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Deficiency of primary cilia in kidney epithelial cells induces epithelial to mesenchymal transition

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ABSTRACT

Primary cilium is a microtubule-based non-motile organelle that plays critical roles in kidney pathophysiology. Our previous studies revealed that the lengths of primary cilia decreased upon renal ischemia/reperfusion injury and oxidative stress, and restored with recovery. Here, we tested the hypothesis that lack of primary cilium causes epithelial to mesenchymal transition (EMT) of kidney tubule cells. We investigated the alteration of length of primary cilia in TGF-β-induced EMT via visualization of primary cilia by fluorescence staining against acetylated α-tubulin. EMT was determined by measuring mesenchymal protein expression using quantitative PCR and indirect fluorescence staining. As a result, TGF-β treatment decreased ciliary length along with EMT. To test whether defect of primary cilia trigger onset of EMT, cilia formation was disturbed by knock down of ciliary protein using siRNA along with/without TGF-β treatment. Knock down of Arl13b and Ift20 reduced cilia elongation and increased expression of EMT markers such as fibronectin, α-SMA, and collagen III. TGF-β-induced EMT was greater as well in Arl13b and Ift20-knock down cells compared to control cells. Taken together, deficiency of primary cilia trigger EMT and exacerbates it under pro-fibrotic signals.

1. Introduction

The primary cilium is evolutionarily conserved organelle that projects from surface of the most mammalian cells [1] performing diverse biological roles including mechano-, chemo-, and photosensation [2]. In the kidney, primary cilia are observed in most part including parietal layer of Bowman's capsule, proximal tubule, distal tubule, and the collecting duct except for intercalated cells [3].

The lengths of primary cilia decrease under physiological condition such as mitosis during cell cycle [4], pathological condition during kidney damage such as kidney transplantation-induced tubular necrosis in human [5] and kidney ischemia/reperfusion (I/R) injury in mice [6]. Primary cilia were restored accompanied with functional recovery following injury [5,6]. Deciliation in the Madin Darby Canine Kidney (MDCK) cells occurred by oxidative stress and primary cilia were restored by ERK activation [6]. Additionally, defects in renal tubular primary cilia cause polycystic kidney disease in which epithelial cell proliferation is out of control [7]. This suggests that defect of primary cilia occurs under pathological microenvironment such as oxidative stress, inflammation, pro-fibrotic signals, and failure to restore primary cilia undergoes irreversible kidney damage such as fibrosis via epithelial to mesenchymal transition (EMT) of kidney tubule cells.

Successful ciliogenesis is critical to achieve functional cilia [8]. Intraflagellar transport protein (Ift) machinery is known to be critical in building cilia of many different organs [9]. Ift complex plays as a vehicle for transporting cargos to regulate cilia assembly, maintenance, and function [10]. Tissue specific inactivation of Ift20 in the mouse kidney collecting duct cells promoted cystic kidneys lacking cilia [11]. ADP ribosylation factor-like GTPase 13b (Arl13b) is also highly enriched within the cilium and essential for the ciliogenesis in zebrafish and mouse [12,13]. Inactivation of Arl13b at the perinatal stage in the distal nephron led to cyst formation and renal

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failure in mice [14].

This research was designed to study whether lack of primary cilia observed in pathological condition is just a result from kidney tubule cell damage or can be a trigger of further damage such as kidney EMT and fibrosis. Therefore, we induced deficiency of primary cilia by inhibition of ciliogenesis via knock down of Arl13b and Ift20 to test the effect of defective cilia on EMT and pro-fibrotic change of kidney tubule cells.

2. Materials and methods

2.1. Cell culture

MDCK cells were purchased from ATCC (Manassas, VA, USA) and cultured in Eagle's Minimum Essential Medium (EMEM) containing 5% fetal bovine serum (Thermo Scientific, Waltham, MA, USA) and 100 U/mL streptomycin/penicillin (S/P) (WelGENE Inc., Daegu, Korea) at 37 °C in a humidified atmosphere containing 5% CO_2. For Fig. 1, 50% confluent cells were treated with TGF-β (R&D Systems, Minneapolis, MN, USA) in final concentration of 5 ng/mL or vehicle for 48 h. The pictures were taken under bright field using a microscope (Leica, Leica instruments, Wetzlar, Germany). For immunofluorescence staining, cells were fixed with 4% paraformaldehyde (PFA) for 15 min. For Fig. 2, 100% confluent cells were incubated in 1% FBS containing EMEM for 2 days, and further incubated with/without TGF-β (R&D Systems, Minneapolis, MN, USA) in final concentration of 5 ng/mL for 16 h.

2.2. Gene silencing

For Figs. 3 and 4, 60–80% confluent MDCK cells were incubated in EMEM containing 5% fetal bovine serum (FBS) without S/P for 2 h and transfected with 25 nmole/L of small interfering RNA (siRNA) for Arl13b (5′-GCAACAGUCGUCAUACAUA-3′, Bioneer, Daegeon, Korea) or Ift20 (5′-GGGUUGAAUAUGAAGCUUU-3′) and scrambled siRNA (SN-1002, Bioneer, Daegeon, Korea) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) for 6 h according to the manufacturer's instructions. The cell culture medium was changed with EMEM containing 1% fetal bovine serum (FBS) without S/P and further incubated for 48 h with/without TGF-β treatment.

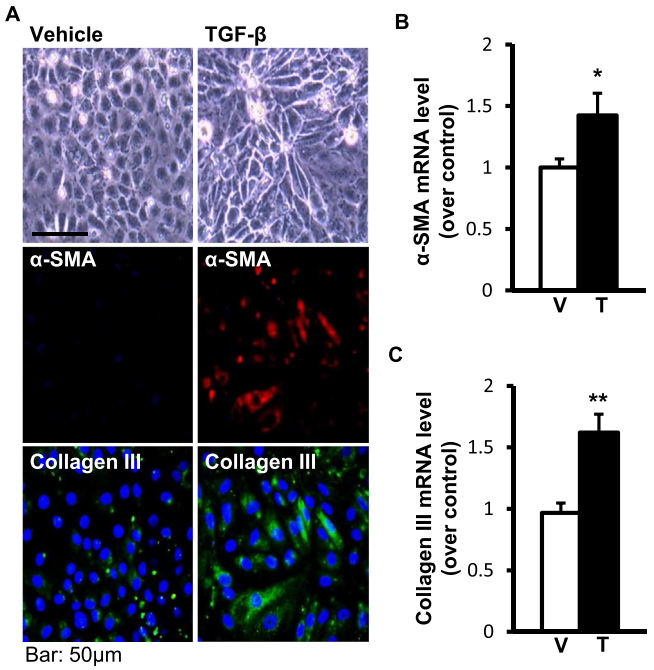

Fig. 1. TGF-β induced epithelial mesenchymal transition of kidney epithelial cells. (A) Representative picture of vehicle or TGF-β-treated MDCK cells of bright field or dual immunofluorescence staining against α-SMA and collagen III with nuclei staining (blue). (B, C) Summary of mRNA expression of α-SMA (B) and collagen III (C) in vehicle (V) or TGF-β (T)-treated MDCK cells. *$P < .05$, **$P < .01$ *vs.* vehicle-treated control. Data are presented as means ± SE. ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

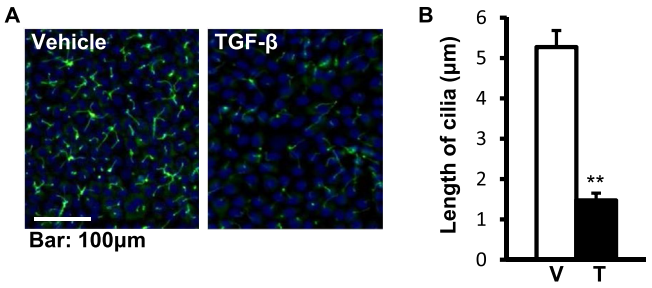

Fig. 2. TGF-β decreased length of primary cilia in kidney epithelial cells. (A) Representative immunofluorescence staining of vehicle or TGF-β-treated MDCK cells. (B) Summary of length of cilia in vehicle (V) or TGF-β (T)-treated MDCK cells. **$P < .01$ *vs.* vehicle-treated control. Data are presented as means ± SE. ($n = 125–151$).

2.3. Immunofluorescence staining

For measurement of lengths of primary cilia, MDCK cells were cultured on the coverslips. For immunofluorescence staining, cells were fixed with 4% paraformaldehyde (PFA) for 15 min. Fixed cells were permeabilized with 0.1% Triton-X 100 for 5 min. Cells were incubated with 3% BSA for 30 min for blocking, then incubated with antibodies against α-SMA (Sigma-Aldrich Corporation, St Louis, MO, USA), Collagen III (Southern Biotech, San Diego, CA, USA), Ac-α-tubulin (Sigma-Aldrich Corporation, St Louis, MO, USA), Arl13b (Protein Tech, Chicago, IL, USA), and Ift20 (Protein Tech, Chicago, IL, USA) diluted at 1:100 overnight at 4 °C. After incubation, cells were washed three times in PBS for 5 min each, incubated with FITC or Texas Red-conjugated secondary antibodies (Vector Laboratories, Inc., Burlingame, CA, USA) for 60 min at room temperature (RT), and washed three times with PBS for 5 min each. To detect the cell nuclei, 4′-6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, MO, USA) was applied. Finally, the cells were observed and pictures were taken using a fluorescence microscope (Leica, Leica instruments, Wetzlar, Germany).

2.4. Measurement of lengths of primary cilia

Cells were processed for immunofluorescence microscopy by staining with anti-acetylated α-tubulin antibody (Sigma-Aldrich Corporation, St Louis, MO, USA) and DAPI (Sigma Aldrich, St. Louis, MO, USA). Pictures were randomly captured using fluorescence microscope (Leica, Leica instruments, Wetzlar, Germany) from three independent experiments. Around 150 cells per group were used to measure lengths of primary cilia. iSolution software (IMT i-Solution, Rochester, NY, USA) was used to trace and measure the length of cilia in captured images.

2.5. Quantitative real-time polymerase chain reaction (qRTPCR) analysis

RNA was extracted using PureHelix™ RNA extraction solution (Nanohelix, Seoul, Korea) from MDCK cells. 1 μg of RNA was used for cDNA synthesis using the DiaStar™ RT Kit (SolGent, Daejeon, Korea). qRTPCR was performed using LightCycler 480 SYBR Green I Master mix and the LightCycler machine (Roche Applied Sciences,

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