Original article

Molecular cloning, recombinant expression, and purification of osteocalcin in sika deer (*Cervus nippon*) antler

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Abstract

Osteocalcin is a major non-collagenous component of the bone extracellular matrix and is considered to be an indicative factor of osteoblast differentiation. In the present study, we detected osteocalcin expression in different antler areas and growth phases by immunohistochemistry. Osteocalcin was highly expressed in all areas during the mineralization period and in mesenchymal cell and chondrocyte areas during the rapid growth period. The nucleotide sequence of the *osteocalcin* gene in sika deer antler was determined. The open reading frame was 303 bp encoding a protein of 100 amino acids. The estimated molecular mass of osteocalcin was 10.38 kDa and the theoretical isoelectric point was 5.37. The *osteocalcin* gene with a 6× His-tag at the *C*-terminus was cloned into the pGEX-4T1 vector and expressed in *Escherichia coli* under optimal conditions. The recombinant soluble protein fused with GST was purified with Ni-NTA resin. The purified osteocalcin protein exhibited a significant increase in HA adhesion and promoted antler chondrocyte proliferation. Osteocalcin is an important factor in regulating the rapid growth and differentiation of deer antlers.

Key words: *Cervus nippon*, osteocalcin, molecular cloning, expression, purification

Introduction

Osteocalcin, also known as bone Gla protein, is synthesized and secreted exclusively by osteoblasts and osteocytes at the late stage of maturation. This protein is a major non-collagenous component of the bone extracellular matrix and is considered to be an indicative factor of osteoblast differentiation (Huang et al. 2016, Li et al. 2016). Osteocalcin undergoes post-translational modification whereby available glutamic acid residues are γ-carboxylated (Hauschka et al. 1975, Price et al. 1976). Mature osteocalcin is secreted into the skeletal microenvironment and then goes through a conformational change that aligns its calcium-binding Gla residues with the calcium ions in hydroxyapatite. This property was initially proposed as a mechanism that enables osteocalcin to initiate the formation of hydroxyapatite (HA) crystals (Ducy et al. 2011). There are two forms of osteocalcin in serum: carboxylated and uncarboxylated (Hauschka et al. 1989).

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Recent data suggest intended roles for osteocalcin in the regulation of energy metabolism, cognition, and reproduction (Booth et al. 2016, Zhou et al. 2017). Undercarboxylated osteocalcin is the hormonally active isoform. It acts in a feed-forward loop to increase the proliferation of β-cells, as well as the production and secretion of insulin. Skeletal muscle and adipose tissue respond to osteocalcin by increasing their sensitivity to insulin (Zoch et al. 2016).

Deer antlers are male skull appendages in all deer species except for reindeer. Deer antlers are unique mammalian organs that are capable of repeated rounds of regeneration (Yao et al. 2012). In early spring, antlers begin to regenerate, and rapid antler growth occurs during late spring and summer. The fastest growth rate achieves 1-2 cm per day. During the growth phase, antlers are covered with a special type of soft velvet skin. In late summer and autumn, the antler attains full size and becomes calcified with loss of blood vessels and nerves, and the velvet skin is shed. In winter, the bony antlers are firmly attached to the living pedicle and are not shed until the next spring (Li et al. 2007, Li et al. 2014). Therefore, deer antlers offer an important model for research mechanisms of organ regeneration and rapid growth. In the present study, we detected osteocalcin expression in different areas and periods by immunohistochemistry. Osteocalcin was highly expressed in all areas during the mineralization period and in mesenchymal cell and chondrocyte areas during the rapid growth period. We expressed and purified recombinant sika deer osteocalcin and characterized its binding to HA and effect on cell proliferation of antler chondrocytes.

Materials and Methods

Materials

Plasmids pGEX-4T1 and pMD19T, competent *E.coli* Rosetta (DE3) and DH5α, restriction endonucleases *Eco*RI and *Not*I, T4 DNA ligase, Taq DNA polymerase, and DNA and protein ladders were purchased from TAKARA Biotechnology Co., Ltd. (Dalian, China). Rabbit anti-osteocalcin (bs-4917R) and rabbit anti-GST (bs-0663R) antibodies were purchased from Bioss Biotechnology Co., Ltd. (Beijing China). Goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase (111-055-003) or horseradish peroxidase (111-035-003) were purchased from Jackson Immunoresearch. HA was purchased from Sigma. Sika deer antler cDNA was prepared as described previously (Wang et al. 2017, Zhang et al. 2016). All chemicals used in this study were of analytical grade.

Immunohistochemical studies

Different stages of antlers were collected from a sika deer farm (Changchun, Jilin Province, China). The antler was cut lengthwise into 5 mm-thick slices. The slices were fixed with 4% paraformaldehyde, embedded in paraffin, deparaffinized, and rehydrated. The slices were then boiled for 2 min in 0.01 M citrate buffer and incubated with 0.3% H_2O_2 in methanol to block endogenous peroxidase. The slices were incubated with the anti-osteocalcin antibody (1:300 dilution), followed by incubation with a secondary antibody (1:1000 dilution) conjugated with peroxidase, and visualized with 0.05% DAB. The slices were then hematoxylin counterstained, dehydrated and sealed.

Construction of the expression plasmid

A candidate *osteocalcin* (OC) gene was identified in transcriptome databases. The gene was amplified from cDNA by RT-PCR using primers 5′-ATGAGAGCCCTTGTGCT-3′ and 5′-TTAGACCGGGCCGTAGA-3′. The PCR product was inserted into the subcloning vector pMD19T to generate cnOC-19T. For protein purification, *Cervus nippon* osteocalcin (cnOC) was labeled with a 6× His tag using primers 5′-CGGAATTCATGAGAGCCCTTGTGCT-3′ and 5′-ATGCGGCCGCTTAATGGTGATGGTGATGATGGACCGGGCCGTAGA-3′. The amplified fragment was digested with *Eco*RI and *Not*I, and inserted into the expression vector pGEX-4T1.

Expression and purification of recombinant cnOC

pGEX-4T1-cnOC-His$_6$ was transformed into *E.coli* Rosetta (DE3). The bacteria were induced to express recombinant protein by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to a culture with an OD_{600} of 0.6-0.8 and then incubating for a further 5 h at 37°C. The cells were harvested by centrifugation at $5000\times g$ for 2 min at room temperature. A portion of the pellet was prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and the remaining pellet was washed with 100 mM Tris-Cl (pH 6.5) twice. The cells were then resuspended in lysis buffer (100 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, pH 6.5, 0.13 mM PMSF, and 0.5 mg/m*l* lysozyme). After mixing on ice for 10 min, sodium deoxycholate (1.33 mg/ml) was added. The suspension was then mixed at 37°C for 20 min. DNase I was added at a concentration of 2000 U/ml with shaking at 150 rpm for 2 h at 37°C. The sample was centrifuged at 12,000×g and 4°C for 20 min.

The suspension was passed through a filter (0.22 μm) and then applied to a 1 ml HisTrap™ FF nickel ion affinity column (GE Healthcare), according to the
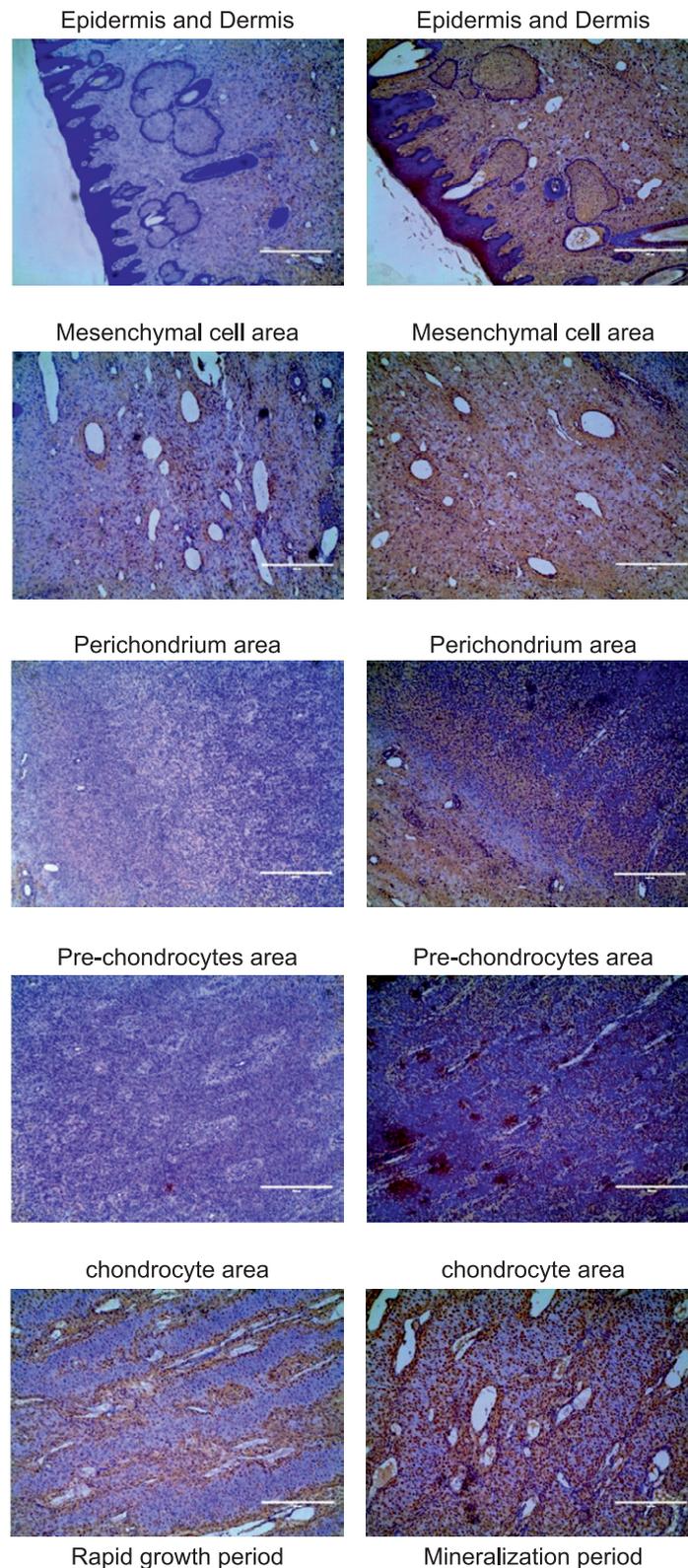
Fig. 1. Immunohistochemical analysis of osteocalcin expression in various parts and growth phases of antlers. Scale bars = 400 μm, magnification ×100.

manufacturer's instructions. After washing the column with binding buffer (200 mM NaCl, 100 mM Tris-Cl, 20 mM imidazole, 10% glycerin, pH 6.5), the fusion protein was eluted with elution buffer containing 250 mM imidazole at a flow rate of 1 ml/min. The eluted recombinant protein was then transferred into a 10 KD Ultra centrifugal filter (Millipore) to remove the high concentration of imidazole at 5000×g for 30 min at 4°C.
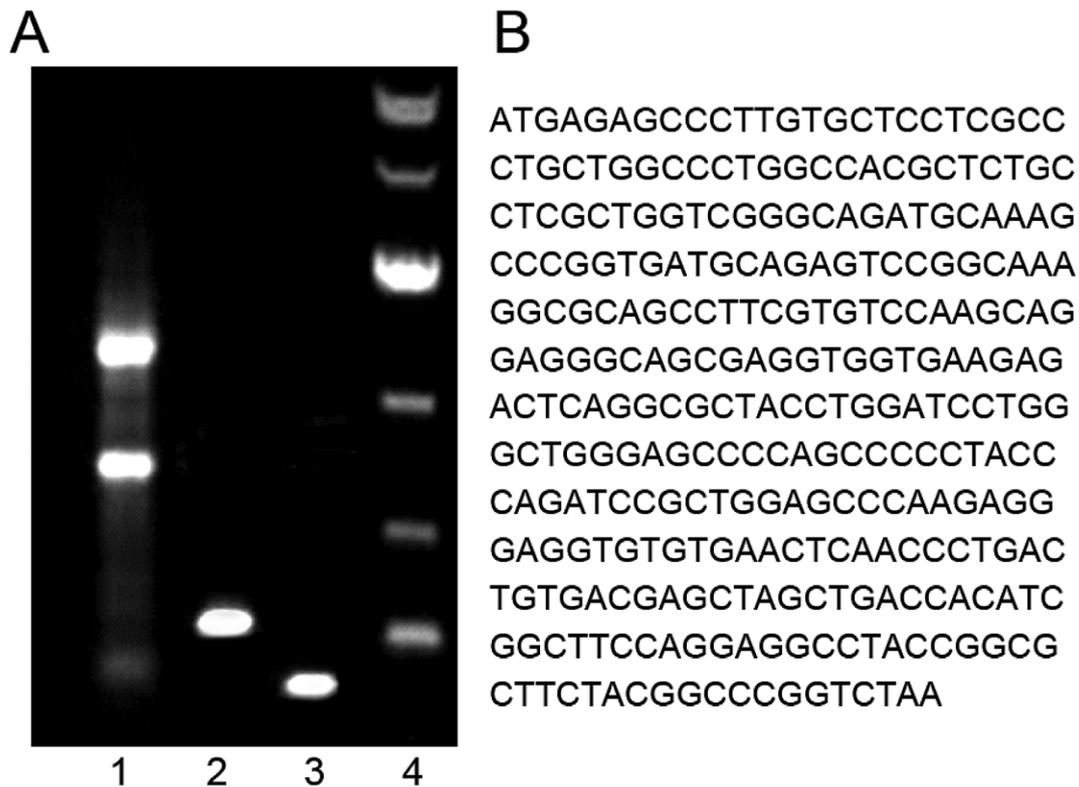
Fig. 2. Amplification of sika deer *osteocalcin* cDNA and sequence analysis. (A) PCR amplification of *cnOC* by RT-PCR from total mRNA of antlers. (B) Nucleotide sequence of *cnOC* cDNA.

Protein analysis

Samples collected from each step of the experiments were analyzed by SDS-PAGE. The gel was then stained with 0.25% Coomassie Blue R-250 and destained. For detection of proteins by Western blotting, samples were resolved by SDS-PAGE and then transferred to a nitrocellulose membrane. Protein was detected with anti-osteocalcin or anti-GST antibodies and anti-rabbit secondary antibodies. Immunoreactions were detected with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride solutions.

HA adhesion assay

The ability of purified cnOC to bind to HA was investigated by a binding assay. HA (50 μg) in 500 μl PBS, pH 6.8, was added to 80 μg cn-osteocalcin recombinant protein or GST protein. Samples were mixed at 37°C for 10 min and then centrifuged at 3000 *g* for 5 min. The supernatants were analyzed by SDS-PAGE and western blotting.

Cell viability assay

Antler chondrocytes were isolated and cultured in 96-well plates for 24 h (Price et al. 1994). The culture medium was removed, and the cells were incubated for 24 h in Dulbecco's modified Eagle's medium with 2% fetal bovine serum. The cells were treated with 160 μg/mL recombinant GST-cnOC or GST protein for 48 h. MTT was added to the cells for 4 h at 37°C with 5% CO_2. After removing the medium, DMSO was added to the wells, followed by incubation at room temperature for 8 min. The absorbance was measured at a wavelength of 590 nm using a microplate reader (Tecan Infinite 200PRO).

Results

Expression of osteocalcin in different parts and periods of antlers

Immunohistochemistry was employed to detect the distribution of cnOC expression in various parts and periods of antlers. Osteocalcin was ubiquitously expressed in most parts during rapid growth and mineralization periods at various levels. High expression was found in all parts in the mineralization period. Relatively high expression was detected in mesenchymal cell and chondrocyte areas in the rapid growth period. Moderate expression was found in the pre-chondrocyte area during the rapid growth period, and low expression in the epidermis, dermis, and perichondrium area during the rapid growth period (Fig. 1).
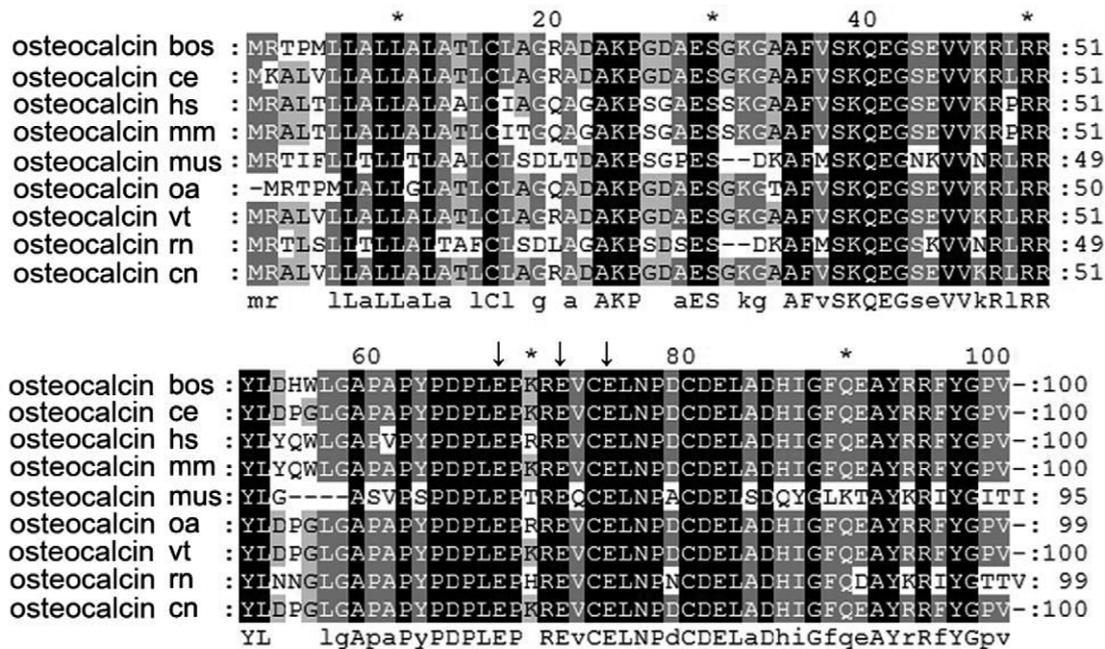
Fig. 3. Alignment of the amino acid sequences of cnOC and OC from other species. Bos: *Bos taurus*; hs: *Homo sapiens*; mm: *Macaca mulatta*; mus: *Mus musculus*; oa: *Ovis aries*; rn: *Rattus norvegicus*; cn: *Cervus nippon*. The black arrows indicate 100% conserved Gla residues.

Characterization and cloning of sika deer osteocalcin

We predicted the *cn-osteocalcin* gene sequence from sika deer transcriptome databases constructed previously (Zhao et al. 2013), and designed oligonucleotide primers based on the predicted sequence. We used these primers to perform RT-PCR for *cn-osteocalcin* and *β-actin* as a control using RNA isolated from sika deer antler chondrocytes (Fig. 2A). Amplified cDNA bands were purified and inserted into the pMD19-T vector for DNA sequencing (Fig. 2B). The *cn-osteocalcin* nucleotide sequence was 303 bp and encoded a protein of 100 amino acids with a predicted molecular weight of 10.83 kDa and theoretical isoelectric point of 5.37. The predicted secondary structure was 58% α-helices, 3% extended strands, 3% β-turns, and 36% random coils. Sequence analysis revealed that cn-osteocalcin shared 95% and 93% identity with osteocalcin in *Bos taurus* and *Ovis aries*, respectively. In addition, osteocalcin between sika deer and other species were highly conserved at the amino acid level. Sika deer osteocalcin containing three glutamic acids at sites known to be γ-carboxylated in other OCs (Fig. 3). The *cn-osteocalcin*-coding sequence was cloned into the prokaryotic expression vector pGEX-4T1, and a C-terminal 6× histidine tag was added to the protein.

Expression and purification of sika deer osteocalcin

After optimizing, the cells were induced with 0.1 mM IPTG and cultivation was continued for another 5 h at 37°C. Bacteria were then pelleted, and the GST-cnOC-His$_6$ protein was evaluated by SDS-PAGE with Coomassie Brilliant Blue staining (Fig. 4A, lane 3). The results showed that the soluble protein was highly expressed (Fig. 4A, lane 4). The GST-cnOC-His$_6$ recombinant protein was purified by Ni-NTA chromatography and eluted using 250 mM imidazole (Fig. 4A, lane 11). The sample was concentrated and desalinated using ultracentrifugal filters. The final yield of the purified protein was ~30 mg/l, and the total purity of GST-cnOC-His6 was >90%. Verification of the identity of the recombinant GST-cnOC-His6 protein after desalting was performed by western blotting. Fig. 4B shows that the purified protein could be detected by anti-osteocalcin and anti-GST antibodies.

Biological activity of sika deer osteocalcin

To analyze the ability of GST-cnOC-His6 protein to bind HA, we compared the adhesion characteristics of GST-cnOC-His6 to HA and that of the GST protein. As shown in Fig. 5, GST-cnOC-His6 protein bound significantly better to HA than GST after absorption to HA.

Antlers contain epidermis, blood vessels, cartilage, bone, nerves, and other tissues. The biological activity
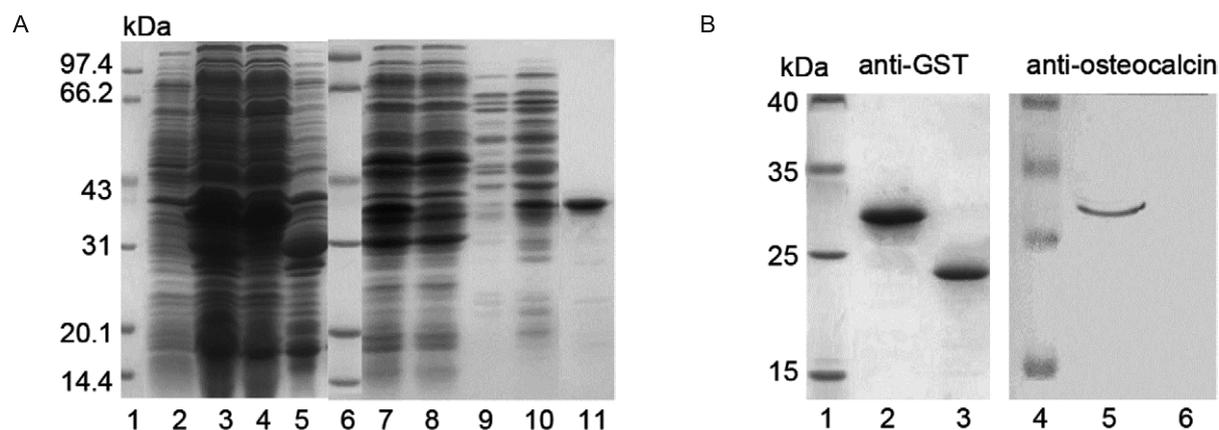
Fig. 4. Expression and purification of GST-cnOC-His6. (A) Samples collected from the purification steps of GST-cnOC-His6 were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining. Lane 1: protein marker; lane 2: without IPTG induction; lane 3: with IPTG induction; lane 4: soluble protein fraction; lane 5: insoluble protein fraction; lane 6: protein marker; lanes 7-10: purification wash fractions; lane 11: purified fraction eluted with 250 mM imidazole. (B) Samples collected from the desalting procedures were analyzed by western blotting with anti-GST and anti-osteocalcin antibodies. Lane 1: protein marker; lane 2: GST-cnOC-His6 protein; lane 3: GST protein; lane 4: protein marker; lane 5: GST-cnOC-His6 protein; lane 6: GST protein.
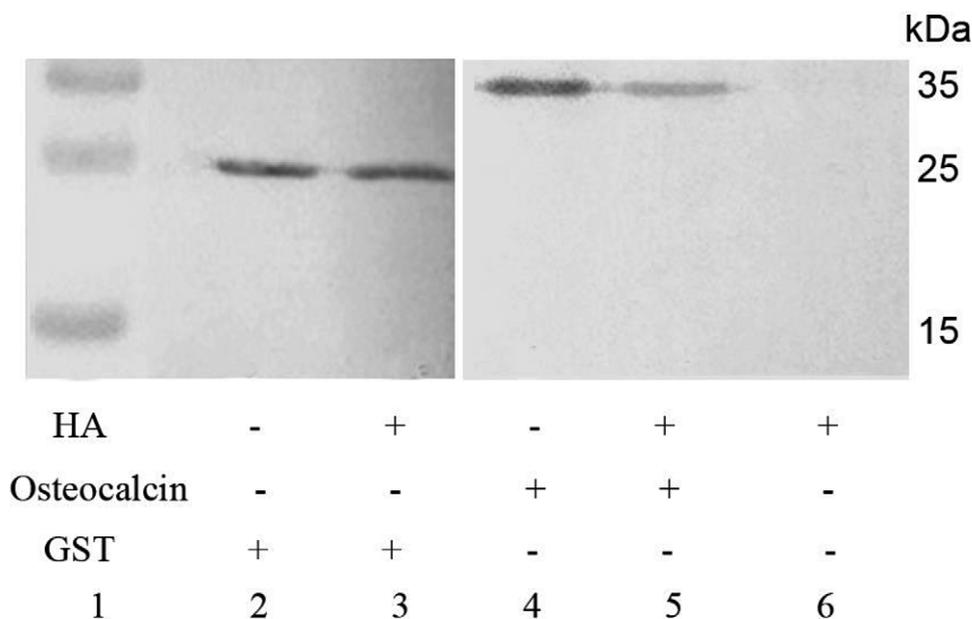
Fig. 5. Binding of GST-cnOC-His6 protein to HA. GST protein served as the control. Results are shown as a western blot.

Table 1. Chondrocyte proliferation activity stimulated by GST-cnOC-His6.

Groups	Concentration (μg/mL)	Absorbance (X±S)
Mock	0	0.558±0.033
GST	160	0.554±0.021
GST-cnOC-His6	160	0.733±0.046[**]

All experiments were independently repeated three times. n=3, ** p<0.01

of the GST-cnOC-His6 protein was determined to assess whether it could promote the growth of antler chondrocytes. The results demonstrate that cell proliferation was enhanced after treatment with GST-cnOC-His6 (Table 1). The rate of cell proliferation was 1.32 times higher than that of the GST negative control. The p-values for the comparison between the cells treated with GST-cnOC-His6 and the control were p<0.01. These results showed that recombinant GST-cnOC His6 protein promoted chondrocyte proliferation.

Discussion

In this study, immunohistochemical analysis confirmed that osteocalcin was differentially expressed in different parts and growth phases of sika deer antler. We cloned osteocalcin cDNA from sika deer antler, and sequence alignment analysis indicated that sika deer osteocalcin was highly conserved with osteocalcin in other mammals. Osteocalcin cDNA was inserted into the pGEX-4T1 expression vector and optimal induction conditions were determined to obtain the highest possible yield. After one-step affinity purification, an apparent pure protein sample was successfully obtained. The recombinant osteocalcin bound to HA. Cell proliferation assays confirmed that the recombinant osteocalcin protein promoted chondrocyte proliferation in vitro.

Deer antler is a unique mammal organ that can regenerate continuously. Such recovery provides a model for research into molecules involved in mammalian organ regeneration (Li et al. 2012). The exact mechanism is unclear. Osteocalcin was highly expressed in all parts during the mineralization period of sika deer antler, suggesting a role in regulation of mineralization. Osteocalcin was also highly expressed in mesenchymal cell and chondrocyte areas during the rapid growth period, indicating that osteocalcin promotes proliferation of the two cell types.

Recent data have emerged that increasingly cast osteocalcin in a new light; these data suggest a broader role for osteocalcin that extends to the regulation of energy metabolism, reproduction, and cognition (Franck et al. 2011, Booth et al. 2016, Zhou et al. 2017). Several groups have now clearly demonstrated a role for osteocalcin in the regulation of glucose metabolism (Ducy et al. 1996, Lee et al. 2007). Whether the osteocalcin affects the rapid growth of antler by regulating glucose metabolism remains to be studied. The GPCR class C group 6 subtype A (Gprc6a) protein is a G protein coupled receptor, expressed by a wide variety of cell types (Kuang et al. 2005). It appears likely that Gprc6a mediates osteocalcin's functions in the tissues including bone, adipose and muscle (Zoch et al. 2016). Osteocalcin induces myoblast proliferation via sequential activation of PI3K/Akt and p38 MAPK pathways, and promotes differentiation through activation of the Gprc6a-ERK1/2 pathway in C2C12 myoblast cells (Liu et al. 2017). Sika deer osteocalcin promoted the proliferation of antler chondrocytes. Whether the role of osteocalcin in the growth of antler is related to Gprc6a is one of the problems to be researched in the future. The reason why the promotive effect was not particularly noticeable may be that the large GST fusion protein affected the protein activity. The specific mechanism is the focus of future research. These results are useful for research of the rapid growth and differentiation of antlers.

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