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Oxymatrine Inhibits Transforming Growth Factor β 1 (TGF- β 1)-Induced Cardiac Fibroblast-to-Myofibroblast Transformation (FMT) by Mediating the Notch Signaling Pathway *In Vitro*

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
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Background: Oxymatrine, a component extracted from the traditional Chinese herb *Sophora japonica* (*Sophora flavescens* Ait.), has various pharmacological effects, especially on the cardiovascular system. However, its cardiac protection effects and the underlying mechanism are still poorly understood. In the present study, we investigated the inhibitory effect and mechanism of oxymatrine on cardiac fibrosis induced by TGF- β 1.

Material/Methods: Cardiac fibroblasts were isolated and purified from neonatal rats. Immunocytochemical staining was used to identify the cells. MTT assay and immunofluorescence staining were used to assess cardiac fibroblasts proliferation and myofibroblasts transformation. Hematoxylin-eosin staining was performed to evaluate morphological changes of cardiac fibroblasts. The secretion of type I and III collagen was assessed by staining with picrosirius red and the hydroxyproline content was determined by colorimetric assay. Cardiac fibroblast migration was examined by scratch assay and DNA content was detected to analyze cell cycle distribution using flow cytometry. Western blot analysis was used to detect the protein expressions of Notch pathway-associated protein in cardiac fibroblasts.

Results: Oxymatrine and Notch signaling pathway inhibitor DAPT could attenuated TGF- β 1 induced the capacity of proliferation and migration increased in cardiac fibroblasts, as well as the secretion of collagen and hydroxyproline colorimetric content in medium. TGF- β 1 induced the biomarker α -SMA of fibroblast-to-myofibroblast transformation (FMT), which was inhibited by oxymatrine and DAPT. Western blotting confirmed that oxymatrine blocked the activation of Notch induced by TGF- β 1.

Conclusions: Oxymatrine is a potential inhibitor FMT induced by TGF- β 1, which is at least in part mediated via inhibition of Notch signaling.

MeSH Keywords: **Fibroblasts • Receptors, Notch • Transforming Growth Factor beta1**

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Background

Cardiovascular disease is a major threat to human health around the world and accounts for nearly 40% of all deaths annually in developed countries [1]. The main causes of the high mortality rate are cardiovascular diseases such as hypertension [2], diabetes [3,4], and hyperlipidemia [5]. Cardiac fibrosis is a common pathogenesis that occurs at a certain stage in a variety of cardiovascular diseases. As the predominant cell type in the heart, cardiac fibroblasts (CFBs) are critically involved in all cardiac fibrotic conditions. CFBs proliferation, migration, and fibroblast-to-myofibroblast transformation (FMT) are significantly increased by pathological stimulation. FMT is a complex pathophysiological process whereby fibroblasts lose their specific markers and acquire myofibroblastic phenotype and express mesenchymal cell products such as α -SMA and collagen [6], as well as disturbed extracellular matrix deposition and degradation, resulting in myocardial stiffness, myocardial diastolic or systolic dysfunction, and ventricular remodeling, and eventually results in heart failure; all of the above contribute to increased morbidity and mortality [7]. Therefore, searching for novel potential agents that could inhibit the FMT in CFBs is important for prevention and cure of cardiac fibrosis.

Oxymatrine (OMT) is an alkaloid derived from extract of *Sophora flavescens* and *Sophora alopecuroides*, which is a traditional Chinese medicine with anti-tumor [8,9], anti-inflammatory [10], and attenuating heart failure properties [11]. OMT is widely applied for various human diseases such as hepatitis B infection and liver fibrosis [12], and provides protection from damage to the liver, intestines, and heart [13–15]. Previous studies have shown that OMT can improve myocardial infarction induced by acute myocardial infarction [16] by attenuating myocardial hypertrophy, left ventricular dysfunction, and heart failure [17]. Previous studies demonstrated that OMT can inhibit aldosterone-induced cardiomyocyte injury [18]. Furthermore, recent evidence reveals that OMT can inhibit the proliferation of CFBs induced by aldosterone [19]. All of this evidence suggests that OMT has potential inhibitory effect on cardiac fibrosis.

The Notch signaling pathway is a highly conserved signaling pathway between adjacent cells. Studies have shown that Notch signaling plays a key role in cell growth, proliferation, differentiation, and apoptosis [20]. In mammals, the Notch signaling pathway is usually composed of the receptor Notch1-4, the homologous transmembrane ligand Jagged-1/2, DLL-4, and the downstream target molecule Hes-1. When the Notch receptor binds to the ligand, the Notch intracellular domain (NICD) is released by protein cleavage through the γ -secretase complex, migrating into the nucleus to activate the Notch target molecule, thereby activating the expression of the relevant

gene [21]. Recent studies have demonstrated that Notch signaling can stimulate fibrosis by activating α -SMA transcription and promoting FMT [22]. Concurrently, previous data confirmed that a highly active γ -secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) can inhibit the Notch signaling pathway, reduce the expression of fibrosis factors such as TGF- β 1, and decrease hydroxyproline content [23].

On the basis of this evidence we tested the hypothesis that OMT inhibits TGF- β 1-induced cardiac fibrosis via the Notch signaling pathway.

Material and Methods

Ethics statement

All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by Guizhou Medical University and was approved by the Bioethics Committee for Animal Studies of Guizhou Medical University.

Materials

OMT (purity, 98%) was purchased from Green Valley Pharmaceutical Co., Ltd., Shanghai, China; Recombinant Human TGF- β 1 was purchased from PeproTech, USA; DAPT was from BioGems, USA; Dulbecco's modified Eagle's medium (DMEM) was from GIBCO, Gaithersburg, USA; Hydroxyproline detection kit was obtained from Jiancheng Bioengineering, Nanjing, China; the Cell Cycle Detection kit was from KeyGEN BioTECH, Nanjing, China; Vimentin, α -SMA and β -actin antibodies were purchased from ProteinTech, USA; Hes-1, Jagged-1, and DLL-4 were purchased from Abcam, USA; and Notch-1 was purchased from Cell Signaling Technology, Beverly, USA.

Cell culture and identification

The isolation and purification of primary CFBs from the neonatal rats was performed according to a modified protocol, as described previously. The hearts of 1- to 3-day-old SD rats were isolated and cut into a size 1 mm³. PBS (6 mL) containing 0.08% trypsin was added and tissues were digested at 37°C for 5 min, which was repeated 5 times. The medium containing suspended cells was removed and an equal volume of DMEM containing 10% fetal bovine serum (FBS) medium was added, centrifuged, and the CFBs were obtained and purified by 1.5-h differential adherence. The purified CFBs were cultured in DMEM with 10% FBS medium at 37°C and 5% CO₂. The second passage was used for further experiments. Cells were cultured in 24-well plates at 37°C with 5% CO₂, and fixed with 4% paraformaldehyde for 15 min, followed by permeabilization

with 0.5% Triton-X-100 for 20 min. Immunocytochemical staining was performed using streptavidin peroxidase (SP) combined with the immunohistochemical 3'-3'-diaminobenzidine (DAB; ZSGB-BIO; OriGene Technologies, Inc., Beijing, China; cat. no. ZLI-9018) staining method. The primary antibody, mouse monoclonal anti-rat vimentin, was added at a dilution of 1: 300 and incubated with CFBs overnight at 4°C, followed by incubation with goat anti-mouse IgG (ZSGB-BIO; OriGene Technologies, Inc.; cat. no. PV-6002; used at the working dilution recommended by the manufacturer) for 1 h at 37°C. This was followed by DAB for 15 s at room temperature. Nuclei were stained with hematoxylin, then observed under a light microscope. Five fields of view were randomly selected to determine the purity of CFBs.

Cell proliferation experiment

The CFBs were cultured in 96-well plates with DMEM containing 10% FBS at 37°C with 5% CO₂. Cells were grown to approximately 80% confluence, then incubated in serum-free DMEM for 24 h, and the cells were exposed to TGF- β 1 (10 ng/mL) for 24 h. or pretreated with DAPT (1 μ mol/L), OMT-L (0.001 mg/mL), and OMT-H (0.05 mg/mL) for 2 h, prior to exposure to TGF- β 1 for 24 h. Then, MTT was added to each well and the plates were incubated at 37°C with 5% CO₂ for 4 h. The medium was discarded and 150 μ L of dimethyl sulfoxide was added to each well to dissolve the formazan. A microplate reader (ELX800; General Electric, Fairfield, CT, USA) was used at 490 nm to detect absorbance.

Hematoxylin-eosin staining

After treatment with different drugs, the cells were fixed with 4% paraformaldehyde for 15 min and incubated with hematoxylin for 10 min. The excess dye was washed with tap water and the cells were stained with eosin for 2 min. Morphological changes were observed under a light microscope.

Immunofluorescence staining

CFBs were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.5% Triton-X-100 for 20 min, blocked in 10% bovine serum albumin at room temperature for 1.5 h, and then incubated with a primary antibodies rabbit monoclonal anti- α -SMA (1: 50 dilution) overnight at 4°C. Subsequently, incubation with secondary antibodies (FITC-conjugated goat anti-mouse IgG, 1: 50 dilution) for 1 h at room temperature. Nuclei were visualized with 4-6-diamidino-2-phenyl indole (DAPI; 5 mg/mL). Images were acquired using a fluorescence microscope (Nikon Corporation, Tokyo, Japan) and the fluorescence absorbance were detected using a plate reader at 550 nm.

Picrosirius red staining

The CFBs from 24-well plates were grouped according to the grouping requirements. After culturing, cells were washed 3 times with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min, and stained with celestine blue liquid dye for 10 min and with picrosirius red liquid dye for 30 min. Hematoxylin was used as a nuclear counter-stain for 15 min. The cells were observed under a light microscope. Type I collagen and type III collagen were stained red.

Scratch experiment

The CFBs migration was examined by scratch assay, as described previously. Briefly, the CFBs were cultured in a 6-well plate at a density of 1×10^5 cells/mL. After the cells were grown to approximately 90% confluence, the medium was changed to serum-free DMEM for 24 h. Cells were treated with designated drugs. Then, an artificial gap was generated by scratching with a pipette tip. The width of the gap was monitored at 24-h intervals.

Hydroxyproline colorimetric assay

At designated time points, the hydroxyproline (Hyp) content of the CFBs supernatants was quantified using a commercial Hyp detection kit. The OD values of the samples were measured at 550 nm using a microplate reader.

Cell cycle assay

DNA content was detected to assess cell cycle distribution using flow cytometry. After the treatment, the CFBs was collected and fixed with pre-cooled 70% ethanol at 4°C overnight. A cell cycle detection kit was used according to the manufacturer's instructions, and the results were obtained using an ACEA NovoCyte instrument. NovoExpress software was used to process and analyze the data.

Western blot analysis

The protein expression levels of Notch-1, Hes-1, DLL-4, and Jagged-1 in CFBs was measure by western blot. After treatment, CFBs were washed with ice-cool PBS and lysed in radio-immune precipitation lysis buffer containing 1% protease inhibitor cocktail on ice. The protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein were subjected to SDS-PAGE on an 8% gel, then transferred to PVDF membrane and blocked with 5% skim milk in TBST at room temperature for 2 h. The membranes were incubated with primary Notch-1 (#3608, Cell Signaling Technology, 1: 1000), Hes-1 (ab108937, Abcam, 1: 1000), DLL-4 (ab183532, Abcam,

1: 1000), Jagged-1 (ab89663, Abcam, 1: 1000), and β -actin (20536-1-AP, ProteinTech, 1: 1000) antibodies overnight at 4°C. After washing with TBST 3 times and incubation with the corresponding secondary antibody (SA00001-2, ProteinTech, 1: 10 000) at room temperature for 1 h, the immunolabeled bands were visualized using Pierce ECL Western blot substrate (7Sea Biotechnology, Shanghai, China).

Statistical analysis

SPSS v13.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Statistical analysis was undertaken using ANOVA and *t* test for 2 groups, and one-way ANOVA for multiple comparisons. All data are presented as the mean \pm SEM. Statistical significance was defined as $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered highly significant. Data from *in vitro* studies were derived from at least 3 independent experiments.

Results

Identification of primary CFBS

Vimentin is a peculiar protein in mesenchymal cells, and in the heart only CFBS are mesenchymal cells. Therefore, vimentin can be used as a biomarker for the identification of CFBS. The CFBS were observed by an inverted microscope (Figure 1A). The negative cell (PBS only) exhibited blue-violet staining with no yellow cytoplasmic staining (Figure 1B). The vimentin-positive cell exhibited a blue-violet nucleus and strong yellow cytoplasmic staining (Figure 1C). These results confirmed that more than 95% of the experimental cell population consisted of CFBS.

OMT and DAPT inhibit proliferation and FMT of CFBS induced by TGF- β 1

The chemical structure of OMT is shown in Figure 1D. The proliferation and FMT induced by TGF- β 1 were analyzed by MTT assay, HE staining, and immunofluorescence staining of the OMT- and DAPT-treated cells. MTT assay showed that TGF- β 1 significantly increased the proliferation of CFBS compared with control group and was significantly inhibited by pretreatment with DAPT and OMT (Figure 1E). TGF- β 1 significantly increased the number of CFBS compared with the control group, which was significantly inhibited by pretreatment with DAPT and OMT (Figure 1F) with HE staining. FMT of CFBS by immunofluorescence staining showed that TGF- β 1 significantly increased the expression of α -SMA in CFBS, whereas both OMT and DAPT attenuated the expression of α -SMA (Figure 1G, 1H).

OMT and DAPT attenuate TGF- β 1-induced CFBS collagen deposition

The pathological synthesis and deposition of collagen is a biomarker of fibrosis, and expression of collagen was analyzed by picrosirius red staining (Figure 2A). The results of the picrosirius red staining showed red staining (collagen) inside the cells and in the extracellular space between the cells. TGF- β 1 increased the presence of collagen compared with the control group, whereas pretreatment with OMT and DAPT decreased the amount of red staining and reduced the number of cells.

OMT and DAPT inhibit migration capacity of FMT induced by TGF- β 1

The scratch migration assay results show that TGF- β 1 induced obvious migration of CFBS compared with the control group. OMT and DAPT pretreatment significantly suppressed CFBS migration to the level of control (Figure 2B, 2C).

OMT and DAPT inhibit Hyp secretion of FMT induced by TGF- β 1

Hyp is a degradation product of collagen, as represented by an index of collagen secretion. To a certain extent, Hyp can indicate cardiac myofibroblast secretion activity. After exposure to TGF- β 1, the Hyp content is significantly increased in medium. Pretreatment with OMT and DAPT significantly alleviated the TGF- β 1 induced Hyp secretion (Figure 2D).

Effect of OMT and DAPT on cell cycle distribution in FMT induced by TGF- β 1

As shown in Figure 3A and 3B, TGF- β 1 induced greater CFBS proliferation by promoting more cells from G1 to S phase compared with the control group. Under TGF- β 1 conditions, pretreatment with OMT and DAPT reduced the proportion of cells in S phase and increased the proportion of cells in G1 phase. There was no difference between groups in G2 phase.

Mechanism of OMT ameliorates FMT induced by TGF- β 1 involved in the Notch signaling pathway

The Notch signaling pathway is mainly mediated by receptor and ligand binding and subsequent activation of the target protein. Western blot analysis revealed that the protein expression levels of Notch-1, Jagged-1, DLL-4, and Hes-1 were significantly increased in the TGF- β 1 group compared with the control group (Figure 4A–4D). Pretreatment with OMT and DAPT decreased the protein expression levels of TGF- β 1-induced Notch-1, Jagged-1, DLL-4, and Hes-1 (Figure 4A–4D). Compared with the OMT + DAPT treated groups, the Notch-1,

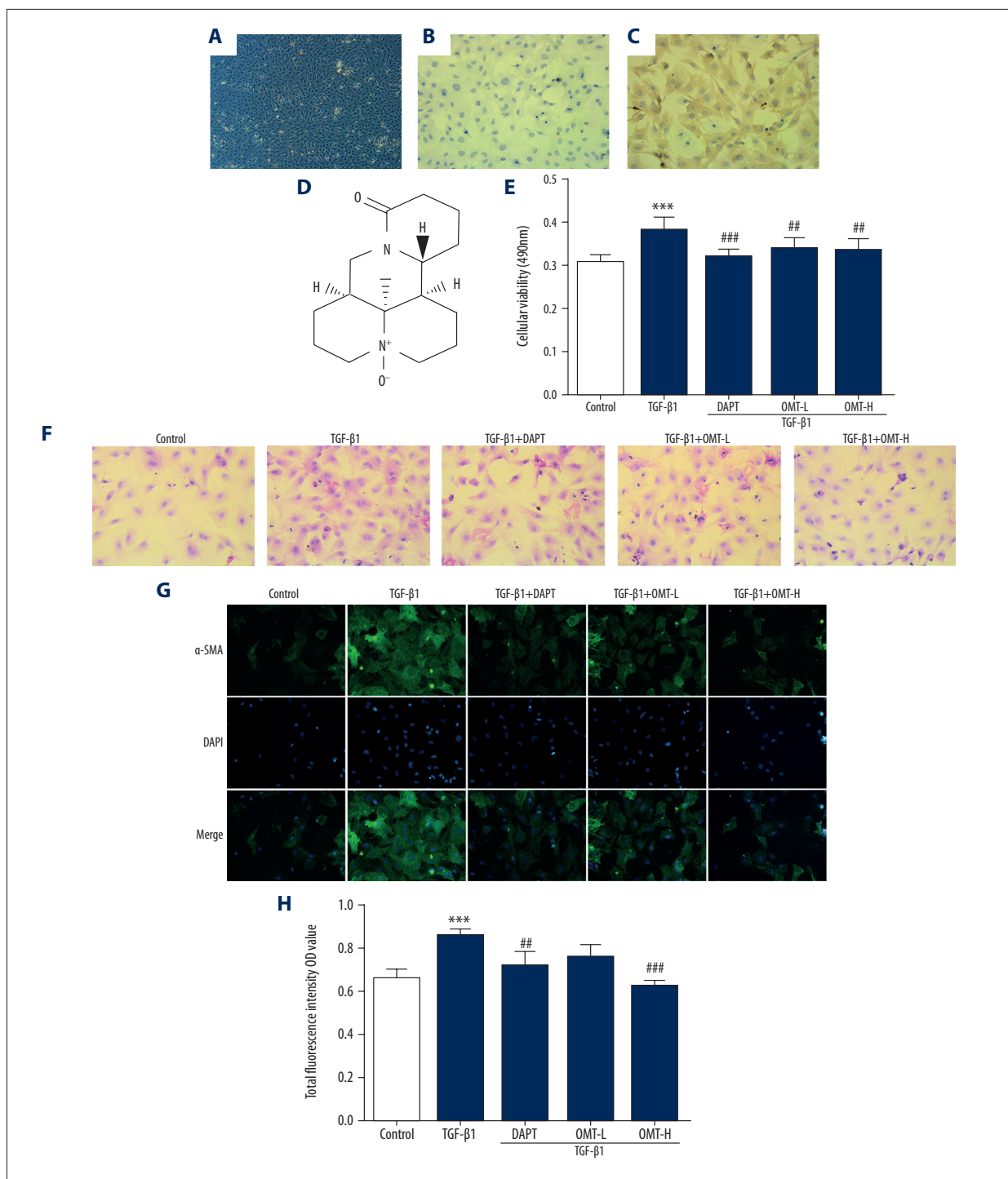


Figure 1. OMT and DAPT inhibit TGF-β1-induced proliferation and differentiation of FMT in CFBs. **(A)** Brightfield image of the primary CFBs (magnification, 50×). **(B)** Representative image of negative control stained cells (PBS was used instead of primary antibody). **(C)** Representative image of cells stained with the anti-vimentin antibody (magnification, 200×). **(D)** Chemical structure of OMT. **(E)** CFBs were pretreated with 1 μmol/L DAPT or 0.005 mg/mL OMT or 0.01 mg/mL OMT for 2 h, and then co-incubated with 10 ng/mL TGF-β1 for 24 h. Cell viability was measured by MTT assay. **(F)** HE staining analysis CFBs morphological change, cytoplasm staining pink and nucleus staining violet (magnification, 200×). **(G)** Immunocytofluorescence results of α-SMA, α-SMA was stained green; nuclei stained with DAPI were blue (magnification, 200×). **(H)** Quantification of total fluorescence intensity OD value of α-SMA. Results are presented as the mean ±SEM (** p<0.01 and *** p<0.001 vs. control; ## p<0.01 and ### p<0.001 vs. TGF-β1).

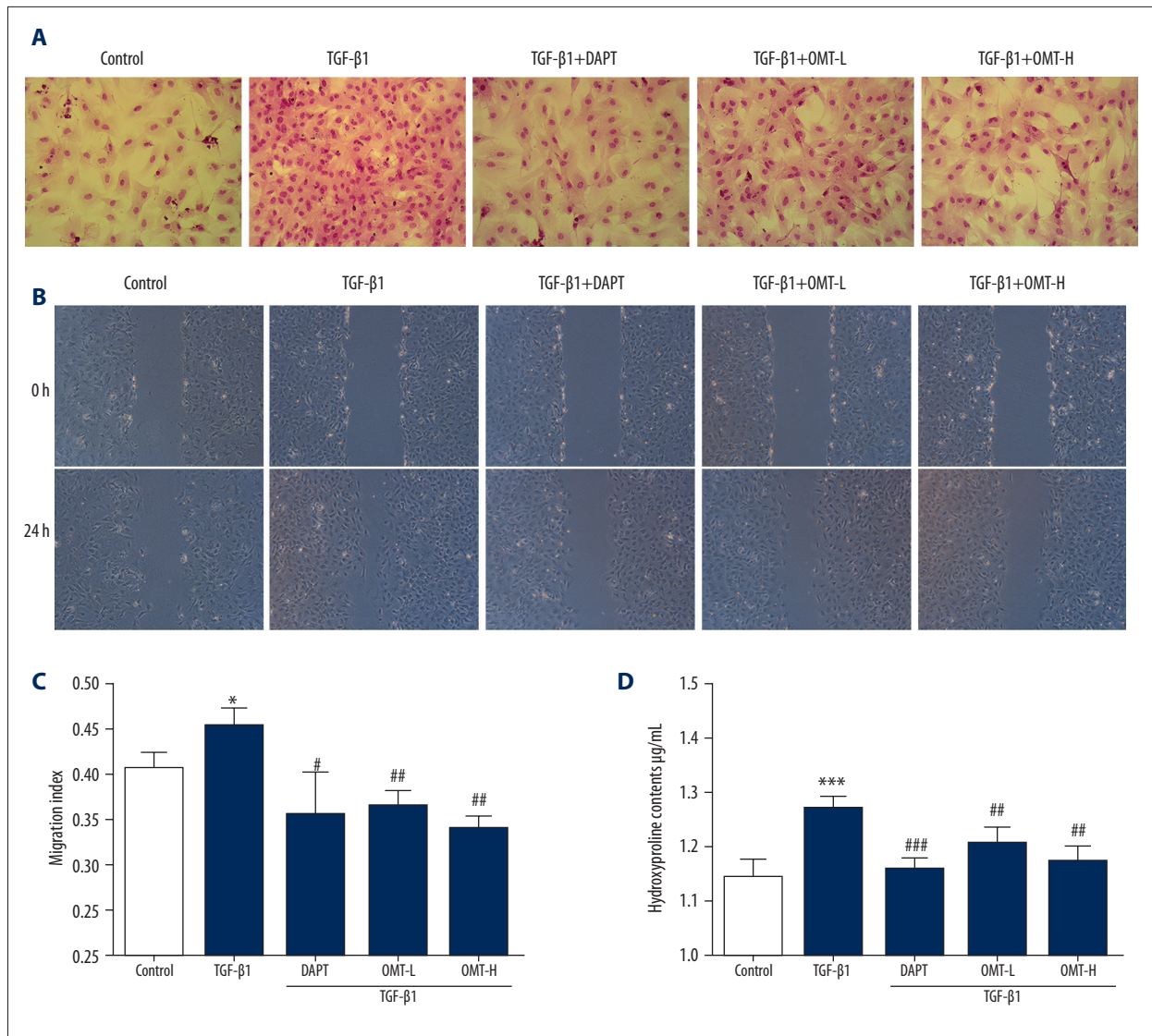


Figure 2. OMT and DAPT inhibit TGF- β 1-induced migration capacity and attenuates hydroxyproline secretion and collagen deposition of FMT in CFBs. CFBs were pretreated with 1 μ mol/L DAPT or 0.005 mg/ml OMT or 0.01 mg/mL OMT for 2 h, and then co-incubated with 10 ng/mL TGF- β 1 for 24h. **(A)** Collagen expression in CFBs examined by picrosirius red staining. The collagen is stained in red (magnification, 200 \times). **(B)** Scratch assay showing that DAPT and OMT can inhibited CFBs migration. **(C)** Quantification of the width of cells migration after scratching. **(D)** Analysis of hydroxyproline content in cell supernatant. Results are presented as the mean \pm SEM (* $p < 0.05$ and *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. TGF- β 1).

Jagged-1, DLL-4, and Hes-1 protein expression exhibited no significant difference between DAPT groups (Figure 4A–4D).

Discussion

Cardiac fibrosis develops in pathological changes to various CVDs, including persistent hypertension, hypertrophic cardiomyopathy, ischemic insults, and congenital heart defects. In various diseases conditions, CFBs respond to stimuli in different

ways, including proliferation and differentiation, and then result in collagen secretion and extracellular matrix deposition, due to the production of cardiac fibrosis. In general, the process is referred to as FMT [24,25]. However, specific pharmaceuticals that directly target fibrosis are unavailable [26]. Previous studies have confirmed that OMT inhibits kidney and liver fibrosis and attenuates acute cardiac infarction-induced cardiac fibrosis. Accumulating evidence suggests that activation of the Notch signaling pathway promotes the expression of fibrosis factors [11,15,22], and TGF- β 1 is a key cytokine that has been

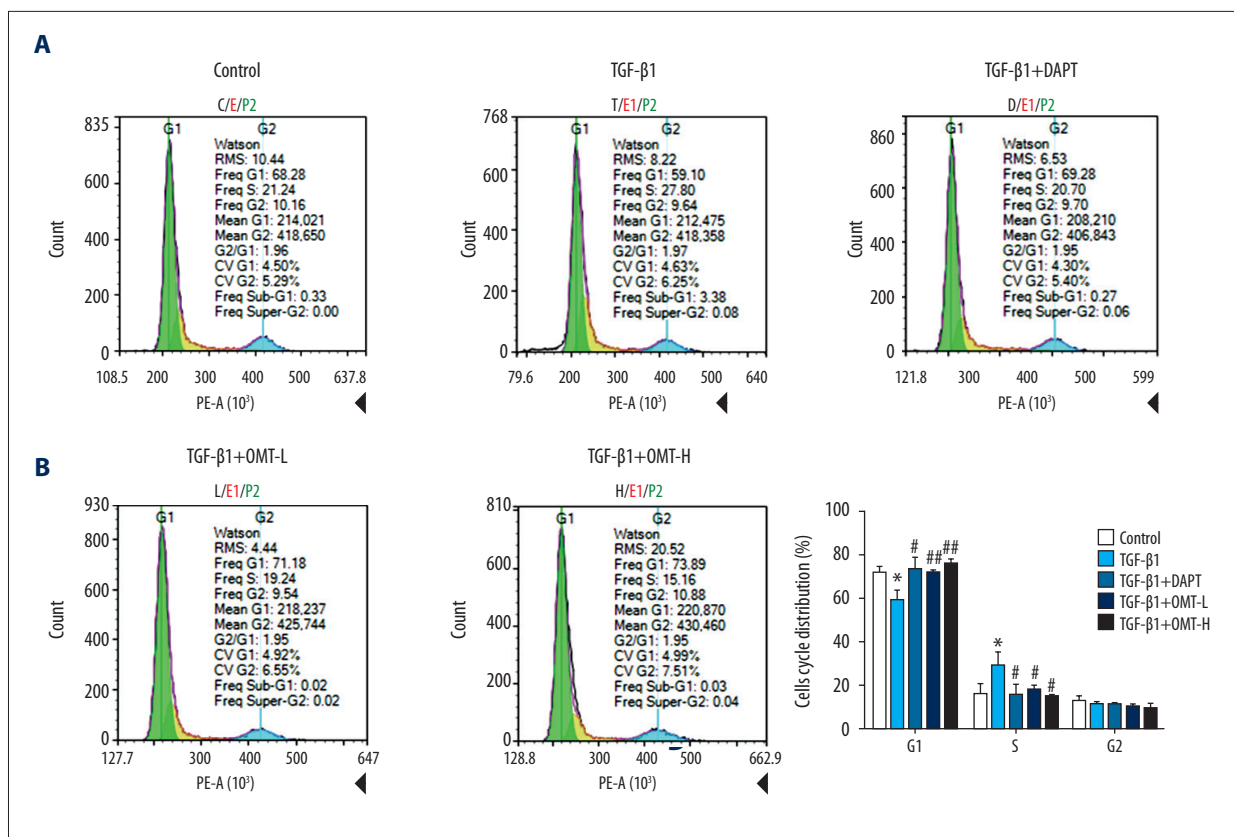


Figure 3. Effect of OMT and DAPT on cell cycle distribution in FMT induced by TGF-β1. CFBS were pretreated with 1 μmol/L DAPT or 0.005 mg/mL OMT or 0.01 mg/mL OMT for 2 h, and then co-incubated with 10 ng/mL TGF-β1 for 24 h. (A) DAPT and OMT induced G1 cell cycle arrest in CFBS. The distribution of cell cycle was assessed by flow cytometry. (B) The percentage of CFBS in each phase. Results are presented as the mean ±SEM (* p<0.05 vs. control; # p<0.05 and ## p<0.01 vs. TGF-β1).

identified to induce FMT. In the present study, the model was reproduced by exposing CFBS to TGF-β1, and then the Notch signaling pathway inhibitor DAPT and OMT intervention was used to determine the role of the Notch signaling pathway in FMT and the inhibitory effect of OMT on cardiac fibrosis.

The pathological properties of cardiac fibrosis are the abnormal proliferation and differentiation of CFBS [27]. In CFBS exposed to TGF-β1, the proliferation significantly increased, whereas pretreatment with OMT and Notch signaling pathway inhibitor DAPT attenuated proliferation. Furthermore, OMT and DAPT caused G1 cell cycle arrest, as the percentage of CFBS in G1 phase increased at the expense of cells in S phase following OMT and DAPT treatment, indicating that OMT intervention and inhibition of Notch signaling inhibits cell proliferation by cell cycle arrest.

FMT is the key pathological process in chronic heart failure [28]. α-SMA is a biomarker of myofibroblasts and was used to determine the extent of FMT [29]. TGF-β1 treatment was reported to promote the spontaneous differentiation of FMT. α-SMA expression was significantly increased by TGF-β1, however,

OMT and DAPT significantly decreased the expression of α-SMA. The migration capacity of myofibroblasts was significantly enhanced compared with fibroblasts. TGF-β1 promoted the migration of CFBS, and OMT intervention and DAPT attenuated cell migration ability. OMT and inhibition of Notch signaling can inhibit FMT, attenuating cardiac fibrosis.

The ECM mainly consists of collagen type I and collagen type III; the most important pathological feature of cardiac fibrosis is the excess production of the ECM [30]. TGF-β1 deteriorates the synthesis and deposition of collagen in CFBS, and OMT intervention and the inhibition of the Notch signaling pathway reversed collagen synthesis and improved collagen deposition. These findings were further confirmed by the Hyp content (a major product of collagen secretion) in medium [31]. After exposure to TGF-β1, the content of Hyp was significantly increased in the TGF-β1 group, and incubation with OMT and DAPT reduced the content of hydroxyproline in medium.

In the developing heart, Notch signaling regulates cardiomyocyte proliferation and valve formation. It has been confirmed that Notch signaling is an important pathway in myofibroblast

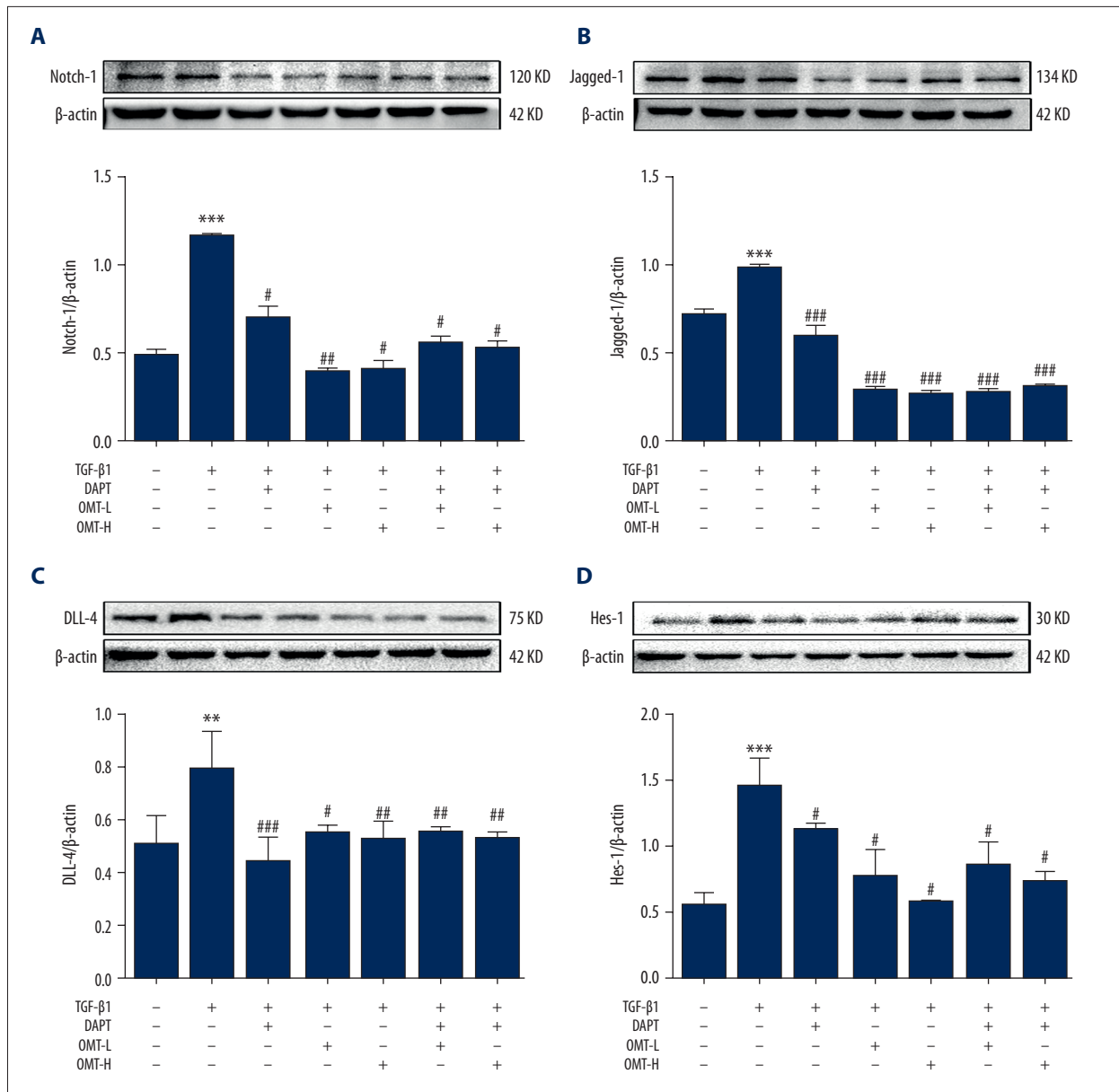


Figure 4. Mechanism of OMT ameliorates FMT induced by TGF- β 1 involving in Notch signaling. CFBs were pretreated with 1 μ mol/l DAPT or 0.005 mg/ml OMT or 0.01 mg/ml OMT for 2 h, and then co-cultured with 10 ng/ml TGF- β 1 for 24 h. Western blot analysis of (A) Notch-1, (B) Jagged-1, (C) DLL-4, and (D) Hes-1 protein expression in CFBs. Results are presented as the mean \pm SEM (** $p < 0.01$ and *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. TGF- β 1).

transformation in various cell types [32], but it remains unclear in FMT. Our results suggest that TGF- β 1 promotes the protein expression of Notch pathway-associated protein, Notch-1, Jagged-1, DLL-4, and Hes-1 in CFBs, and is significantly inhibited after treatment with DAPT, which indicates TGF- β 1 activates the Notch signaling pathway to induced cardiac fibrosis. Inhibition of the Notch signaling pathway can inhibit cardiac fibrosis. Similarly, the expression of these proteins (Notch-1, Jagged-1, DLL-4, and Hes-1) significantly decreased with OMT treatment compared with TGF- β 1 treatment, suggesting that

OMT inhibits TGF- β 1-induced CFBs proliferation and is related to conduction of the Notch signaling pathway. OMT + DAPT also significantly reduced the expression of these proteins in CFBs compared with the TGF- β 1 group, and there was no significant difference compared with the DAPT group. Our results indicate that OMT protects against fibrosis by reducing the expression of proteins involved in the Notch signaling pathway.

Conclusions

Our results demonstrate that OMT intervention and inhibition of Notch signaling can attenuate cell proliferation, differentiation, and collagen synthesis in FMT induced by TGF- β 1. We showed that OMT exerts anti-fibrotic effects by inhibiting the Notch signaling pathway, suggesting that OMT may be a

potential therapeutic agent for effective inhibition of cardiac fibrosis. Further *in vivo* studies are needed to explore the use of OMT for the treatment of cardiovascular disease.

Conflicts of interests

None.

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