positive potentials (Figure 6C) and slowed recovery from inactivation (Figure 6D and Table 3). Conversely, it did not modify the voltage dependence of...
**Figure 5** Pitx2c increased KCNQ1 and KCNE1 transcription. (A) Schematic diagram of the human KCNQ1 region proximal to the TSS (−2186/0). (B) Normalized luciferase activity in CHO cells expressing the pLightSwitch_Prom luciferase expression reporter vector carrying KCNQ1_WT, KCNQ1_mut, or the NPPA minimal promoter cotransfected or not with Pitx2c. (C) Schematic diagram of the human KCNE1 region proximal to the TSS. In (A) and (C), the TAATCC sequences are represented by black boxes. (D) Normalized luciferase activity in CHO cells expressing the PGL3-Basic luciferase expression reporter vector carrying F2 or F7 cotransfected or not with Pitx2c. In (B) and (D), each bar represents the mean ± SEM of five independent batches of cells for each group. **P < 0.01 vs. non-transfected cells. One-way ANOVA followed by Newman–Keuls test (B and D).

**Figure 6** Pitx2c decreases I_{Ba} in HL-1 cells. Current traces recorded by applying the protocol shown at the top (A) and current density–voltage relationships (B) for I_{Ba} recorded in HL-1 cells transfected or not with Pitx2c. (C) Normalized steady-state I_{Ba} activation and inactivation curves. In (C), solid lines represent the fit of a Boltzmann function to the data. (D) Recovery from inactivation data for I_{Ba} recorded by applying the protocol shown in the inset in HL-1 cells transfected or not with Pitx2c. The solid lines represent the fit of a monoexponential function to the data. Each point represents mean ± SEM of more than 15 cells from at least 5 independent batches in each group. In (B), *P < 0.05 vs. non-transfected cells. ANOVA followed by Newman–Keuls test and multilevel mixed-effects model (B–D).
 activation (Figure 6C), kinetics of activation and inactivation, and the reversal potential (Table 3). Additionally, western blot analyses demonstrated that Pitx2c did not significantly modify Cav1.2 expression (Figure 7A and B), suggesting that Pitx2c does not regulate CACNA1C transcription, despite the presence of a TAATCC sequence ~100 bp downstream of the TSS of CACNA1C. In contrast, neither CACNB2 nor CACNA2D minimal promoters exhibit the consensus sequence for Pitx2c binding. From this result, we surmised that the inhibition could be due to post-translational modifications. As shown earlier, Pitx2c activates the NPPA promoter. Moreover, it was previously described that ANP inhibits ICa,L in atrial myocytes through a mechanism involving an increase in cyclic GMP levels. 24 Furthermore, Pitx2c significantly increased ANP concentration in the culture medium of HL-1 cells (n = 6, P < 0.05) (Figure 7C). Therefore, we hypothesized that the effects of Pitx2c on ICa,L could be mediated by ANP. To preliminarily answer this question, the effects of Pitx2c transfection on IBa were analysed in HL-1 cells incubated or not with the ANP type A receptor antagonist A71915 (0.5 μM) for 24 h. 25 As shown in Figure 7D, incubation with the antagonist completely prevented the inhibitory action induced by Pitx2c on IBa, suggesting that ANP was responsible for this effect. It was described that isoproterenol-induced ICa,L potentiation is larger in atrial myocytes from rats with heart failure than in control myocytes. This result was associated to higher levels of ANP in heart failure animals and due to an ANP-induced effect on cGMP-dependent proteins.11 However, recent evidences in mouse models suggest a role of Pitx2c in adult atria and, specifically, in the events that form part of the remodelling process associated with AF. 15,12,15 Chinchilla et al. 11 showed that Pitx2c mRNA expression was markedly decreased in right and left atrial appendages obtained from patients with AF when compared with those obtained from SR patients. More recently, it was demonstrated that Pitx2c expression increased in atrial tissue from patients in AF at the time of sample collection. 10 These discrepancies could be attributed to patient characteristics and AF progression. AF patients included in these two studies were not specifically classified as CAF patients, and the disease duration was not mentioned. More importantly, both studies were conducted using whole atrial appendage samples. It is known that the presence of fibroblasts and other non-myocyte cells in whole atrial samples may complicate the interpretation of gene expression analysis in myocytes. 16 To overcome these issues, we conducted qPCR experiments in isolated myocytes from atrial appendages obtained from patients in SR and patients diagnosed with CAF (>6 month at the time of surgery). Under these conditions, our qPCR experiments showed that Pitx2c mRNA expression was approximately two-fold greater in CAF than in SR myocytes.

4.2 Pitx2c modulates IKs and ICa,L
Up-regulation of IKs and down-regulation of ICa,L are hallmarks of CAF-induced electrical remodelling in humans and critically contribute to the abbreviation of APD and atrial refractory period. 3–5,7,16 Our results confirmed previous observations 5,7 and showed that in atrial myocytes from CAF patients, IKs density was approximately two-fold higher, the activation kinetics was significantly faster, and the conductance curve was shifted to more hyperpolarized potentials when compared with SR myocytes. In contrast, in CAF myocytes, ICa,L density was reduced and the inactivation curves were shifted to more positive potentials in comparison to SR myocytes. Importantly, in human atrial myocytes, increase in Pitx2c expression was positively and inversely correlated with the IKs increase and ICa,L decrease, respectively, suggesting that Pitx2c could be involved in the changes in these currents that characterize CAF-induced electrical remodelling. On the basis of our results, Pitx2c deletion should lead to APD prolongation. However, in mice heterozygous for Pitx2c 15 and in atrial-specific Pitx2c-

Table 3  Electrophysiological properties of IBa recorded in HL-1 cells in the absence and presence of Pitx2c

| IBa | Erev (mV) | τact (ms) | Vact (mV) | kact | τinact (ms) | Vmax (mV) | kmax | τrec (ms) |
|-----|----------|-----------|-----------|------|------------|----------|------|----------|
| Pitx2c (−) | 75.3 ± 1.8 | 1.7 ± 0.1 | 9.2 ± 0.9 | 5.9 ± 0.3 | 167 ± 8.8 | −15.7 ± 6.0 | 10.6 ± 1.9 | 314 ± 14 |
| Pitx2c (+) | 74.0 ± 2.2 | 2.1 ± 0.1 | 10.6 ± 1.0 | 5.8 ± 0.3 | 175 ± 14.5 | −6.6 ± 0.9* | 10.0 ± 0.8 | 480 ± 44* |

Each value represents mean ± SEM of more than 15 experiments in each group.
Erev, reversal potential; τact, time constant of IBa activation measured at +20 mV; τinact, time constant of IBa inactivation measured at +20 mV; τrec, time constant of IBa reactivation measured at −30 mV; Vact and Vmax, midpoint and slope values of the conductance–voltage curves; kact and kmax, midpoint and slope values of the inactivation curves.

* P < 0.05 vs. cells non-transfected with Pitx2c.
deficient mice, atrial APD was shortened and unmodified, respectively, compared with wild-type animals. It is known that atrial action potential repolarization of mouse and human is different. Indeed, mouse atrial myocytes lack functional Kv11.1 + miRP1 and Kv7.1 + minK channels and, thus, $I_{Kr}$ and $I_{Ks}$, respectively, cannot be recorded. Therefore, changes in APD secondary to modulation of Kv7.1 + minK channels would not be expected in mouse. In contrast, repolarizing sustained $K^+$ currents in mice are generated by Kv1.5 and Kv2.1 channels. Therefore, it is possible that Pitx2c-deficient mice display altered expression of Kv2.1 channels or other unknown factors, which may compensate the effects on APD secondary to the $I_{Ca,L}$ increase upon Pitx2c deletion.

To elucidate the molecular mechanism underlying Pitx2c-induced effects, HL-1 cells were used as experimental model. These cells are mouse atrial-derived cells that can be maintained in culture and have been previously used to test Pitx2c actions. Depending on the main outward $K^+$ current recorded, three groups of HL-1 cells were established: $I_{Kr}$-predominant, $I_{Ks}$-predominant, and intermediate (with

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**Figure 7** Pitx2c does not modify Cav1.2 expression in HL-1 cells. (A) Western blot images showing Cav1.2 expression in HL-1 cells transfected (right lane) or not (left lane) with Pitx2c. GAPDH (bottom) was used as loading control. (B) Densitometric analysis of the western blots. Results are presented as mean ± SEM of four batches of cells for each group. (C) ANP concentration measured in the culture medium of HL-1 cells transfected or not with Pitx2c. Bars represent the mean ± SEM of three batches of cells for each group. (D) Current density–voltage relationships for $I_{Ba}$ recorded in HL-1 cells transfected with Pitx2c in the absence or presence of the ANP type A receptor antagonist A71915 (0.5 μM). Each point represents the mean ± SEM of more than 12 cells from at least 3 independent batches in each group. In (C) and (D), $^* P < 0.05$ vs. cells non-transfected with Pitx2c, $^\# P < 0.05$ vs. cells non-transfected with Pitx2c. Unpaired t-test (B and C), ANOVA followed by Newman–Keuls test (D–F, and inset), and multilevel mixed-effects model (D).
Expression of Pitx2c in HL-1 cells increased the percentage of cells without measurable \( I_{\text{Ca,L}} \) reduced the current density, and shifted the voltage dependence of inactivation to positive potentials. Cav1.2 expression was not modified by Pitx2c, even when \( \text{CACNA1C} \) minimal promoter exhibits a TAATCC sequence. Therefore, it can be speculated that the presence of this sequence, 100 bp downstream of the TSS, is not enough for promoting an effect of Pitx2c on \( \text{CACNA1C} \) transcription. This result suggests that the Pitx2c-induced \( I_{\text{Ca,L}} \) inhibition was not due to a direct regulation of channel expression, but involved a post-translational modification of channel function. We surmised that the effect could be produced by a mediator which is a Pitx2c target and is able to reduce \( \text{Ca}^{2+} \) channel function. Pitx2c can target multiple genes, \(^8\) however, we focused on the ANP as it meets both conditions: it is one of the main targets of Pitx2c \(^21\) and inhibits \( I_{\text{Ca,L}} \). \(^24\) As we and others demonstrate, Pitx2c markedly activates NPPA promoter, increases \( \text{NPPA} \) gene transcription, \(^21\) and augments ANP production. In contrast, it has been described that ANP inhibits atrial \( I_{\text{Ca,L}} \) via the accumulation of cGMP, which affects the phosphorylation/dephosphorylation balance of the \( \text{Ca}^{2+} \) channel. \(^24\) Our results demonstrated that the \( I_{\text{Ca,L}} \) density reduction and the shift in the inactivation curve induced by Pitx2c were abolished by the incubation with an antagonist of the ANP receptor (A771915). Therefore, we suggest that Pitx2c regulates \( I_{\text{Ca,L}} \) by a post-translational mechanism involving ANP.

### 4.3 Study limitations

All samples came from right atrial appendages, which could not be representative of the rest of the atria. Furthermore, Pitx2c expression is higher in left than in right atria, \(^8\) and thus it cannot be ruled out that the increase in Pitx2c is more relevant for left atrial myocytes. In this case, it is possible that Pitx2c would play a role in the exacerbation of electrophysiological heterogeneity that characterizes CAF. \(^3\) Pitx2c expression and ion channel function could be influenced by age, sex, pharmacological treatment, and/or underlying cardiac diseases of the patients. Interestingly, \( I_{\text{Ca,L}} \) density and plasma ANP levels are influenced by the clinical history of the donors, in such a way that the ANP levels are higher and \( I_{\text{Ca,L}} \) density is smaller in patients with mitral valve disease or decreased left ventricular function. \(^34\) In our sample, the proportion of patients with ischaemic heart disease alone and combined with valvular cardiomyopathy was equally distributed in both groups. However, more patients were in decompensated heart failure [New York Heart Association (NYHA) III and IV] in the CAF group than in the SR group. Interestingly, multiple linear regression analysis confirmed that CAF influenced both \( I_{\text{Ca,L}} \) and \( I_{\text{Ks}} \) densities, whereas the NYHA class and the presence of mitral valve disease did not (Supplementary material online, Tables S2 and S3).

### 5. Conclusions

We demonstrate that Pitx2c expression increases in human atrial myocytes from CAF patients, and this increase correlates with the \( I_{\text{Ks}} \) increase and \( I_{\text{Ca,L}} \) decrease that characterize CAF-induced electrical remodelling. We propose that through this mechanism Pitx2c is involved in the APD and refractory period shortening that enhances arrhythmia recurrence and maintenance.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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### Conflict of interests

none declared.

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### References

1. Ferrari R, Bertini M, Blomstrom-Lundqvist C, Dobrev D, Kirchhof P, Pappone C, Ravens U, Tamargo J, Tavazzi L, Vicedomini GG. An update on atrial fibrillation in 2014: from pathophysiology to treatment. Int J Cardiol 2015;203:22–29.
2. Prystowsky EN, Camm J, Lip GY, Allessie M, Bergmann JF, Breithardt G, Brugada J, Crijns H, Elinor PT, Mark D, Naccarelli G, Packing D, Tamargo J. The impact of new and emerging clinical data on treatment strategies for atrial fibrillation. J Cardiovasc Electrophysiol 2010;21:946–958.
3. Nattel S, Harada M. Atrial remodeling and atrial fibrillation: recent advances and translational perspectives. J Am Coll Cardiol 2014;63:2335–2345.
4. Van Wagener DR, Pond AL, Lamorgese M, Rossie SS, McCarthy PM, Nerbom J. Atrial L-type Ca\(^{2+}\) currents and human atrial fibrillation. Circ Res 1999;85:428–436.
5. Caballero R, de la Fuente MG, Gómez R, Barana A, Amorós I, Dolz-Gaitán P, Oslona L, Almendral J, Atienza F, Fernández-Avilés F, Pita A, Rodríguez-Roda J, Pinto A, Tamargo J, Delpón E. In humans, chronic atrial fibrillation decreases the transient outward current and ultrarapid component of the delayed rectifier current differentially on each atria and increases the slow component of the delayed rectifier current in both. J Am Coll Cardiol 2010;55:2346–2354.
6. Voigt N, Trausch A, Knaut M, Matschke K, Varras A, Van Wagener DR, Nattel S, Ravens U, Dobrev D. Left-to-right atrial inward rectifier potassium current gradients in patients with paroxysmal versus chronic atrial fibrillation. Circ Arrhythm Electrophysiol 2010;3:472–480.
7. González de la Fuente M, Barana A, Gómez R, Amorós I, Dolz-Gaitán P, Scarcisón S, Atienza F, Pita A, Pinto A, Fernández-Avilés F, Caballero R, Tamargo J, Delpón E. Chronic atrial fibrillation up-regulates β1-adrenoceptors affecting repolarizing currents and action potential duration. Cardiovasc Res 2013;97:379–388.
8. Franco D, Christoffels VM, Campione M. Homeobox transcription factor Pitx2: the rise and fall of a symmetry gene in cardiogenesis and arrhythmogenesis. Trends Cardiovasc Med 2014;24:23–31.
9. Guddbjartsson DF, Arnar DO, Helgadottir A, Gretarsdottir S, Holm H, Sigurdsson A, Jonassonst A, Baker A, Thorleifsson G, Kristjansson K, Palsson A, Blondal T, Sulem P, Backman VM, Hardanson GA, Palsdottir E, Helgason A, Sigurjonsdottir R, Sverrisson JT, Kostulas K, Ng MC, Baum L, So WY, Wong KS, Chan JC, Friels KL, Greenberg SM, Sale M, Kelly P, MacRae CA, Smith EE, Rosand J, Hillert J, Ma RC, Ellinor PT, Thorgeirsson G, Gulcher JR, Kongs A, Thorsteinsdottir U, Stefansson K. Variants conferring risk of atrial fibrillation on chromosome 4q25. Nature 2007;448:353–357.
10. Gore-Panter SR, Hsu J, Hanna P, Gillivon AM, Petterson G, Newton DW, Moravec CS, Van Wagener DR, Chung MK, Barnard J, Smith JD. Atrial fibrillation associated chromosome 4q25 variants are not associated with Pitx2c expression in human adult left atrial appendages. PLoS ONE 2014;9:e86245.
11. Chinchilla A, Daimi H, Lozano-Velasco E, Dominguez JN, Caballero R, Delpón E, Tamargo J, Cincas J, Hove-Madsen L, Aranega AE, Franco D. PITX2 insufficiency leads
to atrial electrical and structural remodeling linked to arrhythmogenesis. Circ Cardiovasc Genet 2011;4:269–279.

12. Yao Y, Zhang M, Li L, Bai Y, Zhou Y, Moon AM, Kaminski HJ, Martin JF. Pitx2, an atrial fibrillation predisposition gene, directly regulates ion transport and intercalated disc genes. Circ Cardiovasc Genet 2014;7:23–32.

13. Wang J, Bai Y, Li N, Ye W, Zhang M, Greene SB, Tao Y, Chen Y, Wehrens XH, Martin JF. Pitx2r–2microRNA pathway that delimits sinoatrial node development and inhibits predisposition to atrial fibrillation. Proc Natl Acad Sci USA 2014;111:9181–9186.

14. Mommersteeg MT, Brown NA, Prall OW, de Gier-de Vries C, Harvey RP, Moorman AF, Christoffels VM. Pitx2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. Circ Res 2007;101:902–909.

15. Kirchhof P, Kahr PC, Kaese S, Piccini I, Vokshi I, Scheld HH, Rotering H, Fortmueller L, Laakmann S, Verheule S, Schotten U, Fabritz L, Brown NA. PITX2c is expressed in the adult left atrium, and reducing Pitx2c expression promotes atrial fibrillation inducibility and complex changes in gene expression. Circ Cardiovasc Genet 2011;4:133–133.

16. Barana A, Matamoros M, Dolz-Gaitán P, Pérez-Hernández M, Amorós I, Núñez M, Sacristán S, Pedraza Á, Pinto Á, Fernández-Avilés F, Tamargo J, Delpón E, Caballero R. Chronic atrial fibrillation increases microRNA-21 in human atrial myocytes decreasing L-type calcium current. Circ Arrhythm Electrophysiol 2014;7:861–868.

17. Gómez R, Caballero R, Barana A, Amorós I, De Palm SH, Matamoros M, Núñez M, Pérez-Hernández M, Iriege I, Rayma J, Delpón E. Structural basis of drugs that increase cardiac inward rectifier KIR2.1 currents. Cardiovasc Res 2014;104:337–346.

18. Clycova WC, Lansen NA Jr, Stallworth BS, Delcarpio JB, Babinski A, Izoo NJ Jr. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci USA 1998;95:2979–2984.

19. Tamargo J, Caballero R, Gómez R, Valenzuela C, Delpón E. Pharmacology of cardiac potassium channels. Cardiovasc Res 2003;62:39–33.

20. Mustapha Z, Pang L, Natalle S. Characterization of the cardiac KCNE1 gene promoter. Cardiovasc Res 2007;73:82–91.

21. Amendt BA, Sutherland LB, Semina EV, Russo AF. The molecular basis of Rieger syndrome. Analysis of Pitx2 homeodomain protein activities. J Biol Chem 1998;273:20066–20072.

22. Ganga M, Espinoza MM, Cox CJ, Morton L, Hijaz TA, Lee Y, Amendt BA. PITX2 isoform-specific regulation of atrial natriuretic factor expression: synergy and repression with Nkx2.5. J Biol Chem 2003;278:22437–22445.

23. Toro R, Saadi I, Kuburac A, Nemer M, Russo AF. Cell-specific activation of the atrial natriuretic factor promoter by PITX2 and MEF2A. J Biol Chem 2004;279:52087–52094.

24. Boixel C, Gonzalez W, Louedec L, Hatem SN. Mechanisms of L-type Ca(2+) currents in mouse atrial cells. Circ Res 2000;87:345–358.

25. Parvez B, Vaglio J, Rowan S, Muñoz-Planillo R, Zipes D, Kucerova G, Stubblefield T, Carter S, Roden D, Darbar D. Symptomatic response to antiarrhythmic drug therapy is modulated by a common single nucleotide polymorphism in atrial fibrillation. J Am Coll Cardiol 2012;60:539–545.

26. Aguirre LA, Alonso ME, Báñez-Careaga C, Rollán I, Arios C, Fernández-Miñana I, López-Jiménez E, Aráneaga A, Gómez-Skarmeta JF, Franco D, Manzanares J. Long-range regulatory interactions at the 4q25 atrial fibrillation risk locus involve PITX2c and EN1/PITX2. BMC Biol 2015;13:26.

27. Brundel BJ, van Gelder IC, Henning RH, Tielemans RG, Tunenburg AE, Wietzes M, Grandjean JG, Van Gilst WH, Crijs HS. Iden channel remodeling is related to intraoperative atrial effective refractory periods in patients with paroxysmal and persistent atrial fibrillation. Circulation 2001;103:684–690.

28. Lai LP, Su MJ, Lin JL, Lin FY, Tsai CH, Chen YS, Tseng YZ, Lien WP, Huang SK. Changes in the mRNA levels of delayed rectifier potassium channels in human atrial fibrillation. Cardiology 1999;92:248–255.

29. Dinnian S, Boixel C, Jun C, Hulot JS, Coulombe A, Rücker-Martin C, Bennet N, Le Grand B, Slama M, Mercader J, Hatem SN. Downregulation of the calcium current in human right atrial myocytes from patients in sinus rhythm but with a high risk of atrial fibrillation. Eur Heart J 2008;29:1190–1197.