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ORIGINAL RESEARCH

Cadherin-11 Deficiency Attenuates Ang-II-Induced Atrial Fibrosis and Susceptibility to Atrial Fibrillation

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Background: Atrial fibrosis serves as a disease initiating mechanism in the development of atrial fibrillation. Angiotensin II (Ang-II), a key mediator for atrial fibrosis, aberrantly activates atrial fibroblasts (AFs) into myofibroblasts, resulting in subsequent excessive synthesis and deposition of extracellular matrix (ECM). Cadherin-11 (CDH11) is essential in the development of non-cardiac fibrotic diseases. In this study, we investigated its role in the pathogenesis and underlying mechanism of atrial fibrillation.

Methods: We obtained left atrial tissues from either patients with atrial fibrillation or Ang-II -induced atrial fibrosis mice. We utilized a global CDH11 knockout mouse ($CDH11^{-/-}$) model to determine the effect of CDH11 on AF cell proliferation, migration, ECM synthesis/deposition. RNA-Seq of isolated AFs from $CDH11^{-/-}$ or normal mice was performed and differential expressed genes were analyzed. The mouse susceptibility to atrial fibrillation was examined by cardiac electrophysiology.

Results: We found that cadherin-11 was significantly up-regulated in fibrotic atrial tissue from patients with atrial fibrillation and Ang-II-induced mice. Both normal and $CDH11^{-/-}$ mice did not develop atrial fibrosis at resting state. However, after Ang-II infusion, unlike severe atrial fibrosis occurred in normal mice, $CDH11^{-/-}$ mice displayed a reduced atrial fibrosis. Atrial fibroblasts with CDH11 deletion from $CDH11^{-/-}$ mice showed reduction in Ang-II-induced cell proliferation, migration and ECM synthesis/deposition, indicating the involvement of CDH11 in atrial fibrosis. Consistently, RNA-Seq of CDH11-null AFs uncovered significant decrease in pro-fibrotic gene expression. In addition, we identified reduction of transcripts associated with Smad2/3, ERK1/2 and JNK pathways. Further, $CDH11^{-/-}$ mice showed a significantly attenuated Ang-II-induced susceptibility to atrial fibrillation.

Conclusion: Our results indicate that CDH11 potentiates Ang-II-induced activation of AFs. The pathogenesis of atrial fibrosis is through CDH11 mediated stimulation of Smad2/3, ERK1/2 and JNK pathways. Thus, CDH11 might serve as a novel therapeutic target for ameliorating the development of atrial fibrillation.

Keywords: atrial fibrillation, atrial fibrosis, atrial fibroblast, cadherin-11, angiotensin-II

Introduction

Atrial fibrillation is one of the most common arrhythmias disorders in the clinical practice. Patients not only have reduced quality of life due to fibrillation itself but also are highly susceptible to stroke and heart failure, leading to increased mortality and morbidity.[1] In the current aging society, atrial fibrillation has become a huge

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burden to health-care and economy.[2] Therefore, research in elucidating the disease pathogenesis of atrial fibrillation is highly needed.

The major pathological feature of atrial fibrillation is atrial structural remodeling, which includes an enlargement in the cavity and fibrosis in the wall tissue.[3,4] Phenotypic change of the atrial fibroblasts (AFs), such as AF differentiation to myofibroblast, is the key initial step and usually occurs under continuous stimulations of endocrine peptide hormones, including angiotensin-II (Ang-II) and transforming growth factor β (TGF-β). Subsequently, excessive myofibroblast proliferation, migration, synthesis/deposition of extracellular matrix (ECM) proteins gradually form fibrotic lesions in local tissue.[5,6] Fibrotic lesions interfere conduction of the cardiac electrical pacing signal and result in re-entry and increased susceptibility to atrial fibrillation.[7,8] Therefore, stopping or slowing the development of atrial fibrosis is a key strategy to prevent pathogenesis of atrial fibrillation.

CDH11 is a type II cadherin family protein and its major function is to mediate homologous (hemophilic) cell adhesion.[9] CDH11 is associated with a mesenchymal phenotype in cells during development and is also involved in many other functions, including tissue morphogenesis, cell proliferation and migration.[10,11] Recent studies have revealed its essential roles in fibrotic diseases of the skin, lung and cardiac valve, as well as in mesenchymal stem cell differentiation.[12–15] However, whether CDH11 plays role in atrial fibrosis and what is the underlying mechanisms remain unknown.

In this study, we found that CDH11 was up-regulated in atrial tissues both from patients with atrial fibrillation and Ang-II-infused atrial fibrosis mice. To determine the role of CDH11 in atrial fibrosis, we utilized CDH11 deletional mutant mice ($CDH11^{-/-}$) we previously generated.[16] In in vitro AFs isolated from $CDH11^{-/-}$ mice, we found deletion of CDH11 attenuated Ang-II-induced proliferation, migration and ECM synthesis/deposition. In vivo, $CDH11^{-/-}$ mice showed significantly attenuated atrial fibrosis, LA dimensions and susceptibility to atrial fibrillation. RNA-Seq analysis of AFs isolated from $CDH11^{-/-}$ mice showed that CDH11 deletion lead to the reduction of transcripts related to pro-fibrosis, compared with that of AFs from normal control mice. In addition, we observed a significant reduction of transcripts pertaining to Smad2/3 and MAPK signaling pathways in differentiated AFs. Taken together, our results indicated that CDH11 plays a role in activating AFs and promotes their proliferation, migration and ECM synthesis/deposition through Smad2/3, ERK1/2 and JNK pathways. Therefore, targeting CDH11 is a novel avenue for drug-discovery for atrial fibrillation therapy.

Materials and Methods

Antibodies and Reagents

The following antibodies were purchased: CDH11 (Abcam, 4442), vimentin (Abcam, ab8978), collagen-III (Abcam, ab34710), α-SMA (Abcam, ab5694), JNK (Cell Signaling, #9252), pJNK1/2 (Cell Signaling, #9251), ERK1/2 (Abclonal, A10613), pERK1/2 (Abclonal, AP0472), P38 (Abclonal, A11340), pP38 (Abclonal, AP0297), Smad2/3 (Cell Signaling), p-Smad2 (Abcam), p-Smad3 (Abcam), Smad4 (Cell Signaling), TGF-β1 (Abclonal), TGF-βR1 (Abclonal), GAPDH (Beyotime, AG019) and goat anti-rabbit IgG H&L (Beyotime, A0423). Angiotensin-II (A9525) was purchased from Sigma-Aldrich and hIgG1 (110-HG) and hCDH11-Fc (1790-CA) were from R&D Systems. LY3200882 (TGF-β-Smad pathway inhibitor), U0126 (ERK1/2 inhibitor), SP600125 (JNK inhibitor) and SB203580 (P38-MAPK inhibitor) were purchased from Selleck Chemicals (USA).

Human Tissues

The study was approved by the ethical review committee of Xinhua Hospital and the clinical study complies with the Helsinki Declaration. Informed consents were provided to all subjects. Human left atrial (LA) appendages were cut out from patients with atrial fibrillation or sinus rhythm (SR) during coronary artery bypass graft surgery and the pathological data were summarized in Supplementary Table 1. Each LA sample was divided into three parts: for immunohistochemistry, quantitative real-time PCR (qRT-PCR) and Western blotting, respectively.

Animal Studies

The study was approved by the ethical review committee of Xinhua Hospital at SJTUSM and all procedures were conducted in compliance with both the Guide for the Animal Care and Use Committee of Xinhua Hospital and the guidance for the care and use of experimental animals published by NIH (the 8th Edition, NRC 2011). Mice with global knock-out of the CDH11 gene was crossbred over seven generations on C57BL/6 background, as described

previously. Six–eight week old male mice of both WT and CDH11$^{-/-}$ were used in this study.

Mice Model of Atrial Fibrosis

Mice were anesthetized intraperitoneally by sodium pentobarbital (50 mg/kg). An osmotic mini-pump (Alzet 2004, USA) was implanted subcutaneously for infusion of Ang-II (750 ng/kg/min) for 28 days, as we reported previously.[17] The control animals received saline infusion. On 28th day, echocardiography (Vivid 7, GE) and cardiac electrophysiology were performed. The mice were then euthanized by intraperitoneal injection with an overdose of pentobarbital sodium (200mg/kg). After sacrifice, mouse cardiac tissues were removed and prepared for appropriate analyses.

AFs Isolation and Cell Culture

AFs were isolated from male mice (C57BL/6) of 6~8 week-old that were sterilized by spraying their body surface with 70% alcohol, followed by sacrificing and harvesting the hearts. After washing off blood in pre-cooled PBS for 3~4 times, we cut the LA into approximately 1mm^3 tissue blocks and immersed the tissue in the digestive solution (1500U/mL collagenase type-II, 0.25% trypsin, and 15 mg/L BSA in DMEM). The digestion was processed in a 75 rpm shaker at 37°C for 20 minutes. Cells were then gently dissociated using an elbow pipetting, the supernatant was collected and mixed with a neutralizing DMEM containing 10% FBS. The cell suspensions were filtered through a 70μm mesh in diameter and were centrifuged at 1000 rpm for 5 minutes to collect the pellets. Finally, the pellets were re-suspended in DMEM supplemented with 10% FBS and were incubated in 37°C for 60 min. AFs were then obtained by differential attachment. AFs were maintained in high glucose Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cultures of AFs in the second passage were used for experiments.

Immunohistochemistry and Immunofluorescence

Atrial tissues from patients or mice were fixed with 10% formalin for 24 h, embedded in paraffin and sectioned with a microtome (4μm thickness). Both immunohistochemistry and immunofluorescence were performed, as described previously.[18] Picrosirius Red (PSR) staining is used to assess the degree of atrial fibrosis. The staining was photographed with a Zeiss fluorescence microscope and was assessed by Image-Pro Plus 6.0.

Flow Cytometry

The AFs were synchronized in serum-free medium for 48 hours. AFs were then stained with 250 μL of nuclear staining solution (10 mg propidium iodide, 0.1 mg trisodium citrate, and 0.03 mL Triton X-100 dissolved in 100 mL H_2O) at room temperature for 30 min in the dark. After adding 750 μL PBS, the DNA contents in each cycle was determined by FACSort and was analyzed using software ModFit (Becton Dickinson, USA). DNA ploidy allows discrimination of cells in G0/G1, S and G2/M phases of the cell cycle.

Treatment of AFs with Recombinant CDH11-Fc Fusion Protein

AFs were seeded at 300–400 cells/mm^2, cultured, and incubated in media containing human IgG1 Fc or human CDH11-Fc fusion protein at 10 μg/mL for 24h. Twenty-four hours later, the cells were harvested for analysis.

Transwell Migration Assay

Cultured AFs were digested by trypsin, followed by seeding on the upper layer of the insert in a serum-free medium at a concentration of 2.0×10^5/mL. After adding 600μl of Ang-II (1 μM)-containing medium to 24-well plate, followed by putting the inserts into wells and kept cultured. On the second day, unattached cells were removed. The inserts were taken out and the cells in the lower layer of the inserts were fixed by 10% formalin, stained with 0.25% crystal violet, and pictured for counting migrated cells from five visual fields for each.

RNA Sequencing

AFs isolated from WT or CDH11$^{-/-}$ mice were cultured and treated with or without Ang-II (1μM) for 24h. Total RNAs were extracted using TRIzol (Takara, Cat# RR036A). Three biological replicates were performed for each group. RNA sequencing (RNA-Seq) was carried out by Beijing Genomics Institute following a standard protocol. The cDNA library was sequenced using a BGISEQ-500, followed by bioinformatics analyses.

Cardiac Electrophysiology

The mice were anesthetized with 2% isoflurane inhalation. In order to maintain a body temperature of 37°C,

the mice were placed on a heated pad and surface ECGs were recorded. A 1.1F octopolar EP catheter (Millar Inc., USA) connected with a #30-gauge subdermal needle electrodes (Grass Technologies) was inserted into the right heart and tested the inducibility of atrial arrhythmias during burst pacing. A burst of electrical stimulation is used to test the inducibility of atrial arrhythmia. The vulnerability of atrial fibrillation was assessed by burst stimulation lasting 5 s, starting with a 50 ms cycle length, gradually shortening to 48, 46, 44, 42, and finally to 10 ms in serial continuous bursts. Atrial fibrillation is defined as a rapid irregular heart rhythm, with irregular R–R intervals exceeding 1 s. If one or more serial bursts induce an atrial fibrillation attack, then this fibrillation is counted. The time from the end-point of pacing, going through the subsequent induced irregular heart rhythm, to the occurrence of the first P-wave is defined as the duration of atrial fibrillation. The number of episodes and the duration of atrial fibrillation were analyzed, as we reported previously.[17]

Quantitative Real-Time PCR

Total RNAs, extracted from atrial tissues of patients and of mice and from cultured cells using TRIzol (Takara, Cat# RR036A), were reversely transcribed into cDNA using the Prime-ScriptTMRT reagent kit (Takara, RR036A). qRT-PCR was performed using specific primers (Supplementary Table 2), Premix Ex Taq™ (Probe qPCR) and normalized to GAPDH expression. ABI 7500 detector (Applied Biosystems) with standard PCR conditions (95°C for 30s, followed by 40 cycles of 95°C for 5s and 60°C for 34s) was used to run the samples.

Western Blotting

Protein samples were prepared from atrial tissues of either patients or mice or from cultured cells. An equal amount of protein (10~30 μg) was resolved by SDS/PAGE and transferred to PVDF membranes. The blots were blocked with 5% non-fat milk in TBST buffer for 1h at room temperature and then incubated with primary antibodies as indicated. The blots were then incubated with secondary antibodies conjugated with horseradish peroxidase (dilution 1:1000) for 1h at room temperature and detected using a chemiluminescence system (Chemi-DocXRS+; Bio-Rad Laboratories, Hercules). Gel Imaging System (Tanon) and AlphaView software were used to image and to analyze the intensity of each band normalized to GAPDH.

Collagen Gel Contraction

Collagen gel contraction assay was performed in a protocol as we described previously.[17]

Data Analysis

Data were analyzed using Graph-Pad prism 6.0 or SPSS 19.0 statistical software and represented as mean ± SD or mean ± SE, as indicated. Two-tailed Student's tests were used for two-group comparisons. ANOVA followed by post hoc Tukey's test was used for multiple group comparisons. A value of $p<0.05$ was considered statistically significant.

Results

Up-Regulated CDH11 and Associated Fibrosis in the LA of Patients with Atrial Fibrillation

We detected a significant increase of CDH11, α-SMA and CTGF in the left atrial appendage tissues of patients with permanent atrial fibrillation compared with those patients with SR in Western blotting analysis (Figure 1A and B). The significantly increase of CDH11 was also revealed by immunohistochemistry (Figure 1C). A picrosirius red staining confirmed that atrial fibrosis was more prominent in patients with atrial fibrillation than that in patients with SR (Figure 1D).

Loss of CDH11 Suppressed Ang-II-Mediated Expression of Pro-Fibrotic Genes in Mouse AFs

To explore the role of CDH11 in AFs activation, we isolated and cultured AFs from both globally $CDH11^{-/-}$-knockout mice[16] and their WT littermates and performed RNA-Seq after Ang-II treatment on those AFs. We conducted differentially expressed gene (DEG) analysis comparing the whole transcriptomes of AFs from $CDH11^{-/-}$ with its control littermate under indicated treatment conditions. Eight hundred and ninety-one DEGs (472 up and 419 down) at resting state and 1263 DEGs (607 up and 656 down) after Ang-II stimulation were identified (Figure 2A). DAVID functional annotation analysis showed that gene functions related to ECM and cell proliferation, especially ECM genes, were significantly down-regulated (Figure 2B). The top 5 enriched pathways between $CDH11^{-/-}$ and WT with or without Ang-II treatment were identified by KEGG (Figure 2C). Those highly enriched signalling pathways

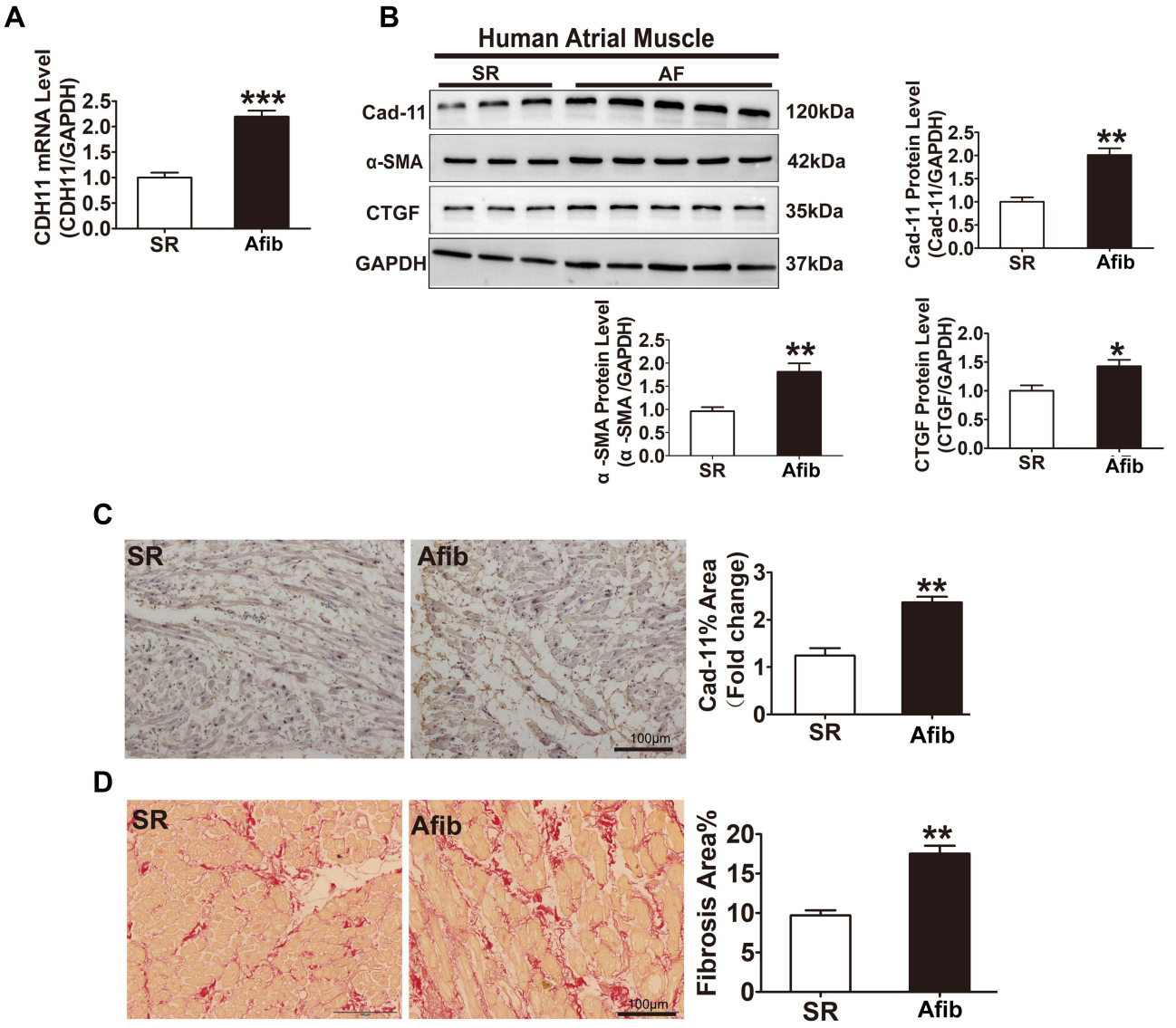

Figure 1 Increased CDH11 expression and fibrosis in the LA appendage tissues of patients with atrial fibrillation. LA tissues were obtained from patients with sinus rhythm (SR) or chronic atrial fibrillation (AFib). (**A**) CDH11 transcripts in the LA tissues of patients with SR or AFib were measured by quantitative RT-PCR. (**B**) Representative Western blotting images showing protein levels of CDH11, α-SMA, CTGF from patient atrial muscle tissues as indicated. GAPDH was used as internal control for protein normalization. Quantification of the band intensity for each protein was summarized in bar graphs. (**C**) Representative images of immunohistochemical staining of LA tissues from patient with SR or AFib as indicated. CDH11 levels were quantified and summarized in the bar graph on the left. Bar=100 μm. (**D**) Representative images of picrosirius red staining of the left atrial tissue for collagen (red staining) from patients with SR or AFib. Bar graph on the left summarized the area of fibrosis. Bar=100 μm. N=6. Data are presented as mean ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs SR.

were for proliferation, fibrosis and inflammation. MAPK and TGF-β pathways occupied the top two most enriched, irrespective of Ang-II treatment. Thirty-nine pro-fibrotic gene transcripts were found to be significantly increased by Ang-II and their expression were attenuated by CDH11 deletion (Figure 2D). Of those fibrosis-related 39 genes, we further validated the expression of eight characteristic pro-fibrotic genes by qRT-PCR. Those genes are Col1A1, Col3A1, CTGF, α-SMA, FGF10, MMP9, fibronectin and BMP9 (Figure 2E).

CDH11 Deletion in Cultured Mouse AFs Compromises Ang-II-Induced AFs' Activation, ECM Protein Synthesis, Migration and Proliferation

We further examined the protein levels of Col1A1, Col3A1, CTGF and α-SMA in cultured AFs by Western blotting. As shown in Figure 3A, CDH11 deletion decreased Col1A1, Col3A1, CTGF and α-SMA protein levels at both resting state and after Ang-II stimulation. In addition, in $CDH11^{-/-}$ mice, Ang-II-induced

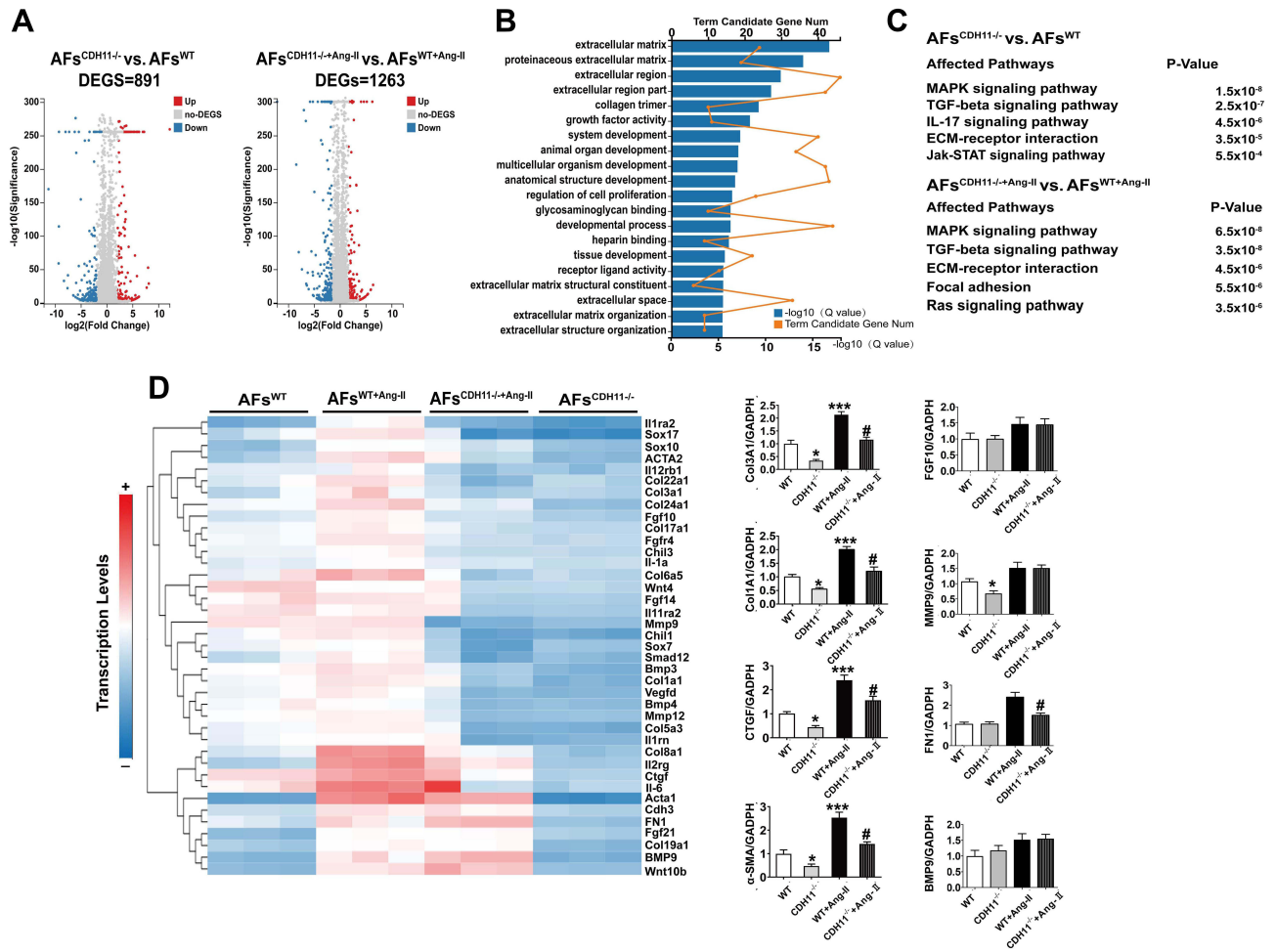

Figure 2 Loss of CDH11 suppressed pro-fibrotic gene expressions in mouse AFs. Atrial fibroblast cells isolated from $CDH^{-/-}$ and WT mice were cultured and treated with Ang-II for 24h, followed by RNA extraction for RNA-Seq analysis. (**A**) Volcano plots of normalized counts comparing the transcriptomes of atrial fibroblasts from $CDH11^{-/-}$ and control WT mice. A total of 891 transcripts were found to differentially expressed (DEGs) between WT and $CDH^{-/-}$ at resting state (*Left*) and 1,263 differentially expressed transcripts after Ang-II stimulation (*Right*). (**B**) DAVID functional annotation analysis showed that gene functions related to ECM were significantly down-regulated. (**C**) KEGG pathway analysis of Ang-II-induced DEGs showed a strong enrichment for signalling pathways associated with proliferation, fibrosis and inflammation. (**D**) Heat map representing color coded expression levels of DEGs, showing that 39 fibrosis-related genes were significantly up-regulated by Ang-II in WT mice, but this up-regulation was abrogated in $CDH^{-/-}$ AFs. € qRT-PCR analysis of eight fibrosis-related gene expression. Data were presented as mean ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs WT; #: $p<0.05$, ##: $p<0.01$ vs WT+ Ang-II.

enlargement in AFs cell size and the formation of stress fibers (Figure 3B), gel contraction (Figure 3C), and migration (Figure 3D) were reduced. These results indicated that CDH11 deficiency suppressed AFs' activation and their pro-fibrotic function.

We next examined the effect of CDH11 in AFs proliferation using CCK-8 cell proliferation assay. As shown Figure 4A, Ang-II significantly promoted cell proliferation in WT AFs. CDH11 deletion greatly attenuated Ang-II-promoted cell proliferation. No difference in cell proliferation rate was observed in AFs from both WT and $CDH11^{-/-}$ mice during serum starvation. CDH11 effect on cell proliferation was corroborated by flow cytometry using PI stained AFs. As shown in Figure 4B, CDH11 deficiency significantly inhibited Ang-II-promoted cell cycle progression. Our findings were consistent with previous reports in vascular smooth muscle cells.[19] These results indicated that CDH11 deletion inhibited Ang-II-induced AFs' proliferation by arresting cell cycle progression at G0/G1 phases.

CDH11 Engagement Induces AFs' Activation and Migration

Engagement of cadherins on the cell surface in homotypic interactions with cadherins on the neighboring cell surface initiates and transduces cell–cell contact signals. To determine whether CDH11 engagement is able to activate AFs, we employed a fusion protein of CDH11 with human Fc

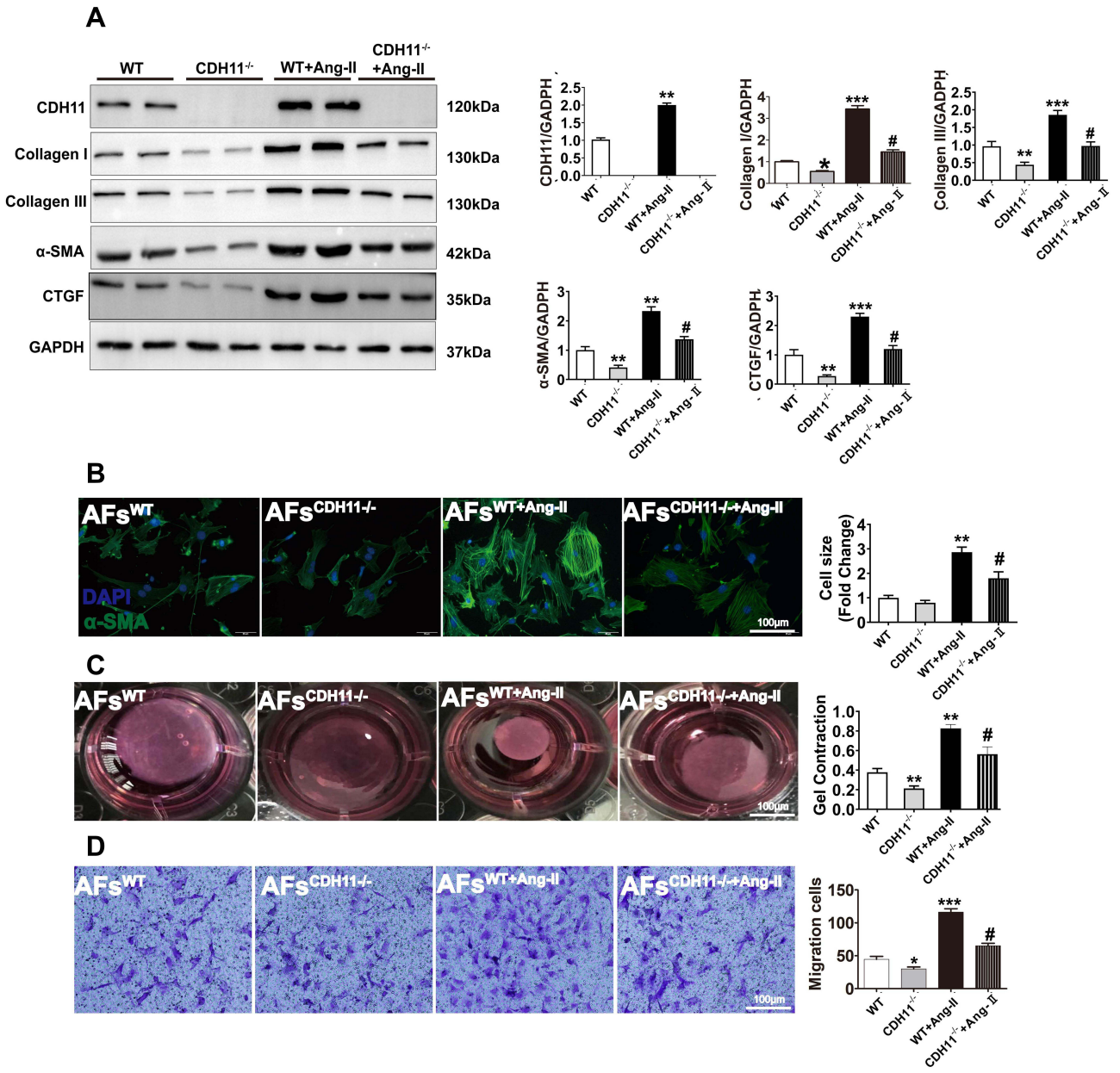

Figure 3 Loss of CDH11 slowed Ang-II-stimulated AFs' activation, ECM synthesis, collagen lattice contraction and migration. AF cells isolated from CDH$^{-/-}$ and WT mice were cultured and treated with Ang-II for 24h, followed by harvesting for the following analyses. (**A**) Expression of CDH11 and four major ECM proteins were measured by Western blotting in WT and CDH11$^{-/-}$ cells with or without Ang-II stimulation (1μM, 24h). (**B-D**) Representative images and bar graph summarization of immunofluorescence staining for α-SMA (**B**), collagen gel contraction assay (**C**), and Burden chamber assay for cell migration (**D**), in mouse AFs with or without Ang-II stimulation. In (**B**) green: α-SMA; blue: DAPI; Bar=100 μm. Data are presented as mean ± SD for (**A-C**) and as mean ± SE for *D*. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs WT; #: $p<0.05$ vs WT +Ang-II.

region of IgG (CDH11-Fc) as CDH11 ligand to initiate cadherin engagement. Compared with human normal IgG (h-IgG1; 10 μg/mL; 24 hours), addition of recombinant human CDH11-Fc protein (hCDH11-Fc; 10 μg/mL; 24 h) activated AFs to differentiate into myofibroblasts, with increased cell size and increased stress fiber numbers (Figure 5A). The findings were substantiated by a more intense gel contraction assay (Figure 5B). The mRNA and protein levels of Col1A1, Col3A1, CTGF and α-SMA were significantly increased (Figure 5C and D). Finally, addition of CDH11-Fc ligand accelerated AFs migrations (Figure 5E). Together, these results suggest that homotypic contact initiated engagement of CDH11 was necessary for AFs activation.

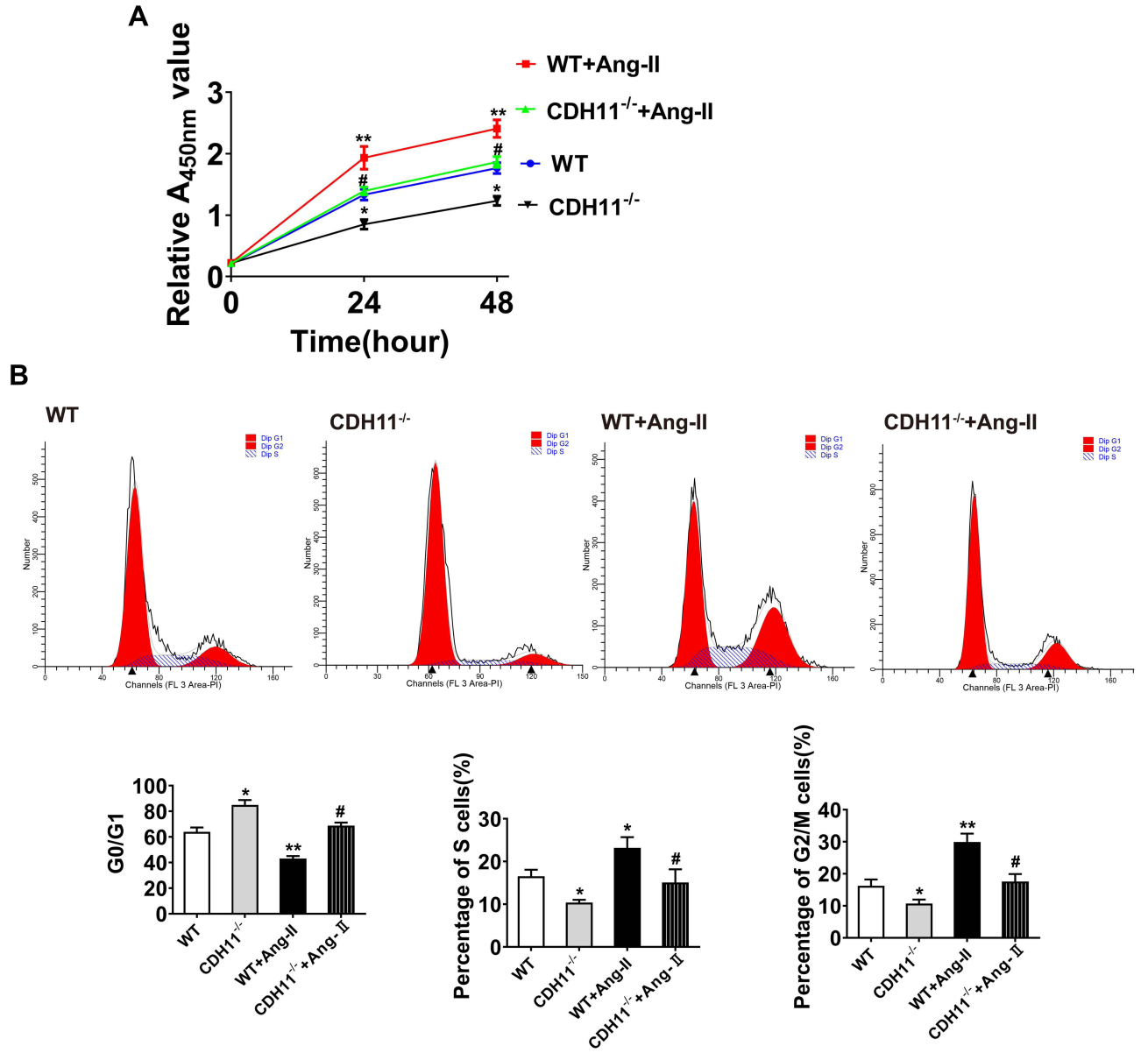

Figure 4 Loss of CDH11 significantly inhibited Ang-II-induced AFs proliferation. AF cells isolated from $CDH^{-/-}$ and WT mice were cultured and treated with Ang-II for 24h, followed by harvesting for multiple analysis. (**A**) Effect of CDH11 null on the viability of AF cells after serum-starvation for 24 hours followed by Ang-II or control treatment as indicated. (**B**) Representative images and summaries of flow cytometry to determine cell cycle stages. AF cells at a density of 6×10^3 cells/cm^2 maintained in DMEM in 0% serum for 24 h to arrest and synchronize the cell growth at G0/G1 phase. Then, the cells were incubated in the DMEM supplemented with Ang-II. Twenty-four hours later, cells were harvested for determinations of the cell cycle progression by flow cytometry after staining of propidium iodide. N=4~6. Data are presented as mean ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs WT. #: $p<0.05$, ##: $p<0.01$ vs WT+Ang-II.

CDH11 Deletion Attenuates Atrial Fibrosis and Susceptibility to Atrial Fibrillation in Ang-II-Induced Mouse Model

Ang-II infusion is the most commonly utilized approach to study atrial fibrosis in vivo.[20] We next set to examine whether loss of CDH11 affect the pathogenesis of atrial fibrosis. Either $CDH11^{-/-}$ or its control mice were continuously infused with Ang-II. On the 28th day of infusion, echocardiography was performed. We observed an Ang-II-induced enlargement in LA cavity in WT control mice, while the LA cavity remained similar to normal size in Ang-II-infused $CDH11^{-/-}$ mice (Figure 6A).

Next, to determine whether the atrial fibrosis induced by Ang-II was associated with a lower threshold for atrial fibrillation, we conducted in vivo electrophysiological studies. No mice showed spontaneous arrhythmias throughout the

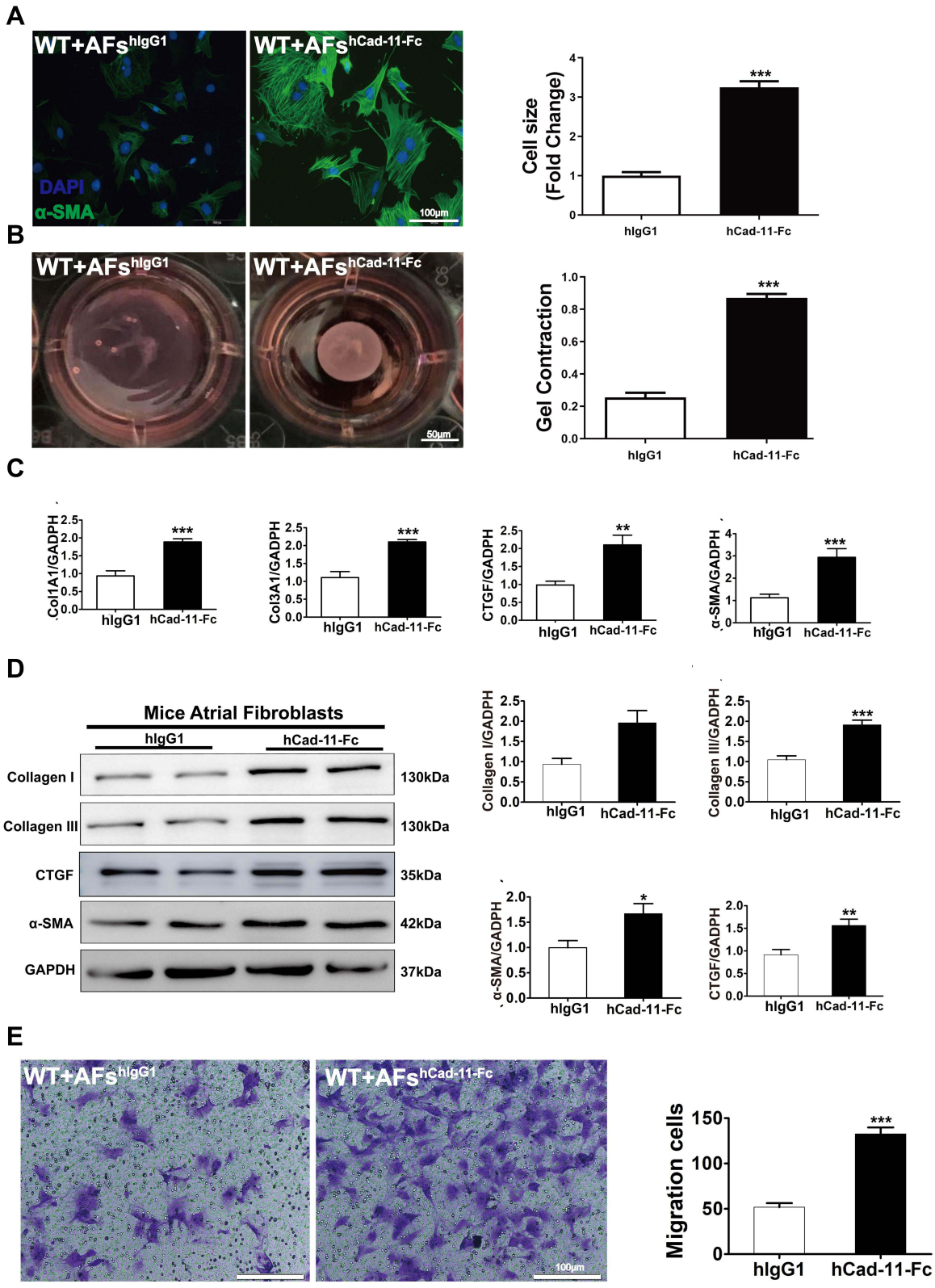

Figure 5 CDH11-Fc induced atrial fibroblast activation and migration. AFs were isolated from WT mice, cultured and serum-starved for 24h, followed by treatment of hCDH11-Fc (10μg/mL; 24h). (**A-E**) Illustrated hCDH11-Fc mediates changes in (**A**) α-SMA expression and cell morphologies by immunostaining; (**B**) contraction of fibroblast-populated collagen lattices by collagen gel contraction assay; (**C**) mRNA levels of Col1A1 and α-SMA by qRT-PCR and (**D**) protein levels of Collagen I/III, α-SMA and CTGF by *Western* blotting. Cell migrations was assayed by transwell (**E**). Data are presented as mean ± SD for (**C** and **D**) and mean ± SE for *E*. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs hIgG1.

electrophysiological study.[21,22] Both WT control and CDH11$^{-/-}$ mice showed normal baseline ECG tracing (Figure 6B). However, after pacing, Ang-II-infused WT mice showed a significantly increased susceptibility to atrial fibrillation, manifested as increased numbers of the episodes and prolonged durations, as compared to saline-infusion. In contrast, CDH11$^{-/-}$ mice exhibited significantly attenuated Ang-II-induced episodes and durations, indicating that CDH11 deficiency attenuated Ang-II-induced susceptibility to atrial fibrillation.

We also examined the impact of loss of CDH11 on collagen synthesis in AFs in vivo (Figure 6C). Ang-II increased the expression of CDH11 and pro-fibrotic expression α-SMA, CTGF, Collagen-I, and Collagen-III in the LA tissue of WT mice. Conversely, loss of CDH11 attenuated the Ang-II-increased ECM gene expressions and fibrosis (Figure 6D). Double immunostaining of cells with antibodies against Vimentin/CDH11 or Vimentin/Collagen-III (Figure 7A) revealed that loss of CDH11 reduced the deposition of Collagen-III and thus severities of atrial fibrosis (Figure 7B). Taken together, these results indicated that loss of CDH11 blocked Ang-II-induced ECM synthesis, atrial fibrosis and thus atrial remodeling.

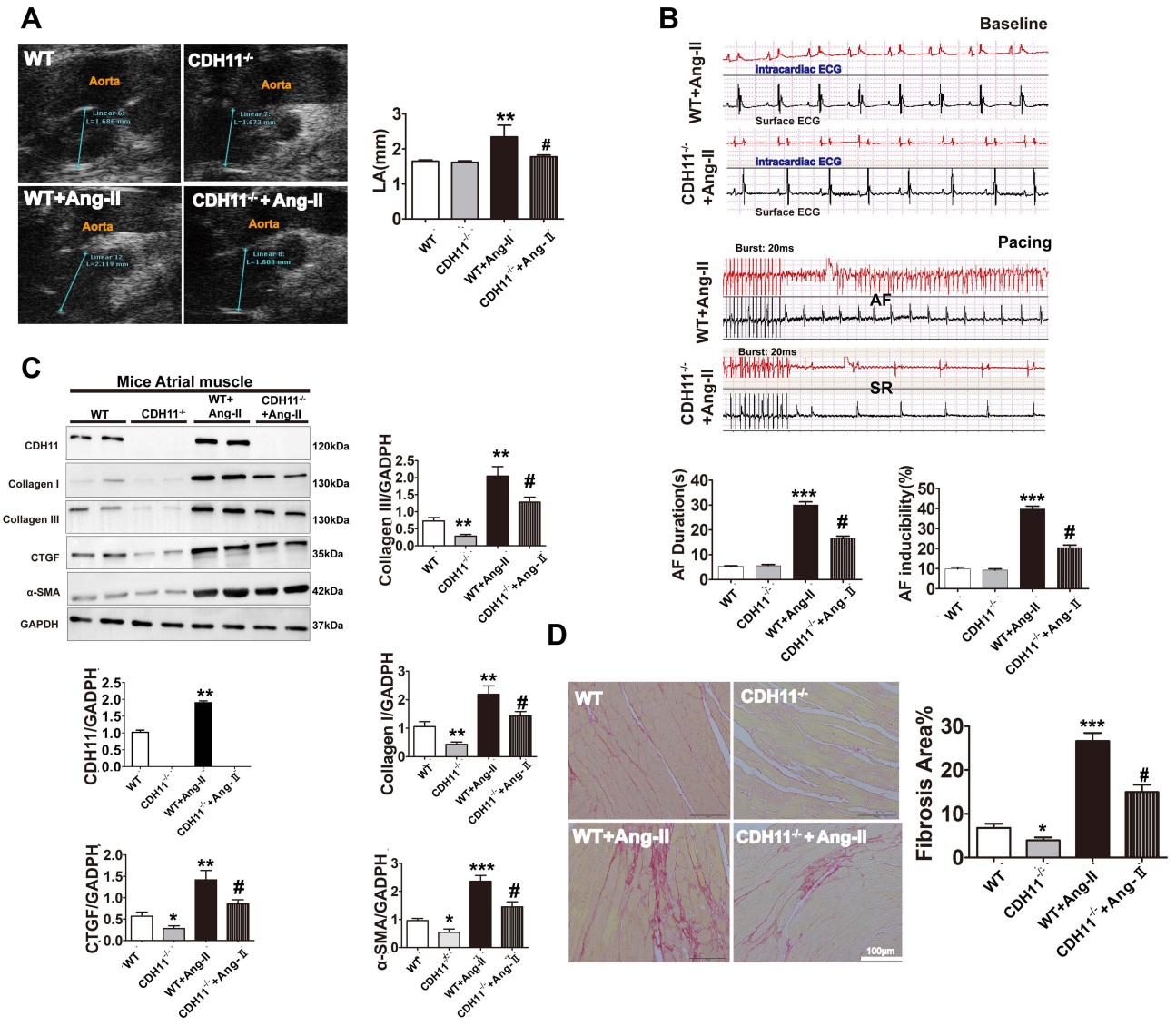

Figure 6 Loss of CDH11 alleviated LA remodeling and susceptibility to atrial fibrillation in Ang-II-infused mice. Both WT and CDH$^{-/-}$ mice were received continuous infusion of saline or Ang-II (1 μM) for 28 days. (**A** and **B**) On the 28th day, the mice were received echocardiography exams (**A**) for measurement of LA dimension and electrophysiological studies for surface and intra-cardiac ECG tracings (**B**). (**C** and **D**) Representative images and bar graph summaries of Western blotting for ECM protein levels (**C**) and picrosirius red staining for collagens (**D**) of the LA tissues. Bar=100 μm; N=6 animals for each group. Data are presented as mean ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs WT. #: $p<0.05$ vs WT+Ang-II.

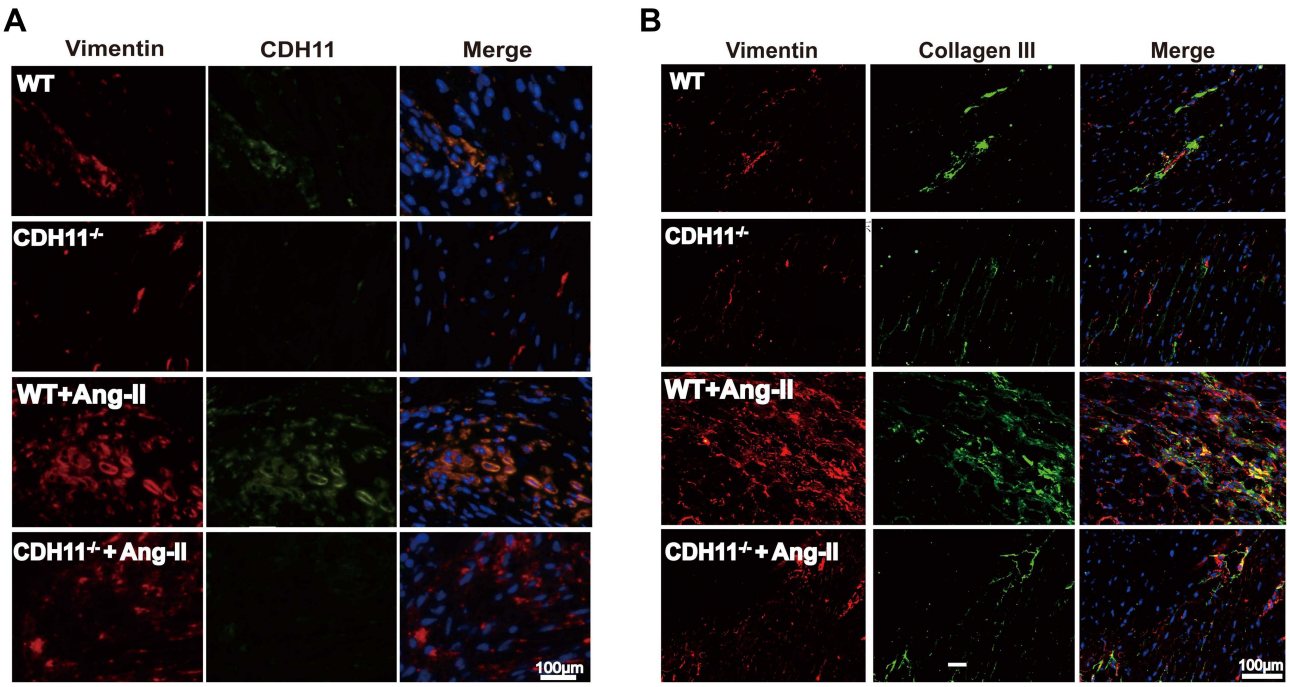

Figure 7 Loss of CDH11 alleviated Ang-II-induced collagen-III synthesis in LA tissue. Both WT and CDH$^{-/-}$ mice were received continuous infusion of saline or Ang-II (1 μM) for 28 days, following by harvesting LA tissues for immunofluorescent staining. Double immunostaining of (**A**) Vimentin (red) and CDH11 (green) or (**B**) Vimentin (red) / Collagen-III (green) were carried out on LA tissues from WT or CDH11$^{-/-}$ mice in presence or absence of Ang-II treatment. Dapi stained for nuclei (blue).

Loss of CDH11 Blocked the Activation of TGF-β-Smad2/3/4 and MAPK Signaling Pathways During Ang-II-Induced AFs Differentiation

Above RNA-Seq results in Figure 2 suggested that CDH11$^{-/-}$ not only abrogated Ang-II-induced pro-fibrotic genes expression, but also blocked activations of both MAPK and TGF-β signaling pathways. Both Ang-II and TGF-β play important roles in the pathogenesis of atrial fibrosis and Ang-II has been shown to up-regulate the expression of TGF-β in the heart.[23–25] In addition to activation of the canonical TGF-β signaling pathway, TGF-β also activates three MAPK pathways: ERK1/2, P38 and JNKs.[26,27] We detected the expression of TGF-β1 ligand and its receptor TGF-β1R1 in cultured AFs and found that Ang-II up-regulated the expression of TGF-β1 and TGFb1R1. Loss of CDH11 significantly attenuated Ang-II-induced TGF-β1 and TGF-βR1 in cultured CDH11$^{-/-}$ AFs (Figure 8A).

To identify the downstream targets of CDH11 deficiency-mediated inhibitions of AFs proliferation, migrations and secretion/deposition of ECM, we measured Smad2/3/4 activities in TGF-β pathway in AFs by examining pSmad2/3 levels (Figure 8B). There is no difference in pSmad2/3 and Smad4 levels in WT cells. Ang-II significantly increased both protein levels, consistent with previous reports.[16,21,40] However, loss of CDH11 greatly attenuated Ang-II-enhanced pSmad2/3 and Smad4 protein levels. We also measured the effect of loss of CDH11 on MAPK signaling activation. We found that at resting state, loss of CDH11 resulted in a slight decrease in all ERK1/2, JNK and P38 activities, compared with WT AF cells (Figure 8C). Ang-II strikingly increased their activities, but CDH11 null attenuated the increased activities of ERK1/2, JNK and P38 signaling molecules.

To substantiate the above hypothesis that Smad2/3 or MAPKs signals are involved in CDH11-mediated fibrosis, we pretreated cultured AFs with the specific inhibitors LY3200882 for TGF-β-Smad, U0126 for ERK1/2, SP600125 for JNK and SB203580 for P38 respectively. AFs activation and differentiation marker α-SMA expression were measured by Western blotting. AFs migration was assayed by Transwell. As shown in Supplementary Figure 1 and Supplementary Figure 2, we found that inhibition of TGF-β-Smad, ERK1/2 and JNK, but not P38, pathways significantly attenuated CDH11-mediated AFs' differentiation and migration.

Furthermore, as shown in Figure 5, initiating CDH11 signalling using hCDH11-Fc as surrogate for cadherin engagement activated AFs' differentiation and migration. We asked whether hCDH11-Fc also activated Smad2/3,

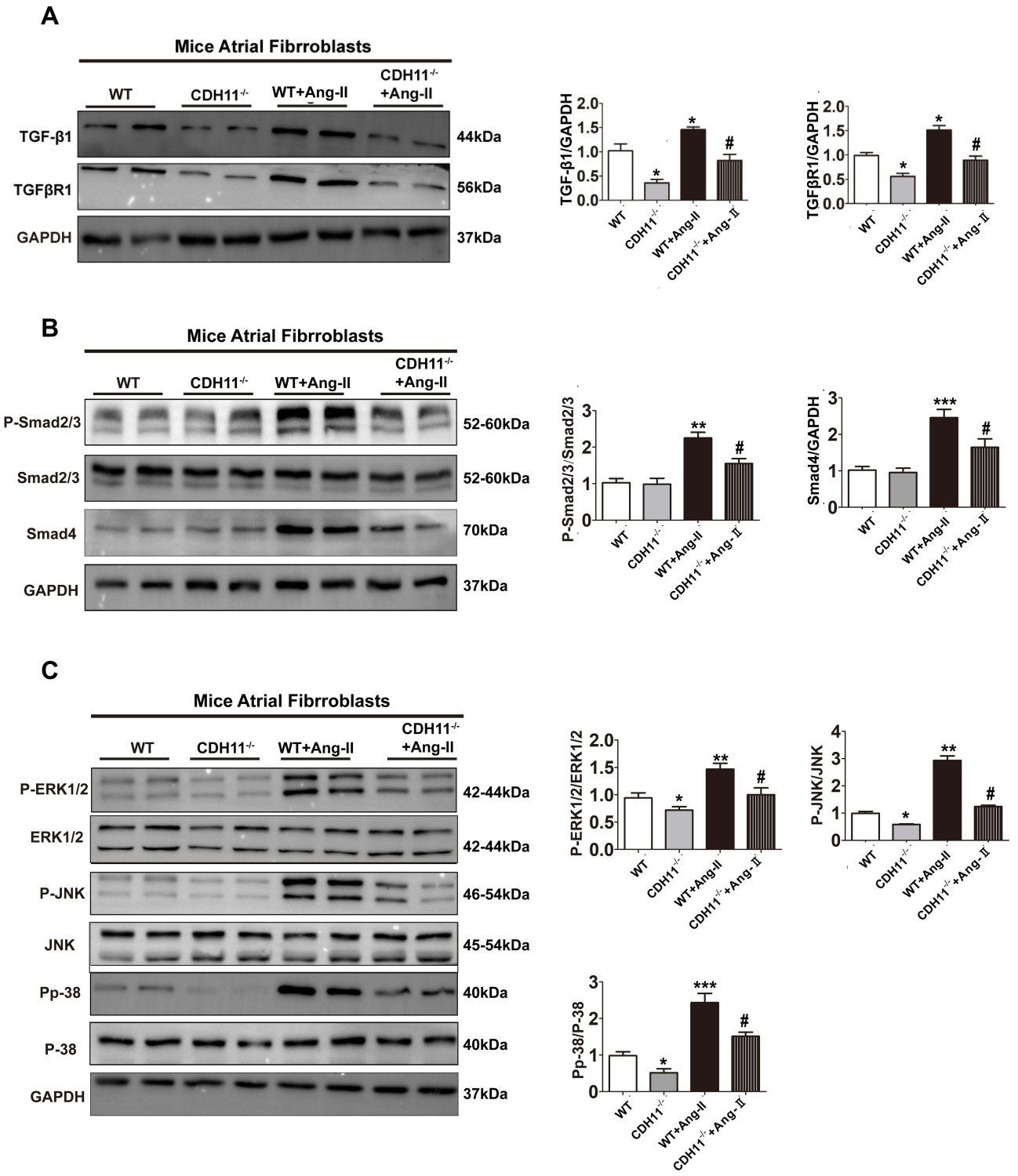

Figure 8 Loss of CDH11 blocked the activation of Smad2/3 and MAPK pathways during Ang-II-induced AFs differentiation. AF cells isolated from CDH$^{-/-}$ and WT mice were cultured and treated with Ang-II for 24h, followed by harvesting for multiple analysis. (**A-B**) Representative Western blotting image showing the protein levels in AFs from control or CDH11-/- mice in presence or absence of Ang-II (1 μM) treatment. Bar graph on the left quantified respective protein level: (**A**) TGF-β1 and TGFRβ1; (**B**) phospho-smad2/3 and smad4; (**C**) phosphor-ERK1/2, phosphor-JNK, and phosphoP38. Data are presented as mean ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs WT: #: $p<0.05$ vs WT+Ang-II.

ERK1/2 and JNK pathways. The phosphorylation status of Smad2/3, ERK1/2 and JNK proteins were examined. AFs were isolated from WT mice, cultured and serum-starved for 24h, followed by treatment of hCDH11-Fc (10 μg/mL; 24h). We found that hCDH11-Fc significantly increased phosphorylation levels of Smad2/3, ERK and JNK, but not p38 (Supplementary Figure 3A and B). Taken together, our results strongly indicated that CDH11 activated both TGF-β-Smad2/3/4 and ERK1/2/JNK pathways during Ang-II-induced AFs activation, differentiation and migration.

Discussion

CDH11 is involved in intercellular adhesion of the interstitial type of cells during development and under various pathological conditions like cancer, rheumatoid arthritis and systemic sclerosis.[28,29] CDH11 promotes the inflammation, migration, invasion and proliferation of many cell types, including mesenchymal cells, vascular smooth muscle cells, cancer cells, fibroblasts and neural cells.[30–33] It is the first factor identified in bone tissue to promote osteogenic differentiation and bone growth and development.[34]

In the field of fibrosis, CDH11 plays important roles in a variety of tissues such as the dermis and lung. In dermis, previous studies reported that the levels of CDH11 mRNA increased in the skin samples from patients with systemic sclerosis.[14] It was found to be localized to the dermal fibroblasts and macrophages. On the other hand, CDH11-deficient mice injected with bleomycin exhibited markedly attenuated dermal fibrosis in the local skin lesions, as quantified by measurements of skin thickness, collagen levels, myofibroblast accumulation, and profibrotic gene expression.[14,35] A therapy with anti-CDH11 neutralizing antibodies decreased the fibrosis at various stages of the bleomycin-induced dermal fibrosis. In vitro studies showed that CDH11 regulated macrophage-derived TGF-β production and fibroblast migration.[11] The role of CDH11 in fibroblast activation in lung tissue has also been documented in CDH11-deficient mice, which displayed a reduced pulmonary fibrosis.[14] The anti-CDH11 antibodies also successfully ameliorated the fibrosis induced by bleomycin.[14] Previous studies also indicated that TGF-β up-regulated CDH11 expression in A549 cells, a human lung cancer cell line, and knockdown of CDH11 expression blocked the TGF-β-induced epithelial-mesenchymal transition (EMT), a key step for fibrosis.[14] In the cardiovascular diseases, CDH11 has been reported so far to potentiate the fibrosis of heart valves and to enhance valvular interstitial cell contractility by regulating focal adhesions and inflammatory cytokine secretion.[36–39] CDH11 provides the necessary cell–cell tension for valve myofibroblast aggregation and calcification. CDH11 also increases stress fibers, compaction and collective migration.[40] In this report, we found a role for CDH11 in the atrial fibrosis and atrial fibrillation through regulating the AFs differentiation. Our studies extended the current understanding of CDH11 function to atrial fibrosis.

In this study, CDH11 was significantly up-regulated in the atrial tissues from patients with atrial fibrillation. In AFs isolated from WT mice, CDH11 transcription and translation were up-regulated by Ang-II. We also demonstrated that activated AFs have enhanced abilities in proliferation, migration and secretion/deposition of ECM. Enhanced proliferation of AFs contributes greatly to structural remodeling of the atria in WT mice, while loss of CDH11 deficiency inhibited cell proliferation and atrial remodeling. Stimulation of AFs with Ang-II enhanced cell migration as well. In contrast, CDH11 deficiency attenuated AFs migration following stimulation of Ang-II. Excessive secretion/deposition of ECM including Collagen I, Collagen-III, CTGF, and α-SMA in Ang-II-infused WT mice in vivo or Ang-II-stimulated WT AFs in vitro, together with the reduced secretion/deposition of the ECM in CDH11-deficient mice also supported the essential role of CDH11 in the atrial fibrosis. Functionally, in WT mice, LA tissues showed a significant increase in CDH11 levels, atrial enlargement, atrial fibrosis, and susceptibility to atrial fibrillation in WT mice after continuous infusion of Ang-II. Whereas in $CDH11^{-/-}$ mice, the loss of CDH11 significantly attenuated Ang-II-induced changes of these three indexes. Mechanistically, we examined transcriptome changes in response to Ang-II in both WT and $CDH11^{-/-}$ AFs and found multiple pro-fibrotic genes were activated by Ang-II. We also demonstrated that CDH11 deficiency blocked the activation of TGF-β-Smad2/3/4 and MAPK signaling pathways in Ang-II-activated AFs.

These novel findings have three important implications. First, CDH11-induced pathogenesis of atrial fibrosis involves up-regulations of multiple profibrotic genes. Second, CDF11-induced activation of TGF-β- and Ang-II-mediated Smad2/3/4 and ERK1/2/JNK pathways contribute greatly to the process of atrial fibrosis. Third, CDH11 is a key regulator of atrial fibrosis and susceptibility of atrial fibrillation and thus CDH11 may become a new therapeutic target for atrial fibrillation.

Author Contributions

Yingze Li provided technical assistance. Yuepeng Wang helped to formulate the hypothesis and revised the manuscript. Qunshan Wang established the project, supervised all experiments, and edited the manuscript. All authors have agreed on the journal to which the manuscript will be submitted, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

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