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A Study on Differentially Expressed Genes in Reserve Mesenchyme of Male and Female Reindeer Antler Tip

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Authors' Contribution
JZ, LG and HL designed the experiments. JZ and LG performed the experiments. JZ, LG and YX analyzed the data, JZ wrote the article.

Key words
Reindeer (*Rangifer tarandus*), Antler, Mesenchyme, mRNA differential display, RT-PCR.

ABSTRACT

Reindeer is the only species in which the females carry antlers in Cervidae, and the differences between the antler tips of the two sexes are one of the most interesting scientific mysteries. The objective of this study was to isolate preliminarily differentially expressed genes in antler mesenchyme of both sexes. The genes were detected with three anchored primers and ten random primers using differential display reverse transcription PCR (DDRT-PCR). Five differentially expressed fragments were obtained through recovery, cloning and sequencing. Three of them (Af1, Ce1, Ci2) presented a higher expression in antler mesenchyme of female reindeer, but the other two (Ae1, Af3) were the opposite. The results of Blast in GenBank showed that they shared high homologies with SMAD5 (95%), COL6A3 (95%), SLC17A5 (93%), TPM4 (99%) and HMGB1 (98%), respectively. Quantitative RT-PCR validation of gene COL6A3 showed that the expression in both sexes was consistent with the results of mRNA differential display, but there was no significant difference between the male and female.

INTRODUCTION

Deer antlers are the only mammalian appendages capable of regeneration, represent one of the fastest rates of organogenesis in the animal kingdom, and provide a model for studying two unique processes: the development of a complete appendage that is delayed until puberty and mammalian organ regeneration. However, the reindeer (*Rangifer tarandus*) is the only species of deer in which both sexes normally produce antlers (Leader-Williams, 1979), and many gaps exist in morphology, growth period in antler of male and female reindeer. The early work mainly focused on the relationship with hormones and antler cycle of male and female reindeer to seek the differences between them. However, there is still a lack in understanding the interplay of differentially expressed genes involved in the antler growth of male and female reindeer. Here, we attempted to isolate and identify preliminarily the differentially expressed genes in antler mesenchyme between the both sexes of reindeer.

Given an outer fibrous perichondrium where type 1 collagen mRNA and protein are highly expressed and an inner, more cellular zone that cells are actively dividing (Price *et al*., 2005), the reserve mesenchyme, therefore, provides an ideal source for exploring the antler growth and may help in understanding the mechanism of the difference in antlers of male and female reindeer. Differential display reverse transcription PCR (DDRT-PCR), introduced firstly by Liang and Pardee (Liang and Pardee, 1992), can identify the total number of genes subject to up- or down-regulation in a particular setting and has been widely used to screen for genes that are differentially expressed (Breyne and Zabeau, 2001; Ahmed *et al*., 2000). Furthermore, Quantitative reverse transcription PCR was proved to eliminate the false positive rate to validate effectively the differential expression of genes.

To study its possible mechanism in response to the molecular machinery in the antlers between the male and female reindeer, we decided to perform a preliminary study to examine differences in the gene expression in relationship to antler mesenchyme of both sexes using the mRNA differential display method.

MATERIALS AND METHODS

Sample collection and preparation

The distal 5 cm from each antler was collected from two anaesthetized healthy reindeer at Grand Farm in Harbin, which were introduced from the Ewenki reindeer herding of Aoluguya, Inner Mongolia, China. The two reindeer are half-sib, the male is 6-year-old, and the female is 7-year-old. Following the established methods (Li *et*

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al., 2002), reserve mesenchyme was rapidly separated from five morphologically defined layers (dermis, reserve mesenchyme, precartilage, transition zone and cartilage; Fig. 1), and stored immediately in liquid nitrogen until further processing.

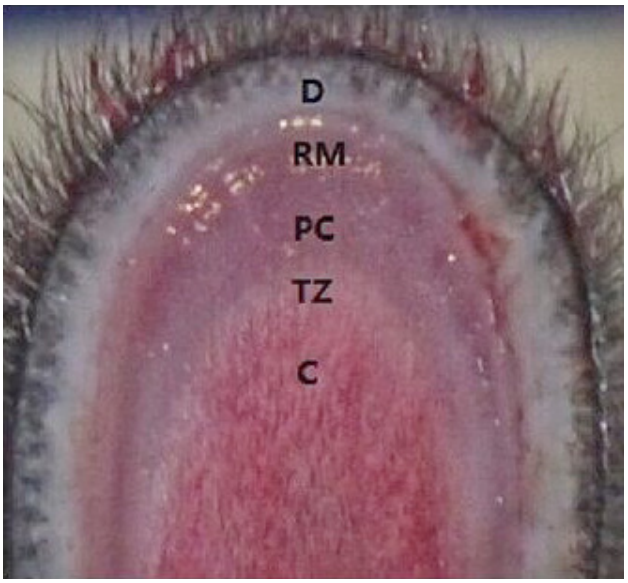

Fig. 1. Layer identification of unstained proliferative zone in a growing reindeer antler tip. D, dermis; RM, reserve mesenchyme; PC, precartilage; TZ, transitional zone; C, cartilage.

Extraction of RNA and synthesis of first-strand cDNA

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacture's instructions. The RNA quality and integrity were examined with ultraviolet spectrophotometer and electrophoresed on 1.0% agarose gels. Three reverse transcription reactions were carried out for each RNA using three anchored primers. In each PCR tube, 4.0μL of total RNA (1μg/μL), 8.0μL 5x RTbuffer, 9.6μL dNTP (2.5mM), 0.5μL RNase inhibitor (10U/μL), 2.0μL anchored primer (10mM), 2.0μL M-MLV, (200U/μL, TOYOBO) were added, and ddH_2O treated by diethypyrocarbonate (DEPC) was filled to the total volume of 40.0μL. The reverse transcription condition were 42°C for 20min, 99°C for 5min, and 4°C for 5min. The products of reverse transcription (cDNA) were detected with ultraviolet spectrophotometer, diluted to 1μg/μL and stored at -20°C for using as template for differential display PCR amplifications.

DDRT-PCR

Each primer pair was composed of an anchored primer and a random primer (Table I, Invitrogen, Shanghai). PCR was carried out in a final reaction volume of 25μL containing 2.5μL 10 x buffer, 2.0μL $MgCl_2$ (25mM), 2.0μL dNTP (2.5mM), 0.125μL rTaq DNA polymerase (5U/μL, TaRaKa, Japan), 4.0μL anchored primer (10mM), 0.8μL random primer (10mM), 2.0μL cDNA (1μg/μL), 11.575μL ddH_2O. Amplifications were performed using the following PCR procedure: an initial denaturation step for 5 min at 94°C, followed by 40 cycles of 94°C for 30 s, 2min at 42°C and 1 min at 72°C, and a final elongation for 10 min at 72°C. PCR products were separated on 8% nondenaturing polyacrylamide gels.

Table I.- Anchored and random primer sequences for DDRT-PCR.

Primer	Sequence (5'-3')
Anchored primers	
A	AAGCTTTTTTTTTG
B	AAGCTTTTTTTTTA
C	AAGCTTTTTTTTTC
Random primers	
a	GTTTTCGCAG
b	GATCAAGTCC
c	GATCCAGTAC
d	GATCACGTAC
e	GATCGTACAC
f	GATCTCAGAC
g	GATCATAGCC
h	GATCAATCGC
i	GATCTAACCG
j	GATCGCATTG

Recovery of differential fragments and their reamplifications

The differentially expressed bands were excised from the gel and washed two times. Then mashed the differentially expressed bands and added 25-35μL ddH_2O to incubate at 100°C for 10 min. After that, the tubes were incubated for 5 min at room temperature and centrifuged at 5000 rpm for 5 min. We transferred DNA into a new PCR tube and stored at -20°C. The reamplification system was in 25μL volume being consistent with the differential display RT-PCR, except that the template was changed to the stored DNA. And PCR condition remained the same. The PCR products were electrophoresed on 1.0% agarose gels, then we carried out large scale amplification of the product with the same size to make the final product 80-100μL. The differentially expressed bands were excised from the gel and extracted by gel extraction kit (GALEN BIOPHARM).

Cloning and sequence analysis of differential fragments

The differential cDNA fragments were cloned into pMD 18-T vector (TaKaRa) according to the manufacture's instructions. The positive clones were sequenced by BGI (Beijing, China). The target sequences were obtained after the vector and primer sequences were kicked off. These target sequences were searched at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The identity of the clones was determined from their similarity in the GenBank.

Quantitative RT-PCR verification of differential fragments

The reverse transcription condition was consistent with synthesis of first-strand cDNA, except that the anchored primer was Oligo dT (10mM). The cDNA was diluted ten times and stored at -20°C. The primer sequences of β-actin (internal reference) and the differential fragments were designed with Primer Premiers 5.0 (Table II). The products of reverse transcription were used as the templates of another PCR reaction in a 25μL volume: 2μL template, 2.5μL 10 x PCR buffer, 2.5μL $MgCl_2$ (25mM), 2μL dNTP (2.5mM), 0.125μL rTaq (5U/μL, TaKaRa), 2μL forward and reverse primers, and 15.375μL ddH_2O. The PCR procedure was 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 1 min at 60°C and 72°C for 1.5 min, and a final elongation for 10 min at 72°C. The PCR products were inspected by electrophoresis on 1.0% agarose gels. PCR without template was performed as the negative control.

Table II.- Primer sequences used in RT-PCR.

Primer	Sequence (5'-3')
COL6A3 Forward	AGCAAGAGGGAGGTCATAAACG
COL6A3 Reversed	CCTGTAACCAGCACCAGAAGC
β-actin Forward	GCGTGACATCAAGGAGAAGC
β-actin Reversed	GGAAGGACGGCTGGAAGA

Quantitative RT-PCR was performed with ABI 7500 and the reaction mixtures (10μL) consisted of 1μL cDNA, 5μL SYBR Premix Ex Taq™ (TaKaRa), 0.4μL forward and reversed primers, 3.6μL ddH_2O. The PCR procedure was 95°C for 5 min, followed 40 cycles of 30s at 95°C, 1min at 60°C and 30s at 72°C, then dropped quickly to 60°C and heated up to 95°C slowly to establish the dissolution curve of products.

Final quantification was calculated by the comparative C_T method (Martha *et al*., 2009). The C_T for the target amplicon and the C_T for the endogenous control were determined for each sample, and differences between them, called ΔC_T, were calculated to normalize for the differences in the RNA extractions and the efficiency of the RT step. The difference between ΔC_T for each experimental sample and ΔC_T of the calibrator was called the $\Delta\Delta C_T$ value. Finally, the amount of target, normalized to the endogenous control and relative to the calibrator, was calculated by $2^{-\Delta\Delta CT}$. The differential genes were highly expressed as long as $2^{-\Delta\Delta CT} \geq 2$ ($P<0.05$).

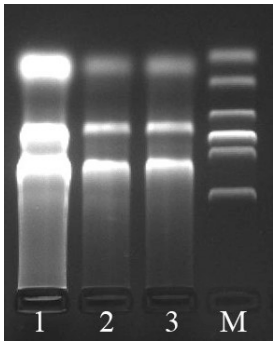

Fig. 2. Electrophoresis of RNA of reindeer antler mesenchyme. 1, the male reindeer; 2 and 3, the female reindeer; M, 2000bp DNA marker.

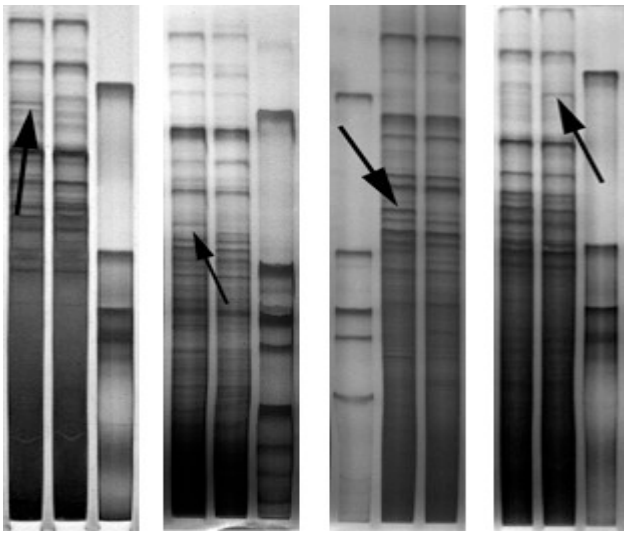

Fig. 3. Electrophoresis of partial products of DDRT-PCR. The lanes represent the DDRT-PCR from reserve mesenchyme of male and female reindeer antler tip. The differentially expressed fragments are indicated by arrows.

RESULTS

Differentially expressed fragments in antlers of both sexes

To identify genes differentially expressed in antlers of male and female reindeer, we compared the mRNA expression profiles in reserve mesenchyme of antlers of both sexes. The total RNA extracted from each sample showed 5S, 18S and 28S bands clearly (Fig. 2), and the A260/A280 value were all around 1.9. Besides, concentration of the male was higher than the female. We isolated 52 differential bands in all DDRT-PCR reactions.

However, only 7 of 52 differential fragments obtained were recovered successfully (Fig. 3), the four (Ae1, Af2, Cc2, Af3) performed a higher expression level in the male than the female, it's worth noting that the other three (Af1, Ce1, Ci2) had stronger expression level in the female reindeer. We recovered, cloned and sequenced successfully five differential fragments (Ae1, Af1, Af3, Ce1 and Ci2, 100bp-400bp; Fig. 4).

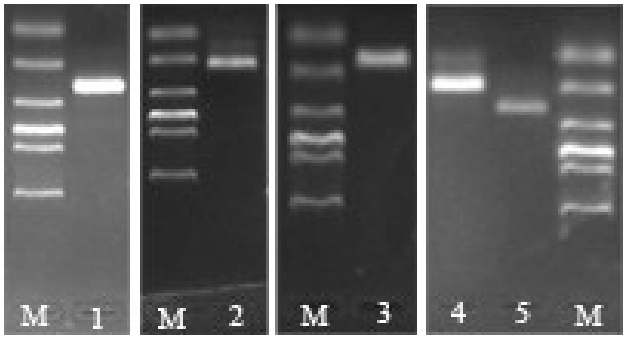

Fig. 4. Electrophoresis of recovered differential fragments. 1 to 5 represented fragments Ci2, Ce1, Ae1, Af1 and Af3, respectively. M, 2000bp DNA marker.

The sequence similarities and characterization of differentially expressed transcripts are summarized in Table III, and BLAST searches in NCBI Genbank revealed that the five differentially expressed fragments (Ae1, Af1, Af3, Ce1, Ci2) displayed significant similarities to known genes. Sequence Ae1 and Af3 shared 99%, 98% homologies with TPM4 gene (NM_001101162) and HMGB1 gene (BT030587), respectively. Sequence Af1 and Ce1 shared 95% homology with SMAD5 gene (XM_012178532) and COL6A3 gene (XM_005971413), respectively. Then sequence Ci2 was 93% homology with SLC17A5 gene (XM_005902349).

Verification of differential fragments with quantitative RT-PCR

To verify the expression profiles of COL6A3 gene in antlers of male and female reindeer, fluorescence-monitored quantitative real-time RT-PCR analysis was employed. The products, which were amplified with the primers of β-actin and COL6A3 genes, were consistent with the target fragments. The products of target gene (COL6A3) and internal reference gene (β-actin) showed a single peak at 83.5°C and 84°C in the dissolution curve, respectively (Fig. 5). Gene COL6A3 expressed both in antlers of male and female reindeer. However, there was no significant difference between antler mesenchymes of the male and female reindeer, following that the relative quantitative value was less than 2 (P>0.05, Table IV and Fig. 6).

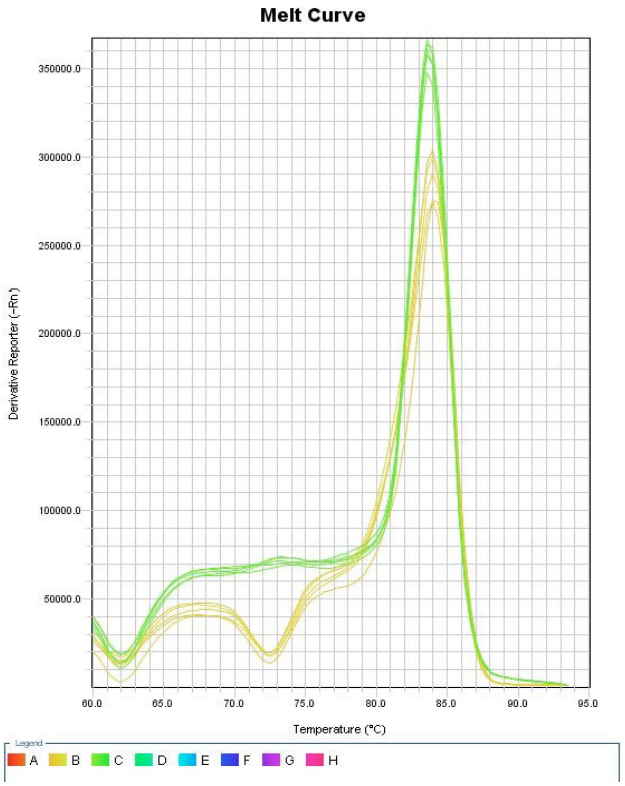

Fig. 5. Melt curve of real-time PCR. Yellow, β-actin gene. Green, COL6A3 gene

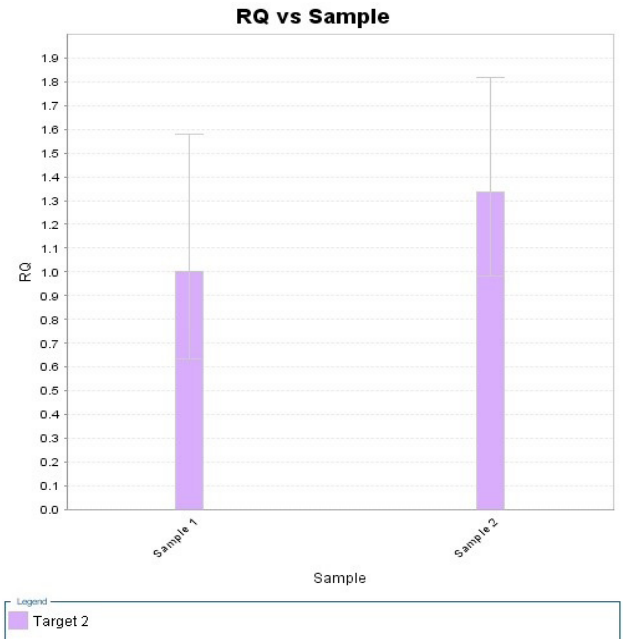

Fig. 6. Relative quantitative of samples. The 1 and 2 mean the relative expression levels of the reserve mesenchyme of the male and the female reindeer antler tip, respectively.

Table III.- Differentially expressed fragments and their Blast results.

Differential sequence	Primer combination	Gene/sequence in GenBank			Homology (%)
		Gene	Accession No.	Characterization	
Ae1	A/e	TPM4	NM_001101162	Bos taurus tropomyosin 4	99
Af1	A/f	SMAD5	XM_012178532	Ovis aries SMAD family member 5	95
Af3	A/f	HMGB1	BT030587	Bos taurus high-mobility group box1	98
Ce1	C/e	COL6A3	XM_005971413	Pantholops hodgsonii collagen, type VI, alpha 3	95
Ci2	C/i	SLC17A5	XM_005902349	Bos mutus solute carrier family 17 (acidic sugar transporter), member 5	93

Table IV.- Differential expression of COL6A3 gene in antler mesenchyme of male and female reindeer.

	C_T of gene COL6A3	C_T of gene β-actin	ΔC_T	$\Delta\Delta C_T$	RQ	RQmin	RQmax
The male	26.9±0.042	24.561±0.41	2.339±0.238	0	1	0.632	1.581
The female	26.516±0.229	24.595±0.156	1.921±0.16	-0.418	1.336	0.982	1.817

DISCUSSION

In our preliminary study, five differential expressed genes were isolated from mesenchyme in antlers of male and female reindeer using mRNA DDRT-PCR method, and they showed strong homology with genes of other species. Most of the genes indicated their alleged role related to the antler growth. Gene Ae1, Af1, Af3 and Ce1 were detected to be associated with the proliferation of antler tip, cellular transport and ossification mechanism, but gene Ci2 provides instructions for producing a protein called sialin that is located mainly on the membranes of lysosomes, compartments in the cell that digest and recycle materials, and further research remains to done to find the relationship with the antler molecular machinery. However, as one of the leading high-throughput technologies screening for changes in gene expression, differential display PCR has a shortcoming that it's not quantitative but qualitative. Therefore, quantitative real-time PCR should be performed to validate the changes in differential expression. Comparing the relative gene expression identified by high throughput assays and real-time could determine if the gene was truly differentially expressed. For this purpose, we used SYBR green detection and product melting curve analysis to validate the results from differential display PCR technologies. Furthermore, we chose SYBR green detection rather than TaqMan probes aiming to increase the flexibility to quickly validate numerous genes identified by the high-throughput screen.

Gene Af1, presented a higher expression in antler mesenchyme of female reindeer than the male reindeer, was found to share a high homology (95%) with the Ovis aries SMAD family member 5 (SMAD5) that related to transforming growth factor (TGF-β). Bone morphogenetic protein (BMP), as a member of TGF-β superfamily, was found to induce the osteogenic activity. Besides, of the nine SMAD protein that were found, SMAD5 and SMAD1 are involved in the signal transduction of BMP (Hild *et al*., 2000). Previous study has reported that the BMP7- induced inhibition of TGF-β-dependent profibrogenic activities in mesangial cells requires SMAD5 (Wang and Hirschberg, 2004). Then Retting and Buer reported that SMAD5 is required for endochondral bone formation (Retting *et al*., 2009). In our study, we found that gene Af1 had a higher expression in antler mesenchyme of the female reindeer, probably due to the difference in antler ossification degree between the male and female reindeer when we sampled, in other words, the antler of female reindeer began to ossify and gene Af1 was up-regulated expression. Further research remains to done to explain the phenomenon.

Gene Ae1 was observed a higher expression in antler mesenchyme of male reindeer, and shared a high homology (99%) of Bos taurus tropomyosin 4 (TPM4), which is one of the four identified tropomyosin (TPM1, TPM2, TPM3, TPM4) involved in the regulation of muscle contraction and cell membrane transport (Spinner *et al*., 2002). It's reported that TMs may be broadly categorized into high- and low-M_r species depending on their size, and suppression of high-M_r tropomyosin (TM) occurs in malignant cells, suggesting a role for these proteins in neoplastic transformation (Raval *et al*., 2003). Besides, regulating the expression of TM could hinder the process of malignant transformation in cells (Prasad *et al*., 1999). We detected a higher expression of gene Ae1 in

antler mesenchyme of male reindeer, and there were two conjectures: the male maybe need a higher expression of gene Ae1 to regulate cell carcinogenesis, and the antler of male reindeer may grow faster than the female.

The differentially expressed gene Af3 shared a high homology (98%) with Bos Taurus high-mobility group box1 (HMGB1), which is an important structural protein mainly distributed in mammalian cell nucleus (Suda *et al*., 2010). HMGB1 acts as a DNA chaperone, chromosome guardian, autophagy sustainer, and protector from apoptotic cell death inside of the cell, on the other hand, it's the prototypic damage associated molecular pattern molecule outside the cell (Rui *et al*., 2014). Therefore, the antler mesenchyme of male reindeer showed a higher expression of gene Af3, this may be one reason that the antler of male reindeer is obviously bigger than the female.

Gene Ce1 shared a high homology (95%) with Pantholops hodgsonii collagen, type VI, alpha 3 (COL6A3), whose primary role is in assembling collagen VI microfibrils (Te-Cheng *et al*., 2013). Collagen VI is the key component of bone and muscle, which mainly has the function of maintaining the integrity of the organization and guiding the signal transduction in the specific cell. Mutation in collagen VI genes cause congentital muscular dystrophy types Bethlem and Ullrich as well as intermediate phenotypes characterized by muscle weakness and connective tissue abnormalities. Collagen families were highly expressed in the antler tips using RNA-sequencing (Yang *et al*., 2015). In our study, gene COL6A3 presented a higher expression in antler mesenchyme of female reindeer, but there was no significant difference. Therefore, further studies to evaluate the function of COL6A3 gene related with antler growth regulation.

This study was designed for the identification of differentially expressed genes of antler mesenchyme between the male and female reindeer. We observed successfully the five differentially expressed genes and screened several candidate genes with respect to the regulatory genes of antler mesenchyme in both sexes reindeer. However, additional evidence is required in future.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Ahmed, N., Siddiqui, A.A. and Ahmed, A., 2000. DDRT-PCR: use of agarose gels for detection of amplified products. *Mol. Vis.*, **6**: 144-147.

Breyne, P. and Zabeau, M., 2001. Genome-wide expression analysis of plant cell cycle modulated genes. *Curr. Opin. Pl. Biol.*, **4**: 136-142. https://doi.org/10.1016/S1369-5266(00)00149-7

Hild, M., Dick, A., Bauer, H., Schulte-Merker, S., Haffter, P., Bouwmeester, T. and Hammerschmidt, M., 2000. The roles of BMPs, BMP antagonists, and the BMP signaling transducers Smad1 and Smad5 during dorsoventral patterning of the zebrafish embryo. *Ernst Schering Res. Found. Workshop*, **29**: 81-106. https://doi.org/10.1007/978-3-662-04264-9_6

Leader-Williams, N., 1979. Age-related changes in the testicular and antler cycles of reindeer, Rangifer tarandus. *J. Reprod. Fertil.*, **57**: 117-126. https://doi.org/10.1530/jrf.0.0570117

Li, C., Clark, D.E., Lord, E.A., Stanton, J.A.L. and Suttie, J.M., 2002. Sampling technique to discriminate the different tissue layers of growing antler tips for gene discovery. *Anat. Rec.*, **268**: 125-130. https://doi.org/10.1002/ar.10120

Liang, P. and Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, **257**: 967-971. https://doi.org/10.1126/science.1354393

Martha, P.G., Cena, M., Childs, L.A., Lacson, J.C., Thomas, P., Betthauser, J.M., Goueleke, P.J., Koppang, R.W., Gail, L. and Patricia, F., 2009. Identification of differentially expressed genes in individual bovine preimplantation embryos produced by nuclear transfer: improper reprogramming of genes required for development. *Nature*, **4**: 708-708.

Prasad, G., Masuelli, L.M. and Harindranath, N., 1999. Suppression of src-induced transformed phenotype by expression of tropomyosin-1. *Oncogene*, **18**: 2027-2031. https://doi.org/10.1038/sj.onc.1202264

Price, J.S., Allen, S., Faucheux, C., Althnaian, T. and Mount, J.G., 2005. Deer antlers: a zoological curiosity or the key to understanding organ regeneration in mammals? *J. Anat.*, **207**: 603-618. https://doi.org/10.1111/j.1469-7580.2005.00478.x

Raval, G.N., Bharadwaj Slevine, E.A., Willingham, M.C., Geary, R.L., Kute, T. and Prasad, G.L., 2003. Loss of expression of tropomyosin-1, a novel class II tumor suppressor that induces anoikis, in primary breast tumors. *Oncogene*, **22**: 6194-6203. https://

doi.org/10.1038/sj.onc.1206719

Retting, K.N., Buer, S., Yoon, B.S. and Lyons, K.M., 2009. BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation. *Development*, **136**: 1093-1104. https://doi.org/10.1242/dev.029926

Rui, K., Chen, R., Zhang, Q., Wen, H., Sha, W., Cao, L., Jin, H., Yan, Y., Fan, X.G. and Yan, Z., 2014. HMGB1 in health and disease. *Mol. Aspects Med.*, **40**: 1-116. https://doi.org/10.1016/j.mam.2014.05.001

Spinner, B.J., Zajdel, R.W., Mclean, M.D., Denz, C.R., Dube, S., Mehta, S., Choudhury, A., Nakatsugawa, M., Dobbins, N. and Lemanski, L.F., 2002. Characterization of a TM-4 type tropomyosin that is essential for myofibrillogenesis and contractile activity in embryonic hearts of the Mexican axolotl. *J. cell. Biochem.*, **85**: 747-761. https://doi.org/10.1002/jcb.10178

Suda, K., Takeuchi, H., Ishizaka, A. and Kitagawa, Y., 2010. High-mobility-group box chromosomal protein 1 as a new target for modulating stress response. *Surg. Today*, **40**: 592-601. https://doi.org/10.1007/s00595-009-4232-1

Te-Cheng, P., Rui-Zhu, Z., Dessislava, M., Machiko, A., Yejia, Z., Sasha, B., Khurana, T.S., Bnnemann, C.G., Birk, D.E. and Mon-Li, C., 2013. COL6A3 protein deficiency in mice leads to muscle and tendon defects similar to human collagen VI congenital muscular dystrophy. *J. biol. Chem.*, **288**: 14320-14331. https://doi.org/10.1074/jbc.M112.433078

Yang, X., Chen, Y., Liu, X., Liu, Q., Pi, X., Liu, Y. and Zheng, D., 2015. De Novo characterization of velvet skin transcriptome at the antlers tips of red deer (*Cervus elaphus*) and analysis of growth factors and their receptors related to regeneration. *Pakistan J. Zool.*, **48**: 151-157.

Wang, S. and Hirschberg, R., 2004. Bone morphogenetic protein-7 signals opposing transforming growth factor in mesangial cells. *J. biol. chem.*, **279**: 23200-23206. https://doi.org/10.1074/jbc.M311998200