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Overexpression of Snail in retinal pigment epithelial triggered epithelial–mesenchymal transition

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ABSTRACT

Snail transcription factor has been implicated as an important regulator in epithelial–mesenchymal transition (EMT) during tumourigenesis and fibrogenesis. Our previous work showed that Snail transcription factor was activated in transforming growth factor β1 (TGF-β1) induced EMT in retinal pigment epithelial (RPE) cells and may contribute to the development of retinal fibrotic disease such as proliferative vitreoretinopathy (PVR). However, whether Snail alone has a direct role on retinal pigment epithelial–mesenchymal transition has not been investigated. Here, we analyzed the capacity of Snail to drive EMT in human RPE cells. A vector encoding Snail gene or an empty vector were transfected into human RPE cell lines ARPE-19 respectively. Snail overexpression in ARPE-19 cells resulted in EMT, which was characterized by the expected phenotypic transition from a typical epithelial morphology to mesenchymal spindle-shaped. The expression of epithelial markers E-cadherin and Zona occludin-1 (ZO-1) were down-regulated, whereas mesenchymal markers a-smooth muscle actin (a-SMA) and fibronectin were up-regulated in Snail expression vector transfected cells. In addition, ectopic expression of Snail significantly enhanced ARPE-19 cell motility and migration. The present data suggest that overexpression of Snail in ARPE-19 cells could directly trigger EMT. These results may provide novel insight into understanding the regulator role of Snail in the development of retinal pigment epithelial–mesenchymal transition.

1. Introduction

Epithelial–mesenchymal transition (EMT) is a fascinating phenotypic change that involves the loss of epithelial characteristics and acquires mesenchymal-like phenotype and migratory properties [1]. EMT was initially described in early embryogenesis; however, EMT has also recently been implicated in cancer progression, tissue repair and organ fibrosis [1–3]. Fibrotic diseases are characterized by the appearance of myofibroblasts, the key cell type involved in the fibrogenic reaction, and by excess accumulation of extracellular matrix with resultant tissue contraction and impaired function [4]. A multitude of studies have identified that myofibroblast and mature fibroblast in a significant portion of renal, ocular, peritoneal mesothelial, liver and pulmonary fibrosis arise from the conversion of epithelial cells through an EMT [2,5]. In one analysis, lineage-tagging experiments and bone marrow transplant studies demonstrated that during the course of kidney fibrosis in mice, about 30% of fibroblasts are derived via EMT from the tubular epithelial cells of the kidney [6]. Additionally, studies using fibrosis tissue from humans have also confirmed EMT. In a study of 133 patients with kidney fibrosis, an EMT was demonstrated in a substantial number of the samples, as evaluated using double labeling of the tubular epithelial cells with cytokeratin, vimentin, α-SMA, or zona occludens 1 (ZO-1) [7]. Similarly, in patients with proliferative vitreoretinopathy (PVR), an EMT was demonstrated in areas of fibrosis in the retina [8,9]. PVR is the leading cause of failure of retinal detachment surgery and sometimes results in the loss of visual function. Retinal pigment epithelial (RPE) cells dedifferentiate and undergo EMT following retinal detachment, playing a key role in formation of fibrous tissue on the detached retina and vitreous retraction [10]. However, the detailed information on the molecular and cellular events of EMT in RPE cells has not been fully clarified.

EMT can be induced by growth factors such as transforming growth factor β (TGF-β), epidermal growth factor (EGF) and transcription factors such as Snail, twist and slug [11,12]. Snail transcription factor, a member of the Snail superfamily, is a zinc finger protein that can mediate EMT through downregulation of cell adhesion molecules such as E-cadherin by binding several

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E-boxes located in the promotor region [13]. The expression of Snail is stimulated by signaling pathways of a number of growth factors including TGF-β [14]. Our previous data demonstrated that Snail was up-regulated when human RPE cells were induced by TGF-β1 to undergo EMT, and that inhibition of activated Snail could reverse TGF-β1-induced EMT in vitro [15]. We ask whether Snail was sufficient to trigger EMT when ectopically expressed in RPE cells.

Snail has been extensively studied in cancers and fibrosis disease such as renal fibrosis; however, its role in ocular fibrosis is not as widely studied, especially in PVR. Based on these findings, we sought to explore whether Snail overexpression in human RPE cells could directly induce EMT and to obtain additional insights into the role of Snail in retinal pigment epithelial–mesenchymal transition in vitro.

2. Materials and methods

2.1. Cell culture and transfection

Human retinal pigment epithelial cell lines ARPE-19 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) at 37 °C under 5% CO_2. For Snail overexpression, ARPE-19 cells were transfected with Snail expression vector (pReceiver; OmicsLink Expression Clones; GeneCopoeia, Inc., USA) or an empty vector as negative control. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to manufacturer's protocols.

2.2. Immunofluorescent staining

ARPE-19 cells were seeded and cultured in 24-well chamber slides in Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, USA) for 24 h and then were transfected with pReceiver-Snail or pReceiver-control plasmids respectively. After 48 h, cells were washed and fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature. Then the fixed cells were incubated with primary antibody against Snail (Abcam Ltd., Cambridge, USA) diluted 1:100 at 37 °C for 2 h, washed three times with PBS followed by treatment with FITC-conjugated secondary antibody (diluted in 1:200) at room temperature for 1 h. Nuclei were stained with 4′6′-diamidino-2-phenylindole hydrochloride (DAPI) for 5 min. Stained ARPE-19 cells were observed using OLYMPUS™ microscope.

2.3. Morphology observation

After ARPE-19 cells were transfected with pReceiver-Snail or pReceiver-control plasmids for 24 and 48 h, the morphology of cells were observed under an inverted phase-contrast microscope (Olympus, Tokyo, Japan) and photographed by a digital camera.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated using TRIzol reagent (Invitrogen-Gibco, Carlsbad, USA) following the manufacturer's protocol. QRT-PCR was performed using TaqMan Universal PCR Master Mix and the 7500 Sequence Detection System (Applied Biosystems, Foster, CA). 18S RNA was used to standardize the mRNA level of the target genes. The sequences of the primers were used as before [15]. The expression of each target gene was defined from the threshold cycle (Ct), and relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method [16] after normalization with reference to expression of 18S RNA. All experiments were performed in triplicate.

2.5. Immunoblotting assay

Preparation of whole cell extracts and immunoblotting assay was performed as previously described [17]. The primary antibodies used were as follows: 1:500 E-cadherin antibody and 1:1000 fibronectin antibody (R&D systems, Inc., USA), 1:1000 Snail antibody (Abcam Ltd., Cambridge, USA), 1:1000 α-SMA antibody (Sigma–Aldrich, MO, USA), 1:1000 ZO-1 antibody (Invitrogen, Carlsbad, CA), and 1:5000 GAPDH (Good HERE, Hangzhou, China). HRP-conjugated sheep anti-mouse or anti-rabbit antibodies were used as secondary antibodies (1:10,000, Jackson, USA). Image Quant LAS 4000 with Image Quant TL 7.0 software (GE Healthcare Life Sciences, Pittsburgh, PA, USA) was used to quantify band intensities.

2.6. RPE cell migration assay

Transwell chamber (8-μm pores, Costar, Conning, USA) was used to determine the effect of forced Snail expression on ARPE-19 cells migration. After ARPE-19 cells were transfected with pReceiver-Snail or pReceiver-control for 48 h, about 5×10^5 cells were plated into the insert in 100 μl DMDM/F12 containing 0.5% FBS and allowed to migrate from upper compartment to lower compartment toward a 10% FBS gradient. The chamber was then incubated at 37 °C for 24 h. After removing the non-migrating cells with a cotton swab, migrated cells on the lower surface of the culture inserts were fixed with 4% paraformaldehyde, stained with H&E, and photographed under a light microscope. Five random fields were chosen in each insert, and the cell number was counted. All the experiments were performed in triplicate.

2.7. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) from at least three independent experiments. Independent sample *t*-test was used for two-group comparisons. Statistical Product and Service Solutions 16.0 software (Chicago, IL) was used for statistical analysis. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Expression of Snail protein in transfected ARPE-19 cells

In order to determine whether Snail alone could induce EMT, ARPE-19 cells were transfected with specific Snail expression vector as described in methods. First, we confirmed the expression of Snail in ARPE-19 cells by the immunofluorescence stain. As shown in Fig. 1, after 48 h of transfection, an increase in the protein levels of Snail was detected in pReceiver-Snail transfected ARPE-19 cells comparing to pReceiver-control transfected cells. Moreover, by immunofluorescence we could observe Snail, as well as its delocalization from the cytoplasm to intracellular compartments.

3.2. Effects of Snail overexpression on ARPE-19 cells morphology

To investigate whether Snail overexpression was associated with ARPE-19 cells phenotype changes, the phase contrast microscope was used. As shown in Fig. 2, cultured normal ARPE-19 cells had a typical cobblestone-like epithelial morphology. After transfected with pReceiver-Snail, the epithelial cells morphology gradually changed to spindle fibroblast-like cells accompanied by loss of