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Effects of macrophage-conditioned medium on sika deer (*Cervus nippon*) antler stem cells

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Abstract

Context. Immune system has been claimed as the 'main switch' of tissue or organ regeneration. Among immune cells, macrophages stand out as important modulators in mutiple regeneration models, such as planarian, axolotl, mammalian hair and liver. As a unique model for mammals, deer antler is considered to ideal for studying complete mammalian organ regeneration. Studies have found that antler regeneration is a stem cell-based process and antler stem cells locate in the pedicle periosteum (PP). Although the regulatory roles of the immune system in other regeneration models have been extensively studied, they remain unstudied in antler regeneration.

Aims. To explore the possible role of macrophages in the PP cells (PPCs).

Methods. We treated PPCs with a macrophage-conditioned medium (MCM) and detected effects of MCM on proliferation, migration and apoptosis of the PPCs, and identified differentially expressed genes by using the RNA-seq technique.

Key results. We found that MCM enhanced proliferation rate and migration rate significantly and stimulated apoptosis of the PPCs. Using the RNA-seq technique, we identified 112 differentially expressed genes in the PPCs (38 downregulated and 74 upregulated) after the MCM treatment. Furthermore, gene-ontology annotation analyses showed that the upregulated genes were mainly involved in cell adhesion, chemotaxis, wound healing, growth factor-stimulated responses, and bone formation, and the downregulated genes were involved in regulation of biosynthesis.

Conclusions. MCM had a great influence on the antler stem cells, and macrophages might regulate antler regeneration through altering the microenvironment and gene-expression profiles of the PPCs.

Implications. We believe that the results of the present study would facilitate the discovery of the roles of immune system in antler stem cells and, thus, mammalian organ regeneration in general.

Additional keywords: antler regeneration, differentially expressed genes, macrophages, RNA-seq.

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Introduction

Immune system and tissue or organ regeneration have been found to be closely related; the former plays an important role in regulation of the latter. Among immune cells, macrophages have been found to be essential for tissue or organ regeneration. Godwin *et al*. (2013) reported that macrophages are indispensible in limb regeneration of axolotl. Using a model of mouse digit-tip regeneration, Simkin *et al*. (2017) found that the regeneration process and wound re-epithelialisation were completely inhibited when macrophages were depleted. These findings collectively showed that macrophages are essential in coordinating the epimorphic regenerative response. However, the immune system can trigger regeneration of a fingertip only, but not the whole finger, in mammals. Antlers offer a novel model to study the entire mammalian organ regeneration.

Antler regeneration occurs in yearly cycles consisting of growth, calcification, velvet-skin shedding and antler casting (Li and Suttie 2003). The full annual regeneration of deer antlers is unique among mammals and is a stem cell-based process (Li *et al*. 2009). Antler regeneration results from proliferation and

differentiation of the pedicle periosteum cells (PPCs; Li *et al*. 2007). The PPCs express key stem-cell markers and can be induced to differentiate into multiple cell lineages, so that the PPCs are termed antler stem cells (Wang *et al*. 2019).

Annual antler regeneration is triggered by the process of hard-antler casting from the pedicle. Thereafter, rough cast surface on the pedicle stump is quickly healed by a rim of skin that is shiny and sparsely populated with hair. At the same time, PP becomes thickened through the active division of cells resident in the PP to form a new antler growth centre. Although regulatory role of the immune system in antler regeneration is not known, we speculated that immune cells, particularly macrophages, would be indispensible. To study the effects of macrophages on antler regeneration, we treated the antler stem cells with macrophage-conditioned medium (MCM) *in vitro* and identified 112 differentially expressed genes (DEGs) by using RNA-seq approach. Some of these DEGs were further confirmed using quantitative polymerase chain reaction (qPCR). We believe that studying the function of these DEGs would facilitate the discovery of the roles of immune system in antler stem cells and, thus, mammalian organ regeneration in general.

Materials and methods

Pedicle periosteum cells were isolated from the sampled PPs of three deer heads and allocated into two groups: Group 1, which was treated with the MCM; and Group 2, which was treated with complete dulbecco modified eagle medium (DMEM).

Culture of PPCs

Pedicle periosteum was collected from the heads of three slaughtered 2-year-old male sika deer (*Cervus nippon*) in a deer slaughter plant, according to the protocol described by Li and Suttie (2003). The primary culture of PPCs was established following the protocol described by Li *et al*. (2012). The PPCs were trypsinised and transferred into T75 flasks (Nest Biotechnology, Hong Kong, China) in the complete medium ((DMEM, Gibco BRL, Life Technologies, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Gibco BRL Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, and 1 mg/mL streptomycin), frozen and stored in liquid nitrogen containing 90% FBS + 10% dimethyl sulfoxide when they reached 80% confluent. PPCs were retrieved from the storage and grown into subconfluence (~85%) in T75 flasks before use.

Collection of macrophages from sika deer

Peritoneal macrophages from sika deer were harvested through lavage of the peritoneal cavity with 500 mL of DMEM basal medium (intraperitoneal injection). The collected cells were washed twice with phosphate-buffered saline (PBS), resuspended in DMEM complete medium, then transferred into T75 flasks and 12-well plates and incubated for 3 h at 37°C, supplemented with 5% CO_2. Non-adherent cells in the flasks were washed away with PBS, and the adherent macrophages were replenished with DMEM complete medium. The cells in the 12-well plates were stained using α-naphthyl acetate staining for the confirmation of macrophages. Medium from macrophage culture was collected, and it was defined as MCM.

MTT cell-proliferation assay

Pedicle periosteum cells were seeded in 96-well plates at a density of 5000 cells/well. The MCM and DMEM complete medium were added to different wells that were allocated to Group 1 and Group 2 respectively, at the final volume of 200 μL. The PPCs were incubated for 24, 48, 72 and 96 h at 37°C, supplemented with 5% CO_2. Each treatment was technically repeated three times. After the incubation, 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) reagent (5 mg/mL, Sigma, St Louis, MO, USA) was added to each well and incubated for further 2 h, until purple precipitate was visible. The medium was then removed and 150 μL of dimethyl sulfoxide was added. Optical density value was measured at 560 nm by using an enzyme-linked immunosorbent assay reader (Tecan, Grodig, Austria).

Migration assay

Migration assay was performed using Ibidi cell-migration plates (Ibidi, InVitro Technologies, Munich, Germany) consisting of silicon-based cell-culture inserts with two reservoirs. The PPCs were trypsinised and diluted to 2×10^5/mL. In total, 70 μL of cell suspension was added to each reservoir. When the cells reached confluence, the inserts were removed and the wells were washed with PBS. The MCM and DMEM complete medium were added to wells of two different groups respectively. Reaction was stopped 24 h after medium addition by removing the culture medium. The cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min, and then stained with crystal violet dye. The migration images were photographed under an inverted microscope 24 h after incubation. Number of the migrated cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Flow cytometry

Apoptosis of the PPCs was evaluated on the basis of staining with both Annexin V-FITC and PI, according to the manufacturers' instructions. Annexin V-FITC staining was used to quantitatively determine the percentage of cells that were undergoing apoptosis. Cells that were positively stained with Annexin V-FITC and negatively stained with PI were considered as apoptotic. Cells that were positively stained with both Annexin V-FITC and PI were considered as necrotic. PPCs were treated with MCM (Group 1) and DMEM complete medium (Group 2) for 48 h, stained with both Annexin V-FITC and PI, according to the instructions of Annexin V-FITC and PI apoptosis detection kit (KGA107, KeyGEN BioTECH, NanJing, China). Flow-cytometry analysis was performed using FACSCalibur (BD Biosciences, Franklin Lake, NJ, USA), and the results were analysed with Cellquest software (BD Biosciences).

RNA preparation and RNA-seq sequencing

Pedicle periosteum cells from three male sika deer (*Cervus nippon*) were cultured in either MCM (PPC-T1-3, Group 1) or

DMEM complete medium (PPC-C1-3, Group 2). Once confluence was reached, total RNA of the PPCs was extracted using PureLink®RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instruction. A total of 20 μg of RNA per sample was used to construct the cDNA library. Agilent Bioanalyzer 2100 system (Agilent, CA, USA) was used for assessing quality of the library. In addition, the Illumina HiSeq 2000 instrument (Illumina, San Diego, CA, USA) was used for sequencing the amplified fragments and 150-bp paired-end reads were obtained.

Sequence analysis

The high-quality clean reads were obtained by filtering out the low-quality and contaminating reads in the raw data. The clean reads were mapped on the white-tailed deer (*Odocoileus virginianus*) genome. DEGs were identified on the basis of the mapped counts by using DESeq2 v2.1.18 R package (Anders and Huber 2010) at $\log_2$(fold change) ≥ 2 and an adjusted *P*-value of 0.001. Gene-ontology (GO) annotation of the DEGs was performed online by using the David database (Version 6.8, http://david.abcc.ncifcrf.gov/, accessed April 2019). The interactive network containing the identified DEGs was constructed using the online String database (Version 10.5, https://string-db.org, accessed July 2019).

Quantitative real-time polymerase chain reaction (qRT–PCR) analyses

Quantitative real-time polymerase chain reaction (qRT–PCR) method was used to validate the differentially expressed mRNA between Group 1 and Group 2. A total of 1 μg of RNA was used to make single-stranded cDNA by using PrimeScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time; TaKaRa, DaLian, China). Primers for qRT–PCR were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesised by Sangon Biotech Co. Ltd (Shanghai, China). By using a SYBR Green-based PCR assay, qPCR was performed with LightCycler 480 II (Roche, Basel, Switzerland). The qPCR mixture system and programs were similar to those described in our previous publication (Ba *et al*. 2016). Expression values of the selected genes were calculated according to $2^{-\triangle\triangle Ct}$ method, after normalisation against values of glyceraldehyde 3-phosphate dehydrogenase.

Statistical analyses

Statistical analyses were performed using Student's *t*-test (GraphPad Prism software 5.0, GraphPad, San Diego, CA, USA). All quantitative data are given as means ± s.d. for at least three technical repeats. Significance was declared at $P < 0.05$.

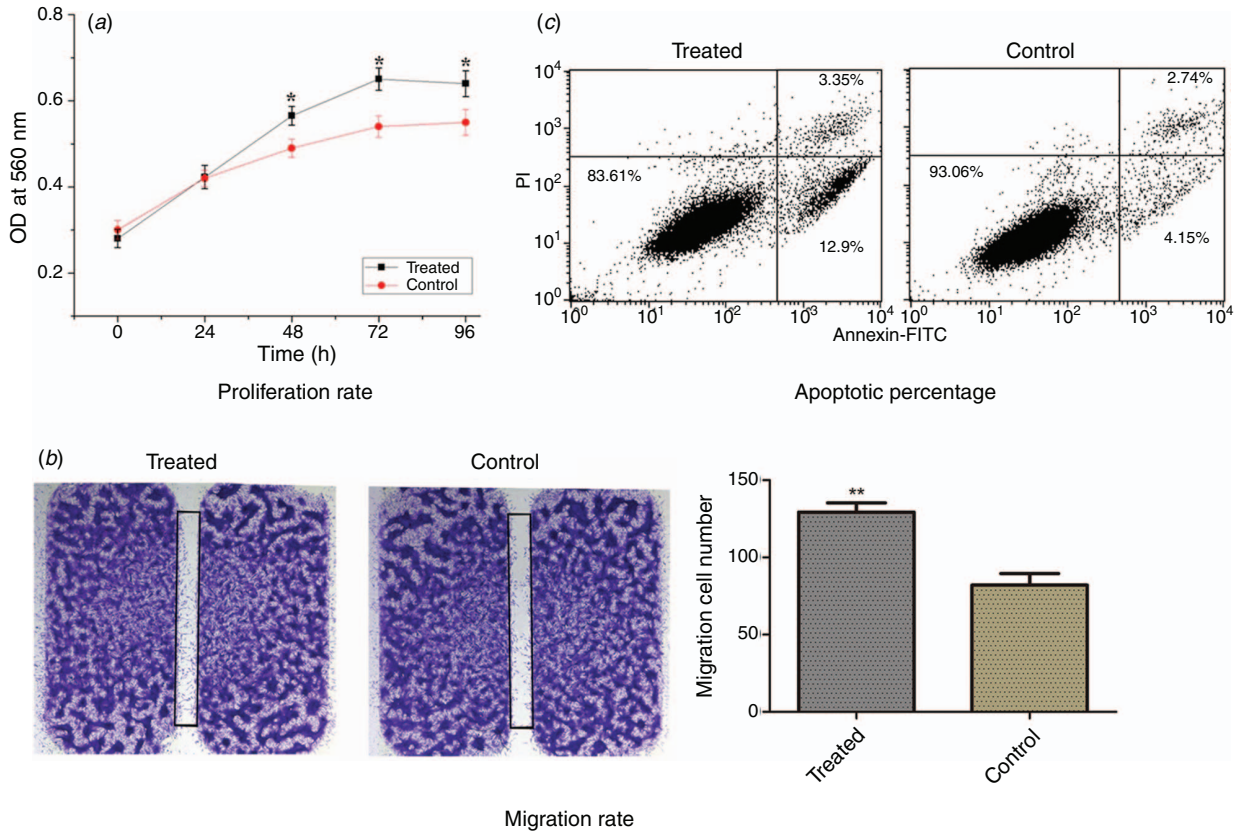

Fig. 1. Influence of macrophage-conditioned medium (MCM) on pedicle periosteum cells (PPCs). (*a*) Proliferation rate. (*b*) Migration rate. (*c*) Apoptotic percentage. Treated, PPCs were treated with MCM; control, PPCs were treated with complete dulbecco modified eagle medium (DMEM). Note that MCM significantly stimulated proliferation rate (*a*), migration rate (*b*) and apoptotic percentage (*c*) of PPCs. Values are means ± s.d. *, $P < 0.05$; **, $P < 0.01$.

Results

Effects of MCM on proliferation, migration and apoptosis of the PPCs

The results of the cell-proliferation assay showed that MCM increased the proliferation rate of the PPCs in both groups during the period of 24 h and 96 h after the treatment, and the difference in proliferation rates between Group 1 and Group 2 became significant ($P < 0.05$) from 48 h onward, till 96 h (Fig. 1*a*).

The results of the cell-migration assay showed that the number of the migrated PPCs in Group 1 was significantly higher than that in Group 2 (Fig. 1*b*), suggesting that MCM has the ability to significantly promote the migration rate of PPCs.

The results of the cell-apoptosis assay showed that the percentage of apoptotic cells in Group 1 was higher than that in Group 2 (8.75%), suggesting that the MCM stimulated apoptosis of the PPCs from 48 h of incubation onward (Fig. 1*c*).

High-throughput sequencing results

High-throughput sequencing of the libraries of Group 1 and Group 2 generated a total of 70 812 796 and 61 326 492 paired-end clean reads respectively. The clean bases from Group 1 and Group 2 aligned successfully with the white-tailed deer (*Odocoileus virginianus*) genome, and accounted for 84.15% and 77.69% of the total reads respectively (Table 1).

On the basis of the results of the hierarchical clustering analysis, two groups could be clearly separated (Fig. 2). The results showed that there was a significant difference in the overall expression pattern between Group 1 and Group 2, indicating that the quality of the sequencing data was high and that the data could be used for the further analysis.

Identified DEGs

In total, 112 DEGs were identified between Group 1 and Group 2. Among the DEGs, 74 were found to be upregulated and 38 downregulated in Group 1 (Fig. 3).

All DEGs in the two groups were subjected to GO annotation by using the David database (online). The main enriched biological functions of GO terms on the upregulated and downregulated genes are presented in Fig. 4. The GO annotation of the upregulated genes in Group 1 was found to be mainly involved in cell chemotaxis, wound healing, growth factor-stimulated responses and bone formation, whereas the downregulated genes were mainly involved in regulation of biosynthesis, the synthesis of protein and lipid.

The interaction network of DEGs was analysed using String database (online), as shown in Fig. 5. In total, 72 of the 112 DEGs were found to be involved in the interaction network (47 upregulated and 25 downregulated).

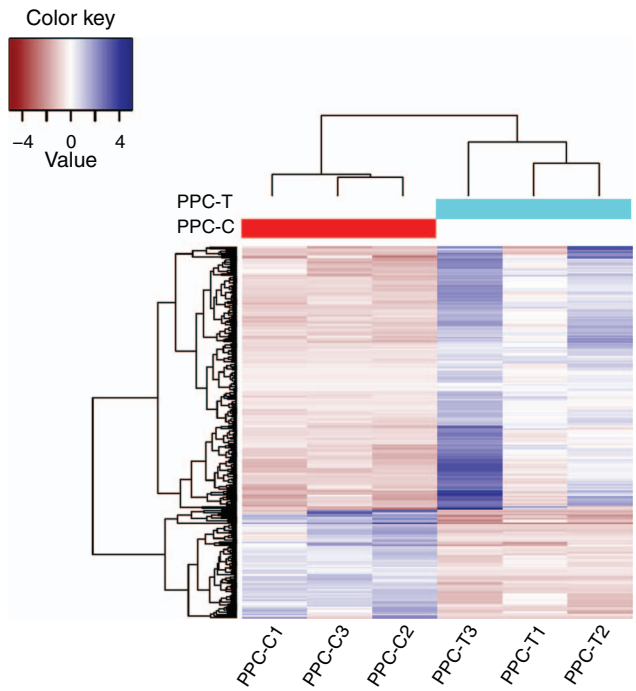

Fig. 2. Results of the hierarchical clustering analysis. Note that the analysis could clearly separate the treated group (PPC-T) from the control group (PPC-C).

Table 1. Statistical results of RNA-seq

PPC-T, treated; PPC-C, control; GC, in clean bases, guanine (G) and cytosine (C) as a percentage of total bases; Q20, in clean bases, the ratio of bases with a quality value greater than 20 to the total number of bases

Group	Sequencing data					Comparison with the reference genome
	Clean reads	Read length (bp)	Clean base	GC (%)	Q20 (%)	Mapped ratio (%)
			Group 1			
PPC-T1	20 926 373	150	6 277 911 900	51.48	99.00	80.69
PPC-T2	21 641 791	150	6 492 537 300	53.94	98.91	85.30
PPC-T3	28 244 632	150	8 473 389 600	53.02	98.93	86.46
			Group 2			
PPC-C1	21 501 368	150	6 450 410 400	52.72	98.99	83.09
PPC-C2	20 447 418	150	6 134 225 400	54.47	98.88	71.89
PPC-C3	19 377 706	150	5 813 311 800	51.51	98.95	78.08

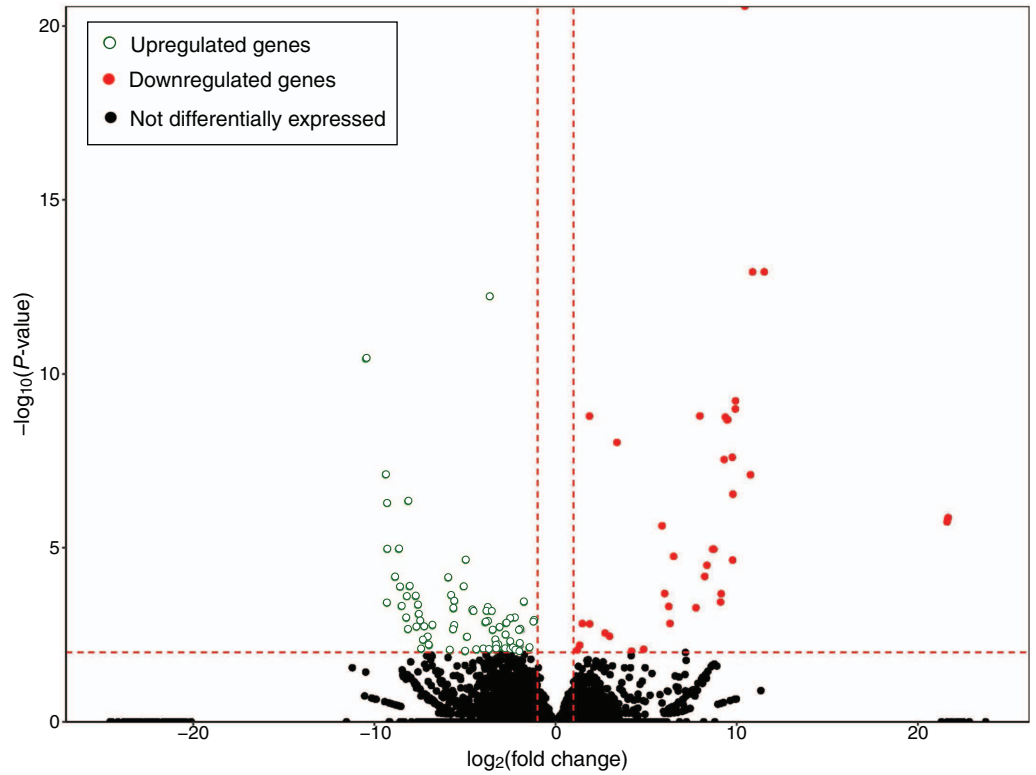

Fig. 3. Differentially expressed genes (DEGs) expressed in a volcano chart. Each dot indicates one gene in Group 1. (○), Upregulated (●), downregulated; (●), not differentially expressed between Group 1 and Group 2.

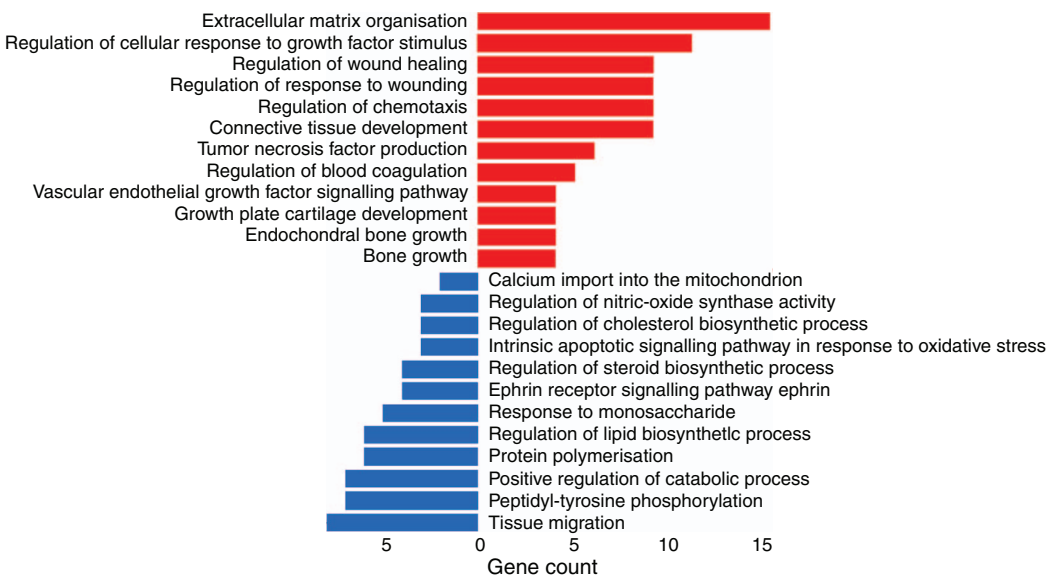

Fig. 4. Differentially expressed genes (DEGs) in Group 1 in the gene-ontology (GO) functional annotation. *x*-axis, number of the enriched genes; *y*-axis, name and classification of GO. (■), Upregulated genes; (■), downregulated genes.

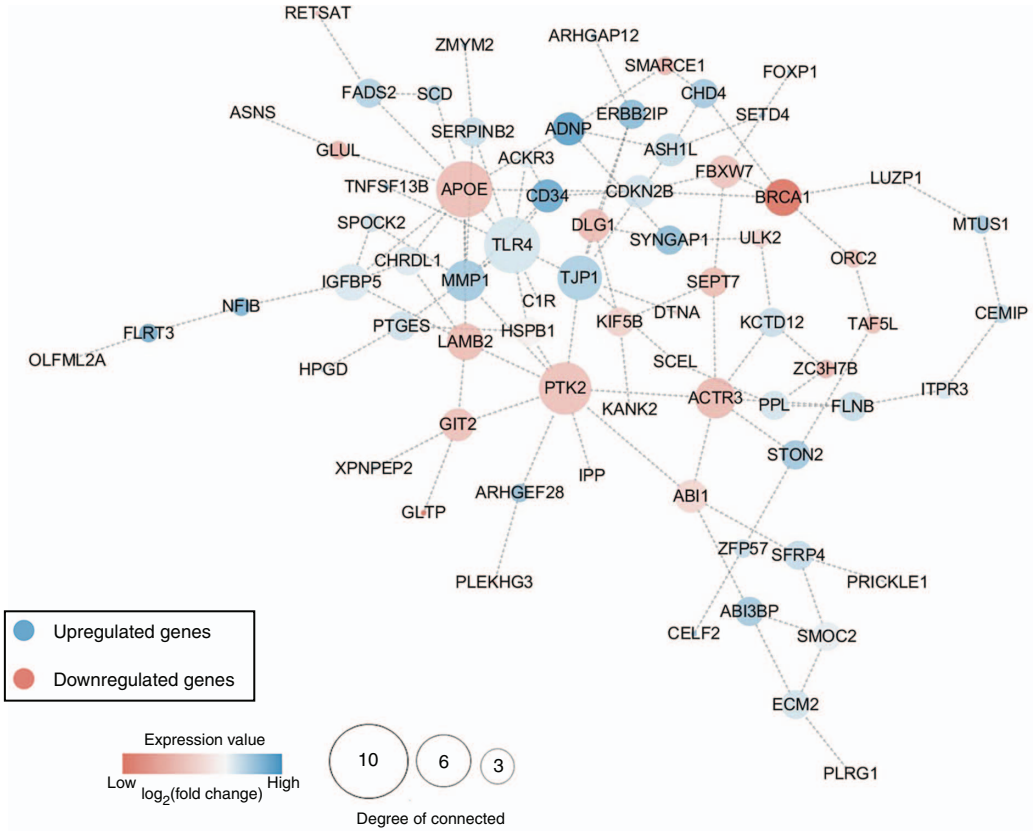

Fig. 5. Interactive network of the differentially expressed genes (DEGs) in Group 1. Nodes represent genes. Interactive network consisted of 72 DEGs, including 47 upregulated and 25 downregulated genes. Value of $\log_2$(fold change) of genes is indicated by the grade of the colour. The size of the node indicates the degree of connected; the larger the node, the higher the degree of connected with others for a given gene.

Confirmation of selected genes by using qPCR

So as to verify the reliability of the RNA-seq data, we selected four DEGs for conducting the qPCR analysis. Expression values were normalised against values of glyceraldehyde 3-phosphate dehydrogenase. The qPCR results showed that the changes in the expression level of the four DEGs between Group 1 and Group 2 were similar to those from the RNA-seq, suggesting that the RNA-seq data were reliable for identification of the DEGs in the present study (Fig. 6).

Discussion

Deer antlers are the unique mammalian appendages that are subject to an annual cycle of epimorphic regeneration. Previous studies have reported that histogenesis of antler regeneration relies on the cells resident in the PP, and that the PP cells (PPCs) are the antler stem cells (Li *et al*. 2009). Rapid antler growth requires rapid development of cartilage and blood vessels. Results of the GO annotation analysis in the present study showed that the upregulated genes in the PPCs that were treated with MCM were mainly involved in biological functions related to cartilage and vascular development, vascular endothelial growth-factor signalling pathway, bone growth, endochondral bone growth and growth-plate cartilage development. Therefore, our results support the notion that the macrophages play an important role in antler regeneration.

Macrophages are one of the major immune cells. When antlers are cast from pedicles, macrophages are likely to be recruited to the wound. Cytokines secreted by macrophages may act on the PPCs. In our study, MCM enhanced the rates of proliferation and migration, and stimulated apoptosis of the PPCs. DEGs (refer to the following described genes) in the PPCs related to immunity and regeneration were also upregulated significantly after the treatment with MCM. These results indicated that macrophages may play an important role in regulating antler regeneration through stimulating growth and gene expression of the PPCs.

TLR4 gene encoding the Toll-like receptor (TLR) family 4 protein plays a fundamental role in pathogen recognition and activation of innate immunity (Yu *et al*. 2018). The TLR family consists of 11 members, each of which can bind to different ligands and play an exclusive role (Kawai and Akira 2010). We found that the *TLR4* gene was upregulated significantly in the PPCs, suggesting that MCM might act on PPCs through binding to TLR4. *ACKR3* gene is also known as *CXCR7*, and encodes a member of the G-protein-coupled receptor family. The CXCR7 is the receptor of CXCL12, which is a kind of chemokine. Recent studies have reported

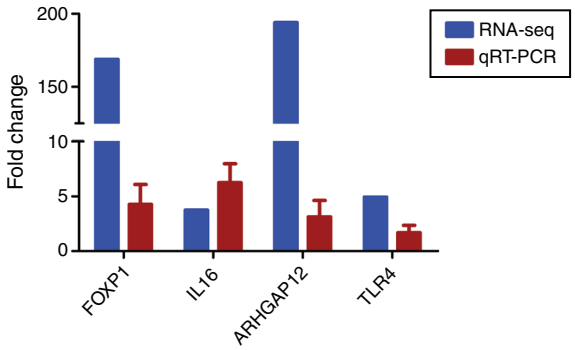

Fig. 6. Four genes from RNA-seq were verified using quantitative polymerase chain reaction (qPCR) analysis. Means of the normalised mRNA expression values (n =3 pools) were calculated and are expressed as fold changes. The quantitative real-time (qRT)–PCR data are expressed as means ± s.d. Note that results of the qPCR analysis had a trend similar to that of the RNA-seq.

that CXCL12 expression in THP-1 monocytes (u-THP-1) can be induced by applying lipopolysaccharides and a TLR ligand (Yu *et al*. 2018). The *CXCR7* gene was upregulated in Group 1 PPCs, suggesting that the PPCs may secrete its ligand CXCL12. CXCL12 can recruit immune cells or other types of cells around the pedicle wound to interact with PPCs and to regulate their growth and differentiation.

ARHGAP12 gene encodes the Rho GTPase, which can activate Rho-type guanosine triphosphate metabolising enzymes (Rho A). Rho A is involved in TGF-β signalling pathway. ERBB2IP is known as an ERBIN, or ERBB2 or HER2 interactive protein. ERBIN negatively modulates TGF-β-dependent cell growth (Wang *et al*. 2015). In our study, *ERBB2IP* gene was downregulated and *ARHGAP12* gene upregulated in Group 1 PPCs. At the same time, *TGFBR2* gene was upregulated, which encodes TGF-β receptor 2. Our results indicated that MCM may play a role in regulating growth of PPCs through the TGF-β pathway.

Recent research has identified ABI3BP as an important protein for mesenchymal stem cells. ABI3BP can promote differentiation of mesenchymal stem cells (Hodgkinson *et al*. 2014). *SMOC2* (SPARC-related modular calcium-binding 2) gene encodes for the matricellular protein SMOC2, which is highly expressed during embryogenesis and cicatrisation (Araujo *et al*. 2017). SMOC2 may function as an angiogenic factor that potentiates the angiogenic effects of growth factors (Song *et al*. 2015). In our study, we found that both *ABI3BP* and *SMOC2* genes were upregulated in Group 1 PPCs, suggesting that MCM may stimulate angiogenesis.

IGFBP5 and *CD34* genes were also upregulated in Group 1 PPCs. IGFBP5 (insulin-like growth factor-binding protein 5) and CD34 were reported to participate in regeneration of blood vessels (Hwang *et al*. 2016; Mathiyalagan *et al*. 2017). According to the analysis of the DEGs, genes including *IL16*, *ROBO1*, *MMP13* were upregulated. Previous studies have shown that those genes are related to immunity and regeneration (Godwin *et al*. 2013; Yang *et al*. 2017; Chen *et al*. 2019).

In adult mammals, response to tissue injury involves inflammation that includes mobilisation of and invasion by macrophages. A key goal in regeneration biology is to prevent pathological outcomes from uncontrolled infection or fibrosis and to enhance restoration of tissue function. Macrophages are essential in protecting the host from infection and have recently been shown to be essential in promoting regenerative responses. The present study has provided convincing evidence that macrophages have significant influence on antler stem cells and, thus, would likely play a role in antler regeneration.

Conclusions

On the basis of the previous reports that immune system plays a critical role in epimorphic regeneration in other model animals and our own discovery that MCM had a great influence on the antler stem cells (the cells for antler regeneration) in the present study, we conclude that immune system would also be indispensable for deer antler regeneration and this process is highly likely to be mediated by macrophages.

Conflicts of interest

The authors declare no conflicts of interest.

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