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## TRPM7-mediated $\text{Ca}^{2+}$ signals confer fibrogenesis in human Atrial Fibrillation

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## Abstract

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

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

**Methods and Results**—We demonstrate that the transient receptor potential melastatin related 7 (TRPM7) is the molecular basis of the major  $\text{Ca}^{2+}$ -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 (shRNA) largely eliminates TRPM7 current and  $\text{Ca}^{2+}$  influx in atrial fibroblasts. More importantly, atrial fibroblasts from AF patients show a striking upregulation of both TRPM7 currents and  $\text{Ca}^{2+}$  influx and are more prone to myofibroblast differentiation, presumably due to the enhanced expression of TRPM7. TRPM7-shRNA markedly reduced basal AF fibroblast differentiation. Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), the major stimulator of atrial fibrosis, requires TRPM7-mediated  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -permeable channel in human atrial fibroblasts, and likely plays an essential role in TGF- $\beta$ 1-elicited fibrogenesis in human AF.

## Keywords

Atrial Fibrillation; TRPM7;  $\text{Ca}^{2+}$  signaling; TGF- $\beta$ 1; fibrogenesis

## INTRODUCTION

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<sup>1</sup>. Atrial fibrillation (AF) is the most sustained clinical arrhythmia and is a major cause of morbidity and mortality<sup>2</sup>. 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 electrical and contractile remodeling to AF substrate<sup>2-6</sup>. Atrial fibrosis is a hallmark feature of arrhythmogenic structural remodeling in clinical AF<sup>7</sup>. Increased amount of fibrosis occurs in not only AF patients associated with other cardiac diseases<sup>8, 9</sup>, but also in those with lone AF<sup>8, 10</sup>. There is a positive correlation between the amounts of fibrosis and the persistence of AF<sup>9</sup>, 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 TGF- $\beta$ 1 increases AF vulnerability<sup>11, 12</sup>. 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 proteins (ECM) synthesized by cardiac fibroblasts or myofibroblasts upon stimulation<sup>13, 14</sup>. Myofibroblasts are not found in normal cardiac tissue<sup>15</sup>. 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<sup>14, 16</sup>. Myofibroblasts might also be derived from endothelial cells in fibrotic heart<sup>17</sup>. By producing growth factors, cytokines, chemokines, ECM proteins, and proteases<sup>18, 19</sup>, myofibroblasts play a pivotal role in fibrosis. 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<sup>2+</sup> signals are essential for diverse cellular functions including differentiation, gene expression, cell proliferation, growth and death<sup>20</sup>. In cardiac fibroblasts, several lines of evidence suggest that Ca<sup>2+</sup> entry is essential for fibroblast functions. Chelating external Ca<sup>2+</sup> by EGTA prevents substance P induced proliferation of cultured rat cardiac fibroblasts<sup>21, 22</sup>. Eliminating external Ca<sup>2+</sup> to prevent Ca<sup>2+</sup> influx attenuates H<sub>2</sub>O<sub>2</sub> induced IL-6 mRNA expression<sup>23</sup>. In mechanical-stimulation or stretch-induced membrane potential studies, both Ca<sup>2+</sup>-entry and Ca<sup>2+</sup>-release are involved in generation of MIP (MIP: mechanically induced potential)<sup>24, 25</sup>. A stretch induced current can be blocked by Gadolinium (Gd<sup>3+</sup>)<sup>26</sup>, a non-selective cation channel blocker. Recently, it has been shown that intracellular Ca<sup>2+</sup> contributes to Angiotensin II (Ang II) induced proliferation of cardiac fibroblasts<sup>27</sup>. In an *in vivo* study, Mibefradil, a Ca<sup>2+</sup> channel blocker, significantly reduced collagen production and fibroblast differentiation in rats treated with AngII or Aldosterone<sup>28</sup>. These studies indicate that Ca<sup>2+</sup> entry through Ca<sup>2+</sup>-permeable ion channels is essential for gene expression and fibrosis promotion. Therefore, understanding the molecular basis of Ca<sup>2+</sup>-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<sup>29-31</sup>. There is no evidence indicating that functional voltage-gated calcium channels exist in the fibroblast<sup>32</sup>. The transient receptor potential (TRP) channels are responsible for Ca<sup>2+</sup> entry in various non-excitable and excitable cells<sup>33-35</sup>. 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<sup>33-35</sup>. 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<sup>2+</sup> signaling in cardiac fibroblasts.

In order to understand Ca<sup>2+</sup> signaling mechanisms in cardiac fibroblasts and potential roles of Ca<sup>2+</sup> signals in the cardiac fibrogenesis cascade, we investigated the molecular basis of Ca<sup>2+</sup>-permeable channels in human atrial fibroblasts isolated from AF patients and normal sinus rhythm patients (NSR), and studied how Ca<sup>2+</sup> influx contributes to TGF-β1 induced fibrogenesis process. We discovered that TRPM7, a Ca<sup>2+</sup>-permeable cation channel which also possesses protein kinase function<sup>36-38</sup>, underlies the Ca<sup>2+</sup> signaling mechanism in human atrial fibroblasts. TRPM7 has been reported to play a vital role in embryonic development<sup>39</sup> and anoxic cell death<sup>40</sup>. We found that TRPM7 is the major Ca<sup>2+</sup>-permeable channel in human atrial fibroblasts. Knocking down TRPM7 by shRNA largely eliminates the endogenous TRPM7 currents as well as Ca<sup>2+</sup> influx in atrial fibroblasts. Strikingly, TRPM7 and TRPM7-mediated Ca<sup>2+</sup> influx are drastically up-regulated in fibroblasts from AF patients. In addition, inhibition of TRPM7-mediated Ca<sup>2+</sup> influx renders fibroblasts less sensitive to TGF-β1 induced proliferation and differentiation, indicating that TRPM7-mediated Ca<sup>2+</sup> signal is necessary for TGF-β1 elicited fibrogenesis. On parallel with myofibroblast differentiation, TRPM7 is up-regulated by TGF-β1 in cultured

fibroblasts. Taken together, our results indicate that TRPM7-mediated  $\text{Ca}^{2+}$  signal contributes to fibroblast differentiation and may mediate the fibrogenic effect of TGF- $\beta$ 1. 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 supplementary methods). 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<sup>41</sup> by using the primers shown in Online Table II. TRPM7-specific shRNAs were designed and constructed into lenti-viral delivery system. The efficiency of the shRNAs was tested in the TRPM7 over-expression 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<sup>42, 43</sup>. Internal  $\text{Mg}^{2+}$ -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 $\text{Ca}^{2+}$ imaging experiments

$\text{Ca}^{2+}$  influx was measured using ratio  $\text{Ca}^{2+}$  imaging system and analyzed using NIS-Elements (Nikon). The F340/F380 ratio in the presence of 20 mM  $\text{Ca}^{2+}$  was normalized to that of 1  $\mu\text{M}$  Ionomycin (Iono) elicited  $\text{Ca}^{2+}$  signal (see details in supplementary methods).  $\text{Ca}^{2+}$  influx was also measured with/without store depletion by 2  $\mu\text{M}$  thapsigargin (Tg) in some experiments as specified in text.

### Immunostaining

Fibroblasts were fixed by 4% formaldehyde and immunostained with  $\alpha$ -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  $\alpha$ -SMA were evaluated by Western blot experiments. GAPDH was used as a loading control (the Online Data Supplement).

### Data Analysis

Pooled data are presented as mean  $\pm$  SEM. Dose-response curves were fitted by an equation of the form  $E = E_{\text{max}} \{ 1 / [1 + (\text{EC}_{50}/C)^n] \}$ , where  $E$  is the effect at concentration  $C$ ,  $E_{\text{max}}$  is maximal effect,  $\text{EC}_{50}$  is the concentration for half-maximal effect and  $n$  is the Hill coefficient. Statistical comparisons were made using two-way ANALYSIS of variance (ANOVA) and two-tailed  $t$ -test with Bonferroni correction. Fisher's exact test was used for

analysis of percentage differences for patients' database.  $P < 0.05$  indicated statistical significance.

## RESULTS

### TRPM7 is the major $\text{Ca}^{2+}$ -permeable channel in human atrial fibroblasts

To understand which  $\text{Ca}^{2+}$  permeable channel is responsible for  $\text{Ca}^{2+}$  entry in cardiac fibroblasts, we first investigated the expression of  $\text{Ca}^{2+}$ -permeable TRP channels. Among the 27 human TRP channel genes<sup>33</sup>, 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 relation (I-V relation) with small inward current and strong outward rectification (Figure 1A) is similar to the typical characteristics of the recombinant TRPM7<sup>36, 37</sup>. 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 concentration of 2-APB<sup>43, 44</sup>. We then tested if the endogenous TRPM7-like current in cardiac fibroblasts has similar properties. The single channel conductance of TRPM7-like current (Figure 1B) is similar to that of TRPM7 over-expressed in CHO cells<sup>43</sup>. TRPM7-like current was significantly potentiated by low extracellular pH (Figure 1C) with an  $\text{EC}_{50}$  of 3.9 pH unit (Online Figure IIB). Like TRPM7, TRPM7-like current was inhibited by low concentrations of 2-APB with an  $\text{IC}_{50}$  of 32.1  $\mu\text{M}$  (Figure 1E-F). 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<sup>42, 43</sup>, 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 over-expressing human TRPM7 (Figure 2A-B). 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 Scr-shRNA (Figure 2C-D), 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 $\text{Ca}^{2+}$ entry in human atrial fibroblasts

We next asked whether TRPM7 is responsible for  $\text{Ca}^{2+}$  entry in atrial fibroblasts. Using ratio  $\text{Ca}^{2+}$  imaging measurements, we found that  $\text{Ca}^{2+}$  influx was decreased by 62% after fibroblasts were treated with TRPM7-shRNA for 5 days, indicating that TRPM7 is the major  $\text{Ca}^{2+}$ -permeable channel in human atrial fibroblasts (Figure 2E-F). To elucidate whether there are other  $\text{Ca}^{2+}$ -permeable channels which may contribute to  $\text{Ca}^{2+}$  influx in human atrial fibroblasts, we used the conditions specific for TRPV2<sup>45</sup>, TRPV4<sup>46</sup> or TRPC6<sup>47, 48</sup> current recording, and tested if 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  $\mu\text{M}$  2-APB in human atrial fibroblasts (Figure 3A), whereas 2-APB readily activated TRPV2 overexpressed in HEK293 cells (Figure 3B-C). The conditions used for activating TRPV4 expressed in HEK293 cells (Figure 3E-F) failed to induce any TRPV4-like current



in atrial fibroblasts. TRPC6 can be elicited by activation of  $G_q$ -like receptor activation (Figure 3H-I). However, neither using LPA to activate EDG7 receptor (Figure 3G) nor including GTP $\gamma$ S in the pipette solution (Online Figure III) was effective in inducing TRPC6-like current in atrial fibroblasts. These results, summarized in Figure 3, indicate that TRPV2, TRPV4, and TRPC6 do not constitute functional currents even though their expression was detectable by RT-PCR (Online Figure I). Therefore, it is unlikely that TRPV2, TRPV4 or TRPC6 contribute to  $Ca^{2+}$  entry in human atrial fibroblasts.

It appears that TRPC1 is abundantly expressed at mRNA level (Online Figure I) in human atrial fibroblasts. Since TRPC1 may form heteromeric channels with TRPC4 or TRPC5<sup>49</sup>, we attempted to record heteromeric currents with I-V relations similar to those of TRPC1/TRPC4 (or TRPC1/TRPC5). However, including GTP $\gamma$ S in the pipette solution failed to induce any TRPC1/TRPC4- or TRPC1/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 Figure 5, store-depletion by 5  $\mu$ M thapsigargin (Tg) 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 Tg, 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.

### TRPM7 is markedly up-regulated in atrial fibroblasts from AF patients

It has been shown that fibrosis associated structural remodeling is a major contributor to AF<sup>7</sup>. However, the  $Ca^{2+}$  signaling mechanism in fibrogenesis is unknown. Our findings that TRPM7 is the major  $Ca^{2+}$ -permeable channel in human atrial fibroblasts provided us an excellent opportunity to explore how  $Ca^{2+}$  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 vs. 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. Since measurement of outward current amplitude gives a more accurate assessment, we used outward current amplitude measured at +100 mV for comparison (Figure 4C-D). The average TRPM7 current density was  $32.5 \pm 2.3$  pA/pF in fibroblasts from AF patients (88 cells from 7 patients) and  $11.1 \pm 0.5$  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 due to the limited amount of tissues and limited number of fibroblasts. However, we observed that whereas we were able to obtain 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 if the increased TRPM7 currents result in enhanced  $\text{Ca}^{2+}$  influx, we used ratio  $\text{Ca}^{2+}$  imaging method to compare the  $\text{Ca}^{2+}$  entry through TRPM7 in fibroblasts isolated from AF and NSR patients. Since TRPM7 channels conduct small inward currents and are constitutively active, we used 20 mM  $\text{Ca}^{2+}$  in order to obtain better signal/noise ratio. In addition, cells were briefly exposed to a nominally divalent free solution (DVF) in order to eliminate any blockade of TRPM7 by external  $\text{Mg}^{2+}$ . As shown in Figure 5, the peak  $\text{Ca}^{2+}$  influx in fibroblasts from AF patients was 10-fold greater than  $\text{Ca}^{2+}$  influx in the fibroblasts from NSR patients. This result not only further indicates that TRPM7 is the major  $\text{Ca}^{2+}$ -permeable channel responsible for  $\text{Ca}^{2+}$  influx in human atrial fibroblasts, but also strongly implies that TRPM7-mediated  $\text{Ca}^{2+}$  signals may play an important role in AF-associated fibrogenesis.

### TRPM7 is required for differentiation of human atrial fibroblasts

As the myofibroblast is the major cell type that synthesizes growth factors, cytokines, chemokines, and extracellular matrix (ECM) proteins, and plays a pivotal role in fibrogenesis<sup>13, 19</sup>, 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 hrs 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 up-regulated 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 hrs in 10% FBS media) for 72 hrs. Fibroblasts were then cultured in 10% FBS media for 24 hr, followed by  $\text{Ca}^{2+}$  influx and differentiation assessment. As shown in Figure 6C-D, TRPM7-shRNA significantly decreased  $\text{Ca}^{2+}$  influx and inhibited basal AF fibroblast differentiation, indicating that TRPM7-mediated  $\text{Ca}^{2+}$  entry contributes to basal differentiation of AF fibroblasts.

We further investigated whether TRPM7 is involved in TGF- $\beta$ 1 induced differentiation. Human atrial fibroblasts from NSR patients were treated with TRPM7-shRNA and scramble shRNA (Scr-shRNA). TGF- $\beta$ 1 was then applied to induce differentiation after cells were serum starved for 24 hours. As shown in Figure 7, without TGF- $\beta$ 1 (10 ng/ml) treatment, there were 30.0% myofibroblasts in the Scr-shRNA treated group and 25.4% myofibroblasts in the TRPM7-shRNA treated group. This higher basal differentiation of NSR fibroblasts in comparison with that in Fig. 6 was due to that the fibroblasts were culture in 10% FBS media for more than 84 hrs, and there is a time-dependent increase in basal differentiation rate when fibroblasts were cultured in 10% FBS media (Online Figure VII). After TGF- $\beta$ 1 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- $\beta$ 1 in TRPM7-shRNA treated fibroblasts suggests that TRPM7 plays a role in TGF- $\beta$ 1 induce atrial fibroblast differentiation.

To understand how TRPM7 may be involved in fibroblast differentiation, we quantified changes of  $\alpha$ -SMA protein in scramble- and TRPM7-shRNA treated cells by Western blot. This experiment was performed by using mouse fibroblasts due to the limited number of human fibroblasts. To rule out other factors such as residual TGF $\beta$ <sup>50</sup> in serum which may influence differentiation, we applied the TGF- $\beta$ 1 receptor blocker SB431542 as a control for TGF- $\beta$ 1 treatment. As shown in Figure 7C, the increased protein expression of  $\alpha$ -SMA induced by TGF- $\beta$ 1 was significantly reduced by TRPM7 shRNA, which is consistent with the immunostaining result in human fibroblasts (Figure 7A-B).

### Role of TRPM7 in TGF- $\beta$ 1 induced fibroblast proliferation

We next investigated the role of TRPM7 in proliferation of fibroblasts. Human cardiac fibroblasts treated with scramble or TRPM7-shRNA were exposed to TGF- $\beta$ 1 (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- $\beta$ 1.

### Regulation of TRPM7 by TGF- $\beta$ 1

As TGF- $\beta$ 1 is the most potent fibrogenesis stimulator, we studied whether expression of TRPM7 is modulated by TGF- $\beta$ 1. In this experiment, fibroblasts were treated with TGF- $\beta$ 1 or the TGF- $\beta$ 1 receptor blocker SB431542. TRPM7 currents were recorded after the human atrial fibroblasts were treated for 24 hr. As shown in Figure 8A-B, TRPM7 current density was increased by about 80% by TGF- $\beta$ 1. Ca<sup>2+</sup>-influx in TGF- $\beta$ 1 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 qPCR using mouse cardiac fibroblasts was increased by 2.8 fold. Although other Ca<sup>2+</sup>-permeable channel genes were also up-regulated by TGF- $\beta$ 1 (Online Figure IX), TRPM7 is the only functional channel which can be measured by current recording in human atrial fibroblasts (Figure 2-3) and in mouse fibroblasts before and after after TGF- $\beta$ 1 treatment (data not shown). Moreover, knocking down the abundantly expressed TRPC1 did not alter Ca<sup>2+</sup> influx or differentiation of human atrial fibroblasts (Online Figure IV). Therefore, it is likely that TRPM7 plays a major role in mediating Ca<sup>2+</sup> in atrial fibroblasts under normal conditions and upon TGF- $\beta$  stimulation. To further understand the correlation between the changes of TRPM7 induced by TGF $\beta$ 1 and fibroblast differentiation, we tested TRPM7 protein and  $\alpha$ -SMA expression levels by using mouse cardiac fibroblasts. Figure 8C-D shows that TGF- $\beta$ 1 increased TRPM7 and  $\alpha$ -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- $\beta$ 1 (Fig. 8E-F). These results further suggest that up-regulation of TRPM7 underlies the Ca<sup>2+</sup> signaling mechanism for TGF- $\beta$ 1 induced fibroblast differentiation and collagen production, and that TRPM7 may play a role in TGF- $\beta$ 1 induced atrial fibrogenesis.

## DISCUSSION

To our knowledge, this is the first study to report a pathophysiological function of the Ca<sup>2+</sup>-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 Ca<sup>2+</sup> signaling in human atrial fibroblasts. Second, we demonstrate that TRPM7 mediated Ca<sup>2+</sup> plays an essential role in atrial fibroblasts proliferation and differentiation. Third, we show that TRPM7 is markedly up-regulated in AF patients, and that up-regulation of TRPM7 confers increased fibroblast differentiation in AF patients. Our results suggest that TRPM7 mediated Ca<sup>2+</sup> may play a pivotal role in fibrogenesis in human AF.

### TRPM7 underlies the molecular mechanism of Ca<sup>2+</sup> signaling in human atrial fibroblasts

Ca<sup>2+</sup> signals are essential for various cellular functions. Although the importance of fibrosis in various cardiac diseases has been well appreciated, the Ca<sup>2+</sup> signaling mechanisms in cardiac fibrogenesis are not known. Here we demonstrate that TRPM7 is abundantly expressed in human atrial fibroblasts, and is the major Ca<sup>2+</sup>-permeable channel which is responsible for Ca<sup>2+</sup> influx in human atrial fibroblasts.

TRPM7 is a Ca<sup>2+</sup>-permeable cation channel<sup>36, 37</sup>. It is constitutively active and brings Ca<sup>2+</sup> into cells under physiological conditions. High intracellular Mg<sup>2+</sup> concentration blocks TRPM7. However, TRPM7 channels show ~50% of their maximum activity at physiological Mg<sup>2+</sup> levels (0.5–1 mM)<sup>36, 51</sup>. Extracellular low pH potentiates TRPM7 inward current<sup>42</sup>, and oxidative stress activates TRPM7<sup>40</sup>. Membrane stretch<sup>52</sup> and shear stress<sup>38</sup> 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<sup>2+</sup>-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<sup>2+</sup> signaling in the fibroblasts. TRPV4 is sensitive to stretch<sup>53</sup>, and can be specifically activated by 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<sub>q</sub>-linked receptor stimulation, DAG or OAG, or by direct application of GTPγS in the pipette solution. We could not activate TRPC6-like current by LPA, which activates the EDG7 receptor<sup>54</sup>, or by including GTPγS 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<sup>48</sup>. We do not know the reason for this discrepancy, but human and rat cardiac fibroblasts may express TRPC6 differently.

Store-depletion activated Ca<sup>2+</sup> current (I<sub>CRAC</sub>) contributes to Ca<sup>2+</sup> influx and plays an important role in cellular functions in a variety of cells<sup>55, 56</sup>. In human atrial fibroblasts, we could not detect I<sub>CRAC</sub> currents, and did not find that store-depletion could induce Ca<sup>2+</sup> influx (Online Figure V) in human atrial fibroblasts. I<sub>CRAC</sub>, TRPV2, TRPV4, TRPC1 and TRPC6 currents were not present in AF fibroblasts. Even though we found that TGF-β1 up-regulates 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 TGF-β1, suggesting that TRPM7 is the major channel mediating Ca<sup>2+</sup> entry in atrial fibroblasts. When TRPM7 was knocked down, Ca<sup>2+</sup> 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<sup>2+</sup> entry in fibroblasts. Taken together, our results indicate that TRPM7 is the major Ca<sup>2+</sup>-permeable channel which is responsible for Ca<sup>2+</sup> influx in human atrial fibroblasts.

### Up-regulation of TRPM7 may underlie TGF-β1 induced fibroblast differentiation in AF patients

We found that TRPM7 is markedly up-regulated in the fibroblasts from AF patients. Both current density and Ca<sup>2+</sup> influx mediated by TRPM7 from AF patients were significantly larger than those from NSR patients (Figure 4). Due to 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. Since the only differences between AF patients and NSR patients is the presence of atrial fibrillation versus normal sinus rhythm (Online Table I), it appears that atrial fibrillation is the only variable that correlates with the up-regulated 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- $\beta$ 1 treatment. This inherent tendency to differentiation could be due to the increased expression level of TRPM7 when the fibroblasts were exposed to the residual amount of TGF $\beta$  in the culture media containing serum<sup>50</sup>. Indeed, knocking-down TRPM7 by TRPM7-shRNA not only markedly reduced basal differentiation of AF fibroblasts (Fig. 6C-D), but also substantially decreased the number of differentiated myofibroblasts and the expression level of  $\alpha$ -SMA induced by TGF- $\beta$ 1, 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  $\text{Ca}^{2+}$  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<sup>14, 16</sup>, among which TGF- $\beta$ 1 is the most potent stimulator in both *in vivo* and *in vitro* conditions. Interestingly, we found that TRPM7 expression was significantly increased by TGF- $\beta$ 1 in cultured fibroblasts (Figure 8). The increased TRPM7 expression level induced by TGF- $\beta$ 1 parallels the fibroblast differentiation as assessed by  $\alpha$ -SMA expression and collagen production (Figure 8C-F). These results indicate that TRPM7 is indispensable for TGF- $\beta$ 1 induced differentiation, and that the up-regulated TRPM7 may synergize with TGF $\beta$ 1 in inducing fibroblast differentiation. It is possible that the up-regulation of TRPM7 by TGF- $\beta$ 1 in cultured fibroblasts represents the major mechanism by which TRPM7 is up-regulated in AF patients *in vivo*. Taken together, our results suggest the following model: In human atria, TGF- $\beta$ 1 is activated by mechanical stretch, oxidative stress, or other stimuli. TGF- $\beta$ 1 up-regulates TRPM7, which in turn brings more  $\text{Ca}^{2+}$  for TGF- $\beta$ 1 to induce differentiation. The differentiated myofibroblasts synthesize ECM and cytokines, and excessive accumulation of ECM forms fibrosis. Cytokines can in turn stimulate TGF- $\beta$ 1 and cause up-regulation of TRPM7, leading to perpetuation of the fibrogenesis process.

### Potential mechanism by which $\text{Ca}^{2+}$ signals are involved in TGF- $\beta$ 1 induced fibrosis

Atrial fibrosis plays an important role in the pathology of AF. Although three interrelated pathways including the renin-angiotensin system, TGF- $\beta$ 1 and the oxidative stress pathways have been shown to be involved in fibrosis<sup>57-63</sup>, many fundamental aspects of fibrogenesis are still to be established<sup>6, 57</sup>. Our results indicate that TRPM7-mediated  $\text{Ca}^{2+}$  signal is another fundamental factor contributing to atrial fibrogenesis cascade.

How does the  $\text{Ca}^{2+}$  signal contribute to fibrosis formation? There are several possibilities. First,  $\text{Ca}^{2+}$ -dependent but TGF- $\beta$ 1-independent pathways may control fibrotic gene expression<sup>64-67</sup>. Second,  $\text{Ca}^{2+}$  signals may influence TGF- $\beta$ 1 via the Smad-independent pathway<sup>68-70</sup>. Third, TGF- $\beta$ 1 can induce  $\text{Ca}^{2+}$  entry<sup>71, 72</sup> or integrate with reactive oxygen species (ROS) associated  $\text{Ca}^{2+}$  influx and therefore influence ECM protein synthesis<sup>73</sup> through a ROS activated calcineurin pathway. As TRPM7 can be activated by ROS<sup>40</sup>, it is possible that the ROS activated calcineurin pathway may have contributed to the up-regulation of TRPM7 by TGF- $\beta$ 1 in human atrial fibroblasts. Fourth, TRPM7-mediated  $\text{Ca}^{2+}$  signals may activate the calcineurin pathway and produce synergistic effect with TGF- $\beta$ 1 (Smad-dependent pathway) in regulating gene expression. Further studies are required to understand how TRPM7 mediated  $\text{Ca}^{2+}$  signals contribute to TGF- $\beta$ 1 induced fibrogenesis. Nevertheless, our findings that TRPM7 is the molecular basis for the major  $\text{Ca}^{2+}$  entry

channel in cardiac fibroblasts provide a necessary tool to unravel the mechanisms by which  $\text{Ca}^{2+}$  signals contribute to fibrogenesis.

### Potential significance

Atrial fibrosis can be triggered by various factors<sup>13</sup> via different pathways<sup>57</sup>. Blocking one pathway may not be able to attenuate or eliminate fibrosis given the complex pathological conditions. The TRPM7-mediated  $\text{Ca}^{2+}$  signals which are necessary for TGF- $\beta$ 1 induced fibrogenesis may serve as a common pathway in the fibrogenesis cascade. If so, inhibition of  $\text{Ca}^{2+}$  entry through TRPM7 may attenuate cardiac fibrosis regardless of the original stimuli.

TRPM7 has been shown to be essential for cell viability<sup>74, 75</sup> and is responsible for  $\text{Ca}^{2+}$  overload induced cell death under anoxia conditions<sup>40</sup>. Although previous studies suggested that TRPM7 is involved in  $\text{Mg}^{2+}$  homeostasis<sup>74, 76</sup>, knocking out of the *TRPM7* gene disrupts embryonic development but does not alter  $\text{Mg}^{2+}$  homeostasis<sup>39</sup>, indicating that  $\text{Ca}^{2+}$  permeation may confer major channel functions of TRPM7. The contribution of the TRPM7-mediated  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -permeable cation channel TRPM7 underlies  $\text{Ca}^{2+}$  signaling mechanisms in the cardiac fibrogenesis, making TRPM7 the first known  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  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.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## NOVELTY AND SIGNIFICANCE

### What is known?

- Atrial fibrosis is a hallmark feature of structural remodeling in atrial fibrillation (AF).
- Previous studies have suggested that  $\text{Ca}^{2+}$  entry is essential for fibroblast function. However, the  $\text{Ca}^{2+}$  signaling mechanisms in cardiac fibrogenesis have not elucidated.

### What new information does this article contribute?

- Here we report that the  $\text{Ca}^{2+}$ -permeable cation channel TRPM7 is the major  $\text{Ca}^{2+}$ -permeable channel in human atrial fibroblasts.
- TRPM7-mediated  $\text{Ca}^{2+}$  entry is essential for TGF- $\beta$ 1-induced fibroblast differentiation.
- TRPM7 is significantly up-regulated in AF fibroblasts and plays a key role in enhanced fibroblast proliferation, differentiation, and collagen production during AF.



## Summary

Atrial fibrosis is a major factor contributing to 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  $\text{Ca}^{2+}$  signaling mechanisms in fibroblasts and how  $\text{Ca}^{2+}$  signals contribute to fibrogenesis in AF. We discovered that TRPM7, a  $\text{Ca}^{2+}$ -permeable cation channel that possesses protein kinase function, is the key  $\text{Ca}^{2+}$ -permeable channel in human atrial fibroblasts. TRPM7 and TRPM7-mediated  $\text{Ca}^{2+}$  signals were drastically up-regulated 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  $\text{Ca}^{2+}$ -permeable channel in cardiac fibroblasts, which helps to elucidate how  $\text{Ca}^{2+}$  signals contribute to fibrogenesis in human AF. Also, our observation that TRPM7-mediated  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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.

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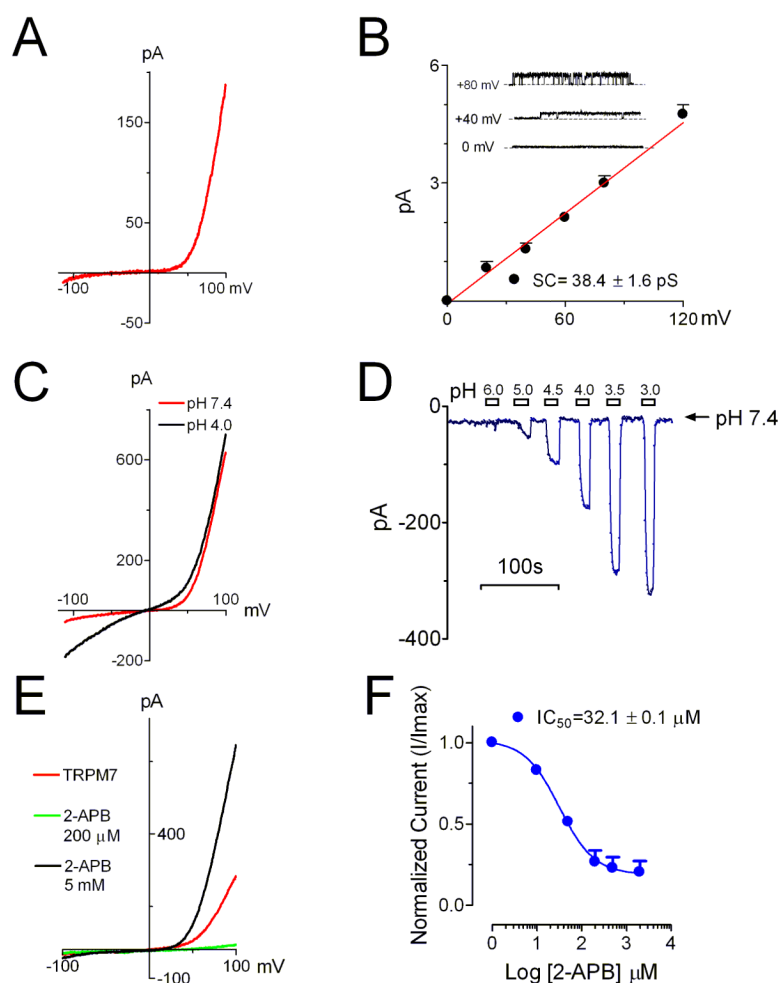
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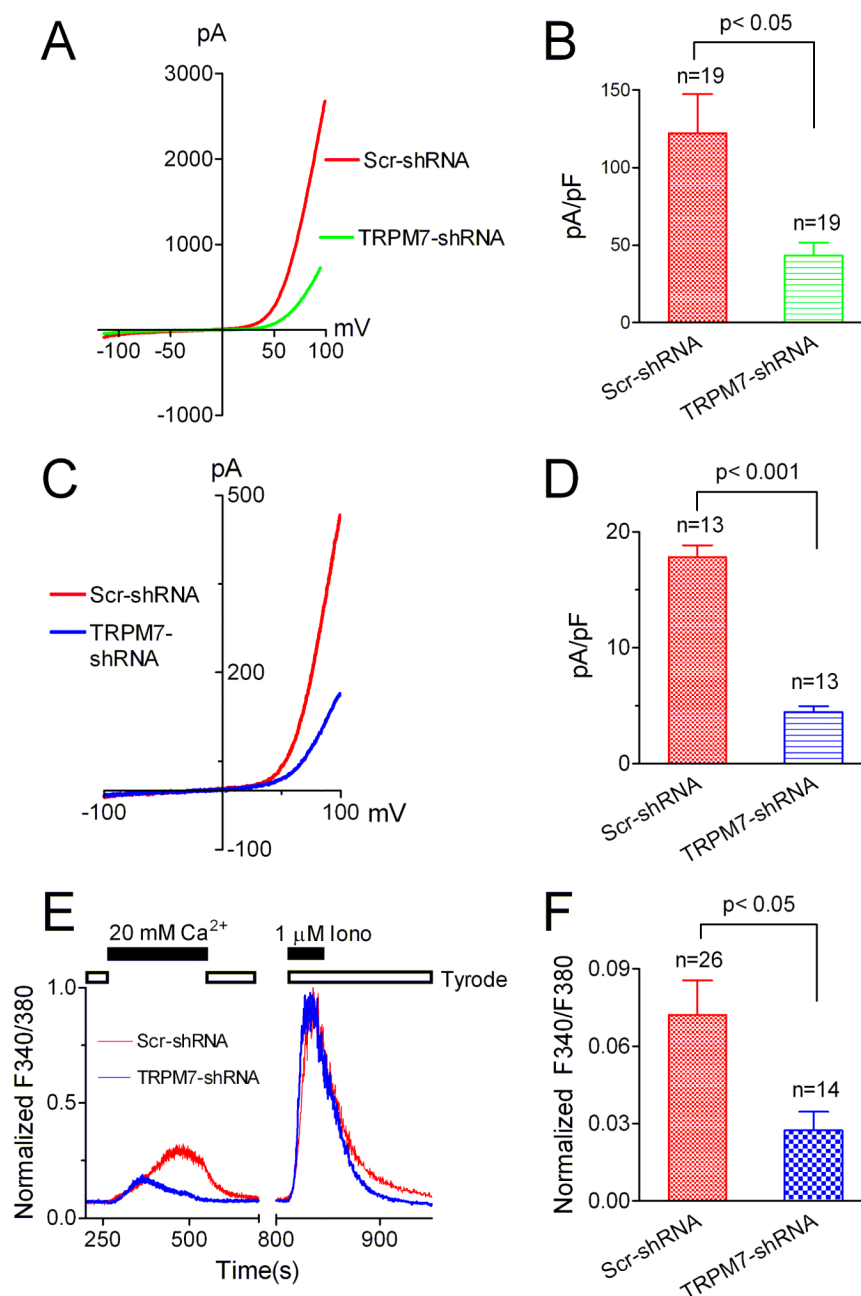
## Non-standard Abbreviations and Acronyms

<b>AF</b>	Atrial Fibrillation
<b>NSR</b>	Normal sinus rhythm
<b>TRPM7</b>	Transient receptor potential melastatin related 7
<b>TRP</b>	Transient receptor potential
<b>TRPV2</b>	Transient receptor potential V2
<b>TRPV4</b>	Transient receptor potential V4
<b>TRPC6</b>	Transient receptor potential C6
<b>CABG</b>	Coronary Artery Bypass Graft
<b>AngII</b>	Angiotensin II
<b>2-APB</b>	2-Aminoethoxydiphenyl borate
<b>TGF-<math>\beta</math>1</b>	Transforming growth factor $\beta$ 1
<b>ECM</b>	Extracellular matrix proteins
<b>shRNA</b>	Small hairpin RNA
<b>4<math>\alpha</math>-PDD</b>	4 $\alpha$ -Phorbol-12,13-didecanoate
<b>DAG</b>	Diacylglycerol
<b>OAG</b>	1-Oleoyl-2-acetyl-sn-glycerol
<b><math>\alpha</math>-SMA</b>	alpha-Smooth Muscle Actin
<b>EDG7</b>	a Lysophospholipid/Lysosphingolipid Receptor
<b>ROS</b>	Reactive oxygen species
<b>Tg</b>	Thapsigargin
<b>CCH</b>	Carbachol
<b>LPA</b>	Oleoyl-lysophosphatidic acid



**Figure 1.**

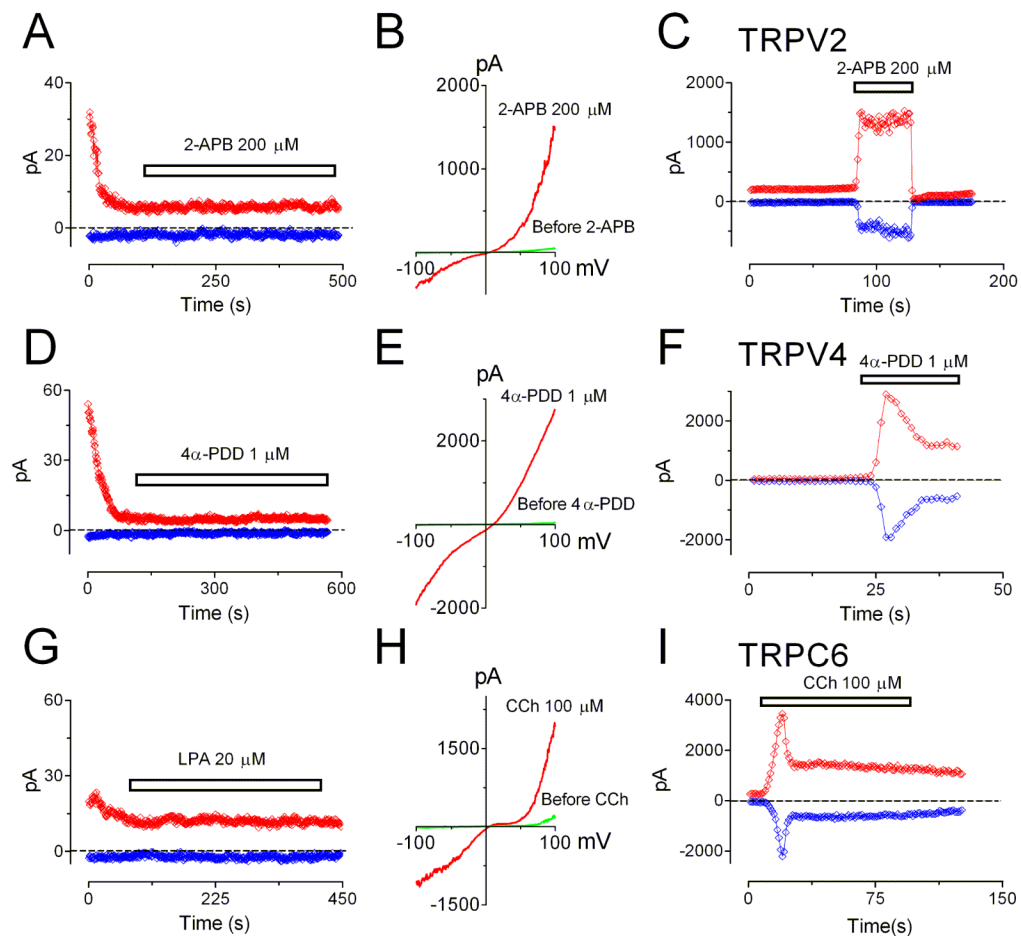
The endogenous TRPM7-like channels in human atrial fibroblasts resemble the characteristics of recombinant TRPM7. A, Representative TRPM7-like currents elicited by a voltage ramp protocol ranging from -120 to +100 mV. B, Single channel conductance of TRPM7-like channel was  $38.4 \pm 1.6$  pS ( $n=6$ ). Representative recordings from inside-out patches are shown in the inset. C, TRPM7-like inward current was potentiated by external acidic pH solution. D, Concentration-dependent effects of external protons on TRPM7-like currents. E, Biphasic effects of 2-APB on TRPM7-like currents, with inhibition and potentiation at low and high 2-APB concentrations, respectively. F, Concentration-dependent inhibition of 2-APB on TRPM7-like currents. Best fit with Hill equation yielded  $IC_{50}=32.1 \pm 0.1$   $\mu$ M,  $n_H=1.1$  (mean  $\pm$  SEM,  $n=5$ ).



**Figure 2.**

TRPM7 underlies the molecular basis of the TRPM7-like channel and that TRPM7-like channel mediated  $\text{Ca}^{2+}$  entry constitutes the major  $\text{Ca}^{2+}$  influx in human atrial fibroblasts. A, Typical recordings of TRPM7 over-expressed in HEK-293 cells after treatment with scramble (Scr)-shRNA and TRPM7-shRNA. B, Average current amplitude of TRPM7. TRPM7-shRNA decreased TRPM7 by about 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,  $\text{Ca}^{2+}$  influx mediated by TRPM7-like channel.  $\text{Ca}^{2+}$  influx was elicited by 20 mM  $\text{Ca}^{2+}$ . The amplitude of  $\text{Ca}^{2+}$  influx was normalized to the peak  $\text{Ca}^{2+}$  influx induced by 1  $\mu\text{M}$  Ionomycin (Iono).

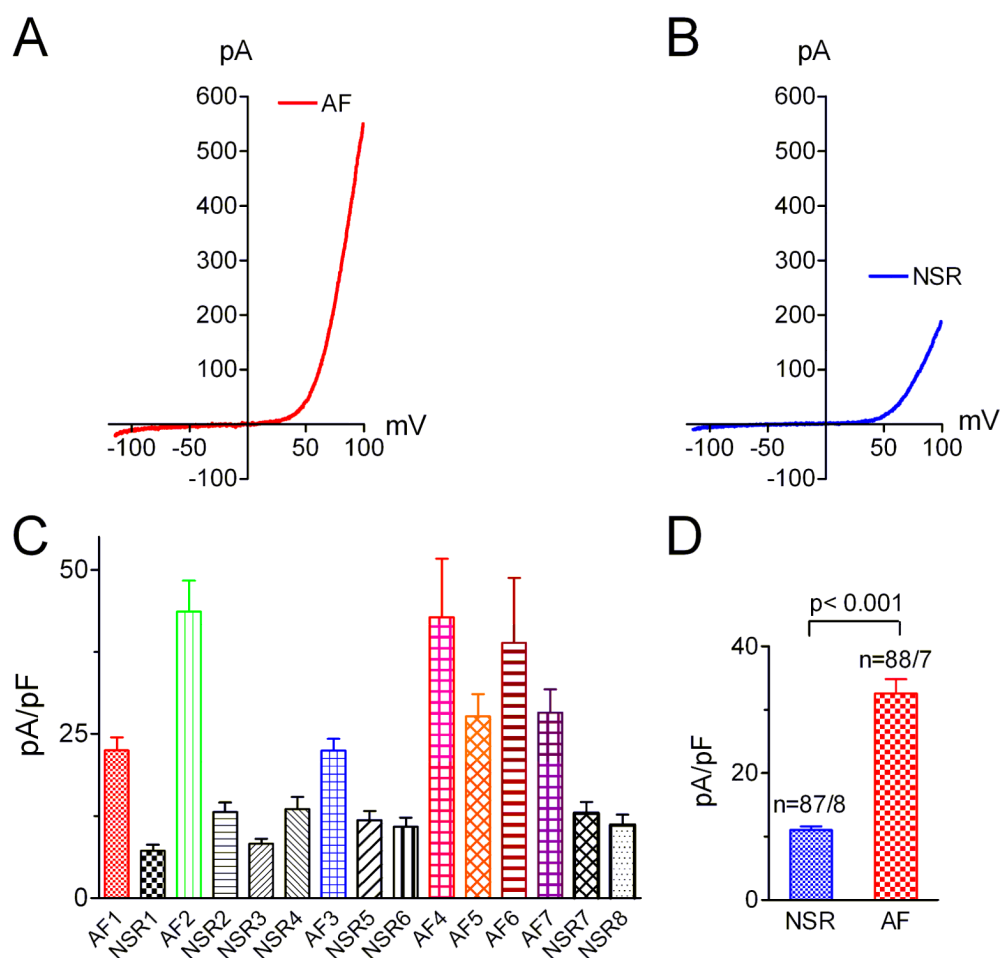
TRPM7-shRNA significantly reduced the  $\text{Ca}^{2+}$  influx (Blue) in comparison with the  $\text{Ca}^{2+}$  influx obtained in scramble shRNA treated cells (Red). F, TRPM7 shRNA reduced  $\text{Ca}^{2+}$  influx in human atrial fibroblasts by 62% measured by normalized peak amplitude.



**Figure 3.**

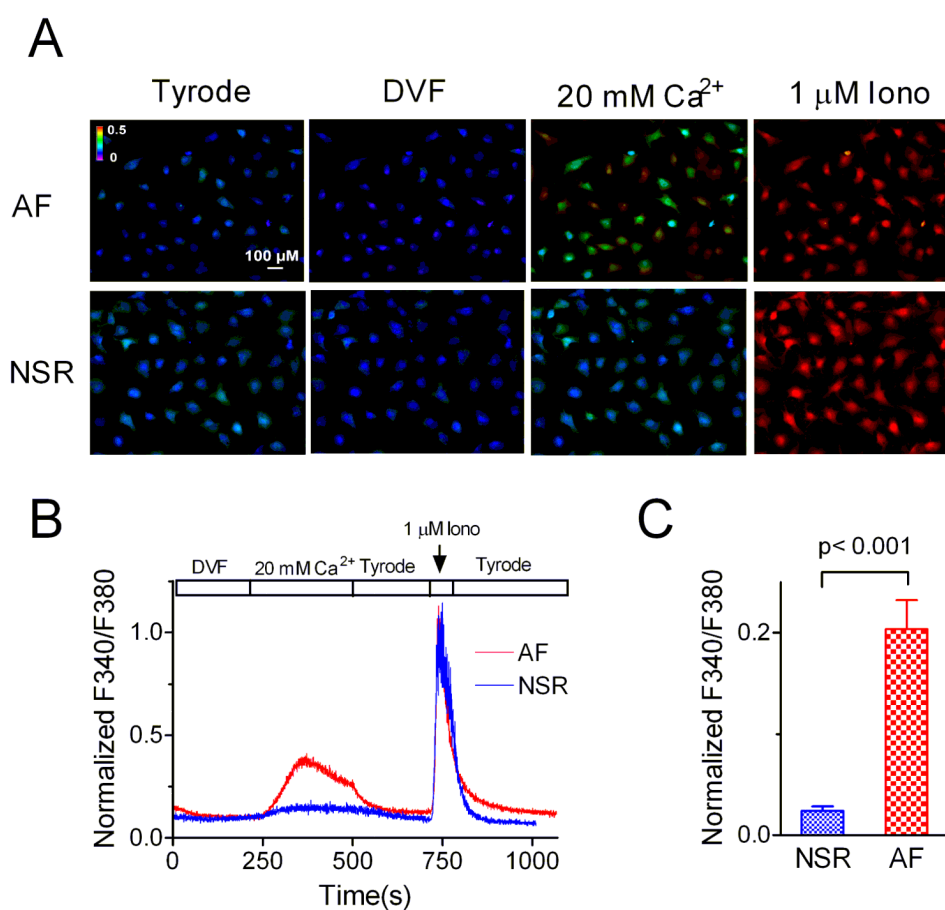
TRPV2, TRPV4 and TRPC6 currents were not detectable in atrial fibroblasts. A, TRPV2-like current was not elicited by 2-APB (200  $\mu$ M) in human atrial fibroblasts. B, Typical recording of TRPV2 over-expressed in HEK293 cells. C, Time-dependent changes of inward and outward currents of TRPV2 in HEK293 cells. D, 4 $\alpha$ -PDD (1  $\mu$ M) failed to induce TRPV4-like current in human atrial fibroblasts. E, Representative recording of recombinant TRPV4 activated by 1  $\mu$ M 4 $\alpha$ -PDD in HEK293 cells. F, Time-dependent changes of inward and outward currents of TRPV4 in HEK293 cells. G, Activation of Gq-linked EDG7 receptors in fibroblasts by Oleoyl-lysophosphatidic acid (LPA) could not activate TRPC6-like current. H, Recombinant TRPC6 current activated by Carbachol (CCh) in HM1 cells (HEK293 cells with over-expression 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 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.



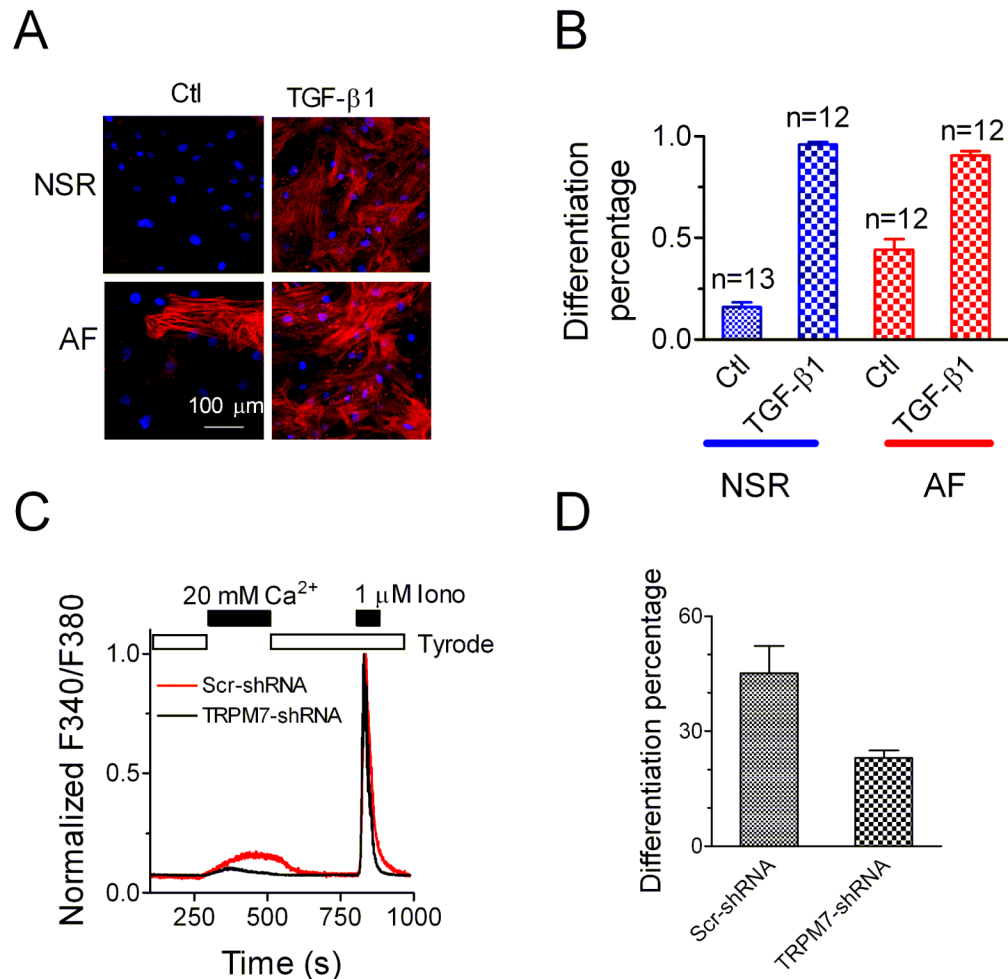


**Figure 4.**

Up-regulation of endogenous TRPM7 in atrial fibroblasts from AF patients. A, Typical TRPM7 recording in a freshly isolated fibroblast from AF patient. B, TRPM7 recorded in a freshly isolated fibroblast from NSR patient. C, Average current density of TRPM7 from each AF and NSR patient. Note that current amplitudes from all the AF patients are larger than the biggest current amplitude in NSR patients. D, Average current density of endogenous TRPM7 from all the fibroblasts in AF and NSR patients.

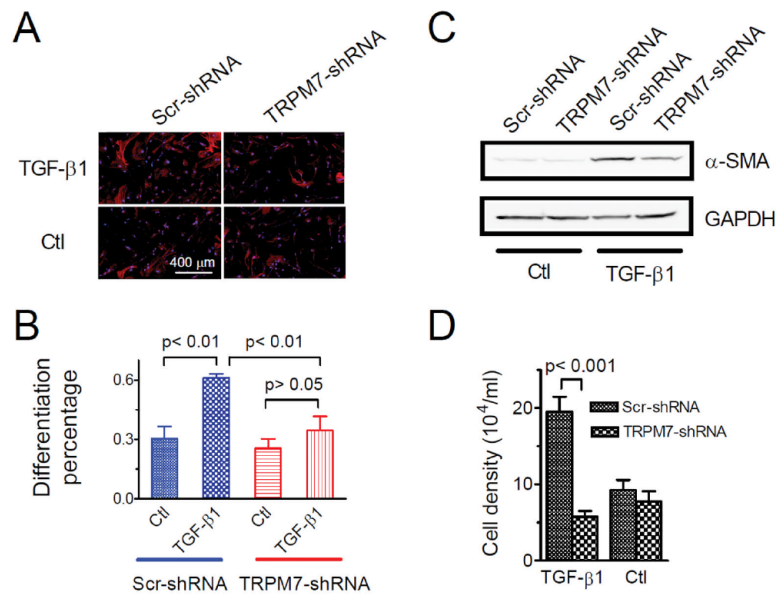


**Figure 5.** Increased  $\text{Ca}^{2+}$  influx in AF fibroblasts in comparison with that in NSR fibroblasts. A, Changes of F340/F380 ratio under indicated conditions. There was more  $\text{Ca}^{2+}$  influx in the fibroblasts from AF patients when the cells were perfused with 20 mM  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  influx was measured after the fibroblasts were cultured for 12 hrs which allows fibroblasts to attach to the coverslips. B, Normalized F340/F380 ratio from a representative experiment using the cells from AF and NSR patients. C, Average ratio of F340/F380 in fibroblasts from AF and NSR patients. The ratio was normalized to the maximal response induced by 1  $\mu\text{M}$  ionomycin.

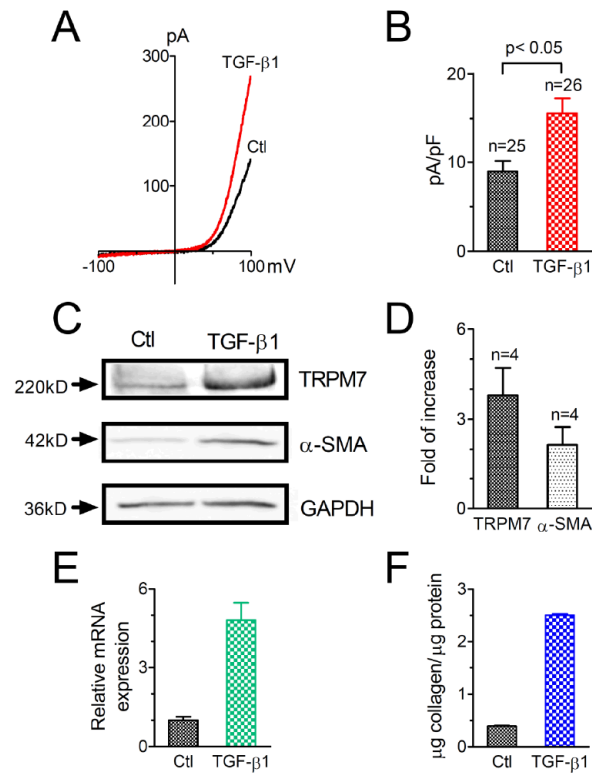


**Figure 6.**

Myofibroblast differentiation in cells from AF and NSR patients. A, Immunostaining with antibody against  $\alpha$ -SMA in fibroblasts from NSR and AF patients, respectively. Fibroblasts from AF patients show a higher percentage of differentiated myofibroblasts in the absence of TGF- $\beta$ 1 treatment ( $P < 0.01$ ). TGF- $\beta$ 1 (10 ng/ml) treatment induced 90% differentiated myofibroblasts in cells from both AF and NSR patients.  $\alpha$ -SMA is in red, and nuclei are labeled in blue by TO-PROIII. B, Mean percentage of differentiated myofibroblasts in AF or NSR patients with or without TGF- $\beta$ 1 treatment. The basal differentiation (without TGF- $\beta$ 1 treatment) in AF patients is higher than that in NSR patients ( $P < 0.01$ ). C, TRPM7-shRNA decreased Ca<sup>2+</sup> influx in atrial fibroblasts from AF patient. The average decrease of Ca<sup>2+</sup> influx by TRPM7-shRNA was 64%. D, The basal differentiation of atrial fibroblasts from AF patient was significantly decreased by TRPM7-shRNA.

**Figure 7.**

Effects of TRPM7 on fibroblast differentiation and proliferation. A, Differentiated myofibroblasts detected by immunostaining of  $\alpha$ -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 Scr- or TRPM7-shRNA treated cells. In TRPM7-shRNA treated cells, differentiation induced by TGF- $\beta$ 1 was not significantly different from that of cells without TGF- $\beta$ 1 induction ( $34.4 \pm 7.2\%$  and  $23.5 \pm 4.8\%$  respectively;  $p > 0.05$ ). In Scr-shRNA treated cells, the differentiation induced by TGF- $\beta$ 1 ( $61.0 \pm 1.9\%$ ) was significantly higher than that in control cells ( $30.4 \pm 6.1\%$ ). 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  $\mu$ M) 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. Please note the significant decrease of  $\alpha$ -SMA in TRPM7-shRNA treated cells. Similar results were obtained in three separate experiments. D, Effects of TRPM7-shRNA on human atrial fibroblast proliferation induced by TGF- $\beta$ 1 (10 ng/ml).



**Figure 8.**

Up-regulation of TRPM7 by TGF-β1. A, Representative recording of TRPM7 in control human atrial fibroblasts and in fibroblasts treated with 10 ng/ml TGF-β1. B, Average current amplitude of TRPM7 after TGF-β1 treatment in comparison with control cells. The current density was  $8.9 \pm 1.2$  (n=25) pA/pF in control and  $15.6 \pm 1.7$  (n=26) pA/pF in TGF-β1 treated group. C, Up-regulation of TRPM7 by TGF-β1 evaluated in mouse cardiac fibroblasts. Top: western blot results of TRPM7 in control and TGF-β1 treated groups; Middle: expression of α-SMA in control and TGF-β1 treated groups; Bottom: GAPDH used as loading control. D, Average increase in TRPM7 and α-SMA expression induced by TGF-β1. TRPM7 protein expression was increased by  $3.8 \pm 0.9$  fold (n=4) and α-SMA was increased by  $2.1 \pm 0.6$  fold (n=4). E-F, Increased collagen production in human atrial fibroblasts induced by TGF-β1 evaluated at mRNA (E) and protein (F) levels. Average data were from three separate experiments.