Wnt3a-regulated TCF4/β-catenin complex directly activates the key Hedgehog signalling genes *Smo* and *Gli1*

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Abstract. The Wnt and Hedgehog signalling pathways serve key roles in diverse developmental processes. However, the molecular associations between these two signalling pathways remains unclear. Previous transcriptome studies on human foreskin fibroblasts have indicated that Wnt signalling activation induces the expression of key Hedgehog signalling genes, including smoothened, frizzled class receptor (*Smo*) and GLI family zinc finger 1 (*Gli1*). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) results revealed that Wnt3a treatment induced the expression of the key Hedgehog signalling genes, including *Smo*, patched (*PTCH*), *Gli1*, *Gli2* and *Gli3*. In addition, western blot analyses demonstrated that Wnt3a treatment resulted in the accumulation of cellular Smo and Gli proteins. Furthermore, promoter sequence analysis revealed that the putative β-catenin/T-cell factor (TCF)-4 complex binding motifs ($^{T}/_{A}{}^{C}/_{G}$AAAG) were located within 1.5 kb of the *Smo* and *Gli1* promoters. Results of the chromatin immunoprecipitation experiments and yeast-one hybrid assays revealed that TCF4 directly binds to the *Smo* and *Gli1* promoters, with two binding sites for *Smo* and a single binding site for *Gli1*. Further analysis showed that the β-catenin/TCF4 complex binds to the *Smo* and *Gli1* promoters. To investigate the functions of TCF4 and β-catenin in transcriptional regulation of *Smo* and *Gli1*, *TCF4* and *β-catenin* were transiently expressed in fibroblast cells. RT-qPCR results demonstrated that overexpression of *TCF4* and *β-catenin* induced the expression of *Smo* and *Gli1*. In addition, small interfering RNA-mediated suppression of *β-catenin* resulted in the downregulation of *Smo* and *Gli1* expression levels, even under Wnt3a treatment. Suppression of *β-catenin* and *Gli1* expression inhibited cell proliferation. Taken together, the results of the present study suggested that the β-catenin/TCF4 complex directly activates *Smo* and *Gli1* by binding to their promoters, which in turn controls cell proliferation in human fibroblasts.

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Introduction

The canonical Wnt signalling pathway, also known as the Wnt/β-catenin or the β-catenin/T-cell factor (TCF) pathway (1), modulates diverse biological processes via signal transduction (2-4). The key step in Wnt/β-catenin signalling pathway is the stabilization and accumulation of cytosolic β-catenin. Under normal conditions without stimulation, β-catenin is constantly phosphorylated by the glycogen synthase kinase-3β (GSK3β) complex (5). Phosphorylated β-catenin is subsequently ubiquitinated and targeted for degradation by the 26s proteasome (5,6). Wnt signalling activation leads to the inhibition of GSK3β activity and β-catenin accumulation in the cytosol and nucleus. In the nucleus, β-catenin forms a complex with T-cell factor/lymphoid enhancer binding factor (TCF/LEF) and induces the expression of downstream genes involved in Wnt signalling (5). A genome-wide scan of TCF/LEF binding sites revealed that TCF/LEF binds to the putative *cis*-elements ($^{T}/_{A}{}^{C}/_{G}$AAAG) in the target gene promoters (7).

Hedgehog signalling plays important roles in both vertebrate and invertebrate development (8). Hedgehog was first identified as a secreted signalling protein whose expression is induced in the *Drosophila melanogaster* embryonic segment (9). The three mammalian *Hedgehog* (*hh)* genes, namely, Sonic hedgehog, Indian hedgehog, and Desert hedgehog, are important in the patterning of many tissues and biological structures (9). In addition, abnormal activation of Hedgehog signalling is required for nearly all basal cell carcinomas, medulloblastomas, and rhabdomyosarcomas; however, overactivation of Hedgehog signalling has also been observed in some tumours (8,10-12). In the absence of Hedgehog stimulation, the transmembrane protein Patched1 (Patch1) interacts with Smoothened (Smo), another transmembrane protein that

maintains inactivity of the Hedgehog pathway. Sonic hedgehog binds to and inactivates Patch1 after secretion, leading to Smo activation (11,13) and subsequent transcription of downstream genes via the GLI-Kruppel family transcription factors (13). Among the three GLI-Kruppel family members (Glis), Gli1 and Gli2 are positive regulators, while Gli3 is a negative regulator (14). Gli1 overexpression was associated with promoting gastric initiation and progression in patients (14). A previous study identified the novel function of Gli1 in modulating E-cadherin/β-catenin-regulated cancer cell properties. Gli1 was demonstrated to interfere with membrane localization of E-cadherin by upregulating MUC5AC, a gel-forming mucin, which in turn weakens E-cadherin-dependent cell-cell adhesion and promotes cell migration and invasiveness in pancreatic ductal adenocarcinoma (15). In addition, *Gli3* deficient showed prevention of premature fusion of calvarial suture in mice via eliminate one allele of a key transcription factor Runx2 (16).

The extracellular matrix (ECM) is the largest component of the dermal skin layer (17). Fibroblasts are known to participate in ECM assembly and remodelling (18,19), demonstrating their important roles in wound repair. More recently, Wnt signalling was found to be involved in human fibroblast repair (20). In our transcriptome study using Wnt3a-stimulated cells, Wnt signalling activation was observed to alter the expression patterns of a large number of genes, including Hedgehog signalling genes (21). In addition, Hedgehog signalling genes have been demonstrated to regulate fibroblast repair and are controlled by β-catenin, a Wnt signalling regulator (22). However, the association between Wnt and Hedgehog signalling pathways remain unclear.

In the present study, we further examined regulation of Hedgehog signalling genes by Wnt signalling by examining the gene expression profiles of Hedgehog signalling genes under Wnt3a stimulation. In addition, promoter sequence analysis combined with ChIP and yeast-one hybrid assays were performed to explore the potential mechanisms that mediate the activation of *Smo* and *Gli1* transcription by β-catenin/TCF4 complex. Next, *TCF4* and *β-catenin* were transiently overexpressed in fibroblasts, after which *Smo* and *Gli1* expression levels were determined. In addition, *β-catenin* expression was suppressed via siRNA transfection, and *Smo* and *Gli1* levels were monitored. In conclusion, our analyses indicated that the β-catenin/TCF4 complex directly regulates *Smo* and *Gli1* transcription, which provided direct evidence for the link between the Wnt and Hedgehog signalling pathways.

Materials and methods

Human foreskin fibroblast cell culture. Human foreskin samples were isolated from 4 patients in the Department of Dermatology, the First Affiliated Hospital, Wenzhou Medical University (Wenzhou, China). The present study was approved by the Ethics Committee of Wenzhou Medical University (Wenzhou, Zhejiang, China) and written informed consent was obtained from all of the patients involved (21). All the procedures followed for purification and culture of human fibroblasts were described by Xuan *et al* (23).

Cell culture. Human foreskin fibroblast cells were cultured for 12 h at 37˚C in an incubator with 5% CO_2 in Petri dishes and subsequently cultured into a monolayer until reaching confluence (23). Afterwards, cells were cultured in DMEM containing 0.5% FBS and treated with Wnt3a (100 ng/ml). Cells were harvested after 0, 1, and 3 h of Wnt3a treatment.

Yeast-one hybrid assay. For the yeast one-hybrid assay, the 1.5-kb promoters of *Smo* and *Gli1* were synthesized by Sangon Biotech (www.sangon.com/) and subsequently cloned into the *pHISi* vector. The *TCF4* ORF sequences were cloned into the *pGAD424* yeast expression vector. The *pGAD424-TCF4* and *pSmo-His, mpSmo-His, pGli1-His,* or *mpGli1-His* were transformed into the yeast one-hybrid bait strain (YM4271). The growth of yeast cells was monitored on synthetic dropout Leu or His.

ChIP assay. Chromatin immunoprecipitation (ChIP) assay was performed using a chromatin immunoprecipitation assay kit (cat no. 17-295, Millipore, Billerica, MA) according to the manufacturer's instructions. Immunoglobulin (1 μg; IgG; Abcam, ab172730), anti-TCF4 antibody (Cell Signaling Technology, 2566), and anti-β-catenin antibody (Abcam, ab32572) were used for immunoprecipitation (24). The immunoprecipitated DNA fragments were analysed via quantitative PCR. Primer sequences used for ChIP-PCR are listed in Table I.

Western blot analysis. Cells were lysed in an ice-cold lysis solution [7 M urea, 2 M thiourea, 2% CHAPS, 40 mM Trizma base, 40 mM dithiothreitol (DTT), and 1% protease inhibitor]. After complete lysis of the cells and centrifugation at 15,000 x g for 15 min, the total protein concentration in the supernatant was measured using a Bradford protein assay kit (Bio-Rad, Richmond, CA, USA). Proteins were separated via SDS-PAGE and electrotransferred onto Immobilon-P Transfer Membranes (Millipore, Tokyo, Japan). Membranes were incubated in TBS containing 5% skim milk and 0.05% Tween-20 for 1 h and subsequently blotted with primary antibodies at 4˚C overnight. Anti-Smo antibody (1:1,000, Abcam, ab72130), anti-Gli1 antibody (1:2,000, Abcam, ab49314), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam, ab8245) were used as the primary antibodies. Membranes were incubated for 1 h with an anti-mouse or anti-rabbit HRP-linked secondary antibody (1:2,000; Cell Signaling Technology).

Total RNA extraction, cDNA synthesis, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA (1 μg) was extracted from human foreskin fibroblast cells and reverse-transcribed using a GoScript Reverse Transcription Kit (Reverse Transcription System, Promega) according to the manufacturer's instructions. Gene expression was quantified via RT-qPCR as previously described (25). A SYBR Green Master Mix kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for performing qPCR on an Illumina Eco 3.0 (Illumina, Inc., San Diego, CA, USA). A typical reaction consisted of an initial denaturation at 95˚C for 3 min, followed by 40 cycles of denaturation for 30 sec at 95˚C, annealing for 30 sec at 55˚C and extension at 72˚C for 30 sec, followed by a final extension at 72˚C for 5 min. The transcription levels were normalized against those of *GAPDH* using the $2^{-\Delta\Delta Cq}$ method (26). Primer sequences used for RT-qPCR are listed in Table I.

Table I. Primer sequences.

Primer	Sequences (5'-3')
Smo F	ACCTATGCCTGGCACACTTC
Smo R	AGGAAGTAGCCTCCCACGAT
PTCH F	CAAACTCCTGGTGCAAACCG
PTCH R	CCGGGATTCTCAGCCTTGTT
Gli1 F	CCAGAGTTCAAGAGCCTGG
Gli1 R	CCTCGCTCCATAAGGCTCAG
Gli2 F	GTTCCAAGGCCTACTCTCGCCTG
Gli2 R	CTTGAGCAGTGGAGCACGGACAT
Gli3 F	GGGTGAACAGCATCAAAATGGAG
Gli3 R	CCGATAGCCATGTTGGTGG
TCF4 F	AGAGCGACAAGCCCCAGAC
TCF4 R	ATTCGCTGCGTCTCCCATC
β-catenin F	TCGCCAGGATGATCCCAGC
β-catenin R	GCCCATCCATGAGGTCCTG
GAPDH F	GACCTGCCGTCTAGAAAAAC
GAPDH R	CTGTAGCCAAATTCGTTGTC
Smo F1 F	CGTTGAGGGAGACTTGCTTA
Smo F1 R	CTTGGATGAATACCTGTGGC3
Smo F2 F	CTCTGAGTGACTCCGAGGTTAT
Smo F2 R	TAGTTGGTCCTAAGGTTGTTTG
Gli1 F3 F	TGAAGTCTTATTCCCTCCCAC
Gli1 F3 R	TCCCTCTACCATTTCTTGTTCT

F, forward; R, reverse; Smo, smoothened, frizzled class receptor; Gli, GLI family zinc finger; TCF4, T-cell factor 4; PTCH, patched.

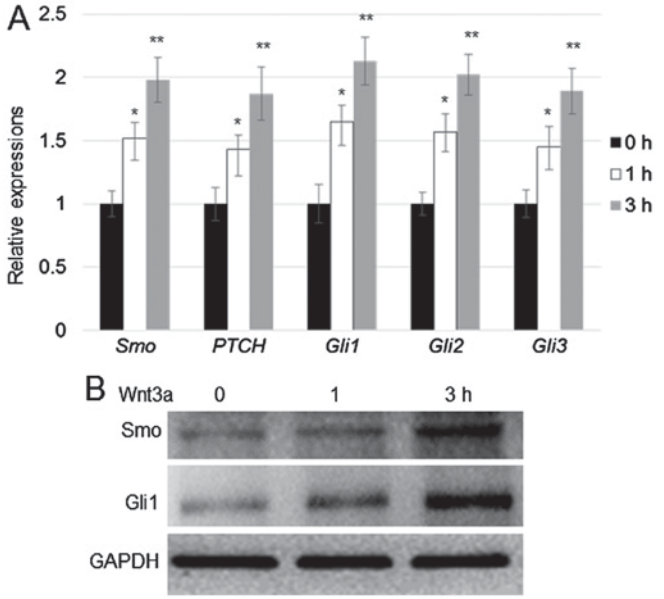

Figure 1. Wnt3a treatment induced the expression of Hedgehog signalling genes. (A) *Smo*, *PTCH*, *Gli1*, *Gli2* and *Gli3* expression levels were analysed following 0, 1 and 3 h of Wnt3a treatment. Data are expressed as means ± standard error (n=3). (B) Western blot analysis was performed to determine Smo and Gli1 levels following 0, 1 and 3 h of Wnt3a treatment. GAPDH was used as the loading control. The experiments were repeated thrice. *P<0.05 and **P<0.01 vs. 0 h. Smo, smoothened, frizzled class receptor; Gli, GLI family zinc finger; PTCH, patched.

Overexpression (OX) and RNA interference. Open reading frame (ORF) regions of *TCF4* (NM_013685.2, NCBI) and *β-catenin* (NM_001165902.1, NCBI) were synthesized and cloned into the *pcDNA3.1 (+)* vector to generate the *TCF4 OX* and *β-catenin OX* constructs. siRNA targeting *β-catenin* (ON-TARGET plus SMART pool, L-004018), siRNA targeting *Gli1* (ON-TARGETplus SMART pool, J-041026-05), and negative control siRNA (ON-TARGETplus Non-targeting Control Pool, D-001810) were purchased from Dharmacon RNA Technologies (Chicago, IL, USA). Fibroblast cells were seeded for 12 h before transfection and allowed to reach 30 to 50% confluence at the time of transfection. Afterwards, 30 nM siRNA duplex and 2 μg of *TCF4 OX* or *β-catenin OX* plasmids were transfected on day 0 using Lipofectamine 2000 (Invitrogen) and Opti-MEM®I Reduced Serum Medium (Gibco) according to the manufacturer's instructions. Cells reached confluence at 24 h after transfection, and the OX or siRNA solutions were replaced with full growth medium. The transfected cells were used for two experiments, namely, cell migration assay (60-80% confluence at the time of cell migration assay on day 2) and RT-qPCR (up to a density of 80-90% confluence at the time of harvest for RNA preparation on day 3).

Cell proliferation assay. Cell proliferation was examined after siRNA treatment. Proliferation ability was measured using a CCK-8 Kit (Dojindo Bio., Japan) according to the manufacturer's instructions. The cell densities of the *β-catenin* and *Gli1* siRNA-transfected cells were analysed relative to those of the control group as previously described (23).

Statistical analysis. Statistical calculations were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean ± standard deviation. Significant differences between groups were analysed by one-way analysis of variance, followed by Bonferroni's multiple comparison post hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Wnt3a induced the expression of Hedgehog signalling genes. To verify the effect of Wnt3a on the expression of hedgehog signalling genes, human fibroblast cells were treated with Wnt3a (100 ng/ml) for 1 and 3 h. RT-qPCR assay was performed to monitor the expression of *Smo*, *PTCH*, *Gli1*, *Gli2*, and *Gli3*. The results indicated that expression of all the above mentioned genes was induced by Wnt3a treatment, and peak expression levels for all genes tested were detected after 3 h of Wnt3a treatment (Fig. 1A). Western blot analysis was performed to verify the results of RT-qPCR analysis. Consistent with the results of RT-qPCR, western blotting results revealed that Smo and Gli1 expression was induced after 3 h of Wnt3a treatment (Fig. 1B).

The β-catenin/TCF4 complex directly activates Smo and Gli1 transcription. *Smo* and *Gli1* are induced by Wnt3a treatment. Therefore, we next determined whether the key Wnt signalling transcription factor complex β-catenin/TCF4 can directly induce the transcription of *Smo* and *Gli1*. Promoter sequence

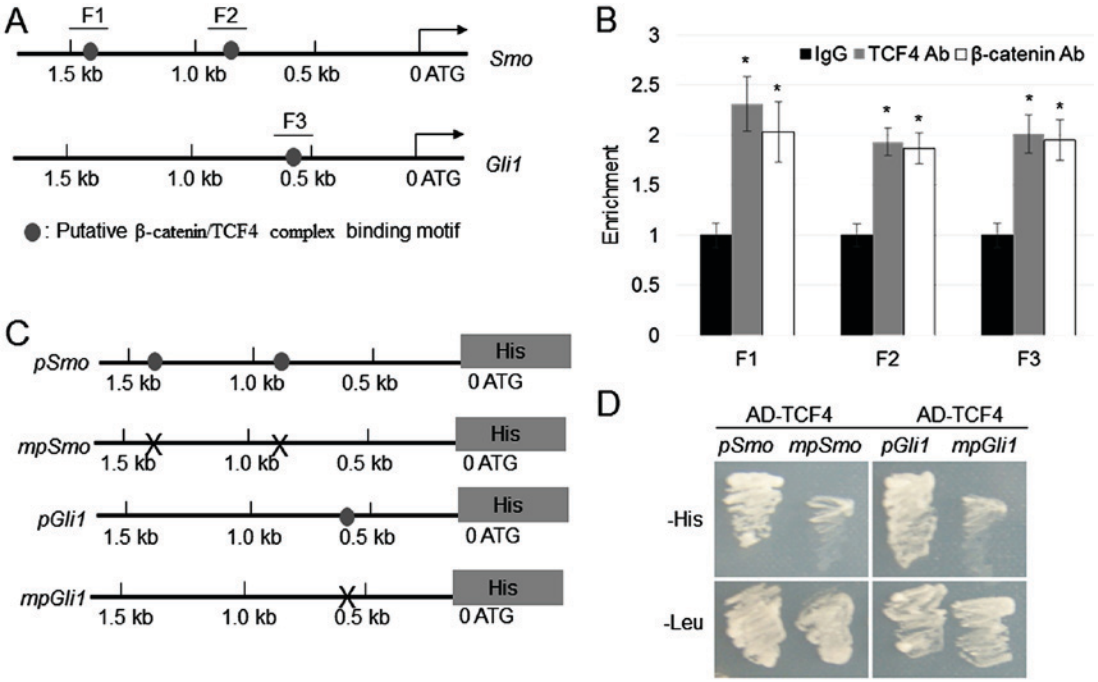

Figure 2. β-catenin/TCF4 complex directly binds to the *Smo* and *Gli1* promoters. (A) Schematic diagram indicating the locations of the putative TCF binding motifs (grey circles) within 1.5 kb of the *Smo* and *Gli1* promoters. (B) ChIP assay was performed by amplifying immunoprecipitated DNA from the F1, F2 and F3 regions; the relative ratios of immunoprecipitated DNA and input DNA were determined via ChIP-polymerase chain reaction. Data are expressed as means ± standard error (n=3). *P<0.05 vs. IgG. (C) The 1.5-kb regions of the *Smo* and *Gli1* promoters with or without mutations at the TCF4 binding motif were cloned into the *pHISi* vector, which utilizes His as a reporter gene. Grey circles indicate TCF4 binding motifs, while 'X' marks indicate mutations in the TCF4 binding sequences. (D) Yeast one-hybrid assay was performed to analyse the binding of tTCF4 with the *Smo* and *Gli1* promoters. Yeast cells harbouring AD-TCF4 and *pSmo*-His and *mpSmo*-His or *pGli1*-His and *mpGli1*-His were grown on synthetic defined medium lacking Leu or His. TCF4, antibody; Ab, antibody; IgG, immunoglobulin G; TCF4, T-cell factor 4; Smo, smoothened, frizzled class receptor; Gli, GLI family zinc finger; ChIP, chromatin immunoprecipitation.

analysis revealed that two and one putative TCF/LEF binding motifs (AGAAAG) (7) was located within 1.5 kb of the *Smo* and *Gli1* promoters, respectively (Fig. 2A). To investigate whether β-catenin/TCF4 directly binds to the putative motifs located in the *Smo* and *Gli1* promoters, ChIP assays were performed using antibodies targeting TCF4 or β-catenin, with IgG as the control. Immunoprecipitated DNA fragments were amplified using the primer pairs targeting the F1, F2, and F3 regions of the *Smo* and *Gli1* promoters (Fig. 2B). Data were normalized using the input DNA as template. ChIP-PCR results showed that β-catenin and TCF4 can bind to the F1, F2, and F3 regions (Fig. 2B). To verify the ChIP findings, yeast one-hybrid assay was performed by co-expressing AD (activation domain)-TCF4, and *pSmo*-His and *mpSmo*-His or *pGli1*-His and *mpGli1*-His (Fig. 2C). The results indicated that cells transfected with AD-TCF4 co-expressing *pSmo*-His or *pGli1*-His were able to grow in the SD medium without histidine, whereas cells transfected with AD-TCF4 co-expressing *mpSmo*-His or *mpGli1*-His did not grow. All transformants were normally grown in SD medium without leucine (Fig. 2D). The above findings indicated that TCF4 activates the transcription of *Smo* and *Gli1* by binding to their promoters in yeast cells.

Overexpression of TCF4 and β-catenin activated Smo and Gli1. Given that TCF4 and β-catenin bind to the *Smo* and *Gli1* promoters, their expression levels were further analysed in the *TCF4*- and *β-catenin*-overexpressing cells. *TCF4* and *β-catenin* were transiently overexpressed (*TCF4 OX* and *β-catenin OX*) in human fibroblasts by transformation of the plasmids *pcDNA3.1-TCF4* or *pcDNA3.1-β-catenin* using Lipofectamine 2000 reagent. Gene expression analysis via RT-qPCR revealed that *TCF4* and *β-catenin* were significantly upregulated after 24 h of transfection (Fig. 3). In addition, *Smo* and *Gli1* expression levels showed 2.2- and 2.8-fold upregulation in *TCF4 OX* cells relative to control cells, respectively (Fig. 3A). In addition, the results showed that *Smo* and *Gli1* expression levels were upregulated by 2.2- and 2.6-fold in *β-catenin OX* cells relative to control cells, respectively (Fig. 3B).

Suppression of β-catenin reduced Smo and Gli1 levels with or without Wnt3a stimulation. Considering that overexpression of *TCF4* and *β-catenin* induced *Smo* and *Gli1* expression, *TCF4* and *β-catenin* levels were further analysed in the *β-catenin* siRNA-transfected human fibroblast cells with or without Wnt3a treatment. RT-qPCR results indicated that transfection with *β-catenin*-specific siRNA significantly downregulated the expression of *β-catenin* independent of Wnt3a treatment (about 60-70%), and Wnt3a treatment induced *β-catenin* levels (Fig. 4). Wnt3a treatment also upregulated *Smo* and *Gli1* expression in cells with or without Wnt3a stimuli; however, the relative increases in expression were lower in *β-catenin* siRNA-transfected cells compared to those of control cells. Furthermore, siRNA suppression of *β-catenin* expression significantly reduced *Smo* and *Gli1* levels in human fibroblasts (Fig. 4).

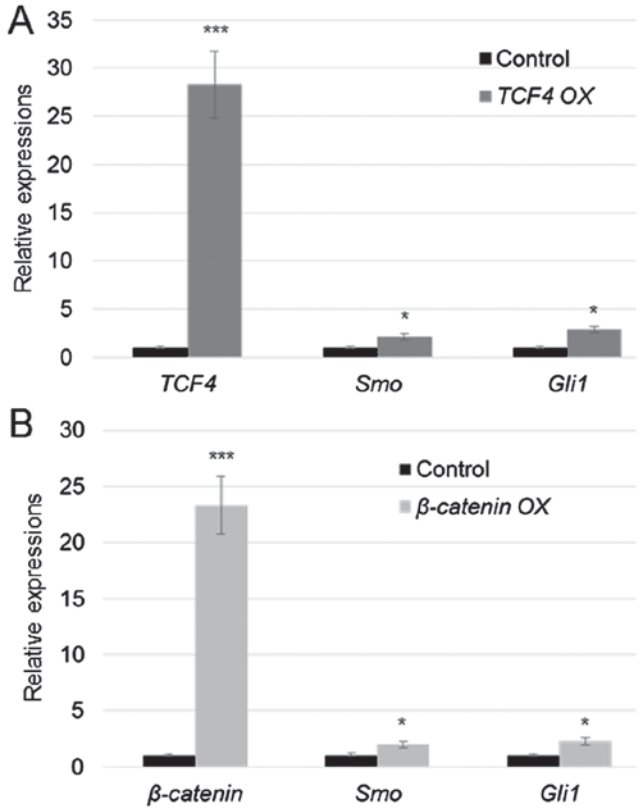

Figure 3. Overexpression of *TCF4* and *β-catenin* activates the transcription of *Smo* and *Gli1*. (A) Full-length open reading frames of *TCF4* and *β-catenin* were transiently expressed using Lipofectamine 2000 reagent, and the transcription levels of (A) *TCF4* and (B) *β-catenin* were monitored in non-transformed (Control) and transformed cells via reverse transcription-quantitative polymerase chain reaction. *Smo* and *Gli1* levels were analysed in control cells and in cells overexpressing *TCF4* or *β-catenin*. Data are expressed as the mean ± standard error (n=3). *GAPDH* was used as an internal control. *P<0.05 and ***P<0.001 vs. control. TCF4, T-cell factor 4; Smo, smoothened, frizzled class receptor; Gli, GLI family zinc finger; OX, overexpression.

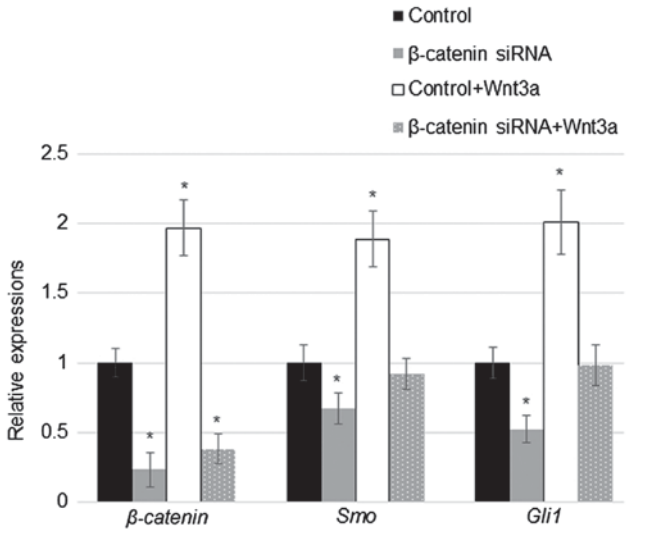

Figure 4. *β-catenin* suppression downregulates the transcript levels of *Smo* and *Gli1*. *β-catenin*, *Smo* and *Gli1* levels were analysed in cells transfected with control (scrambled) siRNA and *β-catenin* siRNA with or without Wnt3a stimulation. Data are expressed as the mean ± standard error (n=3). *GAPDH* was used as an internal control. *P<0.05 vs. control. Smo, smoothened, frizzled class receptor; Gli, GLI family zinc finger; siRNA, small interfering RNA.

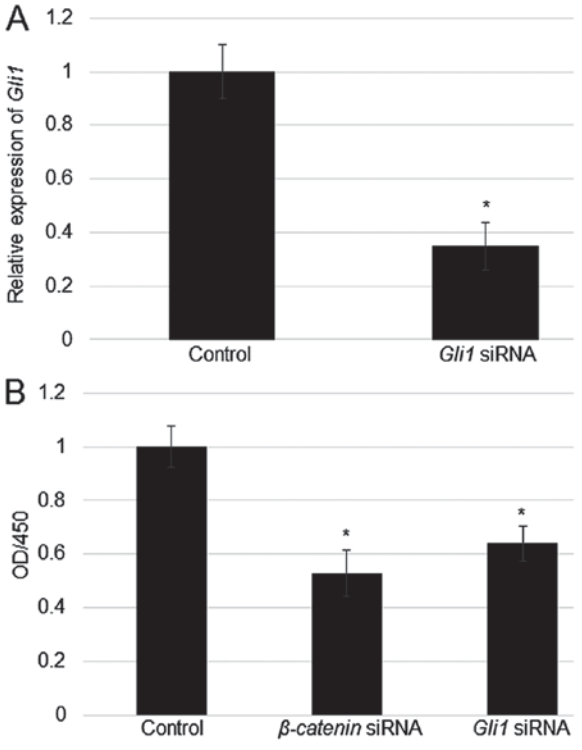

Figure 5. Effects of *β-catenin* and *Gli1* siRNA transfection on the cell proliferation of human foreskin fibroblasts. (A) *Gli1* levels were analysed in cells transfected with control (scrambled) siRNA and *Gli1* siRNA. *Gli1* expression levels were normalized against those of *GAPDH*. (B) Cell proliferation was monitored in *β-catenin* and *Gli1* siRNA-transfected fibroblasts. Data are expressed as the mean ± standard error (n=3). *P<0.05 vs. control. Gli, GLI family zinc finger; siRNA, small interfering RNA; OD, optical density.

Effects of β-catenin and Gli1 suppression on cell proliferation in fibroblasts. Given that the Wnt and Hedgehog signalling pathways have been demonstrated to be involved in cell proliferation, we examined the effects of *β-catenin* and *Gli1* suppression on fibroblast proliferation. Before evaluating cell proliferation ability, fibroblasts were transfected with *Gli1* siRNA and control siRNA, after which *Gli1* expression levels were measured by RT-qPCR. *Gli1* was significantly downregulated in *Gli1*-siRNA transfected cells relative to those of control cells (Fig. 5A). In addition, cell proliferation rates were determined in *β-catenin* and *Gli1* siRNA-transfected cells relative to those of the control group. Cell density measurements evidently showed that suppression of *β-catenin* and *Gli1* inhibited cell proliferation (Fig. 5B).

Discussion

The skin is the tissue layer that protects the body from external damage. Fibroblasts are a cell type that play a major role in wound repair. Extensive studies have been conducted to elucidate the regulatory mechanisms underlying fibroblast cell migration and proliferation (20-23). TCF and β-catenin are master transcriptional regulators of Wnt signalling, and nuclear translocation of β-catenin leads to the formation of a TCF/β-catenin transcription factor complex (2-4). GSK3β-β-catenin signalling was reported to regulate cell migration in human fibroblasts via

feedback regulation of basic fibroblast signalling (20). Another study demonstrated that key regulators of Hedgehog signalling, including *Smo* and *Gli1*, are controlled by β-catenin to modulate fibroblast cell migration (22). In addition, connections between Wnt and hedgehog signalling was reported (27). In colon cancer, GSK3β and CK1α phosphorylate full-length Gli3 (28), leading to the degradation of C-terminal peptides of Gli3 to produce truncated form, Gli3R. Gli3R further inhibits Gli1 activity (29). A negative regulator (kinase) of both Wnt/β-catenin and Hedgehog/Gli signaling pathways, Sufu interacts with β-catenin and Gli1 to modulate their nuclear-cytoplasmic distributions (30,31). However, the relationship between β-catenin and Gli as well as Wnt and hedgehog signalling remains unclear in fibroblasts. Our previous transcriptome study revealed that Wnt3a stimulation in fibroblasts induced the expression of Hedgehog signalling genes, such as *Smo*, *PTCH*, and *Gli* (21). To further confirm the relationship between Wnt and Hedgehog signalling, fibroblast cells were subjected to Wnt3a treatment, and expression patterns of key hedgehog signalling genes were analysed. The results of RT-qPCR and western blot analyses demonstrated that Wnt3a-induced Wnt signalling activation upregulated the expression of *Smo*, *PTCH*, *Gli1*, *Gli2*, and *Gli3* (Fig. 1).

A previous study demonstrated that the TCF/β-catenin complex activates various downstream genes by binding to specific sequences in the promoters of the target genes (7). Interestingly, promoter sequence analysis revealed that the putative TCF/LEF binding motifs (7) to which the TCF/β-catenin complex binds were located within 1.5 kb of the *Smo* and *Gli1* promoters. We next performed ChIP and yeast-one hybrid assays to determine whether the TCF/β-catenin complex directly binds to the *Smo* and *Gli1* promoters. Our findings indicated that the TCF4/β-catenin complex directly binds to two and one TCF/LEF motifs in the *Smo* and *Gli1* promoters, respectively (Fig. 2B).

Given that the TCF4/β-catenin complex binds to the *Smo* and *Gli1* promoters, the expression levels of *Smo* and *Gli1* were examined in the cells overexpressing *TCF4* or *β-catenin*. *TCF4* or *β-catenin* was highly expressed in the cells transfected with *pCDNA3.1-TCF4* or *pCDNA3.1-β-catenin* (Fig. 3). In addition, the results indicated that *TCF4* or *β-catenin* overexpression activated *Smo* and *Gli1* transcription. Moreover, *Smo* and *Gli1* expression levels were examined in *β-catenin* siRNA-transfected cells. siRNA transfection evidently suppressed *β-catenin* levels. In addition, *Smo* and *Gli1* levels were lower in *β-catenin* siRNA-transfected cells compared to those of control cells (Fig. 4), which suggested that β-catenin is located upstream of *Smo* and *Gli1*. To confirm whether Wnt3a stimuli-mediated induction of *Smo* and *Gli1* are mediate by β-catenin, control siRNA- and *β-catenin* siRNA-transfected cells were treated with Wnt3a. The results indicated that Wnt3a induced *β-catenin*, *Smo*, and *Gli1* expression in both the control siRNA- and *β-catenin* siRNA-transfected cells (Fig. 4). However, the fold inductions in *β-catenin* siRNA-transfected cells were lower than those in control cells, suggesting that β-catenin mediates Wnt3a-induced activation of Hedgehog signalling.

We further analysed the biological function of β-catenin as a key regulator of Hedgehog signalling genes. The Wnt signalling pathway is known to regulate cell proliferation (2). Thus, *β-catenin* and *Gli1* expression was suppressed via siRNA transfection of human fibroblasts, and cell proliferation rates were determined. The results suggested that *β-catenin* and *Gli1* participate in the same signalling pathway and act as positive regulators of cell proliferation.

Wnt and Hedgehog signalling play crucial roles in diverse processes involved in mammalian development. In the present study, our findings revealed that the Wnt and Hedgehog signalling pathways directly share a common molecular mechanism, which is the binding of the TCF4/β-catenin complex to *Smo* and *Gli1* promoters in human fibroblasts. Furthermore, *β-catenin* and *Gli1* were demonstrated to positively regulate cell migration and proliferation in human fibroblasts (2; Fig. 5B), thereby demonstrating the strong link between gene regulation and biological function. Previous study showed the post-transcription regulation of Gli family proteins by Wnt signalling regulators GSK3β and CK1α (26-28), but transcriptional regulation between two pathways has not been observed. In this study, we identified a transcriptional regulation between β-catenin and *Smo* or *Gli1*, which extended knowledge of Wnt and hedgehog relationship, which could be important for understanding regulatory networks not only in fibroblasts, but also in cancer cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YPW, PPL and QW performed the experiments. YPW, MQZ and LXP analyzed and interpreted the data. YPW, MQZ and LXP were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Wenzhou Medical University (Wenzhou, Zhejiang, China) and written informed consent was obtained from all of the patients involved.

Patient consent for publication

The patients provided written informed consent for the publication of any associated data.

Competing interests

The authors declare that they have no competing interests.

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