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RESEARCH ARTICLE

Expression and Localization of Stanniocalcin-1 in Bovine Osteoblasts

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ABSTRACT

As a novel glycoprotein hormone, Stanniocalcin-1 (STC-1) was first identified in teleost species, and it is involved in the regulation of mineral homeostasis in bony fish and mammals. STC-1 can not only affect the mammals bone development, but also protect neurons from the damage of ischemia, and stimulate the angiogenic response. Although it is widely expressed in rodent skeletons, whether this hormone is expressed in the skeleton of ruminant animals, like bovines, is still unknown. Here, we investigated the expression of STC-1 in bovine osteoblasts by using immunocytochemical staining and RT-PCR. Our results show that the mRNA and protein of STC-1 are expressed in the bovine osteoblasts during later differentiation periods 10th day *in vitro*. The present data support the notion that STC-1 may play a role in the process of bovine bone development.

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INTRODUCTION

STC-1 is a glycoprotein hormone that was originally described in fish as being secreted by the corpuscles of Stannius, which are unique endocrine glands in bony fish. The primary function of STC-1 in teleost species is to regulate the calcium homeostasis through its ability to inhibit the gill and gastrointestinal tract calcium transport and to enhance phosphate reabsorption in the kidney (Gerritsen and Wagner, 2005).

The mammalian homolog of fish STC-1 was first identified in a human cell line. Subsequently, this hormone was detected in mouse (Chang *et al*., 1996), cow (Paciga *et al*., 2002), and sheep (Song *et al*., 2006). Unlike fishes STC-1, which is present in the blood circulation, the mammalian STC-1 is undetectable in blood under normal conditions unless during gestation and lactation (Miller *et al*., 2006; Tremblay *et al*., 2009). In mammals, STC-1 is expressed in multiple organs, including brain, thyroid, spleen, thymus, parathyroid, lung, heart, skeletal muscle, kidney, pancreas, small intestine, colon, placenta, ovary, testis, and prostate (Chang *et al*., 1995b; Chang *et al*., 1996; Sheikh-Hamad *et al*., 2000). Although its physiological function in mammals in undetermined, the wide existence of STC-1 suggests that it acts as an autocrine or paracrine hormone, particularly in the process of osteoblast development and bone formation (De Niu *et al*., 2000; Deol *et al*., 2000; Yoshiko *et al*., 2000).

There are a number of reports describing rodent skeletal STC-1 expression. STC-1 mRNA was detected in osteoblasts, prehypertrophic and hypertrophic chondrocytes, and undifferentiated intervertebral disc mesenchyme during the murine embryonal development by using *in situ* hybridization (ISH) (Yoshiko *et al*., 1999; Jiang *et al*., 2000; Yoshiko *et al*., 2002). It is also confirmed in rodent calvarial osteoblast cultures via immunocytochemistry (ICC) (Yoshiko *et al*., 2003). Treatment of mature calvaria osteoblast subcultures with recombinant hSTC1 can enhance osteoblast mineralization by inducing expression of the type III NaP_i transporter (P_it1), resulting in the increase of phosphate utilization (Yoshiko *et al*., 2003; Yoshiko *et al*., 2007). These results suggest that STC-1 is involved in the process of mammalian bone formation through an autocrine or paracrine manner.

It has remained unknown whether STC-1 is expressed in the bovine skeleton, although it is widely expressed in the rodent skeleton. In this paper, we investigated the expression of STC-1 in bovine osteoblast cultures which are isolated by a sequential enzymatic digestion from newborn calves costal bone.

MATERIALS AND METHODS

Isolation of bovine osteoblasts: Primary bovine osteoblastic cells were obtained from costal bone of 1-day-old female Chinese Holstein calves by a sequential enzymatic digestion as described previously (Kartsogiannis

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and Ng, 2004). In brief, bovine costal bone was collected at a local slaughterhouse from neonatal Chinese Holstein calves, then placed into cold PBS (4°C) supplemented with penicillin (1000 IU/mL) and streptomycin (1000 IU/mL), maintained in a freezer bottle and transported to the laboratory within 3 h. After removing the muscles and periosteum, the bones were cut into blocks of 1 mm^3 in size and rinsed three times with PBS. The samples were subjected to digestion with 0.1% w/v collagenase B (Roche, Penzberg, Germany) for 20 min at 37°C. This procedure was repeated to yield a total of five digests, rejecting the first digest. The cells isolated from second to fifth digests were cultured in 50 cm^2 flask with Dulbecco's modified eagle's medium (DMEM, HyClone, Beijing, China) containing 10% fetal bovine serum (FBS, HyClone, Beijing, China) and antibiotics (100 mg/mL of penicillin, 100 IU/mL of streptomycin) at 37°C in a humidified incubator with 5% carbon dioxide. The culture media was changed every 2 days.

Once the cells reached confluence, they were washed with PBS and detached by incubation with 0.25% trypsin solution for 3 to 4 min at 37°C. Complete medium (DMEM containing 10% FBS, 100 mg/mL of penicillin and 100 IU/mL of streptomycin) was added to inactivate the trypsin. The mixture was centrifuged at 1200rpm for 3 min, the supernatants were removed, and the cell pellets were re-suspended in 3 to 5 mL complete medium. The cells were counted using a hemocytometer and then plated in 6-well plate at a density of 5×10^4 cells/well.

To determine the morphology of bovine osteoblast, the Giemsa staining was performed as described previously (Li *et al*., 2009). Cells were fixed in 70% ethanol (v/v) for 15 min and stained with a pre-mixed Giemsa staining solution for 30 min at room temperature. The stained cells were rinsed with tap water and air dried. Then, the cells were observed, photographed by Olympus IX71 (Olympus, Tokyo, Japan).

Identification of bovine osteoblasts: The modified Gomori calcium-cobalt method was used to examine intercellular alkaline phosphatase (ALP) to identify osteoblasts (Li *et al*., 2009). Briefly, confluent monolayer cells were fixed with 4% paraformaldehyde for 10 min and then rinsed three times with distilled water. The fixed cells were placed at 37℃ for 4 h in an incubation solution, which consisted of 5mL sodium barbital (20 g/L), 5mL sodium β-glycerophosphate (30 g/L), 10 mL calcium chloride (20 g/L), 0.5 mL magnesium sulfate (20 g/L), and 2.5 mL distilled water. The cells were then dripped into cobalt nitrate solution (20 g/L) for 5min, rinsed with running water, dripped into sulfurated amine solution (10 g/L) for 5 min, and then rinsed with running water, air dried and mounted. For a negative control, sodium β-glycerophosphate was replaced by distilled water in the incubation solution.

To induce mineralization, the second passage cells were inoculated at a density of 5×10^4/well and treated with osteogenic media (consisting of 10% FBS, 10 mM β-glycerol phosphate and 50 μg/mL ascorbic acid) for 21 days (Wada *et al*., 1998). After induction of ossification, Alizarin Red S staining was carried out according to standard technique to detect the mineralized nodules in vitro (Utting *et al*., 2006). Gomori calcium-cobalt and Alizarin Red S staining experiments were repeated at least three times.

Immunocytochemistry assay: After 10 days culture, the samples were fixed with 4% paraformaldehyde for 30 min at 4°C. The endogenous peroxidase was inactivated by 0.75% hydrogen peroxide for 30 min at 37°C and then the cells were incubated with 10% horse serum for blocking nonspecific antigens. The primary anti-rhSTC-1 polyclonal antibody (Santa Cruz Biotechnology, California, USA, 1:100) was incubated with cells for 1 h at 37°C. Following incubation, the cells were washed 3 times for 5 min by PBS. Then biotinylated secondary antibody (Boster, Wuhan, China, 1:500) was added and the cells then were incubated again for 30 min, followed by streptavidin-biotin complex for another 30 min at room temperature, and the visualization was achieved by using Brown Peroxidase Substrate kit (Diaminobenzidine, Boster, Wuhan, China). Finally, the cells were counterstained with haematoxylin for 3 min. Each incubation step was followed by three times washes (5 min each) with PBS.

RT-PCR: Total RNA was extracted from the 10-day-old primary bovine osteoblast cultures using TRIzol reagent (Invitrogen, California, USA) according to the manufacturers instructions and 1 μg RNA was reverse transcribed with oligo (dT18) primer using the Transcript First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China) for complementary DNA (cDNA) synthesis.

PCR amplification primer pairs for STC-1 were sense 5'-GCCTCAGCAAACTACGAT-3' and antisense 5'-ACTGGGGCAACCA.TTCTA0-3' (GenBank No. NM-176669.3, Fragment length 362 bp) and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense 5'-CCTTCATTGACCTTCACTACATG GTCTA-3' and antisense 5'-TGGAAGATGGTGATGGC CTTTCCATTG-3' (GenBank No. U85042, Fragment length 126 bp). An aliquot (2 μL) of the cDNA template was amplified by PCR using 2×Taq PCR MasterMix (Zomanbio, Beijing, China). The PCR mixtures were preheated to 94°C for 5 min followed by 35 cycles of amplification (94°C for 30 s; 57°C for 30 s; 72°C for 1 min), and the PCR reactions were terminated with a final elongation at 72°C for 10min, and then the PCR products were separated on 1% agarose gel stained with ethidium bromide (0.5 μg/mL) and visualized, photographed by G: Box BioImaging Systems (Syngene, Cambridge, UK). Immunocytochemistry assay and RT-PCR assay were repeated at least three times.

RESULTS

Cell culture and identification: After 3 days culture, the cells, isolated from costal bone of neonatal female Chinese Holstein calves, showed spindle, triangular and polygonal morphology (Fig. 1A). As shown in Fig. 1B and 1C, for 7 days culture, cells can be confluent. Most of the cells show spider-like morphology and tight junctions.

The dark brown and black deposits were distributed in the cytoplasm and extracellular matrix, which corresponds to the secretion of ALP by osteoblasts (Fig. 2A and 2B).

Alizarin red staining revealed the presence of multiple red nodules, with variable size, further confirming that these cultured cells were osteoblasts (Fig. 2C).

Localization and expression of STC-1 in the osteoblasts: To identify the STC-1 expression in bovine osteoblasts, immunocytochemistry assays were carried out. As Fig. 3A shows, STC-1 is widely distributed in bovine osteoblast cells, and also brown staining particles were only restricted to localize around the nucleus about some of the cuboidal cells in confluent phase. In contrast, there was none of brown granulae displayed in the control samples, and only a separate blue nucleus can be found (Fig. 3B).

To determine whether the STC-1 mRNA was expressed in bovine osteoblasts, we performed the RT-PCR assays. As Fig. 4, showing, there was a 362 bp STC-1 specific band on the lane, in accordance with the length of amplified cDNA segments by our previously designed STC-1 primers. The housekeeping gene GAPDH was used as normalization control.

DISCUSSION

As a dynamic tissue, apart from the functions of supporting the soft tissue, protecting the internal organs, and housing for bone marrow, the bone also plays a critical role to maintain the mineral balance in the animal's body. Both bone-specific cells, osteoblasts, or bone-forming cell, and osteoclasts, or bone-resorbing cells, are involved in the regulation of mineral homeostasis (Ducy *et al*., 2000). Primary cell cultures and cell lines are extensively used in the bone biology research to test the effect of various physiological and pathological factors on the bone-specific cells *in vitro* (Kartsogiannis and Ng, 2004).

In cell culture, it is difficult to distinguish osteoblasts from fibroblasts, both of them arise from a common pluripotent mesenchymal stem cells and exhibit a similar shape in morphology (Ducy *et al*., 2000). Since ALP expression and mineralization nodules formation are the specific markers of mature osteoblasts, Calcium cobalt

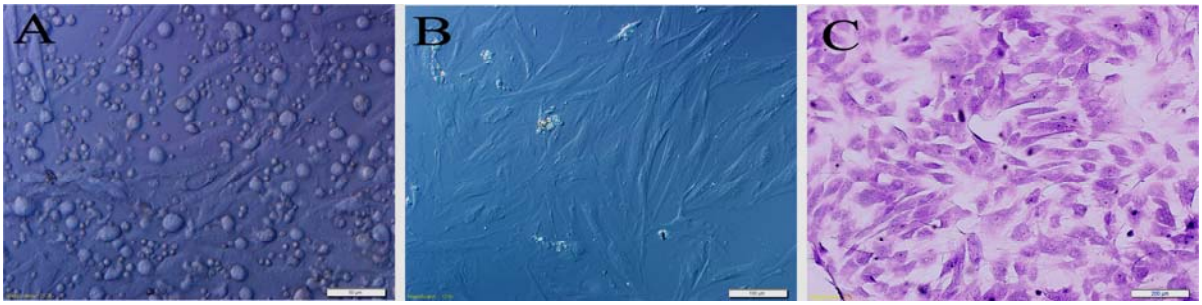

Fig. 1: Morphology of primary culture bovine osteoblasts. A) Cells cultured for 3 days. Scale bar 50 μm. B) The confluent cells at day 7 Scale bar 100 μm. C) Cells stained with Giemsa solution, Scale bar 200 μm.

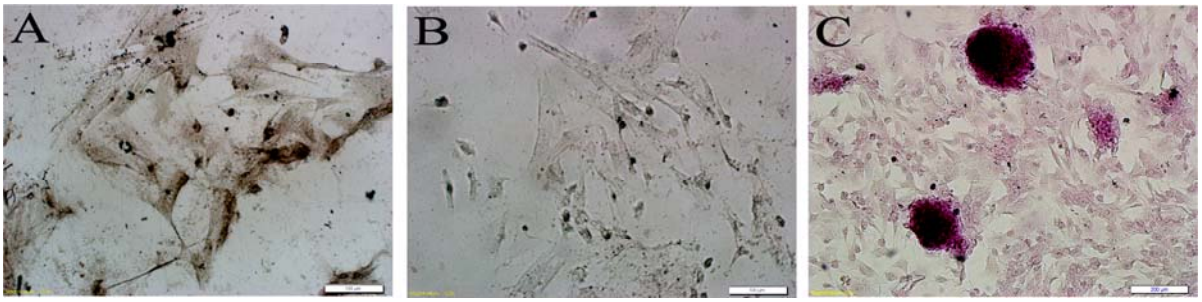

Fig. 2: Identification of bovine osteoblasts. A) Cells stained by modified Gomori calcium-cobalt method for investigate the expression of ALP after 14 days of culture. B) Negative control, Scale bar: 100 μm. C) Bovine osteoblasts form calcified nodules after inducing 21 days, stained by Alizarin red S, Scale bar: 200 μm.

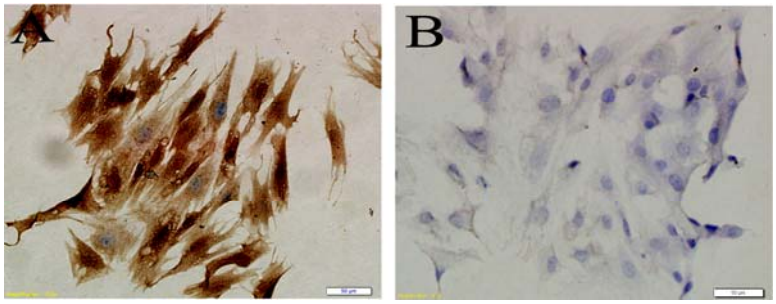

Fig. 3: Immnocytochemical detection of STC-1 in osteoblasts on 10 days of culture. A) The brown staining particles were restricted to localize around the nucleus, Scale bar: 50 μm. B) Negative control, PBS was used as a substitute for anti-rhSTC-1), Scale bar: 50 μm.

staining and Alizarin red S staining assay were employed in the present study for identification of isolated cells (Kartsogiannis and Ng, 2004). Our results show that the cells isolated from costal bone of neonatal female Chinese Holstein calves, could be stained by Gomori calcium-cobalt method and exhibited dark brown and black deposits in the cytoplasm. Moreover, these cells were incubated with the osteogenic media for 21 days followed by staining with Alizarin S red, showing a number of calcified nodules, suggesting these are capable of depositing calcified salts *in vitro*. Hence, consistent with previous reports (Whitson *et al*., 1984; Utting *et al*., 2006; Wang *et al*., 2007; Li *et al*., 2009), the isolated cells in the present study are osteoblasts.

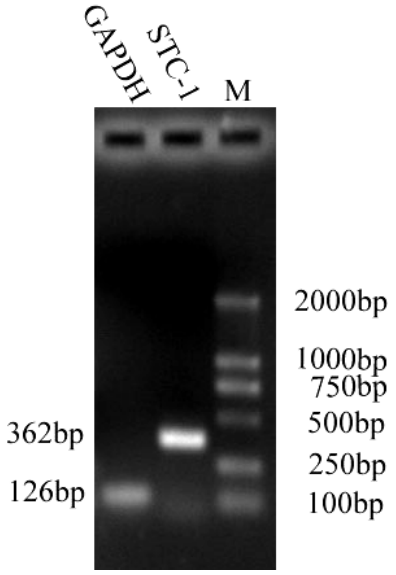

Fig. 4: Amplification of STC-1 and GAPDH gene from bovine osteoblasts by RT-PCR. M: Marker (DL2000);

Mammalian STC-1 was first identified by Chang *et al*. in 1995 (Chang *et al*., 1995a). A large number of studies were performed to determine the physiological function of this hormone in mammals. STC-1 protein and mRNA were localized in the bone and osteoblasts during the developmental period by immunocytochemistry (ICC) (Yoshiko *et al*., 2003) and *in situ* hybridization (Yoshiko *et al*., 1999; Jiang *et al*., 2000; Yoshiko *et al*., 2002), respectively. The overexpression of STC-1 in transgenic mice displayed growth retardation postnatally, elevated the serum calcium or phosphate levels, and decreased the serum ALP levels, indicating that this hormone affects the murine skeletal development, growth, and mineral homeostasis (Filvaroff *et al*., 2002; Varghese *et al*., 2002; Johnston *et al*., 2010).

STC-1 mRNA could be detected at day 4, and increased, remained high levels in the later mineralization period in the MC3T3-E1 cell lines culture (Yoshiko *et al*., 2002). In the later mature stages of rat primary calvaria cell cultures, STC-1 stimulated the type III NaP_i transporter (P_it1) expression and enhanced the inorganic phosphate (P_i) uptake by osteoblasts, favored the formation of mineralize-tion nodules, although the hormone was undetectable in the early primitive progenitor stages (Yoshiko *et al*., 2003; Yoshiko *et al*., 2007). In this study, the 10-day-old culture cells were subjected to detect the expression of STC-1 mRNA and protein. The results showed that both STC-1 mRNA and protein were detected in bovine osteoblasts, consistent with other previous studies that its expression was mainly present in the differentiation and mineralization stages of osteoblasts.

In summary, our data show that STC-1 mRNA and protein were expressed in the bovine osteoblasts, suggesting that STC-1 may affect bovine osteoblast differentiation and formation of bone nodules through an autocrine or paracrine manner, although the underlying molecular mechanism are unknown. Since the STC-1 affects the bone development and mineral homeostasis of mouse (Wagner *et al*., 1997; Yahata *et al*., 2003) and the pig (Madsen *et al*., 1998), respectively, our future studies will analyze the physiological role of STC-1 in bovine osteoblasts and the bovine mineral metabolism.

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