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Full Length Article

Identification of MicroRNA-148a-3p with a Role in Transcriptional Regulation of TGF-β2 and its Relationship with Antler Cell Proliferation

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Abstract

Deer antler is the only regenerative organ with bone tissue among mammals. In the process of angiogenesis, transforming growth factor (TGF-β) plays an important role. The purpose of this study was to explore the microRNA-mediated regulation of TGF-β2 expression during rapid antler growth and antler cell proliferation in sika deer. Total RNA was extracted from cartilage cells and mesenchymal cells, present on the antler tip tissue using Trizol reagent, and deep sequencing of miRNAs was performed via Hiseq. Then, using bioinformatics software, we identified the miRNAs that specifically bind to the TGF-β2 3'-UTR among which the differentially expressed miRNAs were subsequently verified by qPCR. The results indicated that miRNA-148a-3p exhibited highest upregulation among the differentially expressed miRNAs. We also found that the TGF-β2 3'-UTR contains a binding site for miRNA-148a-3p by transfection of wild-type and mutant dual luciferase reporter vectors into antler cartilage cells. Meanwhile, the overexpression of miRNA-148a-3p significantly inhibited the proliferation of cartilage cells *in vitro* and decreased the level of TGF-β2 protein expression. Furthermore, the protein expression of IGF-1 and TGF-βRII, which are associated with TGF-β2, also reduced after transfection of cartilage cells with miRNA-148a-3p mimics. Our results indicate the important role of miRNA-148a-3p in antler cell proliferation and its potential application. Thus, miRNA-148a-3p is a potential novel regulator of TGF-β2 protein in antler development in sika deer. © 2019 Friends Science Publishers

Keywords: microRNA-148a-3p; Antler; Transforming growth factor regulation; Cell proliferation inhibition

Introduction

Antlers are a deer's secondary sexual organ and grow on the forehead during adolescence. Antlers are a multi-tissue organ that contains blood vessels, skin, nerves, cartilage and bone (Li *et al.*, 2004). Uniquely, annual antler shedding stimulates periodic regeneration of the antlers (Gao *et al.*, 2012) and therefore, they are an ideal model for studying organ regeneration (Price and Allen, 2004).

The cycle of antler regeneration progresses via osteogenesis of specialized cartilage, in which the *TGF-β* family members play a significant role (Francis and Suttie, 1998). *TGF-β* is a large protein family consisting of many structurally related polypeptide growth factors, including more than 40 members across three major classes that function in a variety of biological processes such as cell growth regulation, cell differentiation, stromal formation, immune, injury repair and tumorigenesis (Zimmerman and Padgett, 2000). *TGF-β* signaling pathway functions synergistically with the Wnt signaling pathway to promote the transformation of epithelial cells into mesenchymal cells and maintain cells in a mesenchymal state (Yan *et al.*, 2014; Singh *et al.*, 2016; Zepeda-Morales *et al.*, 2016). The early stage of cartilage cell formation in antler tissue involves rapid proliferation of mesenchymal cells. *TGF-β* has three subtypes in mammals, including *TGF-β1*, *TGF-β2* and *TGF-β3* (Shi and Massague, 2003). The activated *TGF-β* ligands bind to *TGF-β* receptors on the cell surface, to initiate signal transmission inside the cell, which further leads to the activation of smad proteins and ultimately causes a nuclear factor response (Derynck and Zhang, 2003).

The TGF-β family and its receptors are expressed in the growing bone tissue and TGF-β2 is mainly expressed in the cartilage area (Horner *et al.*, 1998). Previous studies have reported that *TGF-β1* is highly expressed in red deer, further suggesting that the TGF-β family may play an important regulatory role in antler regeneration (Faucheux *et al.*, 2004). Francis and Suttie (1998) reported that *TGF-β2* mRNA is expressed at the tip of the antler. Our research group has found that the expression level of *TGF-β2* in the cartilage layer of antler was higher than that in the mesenchymal and skin layer in sika deer. Cartilage tissue is the main structure on the tip of deer antlers and the rapid growth of antler is due to the rapid growth of cartilage tissue. Therefore, *TGF-β2* may play an important role in cartilage formation in sika deer.

MicroRNAs (miRNAs) are a class of non-coding, single-stranded RNA that is widely expressed in eukaryotes and exhibits highly conserved temporal expression and tissue specificity between species (Farh *et al.*, 2005). MiRNAs regulate the differentiation and function of osteoblasts,

cartilage and osteoclasts, suggesting that they are one of the key regulators of bone formation, absorption, remodeling, and repair (Lin *et al.*, 2014; Zhao *et al.*, 2015). HiSeq is a recent sequencing technology based on edge-sequencing that reduces sequence loss due to secondary structure. Currently, HiSeq deep sequencing is employed for almost all RNAs and has enabled the annotation of small RNAs by comparisons with sequences in known databases. Differential miRNA expression profiles have been elucidated on the basis of miRNA identification, which facilitates miRNA functional investigations (Ba *et al.*, 2016). The effects of miRNA-148a in regulation of *TGFβ2* and *SMAD2* expression in MNNG-induced gastric cancer of rat and the mechanism of action in GC cells were determined (Zhang and Li, 2016). In addition, miRNAs have been found to inhibit the proliferation of antler cells by modulating *IGF-1*, *IGF-1R*, *VEGF*, *VEGFR*, *TGF-βR* expression and other signalling pathways.

While pathways that participate in the regulation of antler growth and development have been identified, the precise regulatory mechanism still remains unclear. Therefore, the transcriptional regulation of TGF-β2 by miRNA-148a-3p in antlers was explored in this study. We also investigated the role of microRNA-148a-3p in the process of antler cell proliferation and the underlying mechanism. Our study will provide the experimental basis for further investigation of miRNAs in deer.

Materials and Methods

Materials

Deer antler tip tissue samples were obtained from sika deer (*Cervus nippon*), which were provided by the Jilin Agricultural University Deer Farm in June 2015.

Antler Mesenchymal Cell and Cartilage Cell Cultures

According to the method previously described by Li, about 5 cm of antler tip tissue was cut with a sterilized scalpel and the top of the antler was separated into mesenchymal and cartilage tissues. Tissue digestion method was used to isolate the cells from the antler tip tissue for culture. Isolated tissue was cut into 1.0 mm^3 pieces, after which collagenase I, hyaluronidase and collagenase II were added, respectively. Samples were centrifuged at 1000 rpm for 5 min, which resulted in a pellet with a large number of cells from the tissue mass. These cells were resuspended in high-glucose supplemented Dulbecco's modified Eagle medium containing 15% fetal bovine serum, transferred into a cell culture flask and cultured at 37℃. in an incubator with 5% CO_2.

HiSeq Deep Sequencing of Sika Deer Antler Tip Tissue

When the cells reached 90% confluency, total RNA was extracted from the cartilage cells and mesenchymal cells using Trizol reagent. Total RNA was ligated with 3'- and 5'-adaptors and cDNA was reverse transcribed with an RT primer and reverse transcriptase, and the cDNA template was amplified by PCR. The purified PCR product was recovered and sent to Shenzhen Huada Gene Technology Service Co., Ltd. for HiSeq deep sequencing. The miRBase and Rfam database were taken into consideration for annotation and identification of miRNAs. The obtained sequences were subjected to quality control, decontamination and other data cleaning processes. The small RNAs (sRNAs) were annotated by aligning the sequences with those obtained from various known databases, and we determined the coverage of each genomic location, between the sample and the database. For miRNA identification, miRNAs with differential expression among the various tissues were identified through a flexible differential analysis of the sequence dataset.

Quantitative Real-time PCR Validation of Differentially Expressed miRNAs

TargetScan 6.2 bioinformatics software was used to predict the miRNAs that specifically bind to the *TGF-β2* 3'-UTR. miRNAs that were differentially expressed between the cartilage and mesenchymal cells were further screened by small RNA sequencing. Antler chondrocytes were cultured, and when the cell reached 90% confluency, total RNA was extracted by Trizol reagent and reverse transcribed into cDNA. Differentially expressed miRNAs and an internal control (U_6 miRNA amplified fragment of 89bp), were amplified by PCR, and quantitative real-time PCR was used to validate the differentially expressed miRNAs.

Luciferase Activity Assay

In this experiment, we mutated the 3'-UTR sequence of the *TGF-β2* gene and constructed the wild type and mutant luciferase reporter gene vector. When the cells reached 60%-70% confluency, they were transiently co-transfected with the wild-type or mutant dual luciferase reporter gene recombinant plasmids and miRNA-148a-3p mimic and cultured for 24 h, 48 h, or 72 h, in triplicates. After washing with phosphate buffer saline, the cells were lysed with Passive Lysis Buffer for 15 min and centrifuged at 13000 rpm for 5 min, after which the supernatant was collected. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System following the manufacturer's instructions. To each well, 100 μL Luciferase Assay Reagent II was added and chemiluminescence was quantified on a microplate reader, in mode M1. Subsequently, 100 μL Stop reagent was added to each well, and the plate was examined in mode M2. Luciferase expression was defined as the ratio of M1/M2.

Cell Transfection

Antler cartilage cells in the logarithmic phase were seeded into 96-well or 6-well plates containing Dulbecco's modified Eagle's medium supplemented with non-double antibody

and 10% fetal bovine serum. When the cartilage cells reached 60%-70% confluency, miRNA-148a-3p mimic and negative control mimics were transfected into the antler cartilage cells, using the Roche HP transfection reagent. Cells were incubated under a 5% CO_2 atmosphere at 37°C.

Real-time Quantitative PCR for miRNA-148a-3p

Cartilage cells were cultured for either 24 h, 48 h, or 72 h in 6-well plates. Total RNA was extracted from antler cells at 24 h, 48 h and 72 h culture using Trizol reagent and reverse transcribed into cDNA. Using the cDNA template, miRNA-148a-3p and corresponding internal reference, U6 were amplified by PCR, the PCR mixture was composed of 10 μL 2x SYBR Green, 4 μL ddH2O, 1 μL upstream and downstream primers, 2 μL template and 2 μL QN ROX Reference Dye. The reaction conditions were: denaturation at 95°C for 2 min, denaturation at 95°C for 5 s and annealing at 60°C for 10 s, which was repeated for 40 cycles. Relative miRNA-148a-3p expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method.

Cell Proliferation Assay

Cartilage cells were subsequently cultured for either 24 h, 48 h, or 72 h into 96-well plates. MTT solution was added to the 96-well plate at 24 h, 48 h and 72 h post-transfection and incubated at 37°C for 4h. The supernatant was discarded, 200 μL of dimethyl sulfoxide was added to each well and the absorbance was measured at 570 nm.

Western Blot Assay

At 24 h, 48 h and 72 h post-transfection, cartilage cells were harvested and total protein was extracted using IP cell lysate buffer containing Phenylmethylsulfonyl fluoride for lysis, on ice for 30 min, with a shock applied once every 10 min. The supernatant was collected by centrifuging at 13000 rpm for 5 min and protein concentration was determined using the Bicinchoninic Acid protein assay kit. The protein was separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Nonspecific binding was blocked containing Tris-buffered Saline, Tween 20 h, for 2 h, after which polyvinylidene fluoride membranes were placed in primary and secondary antibody solutions and incubated at room temperature for 2 h. After washing thrice with Tris-buffered Saline and Tween 20, immunoreactive bands were visualized using an enhanced Horseradish Peroxidase and Diaminobenzidine chromogenic substrate kit.

Statistical Analysis

T-test was performed to compare the data between different groups. All data are presented as means ± SD. SPSS Statistics 12.0 software was used for all the statistical analyses and P-values < 0.05 were considered to be statistically significant.

Results

Differentially Expressed miRNAs in Mesenchymal and Cartilage Cells

HiSeq deep sequencing was performed to determine the miRNA content in the antler cartilage and mesenchymal cells. The HiSeq deep sequencing scatter plot analysis intuitively revealed differences between miRNA expression levels in the two cell types. Test results showed 1546 and 1545 miRNAs in cartilage cells and mesenchymal cells, respectively, among which 743 miRNAs were upregulated and 147 were downregulated in cartilage cells. On the other hand, 785 miRNAs were up-regulated and 157 were down-regulated in mesenchymal cells. There were 666 miRNAs that co-existed and were significantly expressed in both types of cells (Fig. 1).

Preliminary Screening Reveals the miRNAs Involved in Regulation of TGF-β2 Levels in Sika Deer Antlers

TargetScanHuman 6.2 bioinformatics software was used to identify the miRNAs that specifically bind to the *TGF-β2* 3'-UTR. Our results indicated that 36 miRNAs may target the *TGF-β2* 3'-UTR and inhibit *TGF-β2* expression. These miRNAs include miRNA-141-3p, miRNA-200a-3p, miRNA-153, miRNA-145-5p, miRNA-148a-3p, miRNA-148b-3p, miRNA-152-3p, miRNA-92a-3p, miRNA-133a-3p, miRNA-133b, miRNA-199a-5p. We identified six miRNAs that were differentially expressed between mesenchymal and cartilage tissues, including miRNA-148a-3p, miRNA-148b-3p, miRNA-92a-3p, miRNA-133a-3p, miRNA-133b, miRNA-199a-5p. These results are shown in Table 1.

Endogenous Levels of the Differentially Expressed miRNAs

Real-time PCR analysis was used to determine the relative expression levels of the six miRNAs that were differentially expressed in antler top mesenchymal and cartilage tissues, and calculated by the $2^{-\triangle\triangle Ct}$ method. As shown in Fig. 2, the relative expression levels of miRNA-148a-3p, miRNA-92a-3p, miRNA-199a-5p, miRNA-148b-3p, miRNA-133a-3p and miRNA-133b, differed among both tissues. These results are consistent with those we obtained from the miRNA deep sequencing of antler cartilage tissue.

Luciferase Activity

Luciferase activity assay revealed that, in comparison with that in the negative control group, the relative luciferase activity in the miRNA-148a-3p mimic and wild-type 3'-UTR-transfection groups decreased. Co-transfection of the miRNA-148a-3p mimic with mutant-type *TGF-β2* 3'-UTR did not affect the relative luciferase activity significantly,

Table 1: HiSeq depth sequencing of deer antler mesenchymal cells and chondrocytes differentially expressed miRNA

pairwise	miR-name	SCC-std	SCM-std	fold-change (log2 SCM/SCC)	p-value	sig-lable
SCC-SCM	miR-92a-3p	353.6203	823.6016	1.21974554	0	**
SCC-SCM	miR-148a-3p	736.8719	248.7811	-1.56653695	0	**
SCC-SCM	miR-148b-3p	38.7786	4.8378	-3.00283769	6.18E-89	**
SCC-SCM	miR-133a-3p	9.4601	2.932	-1.68997033	4.88E-12	**
SCC-SCM	miR-133b	8.991	2.7854	-1.69059706	1.63E-11	**
SCC-SCM	miR-199a-5p	41.6714	127.2492	1.61052711	1.20E-131	**

Remarks: Pairwise indicates two samples used for differential comparison. SCC represents antler chondrocytes, and SCM represents antler mesenchymal cells. MiR-name represents the name of the differential miRNA. SCC-std and SCM-std represent the normalized expression of the sample. Fold-change indicates the log2 value of the difference multiple, the molecule is the treatment group, and the denominator is the experimental group. P-value represents the statistical p-value. Sig-lable (**) indicates whether the expression of this miRNA is significantly different between the two samples

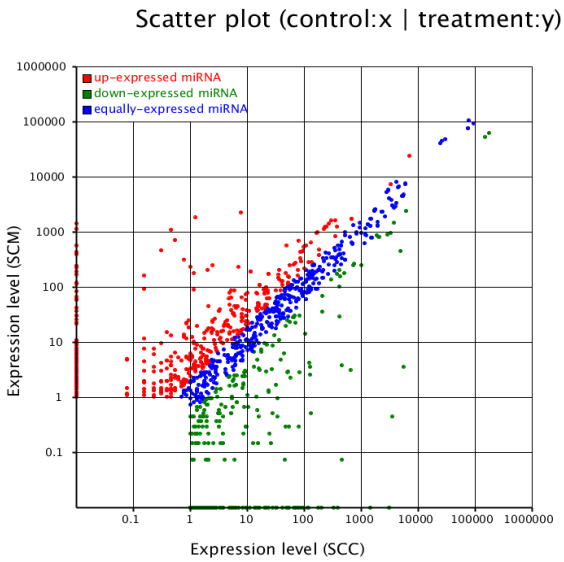

Fig. 1: Comparative analysis of scatter diagram for antler mesenchymal cells (SCM) and cartilage (SCC). Each point in the figure represents a miRNA, and the x-axis is the miRNA expression level in cartilage cells and y-axis is the miRNA expression level in mesenchymal cells. The red and green marks indicate a ratio (the normalized sample expression level divided by the normalized control expression level) of > 2 or < 0.5, respectively. Ratios of miRNA content between the two cell types that were more than double or less than half were considered to indicate differential expression

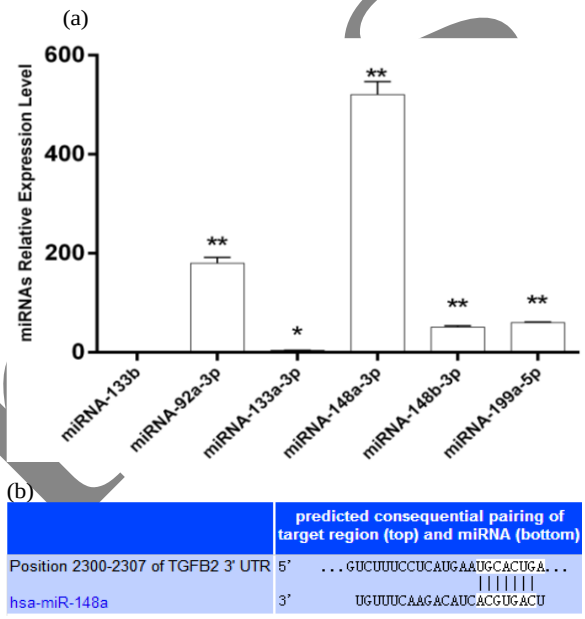

Fig. 2: (a) Comparison of microRNA fold changes. The relative quantitative results for endogenous microRNAs screened in cartilage and mesenchymal cells are shown. The level of endogenous miRNA-148a-3p was differentially expressed in cartilage and mesenchymal cells. Data are presented as the mean±SD (n=3). (b) Conservative analysis of the miRNA binding sites in the 3'-UTR of TGF-β2. In Chinese sika deer antler, TGF-β2 may be a molecular target of miRNA-148a-3p. This figure shows the seed region sequence of miRNA-148a-3p and its conserved target site in the 3'-UTR of TGF-β2, which was downloaded from TargetScan. Predicted miRNA-148a-3p target sequences in the 3'-UTR region of TGF-β2 are presented. As predicted, one section of miRNA-148a-3p bind to the 3'-UTR of TGF-β2, respectively

that is, miRNA-148a-3p was inactive (Fig. 3). These results suggested that there is indeed a binding site for miRNA-148a-3p in the *TGF-β2* 3'-UTR, and it proved that *TGF-β2* is the target gene of miRNA-148a-3p. This interaction may inhibit TGF-β2 protein expression.

miRNA-148a-3p Expression Levels in Cartilage Cells

The relative expression levels of miRNA-148a-3p in antler cartilage cells transfected with miRNA-148a-3p mimic at 24 h, 48 h and 72 h were 392.62, 541.19, and 470.483, respectively. Real-time PCR analysis showed that the expression level of miR-148a-3p in transfected cells was significantly upregulated compared to that in the normal and control cells (Fig. 4).

Proliferative Activity of Antler Cells

The cartilage cell proliferation was investigated using MTT assays at 24 h, 48 h and 72 h post-transfection. Compared with that in the negative control group which was not transfected, the antler cell proliferation was inhibited within 24 h to 72 h after transfection with miRNA-148a-3p minics. Due to the transient nature of miRNA expression *in vitro* transfection, the changes in cell proliferation were observed within the 24 h to 72 h of miRNA transfection. This result of MTT assay is shown in Fig. 5.

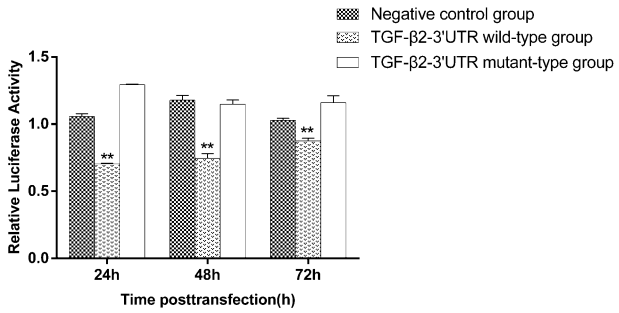

Fig. 3: TGF-β2 is a direct target of miRNA-148a-3p. Sika deer antler cartilage cells were cotransfected with the recombinant firefly luciferase reporter plasmid PmiR-RB-Report™-TGF-β2-3' UTR and the NC or miRNA-148a-3p mimic, as indicated. Luciferase activity decreased compared with the control group when cells were transfected with a mimic of miRNA-148a-3p. *p<0.05; **p<0.01 compared with the control

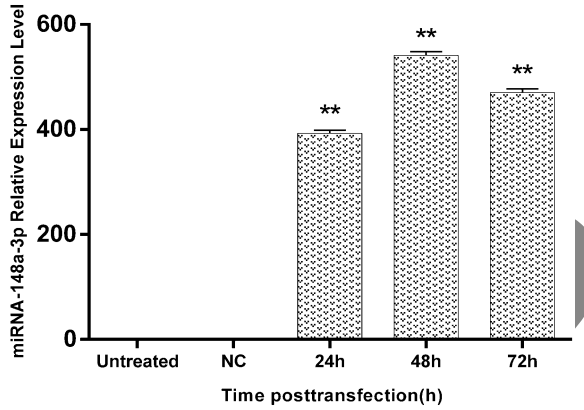

Fig. 4: miRNA-148a-3p content increased markedly following transfection. The content of miRNA-148a-3p in the cartilage cells increased markedly compared with that of the control cells and untransfected cells. Data are presented as the mean±SD(n=3). Similar results were found in three independent experiments. **p<0.01 compared with the control. NG, normal group

Relative TGF-β2, IGF-1 and TGF-βRII Protein Expression Levels

Western blotting was performed to detect the expression levels of *TGF-β2*, *IGF-1* and *TGF-βRII* in antler cartilage cells transfected with miRNA-148a-3p. Western blotting assays showed that *TGF-β2* expression significantly decreased, compared to that in the negative control group, following miRNA-148a-3p mimic transfection into the antler cells. These results indicated that miRNA-148a-3p binding to the *TGF-β2* 3'-UTR leads to the *TGF-β2* gene silencing and reduction of TGF-β2 protein levels. Similarly, the expression of *TGF-βRII* and *IGF-1* also decreased, which indicated interactions between growth factors in the antler tissue. The regeneration and rapid growth of antlers is regulated by many growth factors. These results are shown in Fig. 6 and the optical density analysis results are shown in Table 2.

Table 2: Densitometric data analysis of TGF-β2, IGF-1 and TGF-βRII protein

Group	TGF-β2 protein			IGF-1 protein			TGF-βRIIprotein		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Untreated group	725.65	554.39	602.73	748.79	749.46	706.23	991.74	903.48	961.14
Negative control group	656.74	625.24	740.46	936.09	886.83	861.94	724.77	728.58	680.22
miRNA-148a-3p mimic group	320.59	190.66	105.04	1313.4	689.86	469.93	833.79	564.53	365.04
GAPDH	619.5	773.87	749.53	1077.8	1057.9	1186.1	1280.8	1332.5	1335.1

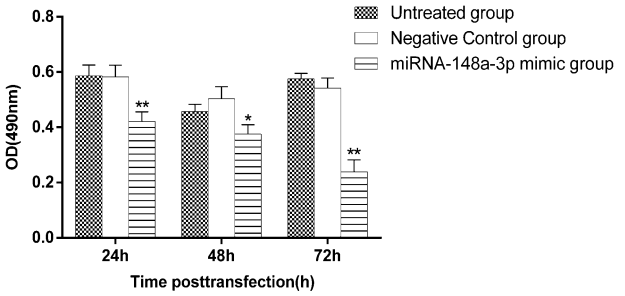

Fig. 5: miRNA-148a-3p inhibits cartilage cell growth. Compared with the control, cell proliferation decreased after 24, 48, and 72h, in cells that were transfected with miRNA-148a-3p mimic. Data are presented as the mean±SD (n=3). *p<0.05; **p<0.01, compared with the control

Discussion

MiRNAs are an important class of biomolecules that induces gene silencing and are involved in the regulation of cell growth and development and gene transcription and translation (Herranz and Cohen, 2010). Recently, many studies have shown that high levels of antler cell growth factors are involved in the regulation of antler regeneration and development (Pita-Thomas *et al.*, 2010). Sika deer *IGF* is a multi-functional protein that regulates cell proliferation (Jones and Clemmons, 1995) and *IGF-1* is an important cell growth factor that promotes rapid antler growth. The eukaryotic expression vector pcDNA6.2-Gw/EmGFP-*IGF1*-miRNA, which targets the *IGF-1* gene, was successfully constructed by Li Mu. After transfection of this plasmid into antler cartilage, antler cell growth was arrested in the G0/G1 phase and *IGF-1* expression decreased, which suggests that miRNAs can regulate the expression of antler growth factors. Further studies have shown that miRNA-1, miRNA-18a and miRNA-18b specifically downregulate *IGF-1* protein expression, by interacting with the *IGF*-1 3'-UTR sequence, to inhibit antler cartilage cell proliferation (Hu *et al.*, 2013; Hu *et al.*, 2014b; Hu *et al.*, 2015). *IGF-1* exerts biological functions mainly through binding to its receptors, and our previous research showed that *IGF-1R* is the direct target of miRNA-let-7a and miRNA-let-7f (Hu *et al.*, 2014a). Overexpression of the two miRNAs *in vitro* results in inhibition of antler cell proliferation and *IGF-1R* protein expression.

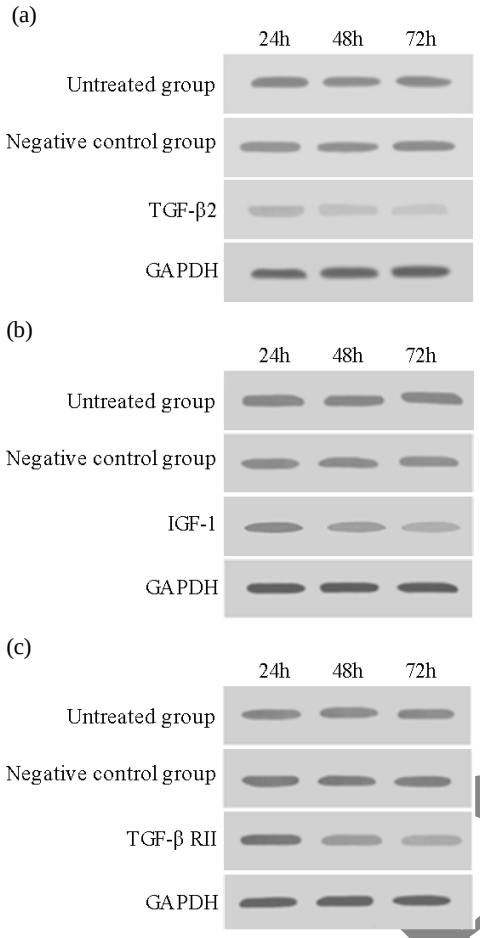

Fig. 6: Detection of the effect of miRNA mimic on the expression of TGF-β2, IGF-1, and TGF-βRⅡ protein by western blotting. (a) miRNA-148a-3p inhibited the expression of the TGF-β2 （50kDa） protein. The expression of TGF-β2 protein and GAPDH in sika deer cartilage cells was detected using western blot analysis. Protein levels of TGF-β2 decreased compared with the control group, when cells were transfected with mimic of miRNA-148a-3p after 24, 48 and 72h. (b) Protein levels of IGF-1（7.7kDa ）decreased compared with the control group when cells were transfected with mimic of miRNA-148a-3p after 24, 48 and 72h. (c) Protein levels of TGF-βRⅡ (62kDa) decreased compared with the control group when cells were transfected with mimic of miRNA-148a-3p after 24 h, 48 h and 72 h

One of the main reasons for the rapid growth, repair, and regeneration of antlers is the formation of blood vessels throughout the cartilage tissue, which provide sufficient nutrients for antler growth (Park *et al.*, 2004). *VEGF* plays a significant role in promoting vascular endothelial cell proliferation, vascular permeability and blood cell formation (Dvorak *et al.*, 1995; Iacovelli *et al.*, 2015). The function of *VEGF* is mediated by its interaction with the *VEGF* receptor (Lau *et al.*, 2001). Our previous research also showed that miRNA-15a and miRNA-15b regulate the expression levels of *VEGFR* proteins in antler cartilage and affect antler cartilage cell proliferation (Liu *et al.*, 2018). In addition, *TGF-β* elicits biological functions via interaction with *TGF-β R II*. Our previous research revealed that *TGF-βR II* is one of the target genes of miRNA-19a and miRNA-19b. The two miRNAs bind to and influence the function of *TGF-βR II*.

Deer antler is a special, periodic mammalian bone organ that originates from the periosteum of the antler area. The antler undergoes the whole process of bone formation from osteogenesis to endochondral ossification, and the rapid antler growth is dependent on the rapid cartilage formation. *TGF-β* is a member of the *TGF* family that plays an important role in the transformation of antler periosteal cells into osteoblasts, osteoblast proliferation, extracellular matrix synthesis, and new bone formation and maturation (Li *et al.*, 2001). In addition, the antler cartilage membrane exhibits high expression of *TGF-β2*, which suggests that *TGF-β2* is involved in cartilage growth and proliferation. Related studies have confirmed that *TGF-β2* induces the transformation of mesenchymal cells into cartilage. *TGF-β2* can promote cartilage proliferation and matrix secretion, and the differentiation of bone marrow stromal cells into osteogenic cells and inhibits cartilage dedifferentiation (Weiss *et al.*, 2010). In addition, *TGF-β2* also promotes chondrocyte extracellular matrix synthesis (Kalwitz *et al.*, 2011).

HiSeq deep sequencing can be used for accurate high-throughput identification of differentially expressed miRNAs in various samples, and is often used for target gene functional predictions and pathway annotations. Cartilage is the main tissue located at the tip of the antler in sika deer and the initial stage of cartilage growth involves rapid proliferation of the mesenchymal cells. Therefore, in this study, the mesenchymal and cartilage tissue from sika deer antlers were selected for HiSeq deep sequencing, to detect differentially expressed miRNAs. Bioinformatics software was to identify the predict miRNAs that specificity interact with the *TGF-β2* 3'-UTR. The deep sequencing results of antler tissue revealed six miRNAs that are differentially expressed and specifically interacted with the *TGF-β2* gene 3'-UTR, among which the expression level of miRNA-148a-3p was the highest. The luciferase assay further confirmed that one of the target genes of miRNA-148a-3p is *TGF-β2*. To validate our hypothesis, miRNA mimics were transfected into antler cells to mimic the endogenous miRNAs. The MTT assay and western blot analysis showed that miRNA-148a-3p inhibits cell proliferation and decreases *TGF-β2* protein expression. These results further demonstrate the functional role of miRNA-148a-3p in inhibition of the proliferation of antler cartilage cells *in vitro* by binding to the *TGF-β2* 3'-UTR.

In the *TGF-β* signalling pathway, signal transduction is carried out through cell-surface *TGF-β* receptors. The type II receptor is maintained in an auto-phosphorylated state and interaction with *TGF-β* catalyzes the phosphorylation of the type I receptor and the formation of a type II-type I receptor signalling complex. The phosphorylated type I receptor

further phosphorylate downstream molecules to transfer signals into the cell (Wrana *et al.*, 1994). Our study has proven that miRNA-148a-3p reduces *TGF-β*2 expression and influences the binding of *TGF-β2* to *TGF- βR II*, which maybe impedes type I receptor phosphorylation, affects signal transduction, and inhibits antler cell proliferation. It is known that *IGF-1* can stimulate chondrocyte division and proliferation and also promote the synthesis of type II collagen and proteoglycans in chondrocytes, which maintain cartilage cell phenotypes (Macrae *et al.*, 2009). Additionally, *IGF-1* promotes articular chondrocyte proliferation and matrix synthesis *in vitro* (Fernandez-Cancio *et al.*, 2008; Kiepe *et al.*, 2008) and the combined application of *IGF-1* and *TGF-β2* significantly promotes cartilage proliferation and extracellular matrix production. The combined effect of *IGF-1* and *TGF-β2* is superior to the effects of either *IGF-1* or *TGF-β2* alone, indicating that cartilage formation is synergistically promoted by these proteins. In our study, after transfection of miRNA-148a-3p, *IGF-1* and *TGF-βRII* protein levels also decreased, and the proliferation rate of antler cartilage was reduced. Our results indicate the potential for interactions between growth factors in antler tissue, which could regulate the growth of antler cells.

In summary, our study found that miRNA-148a-3p inhibits *TGF- β2* protein expression and expression of related growth factors, including *TGF-βR II* and *IGF-1*. Consequently, it affects the proliferation of antler cells. The results of this study further demonstrated the transcriptional regulation of *TGF-β2* by miRNA-148a-3p and its relationship with antler cell proliferation. They also preliminarily revealed the role of miRNA-148a-3p in the rapid growth of antler. There has not been much research on miRNA in sika deer. Our study will provide new theoretical basis and experimental data for further research on the molecular mechanism of miRNA-mediated regulation of antler growth, especially the regulation of antler growth factors.

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