Original Paper

Hypoxia Suppresses TGF- β 1-Induced Cardiac Myocyte Myofibroblast Transformation by Inhibiting Smad2/3 and RhoA Signaling Pathways

Zhankui Yan^{a,b} Daifei Shen^a Jilin Liao^{a,b} Yanmei Zhang^a Yicun Chen^a
Ganggang Shi^a Fenfei Gao^a

^aDepartment of Pharmacology, Shantou University Medical College, ^bDepartment of Pharmacy, The Second Affiliated Hospital, Shantou University Medical College, Shantou, Guangdong, China

Key Words

Hypoxia • TGF- β 1 • Cardiac myocyte • Transformation

Abstract

Background/Aims: Hypoxia modulation of transforming growth factor (TGF)- β -induced signaling during myofibroblast transformation is dependent on the specific cell type. The purpose of this study was to explore the effects of hypoxia on myofibroblast transformation of TGF- β 1-induced cardiomyocyte H9c2 cells. **Methods:** H9c2 cells were cultured for intermittent hypoxia treatment and TGF- β 1 treatment. α -Smooth muscle actin (α -SMA) expression was examined by western blotting and immunofluorescence after treatment. To further explore the possible mechanism for this effect, the effects of hypoxia on three early TGF- β -dependent signaling pathways, i.e. the Smad2/3, RhoA and mitogen-activated protein kinase (MAPK) pathways, were screened by western blotting. **Results:** Intermittent hypoxia induced TGF- β 1 expression, but had no effect on α -SMA expression. Exogenous TGF- β 1 alone upregulated α -SMA expression in H9c2 cells in a concentration- and time-dependent manner. α -SMA expression declined with the duration of hypoxia after intermittent hypoxia and exogenous TGF- β 1 co-treatment. Phospho-JNK and phospho-p38 levels were not significantly altered after TGF- β 1 and hypoxia treatment. However, levels of phospho-ERK increased after TGF- β 1 treatment and continued to increase after hypoxia co-treatment. The activation of phospho-Smad2/3 and phospho-RhoA induced by TGF β 1 was significantly reduced after hypoxia co-treatment. **Conclusion:** Hypoxia can inhibit TGF- β 1-induced H9c2 myofibroblast transformation, based on inhibition of α -SMA expression by suppressing signaling downstream of TGF- β 1, Smad2/3 and RhoA. It suggested that TGF- β -mediated cardiomyocyte transformation is not involved in hypoxia-mediated fibrosis.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Fenfei Gao, M.D., Ph.D.
and Gang-Gang Shi, M.D.,
Ph.D.

Department of Pharmacology, Shantou University Medical College, 22 Xinling Road, Shantou, Guangdong 515041 (China); Tel. +86-754-88900432, Fax +86-754-88566782, E-Mail ffgao@stu.edu.cn, ggshi@stu.edu.cn

KARGER

Introduction

Cardiac fibrosis is a common consequence of heart disease, such as myocardial infarction, hypertrophy, hypertension and ischemia-reperfusion [1-3]. Hypoxia is an important factor in initiating cardiac fibrosis, which can modulate transdifferentiation from fibroblasts into myofibroblasts, proliferation and extracellular matrix composition (ECM) [4-6]. Fibroblasts, and related myofibroblasts, are the principle producers of ECM and contribute significantly to fibrosis in the heart [7, 8]. Myofibroblasts, which are distinguished by the expression of α -smooth muscle actin (α -SMA), are generated from a variety of sources, including resident mesenchymal cells, and epithelial and endothelial cells in processes termed epithelial/endothelial-mesenchymal transition (EMT/EndMT). In addition, myofibroblasts are also derived from circulating fibroblast-like cells, called fibrocytes, which themselves are derived from bone-marrow stem cells [9].

Transforming growth factor (TGF)- β plays a key role in myofibroblast transformation, as well as EMT [10-12]. TGF- β activates a heteromeric complex of type I and type II serine/threonine kinase receptors to induce phosphorylation of Smad2/3 transcription factors [13]. Phospho-Smad2/3 (p-Smad2/3) combines with commonly-expressed Smad4 and enters the nucleus to recruit the coactivator, CBP (CREB binding protein)/p300, to regulate downstream genes and alter ECM expression [14-16]. TGF- β can also activate mitogen-activated protein kinase (MAPK) signaling [17] and Ras homolog gene family, member A (RhoA) pathways [18] either independently or as modulators of Smads.

Zhang, et al. reported that hypoxia increases TGF- β expression and concomitant downstream effectors, Smads, in human umbilical vein endothelial cells [19]. However, Chen et al. found that both TGF- β 1 and hypoxia increased ECM expression in placental fibroblasts, but TGF- β production was not increased under hypoxia, suggesting that ECM production can be stimulated independently by hypoxia and TGF- β 1 [20]. More interestingly, Xing et al. found that hypoxia reduces TGF- β 1-induced corneal keratocyte myofibroblast transformation and α -SMA expression, possibly by reducing the early activation of RhoA [10]. These studies suggest that the profibrotic or anti-fibrotic effects of hypoxia could be dependent on the specific cell type.

Cardiac myocytes, which comprise almost 75% percent of the heart [21], are assumed be the victims of cardiac fibrosis. However, our previous oligonucleotide array-based transcription factor assay (OATFA) screen found that hypoxia enhances E47 (transcription factor-3, TCF-3) and hypoxia-inducible factor (HIF) activity in primary cardiac myocytes of neonatal rats. Several studies concerning the molecular regulation of EMT suggest that E2A transcription factors E12 and E47, which result from alternative splicing, play a critical role in driving alterations in gene expression profile during EMT [22-24]. The promoters of two key genes involved in the EMT process, E-cadherin and α -SMA, contain a number of E-box motifs that enable the binding of E2A transcription factors [22, 25]. Because cardiac myocytes may be induced to undergo myofibroblast transformation, and hypoxia/reoxygenation can increase the expression of fibrosis-related proteins, including fibronectin, collagen I and III in cardiomyocyte H9c2 cells [26], in light of the possibility that the interplay between hypoxia and TGF- β is cell type-specific, we explore the interplay between hypoxia and TGF- β 1 on myofibroblast transformation in cardiomyocyte H9c2 cells.

Materials and Methods

Cell culture

The cardiac myocyte H9c2 cells (ATCC, USA) were inoculated into 60 mm dishes (at approximately 10^5 cells per dish) containing 5 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and cultured under 95% air and 5% CO₂ at 37°C. Culture medium was changed every 2 days.

TGF- β 1 and hypoxia treatment

For TGF- β 1 treatment, recombinant TGF- β 1 (Cell signaling Technology, USA) was added into the culture medium and cells were incubated for various times. For hypoxia treatment, cells were incubated in a hypoxia workstation (Baker Ruskin, UK) equilibrated with 1% oxygen/5% CO₂-balance nitrogen. In consideration of cell viability under long-term hypoxia exposure, cells were exposed to hypoxia for 1-h durations every 6 h, over a 24-h period, concurrently with or without TGF- β 1.

Western blot

Cells were lysed with cell lysis buffer containing a protease inhibitor cocktail, and total protein was measured using a BCA kit (Thermo Fisher). Extracts were denatured by boiling for 5 min in sample buffer. Equal amounts of total protein (50 μ g) were subjected to 8-10% SDS-PAGE, and then were electrophoretically transferred onto nitrocellulose membranes. Nonspecific binding was blocked by incubation of membranes with TBST containing 10% skim milk for 1 h at room temperature. The blot was incubated with antibodies against α -SMA (Sigma); TGF- β 1, Smad2/3, p-Smad2/3, p-ERK, p-JNK, p-p38, RhoA (Cell signaling technology); and p-RhoA (Abcam) overnight at 4°C, then washed 3 times with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000) for 1 h at room temperature. Immunoreactive bands were visualized with chemiluminescence luminol reagent (Thermo Fisher). β -Actin (1:1000, Abcam) was used to confirm equal loading of samples. Individual blots were scanned and the desired proteins quantified using densitometry.

Immunofluorescence staining

Cultured H9c2 were fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized in 0.1% Triton X-100 with phosphate-buffered saline (PBS). Cells were then blocked with 5% BSA for 1 h, stained with mouse α -SMA antibody (1:100) in blocking medium overnight at RT, washed thrice with PBS, and incubated with goat anti-mouse IgG Alexa Fluor 488 (1:100) for 2 h. Cells were washed four times with PBS, then stained with Hoechst 33250 for 10 min, washed with PBS, then photographed under a fluorescence microscope.

Statistical analysis

All data are expressed as mean $\pm$ S.E.M. and analyzed with SPSS 17.0 statistical software (SPSS Inc.). The significance of the differences was determined using one-way ANOVA followed by an L.S.D. test. $P < 0.05$ was considered statistically significant.

Results

The expression of TGF- β 1 and α -SMA after hypoxia treatment

As shown in Fig. 1A, very low expression of TGF- β 1 was detected in control H9c2 cells, but the production of TGF- β 1 increased more than 2-fold at 13 and 19 h after intermittent hypoxia ($p < 0.05$). Therefore, hypoxia induces the expression of TGF- β 1. Strangely, the expression of α -SMA did not significantly change after hypoxia treatment for various times. Since α -SMA, a myofibroblast marker, is representative of the contractile

Fig. 1. Expression of TGF- β 1 and α -SMA in H9c2 cells after hypoxia. (A) Expression of TGF- β 1 after different intermittent hypoxia times in H9c2 cells. (B) Expression of α -SMA after different intermittent hypoxia times in H9c2 cells. Data shown are represented as the means $\pm$ S.E.M. from five separate experiments. * $P < 0.05$ vs. control.

Fig. 2. Effect of exogenous TGF- β 1 on expression of α -SMA in H9c2 cells. (A) Concentration-dependent changes of α -SMA expression after incubation of H9c2 cells with different TGF- β 1 concentrations (1, 5, 10, 20, 30 ng/ml) for 24 h. (B) Time-dependent changes of α -SMA expression after incubating H9c2 cells with 5 ng/ml TGF- β 1 for various times (6, 12, 24, 32, 48 h). Data shown are represented as the means $\pm$ S.E.M. from five separate experiments. * P <0.05 vs control group.

Fig. 3. Effect of combining of hypoxia with TGF- β 1 on the expression of α -SMA in H9c2 cells. (A) Effect of TGF- β 1 and hypoxia on expression of α -SMA in H9c2 cells. Data shown are represented as the means $\pm$ S.E.M. from four separate experiments. * P <0.05 vs. control group; # P <0.05 vs. TGF- β 1 group. (B) Immunofluorescence of α -SMA in H9c2 cells following hypoxia plus TGF- β 1 co-treatment. Green indicates α -SMA, blue indicates nuclei (200 $\times$, bar=100 μ m).

phenotype of myofibroblasts [15, 27], our data suggests that hypoxia induces the expression of TGF- β 1 in cultured H9c2 cells, but does not induce myofibroblast transformation of H9c2 cells.

Effect of TGF- β 1 addition on expression of α -SMA

Following stimulation of H9c2 cells with different concentrations (1, 5, 10, 20, 30 ng/ml) of TGF- β 1 for 24 h, the expression of α -SMA increased with increasing concentration of TGF- β 1 (Fig. 2A), with a statistically significant difference achieved at 5 ng/ml TGF- β 1 compared with the control (p < 0.05). Then we observed the expression of α -SMA at various times (6, 12,

24, 32, 48 h) following addition of 5 ng/ml TGF- β 1 (Fig. 2B). The expression of α -SMA increased with the time, and peaked nearly 3-fold at about 24 h ($p < 0.05$). These results show that the effects of TGF- β 1 addition are different from that of endogenous TGF- β 1 induced by hypoxia.

Effect of hypoxia and exogenous TGF- β 1 co-treatment on expression of α -SMA

We examined the expression of α -SMA after intermittent hypoxia treatment for varying times concurrent with TGF- β 1 (5 ng/ml) treatment for 24 h. As shown in Fig. 3A, exogenous TGF- β 1 alone upregulated α -SMA expression. However, increasing duration of hypoxia treatment increasingly diminished exogenous TGF- β 1-induced α -SMA expression, reduced to the control group level at about 13 h after intermittent hypoxia. To confirm the result, we examined immunofluorescence intensity of α -SMA in groups. Addition of TGF- β 1 alone enhanced the fluorescence intensity of α -SMA. When combined with hypoxia treatment, the fluorescence intensity of α -SMA was increasingly reduced with increases of hypoxia duration in 24 h (Fig. 3B). The immunofluorescence results were consistent with that of western blotting. This suggests that hypoxia suppresses TGF- β 1 signaling.

Fig. 4. Effect hypoxia on expression of TGF- β -induced p-Smad2/3, p-RhoA and MAPK. (A) Effect hypoxia on the expression of TGF- β -induced p-Smad2/3 in H9c2 cells, (B) Effect hypoxia on expression of TGF- β -induced p-RhoA in H9c2 cells. (C) Effect hypoxia on the expression of TGF- β -induced p-ERK, p-JNK, and p-P38 in H9c2 cells. * $P < 0.05$ vs. control, # $P < 0.05$ vs. TGF- β 1.

The effect hypoxia on the expression of TGF- β -induced p-Smad2/3, p-RhoA and MAPKs

As shown in Fig. 4A, compared with the control, expression of p-Smad2/3 in the TGF- β 1-treated cells was increased about 3-fold ($p < 0.05$). However, the expression of p-Smad2/3 in TGF- β 1+hypoxia group was increasingly decreased with increasing hypoxia time compared with cells treated with TGF- β 1 alone ($p < 0.05$). A decrease in expression of p-RhoA was similar to the hypoxia-mediated decrease in p-Smad2/3 (Fig. 4B). TGF- β 1 treatment induced about 5-fold up-regulation of p-ERK. However, co-treatment with hypoxia for 1 h decreased about half of the expression of p-ERK compared with TGF- β 1-treated cells ($p < 0.05$), then p-ERK levels gradually increased with increasing hypoxia exposure (Fig. 4C). The expression of p-JNK and p-p38 was unaffected by TGF- β 1 treatment and hypoxia co-treatment (Fig. 4C).

Discussion

In this study, we demonstrate that hypoxia inhibits TGF- β 1-induced H9c2 cell transformation into myofibroblasts, potentially by suppressing the activation of Smad2/3 and RhoA, a downstream signaling pathway activated by TGF- β 1, consequently reducing the expression of α -SMA. Hypoxia is thought to be one of the most significant causes of tissue fibrosis. Hypoxia causes elevated expression of TGF- β 1 in the myocardium [28, 29]

and stimulates cardiac fibroblast transdifferentiation to myofibroblasts [30]. However, our studies show that in H9c2 cells, although hypoxia induces the expression of TGF- β 1, hypoxia has no effect on α -SMA expression. Then we examined the effects of exogenous TGF- β 1 alone or with hypoxia co-treatment. Exogenous TGF- β 1 induces α -SMA expression in a concentration- and time-dependent manner. However, this TGF- β 1-mediated induction was diminished by hypoxia, indicating that hypoxia reduces TGF- β 1-induced α -SMA expression in H9c2 cells. So, we speculated that hypoxia may suppress the downstream signal pathway of TGF- β 1 to obstruct the α -SMA expression at the same time. It suggested that TGF- β -mediated cardiomyocyte transformation is not involved in hypoxia-mediated fibrosis.

To explore the possible mechanism by which hypoxia inhibits the ability of TGF- β 1 to promote myofibroblast transformation, we observed early events in the signaling pathways induced by TGF- β . It has been reported that Smads are required for TGF- β -induced α -SMA expression in fibroblasts [31, 32]. We found that the expression of p-Smad2/3 increases significantly after exogenous TGF- β 1 stimulation, but gradually decreases with time after hypoxia. This suggests that Smad2/3 might be involved in hypoxia inhibition of TGF- β 1-induced α -SMA in H9c2 cells. Smads require coactivators CBP1 and p300 for transcriptional activity [33]. One possible mechanism for inhibition of TGF- β -induced myofibroblast transformation may be hypoxia-induced HIF, which also requires the same coactivators [34], to bring about competition for CBP/p300 with Smads, thereby altering the TGF- β response.

RhoA is a member of the Ras superfamily of GTP-binding proteins, which is activated by phosphorylation, then subsequently activates downstream effector Rho-kinases (ROCKs). TGF- β can rapidly activate RhoA-dependent signaling pathways to induce EMT [18]. It has been reported that RhoA/ROCK involvement in remodeling of the myocardial matrix in diabetic rats may be associated with activation of the TGF- β /Smad pathway [35]. The changes in p-RhoA expression are similar to our observation of Smads under hypoxia and TGF- β 1 co-treatment. This suggests that the RhoA pathway might also be involved in hypoxia inhibition of TGF- β 1-induced α -SMA in H9c2 cells.

Members of the MAPK family can be activated by TGF- β in a Smad-independent manner and phosphorylate Smad3 [17]. The MAPK family is well-known for signaling in TGF- β 1-induced fibrosis [36, 37]. Liu et al. have demonstrated that reduction of ERK1/2 phosphorylation and Smad-mediated recruitment of transcriptional coactivators inhibits the profibrotic effects of TGF- β in cardiac fibroblasts [38]. We find that the expression of p-JNK and p-p38 in H9c2 cells is unaffected by hypoxia after TGF- β 1 and hypoxia co-treatment, which suggests that JNK and p38 are not likely to be involved in the TGF- β 1-induced myofibroblast transformation in H9c2 cells. Expression of p-ERK is elevated after TGF- β 1 treatment, consistent with previous findings that TGF- β 1 induces lung fibroblast-to-myofibroblast conversion via ERK1/2 activation [36]. Interestingly, co-treatment with hypoxia and TGF- β 1 increases the expression of p-ERK with hypoxia time, same as the effect of TGF- β 1, but less than TGF- β 1 treatment alone at 1 h after hypoxia co-treatment. The pathophysiological effects of p-ERK change in H9c2 cells is unknown and needs to be further investigated, but whatever the effects of p-ERK change are, hypoxia-mediated inhibition of myofibroblast differentiation does not occur through inhibition of ERK phosphorylation. Our results suggest that cardiac myocytes may utilize different signal transduction pathways from fibroblasts for hypoxia-mediated myofibroblast differentiation.

Although the H9c2 cell line was originally derived from embryonic BD1X rat heart tissue, and does not fully represent myocardiocytes, our findings suggest that hypoxia could inhibit TGF- β -induced signaling during myofibroblast transformation in cardiac myocytes. The profibrotic or anti-fibrotic effects of hypoxia are probably dependent on the specific cell type. Hypoxia can induce TGF- β 1 upregulation in cardiac myocytes, but cardiomyocyte TGF- β 1 may exert paracrine actions to stimulate cardiac fibroblast-to-myofibroblast conversion, but have no effect to cardiomyocytes themselves in regard to α -SMA expression. Further experiments are needed to confirm the findings in cardiac myocytes, and to explore the precise mechanisms by which cardiac myocytes are blocked from myofibroblast transformation.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81173048), Distinguished Innovative Project of Guangdong University (2014KTSCX068) and Special Innovation Project of Education Department of Guangdong Province (2018).

Disclosure Statement

No Disclosure Statements exists.

References

- 1 Vilahur G, Juan-Babot O, Pena E, Onate B, Casani L, Badimon L: Molecular and cellular mechanisms involved in cardiac remodeling after acute myocardial infarction. *J Mol Cell Cardiol* 2011;50:522-533.
- 2 Swynghedauw B: Molecular mechanisms of myocardial remodeling. *Physiol Rev* 1999;79:215-262.
- 3 Sabri A, Short J, Guo J, Steinberg SF: Protease-activated receptor-1-mediated DNA synthesis in cardiac fibroblast is via epidermal growth factor receptor transactivation: Distinct par-1 signaling pathways in cardiac fibroblasts and cardiomyocytes. *Circ Res* 2002;91:532-539.
- 4 Agocha A, Lee HW, Eghbali-Webb M: Hypoxia regulates basal and induced DNA synthesis and collagen type I production in human cardiac fibroblasts: Effects of transforming growth factor-beta1, thyroid hormone, angiotensin II and basic fibroblast growth factor. *J Mol Cell Cardiol* 1997;29:2233-2244.
- 5 Su F, Zhang W, Chen Y, Ma L, Zhang H, Wang F: Significance of hypoxia-inducible factor-1alpha expression with atrial fibrosis in rats induced with isoproterenol. *Exp Ther Med* 2014;8:1677-1682.
- 6 Irigoyen M, Anso E, Salvo E, Dotor de las Herreras J, Martinez-Irujo JJ, Rouzaut A: Tgfbeta-induced protein mediates lymphatic endothelial cell adhesion to the extracellular matrix under low oxygen conditions. *Cell Mol Life Sci* 2008;65:2244-2255.
- 7 Eghbali M, Weber KT: Collagen and the myocardium: fibrillar structure, biosynthesis and degradation in relation to hypertrophy and its regression. *Mol Cell Biochem* 1990;96:1-14.
- 8 Carver W, Terracio L, Borg TK: Expression and accumulation of interstitial collagen in the neonatal rat heart. *Anat Rec* 1993;236:511-520.
- 9 Wynn TA: Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008;214:199-210.
- 10 Xing D, Bonanno JA: Hypoxia reduces tgfbeta1-induced corneal keratocyte myofibroblast transformation. *Mol Vis* 2009;15:1827-1834.
- 11 Petrov VV, Fagard RH, Lijnen PJ: Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. *Hypertension* 2002;39:258-263.
- 12 Leask A: Potential therapeutic targets for cardiac fibrosis: Tgfbeta, angiotensin, endothelin, ccn2, and pdgf, partners in fibroblast activation. *Circ Res* 2010;106:1675-1680.
- 13 Wrana JL, Attisano L, Wieser R, Ventura F, Massague J: Mechanism of activation of the tgfbeta receptor. *Nature* 1994;370:341-347.
- 14 O'Kane D, Jackson MV, Kissenpfennig A, Spence S, Damkat-Thomas L, Tolland JP, Smyth AE, Denton CP, Stuart Elborn J, McAuley DF, O'Kane CM: Smad inhibition attenuates epithelial to mesenchymal transition by primary keratinocytes *in vitro*. *Exp Dermatol* 2014;23:497-503.
- 15 MacLean J, Pasumarthi KB: Signaling mechanisms regulating fibroblast activation, phenocconversion and fibrosis in the heart. *Indian J Biochem Biophys* 2014;51:476-482.
- 16 Balogh P, Katz S, Kiss AL: The role of endocytic pathways in tgfbeta signaling. *Pathol Oncol Res* 2013;19:141-148.
- 17 Engel ME, McDonnell MA, Law BK, Moses HL: Interdependent smad and jnk signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* 1999;274:37413-37420.
- 18 Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL: Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a rhoA-dependent mechanism. *Mol Biol Cell* 2001;12:27-36.

- 19 Zhang H, Akman HO, Smith EL, Zhao J, Murphy-Ullrich JE, Batuman OA: Cellular response to hypoxia involves signaling via smad proteins. *Blood* 2003;101:2253-2260.
- 20 Chen CP, Yang YC, Su TH, Chen CY, Aplin JD: Hypoxia and transforming growth factor-beta 1 act independently to increase extracellular matrix production by placental fibroblasts. *J Clin Endocrinol Metab* 2005;90:1083-1090.
- 21 Thavandiran N, Dubois N, Mikryukov A, Masse S, Beca B, Simmons CA, Deshpande VS, McGarry JP, Chen CS, Nanthakumar K, Keller GM, Radisic M, Zandstra PW: Design and formulation of functional pluripotent stem cell-derived cardiac microtissues. *Proc Natl Acad Sci U S A* 2013;110:E4698-4707.
- 22 Perez-Moreno MA, Locascio A, Rodrigo I, Dhondt G, Portillo F, Nieto MA, Cano A: A new role for e12/e47 in the repression of e-cadherin expression and epithelial-mesenchymal transitions. *J Biol Chem* 2001;276:27424-27431.
- 23 Slattery C, McMorrow T, Ryan MP: Overexpression of e2a proteins induces epithelial-mesenchymal transition in human renal proximal tubular epithelial cells suggesting a potential role in renal fibrosis. *FEBS Lett* 2006;580:4021-4030.
- 24 Slattery C, Ryan MP, McMorrow T: E2a proteins: Regulators of cell phenotype in normal physiology and disease. *Int J Biochem Cell Biol* 2008;40:1431-1436.
- 25 Kumar MS, Hendrix JA, Johnson AD, Owens GK: Smooth muscle alpha-actin gene requires two e-boxes for proper expression *in vivo* and is a target of class i basic helix-loop-helix proteins. *Circ Res* 2003;92:840-847.
- 26 Liu Q, Wang Y, Yang T, Wei W: Protective effects of mir-25 against hypoxia/reoxygenation-induced fibrosis and apoptosis of h9c2 cells. *Int J Mol Med* 2016;38:1225-1234.
- 27 Harris WT, Kelly DR, Zhou Y, Wang D, MacEwen M, Hagood JS, Clancy JP, Ambalavanan N, Sorscher EJ: Myofibroblast differentiation and enhanced tgfb signaling in cystic fibrosis lung disease. *PLoS One* 2013;8:e70196.
- 28 Lefer AM, Ma XL, Weyrich AS, Scalia R: Mechanism of the cardioprotective effect of transforming growth factor beta 1 in feline myocardial ischemia and reperfusion. *Proc Natl Acad Sci U S A* 1993;90:1018-1022.
- 29 Shyu KG, Cheng WP, Wang BW, Chang H: Hypoxia activates muscle-restricted coiled-coil protein (murc) expression via transforming growth factor-beta in cardiac myocytes. *Clin Sci* 2014;126:367-375.
- 30 Clancy RM, Zheng P, O'Mahony M, Izmirly P, Zavadi J, Gardner L, Buyon JP: Role of hypoxia and camp in the transdifferentiation of human fetal cardiac fibroblasts: Implications for progression to scarring in autoimmune-associated congenital heart block. *Arthritis Rheum* 2007;56:4120-4131.
- 31 Leivonen SK, Chantry A, Hakkinen L, Han J, Kahari VM: Smad3 mediates transforming growth factor-beta-induced collagenase-3 (matrix metalloproteinase-13) expression in human gingival fibroblasts. Evidence for cross-talk between smad3 and p38 signaling pathways. *J Biol Chem* 2002;277:46338-46346.
- 32 Hu B, Wu Z, Phan SH: Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. *Am J Respir Cell Mol Biol* 2003;29:397-404.
- 33 Ghosh AK, Yuan W, Mori Y, Varga J: Smad-dependent stimulation of type i collagen gene expression in human skin fibroblasts by tgfbeta involves functional cooperation with p300/cbp transcriptional coactivators. *Oncogene* 2000;19:3546-3555.
- 34 Guo Y, Scheuermann T, Partch C, Tomchick D, Gardner K: Coiled-coil coactivators play a structural role mediating interactions in hypoxia-inducible factor heterodimerization. *J Biol Chem* 2015;290:7707-7721.
- 35 Zhou H, Li YJ, Wang M, Zhang LH, Guo BY, Zhao ZS, Meng FL, Deng YG, Wang RY: Involvement of rhoa/rock in myocardial fibrosis in a rat model of type 2 diabetes. *Acta Pharmacol Sin* 2011;32:999-1008.
- 36 Caraci F, Gili E, Calafiore M, Failla M, La Rosa C, Crimi N, Sortino MA, Nicoletti F, Copani A, Vancheri C: Tgf-beta1 targets the gsk-3beta/beta-catenin pathway via erk activation in the transition of human lung fibroblasts into myofibroblasts. *Pharmacol Res* 2008;57:274-282.
- 37 Qin F, Shite J, Liang CS: Antioxidants attenuate myocyte apoptosis and improve cardiac function in chf: Association with changes in mapk pathways. *Am J Physiol Heart Circ Physiol* 2003;285:24.
- 38 Liu X, Sun SQ, Hassid A, Ostrom RS: Camp inhibits transforming growth factor-beta-stimulated collagen synthesis via inhibition of extracellular signal-regulated kinase 1/2 and smad signaling in cardiac fibroblasts. *Mol Pharmacol* 2006;70:1992-2003.