Physiological Research Pre-Press Article

Title: Notch signaling may negatively regulate neonatal rat cardiac fibroblast-myofibroblast transformation

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Short title: Notch signaling and cardiac fibroblast-myofibroblast transformation

Notch signaling may negatively regulate neonatal rat cardiac fibroblast-myofibroblast transformation

Summary

Cardiac fibroblast-myofibroblast transformation (CMT) is a critical event in the initiation of myocardial fibrosis. Notch signaling has been shown to regulate myofibroblast transformation from other kinds of cells. However, whether Notch signaling is also involved in CMT remains unclear. In the present study, expressions of Notch receptors in cardiac fibroblasts (CFs) were examined, effects of Notch signaling inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) and transforming growth factor-β1 (TGF-β1) on CMT were determined by increasing alpha-smooth muscle actin (α-SMA) expression and collagen synthesis, and Notch signaling was examined by analyzing expressions of Notch receptors. The results showed that: (1) Notch receptor 1, 2, 3 and 4 were all expressed in CFs; (2) DAPT promoted CMT in a time-dependent manner; (3) During the period of CMT induced by TGF-β1, expressions of Notch receptor 1, 3 and 4 in CFs were down-regulated, whereas there was no change for Notch receptor 2. Moreover, the downtrends of Notch 1, 3 and 4 were corresponding to the trend growth of α-SMA expression and collagen synthesis. These results suggested that inhibiting of Notch signaling might promote CMT. The down-regulations of Notch receptor 1, 3 and 4 induced by TGF-β1 may facilitate CMT. In conclusion, inhibition of Notch signaling might be a novel mechanism of CMT in myocardial fibrosis.

Keywords

Cardiac fibroblast·Myofibroblast·Transformation·Notch signaling·Notch receptor

Introduction

Cardiac tissue is composed of the cellular and extracellular compartments. Collagens are the major components of extracellular compartments. An appropriate amount of extracellular collagens, produced by cardiac fibroblasts (CFs), play an important role in maintaining structural integrity and normal function of the heart. Excessive collagens, produced and deposited in the heart, lead to myocardial fibrosis. Myocardial fibrosis impedes both contraction and relaxation and impairs electrical coupling of cardiomyocytes, it is the important pathological basis for heart failure, fatal arrhythmia and cardiac sudden death (Caulfield and Janicki 1997, Janicki and Brower 2002, Eghbali and Weber 1990). Thus, prevention of myocardial fibrosis is an important therapeutic target to treat various heart diseases.

The excessive collagens deposited in fibrotic myocardium are produced by myofibroblasts (Teunissen *et al.* 2007, Cucoranu *et al.* 2005, Swaney *et al.* 2005). Myofibroblasts is the specialized CFs formed by irreversible acquisition of expressions of alpha-smooth muscle actin (α-SMA), it has significantly higher capacity of collagen synthesis than CFs has. (Lijnen *et al.* 2002, Petrov *et al.* 2002, Gabbiani 1998). It has been accepted that the phenotypic conversion of CFs to myofibroblasts is the critical event of genesis of myocardial fibrosis, and inhibition of cardiac fibroblast-myofibroblast transformation (CMT) is an effective way to prevent myocardial fibrosis (Brown *et al.* 2005). Although a variety of cytokines, vasoconstrictive factors, and mechanical stimuli have been known to induce CMT so far (Porter and Turner 2009, Long and Brown 2002, Wang *et al.* 2003), little is known about the mechanisms underlying it.

Notch pathway is an evolutionarily conserved cell-to-cell signaling system which regulates cellular differentiation (Nemir and Pedrazzini 2008, Niessen and Karsan 2008). Notch signaling is mediated by the interaction of trans-membrane receptors (Notch 1 - 4) and their corresponding ligands expressed on the surface of adjacent cells. Recently, Notch signaling was found to be involved in myofibroblasts transformation. Activation of Notch signaling induced the transdifferentiation of lung epithelial cells into myofibroblasts (Aoyagi-Ikeda *et al*. 2010, Namba *et al*. 2010); over-expression of Notch1 and Notch3 inhibited myofibroblast transformation from hepatic stellate cells (Mann et al. 2007) and 10T1/2 fibroblasts (Kennard *et al*. 2008) respectively; over-expression of Notch1 facilitates myofibroblast differentiation from lung fibroblasts (Liu *et al.* 2009) and tubular epithelial cells (Saad *et al*. 2010). However, whether Notch signaling is also involved in CMT, and what role it might play in this transformation is still unknown.

In the present study, we sought to (1) identify subtypes of Notch receptors expressed on CFs; (2) determine the effect of Notch signaling inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) on CMT and what role it may play in the process; (3) observe the changes of Notch receptor subtypes in the process of CMT induced by transforming growth factor-β1 (TGF-β1). These data will provide new insights into the mechanisms underlying the pathological CMT in myocardial fibrosis.

Materials and Methods

Culture and identification of neonatal rat CFs

All experiments were carried out under the regulations of the National Institutes of Health Guidelines on the Use of Laboratory Animals. 1- to 3-day-old Sprague–Dawley rats (Experimental Animal Center of Fourth Military Medical University, Xi'an) were anesthetized and hearts were removed under aseptic conditions. The ventricles were minced into 2-3mm^3 fragments. The fragments were digested by four to six 15- minute cycles of incubation with 0.125% trypsin (Sigma, St. Louis, MO). At the end of each cycle, the supernatant was stored on ice after addition of fetal bovine serum (FBS; Hyclone, Ogden, UT) to neutralize trypsin. The dissociated cells were collected by centrifugation at 1000 g for 10 min, re-suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY), supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated in a humidified atmosphere of 5% CO_2 at 37°C. After 1 hour incubation, CFs attached to the dishes, whereas myocardiocytes remaining in the medium were discarded. The attached CFs were further cultured to confluence and then passaged at 1:3 dilution. First passage CFs was used throughout the present experiments. Identification of CFs was performed by immunofluorescence staining using anti-vimentin, anti-desmin and anti-von Willebrand factor antibodies.

Immunofluorescent staining

CFs were cultured at a density of 2×10^5 cells/well on coverslips in six-well culture plates. After growth arrested with serum-free DMEM, CFs were stimulated with serum-free DMEM alone, 75μmol/L DAPT (Alexis Corporation, Lausanne) or 10 ng/ml TGF-β1 (Peprotech, James Square, London) for designated times. CFs were then washed, fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. The

immunofluorescent staining was performed in turn by incubating CFs with 10% normal goat serum, incubating with primary antibodies against α-SMA, vimentin, desmin and von Willebrand factor (Boster Biological Technology, Wuhan; 1:200), washing with PBS, incubating with FITC- or Cy3-conjugated secondary antibody (Santa Cruz, CA; 1:500), washing with PBS again, and finally mounting with aqueous mounting medium. The stained cells were visualized under a fluorescence microscope.

Real-time PCR

CFs were plated in 10-cm culture dishes at a density of 1.2×10^6 cells/well. After CFs were made quiescent at near confluence and stimulated with serum-free DMEM alone or 10ng/ml TGF-β1, total RNA was isolated with TRIZOL Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed with 0.5 μg of total RNA in a 10-μl reaction mixture according to the recommended conditions with a reverse transcription synthesis kit (Takara, Dalian). For real-time PCR, the cDNA was amplified using IQ^5 Real-Time PCR System (Bio-Rad, Hercules, and CA). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the SYBR Premix Ex Taq™ II kit (Takara, Dalian) to allow for quantitative detection of the PCR product in a 20-μl reaction volume. The primer sequences for Notch receptor 1-4 and GAPDH were displayed in Table 1. The temperature profile of the reaction was 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 20 s. Relative mRNA expression levels of Notch 1-4 were normalized to GAPDH.

Table1. The primers sequences used in real time PCR

Gene	Sequence 5'-3'
Notch 1 receptor FW	AATGGAGGGAGGTGCGAAG
Notch 1 receptor REV	ATGGTGTGCTGAGGCAAGG
Notch 2 receptor FW	GATCACCCGAATGGCTATGAATG
Notch 2 receptor REV	CCGGGTCACAGTTGTCGATG
Notch 3 receptor FW	AGGCTACCTTGGCTCTGCTGAA
Notch 3 receptor REV	CAGCCTGTCCAAGTGATCTGTGA
Notch 4 receptor FW	CCTGGACAGCAATGCCAAGA
Notch 4 receptor REV	AGTCCAGCCCTCGTTACACACAC
GAPDH FW	GGCACAGTCAAGGCTGAGAATG
GAPDH REV	ATGGTGGTGAAGACGCCAGTA

FW: forward; REV: reverse.

Western blot analysis

CFs were plated in 6-cm culture dishes at a density of 4.2×10^5 cells/well and made quiescent at subconfluence by incubation in serum-free DMEM. After 24-hour incubation, serum-free DMEM alone, 75μmol/L DAPT or 10ng/ml TGF-β1 was added and additionally incubated for designated times. CFs were then harvested with lysis buffer, freeze-thawed to disrupt the membrane, and centrifuged at 10000 g. Lysates were collected and protein concentrations were determined with bicinchoninic acid reagents. Cellular proteins were supplemented with SDS sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% bovine

serum albumin and then incubated overnight at 4°C with primary antibody against Notch1 (Cell Signaling Technology, MA; 1:1000), Notch2 (Abcam, Cambridge; 1:1000), Notch3, Notch4 (Santa Cruz, CA; 1:200), α-SMA and β-actin (Boster Biological Technology, Wuhan; 1:500) respectively. Afterwards, the membrane was washed and incubated again with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA; 1:3000) for 1 hour. Immunoreactivity of the proteins was detected by using chemiluminescent reagents. Optical densities of the bands were scanned and quantified with image analysis software. Protein levels of Notch 1-4 were standardized by respective levels of β-actin.

Hydroxyproline production assay

CFs were plated at a density of 6.5×10^3 cells per well in 96-well tissue culture dishes. After reaching 90% confluence, CFs were starved for 24 hours in serum-free medium and stimulated with 75μmol/L DAPT or 10 ng/ml TGF-β1. Conditioned media derived from treated CFs were used for hydroxyproline production assay. Hydroxyproline production was determined using hydroxyproline contents assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing) according to the manufacturer's instructions.

Statistical analysis

The results are presented as mean ± SEM. Differences between various treatment conditions were evaluated by one-way ANOVA, followed by LSD-t test for multiple comparisons within treatment groups using the SPSS 11.0 statistical software.

Differences were considered statistically significant at $P < 0.05$.

Results

Characterization of neonatal rat CFs in culture

The neonatal rat first passage CFs cultured in our study presented typical morphological characteristics of fibroblasts. They were of polygonal or spindle shape, or irregularly branched cytoplasm with a large ovoid nucleus. Immunofluorescence staining showed that these cells were positive for vimentin, a marker of fibroblast, and negative for desmin and von Willebrand factors, markers of vascular smooth muscle and endothelial cell respectively. These features indicated that these cells were CFs (Fig. 1).

Effects of blocking activation of Notch signaling on CMT

In order to clarify the role of Notch signaling in CMT, expressions of Notch receptors on CFs were examined. Real-time PCR and western blot showed that Notch receptor 1, 2, 3 and 4 were all expressed on CFs (Fig. 2). Then CFs were cultured with Notch inhibitor DAPT to determine the effects of blocking activation of Notch signaling on CMT. Myofibroblasts formation was determined by α-SMA expression and collagen synthesis through western blot and hydroxyproline production assay respectively. The results showed that 75μmol/L DAPT time-dependently increased α-SMA expression and collagen synthesis in CFs, and there were significant increases at 48 h compared with 0 h ($P < 0.05$). Furthermore，there were no differences for α-SMA expression and collagen synthesis between CFs cultured for 48 h without DAPT and 0 h group ($P > 0.05$) (Fig. 3A

and B). The effects of DAPT on morphological changes of CFs were also examined by immunofluorescence staining. Compared with 0 h group, CFs exposed to DAPT for 48 h exhibited brighter staining for α-SMA, larger cell size and apparent cytoskeletal stress fibers, whereas CFs cultured for 48 h without DAPT presented similar appearance with cells of 0 h group (Fig. 3C).

Effects of TGF-β1 on CMT

Effects of TGF-β1 on CMT were also determined by α-SMA expression and collagen synthesis through western blot and hydroxyproline production assay. 10ng/ml TGF-β1 induced time-dependent increases of α-SMA expression and collagen synthesis of CFs, and there were significant increases at 24 h compared with 0 h ($P < 0.05$). There were no differences of α-SMA expression and collagen synthesis between CFs cultured for 24 h without TGF-β1 and 0 h group ($P > 0.05$) (Fig. 4A and B). Immunofluorescence staining for α-SMA indicated that TGF-β1 notably intensified expression of α-SMA and resulted in distinctive morphological changes in CFs after 24 h stimulation, and CFs cultured for 24 h without TGF-β1 did not exhibit distinctive changes in the expression of α-SMA and morphology (Fig. 4C).

Effects of TGF-β1 on expressions of Notch receptors

Expressions of Notch receptors were measured by real-time PCR and western blot analysis. The results showed that 10ng/ml TGF-β1 down-regulated the expressions of Notch1, 3 and 4 mRNA and proteins time-dependently, whereas Notch2 expression was not changed. However, the downtrends for Notch1, 3 and 4 were different: Notch3

expression decreased significantly after 12 h treatment, and its minimum level was about one third of its untreated level; Notch 1 and 4 expression decreased significantly after 18 h treatment, and their minimum level was about one half of their untreated levels (Fig. 5, 6).

Discussion

Myofibroblasts are characterized by acquisition of α-SMA expression and enhancement of collagen synthesis. It is the major cellular source of excessive collagen in fibrotic myocardium. Myofibroblasts are not observed in normal myocardium (Manabe *et al.* 2002, Sun *et al*. 2002), transformation of myofibroblasts from CFs is a critical event in the initiation and development of myocardial fibrosis. Therefore, inhibition of CMT is considered to be a key target for anti-myocardial fibrosis therapy. However, the regulatory mechanisms underlying this transformation remain unclear.

Notch signaling is an important pathway that determines fates of cells (Kwon *et al*. 2009). It has been found that Notch signaling implicated in myofibroblast transformation from other types of cell (Mann *et al*. 2007, Kennard *et al*. 2008, Liu *et al*. 2009), but whether Notch signaling is involved in CMT remains unclear. In order to elucidate the role of Notch signaling in CMT, the effects of DAPT on CMT were examined. The core procedure of Notch signaling activation is the generation of Notch intracellular domain (NICD), which translocates to the nucleus and activates the expression of its downstream target genes. NICD is cleaved away from the full-length Notch receptor in a two-step proteolytic process, and a γ-secretase complex plays the key role in it (High and Epstein 2008, Sassi *et al*. 2009). It has been demonstrated that DAPT could block the γ-secretase

complex and, as a consequence, efficiently block the generation of NICD, so DAPT is a well used inhibitor of Notch signaling (Shimizu *et al*. 1999, Small *et al*. 2001, Karanu *et al*. 2000). It has been shown that over-expression of NICD could inhibit myofibroblast formation from 10T1/2 fibroblast (Kennard *et al*. 2008), promote lung myofibroblast differentiation from fibroblasts (Liu *et al*. 2009). Inhibition of NICD generation could promote myofibroblasts formation from NIH 3T3 fibroblast (Su *et al*. 2006). These results give us the hint that Notch signaling might be involved in the process of CMT. Our results indicated that DAPT markedly increased α-SMA expression and collagen synthesis in CFs, which suggests that blocking activation of Notch signaling may promote CMT. In other words, Notch signaling may negatively regulate phenotypic switch of CFs into myofibroblasts. However, DAPT is a nonspecific subtype inhibitor of Notch receptors, thus we still could not determine the definite receptor subtypes by which DAPT exerts its facilitative effect on CMT.

TGF-β1 is the most well established cytokine that induces differentiation of CFs into myofibroblasts, and it is the common downstream mediator of myocardial fibrosis in vivo and CMT in vitro induced by various stimulators (Kuwahara *et al*. 2002, Zhao *et al*. 2008, Roy *et al*. 2003). Therefore, we used TGF-β1 as stimulus of CMT in our study to investigate the possible subtype of Notch receptors involved in CMT. Up to now, Signal pathways mediating TGF-β1-induced CMT has not been fully elucidated. Notch signaling is one of the downstream pathways of TGF-β1 (Mitsiadis *et al*. 2003, Zhu *et al*. 2010). Previous studies have shown that Notch receptors involved in myofibroblasts transformation from different kinds of cells induced by TGF-β1 (Kennard *et al*. 2008, Saad *et al*. 2010). Our results revealed that Notch receptors 1 to 4 were all expressed on

CFs, expressions of Notch 1, 3 and 4 on CFs were gradually decreased with TGF-β1 stimulation, whereas expression of Notch 2 was not markedly changed. Moreover, the downtrends of Notch 1, 3 and 4 were corresponding to the trend growth of α-SMA expression and collagen synthesis in the process of TGF-β1 stimulation. Based on the above results together, we infer that Notch receptor 1, 3 and 4 may be involved in the process of CMT, and TGF-β1 might induce CMT by down-regulating Notch 1, 3 and 4 expressions.

However, DAPT promoted CMT alone is not sufficient to prove the cause that there is necessary relationship between the down-regulation of Notch signaling and CMT, since we could not exclude the possibility that DAPT might promote CMT through other pathways except Notch signaling exclusively. Similarly, the down-regulations of Notch receptor 1, 3 and 4 in the process of CMT induced by TGF-β1 is not enough to prove that the facilitation of TGF-β1 on CMT is mediated by the down-regulations of Notch receptor subtypes. The key point is that we could not exclude the possibility that the down-regulations of Notch receptors and CMT are only concomitant phenomenon. In addition, our results also showed that Notch signaling inhibitor DAPT induced CMT in 48 hours, whereas TGF-β1 induced CMT only in 24 hours. The possible reason that TGF-β1 induced CMT more quickly than DAPT did might be: TGF-β1 exerts effects on CMT by multi-ways other than Notch signaling alone. In other words, the down-regulation of Notch receptor subtypes induced by TGF-β1 indicates only the partial rather than the complete mechanism underlying the TGF-β1 induced CMT. Therefore, even if the down-regulation of Notch receptors is indeed involved in CMT induced by TGF-β1, we still could not know whether the down-regulation of Notch receptors is an

important mechanism in the process of TGF-β1-induced CMT. Over-expression of Notch receptors or their corresponding NICDs would help us answer these questions. If over-expressions of Notch receptors or NICDs could inhibit DAPT- and TGF-β1-induced CMT significantly, we would conclude that Notch signaling negatively regulates CMT, and the down-regulation of Notch receptors plays an important role in TGF-β1-induced CMT. Of course, that would be our next work.

In conclusion, we demonstrate for the first time that Notch signaling inhibitor DAPT facilitates CMT, and Notch receptor 1, 3 and 4 are down-regulated in the process of CMT induced by TGF-β1. Further revealing the roles of Notch receptors in the CMT would be helpful to elucidate the essential mechanism of pathological differentiation of CFs into myofibroblasts. The results of the present study may provide a new therapeutic rationale for patients suffering from myocardial fibrosis.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Figure Legends

Fig.1 Identification of CFs. First passage CFs from neonatal SD rat were cultured till near confluence, then the cells were washed, fixed and immunofluorescence stained with antibodies to desmin, von Willebrand factor and vimentin respectively. Bar=20 μm.

Fig.2 Expressions of Notch receptors on CFs. Untreated CFs were cultured till near confluence and then starved for 24 h in serum-free DMEM. A: mRNA expressions of Notch receptor 1-4 in CFs were determined by real-time PCR, GAPDH served as loading control. Data were expressed as mean ± SEM of 6 independent experiments. B: Protein expressions of Notch receptor 1-4 in CFs were determined by western blot analysis, β-actin served as loading control. Upper panel showed a representative immunoblot and lower panel provided the pooled relative values of densitometric scanning. Data were expressed as mean ± SEM of the folds relative to expression of Notch1 in 6 experiments.

Fig.3 Effects of Notch inhibitor DAPT on CMT. CFs were treated with serum-free DMEM alone or 75μmol/L DAPT for designated times. A: Effects of DAPT on α-SMA expression in CFs were determined by western blot, β-actin served as loading control. Upper panel showed a representative immunoblot, and lower panel provided the pooled relative values of densitometric scanning. Data were expressed as mean ± SEM of the folds relative to 0 h cells in 6 experiments. B: Effects of DAPT on collagen synthesis in CFs were determined by hydroxyproline production assay. Data were presented as mean ± SEM of 15 samples from 5 independent experiments. C: Effects of DAPT on morphological changes of CFs were determined by immunofluorescence staining with anti-α-SMA antibody. Pictures shown were representative of 4 independent experiments. Bar =20 μm. *$P<0.05$ compared with 0 h DAPT-treated CFs.

Fig.4 Effects of TGF-β1 on CMT. CFs were treated with serum-free DMEM alone or 10ng/ml TGF-β1 for designated times. A: Effects of TGF-β1 on α-SMA expression in CFs were determined by western blot, β-actin served as loading control. Upper panel showed a representative immunoblot, and lower panel provided the pooled relative values of densitometric scanning. Data were expressed as mean ± SEM of the folds relative to 0 h cells in 6 experiments. B: Effects of TGF-β1 on collagen synthesis in CFs were determined by hydroxyproline production assay. Data were presented as mean ± SEM of 15 samples from 5 independent experiments. C: Effects of TGF-β1 on morphological changes of CFs were determined by immunofluorescence staining with anti-α-SMA antibody. Pictures shown were representative of 4 independent experiments. Bar =20 μm. *P<0.05 compared with 0 h TGF-β1-treated CFs.

Fig.5 Effects of TGF-β1 on mRNA expression of Notch receptor 1-4 in CFs. After CFs were treated with 10ng/ml TGF-β1 for designated times, total RNAs were extracted and the mRNA expressions of Notch1 (A), Notch2 (B), Notch3 (C) and Notch4 (D) were analyzed by real-time PCR, GAPDH served as loading control. All data were expressed as mean ± SEM of 6 independent experiments. *P<0.05 compared with 0 h CFs.

Fig.6 Effects of TGF-β1 on protein expression of Notch receptor 1-4 in CFs. After CFs were treated with 10ng/ml TGF-β1 for designated times, total proteins were extracted and the protein expressions of Notch1 (A), Notch2 (B), Notch3 (C) and Notch4 (D) were analyzed by western blot analysis, β-actin served as loading control. Every upper panel showed a representative immunoblot, and every lower panel provided the pooled relative values of densitometric scanning. Data were expressed as mean ± SEM of the folds relative to 0 h cells in 6 experiments. *P<0.05 compared with 0 h CFs.

Fig.1

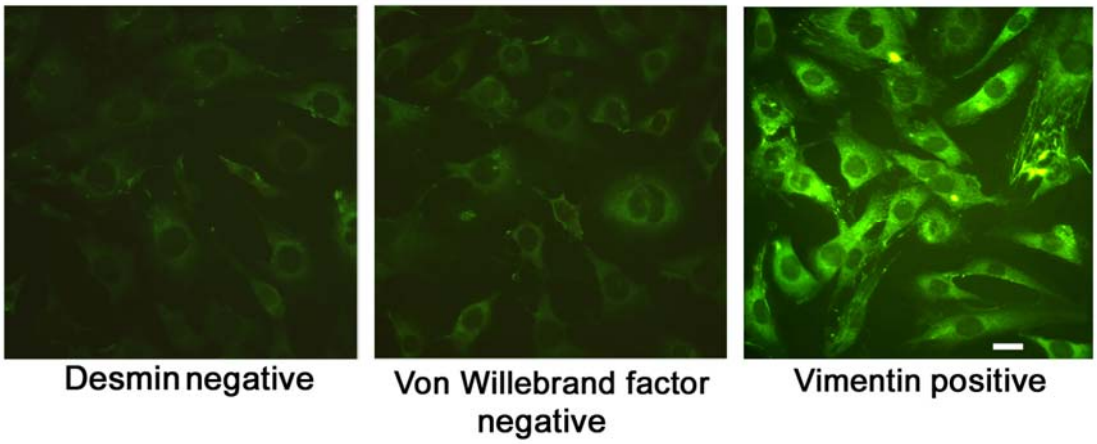

Desmin negative
Von Willebrand factor negative
Vimentin positive

Fig.2

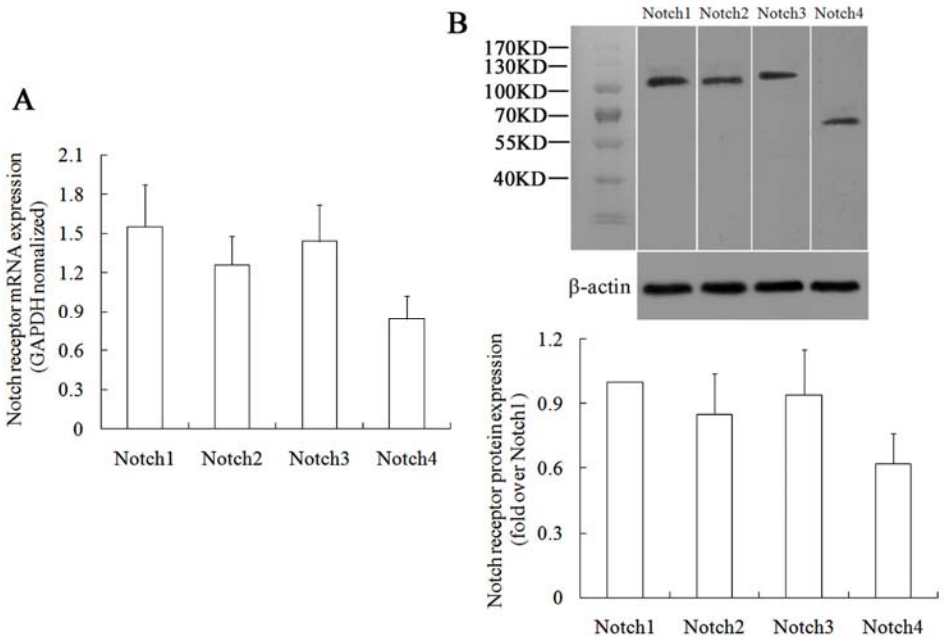

A
Notch receptor mRNA expression (GAPDH nomalized)
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
Notch1
Notch2
Notch3
Notch4
B
Notch1 Notch2 Notch3 Notch4
170KD
130KD
100KD
70KD
55KD
40KD
β-actin
Notch receptor protein expression (fold over Notch1)
1.2
0.9
0.6
0.3
0
Notch1
Notch2
Notch3
Notch4

Fig.3

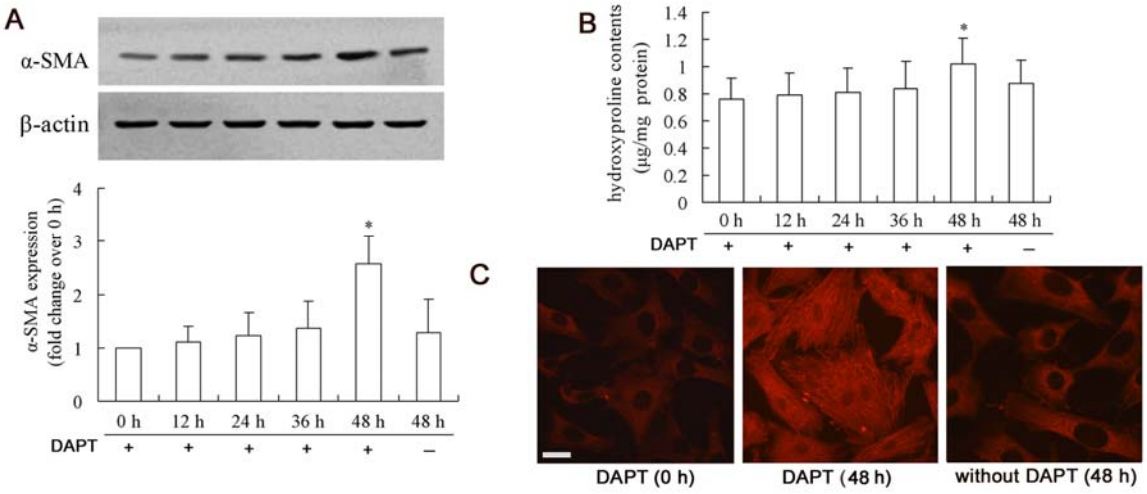

A
α-SMA
β-actin
α-SMA expression (fold change over 0 h)
0 h 12 h 24 h 36 h 48 h 48 h
DAPT + + + + + –
B
hydroxyproline contents (μg/mg protein)
0 h 12 h 24 h 36 h 48 h 48 h
DAPT + + + + + –
C
DAPT (0 h)
DAPT (48 h)
without DAPT (48 h)

Fig.4

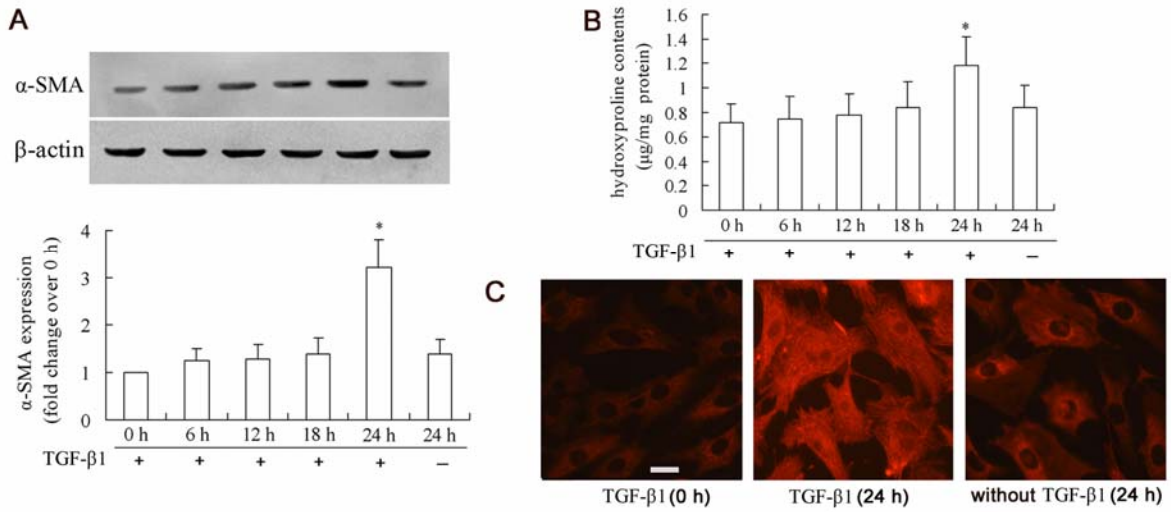

A
α-SMA
β-actin
α-SMA expression (fold change over 0 h)
0 h 6 h 12 h 18 h 24 h 24 h
TGF-β1 + + + + + –
B
hydroxyproline contents (μg/mg protein)
0 h 6 h 12 h 18 h 24 h 24 h
TGF-β1 + + + + + –
C
TGF-β1 (0 h)
TGF-β1 (24 h)
without TGF-β1 (24 h)

Fig.5

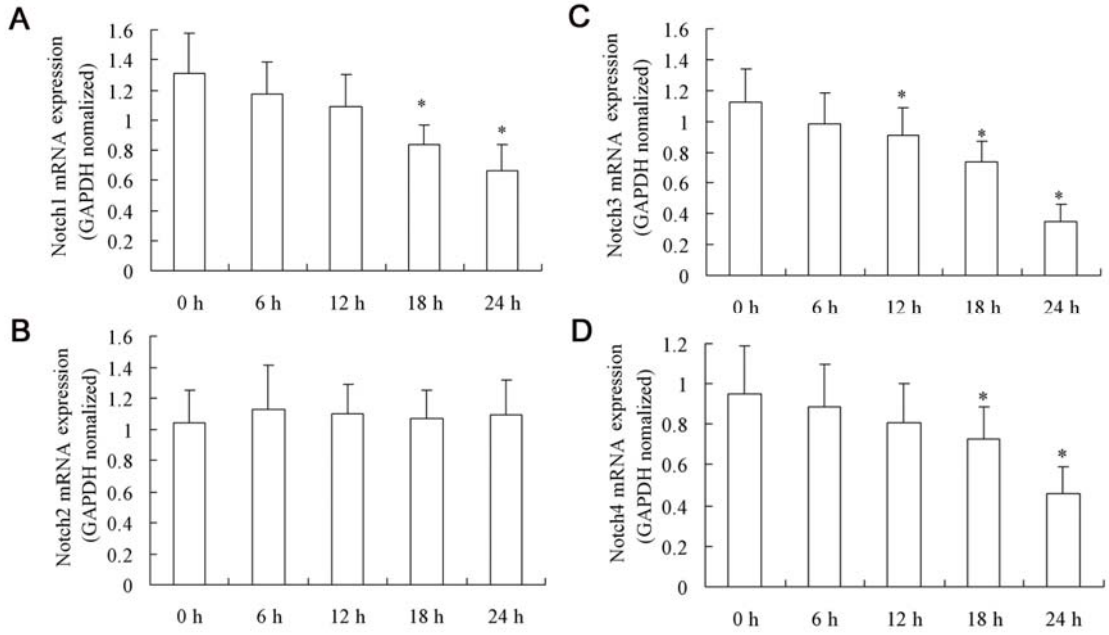
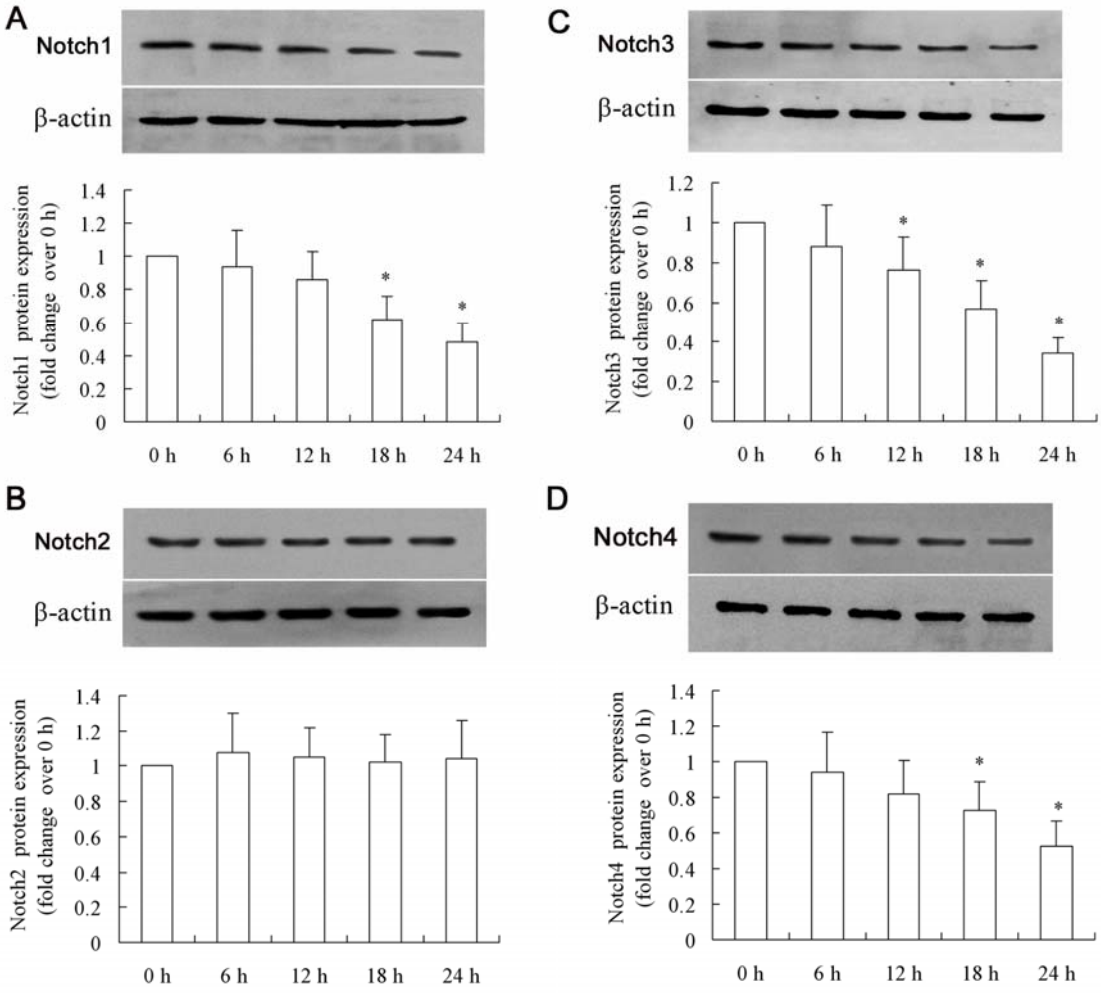

A
Notch1 mRNA expression (GAPDH nomalized)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 h
6 h
12 h
18 h
24 h
*
*
B
Notch2 mRNA expression (GAPDH nomalized)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 h
6 h
12 h
18 h
24 h
C
Notch3 mRNA expression (GAPDH nomalized)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 h
6 h
12 h
18 h
24 h
*
*
*
D
Notch4 mRNA expression (GAPDH nomalized)
1.2
1
0.8
0.6
0.4
0.2
0
0 h
6 h
12 h
18 h
24 h
*
*

Fig.6


A
Notch1
β-actin
Notch1 protein expression (fold change over 0 h)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 h
6 h
12 h
18 h
24 h
B
Notch2
β-actin
Notch2 protein expression (fold change over 0 h)
C
Notch3
β-actin
Notch3 protein expression (fold change over 0 h)
D
Notch4
β-actin
Notch4 protein expression (fold change over 0 h)