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Wnt/β-catenin signaling exacerbates keloid cell proliferation by regulating telomerase

Running title: Role of wnt/β-catenin in telomerase regulation for keloid

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Abstract

This study was designed to investigate the relationship between keloid, telomerase and Wnt/β-catenin signaling. Tissues from keloid patients, keloid progenitor cells (KPC) and skin progenitor cells (SKP) were both included in this study. KPC were divided into different groups: NC, wnt10a siRNA, β-catenin siRNA and TERT siRNA. KPC were injected into BALB/c nude mice to build tumor models. Immunohistochemistry and western blot were performed to detect protein expression whereas RT-PCR was carried out to detect relative mRNA levels. MTT assay and flow cytometry were also conducted for assessing cell proliferation and apoptosis status. β-catenin and telomerase expression levels were significantly higher in keloid tissues ($P < 0.05$). KPC exhibited more dynamic telomerase activity than SKP ($P < 0.05$). After wnt10a/β-catenin signaling pathway was inhibited, the proliferation of KPC was significantly suppressed and the apoptosis rate was remarkably increased ($P < 0.05$). Results from tumor models also validated how wnt10a/β-catenin signaling pathway influenced the activity and length of telomerase. Wnt/β-catenin signaling pathway is able to exacerbate keloid cell proliferation and inhibit the apoptosis of keloid cells through its interaction with telomerase.

Key words: Wnt • β-catenin • telomerase • keloid • cell proliferation • apoptosis

Introduction

Keloid which is characterized by exaggerated response to injury and formation of excessive scar tissues are defined as dermis benign tumors resulted from dysregulated wound healing processes [1]. Many factors contribute to keloids such as race, wound infection and genetic predisposition [2]. African, Asian and Hispanic descents are much more susceptible to keloids compared with individuals from other ethnicities [3]. Keloids usually occur in head, neck and other parts of the body and a high incidence of relapse is observed in patients with keloids [4]. Keloids not only affect one's appearance but also impair skin functions and other parts of the body may be affected to some degree. As a result of this, this disease imposes significant psychological and physical burdens on patients who suffer from this disease. However, most treatments for managing keloids have very limited effectiveness and it is still challenging to cure this disease. Conventional treatments including surgery which may be combined with radiotherapy or corticosteroids have been developed to manage patients with keloid [5]. Several studies have hypothesized that epithelial-to-mesenchymal transition (EMT) plays a vital role in the pathogenesis of keloid or hypertrophic scar [6, 7]. It is well known that EMT can be induced by transforming growth factor-β, other pro-inflammatory cytokines and wingless proteins (Wnt) [8]. Compared with other chronic diseases, keloid pathogenesis is not fully understood and no animal models have been established for investigating how keloids are formed [9]. Therefore, it is worthwhile to further explore the molecular mechanisms of keloids which may provide additional information for the formation of keloids.

Wnt signaling pathway is a sophisticated signaling pathway that is related to cell development. The entire pathway network consists of the independent β-catenin pathway and the dependent β-catenin pathway [10]. Abnormal activation of Wnt is closely related to the development of

cardiovascular disease, hepatic fibrosis and cancer [11, 12]. Wnt/β-catenin signaling pathway which is considered to be the most representative example of Wnt signaling, plays critical roles in diversified cellular functions including cell proliferation, differentiation, migration and apoptosis [13]. Significance of this pathway can be exemplified by the evidence that both over-expression and under-expression of target genes contained in this pathway result in various diseases [14]. Wnt/β-catenin signaling pathway should be appropriately regulated in order to provide cells with their normal functions. It is acknowledged that β-catenin levels are minimized by several destruction complex including Axin, APCGSK3β and casein kinase 1 (CK1) on condition that Wnt is not provoked. As for the nucleus, TCFs block the activation of target genes with the assistance of transcriptional repressors [15, 16]. The accumulated β-catenin then combines with the LEF/TCF family transcription factor, converts them from repressors to activators and thus triggers gene transcription starting from the downstream [17]. As suggested by a study on tumor behavior, tumor cell metastasis was achieved through EMT whose process was driven by Wnt/β-catenin signaling pathway [18].

The telomerase complex which comprises of TERT, an enzymatic subunit and a RNA component, Terc, controls the telomere length and plays an important role in stem cells, aging process and cancer [19]. Telomeres contain specialized genomic structures that are indispensable for genome stability [20]. Chromosome stability of telomere is lost and the length of human telomere starts to downsize after several times of cell cycles in the case of cell proliferation and aging [21]. Telomere/telomerase interplay has been indicated to be an important mechanism which is involved in cellular replicative potential and genomic stability. Besides that, telomere/telomerase dysfunction has significant impact on carcinogenesis [22]. The regulation of telomerase by wnt/β-Catenin Signaling pathway has been studied in both stem cells and cancer cells [23]. Mutations in β-catenin

contributed to an increased Tert expression in cancer and resulted in telomeres stabilization which is a significant tumorigenesis marker. The provoking of telomerase in keloid contributes to the maintenance of telomere length. However, telomerase activity is inhibited in developed keloid [23]. Wnt/β-catenin signaling pathway may activate related gene expressions and thus influences the development of keloid [24]. So far, researches which focus on the mechanism of telomeres and Wnt/β-Catenin Signaling pathway in keloids are still scarce.

This research is designed to employ models in vitro and in vivo for the purpose of investigating Wnt/β-catenin signaling and telomerase activity in keloids. We hypothesized that Wnt/β-catenin signaling exacerbated cell proliferation and apoptosis by regulating telomerase and this mechanism may explain the formation of keloids.

Materials and methods

Ethics statement

Collection of human tissues was authorized by the Institutional Ethics Committee of The First Affiliated Hospital of Harbin Medical University based on the Helsinki Declaration. Informed consent was obtained from patients prior to study commencement. All rat experiments were complied with the Guidance for Care and Usage of Laboratory Animals and were accepted by the National Cancer Institute Animal Care and Use Committee.

Patient samples

Keloid specimens (n = 18), including 12 males and 6 females, were collected from The First Affiliated Hospital of Harbin Medical University between Mar 2014 and Oct 2014. All clinical characteristics of patients were presented in Table 1. Both keloid tissues and related peripheral normal skin tissues were collected from patients, which were used to compare molecule expressions

and isolate stem cells from tissues.

Immunohistochemistry

Immunohistochemical analysis of β-catenin in tissue samples was performed with the two-step EnVision method as described previously [25]. Rabbit polyclonal anti-β-catenin antibody (Zhongshan Biology Company, Beijing) was diluted for 100 times.

Cell isolation and culture

Keloid progenitor cells (KPC) and skin progenitor cells (SKP) were separated from 5 keloid tissues and 5 matched normal skin tissues, respectively as previously described [26]. In brief, aseptical tissues were cut into small pieces and treated with dispase in order to generate scattered cells which were filtered with a 70-μm strainer. Cells were cultured in Dulbecco modified Eagle medium (Gibco, Carlsbad, CA) with 10% fetal bovine serum (Gibco, Carlsbad, CA) at 37℃ in an incubator with 5% CO_2.

Lenti-virus transduction and transfection

Four groups of fragments, separately containing NC, wnt10a siRNA, β-catenin siRNA and TERT siRNA were cloned into the pCDH vector. Then pCDH vectors with other packaging plasmids were co-transfected into cells using Lipofectamine LTX kit（Invitrogen, CA） and the viral particles therein were collected 48h after transfection.

KPC cells were infected with five groups of recombinant lentivirus and 8 ug/ml polybrene: control group (cells without any transfection), NC group (cells transfected with negative vector), wnt10a siRNA group (cells transfected with wnt10a siRNA), β-catenin siRNA group (cells transfected with β-catenin siRNA) and TERT siRNA group (cells transfected with TERT siRNA).

Transplantation in vivo

A total of 20 male BALB/c nude mice (Laboratory Animal Center of The First Affiliated Hospital of

Harbin Medical University) with an average age of 4 weeks and weight of 16-18g were obtained for establishing tumor growth models. Briefly, KPC cells were subcutaneously injected into the neck of mice (1×10^6 cells each mice). Seven days after tumor volumes were quantified, tumor sites of mice in the four groups (5 mice per group) were transfected with four types of lenti-virus expressing vectors (control, wnt10a siRNA, β-catenin siRNA and TERT siRNA) through direct injection (2×10^7 units each time, twice a week) for a period of 14 days. Tumor volume of mice in each group was calculated 28 days after the injection using the following formula: volume = $(A \times B^2)$ /2, where A = the biggest diameter and B = the smallest diameters.

Telomerase mutant sable cells and luciferase promoter-reporter transient transfection experiments

Mutated binding sites (four TCF-4 binding elements) in the telomerase-promoter were established using GeneTailor Site-Directed Mutagenesis System (Invitrogen, USA) and the mutated sequences were showed in Table 2. Then wild type and mutated promoter of telomerase were cloned into the downstream of the psiCHECK-2 luciferase vector (Promega, USA), which was named as wt promoter and mut TBE1-4 promoter, respectively.

KPC cells maintained in 48-well plates were co-transfected with three groups of substances: one group was transfected with the combination of 160ng pcl-neo-β-catenin-D45 encoding expression plasmid, 40ng pCMV-Remnilla and 120 ng wt promoters, while wt promoters in the other two groups were either replaced by mut TBE1-4 promoter or negative control plasmid. The transfected cells were inspected by the Dual-Luciferase Reporter Assay System (Promega) 48h after the transfection.

Chromatin immunoprecipitation (ChIP)

ChIP assay was achived with ChIP-IT kits (Active Motif). In brief, chromatin of KPC cells was cut

into approximately 400 bp by ultra-sound sonification (20-30 sec pause for five times, HTU SONI130), and then immunoprecipitated with antibodies included specific β-catenin antibodies (Sigma) for human cells, as well as mice IgG (Active Motif) as isotype controls. The reverse-crosslinked chromatin precipitates were analysis with real-time PCR (RT-PCR) with 10% precipitates as template and 400 nM of each primer spanning the region of human Tert -2000/-1000/+1000/+2000 (relevant primers were listed in Table 3). Water and input chromatin were listed as negative and positive control, respectively.

Cell proliferation and apoptosis assay

MTT assay was carried out to determine cell proliferation status. A total of 3×10^3 cells were cultured in 96-well plates and incubated for five different periods of time (24h, 48h, 72h, 96h and 120h) and then cultured cells were stained with 0.5 mg/ml MTT for 4h. Subsequently, we discarded the supernatant and added 200ul dimethylsulfoxide for the purpose of dissolving precipitate. Samples were measured at 490 nm by an ELISA reader. Apoptosis rates of samples were calculated using flow cytometry after cells were stained with Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences).

Telomerase activity and telomere length assay

Telomerase activity was quantified using the telomeric repeat amplification protocol (TRAP) in conjunction with the fluorescent telomerase repeat amplification kit (Roche). DNeasy blood and tissue kit (Qiagen) were implemented for telomere length assay and DNA was isolated from cells. Then non-radioactive Tell-TAGGG telomere length assay was conducted to measure telomer length by hybridizing telomere into probes labeled with digoxigenin and telomere length was quantified by photometric enzyme immunoassay.

RNA isolation and RT-PCR

Total RNA from tissues or cells were extracted using the TRIzol reagent (Invitrogen). ReverTra Ace qPCR RT Kit (Toyobo, Japan) was manipulated to reversely transcribe total RNA into cDNA and real time-PCR (RT-PCR) was carried out using THUNDERBIRD SYBR® qPCR Mix (Toyobo, Japan) along with the instrument of CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Relevant primers were listed in Table 4. Target gene expression levels were normalized to those of the control gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) and were calculated using the approach of $2^{-\triangle\triangle CT}$.

Western blot

Tissues and cells were harvested and lysed by radio immunoprecipitation assay (RIPA) buffer. Total proteins were separated and calculated as suggested by the Bradford method [27]. Then total proteins were denatured in boiled water and transferred onto Polyvinylidene Fluoride (PVDF) membranes when sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was completed. Membranes were blocked in Tris Buffered Saline Tween (TBST) with 5% skim milk for 1h and were then treated with primary antibodies against wnt10a, β-catenin and telomerase (1:800 dilution, Zhongshan Biology Company, Beijing) at 4□ overnight. After membranes were washed, incubation with secondary antibodies (horseradish peroxidase-conjugated goat anti-goat, 1:2000 dilutions, Zhongshan Biology Company, Beijing) was carried out. Samples in which reduced GAPDH was set as the endogenous control were ultimately processed with enhanced chemiluminescence and quantified by Lab Works4.5 software (Mitov Software).

Statistical analysis

All statistical analyses were performed with SPSS 18.0 software (Chicago, Illinois, USA). Data were presented in the form of mean ± standard deviation (SD). Two-tailed student's t-test or one-way analysis of variance (ANOVA) was used to carry out between-group comparisons and P <

0.05 provided evidence for statistical significance.

Results

Active Wnt10a, β-catenin and telomerase were expressed in keloid tissues

Results from immunohistochemistry showed that stained Wnt10a was more apparent in the area of stratum granulosum in keloid tissues (Figure 1A) compared with normal tissues (Figure 1B). Besides that, keloid tissues displayed significantly higher mRNA and protein expressions of β-catenin and telomerase in comparison to normal tissues, (all $P < 0.05$, Figure 1C, D). In addition, comparative analysis of telomere length and telomerase activity revealed that keloid tissues exhibited relatively larger telomere length and higher telomerase activity than normal skin tissues (all $P < 0.05$, Figure 1E, F).

KPC cells in keloid tissues exhibited more dynamic telomerase activities

KPC cells were isolated from keloid tissues and SKP cells were collected from normal skin tissues. As shown in Figure 2A, B, KPC cells exhibited significantly higher mRNA and protein expressions of telomerase than SKP cells (all $P < 0.05$). Apart from that, significantly longer telomere length and more dynamic telomerase activities were also identified in KPC cells compared with SKP cells (all $P < 0.05$, Figure 2C, D).

B-catenin bound with TBE sites in the 3' promoter of telomerase

A possible link between β-catenin and telomerase was facilitated by the binding site of TCF-4 binding elements (TBEs). Telomerase gene was analyzed and four TBEs were identified in the 3' promoter of telomerase (Figure 3A). Luciferase activity assay confirmed a direct interaction between β-catenin and telomerase with evidence that co-transfection of β-catenin and 3' promoter of telomerase had significantly higher luciferase activity than the NC group in which 3' promoter of

telomerase was replaced by the plasmid vector ($P < 0.05$, Figure 3B). Moreover, the mutation of TBEs can remarkably down-regulated the luciferase activity ($P < 0.05$, Figure 3B).

Then we further validated the results with ChIP assay. When compared with Tert siRNA group, significantly higher level of chromatin precipitates was identified in WT group at transcriptional start site (TSS) ($P < 0.05$), whereas no significant difference was observed between two groups at -2000/-1000/+1000/+2000 sites ($P < 0.05$, Figure 3C)

Effects of wnt/β-catenin on proliferation and apoptosis of KPC cells

MTT assays revealed that significant differences in KPC cell proliferation and apoptosis among different groups of transfection were identified since day 3 of the cell culture process. The proliferative capability of KPC cells transfected with wnt10a siRNA, β-catenin siRNA and telomerase RNA were notably inhibited in comparison to those of cells transfected with the negative control. Generally, silencing of wnt10a can significantly suppress the proliferation rate compared with the control and NC group, while silencing of both β-catenin and telomerase can further down-regulate the proliferation rate with a greater degree than single silencing of wnt10a (all $P < 0.05$). There was no significant difference in the proliferation rate between the β-catenin siRNA and TERT siRNA group ($P > 0.05$, Figure 4).

Results from flow cytometry illustrated that no obvious difference in cell apoptosis status was observed between the control and NC group. Similarly, no substantial difference in cell apoptosis status was observed between the wnt10a siRNA and β-catenin siRNA group (all $P > 0.05$, Figure 5). Transfection of wnt10a siRNA and β-catenin siRNA can remarkably elevate the apoptosis rate of KPC cells compared with the control and NC group, while TERT siRNA group exhibited the lowest apoptosis rate compared with the control, NC, wnt10a siRNA and β-catenin siRNA group (all $P < 0.05$, Figure 5).

Effects of siRNA on wnt10a, β-catenin and telomerase expressions

No substantial difference in mRNA and protein expressions of wnt10a, β-catenin and telomerase was detected between the control and NC group (all $P > 0.05$). Silencing of wnt10a can significantly decrease mRNA and protein expressions of wnt10a and telomerase as well as β-catenin mRNA, while silencing of β-catenin can remarkably decrease mRNA and protein expression of β-catenin and telomerase ($P < 0.05$). Moreover, silencing of telomerase is able to down-regulate mRNA and protein expressions of telomerase (all $P < 0.05$, Figure 6).

SiRNA of wnt10a, β-catenin and telomerase inhibited telomerase activity in KPC cells

Transfection of the negative control vector did not significantly affect telomere length or telomerase activity in KPC cells (all $P > 0.05$). Cells in the wnt10a siRNA group exhibited shorter telomere compared with the control and NC group, while silencing of β-catenin can further restrict telomere length. Interestingly, silencing of telomerase contributed to the shortest telomere length in comparison to the control, NC, wnt10a siRNA and β-catenin siRNA group (all $P < 0.05$, Figure 7A). In addition, transfection of wnt10a siRNA, β-catenin siRNA and TERT siRNA had similar effects on suppressing the activity of telomerase in KPC cells (all $P < 0.05$, Figure 7B).

Results from animal models

To further investigate the effects of wnt10a siRNA, β-catenin siRNA and TERT siRNA on tumor growth, a human keloid-bearing mice model in vivo was built by injecting KPC cells into mice and hence transplanted tumors were formed. After tumors of mice in each group were separated, we observed that tumor volumes of mice in the wnt10a siRNA, β-catenin siRNA and TERT siRNA group were much smaller than those in the control group, while the TERT siRNA group had significantly smaller tumors compared with the wnt10a siRNA group (all $P < 0.05$, Figure 8).

Similar to the results in vitro, transfection of wnt10 siRNA can significantly down-regulate both

mRNA and protein expressions of wnt10a and telomerase as well as β-catenin mRNA in modeled mice, while silencing of β-catenin can remarkably decrease both mRNA and protein expressions of β-catenin and telomerase. Furthermore, silencing of telomerase is able to down-regulate mRNA and protein expressions of telomerase (all $P < 0.05$, Figure 9).

In addition, the wnt10a siRNA group exhibited smaller telomere length and less dynamic telomerase activity compared with the control group, while silencing of β-catenin can further restrict telomere length and telomerase activity compared with silencing of wnt10a. Of note, TERT siRNA group had the lowest telomere length and telomerase activity compared with the control, wnt10a siRNA and β-catenin siRNA group (all $P < 0.05$, Figure 10).

Discussion

The current study has suggested that knocking-down of Wnt10a/β-catenin signaling significantly blocked proliferation and facilitated apoptosis of KPC cells through its suppressive effect on both telomerase expression and telomere length. Another study conducted by Hoffmeyer *et al.* reported that β-catenin controlled Klf4 expression by interacting with telomerase in mouse embryonic stem cells [23]. Several studies have provided evidence that telomerase played a key role in regulating cell proliferation which may result in various disorders (e.g. endometriosis and colorectal cancer) [28-31]. In fact, Wnt/β-catenin has been documented to exacerbate cell proliferation by binding with the T-cell factor (TCF)/lymphoid-enhancing factor (LEF) family and inducing some oncogene expressions including C-myc and cyclin D1(CCND1) [32]. Additionally, Wnt/β-catenin inhibited cell apoptosis by closing mitochondrial permeability transition pore (mPTP) for avoiding pro-apoptotic factor release [33]. All in all, Wnt/β-catenin signaling manipulated cell proliferation and apoptosis in a direct or indirect manner.

Furthermore，Zhang *et al.* suggested that β-catenin enhanced telomerase transcription in human cancers by directly targeting TCF4 [34]. Our study provided similar evidence that β-catenin directly bound with the TCF4 binding elements in the 3' promoter of telomerase in KPC cells. However, Choi *et al.* argued that telomerase exacerbated epithelial proliferation by transcriptional control of Wnt/β-catenin signaling pathway [35]. Similarly, Park *et al.* also reported that telomerase could directly target Wnt/β-catenin signaling genes in mouse gastrointestinal tract [36]. All of these findings suggested that the activation of Wnt/β-catenin and the transcription of telomerase formed an autocrine loop of the signaling pathway.

Another study conducted by Smith *et al.* observed that inhibitors of the Wnt signaling pathway were reduced in keloid pathogenesis [37]. However, how Wnt/β-catenin signaling pathway is influenced by keloid pathogenesis is still unclear mystery. In our study, wnt10a might be responsible for activating the Wnt/β-catenin signaling pathway in keloids pathogenesis. Wnt10a is a canonical Wnt ligand that initiates telomerase transcription [38]. On the other hand, the Wnt/β-catenin signaling pathway has been reported to be triggered by Wnt5a in keloid pathogenesis [39]. Sato *et al.* indicated that transforming growth factor (TGF)-β stimulated the Wnt/β-catenin signaling pathway in hypertrophic scar and keloid tissues via the Smad3 and p38 MAPK pathways [40]. On top of that, Lee *et al.* demonstrated that the formation and development of keloid was contributed by the mesenchymal transition of dermal microvascular endothelial cells which is caused by wnt3a [41]. Thus, multiple factors might be involved in the activation of Wnt/β-catenin which is related to keloid pathogenesis.

Although this study revealed that telomere length was positively correlated with keloid cell proliferation, the effect of changes in telomere length on keloid is still unfolded. For instance, Granick *et al.* reported that telomere length in the keloid tissues was larger than that in normal

tissues during the early stage of keloid formation, while telomerase activity was suppressed in well-developed keloid tissues [42].Besides that, De Felice *et al.* demonstrated that reactive oxygen species (ROS) stress contributed to the restriction of telomere length in keloid tissues [43]. All of these controversial results may result from different types of cells used in experiments and varied experimental designs, suggesting that telomerase functions are likely to vary across different types of cells. Apart from that, other pathways are likely to play roles in keloid pathogenesis which is affected by telomerase activity. For instance, Xiao *et al.* reported that wild type p53 protein can transiently inhibit the telomerase activity of keloid fibroblasts (KFBs) [44].

For summary, this study provided notion that Wnt/β-catenin signaling pathway regulated cell apoptosis and proliferation in keloid by transcriptional targeting of telomerase. These findings may suggest alternative strategies for managing patients with keloids in the near future.

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Conflict of interest

The authors state no conflict of interest.

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Figure legends

Figure 1 Expression of wnt10a, β-catenin and telomerase in keloid tissues and normal skin. **A-B**: Immunohistochemical staining with wnt10a (stained as yellow) in keloid tissues (A) and normal skin (B). **C**: Western blot analysis of β-catenin and telomerase in tissues with GAPDH as internal control. **D**: Quantitative mRNA and protein expressions of β-catenin and telomerase in keloid tissues and normal skin. **E-F**: Quantitative data of telomere length (E) and telomerase activity (F) in keloid tissues and normal skin. Data were presented as mean ± SD. * $P < 0.05$ versus keloid tissues.

Figure 2 Expression and activity of telomerase in KPC and SKP cells. **A**: Western blot analysis of telomerase in KPC and SKP cells with GAPDH as internal control. **B**: Quantitative mRNA and protein level of telomerase in KPC and SKP cells. **C-D**: Quantitative data of telomere length (C) and telomerase activity (D) in KPC and SKP cells. KPC: keloid progenitor cells; SKP: skin progenitor cells. Data were presented as mean ± SD for three independent experiments. * $P < 0.05$ versus KPC group.

Figure 3 β-catenin interacted with TCF-4 binding elements (TBEs) in telomerase promoter and confers transcriptional activation. **A**: Sequences of telomerase promoter and promoter with mutation in TBEs. **B**: Relative luciferase activity in groups of NC, WT and Mut. NC: negative control vector; WT: wild type of telomerase promoter; Mut: telomerase promoter with mutation in TBEs. Data were presented as mean ± SD for three independent experiments. * $P < 0.05$ versus NC group, # $P < 0.05$ versus WT group. **C**: ChIP analysis between β-catenin and Tert. Data were presented as mean ± SD for three independent experiments. * $P < 0.05$ versus Tert siRNA group.

Figure 4: Effects of wnt10a siRNA, β-catenin siRNA and telomerase siRNA on proliferation of KPC cells by means of MTT assay. Data were presented as mean ± SD for three independent experiments.

Figure 5 Apoptosis rate of cells estimated by flow cytometry. **A-E**: Distribution of apoptotic cells in groups of control (A), NC (B), wnt10a siRNA (C), β-catenin siRNA (D) and telomerase siRNA (E). **F**: Relative apoptosis rate of cells in each group. Data were presented as mean ± SD for three independent experiments. * $P < 0.05$ versus control group，# $P < 0.05$ versus NC group，○ $P < 0.05$ versus wnt10a siRNA group.

Figure 6 Expression of wnt10a, β-catenin and telomerase in KPC cells after transfection. **A**: Quantitative mRNA levels of wnt10a, β-catenin and telomerase in KPC cells among different groups (i.e. control, NC, wnt10a siRNA, β-catenin siRNA and telomerase siRNA). **B**: Western blot analysis of wnt10a, β-catenin and telomerase in KPC cells with GAPDH as internal control. **C**: Quantitative protein level of wnt10a, β-catenin and telomerase in KPC cells. Data were presented as mean ± SD for three independent experiments. * $P < 0.05$ versus control group，# $P < 0.05$ versus NC group，○ $P < 0.05$ versus wnt10a siRNA group，□ $P < 0.05$ versus β-catenin siRNA group.

Figure 7 Quantitative data of telomere length (A) and telomerase activity (B) in KPC cells among different groups (i.e. control, NC, wnt10a siRNA, β-catenin siRNA and telomerase siRNA). Data were presented as mean ± SD. * $P < 0.05$ versus control group，# $P < 0.05$ versus NC group，○ $P < 0.05$ versus wnt10a siRNA group，□ $P < 0.05$ versus β-catenin siRNA group.

Figure 8 Effects of wnt10a siRNA, β-catenin siRNA and telomerase siRNA on tumor growth in vivo. **A-D** Size of transplantations generated from KPC cells in groups of control (A), wnt10a siRNA (B), β-catenin siRNA (C) and telomerase siRNA (D). **E**: Quantitative data of tumor volumes in rat models after transplantation of KPC cells. Data were presented as mean ± SD for five independent experiments. * $P < 0.05$ versus control group，# $P < 0.05$ versus wnt10a siRNA group.

Figure 9 Expression of wnt10a, β-catenin and telomerase in nude rat models with KPC cells transplantation. **A-D**: Immunohistochemical staining with wnt10a (stained as yellow) in tissue

samples from control group (A), wnt10a siRNA group (B), β-catenin siRNA (C) and telomerase siRNA (D). **E**: Quantitative mRNA levels of wnt10a, β-catenin and telomerase in model rats within four groups (i.e. control, wnt10a siRNA, β-catenin siRNA and telomerase siRNA). **F**: Western blot analysis of wnt10a, β-catenin and telomerase in model rats with GAPDH as internal control. **G**: Quantitative protein levels of wnt10a, β-catenin and telomerase in model rats. Data were presented as mean ± SD for three independent experiments. * $P < 0.05$ versus control group，# $P < 0.05$ versus wnt10a siRNA group，○ $P < 0.05$ versus β-catenin siRNA group.

Figure 10 Quantitative data of telomere length (A) and telomerase activity (B) in model rats within four groups (i.e. control, wnt10a siRNA, β-catenin siRNA and telomerase siRNA). Data were presented as mean ± SD. * $P < 0.05$ versus control group，# $P < 0.05$ versus NC group，○ $P < 0.05$ versus wnt10a siRNA group.

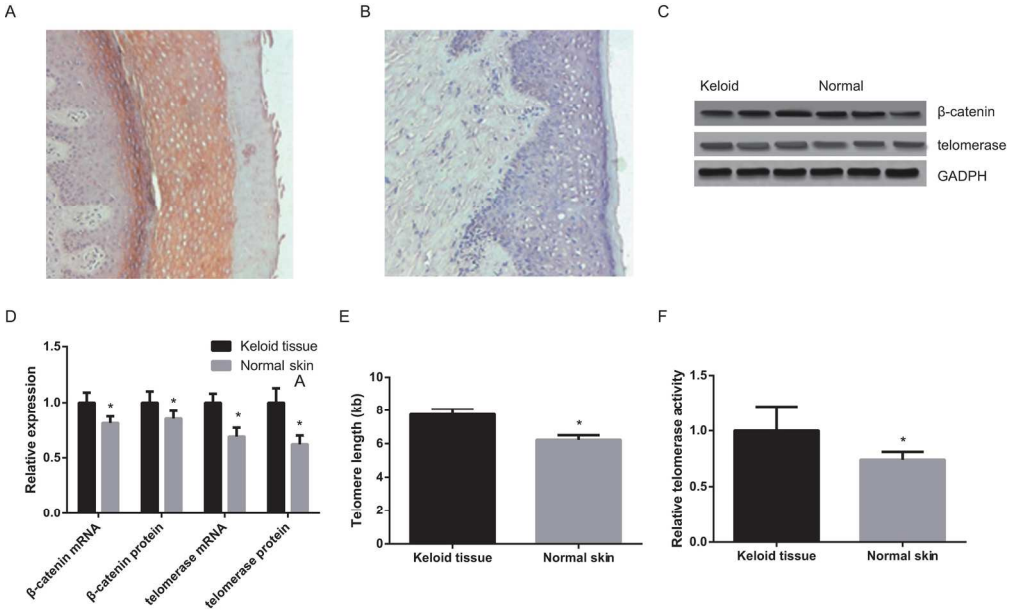

Figure 1
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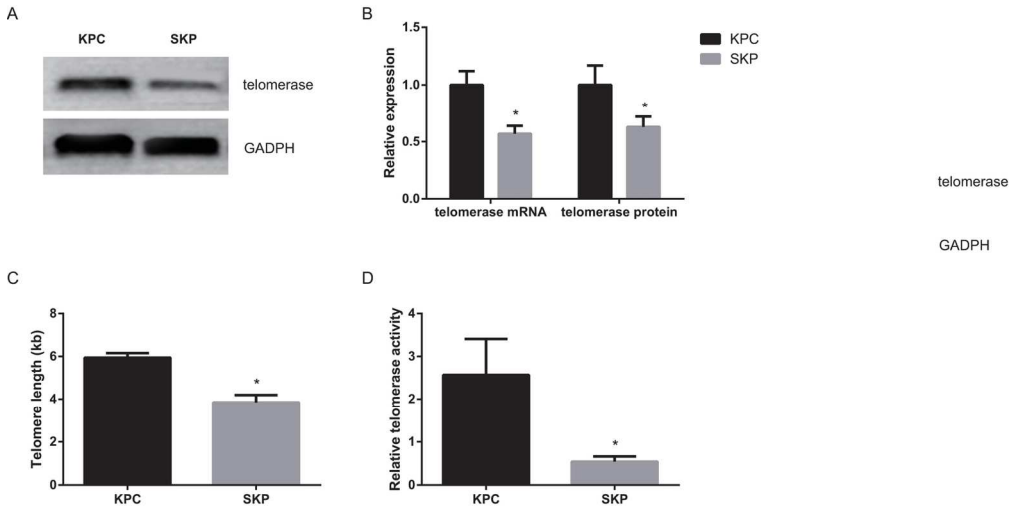

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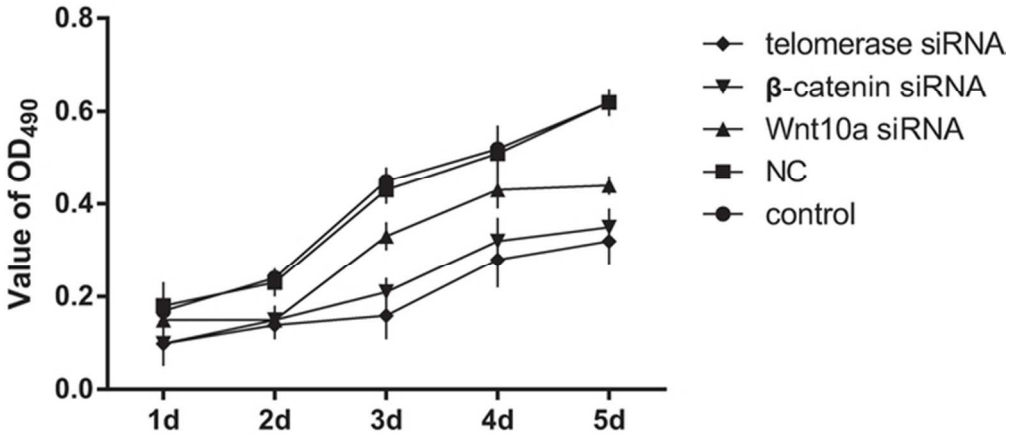

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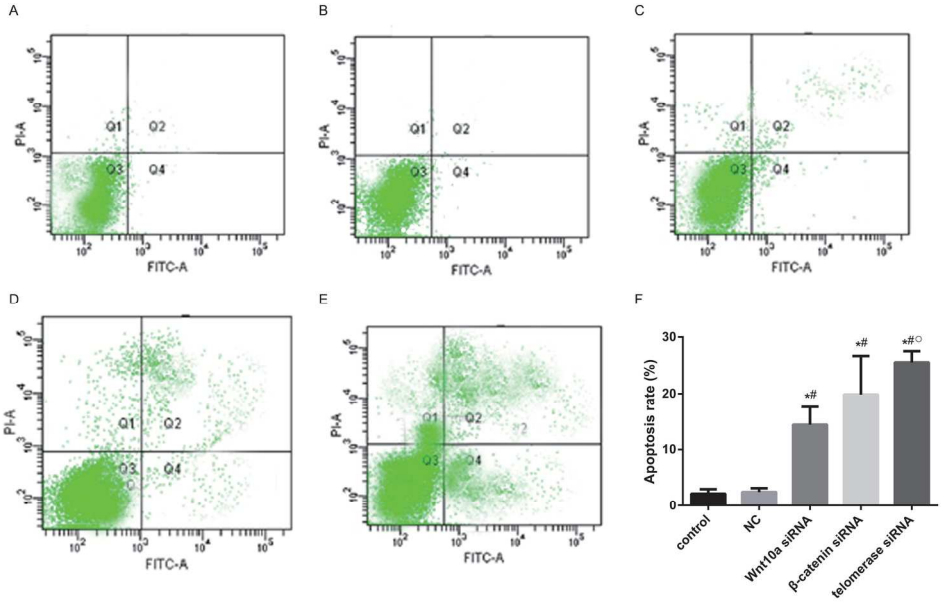

Figure 5
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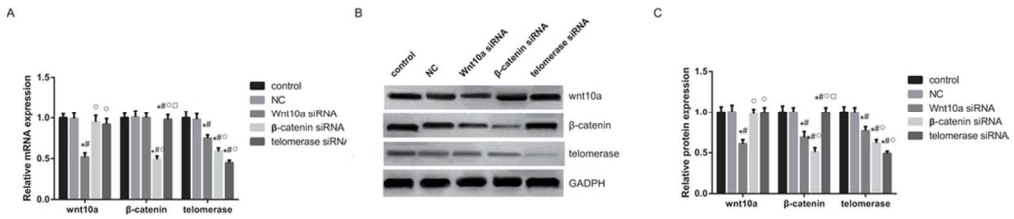

Figure 6
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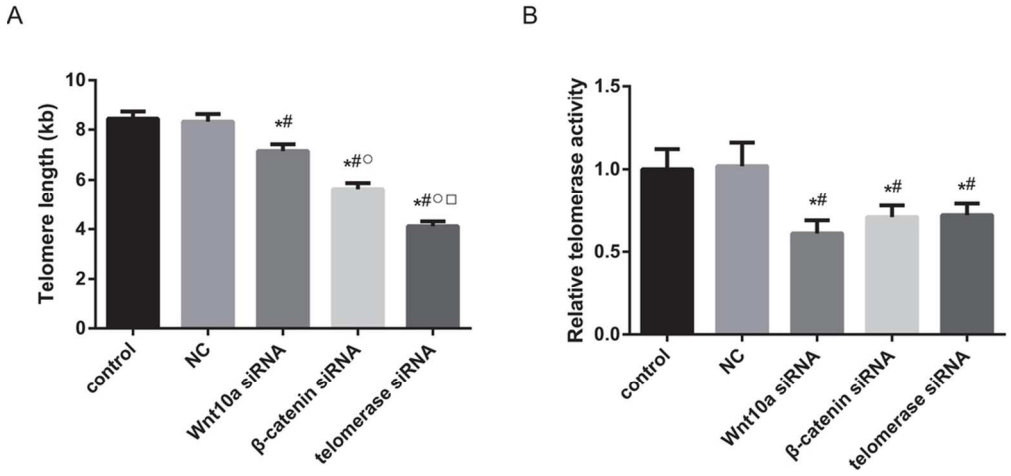

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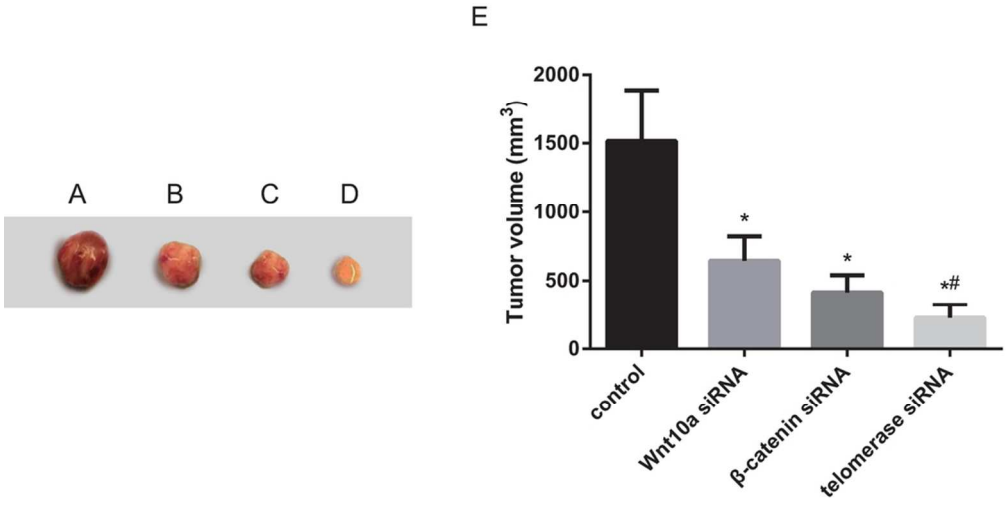

Figure 8
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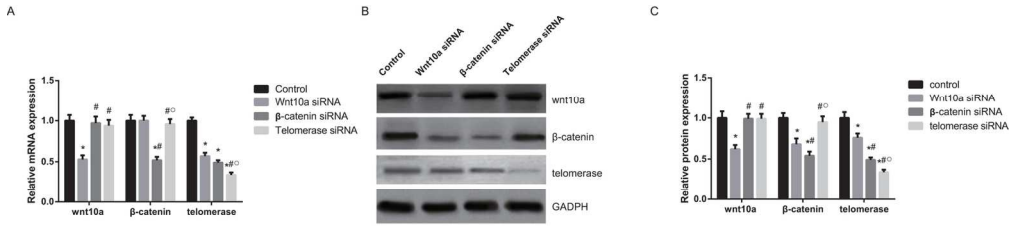

Figure 9
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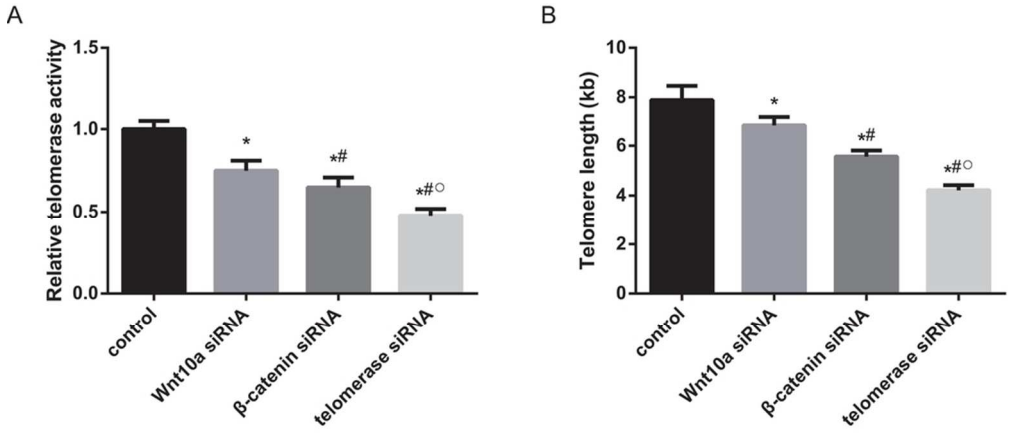

Figure 10
88x38mm (300 x 300 DPI)

Table 1 Clinical data on patients with keloid

No.	Sex	Age (year)	Age of scar (month)	Keloid tissues/ Normal skin					
				β-catenin mRNA	β-catenin protein	Telomerase mRNA	Telomerase protein	Telomere length (kb)	Telomerase activity
1	female	31	10	1.00/0.81	1.00/0.84	0.99/0.68	1.01/0.63	7.77/6.21	0.92/0.75
2	male	29	12	1.01/0.81	1.00/0.84	0.99/0.69	0.97/0.63	7.71/6.22	0.93/0.73
3	male	37	11	1.00/0.81	1.01/0.83	0.99/0.68	0.99/0.61	7.73/6.22	0.94/0.76
4	female	41	10	1.00/0.81	1.01/0.85	1.00/0.70	0.99/0.60	7.75/6.26	1.03/0.75
5	male	25	11	1.00/0.81	1.00/0.84	1.00/0.73	1.03/0.62	7.77/6.24	0.94/0.74
6	male	36	11	1.01/0.81	1.01/0.85	1.00/0.70	1.00/0.61	7.77/6.23	0.97/0.74
7	male	42	12	0.99/0.81	1.00/0.84	1.01/0.70	1.01/0.62	7.76/6.27	0.89/0.73
8	female	52	10	0.99/0.81	1.01/0.86	0.99/0.70	1.02/0.62	7.76/6.22	1.22/0.73
9	female	53	10	0.98/0.81	1.00/0.85	1.01/0.70	0.99/0.63	7.78/6.28	1.19/0.75
10	male	44	11	0.99/0.81	1.00/0.85	0.99/0.70	0.96/0.64	7.77/6.24	0.91/0.74
11	male	49	10	1.01/0.81	0.99/0.87	1.00/0.68	1.04/0.61	7.77/6.21	1.05/0.74
12	female	35	10	1.00/0.81	0.98/0.86	1.01/0.68	1.00/0.64	7.73/6.22	0.94/0.73
13	male	39	12	1.00/0.81	0.97/0.84	1.00/0.70	0.99/0.61	7.79/6.22	1.09/0.73
14	male	58	12	0.99/0.82	0.98/0.87	1.01/0.67	0.97/0.61	7.79/6.22	0.97/0.75
15	female	35	10	1.02/0.81	1.00/0.85	1.01/0.67	0.96/0.61	7.77/6.24	1.02/0.74
16	male	41	12	1.00/0.81	1.00/0.86	1.00/0.68	1.00/0.62	7.79/6.22	0.99/0.73
17	male	28	11	1.01/0.81	1.03/0.85	1.00/0.68	1.08/0.62	7.71/6.21	0.97/0.74
18	male	42	12	0.99/0.80	0.99/0.85	0.99/0.68	1.00/0.62	7.77/6.23	1.04/0.75

Table 2 Sequences of telomerase promoter in luciferase promoter-reporter transient transfection experiments

Gene		WT
TBE3	WT	5'-ATTATTTCAAAACAAAGGTTTACATAAA-3'
	Mut	5'-ATTATTTCAACGCAGAGGTTTACAGAAA-3'
TBE4	WT	5'-GAGTTACCCTCCTTTGATATTTTCTGTA-3'
	Mut	5'-GAGTTACCCTCCTGTGCGATTTTCTGTA-3'

WT: wild type; Mut: mutation.

Table 3 Primer sequences of RT-PCR in ChIP assay

Gene		Primer sequence
Tert -2000	Sense	5'-TGCAAGATGAAGCAAACAGAG-3'
	Antisense	5'-TCTCAGTACCCCTCCCTCAC-3'
Tert -1000	Sense	5'-CCCTATTTCCCAGAGATTCAAA-3'
	Antisense	5'-GGGGCATATGTAATGACACGA-3'
Tert TSS	Sense	5'-ACTTTGGTTGCCCAATGC-3'
	Antisense	5'-AAGGAAAGGTCGGCAGGT-3'
Tert +1000	Sense	5'-CAGGAACTGATGTGGAAGATGA-3'
	Antisense	5'-AGACCAGCCATGCTCACC-3'
Tert +2000	Sense	5'-TGGATAGGAGTTCTGGCACA-3'
	Antisense	5'-CGGGCCCTTACATTAGCTCT-3'

TSS: transcriptional start site.

Table 4 Primer sequences of GADPH, wnt10a, β-catenin and telomerase for implemention of RT-PCR

Gene		Primer sequence
GAPDH	Sense	5'-TGGTATCGTGGAAGGACTCAT-3'
	Antisense	5'-GTGGGTGTCGCTGTTGAAGTC-3'
Wnt10a	Sense	5'-GGGCTCAGGTTCCTACTTCC-3'
	Antisense	5'-AAGGAGAAGCCTCCCAAGAG-3'
β-catenin	Sense	5'-AGCTGACCAGCTCTCTCTTCA-3'
	antisense	5'-CCAATATCAAGTCCAAGATCAGC-3'
Telomerase	Sense	5'-CACGCGAAACCTTCCTC-3'
	antisense	5'-ACCACTGTCTTCCGCAAGTT-3'

GAPDH: glyceraldehyde phosphate dehydrogenase, RT-PCR: real time-polymerase chain reaction