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Wnt2 knock down by RNAi inhibits the proliferation of in vitro-cultured human keloid fibroblasts

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Abstract

To study the effect of knocking down wingless-related MMTV integration site 2 (Wnt2) expression by RNAi on the growth and signaling pathways of ex vitro-cultured keloid fibroblasts (KFB).

Human KFB were isolated from 10 keloid patient specimens. The KFB cells were then transfected with 4 pairs of small interfering RNA (siRNA) targeting human Wnt2, respectively. Reverse transcriptase-polymerase chain reaction and Western blot analysis were conducted to verify the knock down of Wnt2, and the expression of β-catenin glycogen synthase kinase-3β (GSK-3β) and cyclin D1 were examined.

siRNA Wnt2 transfection (siWnt2) resulted in the significant inhibition of Wnt2 expression at both the mRNA and protein levels. The expression of β-catenin, GSK-3β, p-GSK-3β, and cyclin D1 at the protein level also decreased in siWnt2 cells. siWnt2 resulted in a substantially slower growth and significant delay in cell doubling time of the KFB cells compared with control groups. Further, the siRNA knock down of GSK-3β and β-catenin resulted in slower proliferation rates, respectively.

Wnt2 siRNA has an inhibitive effect on keloid fibroblast proliferation, which may be a potential therapeutic approach for keloid and other human fibrotic diseases.

Abbreviations: CCK-8 = cell counting kit-8, GSK-3β = glycogen synthase kinase-3β, KFB = keloid fibroblasts, MOI = multiplicity of infection, RPMI-1640 = Roswell Park Memorial Institute-1640, RT-PCR = real-time reverse transcription polymerase chain reaction, siControl = nonsense sequence siRNA transfection, siRNA = small interfering RNA, siWnt2 = siRNA Wnt2 transfection, TGF-β = transforming growth factor-β, Wnt = wingless-related MMTV integration site.

Keywords: fibroblast, GSK-3β, Keloid, RNAi, Wnt2

1. Introduction

Keloid is a type of dermal fibroproliferative disorder following excessive wound healing in susceptible individuals. The histological characteristics of keloid include hyperplasia of fibroblasts and excessive deposition and disordered arrangement of extracellular matrix, especially collagen.[1] Keloid primarily causes cosmetic concerns to the patients but can also lead to severe itching, pain or pressure. Treatment of keloid remains a challenge to dermatologists or plastic surgeons due to its high rate of recurrence. Many studies have shown that a variety of cytokines and signaling pathways are involved in the pathogenesis of keloid.[2] However, the exact etiology of keloid remains unknown.

The wingless-related MMTV integration site (Wnt) signaling transduction pathway is an important pathway that participates in a series of biological processes including cell growth, proliferation, and apoptosis.[3,4] The Wnt signaling pathway is not only involved in embryonic development but also plays an important role in injury and repair after birth,[5] as well as tumorigenesis.[6] In the canonical Wnt signaling pathway, extracellular Wnt ligands bind to frizzled membrane receptor and lipoprotein-related protein coreceptors. The activation of the receptors then recruits disheveled, axin and GSK3β to the plasma membrane, consequently destabilizing the β-catenin destruction complex mediated by the ubiquitin and 26 S proteasome. The free β-catenin then enters the nucleus to activate transcription targets such as cyclin D1, C-myc, e-fos, finally leading to abnormal cell proliferation.[7–9]

Recent studies reveal a strong correlation between the aberrant activation of the Wnt/β-catenin signaling pathway and the fibrosis in various organs, including the kidney, lung, liver, heart, and skin.[10] Bayle et al found the Wnt 2, Wnt 9, Wnt 10, and Wnt 11 genes to be up-regulated in a mouse model of skin fibrosis through microarray analysis.[10] Studies have demonstrated the important role of the Wnt signaling pathway in keloid pathogenesis,[11] and the down-regulation of β-catenin blocks fibrosis via modulating wingless-related MMTV integration site

2 (Wnt2) signaling in human keloid fibroblasts (KFB).[12] Evidence also indicates that the transforming growth factor-β (TGF-β) signaling pathway is involved in fibrosis.[13] A genome-wide microarray analysis confirmed that TGF-β was spatially enriched in Keloid biopsies and ex vitro-cultured KFB.[14] Finally, there is a cross-talk between the Wnt/β-catenin signaling pathway and the TGF-β signaling pathway in promoting the fibrogenesis and coregulation of fibrogenic gene targets.[15]

In this study, we examined the effects of knocking down Wnt2 expression via siRNA on the growth of human KFB and the associated molecular changes in the Wnt signaling pathway.

2. Materials and methods

2.1. Patients and specimens

Tissue specimens were obtained from 10 patients (4 males and 6 females) who underwent plastic surgery at the Second Affiliated Hospital of Fujian Medical University, China. These 10 samples were collected from the face, chest, back, abdomen, and limbs. Keloid was diagnosed based on clinical signs and pathological examination.

The patients were 30.00 ± 18.83 (range 2–55) years old. The duration of keloid development was 13.70 ± 6.31 (range 6–24) months. Causes of disease include 4 traumas, 3 operations, 1 ear piercing, and 2 infections. These patients had no other skin diseases and no connective tissue or other organic diseases. The patients did not receive any chemotherapy, radiotherapy, laser treatment, or immunological therapy. The collected skin had no ulcers or infections. Normal skin tissue was collected from 9 of the 10 patients around the keloid or the donor site of the patients with skin transplantation. All patients gave informed consent.

2.2. Cell culture and grouping

Human KFB were cultured using the tissue explants adherent method. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, GE Healthcare, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Gibco, New York, NY) and 5% CO_2 in a humidified incubator at 37°C.[16] Culture medium was changed every 3 to 5 days. Cells at passages 3 to 6 were used. There were 5 experimental groups: normal skin cells (B0), keloid cells without treatment (B), keloid cells with mock transfection (M), keloid cells with nonsense sequence siRNA transfection (siControl), and keloid cells with siRNA Wnt2 transfection (siWnt2).

Four siRNA sequences (named siRNA623, siRNA765, siRNA804, siRNA846 based on their start positions) were designed based on the human Wnt2 gene sequence (GenBank accession No. NM_019178) according to the study of Elbashir et al[17] (Table 1). The sequences were queried by BLAST to ensure no homologous genes other than Wnt2 were found. The siRNA sequences were synthesized by Shanghai GenePharma Co, Ltd. (Shanghai, China). A nonsense sequence (siControl) was used as an internal control (Table 1).

Cells at the logarithmic phase were digested gently with trypsin–ethylene diamine tetraacetic acid (Gibco) and harvested as a single cell suspension. The cells were counted with a hemocytometer and then diluted to a concentration of 1.5×10^6/ mL and seeded into 6-well plates for 24 hours at 37°C, 5% CO_2. Each 1 OD_{260} siRNA was dissolved in 150 μL diethyl pyrocarbonate-H_2O to a final concentration of 20 μM. Eight microliters of siRNA was then added into a 1.5-mL Eppendorf tube with 250 μL of Opti- Dulbecco's modified eagle medium. Concurrently, 5 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was added to a 1.5-mL Eppendorf tube with 250 μL and mixed. The tubes were stored at room temperature 5 minutes before combination and were stored at room temperature for 25 minutes following combination. The medium was then removed from the cell culture plate and replaced with 1 mL RPMI-1640 serum-free medium (Gibco). The transfection mixture was added into a 6-well plate dropwise, and cells were cultured in a humidified incubator for 4 to 6 hours. The transfection medium was then replaced with 1.5 mL RPMI-1640 medium supplemented with 10% FBS. The number of transfection-positive cells and the multiplicity of infection (MOI) were calculated 24 and 48 hours later under a fluorescent microscope (Motic, Xiamen, China). MOI = the number of transfection-positive cells/Total cell number × 100%.

2.3. RNA isolation and real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated with Trizol reagent following standard protocols (Invitrogen). First-strand cDNA was reverse-transcribed with MMLV and used for real-time reverse transcription polymerase chain reaction (RT-PCR) following standard protocol. RT-PCR was performed with MX3000P (Stratagene, La Jolla, CA), and the relative expression was analyzed by the method of $2^{-\Delta\Delta Ct}$. Results were presented as means ± standard deviation. Primer sequences of β-catenin, Wnt2, glycogen synthase kinase-3β, (GSK-3β), and cyclin D1 are listed in Table 2.

2.4. Protein isolation and Western blot

Cells were lyzed in Mammalian Protein Extraction Reagent (Pierce, IL), separated by sodium dodecyl sulfate–polyacrylamide

Table 1

siRNA sequences for Wnt2.

Gene names	5′	3′
623	GCCUUUGUUUAUGCCAUCUTT	AGAUGGCAUAAACAAAGGCTT
765	GCUGCAGUGAUAACAUUGATT	UCAAUGUUAUCACUGCAGCTT
804	GCGCAUUUGUGGAUGCAAATT	UUUGCAUCCACAAAUGCGCTT
846	GAGCCCUGAUGAAUCUUCATT	UGAAGAUUCAUCAGGGCUCTT
siControl	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

siControl = nonsense sequence siRNA transfection, siRNA = small interfering RNA, Wnt2 = wingless-related MMTV integration site 2.

Table 2

Primer sequences for quantitative real-time reverse transcription polymerase chain reaction analysis.

Gene name		Primer sequences
β-actin (size: 101 bp)	5′	CTCCCTGGAGAAGAGCTACGAGC
	3′	CCAGGAAGGAAGGCTGGAAGAG
Wnt2 (size: 107 bp)	5′	GGCACAGGTTTCACTGTGGCTAAC
	3′	CCTGCCTCTCGGTCCCTGATAC
GSK-3β (size: 138 bp)	5′	CGGGATATTAAACCGCAGAACCTC
	3′	TGGTGCCCTATAGTACCGAGAACAG
β-Catenin (size: 110 bp)	5′	GTGCTGAAGGTGCTATCTGTCTGC
	3′	TGAACAAGACGTTGACTTGGATCTG
Cyclin D1 (size: 144 bp)	5′	AACAAACAGATCATCCGCAAACAC
	3′	GTTGGGGGCTCCTCAGGTTCAG

GSK-3β = glycogen synthase kinase-3β, Wnt2 = wingless-related MMTV integration site 2.

gels and stained with Coomassie Brilliant Blue. Protein was then transferred to a polyvinylidene difluoride membrane (Millipore, Burlington, MA). The membrane was blocked in 5% nonfat milk and then incubated at 4°C with the first antibody (1:2000) overnight. Wnt2 antibody (sc-50361), β-catenin antibody (sc-7199), p-GSK-3β antibody (sc-81494), and cyclin D1 antibody (sc-753) were purchased from Santa Cruz (Santa Cruz, CA). GSK3β antibody (24198-1-AP) was purchased from Ptglab (Rosemont, IL). The blot was then incubated with the appropriate second antibody, HRP-conjugated goat antirabbit IgG and HRP-conjugated goat antimouse IgG (Jackson Immuno Research Lab, West Grove, PA). Specific binding of the antibody was visualized using SuperSignal West Pico Chemiluminescent Substrates (PIERCE, Pierce, IL). The results were analyzed and quantified using Gel-Pro Analyzer software and corrected by reference to the expression value for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.5. Cell proliferation assay by CCK-8

Cells at the logarithmic phase were harvested and digested gently and then seeded into a 96-well plate at 3×10^6/well and cultured for 24 hours. The culture medium was then replaced with 100 μL fresh medium per well, and 10 μL of cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) per well was added. The cell plates were cultured for another 2.5 hours while protected from light. The plates were then read by an enzyme-linked immunosorbent assay plate reader (Tecan M1000; Tecan, Zürich, Switzerland) at 450 nm at 24, 48, and 72 hours after transfection.

2.6. Statistical analysis

Data were analyzed by SPSS 12.0. Multiple groups were compared using 1-way analysis of variance followed by Fisher least significant difference multiple-comparison post hoc test. $P < .05$ indicated statistical significance.

3. Results

3.1. siRNA successfully knocked down Wnt2 at both the mRNA and protein levels

The knockdown of Wnt2 by siRNA was verified at both the mRNA and protein levels. qRT-PCR analysis showed that the Wnt2 mRNA level was significantly lower compared with the controls (B0, B, M, and siControl groups) at both 24 and 48 hours after transfection (Fig. 1).

Western blot analysis confirmed that siRNA Wnt2 resulted in a decrease in the Wnt2 expression at the protein level (Fig. 2). This decrease was more obvious at 48 hours after transfection than 24 hours. siRNA 623 resulted in the most significant inhibition compared to 765, 804, and 846. Because siRNA Wnt2 623 showed the most significant decrease in Wnt2 levels, it was chosen for the following experiments.

3.2. Decreased expression of GSK-3β, β-catenin, and cyclin D1 at the protein level in siWnt2 cells

We tested the expression of Wnt2 and cyclin D1 at the mRNA level using qRT-PCR in siWnt2 cells. No significant differences were observed in the levels of GSK-3β and β-catenin in siWnt2 KFB cells compared with the siControl group ($P < .05$, Fig. 3). However, cyclin D1 expression decreased significantly compared with the siControl group.

Compared with the siControl, the expressions of β-catenin, Wnt2, cyclin D1, p-GSK3β, and GSK-3β at the protein levels in the siRNA Wnt2 group decreased significantly ($P < .05$, Fig. 4). Among these, decreases in the p-GSK3β and β-catenin levels were the most dramatic.

3.3. siRNA Wnt2 resulted in the decreased proliferation of KFB cells

To test the effects of Wnt2 knockdown on the growth of KFB cells, we measured cell proliferation in different groups using a
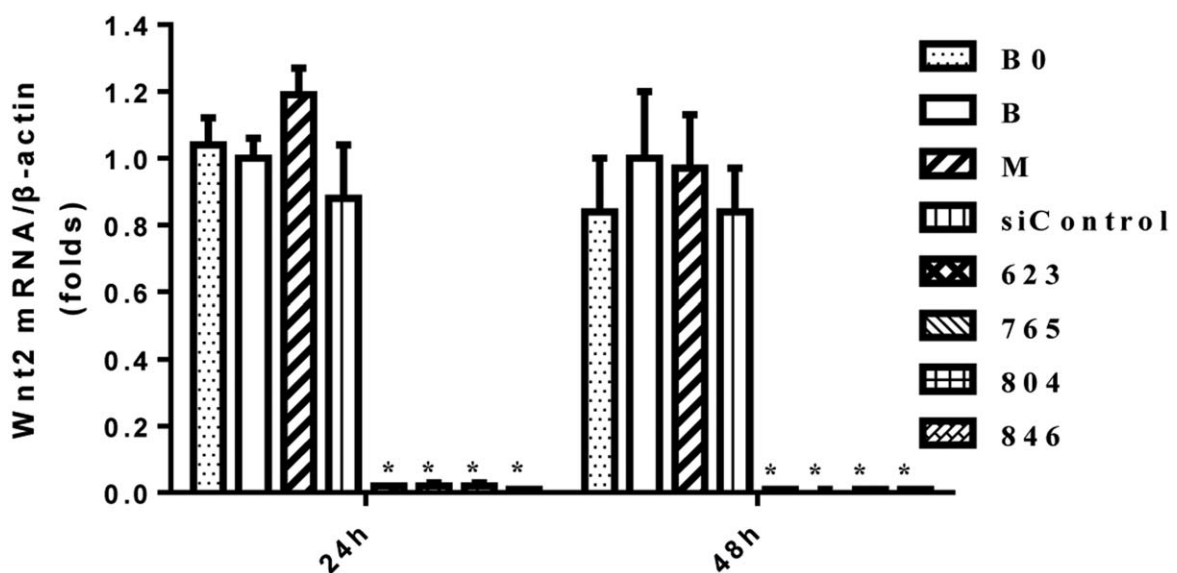

Figure 1. siRNA knockdown of Wnt2 at the mRNA level at 24 or 48 hours by real-time reverse transcription polymerase chain reaction analysis. Four siRNA sequences (623, 765, 804, and 846) were used for siRNA Wnt2 transfection in keloid cells. Data are the means ± standard deviation. B = keloid cells without treatment, B0 = normal skin cells, M = keloid cells with mock transfection, siControl = keloid cells with nonsense sequence siRNA transfection, siRNA = small interfering RNA, Wnt2 = wingless-related MMTV integration site 2. *$P < .05$ versus siControl.
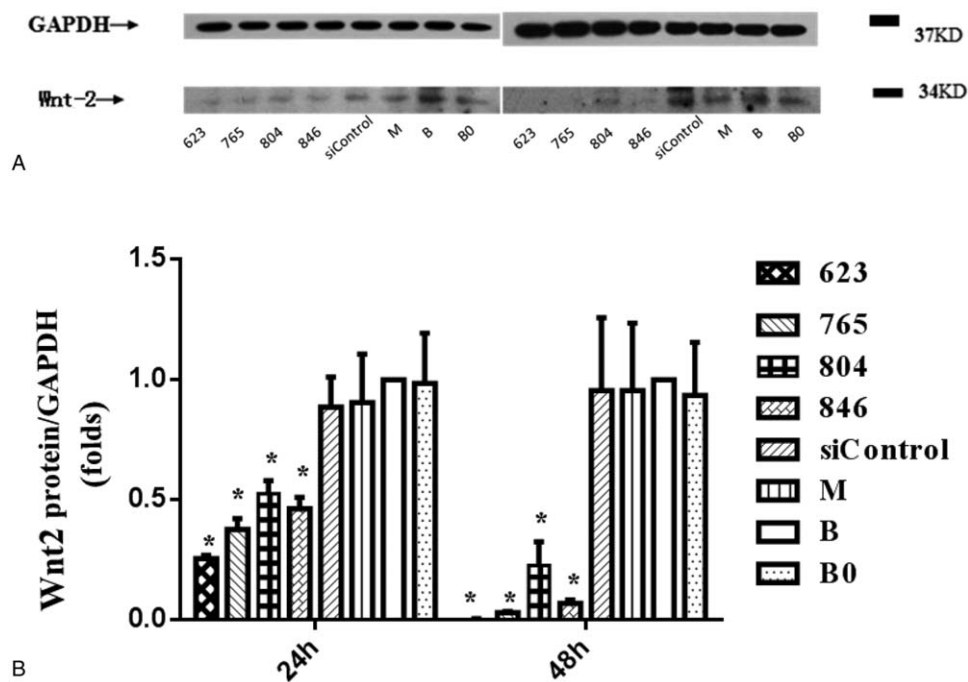
Figure 2. Expression of Wnt2 at the protein level after transfection of siRNA Wnt2 at 24 or 48 hours. Western blot analysis (A) and protein band density quantitative analysis (B). Four siRNA sequences (623, 765, 804, and 846) were used for siRNA Wnt2 transfection in keloid cells. Data are means ± standard deviation. B = keloid cells without treatment, B0 = normal skin cells, M = keloid cells with mock transfection, siControl = keloid cells with nonsense sequence siRNA transfection, siRNA = small interfering RNA, Wnt2 = wingless-related MMTV integration site 2. *$P < .05$ versus siControl.

CCK-8 assay. Our results showed that the growth rate of KFB in Wnt2 siRNA decreased significantly at the logarithmic phase. The decreased growth rate was more significant with prolonged siWnt2 treatment, with the most significant inhibition at 72 hours (Fig. 5, Table S1, http://links.lww.com/MD/C451).

3.4. siRNA GSK-3β and β-catenin resulted in the decreased proliferation of KFB cells

The knockdown of GSK-3β and β-catenin by siRNA were separately verified at both the mRNA and protein levels (Table S2, http://links.lww.com/MD/C451). Similarly, the
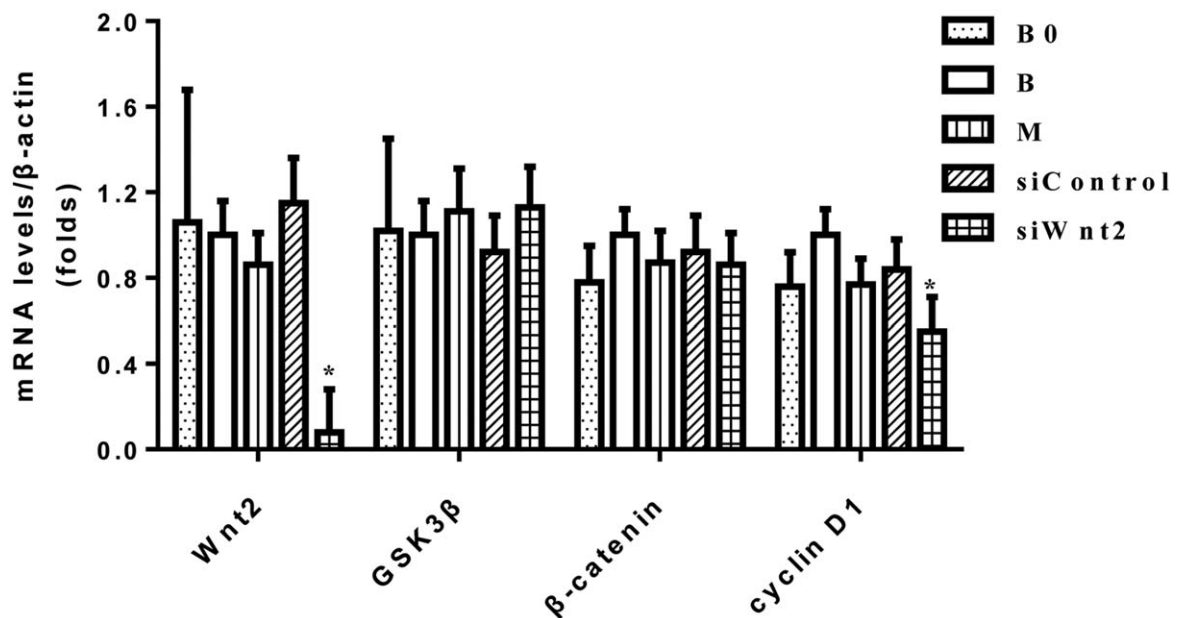

Figure 3. Expression of GSK-3β, β-catenin, and cyclin D1 in siRNA Wnt2-transfected cells by real-time reverse transcription polymerase chain reaction analysis. Data are means ± standard deviation. B = keloid cells without treatment, B0 = normal skin cells, GSK-3β = glycogen synthase kinase-3β, M = keloid cells with mock transfection, siControl = keloid cells with nonsense sequence siRNA transfection, siRNA = small interfering RNA, Wnt2 = wingless-related MMTV integration site 2. *$P < .05$ versus siControl.
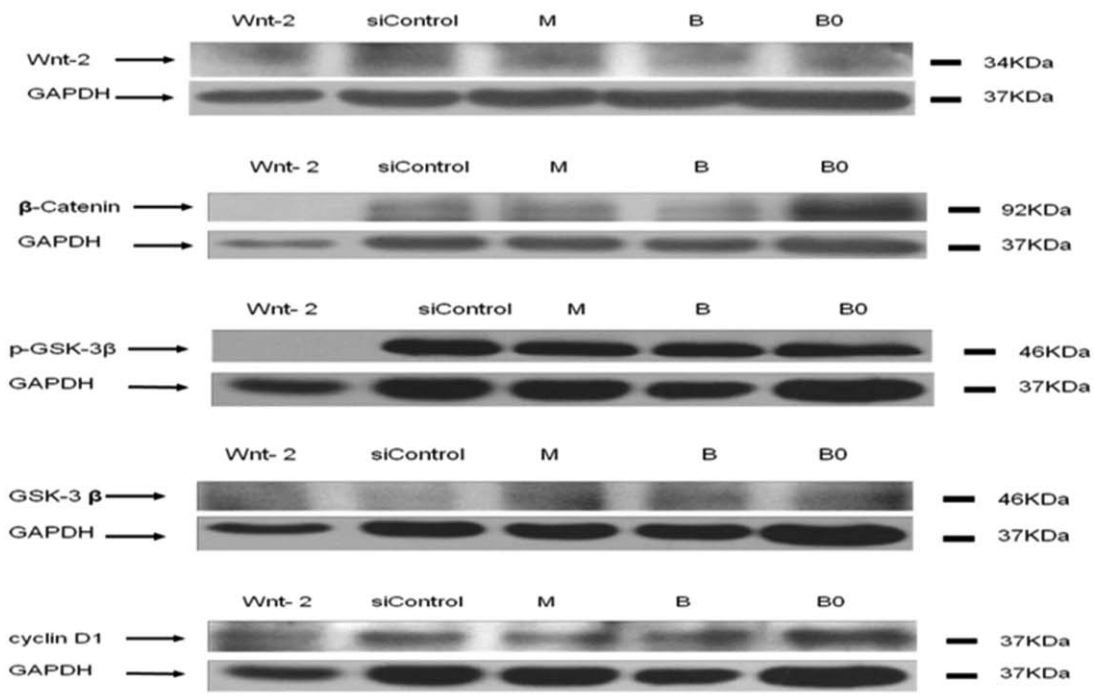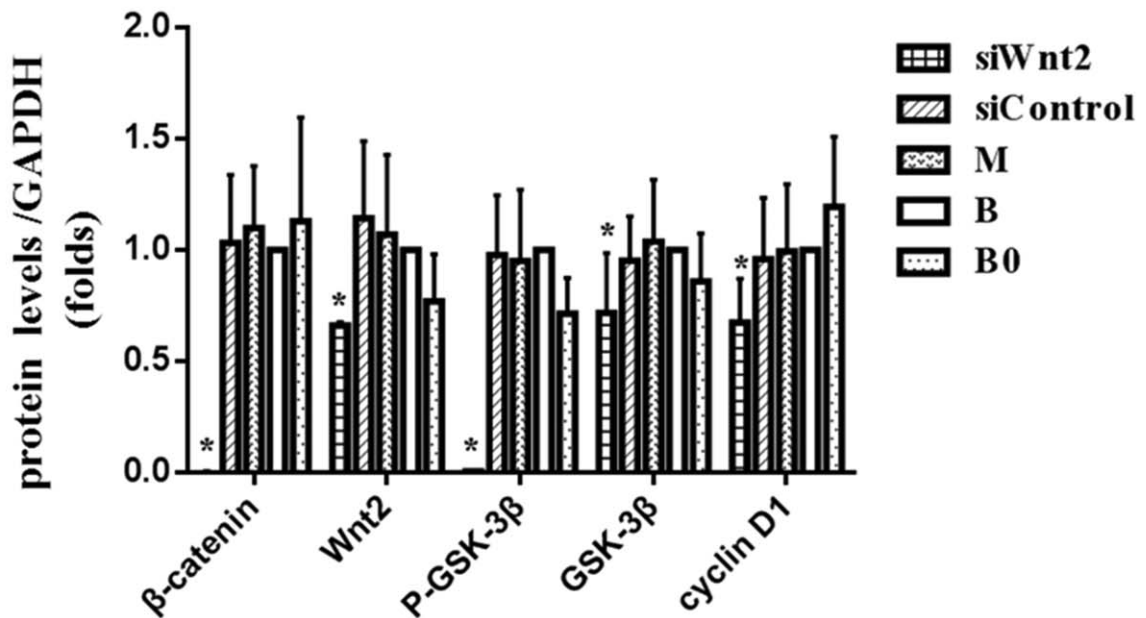
Figure 4. The expressions of β-catenin, GSK-3, P-GSK-3, and cyclin D1 in siRNA Wnt2-transfected cells. Western blot analysis (A) and protein band density quantitative analysis (B). Data are the means ± standard deviation. B = keloid cells without treatment, B0 = normal skin cells, M = keloid cells with mock transfection, siControl = keloid cells with nonsense sequence siRNA transfection, siRNA = small interfering RNA, Wnt2 = wingless-related MMTV integration site 2. *P < .05 versus siControl.

growth rate of KFB was significantly inhibited by siGSK-3β as well as siβ-catenin with prolonged culture (Fig. 5, Table S1, http://links.lww.com/MD/C451).

4. Discussion

Wnt2 gene is a member of the Wnt family, which is located on chromosome 7q31. Several transcription factor binding sites are

located at the 5′ end of the human Wnt2 gene, such as GA-TA-a, AP-2, TCF-1, BHLH, MBF-1, p53, and HNF-5.[18] Wnt2 is highly expressed in breast cancer, pancreatic tumor tissue, and serum, and plays an important role in promoting tumor proliferation and migration.[19,20] Wnt2 knockdown by siRNA suppresses the accumulation of β-catenin in the nucleus, while the transient overexpression of Wnt2 increases nuclear β-catenin.[21] These studies highlight the essential role of Wnt2 in stem cell
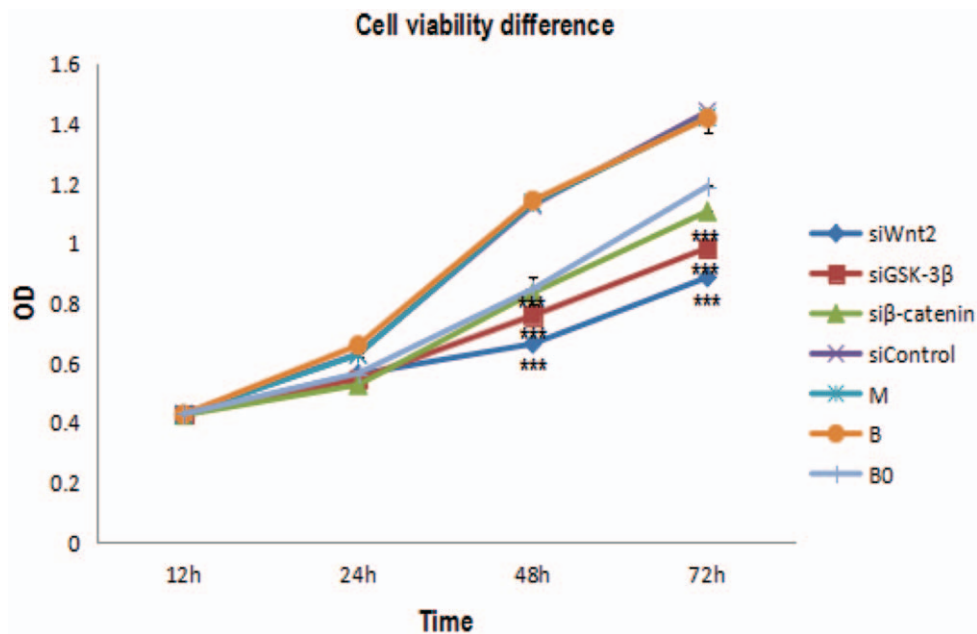
Figure 5. Knockdown of Wnt2 and GSK-3β of β-catenin resulted in a decreased cell proliferation rate of KFB cells. Data are the means ± standard deviation. B = keloid cells without treatment, B0 = normal skin cells, GSK-3β = glycogen synthase kinase-3β, KFB = keloid fibroblasts, M = keloid cells with mock transfection, siControl = keloid cells with nonsense sequence siRNA transfection, siRNA = small interfering RNA, Wnt2 = wingless-related MMTV integration site 2. *$P < .05$ versus siControl.

reprogramming as well as in promoting cell proliferation, which is consistent with our findings that siWnt2 slows the growth of keloid cells.

In this study, we employed an RNA interference technique (RNAi) and designed and synthesized several specific interference sequences that inhibit Wnt2 expression. Among the 4 siRNA sequences, Wnt2-623 had the most dramatic knock down effect compared with the control sequence. The CCK-8 assay showed that growth rate of KFB transfected by Wnt2 siRNA decreased at the logarithmic phase. At the same time, the expression of GSK-3β, β-catenin, and cyclin D1 at the protein level also decreased in siWNT2 KFB. Notably, the expression of p-GSK-3β and β-catenin decreased greatly. This result shows that the activity of Wnt signaling pathways was inhibited in siWnt KFB. The dramatic decrease in the phosphorylation of GSK-3β led to the enhanced phosphorylation of β-catenin and promoted the degradation of β-catenin, resulting in the decreased level of free β-catenin. The protein level of its downstream target gene, cyclin D1 (which is related to cell cycle regulation), consequently decreased, resulting in a delay in the G1-S phase transition in KFB and growth inhibition. In short, our study suggests that the canonical Wnt2/β-catenin signaling pathway is involved in the pathogenesis and development of keloid, which is in agreement with the study by Igota et al.[11]

RNA interference is the process of gene silencing at the transcription level triggered by double-stranded RNA. Because of high specificity and the efficiency of RNA interference, it can be used not only as an important tool to study gene function, but also as a potential treatment option for human cancer.[22] Cai et al[12] found that targeting β-catenin with siRNA inhibited the proliferation and increased apoptosis of fibroblast cells, accompanied by down-regulation of Wnt2 in the keloid fibrosis, suggesting a crucial role of Wnt2/β-catenin in the regulation of fibroproliferation after wound healing. In this study, we investigated the effects of the inhibition of the canonical

Wnt2/β-catenin signaling pathway on the growth of keloid. KFB was cultured in vitro and separately transfected with Wnt2, GSK-3β, and β-catenin siRNA. The effects of these genes on the growth and proliferation of KFB were measured by CCK-8. We found that compared with the siControl group, the proliferation rate was decreased at the logarithmic phase in all 3 groups. The proliferation rate of the Wnt2 group was the slowest, followed by the β-catenin group and GSK-3β group. Why Wnt2 knockdown resulted in the best growth inhibition in KFB compared with β-catenin and GSK-3β remained unclear. Further study is needed to confirm and expand our preliminary findings.

Taken together, our study showed that the down-regulation of Wnt2 by siRNA led to the decreased expression of GSK-3β, β-catenin, and cyclin D1 and the slower proliferation of ex vitro cultured KFB. Our study provides further understanding of the pathogenesis of keloid and valuable insight into the therapeutic treatment of keloid through the down-regulation of the Wnt signaling pathway.

Author contributions

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Validation: Yumei Cai.
Visualization: Yumei Cai.
Writing – original draft: Yumei Cai.
Writing – review & editing: Yumei Cai.

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