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Screening of Transcriptional Differential Genes in Antler After Top Pruning and Expression Characteristics of *BVES* Gene

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CMH, QHG provided theme, scope, and guidance. HC conceived the study, prepared the figures and tables, and wrote the manuscript. JWL conducted the part of data analysis. CL, HL performed sample collection and total RNA preparation. JYZ and XDJ performed the qRT-PCR validation. All authors read and approved the final manuscript.

Key words
Antler, Top pruning, RNA-seq, Transcriptional differential genes, BVES gene

ABSTRACT

The unique mammalian appendage antler is a good model for studying tissue regeneration and its related mechanism. This study explored the mechanism of the rapid growth of regenerated antlers after top pruning damage using transcriptome sequencing technology. A damage repair model was constructed by top pruning treatment on the left antlers of three 10-month-old Tarim red deer, and RNA-seq analysis was performed on the Illumina platform to compare the transcriptome sequencing results of the left regenerated antlers with the right healthy antlers. Among a total of 56 differentially expressed genes (DEGs) that were screened, 37 were up-regulated and 19 were down-regulated; the most significant changes were seen for the BVES gene (up-regulated gene); thus, this gene was further explored. Real-time fluorescence quantitative PCR (qRT-PCR) and immunohistochemistry further indicated a higher expression of *BVES* in the mesenchymal tissues of regenerated antlers than in the antler skin, cartilage, and bone tissues after top pruning treatment ($P < 0.05$); the brown immunohistochemical reaction products were concentrated in the mesenchymal cell membranes and intercellular matrix of healthy and regenerated antlers. Our results suggested that the repair process of antler damage after top pruning treatment mainly promotes the proliferation and differentiation of antler chondrocytes, osteoblasts, and T lymphocytes through biological signals such as WNT, IHH, and IL2RA to ensure the development of antler tissue again after top pruning. To sum up, our data implied that *BVES* gene regulated mesenchymal stem cells' proliferation and differentiation activities in response to ischemic stimuli to promote the healing and rapid growth of wounded antlers.

INTRODUCTION

Tarim red deer is a species native to Central Asia that lives in harsh environmental conditions in this basin, including high temperature, aridity, and poor nutritional conditions. At present, the Tarim red deer are mainly kept in captivity in the second division of the Xinjiang construction corps, with a stock of around 4,000-5,000 head. They are resistant to rough feeding and have a high antler yield. Compared to the Sika deer, their antler is thicker and more branched (The 6-8 branches in Tarim vs. 4 branches in Sika); the average annual fresh antler yield of adult Tarim male deer is up to 12.61 kg (Liu *et al*., 2018).

The top pruning technique is often used to increase antler production by sawing off 1-2 cm from the top when the antler is 3-5cm high; this procedure stimulates the growth point, which allows the developing antler to grow at an alarming rate to a two-bar or three-branch antler (Zhao, 2002). In addition, this technique not only increases the weight of the antler but also makes the fully grown antler more beautiful, which undoubtedly brings more profit to the breeder and turns deer farming into an important economic industry.

The unique mammalian appendage antler is a potential model for studying tissue regeneration due to its ability to regenerate completely. Not only do deer antlers regenerate the following year after natural growth, ossification, and shedding, but they also continue to repair wounds and regrow after artificial cuts. Interestingly, antler damage that occurs in natural environments from deer fighting or

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collisions between deer heals faster, and the antler's weight exceeds that of naturally growing antlers. However, the molecular mechanism through which antlers can repair themselves and rapidly and circumferentially regenerate remains unknown.

Transcriptome sequencing analysis is a method for direct sequencing of RNA molecules present in a given sample and is widely used to screen for differential genes, identify candidate genes, analyze metabolic pathways, and predict the relationship between genes and target organs (Marguerat and Bahler, 2010; Ramayo-Caldas *et al*., 2012). Its main advantages are higher dynamic range, specificity, and sensitivity (Marioni *et al*., 2008). This technique has been developed to provide new tools for transcriptome characterization and gene expression profiling. Using whole transcriptome sequencing analysis, Han *et al*. (2020) constructed the first regulatory network of ceRNAs associated with antler development and showed that coding and non-coding RNAs regulate antler development through interactions and competition. Furthermore, Chen *et al*. (2022) conducted a comprehensive analysis of the transcriptome and proteome of antler cartilage tissues at different growth stages, revealing that gene 13546 annotated in the Wnt signaling pathway and its encoded protein 13546 may have important biological functions in the rapid growth of antlers. In this study, the transcriptome sequencing technology was used to analyze the differences between regenerated antlers and normally developing healthy antlers after top pruning treatment in Tarim red deer, and the resulting database was compared to screen key regulatory genes in the regeneration process of antlers after top pruning treatment, which lays the foundation for further explaining the molecular mechanism of rapid antler growth and development in damaged deer.

MATERIALS AND METHODS

Sample collection

Tarim deer antler tissues were collected from the deer farm of the Thirty-first Group, Second Division, Xinjiang Production, and Construction Corps, and three healthy male deer at 10 months of age with the same feeding environment were randomly selected. When the antlers reached 3-5cm, the left antler of each of the three deer was exposed to top pruning. When the antlers reached 53 days of growth, the deer were first anesthetized with xylazine hydrochloride injection (1.0mL/100kg), and the antler roots were tied with straw rope to prevent excessive blood loss. The whole antler was then cut off 2-3 cm above the antler shank using the sawing method, and the hemostatic drug was applied to the section of the stubble deer antler to stop bleeding. After the blood had clotted and the anesthetic wore off, and the deer could stand up, the bailer was opened, and the deer was released. The antler was quickly cleaned of surface dirt and disinfected with 75% ethanol. Then, the top 5 cm of antler tissue was cut on an ultra-clean workbench. According to the tissue dissection method proposed by Li and Suttie (2003), 100 mg/each of antler skin, mesenchyme, cartilage, and bone tissues were isolated and labeled as trauma group (R1, R2, R3), and healthy antler group (N1, N2, N3). The tissue blocks from the two sample groups were selected and placed flat in a freezing mold. OCT embedding agent was then added and placed in liquid nitrogen for rapid cooling. When free of water vapor, samples were taken out and placed on the freezing bath of the slicer and sliced to 6μm; the slices were absorbed on the rough side of the slide and left at room temperature for 2 h and then placed at -80°C for use (Dong *et al*., 2021). The remaining tissue blocks were placed in liquid nitrogen and used for RNA extraction from each tissue.

Total RNA extraction, transcriptome library construction, and sequencing

Total RNA was extracted from regenerated antler and healthy antler tissues using the Trizol method. RNA concentration and quality were measured by NanoDrop 2000 spectrophotometry. RNA integrity was measured by agarose gel electrophoresis, and after passing the test, the cDNA library and transcriptome sequencing were sent to Novogene Bioinformatics Technology Co. Ltd.

Transcriptome sequencing data processing and analysis

Raw reads of the trauma and health groups were generated on the Illumina Nova Seq 6000 sequencing platform, and high-quality filtered reads were obtained after passing data quality control (Clean Reads). HISAT2 (2.0.5) (Kim *et al*., 2019) software was used to compare Clean Reads to the reference genome (*Cervus elaphus hippelaphus* genome assembly, CerEla1.0). Feature counts (1.5.0-p3) tool was used for quantitative analysis (Liao *et al*., 2014), and gene expression values for RNA-seq were generally calculated using Fragments per Kilobase transcript per Million Mapped Fragments (FPKM) (Zhao *et al*., 2021).

Transcriptome differentially expressed genes and functional annotation

Using log2 (Fold change) ≥ 1 and Padj ≤ 0.05 as thresholds, the software DESeq2 (1.16.1) was used (Love *et al*., 2014). Differentially expressed genes (DEGs) were compared between the trauma and healthy groups to obtain up-regulated and down-regulated genes. Enrichment analysis of DEGs was performed using the R package and combined with GO (Gene Ontology) functional annotation.

Table I. Amplification primer information.

Genes	Primer sequences (5′→3′)	Annealing temperature/ °C	Fragment length/bp
BVES	F: TGACGACCGTCTGAGTATTCTCCTG	61	133
	R: TCACCTTTGTGCATCTGGGTTGATC		
IHH	F: CCAGAACTGCCCACATGAGT	60	210
	R: GAGAGACCATGCCCCATCAC		
IL2RA	F: GTGCATAAGTGAAGGGGCGAACG	57	132
	R: GTGGGCTTCTGGAAATCTGTGGT		
LOC122686105	F: GGAGCCCCAGAATAGAAGCAAGATG	61	137
	R: CCACGGACCTATGCCCTTTCAAG		
GAPDH	F: TGTTTGTGATGGGCGTGAACCA	58	154
	R: ATGGCGTGGACAGTGGTCATAA		

Validation and analysis of real-time fluorescent quantitative PCR (qRT-PCR) for differential genes

Four genes were randomly selected from the differential genes, and specific primers were designed using Primer 6.0. Bovine *GAPDH* gene sequence in GenBank (accession number NM_001034034.2) was used as the internal reference gene (Table I). Each cDNA sample was diluted 1:4 with ddH_2O according to the 2×S6 Universal SYBR qPCR Mix (EnzyArtisan) kit instructions. Reaction system: 5 μL of 2×S6 Universal SYBR qPCR Mix, 0.2 μL of PCR forward primer, 0.2 μL of PCR reverse primer, 1 μL of cDNA, and 3.6 μL of enzyme-free water. PCR amplification parameters were 95°C for 2 min, (95°C for 15s, 61°C for 20s,72°C for 20s) x 34 cycles.

The relative expression was calculated in qRT-PCR using the $2^{-\Delta\Delta Ct}$ method. Statistical analysis was performed using SPSS23 with a t-test for the significance of differences, with $P < 0.01$ indicating highly significant differences and $P < 0.05$ indicating significant differences.

Detection of BVES *gene expression in different tissues of antler*

The BVES expression levels in each tissue of the regenerated and healthy antler by real-time fluorescent quantitative PCR (qRT-PCR).

Immunohistochemical analysis

Operations were performed following Upender *et al*. (2009) and Sun *et al*. (2012) methods. Frozen sections were fixed in cold acetone at 4°C for 10 min, incubated at room temperature for 10 min with an appropriate amount of endogenous peroxidase blocker, and then sealed in a wet box with 10% goat serum for 10 min to block non-specific binding. Next, samples were incubated with primary antibody-rabbit anti-BVES polyclonal antibody (diluted 1:50) overnight at 4°C and then with enzyme-labeled goat anti-rabbit IgG polymer for 20 min at room temperature. Finally, DAB staining (Solarbio) and hematoxylin staining were performed, and samples were observed using an inverted microscope.

RESULTS

Sequencing data analysis

Figure 1 shows that RNA bands were clear and bright with good integrity. The RNA concentration ranged between 1069ng/μl-1350ng/μl and OD260/OD280 between 1.8-2.0, all meeting the transcriptome sequencing requirements.

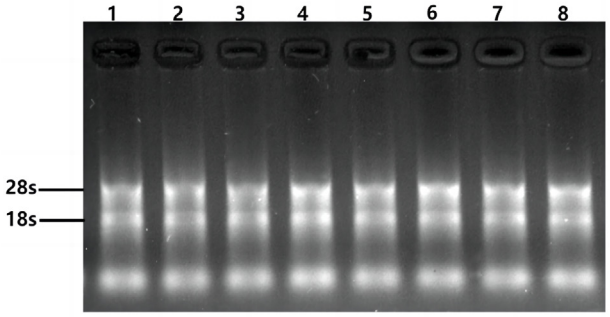

Fig. 1. Electrophoresis of total RNA extraction results from deer antlerissue. Lanes 1-4 are normal antler tissue samples, Lanes 5-8 are damaged antler samples.

High-throughput sequencing technology was used to sequence RNA from trauma and health group samples. The raw sequencing data were quality-controlled to obtain Clean Reads by excluding data containing splice data, data containing unidentifiable bases, and low-quality data from Raw Reads. The readsQ30 of the six samples sequenced in the two groups was greater than 93%, and the efficiency

of comparison between the valid data of each sample and the reference genome ranged from 79.13% to 80.22% (Table II). Comprehensive results showed that the quality of library construction and sequencing was good and met the requirements of subsequent analysis.

Table II. Results of data filtering and comparison statistics.

Sample	Raw reads	Clean reads	Percentage (%)	Q30	Total map(%)
R1	45 702 356	44 569 852	97.52	94.06	79.42
N1	43 451 750	42 298 546	97.35	93.98	79.56
R2	42 550 680	40 937 202	96.21	94.01	79.31
N2	45 801 344	44 146 098	96.39	93.90	79.13
R3	44 904 562	43 564 691	97.02	94.10	79.69
N3	42 106 854	41 597 605	98.79	93.75	80.22

R, trauma group; N, healthy group; Q30, percentage of bases with Phred values greater than 30 out of the total bases; Total map, number of reads compared to the genome and their percentage.

DEGs analysis

The transcriptomic data from the trauma and healthy groups were compared, and 56 DEGs were screened, among which 37 genes were significantly up-regulated and 19 genes were significantly down-regulated in the trauma tissue. The number of up-regulated genes was higher in the trauma group than in the healthy group. The most significant changes were seen for the *BVES* gene (up-regulated gene); thus, this gene was further explored.

GO functional enrichment analysis of DEGs

Fifty-six DEGs were annotated to the GO database. At $p<0.05$, 31 significantly enriched secondary entries were found (Fig. 2). The GO annotation results involved biological process (BP) twenty-five secondary items, including skeletal system development (GO: 0001501), cartilage development (GO: 0051216), three secondary entries for cellular component (CC) including extracellular region (GO:0005576), molecular function (MF) 3 secondary entries including protein binding (GO:0005515). *BVES* genes were significantly enriched in GO entries for epithelial cell-cell adhesion, regulation of cellular processes, developmental tissue processes, and response to ischemic stimuli.

Reliability analysis of transcriptome sequencing results

To verify the reliability of the RNA sequencing results, four genes, including *IL2RA, BVES, IHH,* and *LOC122686105,* were randomly selected from the DEGs for qPCR amplification and compared with the sequencing results (Fig. 3). The results showed that the trends of the two methods were consistent, indicating that the transcriptome sequencing results were reliable.

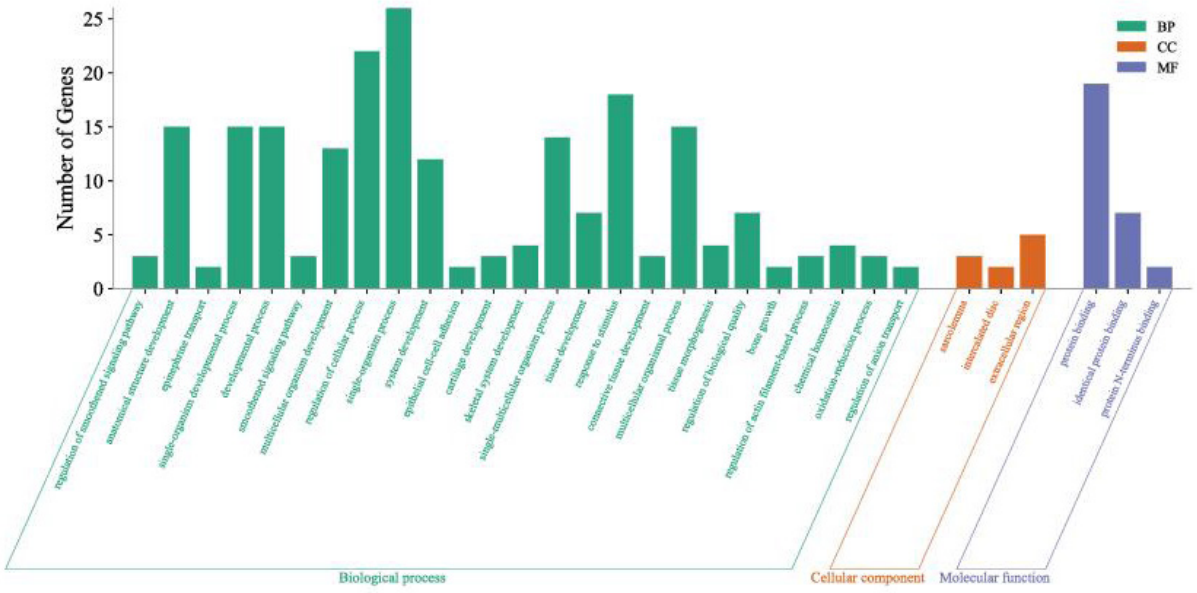

Fig. 2. Functional classification of genes in the trauma and normal groups.

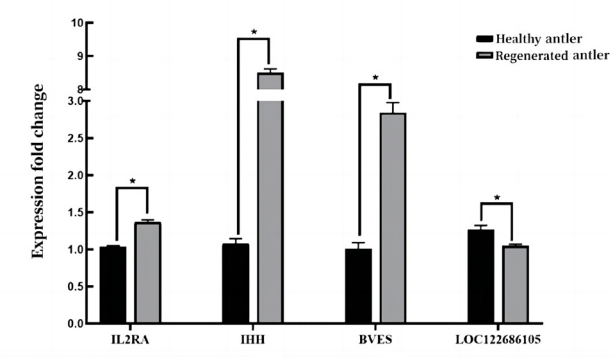

Fig. 3. qRT-PCR validation of differentially expressed genes (* indicates significant difference p<0.05).

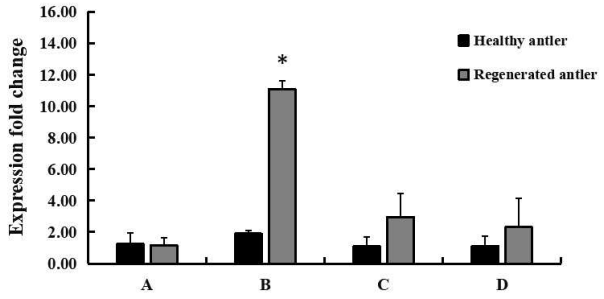

Fig. 4. Relative expression of *BVES* genes in different tissues of healthy antler and regenerated antler.

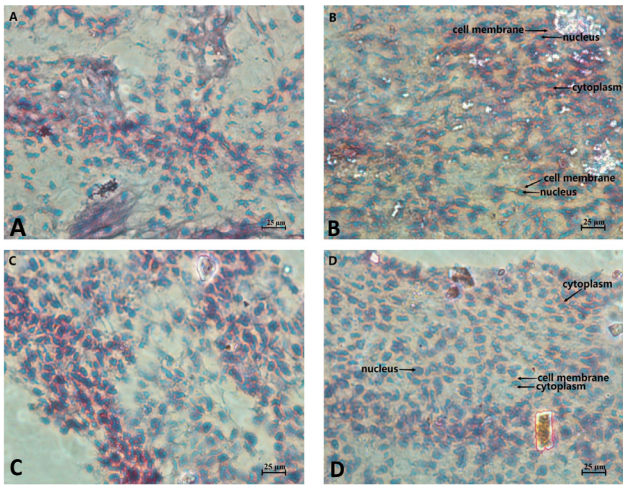

Fig. 5. *BVES* expression in regenerated and healthy antler mesenchymal tissues of the Tarim red deer.
A, negative control of regenerated antler mesenchymal tissue; B, positive expression of regenerated antler mesenchymal tissue; C, negative control of healthy antler mesenchymal tissue; D, positive expression of regenerated antler mesenchymal tissue.

Expression of the BVES *gene in various tissues of healthy antler and regenerated antler after a top pruning*

The expression of the *BVES* gene was significantly higher in regenerated antler tissues after pruning than in naturally grown healthy antlers ($P < 0.05$). The gene was expressed in antler skin, mesenchymal, cartilage, and bone tissues. When comparing the expression of each tissue, the *BVES* gene was not significantly different in healthy and regenerated antlers ($P > 0.05$). However, the expression of the *BVES* gene mRNA was significantly up-regulated ($P < 0.05$) in the regenerated antler mesenchymal tissue after the top pruning treatment (Fig. 4).

The presence of a large number of immunohistochemical brown reaction products in both the cell membrane and intercellular stroma of regenerated antler mesenchymal tissue was observed under the microscope (Fig. 5A, B), whereas the positive expression in healthy antler mesenchymal tissue was weak and low (Fig. 5C, D). No immunoreactivity was observed in antler skin, cartilage, or bone tissues.

DISCUSSION

The transcriptome sequencing results showed a significant increase in up-regulated genes in regenerated antlers after top pruning treatment. Top differential genes, *BVES*, *WNT5B*, *IHH*, *AXIN1*, and *IL2RA,* were mainly enriched in biological processes such as bone development and regulation of cellular processes, indicating that top pruning treatment is a stimulus that can activate a large number of genes and regulate cellular physiological processes. Significant up-regulation of connective tissue development and cartilage development suggests that the tendency of connective tissue, cartilage, and bone development in antlers enhanced under traumatic conditions may be one of the reasons for the increased yield of forked antler after top pruning injury in actual production. Guo (2021) reported enriched bone morphogenesis at the initial stage of antler regeneration, suggesting that bone development accompanies both the initial as well as rapid developmental stages of antler regeneration. At the same time, differential genes were found to be predominantly classified as protein binding within the various subcategories of molecular function (MF), suggesting that antler growth and development are closely related to ligand/receptor interactions, including hormone/receptor interactions and signaling molecule/receptor interactions. This is consistent with results of Saito *et al*. (2002), who first reported homing abilities of MSCs and that chemically injured tissues can attract MSCs, which can be homing to damaged tissues where they exert their therapeutic effects. Thus, we speculated

that there might be a large number of influencing factors driving the migration of MSCs to damaged sites during wound healing. When MSCs are mobilized in the antler tip, they can repair the damage and simultaneously continue to differentiate and proliferate, which is the basis for the rapid growth of regenerated antlers after trauma.

Among all DEGs that were different in regenerated antler tissue compared to healthy groups, the most significant was seen for the *BVES* gene expression, which was significantly up-regulated after top pruning. *BVES* was discovered by the gene cloning study of heart tissue in 1999 and was later named the vascular epicardial active substance (Reese and Bader, 1999; Reese *et al*., 1999). Previous studies have revealed that BVES proteins accumulate at the site of cell-to-cell contact (Hager and Bader, 2009), can control the morphology and movement of normal cells, and are a novel cell adhesion molecule (Wada *et al*., 2001). Osler *et al*. (2005) found that BVES not only participates in establishing and maintaining epithelial cell integrity by regulating the formation of tight junctions between cells but also promotes the differentiation of epithelial cells (Russ *et al*., 2011). In addition, BVES can attenuate myocardial ischemic and oxidative damage and have a positive role in myocardial ischemic tolerance (Alcalay *et al*., 2013). In the present study, *BVES* genes were significantly enriched in biological processes such as epithelial cell-cell adhesion, regulation of cellular processes, tissue development processes, and response to ischemic stimuli, suggesting that *BVES* genes may promote antler growth and development by regulating antler cell physiological activities and participate in the healing process of wound tissue repair, which is consistent with most of the previous findings.

BVES gene expression has been reported to be low in cancers, including liver tumors (Han *et al*., 2015) and colon cancer (Williams *et al*., 2011), suggesting that *BVES* has a cancer-suppressive effect. Williams *et al*. (2011) found that overexpression of BVES in uveal melanoma cells can impair the proliferation of tumor cells, while its down-regulation promotes the invasion and metastasis of liver cancer cells (Han *et al*., 2015).

The proto-oncogene c-Myc is a transcription factor whose role is to regulate cell growth, differentiation, metabolism, and death; however, it is frequently dysregulated and overexpressed in many human cancers (Koo *et al*., 2000; Nair *et al*., 2003; Sorolla *et al*., 2020; Toon *et al*., 2014), being an important cause of cancer and tumourigenesis in humans. Recent studies have found that BVES is an important regulator of the inflammatory carcinogenesis program; it can promote c-Myc degradation through interaction with the PR61α-PP2A protein complex, decreasing cellular c-Myc protein levels, thus reducing the probability of carcinogenesis (Parang *et al*., 2017). Accordingly, BVES can act as an important suppressor of inflammatory tumorigenesis by attenuating excess c-Myc levels.

One of the typical characteristics of deer antler, which is widely known for its traditional medicinal value, is its fast growth rate. Although deer antler cells proliferate faster than cancer cells, they do not proliferate indefinitely like cancer cells (Han *et al*., 2021). Therefore, antler growth is a more complex regulatory process regulated by various signaling pathways and growth factors (Lord *et al*., 2007). Recent studies on rapid antler growth have focused on genetic aspects. For example, the proto-oncogene c-Myc is expressed in antler tip tissues at 30, 60, and 90d of development, but the relative expression levels are weaker compared to other growth factors (Francis and Suttie, 1998). Another study explored the expression of c-Myc genes in different antler tissues of Tarim red deer at different growth stages (Han *et al*., 2012); the *c-Myc* gene was found to be involved in the proliferation and differentiation of antler skin during the rapid growth phase and was highly expressed in cartilage tissue during the late growth phase to regulate antler cartilage development and bone formation, while c-Myc gene expression was lower in the mesenchymal layer at different times. In this study, *BVES* genes were expressed in all tissues of 53d healthy antler and regenerated antler; yet, their expression was significantly higher in regenerated antler tissues, suggesting that *BVES* genes may inhibit the overexpression of proto-oncogenes such as c-Myc in cancer-like growing antlers so that fast-growing antlers do not become cancerous. In addition, after top pruning, antlers that experienced trauma may be more susceptible to disease during the repair and healing process. The *BVES* gene is up-regulated when there is a tendency of pathological changes to prevent pathological changes in response to dangerous signals occurring in antlers. Surprisingly, the relative expression of *BVES* genes was significantly higher in regenerated antler mesenchymal tissues than in antler skin, cartilage, and bone tissues after the same period of pruning, suggesting that BVES may regulate the proliferation and differentiation activities of antler stem cells, but the mechanism of regulation is not yet clear. Therefore, understanding the biological roles of *BVES* genes in antler stem cells is of great significance for antler growth development and regeneration.

CONCLUSION

Top pruning treatment of the antler can promote the proliferation and differentiation of antler chondrocytes, osteoblasts, and T lymphocytes through upregulation

of biological signals such as WNT, IHH, and IL2RA, thus ensuring the re-development of antler tissue after top pruning treatment. Also, a series of screenings and experiments have confirmed that the *BVES* gene may regulate the proliferation and differentiation of antler mesenchymal stem cells after pruning by responding to ischemic stimuli, promoting antler growth and development, and increasing antler production.

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IRB approval

All animal handling practices are approves by the University of Tarim Animal and Use Committee (NO.DTU20221210).

Ethical statement

This study was conducted in accordance with the specifications of the Ethics Committee of the Tarim University.

Statement of conflict of interest

The authors have declared no conflict of interest.

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