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Original Article

DKK1 overexpression promotes the proliferation and cartilage differentiation of mesenchymal stem cells (MSCs) of antler

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Abstract: Wnt signaling plays multiple roles in mammalian mesenchymal stem cells (MSCs). This study aimed to examine the effect of Wnt signaling on the proliferation and differentiation of MSCs of antler. Antlers were harvested at 60 days of growth and MSCs were isolated. An adenovirus for DKK1 overexpression was constructed. Cartilage differentiation was evaluated using Alcian blue, alizarin red, and immunohistochemistry. MSCs were evaluated by qRT-PCR for DKK1, collagen I, collagen II, β-catenin, cyclinD1, c-Myc, and Runx2. On days 3 and 6, Alcian blue staining in Ad-DKK1 cells was positive, but disappeared from day 9. On day 9, collagen II immunohistochemistry was weakly positive. On day 11, alizarin red staining showed positive stain. On day 11, the expression of collagen I in the Ad-DKKI group was higher than that in the empty adenovirus group, and was higher than that on days 6 and 9 (all $P<0.05$); the expression of collagen II on day 11 was significantly higher (all $P<0.05$). The expression of the DKK1 gene was increased from day 9 ($P<0.05$ *vs.* day 6). The expression of the β-catenin gene was decreased from day 9 ($P<0.05$). The expression of the CyclinD1 gene was also declined on day 11 ($P<0.05$). The expression of the Runx2 gene was higher from day 6 (all $P<0.05$). Wnt signaling is involved in the proliferation and cartilage differentiation of MSCs of antler. These results provide a theoretical basis for the growth and regeneration of antler.

Keywords: Wnt signaling, mesenchymal stem cells, antler, proliferation, differentiation, cartilage

Introduction

Tarim Wapitis is a unique animal that inhbits exclusively in Tarim Basin. The antlers of Tarim Wapitis can regenerate with an astonishing growth rate even faster than that of tumor tissues. And this regeneration is tightly controlled [1-3]. Therefore, the regeneration of Tarim Wapitis' antlers represents an ideal model for deciphering the underline mechanism of the regeneration and remodeling of mammalian bone tissues.

Antler is currently the only mammalian organ that can regenerate and its growth, development, and regeneration is a subject of research [4]. The growth and development of antler are accomplished through intramembranous ossification and endochondral ossification, which are both based on stem cells [5]. Cartilage differentiation of antler is used as a study model of the development of human cartilage. Studies have found that mesenchymal stem cells (MSCs) of antler are from the periosteal stem cells of deer spine [5, 6]. The growth of these MSCs form fibroblasts colonies that differentiate into cartilage cells to form osteoblasts [5, 7]. Therefore, MSCs are one of the sources that maintains the growth and development of antler [5, 7], but the exact regulation of MSCs during antler development remains unclear.

The thickness of the mesenchymal cell layer is changing at different grow stages of MSCs of antler, which experiences hyperblastosis and then gradually thins from the early growth stage to the peak growth stage as well as the late growth stage (the ossification stage). Through *in vitro* experiments, MSCs of antler have been proved to have the abilities of differentiating into osteoblasts, adipocytes, and chondrocytes [6]. The stem cells of antler cannot be separat-

ed from their microenvironment (niche) for the growth and development of antler, and many signaling pathway play important role in the proliferation and differentiation of the stem cells, including Wnt signaling [4, 8]. It has been found that the Wnt signaling pathway can regulate the growth and differentiation of MSCs in mammalians through self-renewal and differentiation [9].

Specific inhibitors of Wnt signaling was used to inhibit Wnt signaling in human bone marrow MSCs and found that downstream proliferation-related gene expression can be increased and cell proliferation can be promoted [10]. Overexpression of the Wnt3a gene in pancreatic stem cells showed that the expression of cell proliferation-related genes such as c-Myc can be significantly upregulated, which promoted the proliferation of pancreatic stem cells and delayed cell senescence [11]. In addition, a number of studies confirmed that the Wnt/β-catenin classical pathway was involved in the regulation of the bone differentiation of pluripotent stem cells and human MSCs [12-15]. It was observed that proteins of the β-catenin/Wnt signaling pathway were expressed in MSCs of antler, while it was lowly expressed in cartilage and osteoblasts by Immunohistochemistry [8]. They used epigallocatechin-3-gallate (EGCG) to block Wnt signaling and found that the number of cells was decreased while the activity of alkaline phosphatase (ALP) was increased. On the other hand, lithium chloride (LiCl) was used to activate Wnt signaling and showed that the number of cells was increased but ALP activity was decreased [8]. It has been preliminarily considered that the β-catenin gene plays a key role in the growth and bone formation of antler [8].

The Dickkopf-related protein 1 (DKK1) was first isolated in 1999 and it was found to be located upstream of the Wnt/β-catenin signaling pathway [16]. DKK1 can inhibit Wnt signaling through combining Wnt and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) [16, 17]. Nevertheless, the role of DKK1 in antler development is unknown.

Therefore, the aim of the present study was to examine the proliferation and differentiation of MSCs of antler after inhibiting Wnt signaling, as well as the regulatory mechanisms of the Wnt signaling pathway on the proliferation and cartilage differentiation of MSCs of antler.

Materials and methods

Animals

Two Tarim Wapitis aged 3-4 years were selected at the Experimental Station of Tarim University (Xinjiang, China). At 60 days, of the growth period of the antler, the Wapitis were anesthetized using an air gun dart loaded with xylazine and dihydroetrphine at 6-7 O'clock in the morning, when the weather was cool. The stag was placed under general anesthesia (the recommend dose for anesthesia of Tarim Wapitis is 0.01-0.015 ml per kilogram of body weight) with an intramuscular injection of an aqueous mixture of 2 mL of xylazine and dihydroetrphine (0.1 g/ml xylazine and 40 ug/ml dihydroetrphine. Quartermaster University of PLA, Changchun, China). After the animals were anesthetized, the base of the antler was tied tightly with a grass rope to avoid excessive bleeding. Antlers were cut off using a hand saw. Then the styptic powder (Quartermaster University of PLA, Changchun, China) is applied immediately on the wound to stop bleeding and promote the healing [18, 19]. Contamination on the tissue surface was quickly cleaned. Tissues that were 5 cm from the top of the antler were sampled using a scalpel, and longitudinally dissected; then, mesenchymal cells were separated under a dissecting microscope, according to the literature [20, 21]. The transition part between the antler cortex and the chondrocyte layer was cut off as much as possible. The experiments were approved by the Ethics Committee of Tarim University (Xinjiang, China).

Culture of primary cells from the antlers

Antler MSCs were isolated as previously described [6]. MSCs from antler were washed with PBS thrice and placed in a sterile 90-cm^2 culture dish. The MSCs were washed with D-Hanks containing 400 IU/mL penicillin-streptomycin. The sample was cut into 1-mm^2 pieces and transferred into a 10-mL culture flask. Trypsin (0.25%, 7 mL; Sigma, St Louis, MO, USA) was added and the cells were placed in a CO_2 incubator at 37°C for 1 h. Then, 5 mL of 0.1% type II collagenase (Sigma, St Louis, MO, USA) was added and incubated for 5 h. The supernatant was transferred to a 10-mL centrifuge tube and 3 mL of DMEM medium (Sigma, St Louis, MO, USA) containing 10% FBS (Sigma, St Louis, MO, USA) was used to wash the tissue blocks, followed by centrifugation at 800 rpm

Table 1. PCR primers and reaction conditions for quantitative detection of gene expression

Genes	Primer sequence (5'->3')	Annealing temperature (°C)	Amplified fragment (bp)
DKK1	F: CGGGGTTGTGTTGTGCTAGA R: GTTGGGTAACGCCAGGGTTT	60	315
DKK1 ORF	F: ataggatccgccaccATGACGGCTCTGGGCACAGCG R: atagaattcGTGTCTCTGACAGGTGTGAA	60	819
COL I	F: CTGACTTTCTGCGCCTGAT R: AGCGGAGTTCTTTGGTGGTT	60	261
COL II	F: GGTGAGCTATGATCCGCCT R: CTGGGTCCTTGTTCACCTGC	65	202
β-catenin	F: TGCTCAGGACAAGGAAGCTG R: AGCAGTCTCATTCCAAGCCA	60	220
CyclinD1	F: GCGCAGACCTTCGTTGCCCT R: GCCGTTGGCGCTTCCCAGAT	60	228
c-Myc	F: CAAATGTGCCAGCCCAAGGTTTTC R: CTCTGGGATCTGGTCACGAAGAGCA	63	130
Runx2	F: TCAGAACCCACGGCCCTCCC R: GACAGCGGCGTGGTGGAGTG	65	186
GAPDH	F: GGTGCTGAGTATGTGGTGGA R: GGCATTGCTGACAATCTTGA	63	180

F: Forward; R: Reverse.

for 5 min; the supernatant was discarded. Complete culture medium (5 mL) was added and incubated at 37°C under 5% CO_2. After the tissue blocks were attached to the wall, the flask was gently flipped over, and the tissue blocks were soaked in the complete culture medium. The medium culture was replaced every 2-3 days. When a large number of cells were observed under an inverted microscope to have migrated from the edge of the tissue block and proliferated, the tissue blocks were gently removed.

Cell culture

Passage was performed at 70% confluence. D-Hanks solution was used to wash thrice the cells that were attached to the wall. Trypsin (0.25%) was added to detach the cells. The cells were collected in a 10-mL centrifuge tube and centrifuged at 800 rpm for 5 min. The supernatant was discarded. The cell density was adjusted to 1×10^5 cells/mL with complete culture medium. The cells (5 mL) were inoculated in a flask, and incubated. The medium was completely replaced after 1 day, and then every 3 days.

DKK1 overexpressing adenovirus

The bovine DKK1 gene sequence was obtained from Genbank (accession number NM_001205544.1). PCR primers in the ORF region were designed using the Primer 5.0 software. The restriction endonucleases BamHI and EcoRase (Takara Bio, Otsu, Japan) were used to introduce restriction sites at the two ends of the primers. cDNA from antler was used as the template to amplify the DKK1 gene. The reaction conditions are shown in **Table 1**. The purified DKK1 PCR products were ligated to the pMD18-T vector (Takara Bio, Otsu, Japan). The vectors were transformed into DH5a bacteria. After the plasmids were extracted, they were sent to Sangon Biotech (Shanghai, China) to be sequenced. After analyzing the sequencing results, the recombinant plasmid pMD18-T-DKK1 and the vector pHBAd-MCMV-GFP (Hanbio Biotechnology Co., Ltd., Shanghai, China) were digested. The recovered digested DKK1 segments were purified to be ligated to the digested vector pHBAd-MCMV-GFP. The DKK1 recombinant plasmids were sent to Sangon Biotech (Shanghai, China) to be sequenced. After analyzing and determining the sequencing results, the DKK1 recombinant shuttle plasmid pHBAd-BHG (Hanbio Biotechnology Co., Ltd,. Shanghai, China) was used to transfect the 293A cell line no containing the E1A region of adenovirus, and was routinely cultured in DMEM 90% medium with 10% FBS. All the above-mentioned cell lines were kept at 37°C in a humidified atmosphere of 5% CO_2. Finally, DKK1 overexpressing adenovirus was obtained.

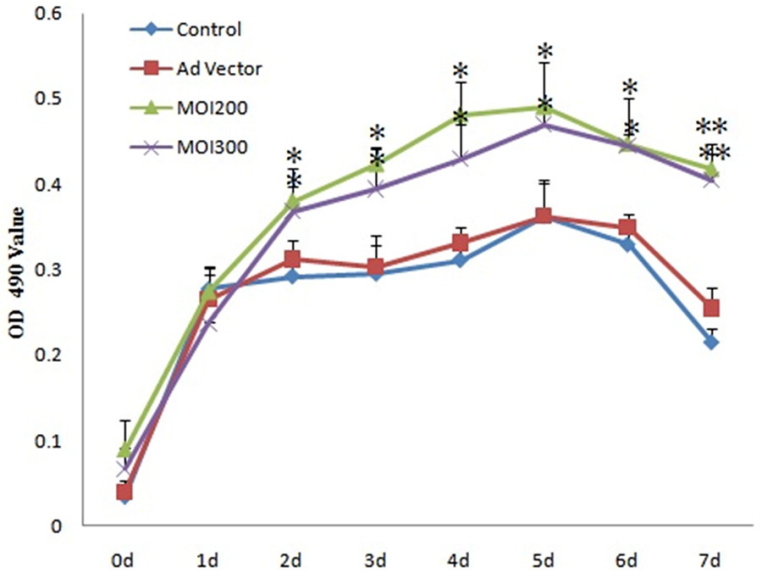

Figure 1. Cell proliferation curve (MTT assay) of antler mesenchymal stem cells (MSCs) infected with a DKK1 overexpressing adenovirus. Cells were treated with no adenovirus (control), empty adenovirus (Ad vector), with DKK1 adenovirus at multiplicity of infection (MOI) of 200, and with DKK1 adenovirus at MOI 300. *p<0.05 *vs*. control at the same time point. **P<0.01 *vs*. control at the same time point.

Self-proliferating characteristics

Four 24-well plates were used; 84 wells were divided into four groups (n=21/group): the adenovirus transduction group with multiplicity of infection (MOI) of 200 (2.0×10^2 PFU/cell); adenovirus transduction group with MOI of 300 (3.0×10^2 PFU/cell); empty adenovirus transduction group (2.0×10^2 PFU/cell); and the blank group. Each well was seeded with about 5.0×10^4 cells, and 0.5 mL of complete culture medium was added and incubated at 37°C under 5% CO_2. Half of the medium was replaced every 2 days. When cells were 50-60% confluent, the experimental groups were infected with DKK1 overexpressing adenoviruses and empty adenoviruses. Three wells were randomly selected from each group every day. MTT solution (20 μL) was added and cultured for 4 h. DMSO (80 μL) was added and the absorbance was read at 490 nm. The cell growth curve was plotted.

Cartilage differentiation

Four 6-well plates were used and the 24 wells were divided into the transduction group and the empty adenovirus transduction group (n=12/group). Each well was seeded with 1.0×10^5 cells, 1 mL of complete culture medium was added, and the plates were incubated at 37°C under 5% CO_2. Half of the medium was replaced every 2 days. When the cells were 50-60% confluent, each well of the transduction group was added with DKK1 overexpressing adenovirus at MOI of 200. At days 3, 6, 9, and 11, cells from three wells were collected from each group and the culture medium was removed. After washing with 500 μL of PBS, the cells were fixed with 4% paraformaldehyde phosphate buffer (PBS, 0.1 M, pH 7.4) overnight at 4°C, followed by Alcian blue staining, alizarin red staining, and immunohistochemistry.

For immunohistochemistry, the fixed cells were washed three times with PBS. The Eradication Staining Kit and DAB Chromatography Kit (Boster Bioengineering Co., Wuhan, China) were used according to the manufacturer's instructions.

Quantitative PCR

Nine 6-well plates were used and the 54 wells were divided into the transduction group, the empty adenovirus transduction group, and the blank group (n=18/group). Each well was seeded with 1.0×10^5 cells; 1 mL of complete culture medium was added and incubated at 37°C under 5% CO_2. Half of the culture medium was replaced every 2 days. When the cells were 50-60% confluent, each well in the transduction group was added with DKK1 overexpressing adenovirus at MOI of 200. After 3, 6, 9, and 11 days, the cells from three wells were harvested from the three groups. RNA was extracted using TRIZOL (1 mL; Invitrogen Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was diluted at 1:10. Based on the Genebank database, Primer 5.0 software was used to design PCR primers (**Table 1**). The reaction conditions of qPCR included 95°C for 5 min; and 40 cycles at 95°C for 10 s and 60°C for 30 s. The melting curve was 55-95°C. Semi-quantitative PCR

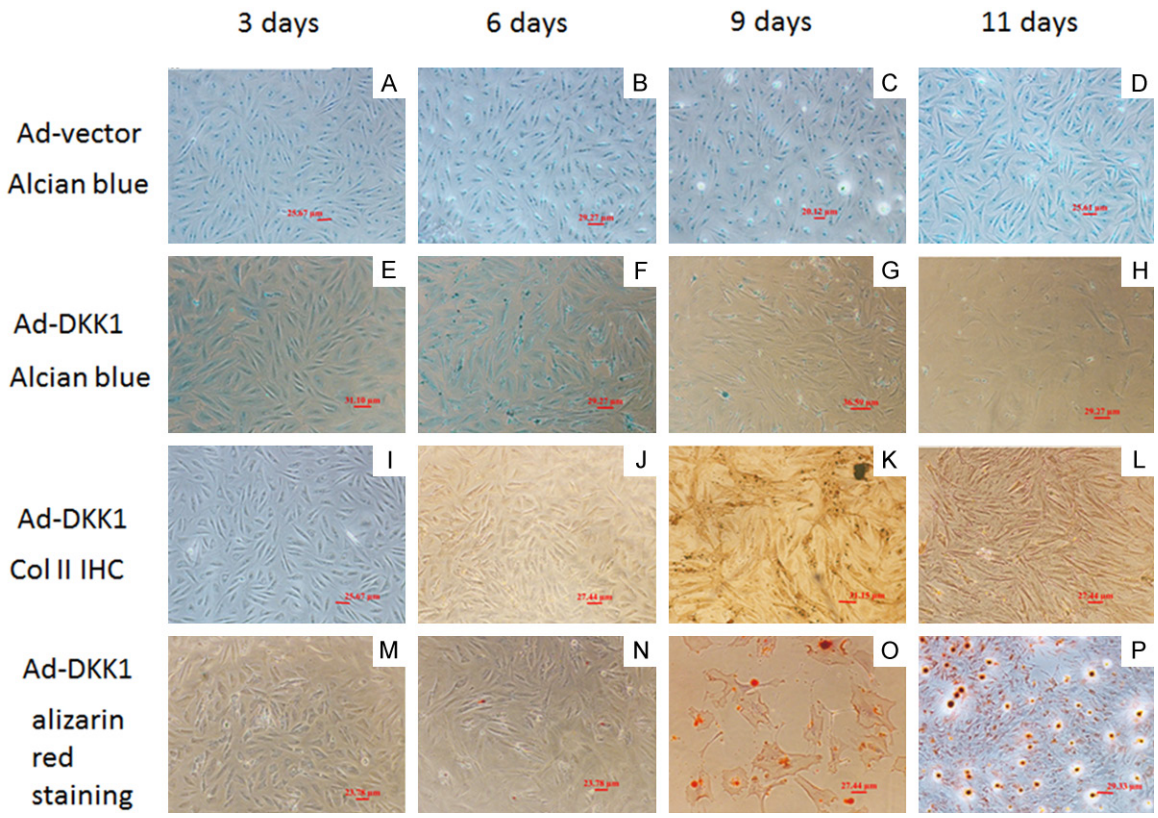

Figure 2. The effect of DKK1 overexpression on the self-differentiation of antler mesenchymal stem cells (MSCs) (magnification 100×). A-D. Alcian blue staining of the empty adenovirus group at 3, 6, 9, and 11 days. There was no obvious change in cell morphology over time. E-H. Alcian blue staining in the Ad-DKK1 transduction group. Cell morphology on day 3 began to change from a spindle to a triangle, and there was light blue positive staining. Staining was significant on day 6, but gradually disappeared from day 9. I-L. Collagen II immunohistochemistry in the Ad-DKK1 transduction group. The staining was weakly positive on day 9 and stronger on day 11. M-P. Alizarin red staining in the Ad-DKKI transduction group. There were spot-like staining on days 9 and 11, indicating Ca^{2+} deposition.

reaction conditions included 95°C for 5 min; and 28 cycles at 95°C for 10 s; 65°C for 30 s, and 72°C for 2 min. The relative expression of proliferation- and differentiation-related genes of MSCs of antler (**Table 1**) was examined. Relative expression was evaluated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Differences among groups were evaluated using ANOVA with the LSD post hoc test. SPSS 17.0 (IBM, Armonk, NY, USA) was used for data analysis. Two-sided *P*-values <0.05 were considered statistically significant.

Results

Effect of Ad-DKKI adenovirus on the proliferation of MSCs from antler

MSCs of antler were transfected with Ad-GFP adenovirus with MOI values of 200 and 300. The cells grew continuously from day 1 of transduction in the blank, empty adenovirus, and treatment groups. Cell proliferation reached a peak on day 5 and gradually decreased thereafter. From days 0 to 7, there were no significant differences in MTT value between the blank and empty adenovirus groups, while the MTT values in the group 200 and 300 MOI were significantly higher than that in the blank and empty adenovirus groups from day 2 of transduction (P<0.05) (**Figure 1**). When the cell proliferation started to decrease (on day 7), the MTT values in the two transduction groups were still significantly higher than in the blank and empty adenovirus groups (P<0.001) (**Figure 1**).

Effects of transfected Ad-DKK1 adenovirus on cartilage differentiation

On days 3, 6, 9, and 11 after antler MSCs were transfected with Ad-DKK1, Alcian blue staining,

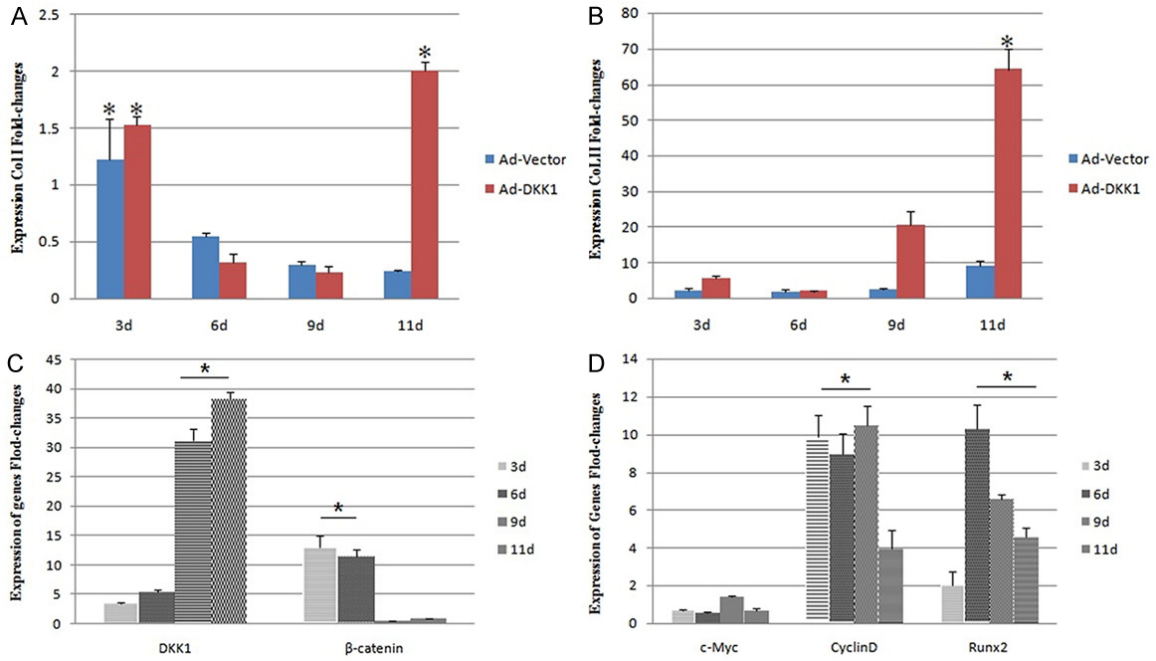

Figure 3. The expression of collagen I (A) and collagen II (B) genes after antler mesenchymal stem cells (MSCs) were transfected with DKK1 adenovirus. There was no significant difference in collagen I gene on day 3 in the empty adenovirus and transduction groups, but the expression in the Ad-DKK1 group on day 11 was significantly higher than in the empty vector group and at other time points (all $P<0.05$). The expression of the collagen II gene on day 11 was significantly higher than in the empty adenovirus group and the transduction group at other time points ($P<0.05$). *$P<0.05$ *vs*. the average value. (C) Expression changes of the DKK1 and β-catenin genes of antler MSCs after transduction with an adenovirus overexpressing DKK1. *$P<0.05$. (D) Expression changes of the genes of antler MSCs downstream of the Wnt signal after transduction with an adenovirus overexpressing DKK1. *$P<0.05$.

alizarin red staining, and collagen II immunohistochemistry were used to detect the differentiation of MSCs. On day 3, Alcian blue staining in Ad-DKK1 cells was weakly positive (**Figure 2E**); the staining was more obvious on day 6 (**Figure 2F**), but disappeared from day 9, indicating that proteoglycans were no longer secreted (**Figure 2G** and **2H**). On day 9, collagen II immunohistochemistry was weakly positive (**Figure 2K** and **2L**). On day 11, alizarin red staining showed that there was significant Ca^{2+} accumulation (**Figure 2P**).

Collagen I and collagen II expression after DKK1 overexpression in antler MSCs

The expression of collagen I and collagen II genes detected by qRT-PCR showed that the expression of the collagen I gene was high on day 3, but without significant difference between the Ad-DKK1 and the empty adenovirus groups. The expression was decreased significantly on days 6 and 9. On day 11, the expression in the Ad-DKK1 group was significantly higher than in the empty adenovirus group, and was also significantly higher than the expression on days 6 and 9 (all $P<0.05$) (**Figure 3A**). The expression of the cartilage surface marker collagen II on day 11 was significantly higher than the expression in each group at all time points (all $P<0.05$) (**Figure 3B**).

Self-proliferation of antler MSCs and expression of downstream genes of the Wnt pathway after transduction with Ad-DKK1

Quantitative PCR and western blot were used to detect the expressions of DKK1, β-catenin, c-Myc, CyclinD1, and Runx2 on days 6, 9 and 11 after transduction. The expression of the DKK1 gene was sharply increased from day 9 ($P<0.05$ *vs*. day 6) (**Figure 3C**). The expression of the β-catenin gene was significantly decreased from day 9 ($P<0.05$) (**Figure 3C**). The expression of the c-Myc gene downstream of Wnt signaling showed no difference after transduction. The expression of the CyclinD1 gene was significantly decreased on day 11 ($P<0.05$). The expression of the Runx2 gene was increased from day 6 (all $P<0.05$).

Discussion

Wnt signaling plays multiple roles in mammalian MSCs. This study aimed to examine the proliferation and differentiation of MSCs of antler. The results showed that Wnt signaling is involved in the proliferation and cartilage differentiation of MSCs of antler. These results provide a theoretical basis for the growth and regeneration of antler.

Two days after antler MSCs was transduced with the DKK1 adenovirus, the cells began to differentiate into chondrocytes and cell morphology changed. Alcian blue staining (which indicates glycisaminoglycans in cartilage) became positive, consistent with cartilage differentiation. Alcian blue staining then became negative while alizarin staining (which indicate Ca^{2+} accumulation) became positive. Consistently, collagen II immunohistochemistry became positive on day 11. DKK1 overexpression was used to analyze the effect of Wnt signal on human MSCs and cartilage differentiation. Their results showed that overexpression of DKK1 and sFRP-1 genes significantly increased the secretion of proteoglycans and upregulated the expression of SOX-9 and collagen II [15], which was consistent with the results of the present study. Itshowed that the controlled activation of the Wnt/β-catenin signaling enhanced the stem and progenitor activities when regeneration is needed [22], supporting the present study.

Antlers and tumors share similar growth characteristics in that antlers can regenerate after being cut off and the growth rate is extremely fast [20]. The growth and development of antlers is dependent upon MSCs. Mount *et al*. [8] has preliminarily analyzed the effect of Wnt signaling on the proliferation of antler stem cells at the protein level, but the mechanisms are still unclear. In the present study, Ad-DKK1 overexpression was used to inhibit Wnt signal. DKK1 overexpression did not affect the proliferation of antler MSCs, which was different from the study by Mount *et al*. in 2006 [8]. Here, DKK1 overexpression downregulated the expression of β-catenin, but it had no inhibitory effect on c-Myc and CyclinD1 genes. The c-Myc and CyclinD1 genes are located downstream of Wnt, and the c-Myc gene plays roles in cell cycle regulation [23]. Increased expression of Myc genes increase the activity of Cyclins, thereby promoting cell cycle progression of pluripotent stem cells (PSCs) and accelerating cell division [24]. Satoh *et al*. [25] found that the number of hematopoietic stem cells was reduced when c-Myc was silenced. Laurenti *et al*. [26] and Wilson *et al*. [27] observed that the number of stem cells was increased when c-Myc and N-Myc were co-expressed. These studies demonstrated that c-Myc plays a positive role in cell division and proliferation.

In cancer, the c-Myc and CyclinD1 genes are upregulated to promote cell proliferation. CyclinD1 binds to cyclin-dependent kinase (CDK) to form a CyclinD/CDK complex that activates specific CDK and phosphorylates various proteins to regulate the procession of the cell cycle [28, 29]. In the present study, after blocking Wnt signaling by overexpressing DKK1, the expression of c-Myc and CyclinD1 genes was unaffected. It could be hypothesized that the expression of these two genes is not only affected by Wnt signaling, but also by other regulatory factors. The FBS in the culture medium contains a small amount of growth factors such as IGF and EGF, and these growth factors could promote the cell cycle through regulating the expression of c-Myc by the MAPK signaling pathway [30, 25]. Using IGF1 at different concentrations to stimulate antler MSCs, it was demonstrated that concentrations of IGF1 as low as 10 nmol could stimulate the rapid division of antler MSCs [31]. However, the relationship between the proliferation of antler MSCs and the signal pathway of MAPK needs to be further investigated.

Runx2 gene is an osteogenic-specific transcription factor that plays an important role in osteogenesis of human bone marrow MSCs and promotes cartilage and osteoclast functions [32]. In the present study, the expression of Runx2 was upregulated on day 11, and alizarin red staining detected that Ca^{2+} deposition occurred in chondrocytes, which suggested cartilage differentiation of antler MSCs and that the osteolysis of cartilage also started at the same time. These results are consistent with the results of the previous studies [8], which showed that inhibitory signals promoted cartilage differentiation of antler stem cells.

The main limitation of the present study is that only a small number of genes and proteins were evaluated in MSCs. In addition, only DKK1 over-

expression was studies. Additional studies are necessary to comprehensively determine the mechanisms involved in the regeneration of antlers, which is the only regeneration encountered in mammals. Among others, microRNAs should be further studied [4], as well as Hedgehog and NELL1 signaling [33].

In conclusion, Wnt signaling is involved in the proliferation and cartilage differentiation of MSCs of antler. These results provide a theoretical basis for the growth and regeneration of antler.

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Disclosure of conflict of interest

None.

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