International Journal of Latest Research in Science and Technology **ISSN (Online):2278-5299**
Volume 6, Issue 1: Page No.61-65,January-February 2017
https://www.mnkpublication.com/journal/ijlrst/index.php

γ -SCHISANDRIN INHIBITS EPITHELIAL-MESENCHYMAL TRANSITION OF HUMAN RENAL TUBULAR EPITHELIAL HK-2 CELLS INDUCED BY TRANSFORMING GROWTH FACTOR-ß1

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Abstract – ***Renal injury and renal fibrosis are closely associated with the progression of chronic kidney disease (CKD). The epithelial-to-mesenchymal transition (EMT) takes an indispensable place of human tubular epithelial cells in renal interstitial. The transforming growth factor TGF- ß1 induced EMT in human kidney proximal tubular epithelial cell line (HK-2 cells). At the same time, the morphology of HK-2 cells altered along with the change of treated methods clearly. These evidences suggest the abduction of EMT by TGF- ß1. Although the involvement in several physiological function of γ-Schisandrin had been discovered, the medical function of TGF- ß1-induced renal fibrosis is still undiscovered. As important elements of EMT, the increased expression of collagen type Ⅰand Ⅳ decreased significantly after pre-treated with γ-Schisandrin. In addition, the gene expression of Snail, which represents myofibroblast, reduced obviously. In the present investigation, it was suggested that Sch might be capable to antagonize TGF- ß1-induced renal fibrosis and EMT in HK-2 cells. Overall, the application of traditional Chinese medicine (Sch) for the treatment of renal fibrosis patients provides an original therapeutic ideas and bright prospects to patients families.***

Keywords—epithelial-to-mesenchymal transition (EMT), renal fibrosis, HK-2 cell, TGF- ß1, γ-Schisandrin

I. INTRODUCTION

Chronic kidney disease (CKD) has become a worldwide problem, which is along with high level of consumption, high mortality and high morbidity rate[1]. It had reported that, in the next 20 years, the incidence rates of CKD are forecasted to increase constantly[2]. Relative researchers said they have found a close link among CKD and hospitalization, death and cardiovascular events[3] . In the meantime, in order to delay the progression and increase the morbidities of CKD, more health costs and focus of attention will be stimulated instantly. The patients' family suffered a white elephant prolonged their lives by means of Kidney Dialysis(KD).

Renal injury is commonly observed in clinical manifestations and relates to the development of renal fibrosis closely[4]. Renal fibrosis is a significant health problem resulted from chronic damage to Normal kidney parenchymal destruction due to scarring (fibrosis) is the key of progressive renal injury which considered as the major cause of chronic kidney disease (CKD)[5]. In end-stage renal disease, little by little, extracellular matrix (ECM) is the symbol of renal fibrosis instead of normal tissue architecture. Increase of ECM destroys the normal function of kidney[6].

Transforming growth factor TGF-ß1 is the major regulators of renal epithelial cell plasticity in the kidney[7]. TGF- ß1 plays an important role in inducing epithelial-to-mesenchymal transition (EMT)[8]. EMT is kind of description of phenotypical change that lose their cell-cell basement membrane contacts. This change is induced in epithelial cells. At the same time, the epithelial cells are similar to mesenchymal/myofibroblast cell in, and the cells' structural polarity turn to spindle-shaped[9]. Generally acknowledged morphology, in renal, liver and pulmonary fibrosis injury models, the major mechanism has been proved to EMT of extracellular matrix for the deposition[10].

In our study, we hope to explore some drug which can reverse the EMT procession. Schisandrin (Sch) is the main active ingredient of the fruit of Schisandra chinensis Baill[11] , which has been referred to multiple physiological function, such as anti-cancer, anti-inflammatory and anti-fibrosis[12-15]. γ-Schisandrin is a kind of lignan and an active ingredient which extracted from Schisandra[16]. In the lung tissue, it has been reported that schisandrin can reduce the pulmonary fibrosis[17, 18]. In another research, evidences demonstrated that TGF- ß1-induced EMT of human breast cancer could be suppressed by Sch[19]. Sch inhibited the EMT, pro-fibrotic activity and the accumulation of ECM proteins in AML12 cells for liver fibrosis[13]. However, the pharmacologic effects and the researches of schisandrin inhibits renal fibrosis are still poorly understood. We expected that these findings can attract persons' attention of the reform and innovation in CKD' therapeutic schedules. Therefore, we investigated the phenomenon of γ-Schisandrin on renal fibrosis and EMT induced by TGF- ß1, aimed at contributing something useful to the clinic treatment.

Publication History
Manuscript Received : 23 February 2017
Manuscript Accepted : 25 February 2017
Revision Received : 27 February 2017
Manuscript Published : 28 February 2017

II. METERIALS AND MERHODS

A. Cell Culture

Human kidney proximal tubular epithelial cell line(HK-2 cells) ,which immortalized by transduction with human papilloma virus (HPV) 16 E6/E7 genes, were purchased from Institute of Basic Medical Sciences & Chinese Academy of Medical Sciences(Beijing, China).HK-2 cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient MixtureF-12(DMEM-F12, HyClone; USA) supplemented with 10% fetal bovine serum(FBS) and 1% penicillin/streptomycin (Thermo Fisher SCIENTIFIC; New York, USA)at 37℃ in 5% CO2.When they reached 80-90% confluence, cells were passaged by a 1:4 spilt ratio .All the experiments were conducted using the HK-2 cells.

B. Cytotoxicity Assays

We tested the cell viability with Cell Counting Kit-8(CCK8, DOJINDO; Japan), following the manufacturer's specifications. HK-2 cells were seeded overnight in 96-well plates with the density of 5×10^3 cells per well(100 µl medium).

Subsequently, cells were treated with γ-Schisandrin (National Institutes for Food and Drug Control, China) at different concentrations for 24h, then adjusted the CCK-8 solution volume per well to 10 % o the total volume. Next incubated the cells for about 2 h at 37 ℃ in 5% CO2. At last, measure the absorbance at 450 nm with a microplate reader (BioTek Instruments; Vermont, USA). Each measurement was repeated in triplicate at least. Every independent concentration had six replicates per experiment.

C. Cell Treatment

HK-2 cells were seeded overnight in 6-well plates with 2×10^5 cells per well. Next HK-2 cells were pre-treated with γ-Schisandrin (5 µM, 25 µM). After 2h, the cells were co-cultured with TGF- ß1(5 ng/ml) for 24 h at 37 ℃ in 5% CO2.

D. Morphological Observation of HK-2 cells

HK-2 cells were incubated with TGF- ß1(5 ng/ml) for 24 h, then cells were washed with phosphate buffed saline (PBS) twice. The morphological changes in the cells were observed under an inverted phase-contrast microscope (Leica, Germany). All of the photographs were taken at 100× magnifications using a digital camera. At last, all micrographs were processed by Adobe Photoshop software.

E. Total RNA Isolation and cDNA Synthesis

HK-2 cells were seeded in six-well plates, with a density of 2×10^5 cells/well. Total RNA was isolated from HK-2 cells and extracted with TRIzol reagent (Invitrogen Life Technologies; CA, USA), and the total RNA was extracted using chloroform extraction and isopropanol precipitation, following the manufacturer's specifications[20]. The RNA samples' concentration was quantified spectrophotometrically (Thermo Scientific, Wilmington, DE, USA), at OD 260 nm, assessed the purity by determining the OD 260/OD 280 ratio, where a ratio between 1.8 to 2.0 is usually considered to be an acceptable indicator of good RNA[21]. Then the RNA was dissolved in 30 µl RNase - Free Water. 1µg of total RNA was reverse transcribed to cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Bio; Tokyo, Japan) as per manufacture's protocol in a 10 µl volume. cDNA stored at -20 □ for a long time.

F. Quantitative real-time PCR

Quantitative real-time PCR was performed in a CFX 96 Touch™ detection system (Bio-Rad Laboratories) using SYBR Green PCR Kit (TaKaRa Bio; Tokyo, Japan). Each measurement was repeated in triplicate at least. Primer sequences and PCR product sizes of primer pairs were provided in table. The fold change of the target mRNA was calculated using the $2^{-\Delta\Delta Ct}$ Method. Expression of the housekeeping gene glyceraldehyde-3-phospgate-dehydrogenase (GAPDH) was used to normalize the amount of cDNA between different samples. The primers and their sequences are listed in Table 1.

All datas were analyzed with Prism GraphPad statistical software 6.01. The significant differences between two groups of data were analyzed by unpaired (Student 's) *T*-test. The multiple comparisons between different Numerical data were presented as mean ± standard deviation (SD). $P < 0.05$ was regarded as statistically significant. All the experiments were done in triplicates.

G. Statistical Analysis

Table 1. Primers used for Quantitative real-time PCR amplification in the present study

Gene	Primer Sequence(5' to 3')	Product length
Col1a1	F: 5'- GAGGGCCAAGACGAAGACATC-3'	140
	R: 5'- CAGATCACGTCATCGCACAAC-3'	
Col4a1	F: 5'- GGACTACCTGGAACAAAAGGG-3'	240
	R: 5'-GCCAAGTATCTCACCTGGATCA-3'	
E-cadherin	F: 5'- CATGAGTGTCCCCCGGTATC-3'	89
	R: 5'- CAGTATCAGCCGCTTTCAGA -3'	
Vimentin	F: 5'- GACGCCATCAACACCGAGTT-3'	238
	R: 5'-CTTTGTCGTTGGTTAGCTGGT-3'	
Slug	F: 5'- CGAACTGGACACACATACAGTG-3'	87
	R: 5'-CTGAGGATCTCTGGTTGTGGT-3'	
Snail	F: 5'- ACTGCAACAAGGAATACCTCAG-3'	242
	R: 5'- GCACTGGTACTTCTTGACATCTG-3'	
GAPDH	F: 5'- ACAACTTTGGTATCGTGGAAGG-3'	101
	R: 5'- GCCATCACGCCACAGTTTC-3'	

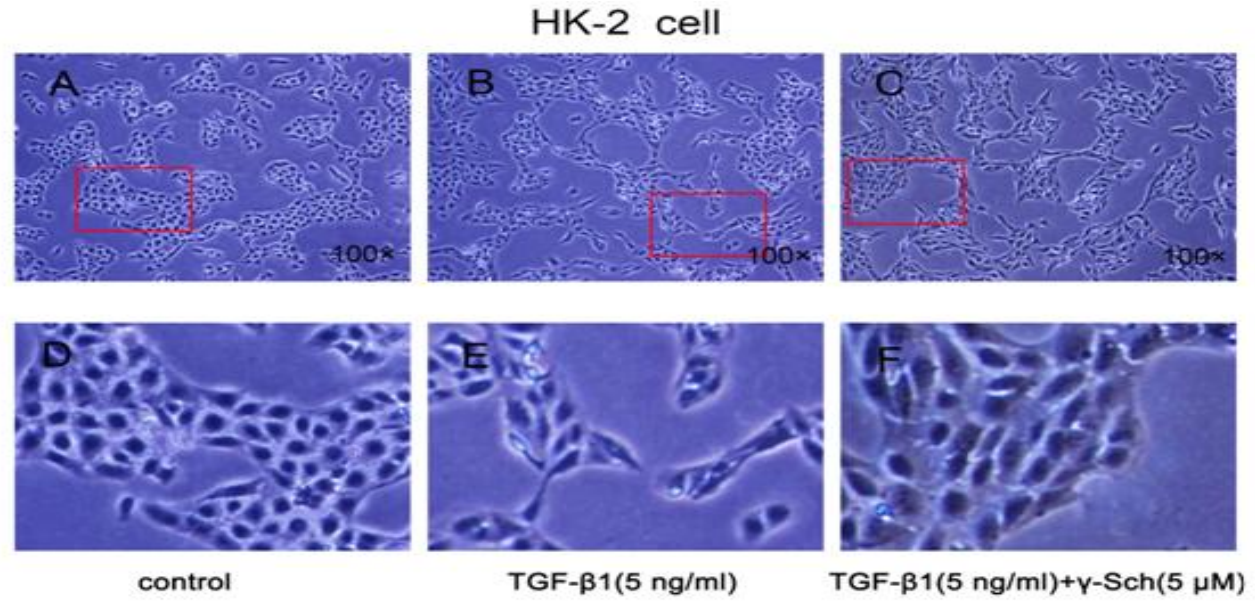

Figure. 1. **Effects on the Morphological Changes Resulting from TGF-β1-Induced HK-2 Cell Injury.** Phase-contrast microscopy images are presented, showing HK-2 cells morphology untreated (A, D), treated with TGF-β1(5 ng/ml) for 24 h (B, E) and pre-treated with γ-schisandrin (5 μM) for 2h (C, F). We found out the cells with fibroblasts-like morphology under TGF-β1 and the cells that retain or recover to their cobblestone morphology under complete medium. Original magnification,×100. HK-2, human kidney proximal tubular epithelial cell; TGF-β1, transforming growth factor- β1.

A. Characterization of cultured human kidney proximal tubular epithelial cells (HK-2 cells) and TGF- β1 induced interstitial cell morphology

This research used human kidney proximal tubular epithelial cells (HK-2 cells) to study the mechanisms of TGF- ß1 mediated EMT. The control cells showed a typical epithelial cuboidal shape, with a cobblestone morphology, as shown in Fig 1(A and D). On the contrary, addition of TGF- ß1 resulted in morphological change to a fibroblast-like shape identifiable by the presence of elongate lamellipodia and a spindle shape, as shown in Fig 1(B and E). After combining with TGF- ß1 and γ-Schisandrin, the morphological of cells went back to cobblestone shape, as shown in Fig 1(C and F).

B. TGF- β1 induced the expression of ECM and EMT in human kidney proximal tubular epithelial cells (HK-2 cells)

*T*GF- ß1 is a recognized important mediator of fibrosis including EMT[22]. In order to ascertain whether TGF- ß1 could induce the expression of ECM proteins and EMT markers, we selected Quantitative real-time PCR. Human kidney epithelial HK-2 cells were treated with TGF- ß1 (5 ng/ml) at the specific arranged time points. As shown in Fig 2(A and B). ECM proteins include collagen type Ⅰ and Ⅳ were induced by TGF- ß1. Then, TGF- ß1 decreased E-cadherin and increased Vimentin protein expression in HK-2 cells, as shown in Fig 2(C and D). Fibrosis is associated with the expression of transcription factors. TGF- ß1 increased Snail and Slug levels, as shown in Fig 2(E and F).

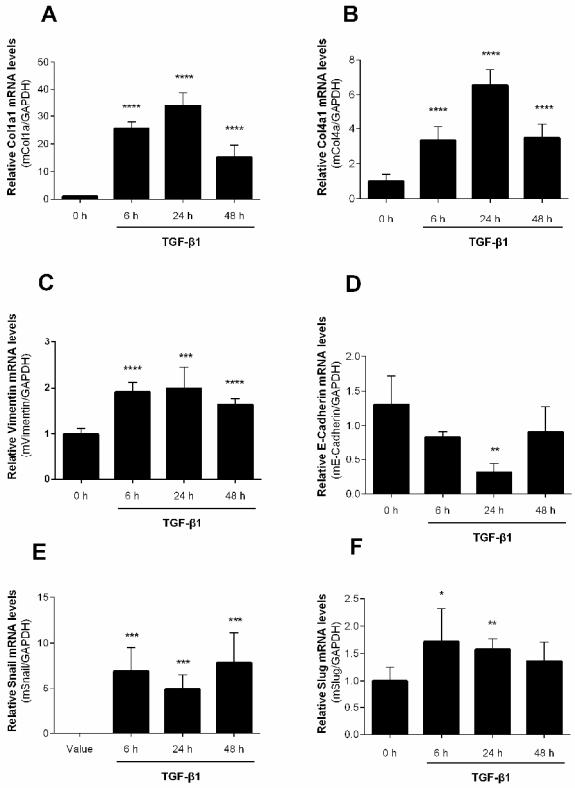

Figure. 2. **TGF-β1 Stimulated EMT in HK-2 cells.**HK-2 cells were stimulated with recombinant human TGF-β1(5 ng/ml), and gene expression was assessed 6, 24 and 48 h later by quantitative real-time PCR for EMT-related genes. Including mesenchymal marker Col1a1 (A), Col4a1 (B), Vimentin (C), Snail 1 (D), and Slug (E), as well as epithelial marker E-cadherin (F) ,values are presented as the mean±SD from three independent experiments, and each was conducted in duplicate.*P＜0.05， **P＜0.01,***P＜0.001,****P＜0.0001 vs. control group. HK-2, human kidney

proximal tubular epithelial cell; TGF-β1, transforming growth factor- β1; EMT, epithelial-mesenchymal transition.

C. γ-Schisandrin inhibited TGF- ß1-induced overexpression of ECM and EMT in human kidney proximal tubular epithelial cells (HK-2 cells)t

As we all known, TGF- ß1 can induce ECM and EMT. We examined the cell viability of γ-Schisandrin to HK-2 cells at different concentrations (Fig 3, A). Though interpretation of the results, we determined cencentration 5 μM, 25 μM as our working concentrations. We used Quantitative real-time PCR to examine the gene expression of collagen type Ⅰ (Fig 3, B) and Ⅳ(Fig 3, C), Snail(Fig 3, D) in HK-2 cells. Snail is the most important EMT transcriptional regulator and E-cadherin repressor, which is a zinc finger –type transcription factor. To analyze the effect of TGF- ß1 on collagen type Ⅰ and Ⅳ, Snail protein expression, HK-2 cells were pretreated with γ-Schisandrin (5 μM, 25 μM) for 2 h. Subsequently, cells were exposed to 5 ng/ml TGF- ß1 for 24h. After treatment with γ-Schisandrin, it is obviously observed that the relative expression levels of ECM and EMT decreased. Our data showed an apparent change of the levels, as shown in Fig 3(B-D).

IV. DISCUSSION

As renal fibrosis always associates with chronic kidney disease (CKD) and ends up with end-stage failure[23], to suppress EMT is a main strategy to combat renal fibrosis. Research previously reported that renal tubular epithelial cells lose their epithelial phenotype, replaced with characteristics of mesenchyme in renal injury and fibrosis[9].

TGF- ß1 is the most established mediator of fibrosis. Although it is known that TGF- ß1 can induce EMT[24], there has been very little research exploring the inhibitory action of fibrosis and EMT in HK-2 cells. In our study, HK-2 cells induced EMT by being treated with transforming growth factor TGF-ß1. The change of morphology indicates that HK-2 cells transformed into myofibroblast cell potentially. Those evidences showed that TGF- ß1 could induce HK-2 cells EMT. On this regard, it is time to develop a kind of ideal drug that has few systematic toxicities to patients.

This could be confirmed that the high-safety of compound had been revealed by amount of laboratories[17, 25].Several studies have provided evidence that γ-Schisandr in possesses a lot of biological properties[12, 13, 15, 17, 26, 27], including anti-inflammatory, anti-tumor and so on. Fructus Schisandrae has long been addicted by all kinds of human beings for sustaining health[28]. Previous studies have reported that Schisandrin attenuates breast cancer and hepatic fibrosis. However, the specific effects on preventing the progression of chronic renal diseases are still lacking, it is necessary to explore the relationship between γ-Schisandrin and the prevention of EMT. Also, we found that Schisandrin can reduce the expression of EMT-related stromal cell marker factors, including collagen type-Ⅰ, type-Ⅳ collagen and Snail in renal fibrosis. In summary of the present study, we evaluated the γ-Schisandrin plays an important role in the inhibitory on EMT process in renal injury and fibrosis.

In conclusion, the present study demonstrated that γ-Schisandrin suppressed TGF- ß1-induced renal fibrosis. γ-Schisandrin inhibited EMT progress so that renal injury and fibrosis were attenuated. Our findings might supply a basis for investigations aimed at developing positive therapeutic strategies in reversal of human renal fibrosis with no severe side effects in future.

V. ACKNOWLEDGEMENTS

This study was supported by the Science and Technology Planning Project of Guangdong Province (2014A030313382).

VI. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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