

## Original Article

# Glioma-associated oncogene homolog 1 promotes epithelial-mesenchymal transition in human renal tubular epithelial cell

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**Abstract:** Sonic hedgehog (Shh) signaling critically regulates embryogenesis and tissue homeostasis. Here, we investigated the role of Shh signaling in mediating epithelial-mesenchymal transition (EMT) in human renal tubular epithelial cells HKC-8. Our RT-PCR assays demonstrated that TGF- $\beta$ 1 induced time-dependent changes in the mRNA transcript levels of Shh, with a steady rise from one hour post TGF- $\beta$ 1 treatment and a peak at four hours post TGF- $\beta$ 1 treatment. Furthermore, TGF- $\beta$ 1 induced a time-dependent increase in the mRNA transcript levels of Gli1. Pre-treatment with 2 or 5  $\mu$ M cyclopamine significantly attenuated TGF- $\beta$ 1-induced rise in the mRNA transcript levels of Gli1, but failed to attenuate TGF- $\beta$ 1-induced rise in Shh mRNA transcript levels. Additionally, immunoblotting assays and immunofluorescence staining demonstrated that inhibition of Shh signaling by cyclopamine significantly attenuated TGF- $\beta$ 1-induced increase in the mRNA transcript levels of  $\alpha$ -SMA, collagen I, and fibronectin. Gli1 over-expression induced Snail1 expression. Moreover, *Gli1*<sup>-/-</sup> mice that had undergone unilateral ureteral obstruction for seven days showed significant reduction in the mRNA transcript levels of Snail1 compared to the wildtype controls. In conclusion, the current study provides novel insight into the regulation of EMT by the Shh/Gli1 signaling pathway, suggesting a critical role of Shh/Gli1 signaling in EMT of human renal tubular epithelial cells.

**Keywords:** Sonic hedgehog (Shh) signaling, Gli1, Snail1, human renal tubular epithelial cells, epithelial-mesenchymal transition

## Introduction

Hedgehog signaling regulates a diverse array of biological processes such as embryonic development, tissue homeostasis, injury repair and tumorigenesis [1, 2]. Hedgehog transmits its signal through binding to the plasma membrane receptor, Patched 1 (Ptch1), resulting in the de-repression of Smoothened (Smo), which then leads to activating the glioma-associated oncogene homolog (Gli) family of transcription factors. Gli proteins are known to enter the nucleus and initiate the transcription of their target genes [3]. Direct targets of hedgehog signaling include several major components in its own pathway such as Gli1, providing positive feedback to ensure delicate regulation of this system.

Recent studies also implicate Shh signaling in regulating injury repair and wound healing after tissue damage such as in chronic liver and lung diseases [4, 5]. Shh promotes epithelial to

mesenchymal transition (EMT) during liver fibrosis [6]. EMT has been increasingly recognized to occur during the progression of renal fibrosis. It has been proposed that EMT is one of the key mechanisms in the pathogenesis of renal fibrosis [8, 9]. We also previously demonstrated that Shh signaling was induced during renal fibrosis in a mouse model of obstructive nephropathy, and inhibition of Gli1 expression attenuated matrix gene expression and mitigated renal fibrotic lesions [7]. However, the mechanisms still remain unknown. In this study, we further delineated the role of Gli1 and the interaction among GLI1, TGF- $\beta$ 1 and Snail1 in the context of the EMT using human renal tubular epithelial cell line (HKC-8) treated with the fibrotic factor TGF- $\beta$ 1.

## Materials and methods

### Cell culture and treatment

HKC-8 cells were provided by Dr. Youhua Liu (Pittsburgh University, Pennsylvania, PHD) and

## Shh promotes renal fibrosis

**Table 1.** Sequences of primers used in this study

Gene	Forward	Reverse	Size
$\alpha$ -SMA	CCCAGC CAAGCACTGTCA	TCCAGAGTCCAGCACGATG	516
SNAIL	AGGATCTCCAGGCTCGAAAG	GTAGCAGCCAGGGCCTAGAG	150
SHH	GCTCGGTGAAAGCAGAGAAC	CCAGGAAAGTGAGGAAGTCG	180
GLI1	TTCTGCCACCAAGCTAACCT	CGACAGAGGTGAGATGGACA	150
COL1	CCAAATCTGTCTCCCCAGAA	TCAAAAACGAAGGGGAGATG	214
$\beta$ -actin	TCAAGATCATTGCTCCTCTGAGC	TGCTGTACCTTCACCGTCCAGT	318

cultured in DMEM-Ham's F12 medium supplemented with 5% fetal bovine serum (FBS). Cells were typically seeded at ~70% confluence in complete medium containing 5% FBS for 24 h and then serum-starved for 16 h, followed by incubation with 2 ng/mL recombinant TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN) for 48 h except as otherwise indicated. For Shh signaling inhibition, cells were pretreated with 2 or 5  $\mu$ M cyclopamine (Sigma, St. Louis, MO) or vehicle (0.1% DMSO) for 30 min, followed by incubation with 2 ng/mL TGF- $\beta$ 1.

### Real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). First strand cDNA synthesis was carried out using 2  $\mu$ g RNA in a 20  $\mu$ L reaction using AMV-RT (Promega, Madison, WI) and random primers at 42°C for 30 min. PCR was carried out using a standard PCR kit and 1  $\mu$ L aliquot of cDNA, HotStar Taq polymerase (Qiagen, Valencia, CA) with specific primer pairs (Table 1). For quantitative determination of mRNA levels, real-time RT-PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), as described previously. The PCR mixture in a 25  $\mu$ L volume contained 12.5  $\mu$ L 2 x SYBR Green PCR Master Mix (Applied Biosystems), 5  $\mu$ L diluted RT product (1:10) and 0.5  $\mu$ M sense and antisense primer sets (Table 1). PCR was run by using standard conditions. After sequential incubations at 50°C for 2 min and 95°C for 10 min, respectively, the amplification protocol consisted of 50 cycles of denaturing at 95°C for 15 sec, annealing and extension at 60°C for 60 sec. The mRNA levels of various genes were calculated after normalizing with  $\beta$ -actin.

### Immunofluorescence staining

Indirect immunofluorescence staining was performed using an established procedure<sup>1</sup>.

Briefly, cells cultured on coverslips were fixed with cold methanol: acetone (1:1) for 10 min and blocked with 10% donkey serum in phosphate buffered saline (PBS) for 30 min. Sections were then incubated with anti-fibronectin (cat. no. 610078, BD Transduction Laboratories, San Jose, CA) and anti-zo-1 (61-7300; Invitrogen, Carlsbad, CA) antibodies. To visualize the primary antibodies, sections were stained with cyanine Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were also stained with DAPI (4',6-diamidino-2-phenylindole, HCl) to visualize the nuclei.

### Western blot analysis

Western blot analysis for specific protein expression was performed essentially according to an established procedure<sup>2</sup>. The primary antibodies used were as follows: anti-Gli1 (ab92611; Abcam, Cambridge, MA), anti- $\alpha$ -SMA (A2547; Sigma), anti-fibronectin (F3648; Sigma), anti-snail (ab180714; Abcam) and anti-glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX) antibodies.

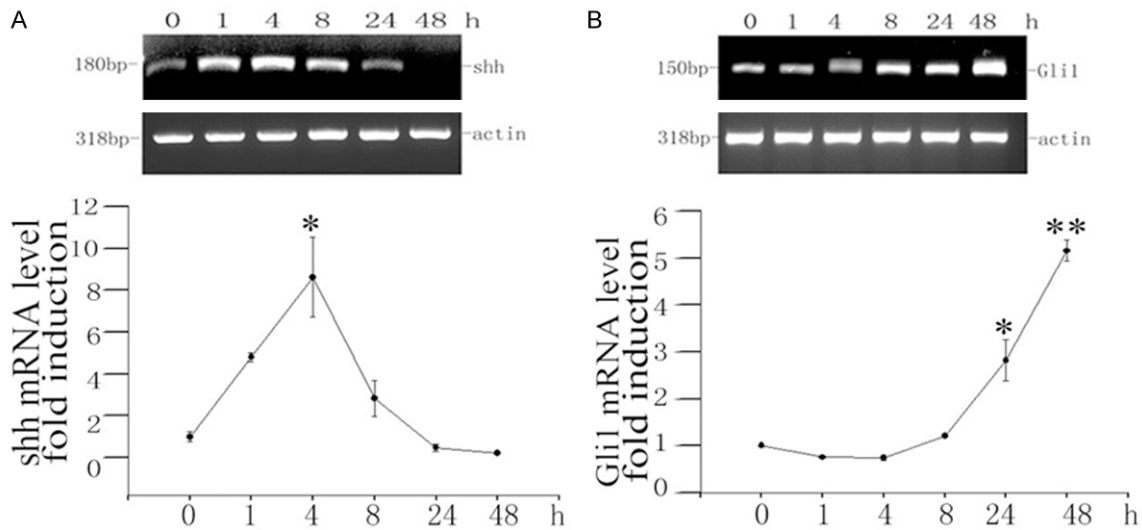
### Plasmid transfection

For transient transfection, HKC-8 cells were seeded in six-well plates at  $5 \times 10^5$  cells/well. The cells were then transfected with Gli plasmid (provided by Dr. Yoshinari Asaoka, University of Tokyo, Tokyo, Japan) for 48 h. Then, cells were collected for Western blotting assays. The empty pcDNA3 vector was used as a mock transfection control.

### Animal model

*Gli1*<sup>lacZ</sup> mutant mice, which harbor a  $\beta$ -galactosidase 'knock-in' mutation and in which endogenous *Gli1* gene function is abolished, was obtained from the Jackson Laboratory (Bar Harbor, ME). Heterozygous mice were mated, and the off-springs were genotyped by PCR according to the protocol specified by the Jackson Laboratory. Mutant mice (*Gli1*<sup>-/-</sup>) and their wild-type (*Gli1*<sup>+/+</sup>) littermates at the age of 5 weeks underwent unilateral ureteral obstruction (UUO). UUO was performed using an estab-

## Shh promotes renal fibrosis



**Figure 1.** Renal tubular epithelial cells HKC-8 cells were serum starved for 24 hours and then treated with TGF- $\beta$ 1 (2 ng/mL) for 0, 1, 4, 8, 24, and 48 hours. RT-PCR assays were performed to determine the mRNA transcript levels of Shh (A) and Gli1 (B). The data are expressed as mean  $\pm$  SD of at least three independent experiments. \* $P$ <0.01 and \*\* $P$ <0.05 vs. controls.

lished protocol as described previously [3] and kidney tissues were collected 7 days after UUO.

### Statistical analyses

All data were expressed as mean  $\pm$  SEM and analyzed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparison between groups was made using one-way ANOVA, followed by Student-Newman-Keuls test.  $P$ <0.05 was considered statistically significant.

### Results

#### *Shh signaling is induced by TGF- $\beta$ 1 in human renal tubular epithelial cells*

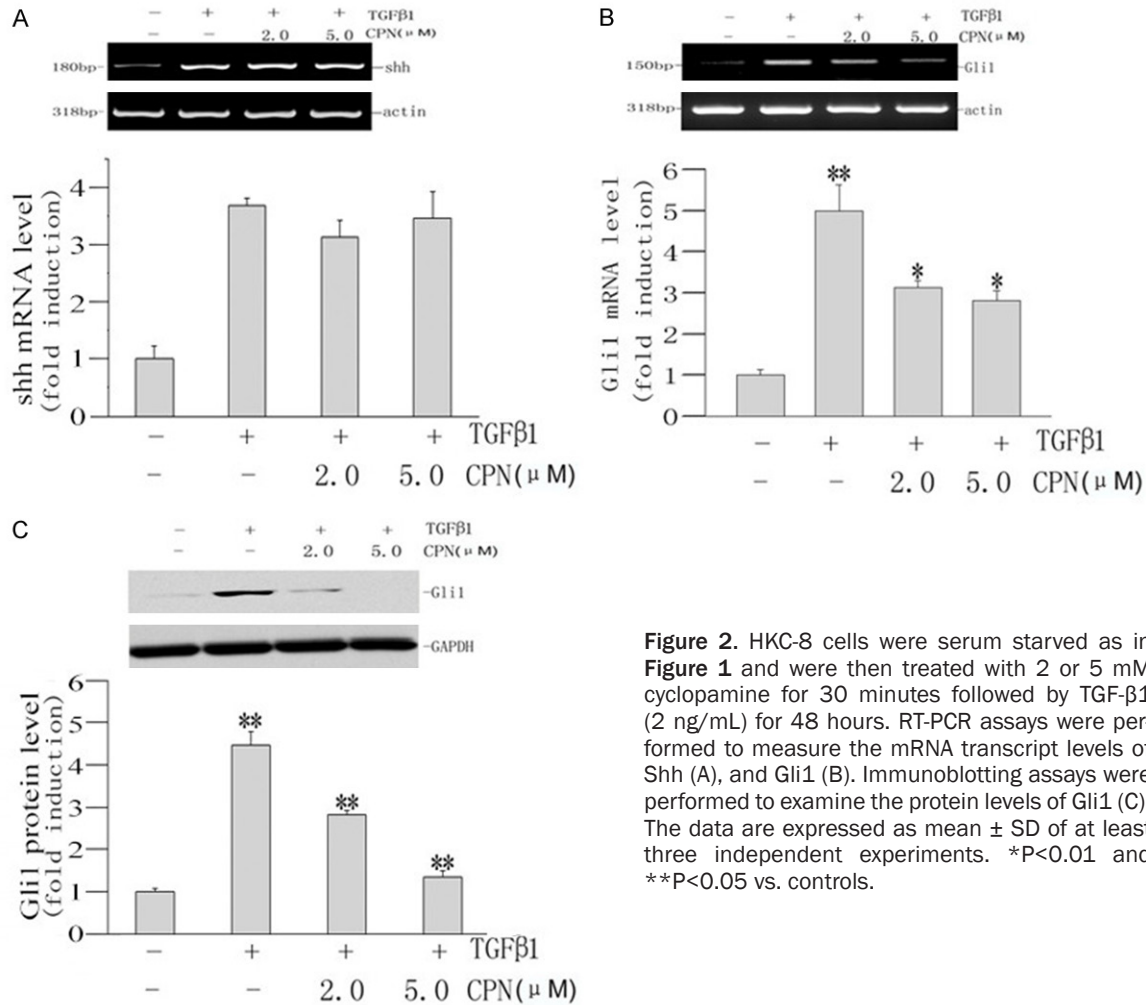
To investigate the potential role of Shh signaling in human renal tubular epithelial cells, we first treated HKC-8 cells with TGF- $\beta$ 1 (2 ng/mL) for 0, 1, 4, 8, 24, and 48 hours. We then examined the mRNA transcript levels of Shh and Gli1. Our RT-PCR assays demonstrated that TGF- $\beta$ 1 induced time-dependent changes in the mRNA transcript levels of Shh, with a steady rise from one hour post TGF- $\beta$ 1 treatment (Figure 1A). Furthermore, Shh mRNA expression peaked at four hours post TGF- $\beta$ 1 treatment with a more than 8-fold increase over the baseline ( $P$ <0.05). Thereafter, this was followed

by a steady decline in Shh mRNA transcript levels, which returned to the baseline levels 48 hours post TGF- $\beta$ 1 treatment. On the other hand, we found a time-dependent increased in the mRNA transcript levels of Gli1 following TGF- $\beta$ 1 treatment. At 48 hours post TGF- $\beta$ 1 treatment, the mRNA transcript levels increased by approximately 5 fold over the baseline ( $P$ <0.01) (Figure 1B).

#### *Inhibition of Shh signaling significantly attenuates TGF- $\beta$ 1-induced rise in Gli1 expression in human renal tubular epithelial cells*

We next sought to explore whether pharmacological inhibition of Shh signaling inhibited Shh signaling in human renal tubular epithelial cells. We pre-treated HKC-8 cells with 2 or 5  $\mu$ M cyclopamine for 30 minutes followed by TGF- $\beta$ 1 (2 ng/mL) for 48 hours. Our RT-PCR assays revealed that cyclopamine at both doses failed to attenuate TGF- $\beta$ 1-induced rise in Shh mRNA transcript levels (Figure 2A). By contrast, pretreatment with cyclopamine significantly attenuated TGF- $\beta$ 1-induced rise in the mRNA transcript levels of Gli1 ( $P$ <0.01) (Figure 2B). Our immunoblotting assays further revealed that pretreatment with cyclopamine also markedly attenuated TGF- $\beta$ 1-induced increase in the protein levels of Gli1 (Figure 2C). As Gli1 transcription is a reliable readout of the hedgehog sig-

## Shh promotes renal fibrosis



**Figure 2.** HKC-8 cells were serum starved as in **Figure 1** and were then treated with 2 or 5 mM cycloamine for 30 minutes followed by TGF-β1 (2 ng/mL) for 48 hours. RT-PCR assays were performed to measure the mRNA transcript levels of Shh (A), and Gli1 (B). Immunoblotting assays were performed to examine the protein levels of Gli1 (C). The data are expressed as mean ± SD of at least three independent experiments. \*P<0.01 and \*\*P<0.05 vs. controls.

naling, these results indicated that cycloamine was effective in blocking Shh signal transduction by targeting Gli1.

### *Blockade of Shh signaling aborts TGF-β1-induced EMT in human renal tubular epithelial cells*

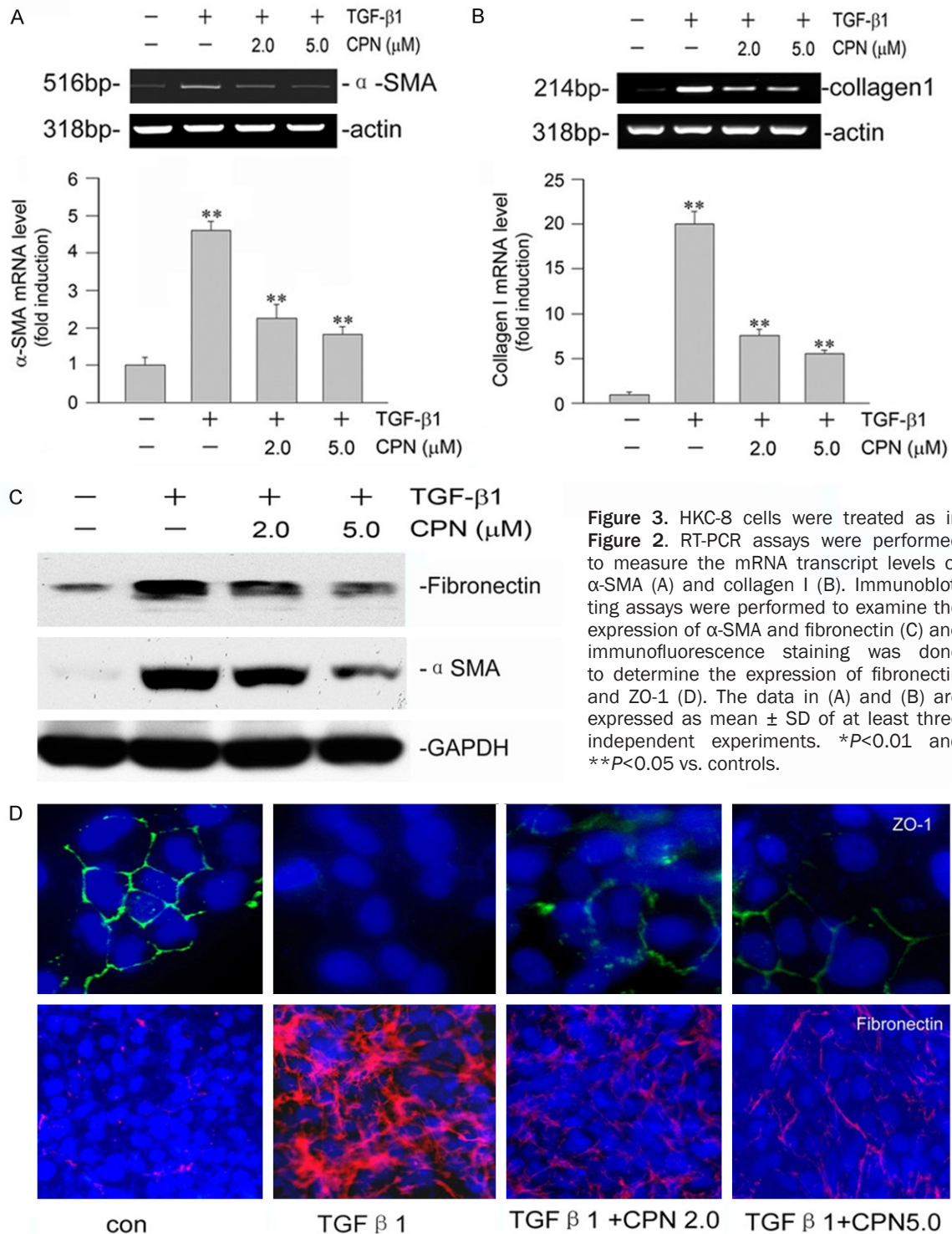
We further investigated the effect of Shh signaling inhibition on EMT. We pre-treated HKC-8 cells with cycloamine followed by TGF-β1 as described above. Consistent with previous literature, TGF-β1 promoted EMT, with significant increase in the mRNA transcript levels of α-SMA (**Figure 3A**) and collagen I (**Figure 3B**). These findings were further confirmed by immunoblotting assays which showed noticeably increased expression of α-SMA and fibronectin (**Figure 3C**) and by immunofluorescence staining which demonstrated elevated expression of fibronectin. Immunofluorescence staining, on the other

hand, revealed a significant decrease in the expression of ZO-1 (**Figure 3D**). Inhibition of Shh signaling by cycloamine significantly attenuated TGF-β1-induced increase in the mRNA transcript levels of α-SMA, collagen I, and fibronectin (**Figure 3A-C**). Immunofluorescence staining also revealed cycloamine attenuated TGF-β1-induced increase in fibronectin expression and TGF-β1-induced decrease in the expression of ZO-1 (**Figure 3D**). These findings suggested that inhibition of Shh signaling attenuated TGF-β1-induced EMT in human renal tubular epithelial cells.

### *Shh/Gli1 signaling regulates EMT in human renal tubular epithelial cells by modulating Snail1 expression*

We further sought to investigate the mechanism whereby blockade of Shh signaling suppressed TGF-β1-induced EMT in human renal

## Shh promotes renal fibrosis

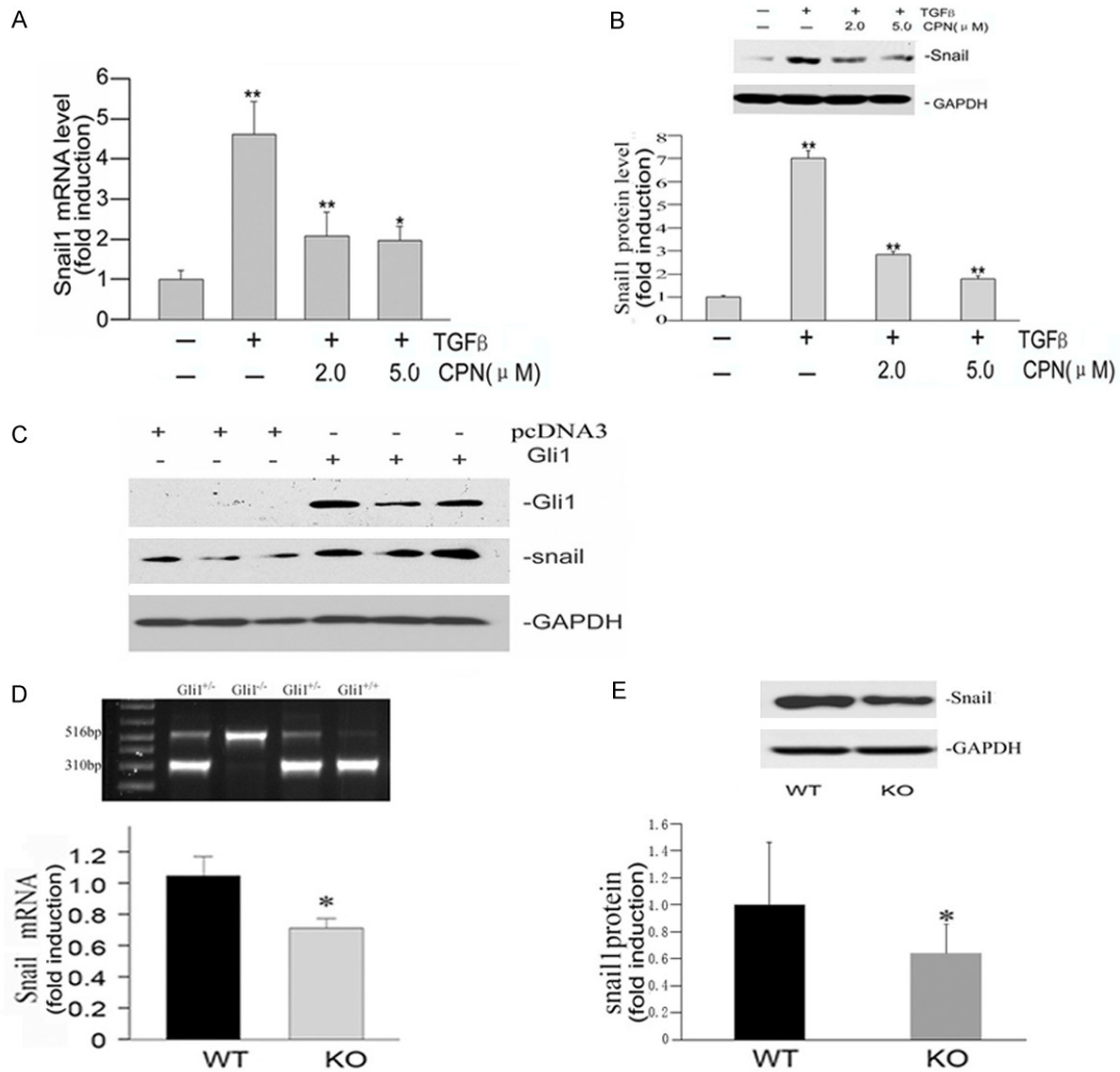


**Figure 3.** HKC-8 cells were treated as in **Figure 2**. RT-PCR assays were performed to measure the mRNA transcript levels of  $\alpha$ -SMA (A) and collagen I (B). Immunoblotting assays were performed to examine the expression of  $\alpha$ -SMA and fibronectin (C) and immunofluorescence staining was done to determine the expression of fibronectin and ZO-1 (D). The data in (A) and (B) are expressed as mean  $\pm$  SD of at least three independent experiments. \* $P$ <0.01 and \*\* $P$ <0.05 vs. controls.

tubular epithelial cells by examining the effect of Shh signaling blockade on the expression of Snail1, an important transcription factor for EMT and a downstream target of the Shh/Gli1 signaling pathway. Our RT-PCR assays showed

that TGF- $\beta$ 1 treatment caused an approximately 4-fold increase in the mRNA transcript levels of Snail1 over the baseline ( $P$ <0.01) (**Figure 4A**). Blockade of Shh signaling by cyclopamine, however, significantly attenuated TGF- $\beta$ 1-

## Shh promotes renal fibrosis



**Figure 4.** HKC-8 cells were treated as in **Figure 2**. RT-PCR assays were performed to measure the mRNA transcript levels of Snail1 (A) and immunoblotting assays were done to detect Snail1 expression (B). HKC-8 cells were transfected with Gli1 plasmid and Snail1 expression was examined by immunoblotting assays (C). Expression of Gli1 in Gli1 null mice (*Gli1<sup>-/-</sup>*) and the wild-type (*Gli1<sup>+/+</sup>*) mice that had undergone UUO for 7 days was examined by real time RT-PCR assays (D), and by immunoblotting assays (E). The data in (A), (B), (D) and (E) are expressed as mean  $\pm$  SD of at least three independent experiments. \* $P < 0.01$  and \*\* $P < 0.05$  vs. controls.

induced increase in the mRNA transcript levels of Snail1 ( $P < 0.01$  TGF- $\beta$ 1 plus cyclopamine vs. TGF- $\beta$ 1). These findings were further supported by the immunoblotting assays (**Figure 4B**).

Furthermore, transient transfection of HKC-8 cells with Gli1 plasmid showed that overexpression of *Gli1* increased Snail1 expression (**Figure 4C**). We further examined the expression of Gli1 in Gli1 null mice (*Gli1<sup>-/-</sup>*) and the wild-type (*Gli1<sup>+/+</sup>*) mice that had undergone UUO for 7 days. Real time RT-PCR assays showed that UUO *Gli1<sup>-/-</sup>* mice showed significant reduction in the

mRNA transcript levels of Snail1 compared to the wildtype controls (**Figure 4D**), which was confirmed by the results of the immunoblotting assays (**Figure 4E**). These results suggested that Shh/Gli1 signaling regulated EMT in human renal tubular epithelial cells by modulating Snail1 expression.

### Discussion

The SHH signaling pathway is critical in the regulation of normal cell growth and differentiation. Our previous study has demonstrated that

## Shh promotes renal fibrosis

the Shh/Gli1 signaling pathway mediates epithelial-mesenchymal communication in interstitial fibroblasts after mouse kidney injury [4]. In the current study, we have shown that the Shh/Gli1 signaling pathway is implicated in TGF- $\beta$ -induced EMT in human renal tubular epithelial cells. We have further demonstrated that blockade of Shh signaling significantly attenuates TGF- $\beta$ 1-induced rise in Gli1 expression and TGF- $\beta$ 1-induced EMT in human renal tubular epithelial cells. We have also shown that Shh/Gli1 signaling regulates EMT by modulating Snail1 expression in human renal tubular epithelial cells. EMT has implicated in the progression of renal fibrosis [11, 12]; however, the underlying mechanisms still remain unknown. The current study provides novel insight into the regulation of EMT by the Shh/Gli1 signaling pathway in human renal tubular epithelial cells, suggesting a critical role of Shh/Gli1 signaling in EMT of human renal tubular epithelial cells.

A recent study showed that the epithelial cell is an active player in fibrosis by controlling fibroblasts [5]. However, little is known about the expression of the Shh/Gli1 signaling pathway components and how those components contribute to fibrosis formation in epithelial cells. In the current study, we confirmed the expression of SHH and GLI1 in epithelial cells at both the transcriptional and translational level. Our findings have raised some questions for future studies, such as how epithelial cells communicate with fibroblasts and whether Shh signaling is involved in this communication.

The EMT is essential for normal development, and also takes place in such pathological conditions as in cancer and fibrosis progression [6, 7]. Although EMT can be stimulated by many extracellular ligands, TGF- $\beta$  has emerged as the major inducer of this transdifferentiation process in both development and pathological contexts [8-10]. Consistently, our results presented here demonstrated TGF- $\beta$  can induce EMT in epithelial cells in a Shh/Gli1 dependent manner, which is partially aborted by Shh signaling inhibitor cyclopamine. Cyclopamine, by inhibiting SMO, did not suppress the expression of Shh, but remarkably decreased Gli1 expression in epithelial cells, which is in line with our previous findings in fibroblasts [4].

In addition to Gli1, Snail, an important transcription factor, is the other well-known down-

stream mediator of Shh signaling in the modulation of EMT [11, 12]. Thus, we questioned and investigated whether EMT induction by TGF- $\beta$  is through stimulating both Shh target genes. Interestingly, TGF- $\beta$  did increase these two known Shh target genes and also induced the expression of genes that are directly implicated in fibroblast activation and interstitial matrix production, including  $\alpha$ -SMA, fibronectin, and collagen I. The ZO-1 gene, a known marker of tight junctions in epithelial cells, can be repressed by TGF- $\beta$  [13]. Surprisingly, we found that decreased ZO-1 expression induced by TGF- $\beta$  was partially rescued by co-administration with cyclopamine, implying a critical role of Shh signaling in the TGF- $\beta$ -induced EMT in epithelial cells. Furthermore, we also observed that overexpression of Gli1 in epithelial cells rendered positive induction of Snail expression.

Taken together, our findings in this study provide a novel mechanistic insight into EMT induced by TGF- $\beta$  in human renal tubular epithelial cells. These findings may also provide useful guidance for future therapeutic designs to prevent fibrosis in human renal tubular epithelial cells by targeting the Shh/Gli signaling pathway.

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### Disclosure of conflict of interest

None.

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