TRPM7-Mediated Ca\(^{2+}\) Signals Confer Fibrogenesis in Human Atrial Fibrillation

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Rationale: Cardiac fibrosis contributes to pathogenesis of atrial fibrillation (AF), which is the most commonly sustained arrhythmia and a major cause of morbidity and mortality. Although it has been suggested that Ca\(^{2+}\) signals are involved in fibrosis promotion, the molecular basis of Ca\(^{2+}\) signaling mechanisms and how Ca\(^{2+}\) signals contribute to fibrogenesis remain unknown.

Objective: To determine the molecular mechanisms of Ca\(^{2+}\)-permeable channel(s) in human atrial fibroblasts, and to investigate how Ca\(^{2+}\) signals contribute to fibrogenesis in human AF.

Methods and Results: We demonstrate that the transient receptor potential (TRP) melastatin related 7 (TRPM7) is the molecular basis of the major Ca\(^{2+}\)-permeable channel in human atrial fibroblasts. Endogenous TRPM7 currents in atrial fibroblasts resemble the biophysical and pharmacological properties of heterologous expressed TRPM7. Knocking down TRPM7 by small hairpin RNA largely eliminates TRPM7 current and Ca\(^{2+}\) influx in atrial fibroblasts. More importantly, atrial fibroblasts from AF patients show a striking upregulation of both TRPM7 currents and Ca\(^{2+}\) influx and are more prone to myofibroblast differentiation, presumably attributable to the enhanced expression of TRPM7. TRPM7 small hairpin RNA markedly reduced basal AF fibroblast differentiation. Transforming growth factor (TGF)-\(\beta\)1, the major stimulator of atrial fibrosis, requires TRPM7-mediated Ca\(^{2+}\) signal for its effect on fibroblast proliferation and differentiation. Furthermore, TGF-\(\beta\)1-induced differentiation of cultured human atrial fibroblasts is well correlated with an increase of TRPM7 expression induced by TGF-\(\beta\)1.

Conclusions: Our results establish that TRPM7 is the major Ca\(^{2+}\)-permeable channel in human atrial fibroblasts and likely plays an essential role in TGF-\(\beta\)1-elicited fibrogenesis in human AF. (Circ Res. 2010;106:992-1003.)

Key Words: atrial fibrillation ■ TRPM7 ■ Ca\(^{2+}\) signaling ■ TGF-\(\beta\)1 ■ fibrogenesis

Cardiac fibrosis is a detrimental factor that results in abnormalities in cardiac conduction, stiffening of the ventricular walls, and loss of contractility, thereby contributing to a variety of heart diseases, including hypertrophy, heart failure, and arrhythmia. 1 Atrial fibrillation (AF) is the most commonly sustained clinical arrhythmia and is a major cause of morbidity and mortality. 2 Recent studies have demonstrated that structural remodeling as a result of fibrosis is one of the fundamental mechanisms underlying the perpetuation of AF, and contributes synergistically with electric and contractile remodeling to AF substrate. 2-6 Atrial fibrosis is a hallmark feature of arrhythmogenic structural remodeling in clinical AF. 7 Increased amount of fibrosis occurs in not only AF patients associated with other cardiac diseases, 8,9 but also in those with lone AF. 8,10 There is a positive correlation between the amounts of fibrosis and the persistence of AF, 9 suggesting that atrial fibrosis results in structural remodeling to promote AF. The causal importance of fibrosis in occurrence and persistence of AF is strongly supported by studies in animal models in which selective atrial fibrosis caused by overexpression of transforming growth factor (TGF)-\(\beta\)1 increases AF vulnerability. 11,12 Thus, understanding the mechanism of fibrogenesis is important in developing new therapeutic strategies aimed at preventing or reversing structural remodeling in AF.

Fibrosis represents excessive deposition of extracellular matrix (ECM) proteins synthesized by cardiac fibroblasts or myofibroblasts upon stimulation. 13,14 Myofibroblasts are not found in normal cardiac tissue. 15 In response to pathological stimuli, such as myocardial injury, oxidative stress, mechanical stretch, autocrine-paracrine mediators, and inflammatory stimuli, fibroblasts proliferate, migrate and undergo phenotypic changes and differentiate into myofibroblasts. 14,16 Myofibroblasts might also be derived from endothelial cells in fibrotic heart. 17 By producing growth factors, cytokines, chemokines, ECM proteins, and proteases, 18,19 myofibroblasts play a pivotal role in fibrogenesis. Inhibition of myofibroblast differentiation may prove to be a common and effective approach to attenuate cardiac fibrosis, regardless of
the initial stimuli. However, little is known about the molecular mechanisms underlying fibroblast differentiation.

Ca$^{2+}$ signals are essential for diverse cellular functions including differentiation, gene expression, cell proliferation, growth and death. In cardiac fibroblasts, several lines of evidence suggest that Ca$^{2+}$ entry is essential for fibroblast functions. Chelating external Ca$^{2+}$ by EGTA prevents substance P induced proliferation of cultured rat cardiac fibroblasts. Eliminating external Ca$^{2+}$ to prevent Ca$^{2+}$ influx attenuates H$_2$O$_2$-induced interleukin-6 mRNA expression. In mechanical-stimulation or stretch-induced membrane potential studies, both Ca$^{2+}$-entry and Ca$^{2+}$-release are involved in generation of MIP (mechanically induced potential). A stretch induced current can be blocked by gadolinium (Gd$^{3+}$), a nonselective cation channel blocker. Recently, it has been shown that intracellular Ca$^{2+}$ influx contributes to angiotensin II–induced proliferation of cardiac fibroblasts. In an in vivo study, mibebradil, a Ca$^{2+}$ channel blocker, significantly reduced collagen production and fibroblast differentiation in rats treated with angiotensin II or aldosterone. These studies indicate that Ca$^{2+}$ entry through Ca$^{2+}$-permeable ion channels is essential for gene expression and fibrosis promotion. Therefore, understanding the molecular basis of Ca$^{2+}$-permeable channels is crucial for elucidating the molecular mechanisms of proliferation and differentiation of cardiac fibroblasts.

Fibroblasts have been reported to have depolarized resting membrane potentials. Measured by standard microelectrode techniques in multicellular tissues, the resting membrane potential of atrial fibroblasts is between -31 to -16 mV. There is no evidence indicating that functional voltage-gated calcium channels exist in the fibroblast. The transient receptor potential (TRP) channels are responsible for Ca$^{2+}$ influx in various nonexcitable and excitable cells. TRP channels are non–voltage-gated but are activated by a variety of different stimuli including receptor activation, oxidative stress, mechanical stretch, cell metabolites, and thermal or sensory stimuli. TRP channels also enable individual cells to sense changes in their local environment. Given that cardiac fibroblasts encounter a variety of pathological conditions, it seems that TRP channels could be the potential candidates for Ca$^{2+}$ signaling in cardiac fibroblasts.

To understand Ca$^{2+}$ signaling mechanisms in cardiac fibroblasts and potential roles of Ca$^{2+}$ signals in the cardiac fibrogenesis cascade, we investigated the molecular basis of Ca$^{2+}$-permeable channels in human atrial fibroblasts isolated from AF patients and normal sinus rhythm (NSR) patients, and studied how Ca$^{2+}$ influx contributes to TGF-β1–induced fibrogenesis process. We discovered that TRP melanostatin related (TRPM)7, a Ca$^{2+}$-permeable cation channel which also possesses protein kinase function, underlies the Ca$^{2+}$ signaling mechanism in human atrial fibroblasts. TRPM7 has been reported to play a vital role in embryonic development and anoxic cell death. We found that TRPM7 is the major Ca$^{2+}$-permeable channel in human atrial fibroblasts. Knocking down TRPM7 by short hairpin (sh)RNA largely eliminates the endogenous TRPM7 currents as well as Ca$^{2+}$ influx in atrial fibroblasts. Strikingly, TRPM7 and TRPM7-mediated Ca$^{2+}$ influx are drastically up-regulated in fibroblasts from AF patients. In addition, inhibition of TRPM7-mediated Ca$^{2+}$ influx renders fibroblasts less sensitive to TGF-β1–induced proliferation and differentiation, indicating that TRPM7-mediated Ca$^{2+}$ influx is involved in atrial fibroblast differentiation. In parallel with myofibroblast differentiation, TRPM7 is upregulated by TGF-β1 in cultured fibroblasts. Taken together, our results indicate that TRPM7-mediated Ca$^{2+}$ influx contributes to fibroblast differentiation and may mediate the fibrogenic effect of TGF-β1. This study suggests that inhibition of TRPM7 may prove to be an effective approach to reduce fibroblast differentiation and therefore attenuate fibrosis during human AF.

### Methods

**Human Cardiac Tissue Sample Collection and Fibroblasts Isolation**

Myocardial samples of the right atria were collected during cardiac surgery. All procedures involving human tissue use were approved by the institutional review boards of the University of Connecticut Health Center. Atrial fibroblasts were isolated by collagenase (see details in the Online Data Supplement, available at http://circres.ahajournals.org). Freshly isolated fibroblasts from AF and NSR patients were used for patch-clamp experiment to compare the TRPM7 current amplitude.

**Molecular Biology**

RT-PCR was performed as we previously reported by using the primers shown in Online Table II. TRPM7-specific shRNAs were designed and constructed into lentiviral delivery system. The efficiency of the shRNAs was tested in the TRPM7 overexpression HEK-293 cells. The one with higher efficiency was used in the experiments (see the Online Data Supplement).

**Electrophysiology**

Whole-cell and single-channel currents were recorded as we reported previously. Internal Mg$^{2+}$-free solution was used for whole-cell current recording and inside-out configuration was applied for single channel current recording. For details, see the Online Data Supplement.

**Ratio Ca$^{2+}$ Imaging Experiments**

Ca$^{2+}$ influx was measured using ratio Ca$^{2+}$ imaging system and analyzed using NIS-Elements (Nikon). The F340/F380 ratio in the presence of 20 mmol/L Ca$^{2+}$ was normalized to that of 1 µmol/L ionomycin elicited Ca$^{2+}$ signal (see details in the Online Data Supplement). Ca$^{2+}$ influx was also measured with/without store
depletion by 2 μmol/L thapsigargin in some experiments as specified in the text.

**Immunostaining**

Fibroblasts were fixed by 4% formaldehyde and immunostained with α-smooth muscle actin (α-SMA) antibody. The immunostained cells were analyzed using a Zeiss LSM 510 confocal microscope. Details are described in the Online Data Supplement.

**Western Blot**

The protein expression levels of TRPM7 and α-SMA were evaluated by Western blot experiments. GAPDH was used as a loading control (see the Online Data Supplement).

**Data Analysis**

Pooled data are presented as means±SEM. Dose–response curves were fitted by an equation of the form \( E = E_{\text{max}} \left( \frac{1}{1 + \left( \frac{C}{C_{50}} \right)^n} \right) \), where \( E \) is the effect at concentration \( C \), \( E_{\text{max}} \) is maximal effect, \( C_{50} \) is the concentration for half-maximal effect, and \( n \) is the Hill coefficient. Statistical comparisons were made using 2-way ANOVA and 2-tailed \( t \) test with Bonferroni correction. Fisher’s exact test was used for analysis of percentage differences for database of the patients. \( P<0.05 \) indicated statistical significance.

**Results**

**TRPM7 Is the Major Ca\(^{2+}\)-Permeable Channel in Human Atrial Fibroblasts**

To understand which Ca\(^{2+}\) permeable channel is responsible for Ca\(^{2+}\) entry in cardiac fibroblasts, we first investigated the expression of Ca\(^{2+}\)-permeable TRP channels. Among the 27 human TRP channel genes,\(^{33} \) we found that TRPM7 is abundantly expressed in human atrial fibroblasts (Online Figure I). To study whether TRPM7 constitutes functional channels in the fibroblasts, we applied voltage-clamp to investigate the current characteristics. As shown in Figure 1, a TRPM7-like current was readily elicited by a voltage ramp protocol ranging from -120 to +100 mV. The current–voltage relationship with small inward current and strong outward rectification (Figure 1A) is similar to the typical characteristics of the recombinant TRPM7.\(^{36,37} \) Heterologous expressed TRPM7 exhibits several unique features, including single channel conductance, potentiation by low external pH, inhibition by low concentrations of 2-aminoethoxydiphenyl borate (2-APB) but potentiation by high concentrations of 2-APB.\(^{43,44} \) We then tested whether the endogenous TRPM7-like current in cardiac fibroblasts has similar properties.
single channel conductance of TRPM7-like current (Figure 1B) is similar to that of TRPM7 overexpressed in CHO cells.43 TRPM7-like current was significantly potentiated by low extracellular pH (Figure 1C) with an EC50 of 3.9 pH unit (Online Figure II, B). Like TRPM7, TRPM7-like current was inhibited by low concentrations of 2-APB with an IC50 of 32.1 μmol/L (Figure 1E and 1F). High concentrations of 2-APB enhanced TRPM7-like current (Figure 1E), but the concentration dependence of the effect was not assessed because of poor solubility of 2-APB at high concentrations. All of these features of the endogenous TRPM7-like currents in human atrial fibroblasts are indistinguishable from those of exogenously expressed TRPM7,42,43 suggesting that the TRPM7-like current in the human cardiac fibroblasts is encoded by TRPM7.

To further confirm that TRPM7 constitutes TRPM7-like currents, we applied TRPM7-specific shRNA to the fibroblasts (Figure 2). The efficiency of TRPM7-shRNA was first examined in HEK-293 cells overexpressing human TRPM7 (Figure 2A and 2B). The current amplitude of TRPM7 was decreased by ~70% after TRPM7-shRNA treatment. Similarly, after treatment for 5 days, the current amplitude of TRPM7-like currents in human fibroblasts was significantly reduced by TRPM7-shRNA in comparison with the cells treated with scramble (Scr)-shRNA (Figure 2C and 2D), indicating that TRPM7 is indeed the molecule encoding TRPM7-like currents in human atrial fibroblasts. Based on these findings, we refer to the TRPM7-like currents as “TRPM7” in the following text.

TRPM7 Is Responsible for Ca2+ Entry in Human Atrial Fibroblasts

We next asked whether TRPM7 is responsible for Ca2+ entry in atrial fibroblasts. Using ratio Ca2+ imaging measurements, we found that Ca2+ influx was decreased by 62% after fibroblasts were treated with TRPM7-shRNA for 5 days, indicating that TRPM7 is the major Ca2+-permeable channel in human atrial fibroblasts (Figure 2E and 2F). To elucidate whether there are other Ca2+-permeable channels which may contribute to Ca2+ influx in human atrial fibroblasts, we used the conditions specific for TRPV2,45 TRPV446 or TRPC647,48

Figure 2. TRPM7 underlies the molecular basis of the TRPM7-like channel and that TRPM7-like channel–mediated Ca2+ entry constitutes the major Ca2+ influx in human atrial fibroblasts. A, Typical recordings of TRPM7 overexpressed in HEK-293 cells after treatment with scramble (Scr)-shRNA and TRPM7-shRNA. B, Average current amplitude of TRPM7. TRPM7-shRNA decreased TRPM7 by ~70%. C, Current amplitude of TRPM7-like current recorded in human atrial fibroblasts in a TRPM7-specific shRNA-treated cell (blue) in comparison with that of a Scr-shRNA–treated cell (red). D, Mean current density of TRPM7-like current in TRPM7-shRNA– and Scr-shRNA–treated cells. E, Ca2+ influx mediated by TRPM7-like channel. Ca2+ influx was elicited by 20 mmol/L Ca2+. The amplitude of Ca2+ influx was normalized to the peak Ca2+ influx induced by 1 μmol/L ionomycin (lone). TRPM7-shRNA significantly reduced the Ca2+ influx (blue) in comparison with the Ca2+ influx obtained in scramble shRNA–treated cells (red). F, TRPM7 shRNA reduced Ca2+ influx in human atrial fibroblasts by 62% measured by normalized peak amplitude.
which were inhibited by Mg^{2+} when the pipette solution dialyzed into the cell. Inward and outward currents in C, F, and I were measured at -100 and +100 mV, respectively.

current recording, and tested whether functional currents of these channels are present in human atrial fibroblasts. As shown in Figure 3, TRPV2-like currents could not be elicited by 200 μmol/L 2-APB in human atrial fibroblasts (Figure 3A), whereas 2-APB readily activated TRPV2 overexpressed in HEK-293 cells (Figure 3B and 3C). The conditions used for activating TRPV4 expressed in HEK-293 cells (Figure 3E and 3F) failed to induce any TRPV4-like current in atrial fibroblasts. TRPC6 can be elicited by activation of Gq-like receptor activation (Figure 3H and 3I). However, neither using oleoyl-lysophosphatidic acid to activate EDG7 receptors in fibroblasts by oleoyl-lysophosphatidic acid (LPA) nor including GTPγS in the pipette solution (Online Figure III) was effective in inducing TRPC6-like current. H, Recombinant TRPC6 current activated by carbachol (CCh) in HM1 cells (HEK293 cells with overexpression of type 1 muscarinic receptor). I, Time-dependent changes of inward and outward TRPC6 currents in HM1 cells. Please note that the currents recorded in the beginning (in A, D, and G) were endogenous TRPM7.

TRPM7 Is Markedly Upregulated in Atrial Fibroblasts From AF Patients

It has been shown that fibrosis associated structural remodeling is a major contributor to AF.7 However, the Ca^{2+} entry in human atrial fibroblasts.

TRPC5-like current (data not shown), suggesting that TRPC1 may not be able to form functional heteromeric channels in atrial fibroblasts. Knocking down hTRPC1 by TRPC1 siRNA did not influence Ca^{2+} influx or fibroblast differentiation (Online Figure IV), further indicating that TRPC1 may not be able to contribute Ca^{2+} influx in human atrial fibroblasts.

We next investigated whether store depletion–activated Ca^{2+} channel (I_{CRAC}) may influence Ca^{2+} entry in atrial fibroblasts. As shown in Online Figure V, store depletion by 5 μmol/L thapsigargin did not induce any current, suggesting that store depletion activated channels are not functionally expressed in human atrial fibroblasts. Moreover, Ca^{2+} influx was not changed by thapsigargin, further suggesting that store-depletion does not contribute to Ca^{2+} signals in human atrial fibroblasts. We also investigated whether TRPV2, TRPV4, TRPC1/TRPC4, TRPC6, and I_{CRAC} currents are present in fibroblasts from AF patients, and found that TRPM7 current is the only current that we were able to record in fibroblasts from both NSR and AF patients. Therefore, it appears that endogenous TRPM7 is the major channel which mediates Ca^{2+} entry in human atrial fibroblasts.
signaling mechanism in fibrogenesis is unknown. Our findings that TRPM7 is the major Ca\(^{2+}\)/H\(^{+}\)-permeable channel in human atrial fibroblasts provided us an excellent opportunity to explore how Ca\(^{2+}\)/H\(^{+}\) signals are involved in fibrosis associated AF. The left atrial size of AF patients was similar to that in NSR patients among those enrolled in the current study (Online Table I), although a larger group of AF and NSR patients, including those who were not enrolled in the fibroblast study, showed a larger left atrial size in the AF versus NSR patients (data not shown).

We used freshly isolated fibroblasts from biopsy specimens for current recording. This eliminated cell culture related factors which could interfere with the experimental results. We found that TRPM7 currents recorded from freshly isolated fibroblasts in AF patients were 3- to 5-fold larger than those from NSR patients (Figure 4). Both inward and outward currents were increased significantly. Because measurement of outward current amplitude gives a more accurate assessment, we used outward current amplitude measured at +100 mV for comparison (Figure 4C and 4D). The average TRPM7 current density was 32.5±2.3 pA/pF in fibroblasts from AF patients (88 cells from 7 patients) and 11.1±0.5 pA/pF in fibroblasts from NSR patients (87 cells from 8 patients), respectively. The increase in TRPM7 current density appears to be resulted from increased membrane expression of TRPM7 channel proteins, as the single channel conductance and open probability of TRPM7 were indistinguishable between the NSR fibroblasts and AF fibroblasts (Online Figure VI). We were not able to do western blot experiments to determine TRPM7 protein levels in NSR and AF fibroblasts because of the limited amount of tissues and limited number of fibroblasts. However, we observed that whereas we were able to obtained single channel recordings from more than 95% of patches in the AF fibroblasts, less than 50% of patches from NSR fibroblasts displayed single channel openings, suggesting that membrane protein level of TRPM7 was higher in AF fibroblasts than that in NSR fibroblasts.

To study whether the increased TRPM7 currents result in enhanced Ca\(^{2+}\) influx, we used ratio Ca\(^{2+}\) imaging method to compare the Ca\(^{2+}\) entry through TRPM7 in fibroblasts isolated from AF and NSR patients. Because TRPM7 channels conduct small inward currents and are constitutively active, we used 20 mmol/L Ca\(^{2+}\) to obtain better signal/noise ratio. In addition, cells were briefly exposed to a nominally divalent free solution (DVF) to eliminate any blockade of TRPM7 by external Mg\(^{2+}\). As shown in Figure 5, the peak Ca\(^{2+}\) influx in fibroblasts from AF patients was 10-fold...
greater than Ca\textsuperscript{2+} influx in the fibroblasts from NSR patients. This result not only further indicates that TRPM7 is the major Ca\textsuperscript{2+}-permeable channel responsible for Ca\textsuperscript{2+} influx in human atrial fibroblasts, but also strongly implies that TRPM7-mediated Ca\textsuperscript{2+} signals may play an important role in AF-associated fibrogenesis.

**TRPM7 Is Required for Differentiation of Human Atrial Fibroblasts**

Because the myofibroblast is the major cell type that synthesizes growth factors, cytokines, chemokines, and ECM proteins, and plays a pivotal role in fibrogenesis,\textsuperscript{13,19} we tested whether there was a higher percentage of myofibroblasts in the cells isolated from atrial samples from AF patients than those from NSR patients. As shown in Figure 6, after 24 hours culture, without TGF-\(\beta\)1 treatment, cells from AF patients displayed 44% myofibroblasts, whereas only 16% cells from NSR patients were myofibroblasts. Such data suggest that fibroblasts from AF patients are more prone to differentiation into myofibroblasts. The increased vulnerability to differentiation in the fibroblasts from AF patients might result from an upregulated TRPM7 level. After TGF-\(\beta\)1 (10 ng/mL) induction, 90% of fibroblasts differentiated into myofibroblasts in both NSR and AF groups.

To determine the contribution of TRPM7 to basal differentiation of fibroblasts from AF patients, AF fibroblasts were treated with TRPM7-shRNA or Scr-shRNA in 1% FBS media after atrial fibroblasts attached to the coverslips (by culturing for 12 hours in 10% FBS media) for 72 hours. Fibroblasts were then cultured in 10% FBS media for 24 hour, followed by Ca\textsuperscript{2+} influx and differentiation assessment. As shown in Figure 6C and 6D, TRPM7-shRNA significantly decreased Ca\textsuperscript{2+} influx in TRPM7-shRNA–treated cells, differentiation induced by TGF-\(\beta\)1 was not significantly different from that of cells without TGF-\(\beta\)1 induction (34.4±7.2% and 23.5±4.8% respectively; \(P>0.05\)). In Scr-shRNA–treated cells, the differentiation induced by TGF-\(\beta\)1 (61.0±1.9%) was significantly higher than that in control (Ctl) cells (30.4±6.1%).

**Figure 7. Effects of TRPM7 on fibroblast differentiation and proliferation.** A, Differentiated myofibroblasts detected by immunostaining with antibody against \(\alpha\)-SMA (red). Human atrial fibroblasts were isolated from NSR patients and treated with Scr- or TRPM7-shRNA. Differentiation was induced by 10 ng/mL TGF-\(\beta\)1. Differentiation percentage was obtained by counting the number of differentiated cells (red) divided by the total number of cells as identified by the nuclei (blue). B, Average percentage of differentiated myofibroblasts in Ctl– or TRPM7-shRNA–treated groups. Ctl indicates control. C, Changes in \(\alpha\)-SMA induced by 10 ng/mL TGF-\(\beta\)1 in TRPM7- or Scr-shRNA–treated mouse cardiac fibroblasts. TGF-\(\beta\) receptor blocker SB431542 (10 \(\mu\)mol/L) was applied to the control groups to minimize differentiation at basal level. \(\alpha\)-SMA protein identified by \(\alpha\)-SMA antibody; GAPDH was used as loading control. Note the significant decrease of \(\alpha\)-SMA in TRPM7-shRNA–treated cells. D, Effects of TRPM7-shRNA on human atrial fibroblast proliferation induced by TGF-\(\beta\)1 (10 ng/mL).
VII). After TGF-β1 stimulation, the differentiated myofibroblasts in the Scr-shRNA-treated group increased to 61.0%, whereas, knocking down of TRPM7 by TRPM7-shRNA significantly reduced the differentiated myofibroblasts to 34.4%, a level similar to the control group (Figure 7B). The loss of sensitivity to TGF-β1 in TRPM7-shRNA–treated fibroblasts suggests that TRPM7 plays a role in TGF-β1 induce atrial fibroblast differentiation.

To understand how TRPM7 may be involved in fibroblast differentiation, we quantified changes of α-SMA protein in Scr- and TRPM7-shRNA–treated cells by Western blot. This experiment was performed by using mouse fibroblasts because of the limited number of human fibroblasts. To rule out other factors such as residual TGFβ in serum which may influence differentiation, we applied the TGF-β1 receptor blocker SB431542 as a control for TGF-β1 treatment. As shown in Figure 7C, the increased protein expression of α-SMA induced by TGF-β1 was significantly reduced by TRPM7-shRNA, which is consistent with the immunostaining result in human fibroblasts (Figure 7A and 7B).

Role of TRPM7 in TGF-β1–Induced Fibroblast Proliferation

We next investigated the role of TRPM7 in proliferation of fibroblasts. Human atrial fibroblasts treated with Scr- or TRPM7-shRNA were exposed to TGF-β1 (10 ng/mL). As shown in Figure 7D, TRPM7-shRNA significantly decreased the number of fibroblasts, suggesting that TRPM7 is also involved in cardiac fibroblast proliferation caused by TGF-β1.

Regulation of TRPM7 by TGF-β1

Because TGF-β1 is the most potent fibrogenesis stimulator, we studied whether expression of TRPM7 is modulated by TGF-β1. In this experiment, fibroblasts were treated with TGF-β1 or the TGF-β1 receptor blocker SB431542. TRPM7 currents were recorded after the human atrial fibroblasts were treated for 24 hour. As shown in Figure 8A and 8B, TRPM7 current density was increased by ~80% by TGF-β1. Ca2+- influx in TGF-β1–treated fibroblasts was significantly larger than that of control group (Online Figure VIII). Consistent with the increased current amplitude, TRPM7 expression at mRNA level assessed by quantitative PCR using mouse cardiac fibroblasts was increased by 2.8 fold. Although other Ca2+-permeable channel genes were also upregulated by TGF-β1 (Online Figure IX), TRPM7 is the only functional channel which can be measured by current recording in human atrial fibroblasts (Figures 2 and 3) and in mouse fibroblasts before and after TGF-β1 treatment (data not shown). Moreover, knocking down the abundantly expressed TRPC1 did not alter Ca2+ influx or differentiation of human atrial fibroblasts (Online Figure IV). Therefore, it is likely that TRPM7 plays a major role in mediating Ca2+ in atrial fibroblasts under normal conditions and up TGF-β1 stimulation. To further understand the correlation between the changes of TRPM7 induced by TGF-β1 and fibroblast differentiation, we tested TRPM7 protein and α-SMA expression levels by using mouse cardiac fibroblasts. Figure 8C and 8D shows that TGF-β1 increased TRPM7 and α-SMA protein expression by 3.8- and 2.1-fold, respectively. The mRNA level of collagen (type I) as well as collagen production measured in human atrial fibroblasts was markedly increased by TGF-β1 (Figure 8E and 8F). These results further suggest that upregulation of TRPM7 underlies the Ca2+ signaling mechanism for TGF-β1–induced fibroblast differentiation and collagen production and that TRPM7 may play a role in TGF-β1–induced atrial fibrogenesis.

Discussion

To our knowledge, this is the first study to report a pathophysiological function of the Ca2+-permeable cation channel TRPM7 in human disease. We made several new findings in this study. First, our results reveal that TRPM7 underlies the molecular mechanism of Ca2+ signaling in human atrial fibroblasts. Second, we demonstrate that TRPM7 mediated Ca2+ plays an essential role in atrial fibroblasts proliferation and differentiation. Third, we show that TRPM7 is markedly upregulated in AF patients, and that upregulation of TRPM7 confers increased fibroblast differentiation in AF patients.
Our results suggest that TRPM7 mediated Ca\(^{2+}\) plays a pivotal role in fibrogenesis in human AF.

**TRPM7 Underlies the Molecular Mechanism of Ca\(^{2+}\) Signaling in Human Atrial Fibroblasts**

Ca\(^{2+}\) signals are essential for various cellular functions. Although the importance of fibrosis in various cardiac diseases has been well appreciated, the Ca\(^{2+}\) signaling mechanisms in cardiac fibrogenesis were unknown. Here we demonstrate that TRPM7 is abundantly expressed in human atrial fibroblasts, and is the major Ca\(^{2+}\)-permeable channel which is responsible for Ca\(^{2+}\) influx in human atrial fibroblasts.

TRPM7 is a Ca\(^{2+}\)-permeable cation channel.\(^{36,37}\) It is constitutively active and brings Ca\(^{2+}\) into cells under physiological conditions. High intracellular Mg\(^{2+}\) concentration blocks TRPM7. However, TRPM7 channels show \(\approx 50\%\) of their maximum activity at physiological Mg\(^{2+}\) levels (0.5 to 1 mmol/L).\(^{36,51}\) Extracellular low pH potentiates TRPM7 inward current,\(^{42}\) and oxidative stress activates TRPM7.\(^{40}\) Membrane stretch\(^{32}\) and shear stress\(^{38}\) have been reported to activate TRPM7. All of these characteristics of TRPM7 suggest that TRPM7 plays a role under pathological conditions, such as ischemia or oxidative stress, which may initiate the cardiac fibrogenesis cascade.

Our results indicate that TRPM7 is encoded by *TRPM7* and that TRPM7 is the major functional Ca\(^{2+}\)-permeable channel in human atrial fibroblasts. Although *TRPV2*, *TRPV4*, *TRPC1*, and *TRPC6* were detectable by RT-PCR, current recording results indicate that there were no functional *TRPV2*, *TRPV4*, or *TRPC6* channels in the fibroblasts, making them unlikely to contribute to Ca\(^{2+}\) signaling in the fibroblasts. *TRPV4* is sensitive to stretch\(^{39}\) and can be specifically activated by 4α-phorbol-12,13-didecanoate (4α-PDD). We found that in both freshly isolated and cultured fibroblasts, 4α-PDD was unable to activate *TRPV4*-like currents (Figure 3). *TRPC6* is activated by G\(_{q}\)-linked receptor stimulation, diacylglycerol, or OAG (1-oleoyl-2-acetyl-sn-glycerol), or by direct application of GTP\(_\gamma\)S in the pipette solution. We could not activate TRPC6-like current by oleoyl-lysophosphatidic acid, which activates the EDG7 receptor,\(^{54}\) or by including GTP\(_\gamma\)S in the pipette solution (Online Figure III). These results indicate that TRPC6 is not functionally expressed in human atrial fibroblasts. A previous study reported that TRPC6 was activated by C-type natriuretic peptide through PLC activation or directly by OAG in rat cardiac fibroblasts.\(^{48}\) We do not know the reason for this discrepancy, but human and rat cardiac fibroblasts may express TRPC6 differently.

Store-depletion activated Ca\(^{2+}\) current (\(I_{\text{CRAC}}\)) contributes to Ca\(^{2+}\) influx and plays an important role in cellular functions in a variety of cells.\(^{55,56}\) In human atrial fibroblasts, we could not detect \(I_{\text{CRAC}}\) currents, and did not find that store-depletion could induce Ca\(^{2+}\) influx (Online Figure V) in human atrial fibroblasts. \(I_{\text{CRAC}}\) is not present in AF fibroblasts. Even though we found that TGFB-1 upregulates mRNA expression of *TRPM7*, *TRPV2*, *TRPC1*, and *TRPC6* currently were not present in AF fibroblasts. Even though we found that TGFB-1 upregulates mRNA expression of *TRPM7*, *TRPV2*, *TRPC1*, *TRPC3*, *TRPC4*, and *TRPC6* (Online Figure IX), TRPM7 current is the only functional current recorded in the fibroblasts treated with TGFB-1, suggesting that TRPM7 is the major channel mediating Ca\(^{2+}\) entry in atrial fibroblasts. When TRPM7 was knocked down, Ca\(^{2+}\) influx was substantially decreased in TRPM7-shRNA-treated atrial fibroblasts in comparison with Scr-shRNA-treated atrial fibroblasts, indicating that TRPM7 is needed for Ca\(^{2+}\) entry in fibroblasts. Taken together, our results indicate that TRPM7 is the major Ca\(^{2+}\)-permeable channel which is responsible for Ca\(^{2+}\) influx in human atrial fibroblasts.

**Upregulation of TRPM7 May Underlie TGF-β1–Induced Fibroblast Differentiation in AF Patients**

We found that TRPM7 is markedly upregulated in the fibroblasts from AF patients. Both current density and Ca\(^{2+}\) influx mediated by TRPM7 from AF patients were significantly larger than those from NSR patients (Figure 4). Because of the limited amount of tissue and numbers of human atrial fibroblasts, we could not compare the TRPM7 expression in AF and NSR patients by measuring protein levels using Western Blot.

Because the only differences between AF patients and NSR patients is the presence of AF versus NSR (Online Table I), it appears that AF is the only variable that correlates with the upregulated TRPM7. To understand the causal importance of TRPM7 in AF, we investigated whether TRPM7 influences fibroblast differentiation. We found that fibroblasts isolated from AF patients are more prone to differentiation under culture conditions even without TGF-β1 treatment. This inherent tendency to differentiation could be attributable to the increased expression level of TRPM7 when the fibroblasts were exposed to the residual amount of TGFβ in the culture media containing serum.\(^{50}\) Indeed, knocking down TRPM7 by TRPM7-shRNA not only markedly reduced basal differentiation of AF fibroblasts (Figure 6C and 6D), but also substantially decreased the number of differentiated myofibroblasts and the expression level of α-SMA induced by TGF-β1, indicating that TRPM7 is necessary for fibroblast differentiation. As the myofibroblasts are the primary cells that synthesize ECM and cytokines, which in turn stimulate the fibrogenesis cascade, the requirement of TRPM7 for myofibroblast differentiation indicates that TRPM7-mediated Ca\(^{2+}\) signals play a pivotal role in fibrosis formation.

Fibrogenesis can be triggered by various stimuli, including mechanical stretch, oxidative stress, hormones, myocardial injury, and autocrine–paracrine mediators,\(^{14,16}\) among which TGF-β1 is the most potent stimulator in both in vivo and in vitro conditions. Interestingly, we found that TRPM7 expression was significantly increased by TGF-β1 in cultured fibroblasts (Figure 8). The increased TRPM7 expression level induced by TGF-β1 parallels the fibroblast differentiation as assessed by α-SMA expression and collagen production (Figure 8C through 8F). These results indicate that TRPM7 is indispensable for TGF-β1–induced differentiation, and that the upregulated TRPM7 may synergize with TGF-β1 in inducing fibroblast differentiation. It is possible that the upregulation of TRPM7 by TGF-β1 in cultured fibroblasts represents the major mechanism by which TRPM7 is upregulated in AF patients in vivo. Taken together, our results suggest the following model: In human atria, TGF-β1 is activated by mechanical stretch, oxidative stress, or other stimuli. TGF-β1 upregulates TRPM7, which in turn brings more Ca\(^{2+}\) for TGF-β1 to induce differentiation. The differentiated myofibroblasts synthesize ECM and cytokines, and...
excessive accumulation of ECM forms fibrosis. Cytokines can in turn stimulate TGF-β1 and cause upregulation of TRPM7, leading to perpetuation of the fibrogenesis process.

**Potential Mechanism by Which Ca\(^{2+}\) Signals Are Involved in TGF-β1–Induced Fibrosis**

Atrial fibrosis plays an important role in the pathology of AF. Although 3 interrelated pathways, including the renin–angiotensin system, TGF-β1, and the oxidative stress pathways, have been shown to be involved in fibrosis,\(^{57–65}\) many fundamental aspects of fibrogenesis are still to be established.\(^{6,57}\) Our results indicate that TRPM7-mediated Ca\(^{2+}\) signal is another fundamental factor contributing to atrial fibrogenesis cascade.

How does the Ca\(^{2+}\) signal contribute to fibrosis formation? There are several possibilities. First, Ca\(^{2+}\)-dependent but TGF-β1–independent pathways may control fibrotic gene expression.\(^{64–67}\) Second, Ca\(^{2+}\) signals may influence TGF-β1 via the Smad-independent pathway.\(^{68–70}\) Third, TGF-β1 can induce Ca\(^{2+}\) entry\(^{71,72}\) or integrate with reactive oxygen species (ROS) associated Ca\(^{2+}\) influx and therefore influence ECM protein synthesis\(^{73}\) through a ROS activated calcineurin pathway. As TRPM7 can be activated by ROS,\(^{49}\) it is possible that the ROS activated calcineurin pathway may have contributed to the upregulation of TRPM7 by TGF-β1 in human atrial fibroblasts. Fourth, TRPM7–mediated Ca\(^{2+}\) signals may activate the calcineurin pathway and produce synergistic effect with TGF-β1 (Smad-dependent pathway) in regulating gene expression. Further studies are required to understand how TRPM7 mediated Ca\(^{2+}\) signals contribute to TGF-β1–induced fibrogenesis. Nevertheless, our findings that TRPM7 is the molecular basis for the major Ca\(^{2+}\) entry channel in cardiac fibroblasts provide a necessary tool to unravel the mechanisms by which Ca\(^{2+}\) signals contribute to fibrogenesis.

**Potential Significance**

Atrial fibrosis can be triggered by various factors\(^{3}\) via different pathways.\(^{57}\) Blocking one pathway may not be able to attenuate or eliminate fibrosis given the complex pathological conditions. The TRPM7–mediated Ca\(^{2+}\) signals which are necessary for TGF-β1–induced fibrogenesis may serve as a common pathway in the fibrogenesis cascade. If so, inhibition of Ca\(^{2+}\) entry through TRPM7 may attenuate cardiac fibrosis regardless of the original stimuli.

TRPM7 has been shown to be essential for cell viability\(^{74,75}\) and is responsible for Ca\(^{2+}\) overload–induced cell death under anoxia conditions.\(^{49}\) Although previous studies suggested that TRPM7 is involved in Mg\(^{2+}\) homeostasis,\(^{74,76}\) knocking out of the TRPM7 gene disrupts embryonic development but does not alter Mg\(^{2+}\) homeostasis,\(^{39}\) indicating that Ca\(^{2+}\) permeation may confer major channel functions of TRPM7. The contribution of the TRPM7–mediated Ca\(^{2+}\) signal in human atrial fibrogenesis shown in this study is the first demonstration of an important role of this unique channel in human diseases.

**Conclusions**

In this study, we establish that the Ca\(^{2+}\)-permeable cation channel TRPM7 underlies Ca\(^{2+}\) signaling mechanisms in the cardiac fibrogenesis, making TRPM7 the first known Ca\(^{2+}\)-permeable channel involved in the genesis of cardiac fibrosis. More importantly, our results demonstrate that TRPM7 plays an essential role in fibrogenesis in human AF. Our study opens a new avenue to explore the potential roles of Ca\(^{2+}\) signaling in fibrogenesis, which will lead to better understanding of the mechanism of cardiac fibrogenesis, and could ultimately results in development of more effective approaches for treatment of AF.

**Acknowledgments**

We thank Drs Stanley Nattel, Laurinda Jaffe, Achilles Pappano, and Haoxing Xu for constructive suggestions and comments. We also thank Philip Batista, Mariela Agosto, and Dorota Pawlak for help with the patient database. We also appreciate helpful comments from other members in the laboratory.

**Sources of Funding**

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is Known?**

- Atrial fibrosis is a hallmark feature of structural remodeling in AF.
- Previous studies have suggested that Ca2+ entry is essential for fibroblast function. However, the Ca2+ signaling mechanisms in cardiac fibrogenesis have not elucidated.

**What New Information Does This Article Contribute?**

- Here, we report that the Ca2+-permeable cation channel TRPM7 is the major Ca2+-permeable channel in human atrial fibroblasts.
- TRPM7-mediated Ca2+ entry is essential for TGF-beta1-induced fibroblast differentiation.
- TRPM7 is significantly upregulated in AF fibroblasts and plays a key role in enhanced fibroblast proliferation, differentiation, and collagen production during AF.

Atrial fibrosis is a major factor contributing to atrial fibrillation (AF). Understanding the mechanism of fibrogenesis is crucial for developing new therapeutic strategies for attenuating or reversing fibrosis-associated AF. The aim of this study was to elucidate the Ca2+ signaling mechanisms in fibroblasts and how Ca2+ signals contribute to fibrogenesis in AF. We discovered that TRPM7, a Ca2+-permeable cation channel that possesses protein kinase function, is the key Ca2+-permeable channel in human atrial fibroblasts. TRPM7 and TRPM7-mediated Ca2+ signals were drastically upregulated in AF patients, which correlated with enhanced fibrogenesis in cells from these patients. This study identifies for the first time the molecular nature of the major Ca2+-permeable channel in cardiac fibroblasts, which helps to elucidate how Ca2+ signals contribute to fibrogenesis in human AF. Also, our observation that TRPM7-mediated Ca2+ entry contributes to human AF represents the first report of an important role of TRPM7 in human diseases. Our study opens a new avenue to explore the potential role of Ca2+ signals in fibrogenesis, which should lead to better understanding of the mechanisms of cardiac fibrogenesis and could ultimately result in the development of more effective approaches for the treatment of AF.
TRPM7-Mediated Ca\textsuperscript{2+} Signals Confer Fibrogenesis in Human Atrial Fibrillation
Jianyang Du, Jia Xie, Zheng Zhang, Hiroto Tsujikawa, Daniel Fusco, David Silverman, Bruce Liang and Lixia Yue

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SUPPLEMENT MATERIAL

TRPM7-mediated Ca\textsuperscript{2+} signals confer fibrogenesis in human Atrial Fibrillation

METHODS

Human Cardiac Tissue Sample Collection
Myocardial samples of the right atria were collected during cardiac surgery. All procedures involving human tissue use were approved by the institutional review boards of the University of Connecticut Health Center. Consent was obtained from patients before tissue harvest. Atrial samples were obtained from normal sinus rhythm patients (NSR) and permanent or paroxysmal AF patients (see Online Table I). On excision the samples were immediately placed in cold and oxygenated nominally calcium-free HBSS solution for transport to the laboratory.

Isolation of cardiac fibroblasts from human atrial samples and mouse heart
Atrial samples were minced and incubated collagenase (150–200 U/ml CLS II, Worthington Biochemical, Freehold, NJ 300 U/mg) in a shaking water bath at 37°C. Isolated cells were harvested after each 10 min digestion period. After 5 digestion periods, all the isolated cells were then centrifuged at 1000 rpm for 10 min. Fibroblasts were re-suspended and cultured in DMEM media containing 10% FBS, or used freshly for patch-clamp experiments.

Mouse fibroblasts were isolated using the same method. We did not separate fibroblasts from mouse atrium and ventricle. Therefore, we used the term “cardiac fibroblasts” for the the experiments when mouse cardiac fibroblasts were used.

Molecular Biology

RT-PCR
Gene specific primers were designed for human TRP channel genes (see Online Table II). Total RNA was extracted from cultured human atrial fibroblasts by using TRizol (Invitrogen). RT-PCR was performed as we previously reported\textsuperscript{1}. Ribosome RNA 18 s was used as a positive control.

Real-Time RT-PCR
Cardiac fibroblasts were isolated and seeded in 10 cm dish. For the experiment of measuring collagen mRNA, fibroblasts were cultured in media containing 1% serum in the presence or absence of TGF-β1 (10 ng/ml). After 48 hrs, fibroblasts were then collected and total RNA was extracted by Trizol (Invitrogen). Real-time PCR was performed with SYBR Green method following the protocol suggested by vendor (ABI), with β-actin as the internal control. Primers used for real-time PCR are summarized in Online Table III.

shRNA generation and treatment of cardiac fibroblasts
We have designed two TRPM7-specific shRNAs against for both murine and human TRPM7 respectively. For murine TRPM7, the first one (CAGGCTATGCTTGATGCTC) corresponds to the murine TRPM7 coding region 1320-1338 (denoted shRNAn), and second one (CCTGATGAGGTTGTCACAG) corresponds to the coding region 4676-4894 (denoted shRNAc), relative to the first nucleotide of the start codon of murine TRPM7 (Genebank: AF3760520\textsuperscript{2}). For human TRPM7 shRNA, the first one (GTCTTGCCATGAAATACTC) corresponds to 169-187 (denoted hTRPM7-shRNAn), and the second one (CTGCGACAGAGACTACATG) corresponds to 4092-4110 (denoted hTRPM7-shRNAc), relative to the first nucleotide of the start codon of human TRPM7 (Genebank: AY032950). The scramble shRNA (TGTCCTCCGAACGTGTAGT) was used as a negative control. Based on the chosen 19-nucleotide...
shRNA sequence, two DNA oligos (64 nt) were designed as the shRNA insert sequence. In the forward oligonucleotide, the 19-nucleotide sense shRNA sequence was linked to the reverse complementary anti-sense shRNA sequence by a 9-nucleotide hairpin loop sequence (TTCAAGAGA). Five Ts were added to the 3′-end of the reverse complementary anti-sense shRNA sequence. The MluI overhangs were added to the 5′ end of the forward oligonucleotides. In the reverse 64 oligonucleotides, the ClaI overhangs were added to the 5′ end. The 64 nt oligos were ordered from IDT. After annealing, the shRNA insert was cloned into lentiviral vector (pLVTHM) 3 pre-cut with MluI and ClaI. The shRNA insert was confirmed by sequence analysis. The resulting RNA transcript would fold and form a hairpin loop structure.

Lentiviral vector plasmid and packaging vector plasmids were transfected into AAV-293 cells cultured in 0.4% to 1% FBS media to produce shRNA viruses. Viruses were harvested and used to infect cardiac fibroblasts or HEK-293 cells over-expressing TRPM7. Cells were incubated with viruses for three to five times; each incubation with viruses lasted for 12 hrs. Between virus incubation, cells were cultured in normal media containing 10% FBS for 12 hrs. Infected cells can be identified by GFP fluorescence. Experiments were performed after 60 or 72 hrs of shRNA treatment. Two different shRNAs were tested in the HEK-293 cells, and the one with higher efficiency for knocking down TRPM7 was used in cardiac fibroblast. For both human and murine TRPM7 shRNAs, shRNAc was chosen for the experiments in cardiac fibroblasts. In the text, we use the term “TRPM7 shRNA” for simplicity.

hTRPC1 siRNA

The hTRPC1 specific siRNA was purchased from Dharmacoa. The siRNA duplexes (sense sequences: GAGAAGAACUGCAGUCCUUUU ) of hTRPC1 was synthesized by Dharmacoa, and the siControl non-targeting siRNA (Dharmacon, #D-001206) containing a pool of non-targeting siRNA was used as control (Ser-siRNA)(Dharmacon, #D-001206). For transfection, 1nmol of siRNA and 20 ul of Dharmafect (T-2001-03) was incubated separately in 500ul of OPTI-MEM for 5 min, and then mixed together and incubated for 20 min. The mixture was mixed with 1ml of additional OPTI-MEM and added to cells in 60 cm dishes. OPTI-MEM was replaced by 10% FBS DMEM 8 hours later. After 48 hours, cells were collected for experiments. The specificity and efficiency of hTRPC1-siRNA were tested and the results are shown in sFigure 4.

Electrophysiology

Whole-cell and single-channel currents were recorded using an Axopatch 200B amplifier. Data were digitized at 5 or 10 kHz, and digitally filtered off-line at 1 kHz. Patch electrodes were pulled from borosilicate glass and fire-polished to a resistance of ~3 MΩ when filled with internal solutions. Series resistance (Rs) was compensated up to 90% to reduce series resistance errors to <5 mV. Cells with Rs bigger than 10 MΩ were discarded 5.

For whole cell currents recording, voltage stimuli lasting 250 ms were delivered at 1 s intervals, with either voltage ramps or voltage steps ranging from -120 to +100 mV. A fast perfusion system was used to exchange extracellular solutions, with complete solution exchange achieved in about 1 to 3 s 6. The internal pipette solution for TRPM7 whole cell current recordings contained (in mM) 145 Cs-methanesulfonate (CsSO3CH3), 8 NaCl, 10 EGTA, and 10 HEPES, with pH adjusted to 7.2 with CsOH. Ca2+ was adjusted to various concentrations based on calculation using MaxChelator (http://www.stanford.edu/~cpatton/webmaxC.htm). The standard extracellular Tyrode’s solution for whole cell recording contained (mM): 145 NaCl, 5 KCl, 2 CaCl2, 10 HEPES and 10 glucose; pH was adjusted to 7.4 with NaOH. Divalent-free solution (DFV) contained (mM) 145 NaCl, 20 HEPES, 5 EGTA, 2 EDTA and 10 glucose, with estimated free [Ca2+] < 1 nM and free [Mg2+] ≈10 nM at pH 7.4. MaxChelator was used to calculate free Ca2+ and free Mg2+ concentrations.
High Mg\(^{2+}\) concentration (3 mM free Mg\(^{2+}\)) was used in the pipette solution to inhibit TRPM7 current in the fibroblasts (sFig. 2). Oleoyl-lysophosphatidic acid (LPA) was used to activate G\(_o\)-linked EDG-7 receptor in the fibroblasts\(^7\). 2-APB was used to activate TRPV2 and 4\(\alpha\)-PDD was used to induce TRPV4 currents. All chemicals were from Sigma.

Single channel current recording was conducted under inside-out configuration. The pipette solution contained (mM): 140 NaSO\(_3\)CH\(_3\), 8 NaCl, 10 HEPES, 10 EGTA, 2 EDTA, 10 Glucose (pH 7.4 adjusted with NaOH); and the bath solution contained (mM): 140 NaSO\(_3\)CH\(_3\), 8 NaCl, 10 EGTA, 2 EDTA, 10 HEPES (pH 7.2 adjusted with NaOH).

**Ratio Ca\(^{2+}\) imaging experiments**

Cells plated on 25 mm glass coverslips were loaded with 5 \(\mu\)M Fura-2 for 45 minutes. Non-incorporated dye was washed away using a HEPES-buffered Saline Solution (HBSS) containing (in mM) 20 HEPES, 10 glucose, 1.2 MgCl\(_2\), 1.2 KH\(_2\)PO\(_4\), 4.7 KCl, 140 NaCl, 1.3 Ca\(^{2+}\) (pH 7.4). Ca\(^{2+}\) influx was measured by perfusing the cells with Tyrode’s solution containing 20 mM Ca\(^{2+}\) after the cells were perfused with nominal Ca\(^{2+}\) and Mg\(^{2+}\) free solution for 2 minutes. Ionomycin (Iono) at 1 \(\mu\)M was applied as an internal control. Fluorescence intensities at 510 nm with 340 nm and 380 nm excitation were collected at a rate of 1 Hz using CoolSNAP HQ2 (Photometrics) and data were analyzed using NIS-Elements (Nikon). The 340:380 nm ratio in the presence of 20 mM Ca\(^{2+}\) was normalized to that of the Ca\(^{2+}\) signal elicited by 1 \(\mu\)M Ionomycin (Iono) as we previously reported\(^8\). Ca\(^{2+}\) influx was measured with/without store depletion by 2 \(\mu\)M thapsigargin (Tg).

**Immunostaining**

Cells were fixed by 4% formaldehyde for 20 min, and permeabilized with 0.3% TritonX-100 in the presence of 10% of FCS to minimize non-specific binding. Cells were then incubated with \(\alpha\)-SMA antibody (1:300 dilution, Sigma) at room temperature for 2 hrs, washed with PBS, and incubated with secondary antibody (1:2000, Invitrogen) for an additional 1 hr. The immunostained cells were analyzed using a Zeiss LSM 510 confocal microscope. Images were taken with a 20x objective by using the same settings for pinhole width, laser intensity and detector gain\(^9\).

**Western Blot**

The cardiac fibroblasts treated with TRPM7 shRNA or scramble shRNA were induced for differentiation by 10 ng/ml TGF-\(\beta\)-1, or TGF-R blocker to minimize basal differentiation presumably resulted from FBS. Cells were collected 48 hours after TGF-\(\beta\)-1 or TGF-R blocker treatment. Cell lysate was used to blot with \(\alpha\)SMA antibody (Sigma) or GAPDH antibody (Santa Cruz, sc-25778) as an internal control. For TRPM7 western blot experiments, TRPM7 protein was first immunoprecipitated and then blotted with TRPM7 antibody (NeuroMab). The ECL chemiluminescence was detected and captured by FUJI LAS-3000 Imaging System.

**Measurement of Collagen Content**

Collagen content was determined through a colorimetric reaction against picrosirius red based on the manufacture’s instruction (Biocolor, UK. www.biocolor.co.uk). Briefly, cardiac fibroblasts were cultured in 1% serum media for 48 hours in the presence or absence of TGF-\(\beta\)-1. 100 \(\mu\)l culture media was added to 1ml of picric acid containing 0.1% sirius red, and centrifuged at 12,000 rpm after incubating at room temperature for 1 hour. The pellet was washed by 0.1M HCl and dissolved in 0.5M NaOH. The collagen content was converted from the absorbance of the soluble dye at 540 nm based on the standard curve.
SUPPLEMENTARY TABLES

Online Table I. Clinical Characteristics of Study Population

<table>
<thead>
<tr>
<th></th>
<th>NSR Mean ± SD or Number (%)</th>
<th>Atrial Fibrillation Mean ± SD or Number (%)</th>
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<tr>
<td>Male</td>
<td>6 (75%)</td>
<td>3 (43%)</td>
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<td>Isolated CABG</td>
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<td>β-blocker</td>
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<td>Statin</td>
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<td>Aspirin</td>
<td>6 (75%)</td>
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The category of surgery-others includes patients who underwent CABG (Coronary Artery Bypass Graft) plus valve or isolated valve operations. (%) was the fraction of subjects with a specific characteristic. Unpaired t test was used for continuous variables and Fisher’s exact test was used for categorical variables. NSR: normal sinus rhythm; LA: left atrium; LVEF: left ventricular ejection fraction; ARB: angiotension II receptor blocker; ACEI: angiotensin converting enzyme inhibitor.
Online Table II. Oligonucleotide sequences used as qPCR primers.

<table>
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<tr>
<th>Gene name</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Anticipated size</th>
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### Online Table III. Oligonucleotide sequences used as qPCR primers.

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<tbody>
<tr>
<td>mTRPC1</td>
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REFERENCES


Online Figure I. Expression of TRP channels detected by RT-PCR in human atrial fibroblasts. Total RNA was isolated from cultured atrial fibroblasts. A reaction without reverse transcriptase was used as negative control (Ctl), and expression of ribosome RNA 18s was used as a positive control. TRPC1, TRPC6, TRPV2, TRPV4, TRPM4 and TRPM7 were detected in the human atrial fibroblasts. TRPM7 is abundantly expressed in human atrial fibroblasts.
**Online Figure II.** Concentration-dependent effects of external protons on endogenous TRPM7 currents. A, Representative recordings of endogenous TRPM7 at various external pH. Note the increase in inward current at the indicated pH. B, Dose-response curve of low pH effects on TRPM7. The best fit of the curve with the Hill equation yielded an EC$_{50}$ of 3.9 ± 0.05 pH unit (n=9, n$_H$=1.2).
Online Figure III. TRPC6 currents could not be activated by GTP-γ-S in human atrial fibroblasts. A, Time-dependent changes in inward and outward currents of hTRPC6 over-expressed in HM1 cells (HEK-293 cells stably expressing muscarinic M1 receptor). B, Representative recording of recombinant hTRPC6 expressed in HM1 cells. TRPC6 was activated by 100 mM GTP-γ-S in the pipette solution. The pipette solution contained 100 nM Ca^{2+}, 0.2 mM ATP, 0.2 mM GTP, and 100 mM GTP-γ-S. C, Time-dependent changes of inward and outward current recorded in a human atrial fibroblast. 500 μM GTP-γ-S was included in the pipette solution. Note 500 μM GTP-γ-S dialyzed into the cells did not elicit TRPC6-like currents, but blocked endogenous TRPM7 current. D, Representative recordings of currents after rupture of the cell and after GTP-γ-S was dialyzed into the cells. TRPM7 was recorded in the beginning but was blocked by GTP-γ-S.
Online Figure IV. Effects of TRPC1 on Ca\textsuperscript{2+} influx and human atrial fibroblast differentiation. A-B. hTRPC1 specific siRNA did not change Ca\textsuperscript{2+} influx after the cells were treated with hTRPC1-siRNA for 48 hours in comparison with the cells treated with Scr-siRNA. The mean normalized F340/F380 was 0.3±0.03 and 0.35±0.01 for Scr-shRNA and hTRPC1-siRNA treated groups, respectively (from 3 separate experiments, p>0.05). C, hTRPC1-siRNA specifically inhibited hTRPC1 expression. TRPM7 expression was not altered by hTRPC1-siRNA. D, knocking-down hTRPC1 did not change atrial fibroblasts differentiation induced by culturing the fibroblasts in 10% FBS for 24 hours after siRNA treatment.
**Online Figure V.** Ca\(^{2+}\) release does not elicit any channel activation in human atrial fibroblasts. Intracellular Ca\(^{2+}\) concentration was buffered to 10 nM, and 3 mM Mg\(^{2+}\) was included in the pipette solution to eliminate endogenous TRPM7. A, Application of 5 µM Thapsigargin (Tg) after inactivation of TRPM7 did not induce any current. B, Representative recordings at time points right after rupture of the cell, and before and after application of 5 µM Tg. Note that Tg did not activate any current. C, Time-dependent changes of current amplitude in divalent free (DVF) solution and after perfusion with 20 mM Ca\(^{2+}\). Note that after TRPM7 inactivation, no other current was induced by application of 20 mM Ca\(^{2+}\). D, Representative recordings obtained in DVF and 20 mM Ca\(^{2+}\) solutions. E, Ca\(^{2+}\) influx under different conditions. Cells were perfused with DVF or DVF/Tg before 20 mM Ca\(^{2+}\) perfusion. Ionomycin was used as an internal control. Ca\(^{2+}\) influx upon perfusion with 20 mM Ca\(^{2+}\) in cells perfused with DVF/Tg was similar to that in cells perfused with DVF, indicating that store depletion by Tg did not contribute to Ca\(^{2+}\) influx. F, Average ratio of F340/F380 in cells perfused with DVF and DVF/Tg. No statistical difference was obtained between the two groups.
Online Figure VI. Single channel properties of TRPM7 in atrial fibroblasts from NSR and AF patients. A-B, Typical recordings of single channel currents at +60 and +100 mV in inside-out patches in atrial fibroblasts from NSR (A) and AF (B) patients, respectively. C, Current amplitude at indicated voltages. Linear regression fit yielded single channel conductance of 37.1±0.7 pS in cells from NSR patients (n=9), and 35.0±0.8 pS (n=13) in cells from AF patients. No statistical difference was obtained. D, Open probability of TRPM7 in atrial fibroblasts from NSR patients (n=6) is similar to that from AF patients (n=12).
Online Figure VII. Time-dependent differentiation of atrial fibroblasts from NSR patients in 10% FBS media.
Online Figure VIII. Increased Ca\(^{2+}\) influx induced by pre-treatment with 10 ng/ml TGF-β1. A-B, Ca\(^{2+}\) entry in Ctl and TGF-β1 treated fibroblasts for 24 hr. Acute application of TGF-β1 in the perfusion solution did not alter Ca\(^{2+}\) influx in Ctl or TGF-β1 treated atrial fibroblasts. C, Ca\(^{2+}\) influx in fibroblasts pre-treated with TGF-β1 was significantly larger than that of Ctl fibroblasts.
Online Figure IX. Regulation of Ca\(^{2+}\) signaling related gene expression by TGF-β1. Mouse cardiac fibroblasts were treated with 10 ng/ml TGF-β1 or 10 μM SB431542 (Ctl). Expression level of RNA was assessed by qPCR. Relative expression of each gene in TGF-β1 treated group (T) was normalized to control group (C).