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Original Article

microRNA-196a promotes osteogenic differentiation and inhibit adipogenic differentiation of adipose stem cells via regulating β-catenin pathway

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Abstract: microRNAs play important roles in proliferation and differentiation of stem cells, but mechanisms by which microRNAs regulate osteogenic or adipogenic differentiation of adipose stem cells (ASCs) are still poorly understood. In the present study, results showed up-regulation of microRNA-196a was able to promote the osteogenic differentiation of ACSs, but down-regulation of microRNA-196a induced adipogenic differentiation. Further investigation indicated microRNA-196a could regulate Wnt signaling pathway to affect osteogenic or adipogenic differentiation of ASCs, addition of Wnt agonist 1 was able to reverse the down-regulated osteogenic differentiation of ASCs caused by microRNA-196a deficiency and inhibition of Wnt signaling pathway with XAV939 promoted the adipogenic differentiation of ASCs. Taken together, microRNA-196a may regulate Wnt signaling pathway to promote the osteogenic differentiation and inhibit the adipogenic differentiation of ASCs.

Keywords: microRNA, adipose stem cell, Wnt signaling pathway, osteogenic differentiation

Introduction

Adipose stem cells (ASCs) have multi-directional differentiation potential and can differentiate into adipogenic cells, osteogenic cells, and chondrogenic cells [1]. ASCs secret a large amount of cytokines to promote tissue repair [2, 3], and thus increasing attention has been paid to the usage of ASCs. To date, ASCs have been widely used in the clinical treatments of some diseases including myocardial ischemia [4], hepatic fibrosis [5], arthritis [6], and diabetic foot [7], and they have been one of seed cells with most applicable prospect in regenerative medicine. However, the proliferation and differentiation potentials of ASCs reduce with the passage. The osteogenic differentiation is significantly inhibited, while the adipogenic differentiation remains unchanged. Thus, to deeply investigate the mechanisms by which the osteogenic and adipogenic differentiation of ASCs is regulated is helpful to improve the efficiency of osteogenic differentiation of ASCs, therefore increasing the therapeutic efficacy.

microRNAs are a group of short-chain non-coding RNA that can regulate gene expression [8]. Studies have shown that microRNAs play important roles in the self-renewal and differentiation of adult stem cells. Oskowitz and his colleagues [9] knocked out Dicer and Dorsha (two key enzymes in the maturation of microRNAs) in the bone marrow stromal cells and found the osteogenic and adipogenic differentiation of stromal cells was significantly inhibited. Ting et al [10] found microRNA-27 could inhibit the PHB activity to block the adipogenic differentiation of ASCs. Chen et al [11] confirmed that mciroRNA-375 was able to promote the osteogenic differentiation of ASCs in vivo and in vitro. However, studies reveal microRNA-21, microRNA-30 and microRNA-106 are able to induce the adipogenic differentiation of ASCs. Taken together, microRNAs are crucial for the regulation of osteo-

genic and/or adipogenic differentiation of stem cells.

microRNA-196a is a potential tumor marker [12, 13]. Recent studies indicate that microRNA-196a can facilitate the occurrence and development of cervical cancer [14], head and neck cancer [15], colorectal cancer [16] and NSCLC [17]. In addition, there is evidence showing that microRNA-196a also has multiple bioactivities including enhancement of neuron morphology and neuroprotective effect [18], induction of osteogenesis [19] and regulation of bone density [20]. These findings remind us that microRNA-196a may facilitate the osteogenic differentiation of ASCs. Kim et al also confirmed this hypothesis [21]. It was found the osteogenic and adipogenic differentiation of ASCs determined the fate of ASCs during their development and aging, the up-regulation of osteogenic differentiation of ASCs is inevitably accompanied by the down-regulation of their adipogenic differentiation, and microRNA-196a may regulate the fate of ASCs. In the present study, loss of function and gain of function were employed to investigate the effects of microRNA-196a on the osteogenic and adipogenic differentiation of ASCs. Results showed microRNA-196a could promote the osteogenic differentiation of ASCs, but concomitantly inhibit their adipogenic differentiation. Further investigation revealed microRNA-196a could regulate Wnt/β-catenin signaling pathway to promote the osteogenic differentiation of ASCs and inhibit their adipogenic differentiation.

Materials and methods

Isolation of ADSC and cell culture

All protocols involved in this study were approved by the Ethics Committee of Shanghai Tenth People's Hospital and written informed consent were collected from all the patients. Methods for the isolation of ASCs were established as previously reported [22]. Briefly, adipose tissues were incubated with 0.1% collagenase I for 60 min in 37°C. After neutralization with fetal bovine serum (FBS), the resultant solution was filtered through 100-μm mesh, and ASCs were collected by centrifugation at 1500 rpm 10 min. Then, cell sediment was suspended in DF12 medium supplemented with 10% FBS and 1% penicillin/streptomycin.

Osteogenic induction and quantification

The osteogenic differentiation was performed when ASCs reached 80% confluence. Osteogenic induction medium was used to induce osteogenic differentiation. Induction medium was refreshed once every other day. After 7-day induction, alkaline phosphatase was detected by BCIP/NBT alkaline phosphatase detection Kit. Osteogenic differentiation was completed after 21-days induction and confirmed by alizarin red staining. Alizarin red was dissolved for quantifications previously reported [22].

Adipogenic induction and quantification

The adipogenic induction was performed when ASCs reached 80% confluence. Adipogenic induction medium was utilized to induce adipogenic differentiation. Induction medium was refreshed once every other day. Adipogenic differentiation was completed after 21-day induction and confirmed by oil red staining. Oil red was dissolved for quantification as previously reported [22].

Real-time quantitative PCR

For gene expression detection, total RNA was isolated by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Then, reverse transcription was performed to synthesize the first strand of cDNA. Quantification of targeted gene was performed via real-time quantitative PCR by using SYBR Green PCR Kit. GAPDH and 18S were loaded as internal controls. The primers used in this study are listed in **Table 1**. The gene expression was determined with $2^{-\Delta\Delta Ct}$ method.

Western blotting

Cells were lysed in RIPA buffer supplemented with 1% PMSF. Protein concentration was determined with BCA method. Primary antibodies against Wnt5a, β-catenin, pho-β-catenin and GAPDH (1:1000) were independently incubated with the membrane transferred with proteins at 4°C for 16-h. Then, the membrane was incubated with mouse or rabbit secondary antibodies conjugated with HRP at room temperature for 2 h. The visualization was performed with ECL kit and protein bands were quantified using ImageJ software. The expression of target proteins was normalized to that of GAPDH.

Table 1. Primer used for real-time qPCR

Gene	Sense	Antisense
GAPDH	TGGTACGACAACGAGTTTGGC	GTCTCACCCCATTCTACCGC
PPAR-γ	GCGAACGATTCGACTCAAGC	CATCCCGACAGAAAGGCACT
PGC1-α	CTTTGGAGGCAAGCAAGCAG	GCTTGACTGGGATGACCGAA
ALP	CTCATCTCCTGACCCTCCCA	AGTGAGTGAGTGAGCAAGGC
Runx2	CCACCGAGACCAACAGAGTC	TCACTGTGCTGAAGAGGCTG
OPN	GGAAGTTCTGAGGAAAAGCAGC	TACTTGGAAGGGTCTGTGGGG
RSPO2	CCAAGGCAACCGATGGAGAC	CATACTGGCGCATCCCTTCT
RNF43	ATGGGGCACTTGGTTTGGTT	TCAAGTTGCCAGGCACTGTT
WNT5a	TTCGGCTACAGACCCAGAGA	TGTACCACTACTCAACTGTGGC
LRP5	ACAACATGGAGGCAGCG	GACTCCAGCTTGACTCCGC
LRP6	CCGGTGAGAGAAGAGAACGC	ATCGCAAGTCCCGTCTGTTT
CTNNB1	CTGAGGAGCAGCTTCAGTCC	GGCCATGTCCAACTCCATCA

Immunofluorescent staining

Immunofluorescent staining was performed as previously described [22]. Cells were fixed in 4% PFA, and blocked with 3% BSA in 0.1% PBST (Triton X-100). Incubated with primary antibodies, including anti-PPAR γ (1:1000, abcam, ab-209350), anti-OPN (1:1000, abcam, ab8848), overnight. After three times wash, incubated the cell with secondary antibody anti-Rb cy3. Nuclei were stained with DAPI. Images were captured by Nikon A1R microscope. And ImageJ software was used to analyzed the data.

Use of microRNA-196a mimic and inhibitor

microRNA-196a mimic, microRNA-196a inhibitor, and microRNA-196a negative control were purchased from Ruibo (Guangdong, China). Working solution of microRNA-196a mimic, inhibitor, and negative control were prepared according to the manufacturer's instructions. Then, cells were independently treated with culture medium supplemented with microRNA-196a mimic, inhibitor, and negative control.

Alkaline phosphatase (ALP) staining

At the predesigned time points, cells were processed for ALP staining by using an NBT/BCIP staining kit. The total protein concentration was measured with BCA method. ALP activity was normalized to the total protein concentration.

Microarray and bioinformatics analysis

To explore the whole microRNA expression during osteogenic differentiation and adipogenic differentiation, a microRNA microarray dataset GSE72429 was downloaded. In this dataset, the changes in microRNA expression during osteogenic differentiation and adipogenic differentiation of ASCs were profiled by microarray. Then, 40 genes with most significant changes in osteogenic or adipogenic differentiation of ASCs were identified by Classic Student *t* test.

Statistical analysis

Experiments were repeated three times, and data are expressed as mean ± standard deviation (SD). The statistical analysis was performed with Graphpad Prism 5. A value of $P<0.05$ was considered statistically significant.

Results

microRNA-196a is up-regulated during the osteogenic differentiation

To investigate the microRNA expression profile in ASCs during osteogenic differentiation, microarray GSE72429 data of microRNA during the osteogenic and adipogenic differentiation of ASCs were obtained from NCBI GEO. On the basis of microRNA level in the microarray GSE72429, 40 microRNAs with the most significant changes in their expression during osteogenic differentiation were selected (**Figure 1A**). Results showed the expression of some microRNAs including microRNA-196a increased gradually during the osteogenic differentiation of ASCs. Further real time qPCR was employed for confirmation, and results showed the expression of Runx2, OPN, and ALP, biomarkers of osteogenic differentiation, increased gradually after osteogenic induction, suggesting osteogenic differentiation (**Figure 1B**). During the osteogenic differentiation of ASCs, the microRNA-196a expression increased gradually, and remained at a high level at a specific time point of osteogenic differentiation (**Figure 1B**).

microRNA-196a is down-regulated during adipogenic differentiation

In addition, adipogenic induction was also performed in ASCs, and the microRNA-196a ex-

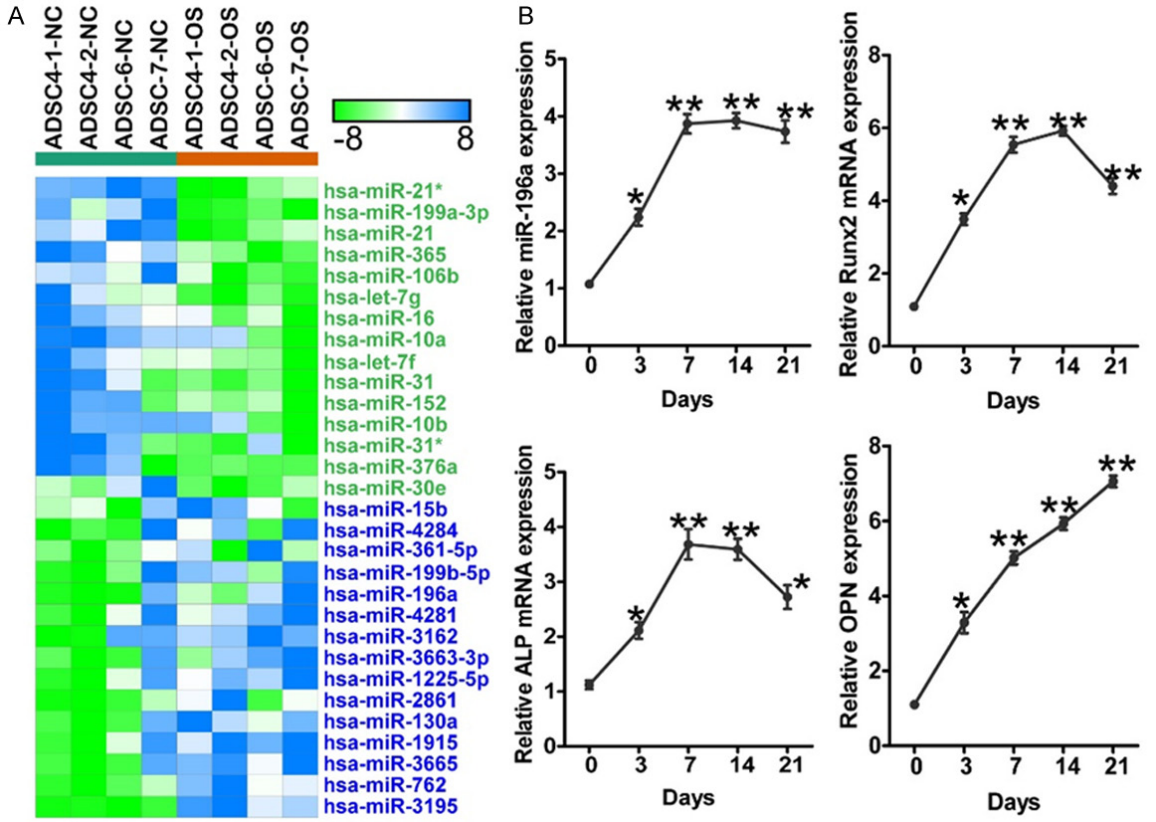

Figure 1. microRNA-196a expression was significantly up-regulated during the osteogenic differentiation of adipose stem cells. A. Microarray showed microRNA-196a expression was up-regulated during the osteogenic differentiation of adipose stem cells. B. Real time qPCR confirmed the expression of microRNA-196a and genes related to osteogenic differentiation was also up-regulated after the osteogenic induction in adipose stem cells (*, $P<0.05$, **, $P<0.01$).

pression was also detected during the adipogenic differentiation of ASCs. Oil red staining (**Figure 2A**) and real-time qPCR of genes related to adipogenic differentiation indicated evident adipogenic differentiation of ASCs after 21-day adipogenic induction. Moreover, the microRNA-196a expression was also detected by real time qPCR, and results confirmed the gradual reduction in microRNA-196a expression during the adipogenic differentiation of ASCs (**Figure 2B**).

microRNA-196a promotes the osteogenic differentiation of hADSC in vitro

To investigate the effect of microRNA-196a on the osteogenic differentiation of ASCs, microRNA-196a mimic was transfected into ASCs to up-regulate microRNA-196a expression. Alizarin red staining and ALP staining showed microRNA-196a significantly promoted the mineral deposition and elevated the ALP activity of ASCs after transfection, suggesting that microRNA-196a is able to promote the osteogenic differentiation of ASCs (**Figure 3A**, *P<0.05). However, after inhibition of micro-RNA-196a, the osteogenic differentiation of ASCs was significantly suppressed, which was characterized by reduced mineral deposition and decreased ALP activity (**Figure 3A**, **P<0.01). Immunofluorescence staining showed microRNA-196a transfection significantly up-regulated the expression of OPN, a key protein involved in the osteogenic differentiation of ASCs, but inhibition of microRNA-196a dramatically inhibited OPN expression (**Figure 3B**). Real time qPCR confirmed microRNA-196a transfection significantly up-regulated the expression of genes related to the osteogenic differentiation of ASCs (**Figure 3C**, *P<0.05, **P<0.01). Taken together, microRNA-196a has the potential to promote the osteogenic differentiation of ASCs.

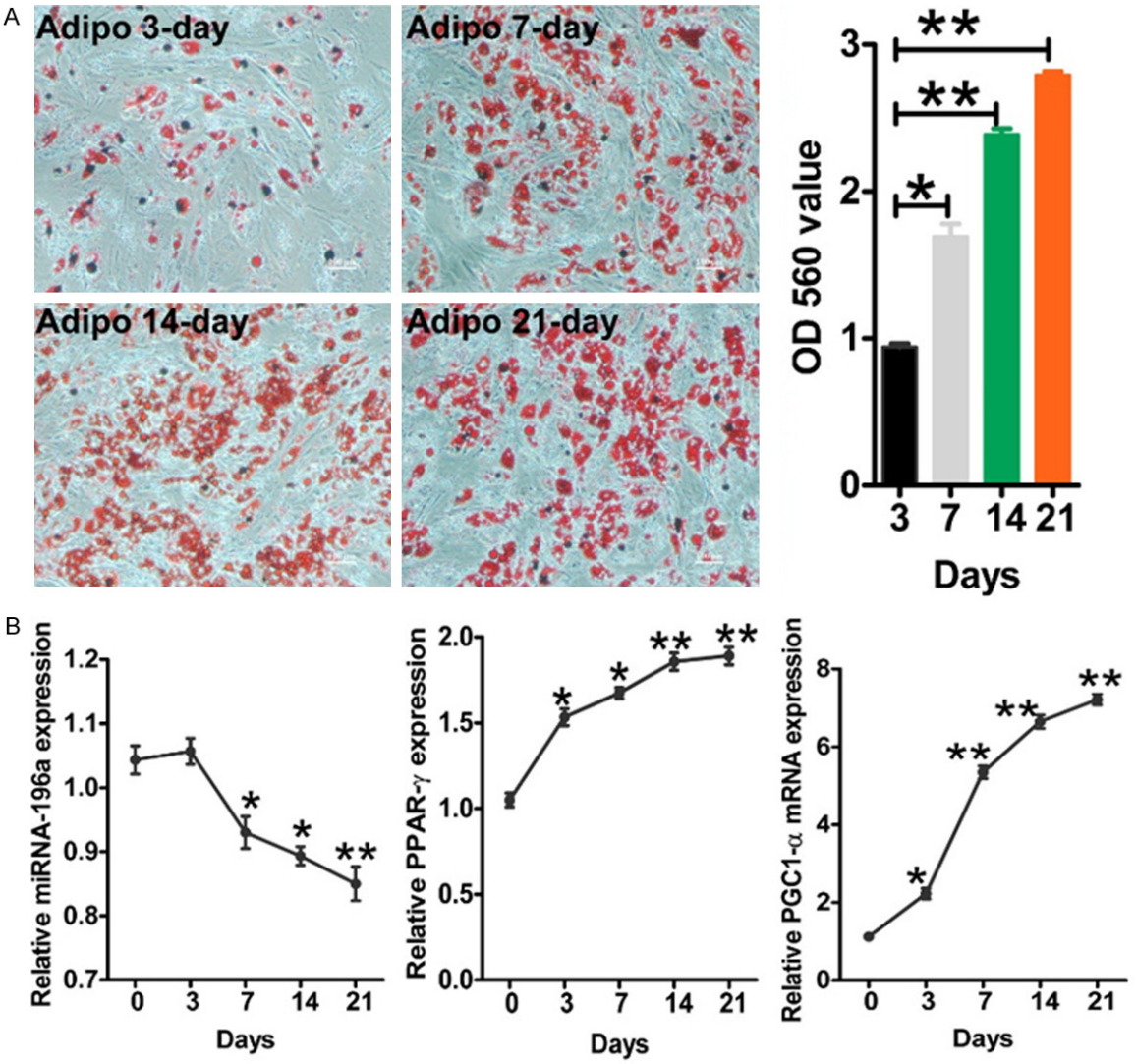

Figure 2. microRNA-196a expression is significantly down-regulated during the adipogenic differentiation of adipose stem cells. A. Adipogenic differentiation process of adipose stem cells. Oil red staining and real-time qPCR of genes related to adipogenic differentiation indicated evident adipogenic differentiation of ASCs after 21-day adipogenic induction. B. Real time qPCR showed microRNA-196a expression reduced gradually during the adipogenic differentiation of adipose stem cells, and the expression of genes related to adipogenic differentiation was significantly up-regulated (*, $P<0.05$, **, $P<0.01$).

microRNA-196a suppresses the adipogenic differentiation of hADSC in vitro

Osteogenic and adipogenic differentiations are two different directions of differentiation. Results showed microRNA-196a could promote the osteogenic differentiation of ASCs and thus it might also inhibit the adipogenic differentiation of ASCs. Oil red O staining showed the adipogenic differentiation of ASCs was inhibited by microRNA-196a, but addition of microRNA-196a inhibitor significantly up-regulated the adipogenic differentiation of these ASCs (**Figure 4A**, *P<0.05). Immunofluorescence staining was employed to detect the expression of PPAR-γ, a key transcription factor in the adipogenic differentiation. Results showed microRNA-196a significantly reduced the number of PPAR-γ positive cells (**Figure 4B**, *P<0.05). Real time qPCR also confirmed microRNA-196a markedly inhibited the expression of PPAR-γ (**Figure 4C**, *P<0.05). Taken together, microRNA-196a has the capability to inhibit the adipogenic differentiation of ASCs.

microRNA-196a regulates adipogenic differentiation through Wnt pathway

Wnt signaling pathway plays an important role in the osteogenic differentiation of ASCs. Thus,

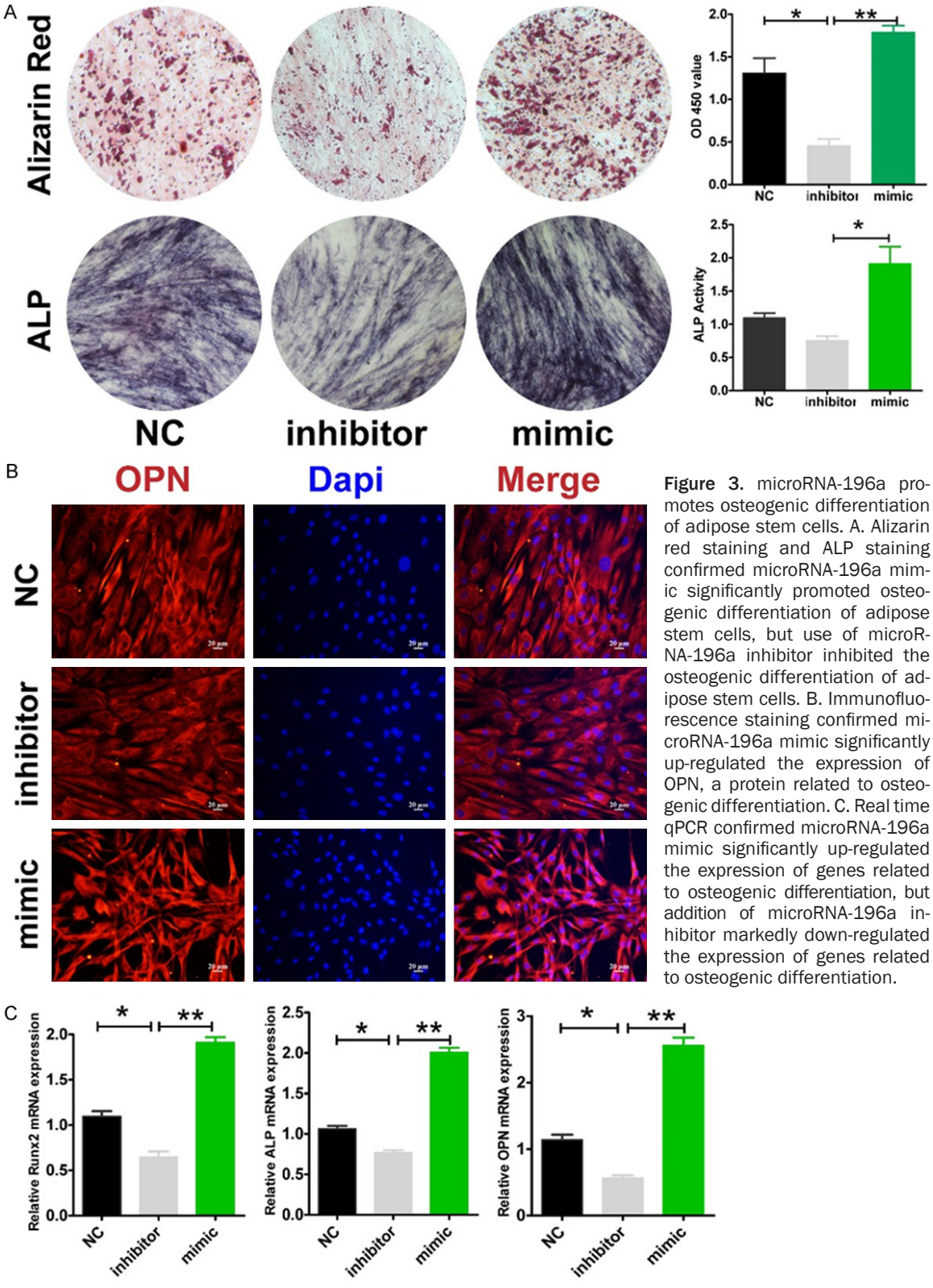

Figure 3. microRNA-196a promotes osteogenic differentiation of adipose stem cells. A. Alizarin red staining and ALP staining confirmed microRNA-196a mimic significantly promoted osteogenic differentiation of adipose stem cells, but use of microRNA-196a inhibitor inhibited the osteogenic differentiation of adipose stem cells. B. Immunofluorescence staining confirmed microRNA-196a mimic significantly up-regulated the expression of OPN, a protein related to osteogenic differentiation. C. Real time qPCR confirmed microRNA-196a mimic significantly up-regulated the expression of genes related to osteogenic differentiation, but addition of microRNA-196a inhibitor markedly down-regulated the expression of genes related to osteogenic differentiation.

it was hypothesized that microRNA-196a could regulate the osteogenic and adipogenic differentiation of ASCs via Wnt signaling pathway. Western blotting showed microRNA-196a significantly up-regulated Wnt5a expression, but inhibition of microRNA-196a markedly reduced

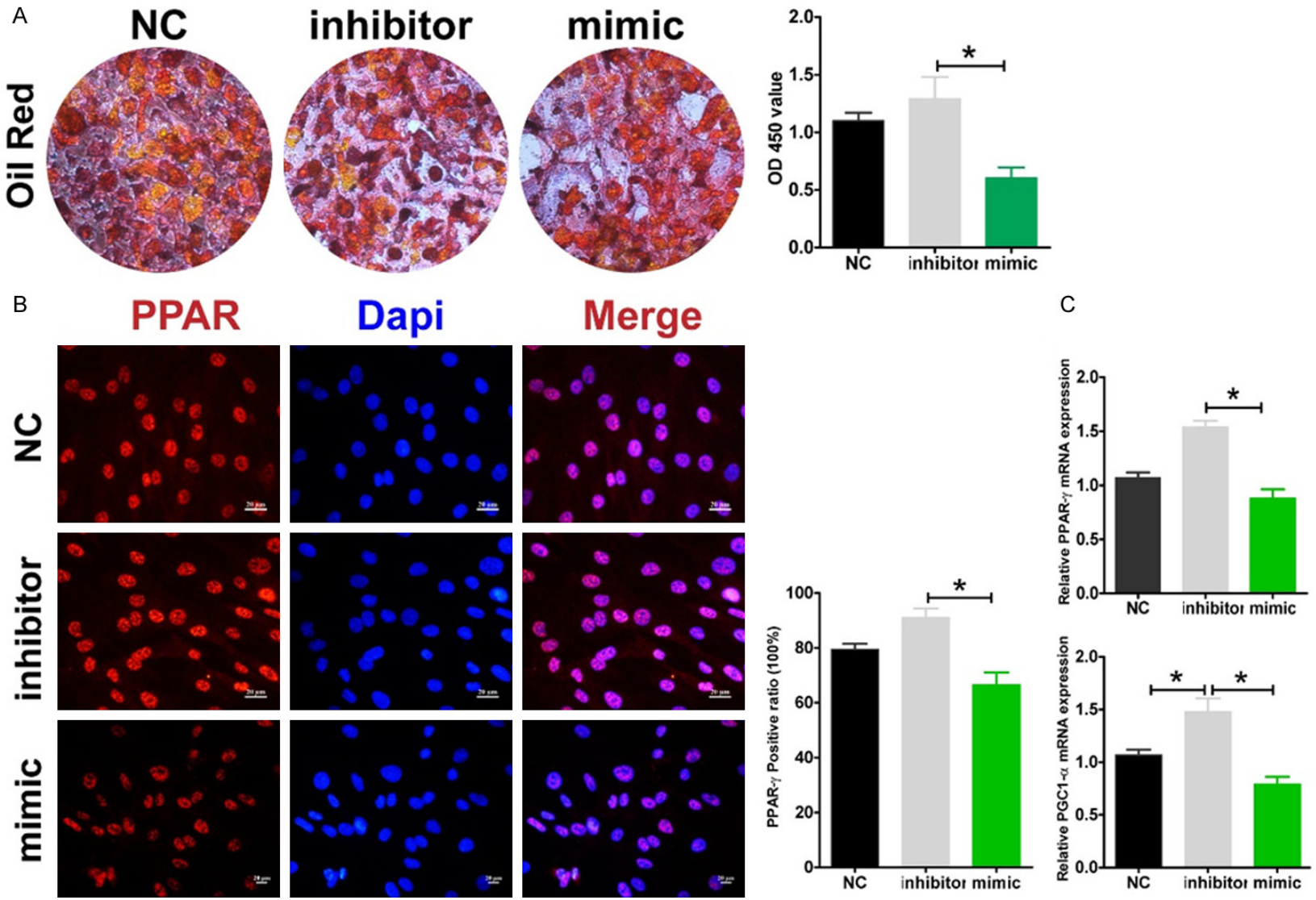

Figure 4. microRNA-196a inhibits the adipogenic differentiation of adipose stem cells. A. Oil red O staining showed microRNA-196a mimic significantly inhibited the adipogenic differentiation of adipose stem cells, but microRNA-196a inhibitor promoted the adipogenic differentiation of adipose stem cells. B. Immunofluorescence staining showed microRNA-196a inhibited the expression of PPAR-γ, the key transcription factor in the adipogenic differentiation. C. Real time qPCR showed microRNA-196a inhibited the expression of genes related to adipogenic differentiation (*P<0.05).

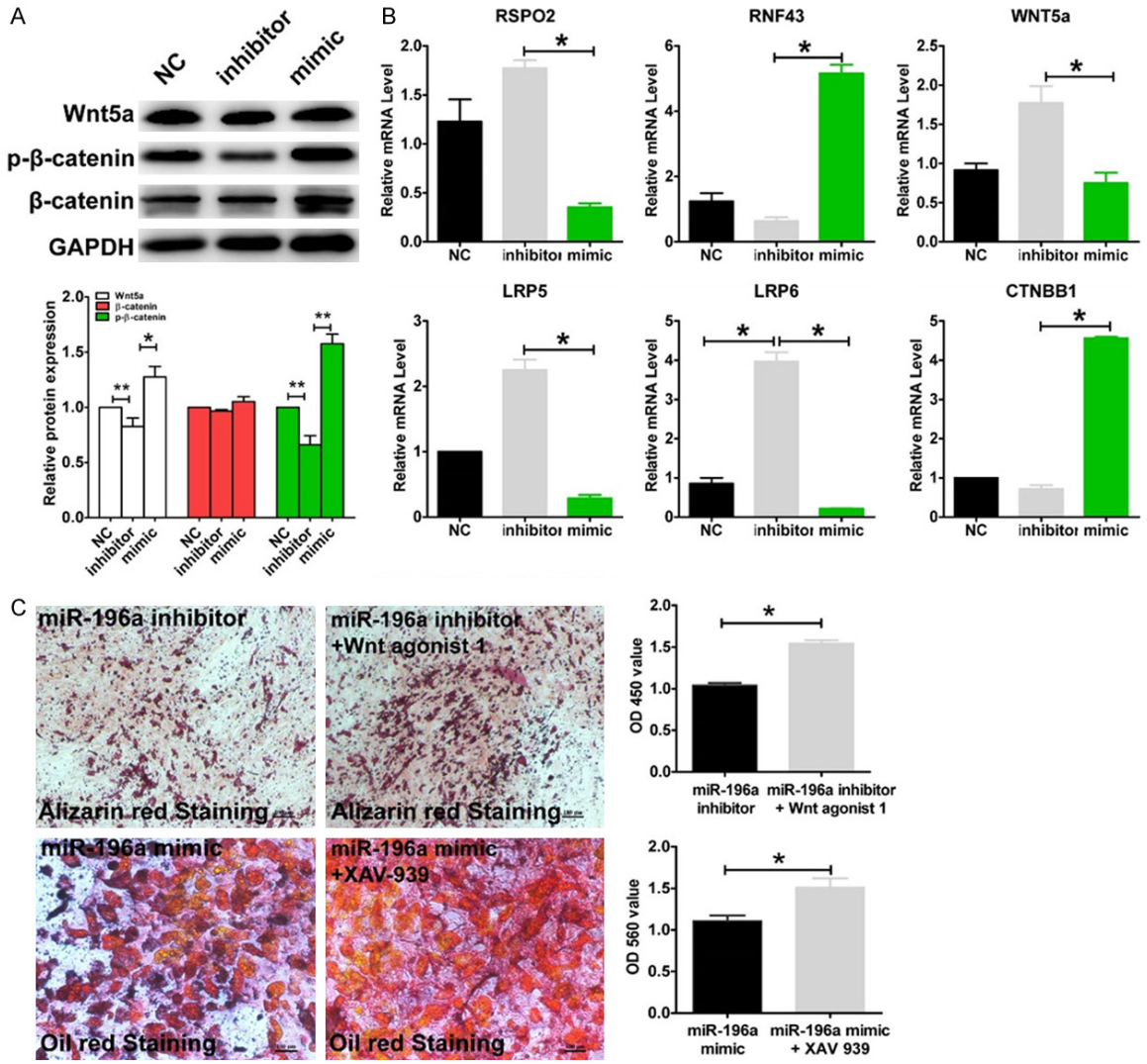

Figure 5. microRNA-196a regulates osteogenic and adipogenic differentiation of adipose stem cells via Wnt signaling pathway. A. microRNA-196a enhanced Wnt5a expression and promoted β-Catenin phosphorylation. B. Real-time qPCR showed microRNA-196a promoted RNF43 and CTNBB1 gene expression, inhibited RSPO2, WNT5a, LRP5 and LRP6 gene expression. C. Wnt agonist 1 was an agonist of Wnt signaling pathway, which could renovate down-regulated osteogenic potency induced by microRNA-196a inhibitor. XAV-939 was an inhibitor of Wnt signaling pathway, which could enhance adipogenic potency of adipose stem cells. (*P<0.05, **P<0.01).

the Wnt5a expression. Furthermore, the expression of downstream molecules of Wnt signaling pathway was also detected, and results showed microRNA-196a was able to up-regulate β-Catenin activity, but inhibition of microRNA-196a markedly suppressed the β-Catenin activity (**Figure 5A**). Meanwhile, real-time qPCR was adopted to detect key gene expression in Wnt pathway (Figure S1). Results showed that microRNA-196a significantly inhibited RSPO2 expression, and promoted RNF43 expression, which further inhibited downstream LRP5/6 expression. microRNA-196a inhibited WNT5a gene expression also (**Figure 5B**). Thus, it was believed that microRNA-196a had the potency of regulating Wnt/β-Catenin pathway. Besides, it was speculated that microRNA-196a regulated osteogenic differentiation of ASCs via Wnt/β-Catenin pathway. As above mentioned, microRNA-196a was able to promote the osteogenic differentiation of ASCs. Thus, the role of microRNA-196a regulated Wnt/β-Catenin in the osteogenic differentiation of ASCs was further investigated, in which microRNA-196a inhibitor was used to inhibit microRNA-196a expression in ASCs, and Wnt pathway specific agonist was employed to activate Wnt signaling pathway. Results showed that osteogenic dif-

ferentiation potency of ASCs was significantly down-regulated after inhibition of microRNA-196a. Wnt pathway agonists could renovate down-regulated osteogenic potency induced by microRNA-196a inhibitor via activating Wnt pathway. Meanwhile, it was believed that microRNA-196a inhibited adipogenic differentiation potency of ACSs via Wnt pathway. Thus, adipogenic differentiation potency of ACSs was significantly upregulated after inhibitor of Wnt pathway was added (**Figure 5C**). Taken together, it was speculated that microRNA-196a may promote the osteogenic differentiation of ASCs and inhibit their adipogenic differentiation via Wnt signaling pathway.

Discussion

ASCs are the important seed cells in regenerative medicine and have following advantages: they can differentiate into different types of adult tissue cells to promote tissue regeneration; they can secrete multiple cytokines to promote tissue repair; they possess the immunoregulative capability and can improve the immunomicroenvironment. Thus, increasing attention has been paid to the ASCs in recent years. Currently, most studies on ASCs focus on the treatment of diseases with ASCs, but the mechanisms by which the fate of ASCs is regulated are poorly understood. In the present study, it was investigated whether microRNA-196a was involved in the osteogenic and adipogenic differentiation of ASCs. Results showed microRNA-196a was able to promote the osteogenic differentiation, but inhibition of microRNA-196a promoted the adipogenic differentiation of ASCs. These findings suggest that microRNA-196a play important roles in the regulation of osteogenic and adipogenic differentiation of ASCs.

microRNA-196a has been found to be involved in multiple processes, including cell proliferation, but little is known about the role of microRNA-196a in the osteogenic differentiation of stem cells. In the present study, results showed microRNA-196a expression was significantly up-regulated during the osteogenic differentiation, but markedly down-regulated during the adipogenic differentiation. Moreover, over-expression of microRNA-196a was found to promote the osteogenic differentiation of ASCs and inhibit their adipogenic differentiation. These findings were inconsistent with previously reported that microRNA-196a had no influence on the adipogenic differentiation of ASCs [21]. This discrepancy might be related to the difference in the medium for adipogenic induction. In the present study, the medium used for adipogenic induction undergoes optimization for several years and insulin is added to the medium, which significantly improve the efficiency of adipogenic differentiation of ASCs and therefore is helpful for the assessment of role of microRNA-196a in adipogenic differentiation.

To date, more than 29000 articles have been published on β-catenin. β-catenin signaling pathway is very complex. Studies have shown that more than 500 genes have interactions with β-catenin [23]. β-catenin is a transcription factor in the Wnt signaling pathway and play key roles in the differentiation and proliferation of stem cells [23]. Yao et al [24] found microRNA-185 could target PTH to down-regulate β-Catenin pathway, which inhibited the proliferation and growth of osteoblasts during the bone repair. Abnormal β-Catenin signaling pathway may cause hyperplasia, resulting in tumorogenesis. There is evidence showing that elevated expression of microRNA-26a [25], microRNA-374a [26] and microRNA-155 may cause over-activation of β-Catenin signaling pathway, promoting the tumorogenesis. Results showed microRNA-196a was also able to regulate β-Catenin signaling pathway. Western blotting and loss/gain of function assay of Wnt pathway confirmed up-regulated expression of microRNA-196a was able to up-regulated the activity of Wnt signaling pathway, which promoted the osteogenic differentiation of ASCs and inhibited their adipogenic differentiation. To date, no study has been conducted to investigate the effect of microRNA-196a on the Wnt signaling pathway during the differentiation of stem cells.

Taken together, results indicate microRNA-196a has the potential to promote the osteogenic differentiation of ASCs, but inhibit their adipogenic differentiation, via activating β-Catenin signaling pathway.

Disclosure of conflict of interest

None.

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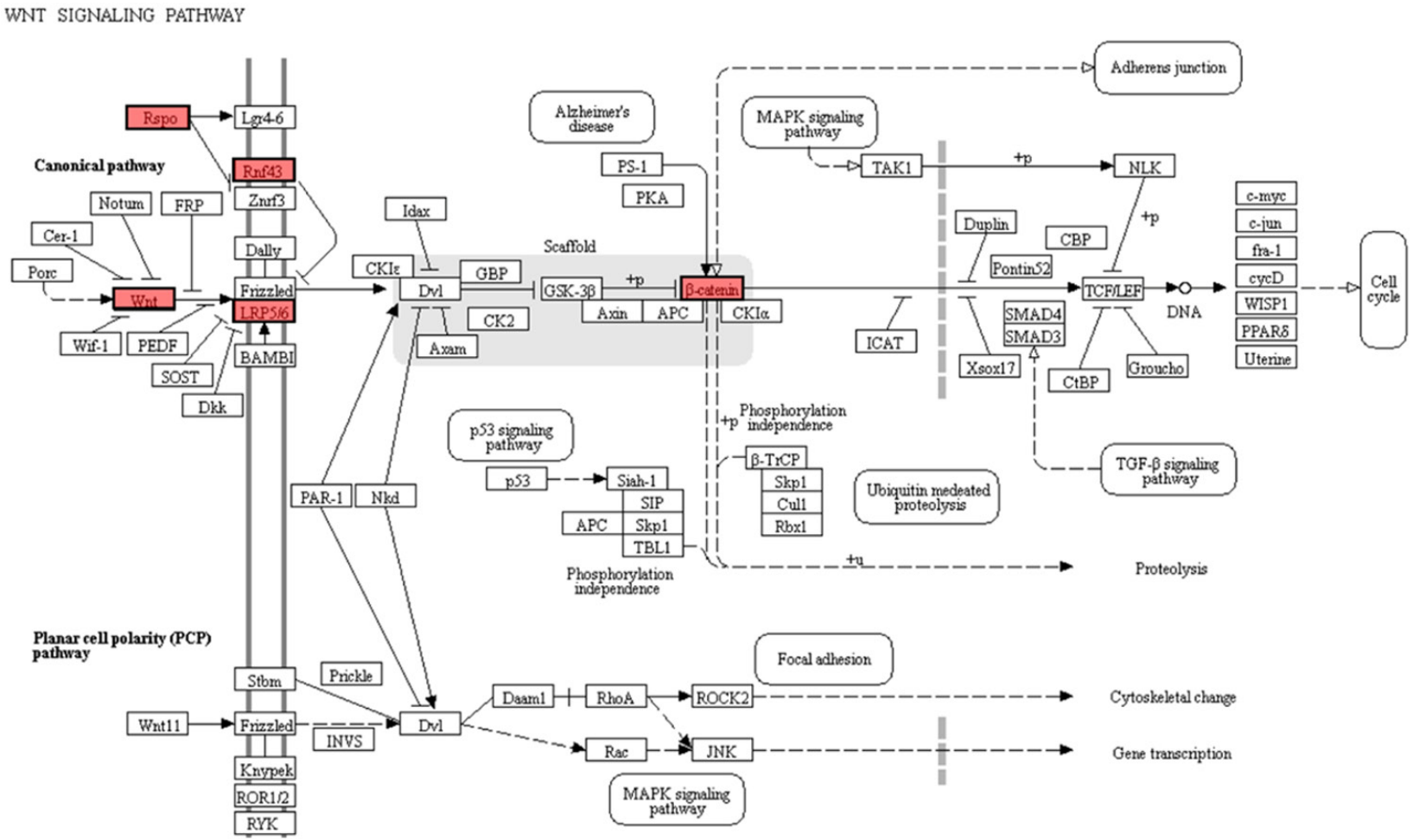

Figure S1. Real-time qPCR was adopted to detect key gene expression in Wnt pathway.