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**Japanese Journal of Veterinary Research**

**HOKKAIDO UNIVERSITY**
Gene expression profile of bovine bone marrow mesenchymal stem cell during spontaneous chondrogenic differentiation in pellet culture system

Darko Bosnakovski¹*, Morimichi Mizuno², Gonhyung Kim³**, Satoshi Takagi⁴, Masahiro Okumura⁴ and Toru Fujinaga⁵

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Abstract

Bovine bone marrow mesenchymal stem cells (MSCs) cultured in condensate culture, spontaneous and independent for any external biostimulants, undergo chondrogenic differentiation. In the present study, the bovine MSC chondrogenesis pathway was studied by analyzing stage-specific gene expression using quantitative “Real Time” reverse transcriptase polymerase chain reaction (qRT-PCR). Results showed that bovine MSCs underwent complete chondrogenesis; the initial stage was characterized by expression of sox9 messenger ribonucleic acid (mRNA), followed by high transcription of chondrocyte specific genes, collagen type II and IX, biglycan and cartilage oligomeric matrix protein, and the final prehypertrophic and/or hypertrophic stage was distinguished by increased expression of collagen type X. From day 7 to day 14 of differentiation increased mRNA expression of the transforming growth factors β1 and β2, basic fibroblast growth factor (FGF 2), bone morphogenic protein 6 (BMP 6), insulin-like growth factors 1, parathyroid hormone related peptide and indian hedgehog (Ihh) were detected. These results suggest that these well know chondrogenic growth factors may play a role in bovine chondrogenesis in autocrine and/or paracrine manner. On day 21 of the culture, FGF 2, BMP 6 and Ihh were highly expressed, compared to cells cul-

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Introduction

Differentiation of mesenchymal stem cells (MSCs) into chondrocytes is a multistep pathway; starting from the recruitment of mesenchymal progenitor cells, which subsequent undergo condensation, followed by synthesis of the extracellular matrix (ECM) characteristic for cartilage [7]. In the growth plate of skeletal elements, several layers of chondrocytes become flattened and extensively proliferate, resulting into the longitudinal growth of the bones. In the terminal stage, these cells stop proliferation, change their genetic program and become hypertrophic cells surrounded by a mineralized ECM [7, 9]. The progression of undifferentiated MSCs to hypertrophic chondrocytes requires both intrinsic and extrinsic factors created by cells, systemic factors and other signaling molecules that can modulate the cellular metabolism in an autocrine and/or paracrine manner.

MSCs isolated from adult bone marrow have potential for extensive in vitro expansion and can undergo multilineage differentiation under adequate conditions. In vivo chondrogenic differentiation of MSCs has been demonstrated by cell transplantation into artificial osteochondral defect and by implantation subcutaneously in SCID mice [10]. For inducing chondrogenesis in vitro, strong cell to cell interaction, growth factors or cytokines possessing chondrogenic potentiality and structure which support three dimensional (3-D) cell orientations were reported to be necessary. Strong cell to cell interaction mediated by cell adhesion molecules such as N-cadherin and integrins allowed MSC conversion to prechondroblasts at the precartilage mesenchymal condensation stage during limb development [13]. This condition in vitro can be obtained by pellet or micromass culture systems. Various bioactive factors, such as basic fibroblast growth factor (FGF 2), transforming growth factors (TGF) β1, β2 and β3, bone morphogenic proteins (BMPs) 2, 6, and 9; and insulin-like growth factors 1 (IGF 1) have been reported to induce or maintain chondrogenesis [15, 28]. Materials such as agarose, collagen, fibrin, alginate, and biopolymers have all been used as scaffolds to support 3-D orientation of chondrocytes and MSCs [21].

Previously we demonstrated the chondrogenic effect of TGF β1 on bovine MSC in different culture conditions (monolayer, pellet, hydrogel) [5, 6]. In addition, we have shown that bovine MSCs have species-specific characteristic for spontaneous chondrogenic differentiation in in vitro conditions, which allowed strong cell to cell interaction (pellet culture) [5]. The present work is a further approach of the previous studies and has the purpose of contributing to the understanding of this specific bovine MSCs chondrogenesis, which occurs independently from any exter-
nally added chondrogenic biostimulants. For this reason, we screened the expression of chondrocytes-specific genes and gene expression of potential chondrogenic growth factors during bovine MSCs pellet culture, and tried to determine which of them in autocrine and/or paracrine manner may have an influence on the chondrogenesis.

**Materials and Methods**

**Harvesting and isolation of bovine MSCs and chondrocytes:** Bone marrow was aspirated from three calves (2 months old). Bovine MSCs were isolated by previously described methods [5, 6]. Briefly, the bone marrow sample was washed twice with phosphate-buffered saline (PBS) and twice with Dulbecco’s Modified Eagle Medium (DMEM; GIBCO BRL, Grand Islands, NY, USA). Following the determination of cell viability and number, nucleated cells \( (5 \times 10^6/cm^2) \) were plated in T-75 culture flasks with DMEM (low glucose) containing penicillin G 100 U/ml, streptomycin 100 µg/ml, amphotericin B 0.25 µg/ml, HEPES 2.4 mg/ml, NaHCO3 3.7 mg/ml, and 10% fetal bovine serum (FBS; lot no. 5300C; ICN, Biomedicals, ND, USA). The cells were incubated at 37°C in 5% CO₂ humidified atmosphere. After being cultured for 4 days, non-adherent cells were removed by changing the culture medium. Following the initial 4 days, which was required for the cells to attach to the flask, the medium was changed every 2 to 3 days. On day 12 or 13, cells were detached by using 0.25% trypsin in 0.1% ethylene diamine tetraacetic acid and either used in experiments or stored by deep-freezing in 10% dimethylsulfoxide until further use.

Chondrocytes were isolated from the articular cartilage harvested from the same calf, from which bone marrow was aspirated. Briefly, cartilage pieces were digested with 0.1 mg/ml of collagenase (Wako Pure Chemical, Osaka, Japan) in DMEM/10% FBS for 18 hr at 37°C with gentle stirring followed by filtering through a 100 µm nylon mesh. Isolated primary chondrocytes were plated overnight in 6-well plate in DMEM/10% FBS and cultured overnight.

**Cell culture:** Cells from the first or second passage were resuspended in the serum free-chemically defined medium consisting of DMEM (high-glucose), insulin 6.25 µg/ml, transferrin 6.25 µg/ml, selenious acid 6.25 µg/ml, bovine serum albumin 1.25 mg/ml, pyruvate 1 mM, linoleic acid 5.35 µg/ml and ascorbate 2-phosphate 50 µg/ml (all from Sigma Chemicals Co, St. Louis, MO, USA). For preparation of each pellet, \( 1 \times 10^6 \) cells were resuspended in 1 ml of defined medium, spun down at 500 \( \times g \) for 10 min in a 15 ml polypropylene conical tube and cultivated at 37°C in a humidified atmosphere containing 5% CO₂ for 21 days by changing the medium every 2 days. For monolayer culture, aliquots of the MSCs used for pellet preparation were plated in T-75 flasks or 4-well chamber slides \( (5 \times 10^5 \text{cells/cm}^2) \) and cultured under standard culture conditions.

**Histological and immunohistochemical analysis:** Pellets were harvested after 21 days of culturing, fixed in 10% buffered formalin for 2 hr, and then in 70% ethanol overnight. Samples were embedded in paraffin and 5 µm sections were cut.

MSCs and chondrocytes cultured in 4 wells chamber slides were rinsed twice in PBS, fixed with 3.7% paraformaldehyde for 15 min, and then rinsed three more times in distilled water before staining.

Hematoxylin and Eosin (H&E) staining was done for evaluation of cell morphology in pellet and monolayer culture. Sulfated gli-
cosaminoglicans (GAGs) were visualized by staining with 0.5% alcian blue (pH 1.0) for 10 min. Coll 2 was detected by immunostaining using polyclonal antibodies (LSL Co., Tokyo, Japan). Briefly, after deparaffinization, sections were predigested with trypsin at 37°C for 30 min to facilitate antibody access, endogenous peroxidase was quenched by the treatment of 3% H2O2 in methanol at room temperature for 30 min and nonspecific antibody binding was blocked by incubation of sections in 10% normal goat serum at 37°C for 30 min. Rabbit anti-bovine coll 2 diluted 1:500 in 0.01 M PBS (pH 7.4) was applied as a primary antibody at 4°C overnight. Sections were then incubated with the secondary antibody, swine anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark), for 60 min followed by rabbit PAP kit (DAKO). Coll 2 was visualized by the reactions with 0.05% diaminobenzidine containing 0.01% H2O2.

**Measurement of messenger ribonucleic acid (mRNA) level by “Real Time” reverse transcriptase polymerase chain reaction (qRT-PCR) method**: Total RNA was isolated from MSCs cultured in pellet and monolayer culture for 21 days and from freshly harvested chondrocytes (cultured overnight) by using Trizol (Invitrogen, Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instruction. After the samples were treated with DNase (Invitrogen) to remove possible contamination by genomic deoxyribonucleic acid (DNA), first-strand complementary (c) DNA was synthesized from 2 µg total RNA by using M-MLV reverse transcriptase (Invitrogen) with oligo (dT) as a primer in 20 µl reaction mixture. The amount of cDNA was measured by the qPCR method using the Smart Cycler System (Cepheid, CA, USA). PCR was carried out in a 25-µl final volume containing PCR buffer, 3 mM MgCl2, 0.3 mM dNTP mixture, 0.3 µM each of primer (Table 1), and Taq polymerase (1.25 units/tube; Takara Biomedicals, Otsu, Japan). Forty-five cycles of thermal cycling was carried out at 95°C for 5 s, 55-60°C for 15 s, and 75°C for 15 s. The amount of PCR product was estimated by measurement of the intensity of fluorescence of SYBR Green I interacting with the PCR product. Expression levels of target genes were normalized by dividing with the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quality of the PCR products was checked by melting curve analysis and electrophoresis.

**Statistical analyses**: Data from a minimum of four independent experiments were evaluated and expressed as mean ± standard deviation (SD). An unpaired Student’s t test was used to t test significance between the two groups. The results were considered significantly different at p < 0.05.

**Results**

**MSCs morphology in monolayer and pellet culture**: MSCs from the first or second passage were used in the experiments. There were not notable differences (on histological examination) on chondrogenic potential among cells from different passages, or reconstructed cells after deep freeze in liquid nitrogen. Within one or two days of samples preparations, cells formed compact pellet, which increased in size during culturing (Fig. 1).

Cells cultured in monolayer manner with serum free-chemically defined medium had bipolar to polygonal fibroblastic cell-shape and grew in uniform monolayer (Fig. 2). On histological and immunohistochemical examination, positive metachromatic alcian blue staining and positive immunoreactivity for coll 2 were not detected (Fig. 2). Opposite of that, cells cultured in pellet culture had
plump, round cell-shape, located in lacunae and surrounded with notable newly synthesized ECM (Fig. 2). Cell shape and structure of the newly generated tissue had typical characteristics of chondrocytes and cartilage. Only cells in the few periphery layer of the pellet had elongated, fibroblast-like shape. Positive metachromatic staining was detected through entire pellet, with notable stronger intensity in the periphery on the lacunae. The evidence of chondrogenesis was supported by detecting *coll 2* in ECM of the entire pellet (Fig. 2).

**Chondrocytes-specific gene expression profile of MSC in the terminal stage of chondrogenesis (day 21):** Complete process of chondrogenic differentiation, from MSCs to prehypertrophic or/and hypertrophic chondrocytes, was demonstrated by analyzing genes expression, which is characteristic for every step of chondrogenesis. One of the markers for the

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early stage of chondrogenesis is chondrogenic transcription factor sox9. The expression of this factor in MSCs cultured in pellet culture, on day 21, was 2.2-fold higher compared with MSCs in monolayer culture and 5.7-fold higher than in freshly isolated chondrocytes (Fig. 3a). Significant \((p < 0.5)\) 21-, 2.6- and 2-fold higher expression of the typical chondrogenic markers, \(coll\ 2\), biglycan \((\text{big})\) and cartilage oligomeric matrix protein \((\text{COMP})\) respectively, was detected in pellet than in the monolayer (Fig. 3). In addition, clear difference was found in expression of the sensitive chondrogenic marker collagen type IX \((coll\ 9)\). \(Coll\ 9\) mRNA was detected only in the MSCs from pellets and in the chondrocytes, but not in the MSCs from the monolayers (Fig. 3). Finally, to detect if chondrogenesis reached final, hypertrophic stage, we analyzed the expression of collagen type X \((coll\ 10)\) gene, which is characteristic markers for prehypertrophic and hypertrophic chondrocytes. \(Coll\ 10\) was significantly \((5.7\text{-fold})\) up-regulated in the pellets compare to the monolayer, and was expressed constantly at low level in freshly iso-
lated chondrocytes (Fig. 3a). Credibility of MSC chondrogenesis was supported by the analyses of collagen type I (coll 1) mRNA, which is marker of de-differentiated chondrocytes and osteoblasts. Expression of coll 1 was significantly, 5- and 25-fold, lower in MSCs from pellet and chondrocytes than in MSCs from monolayer (Fig 3b).

Since chondrogenesis of bovine MSCs in pellet culture system without stimulation of any external biostimulants was proved, we investigated which of the known potential chondrogenic biofactors at transcriptional level in autocrine and/or paracrine manner participate in this mechanism. At the terminal stage of differentiation the most of the analyzed growth factors (TGF β1 and β2, PTH-rP and IGF 1) were down-regulated in pellets compared to monolayer (Fig. 4b). Among them, TGF β1 was only significantly (p < 0.5) 1.6-fold lower expressed in pellet than in monolayer (Fig. 4b). Growth factors that were determined to be significantly up-regulated, more than 2-fold, in pellet then in monolayer were FGF 2, BMP 6 and indian hedgehog (Ihh) (Fig. 4a). mRNA level of all of analyzed growth factors, except FGF 2, was expressed notably at low level in freshly isolated chondrocytes than in MSCs from pellet and monolayer culture (Fig. 4).
**Chondrocytes-specific gene expression profile of MSC during chondrogenesis:** Additionally to the analyses of the gene expression in terminal stage of differentiation, which was subsequently compared to the counterpart cells cultured in monolayer and freshly isolated chondrocytes, we analyzed chondrocytes specific genes alteration correlated with growth factors gene expression during 21 days of pellet culture. *Sox9* was firstly detected on day 7, and its expression increased in the following 7 days of culture, when reaches the maximum, and then decreased at the terminal stage (Fig. 5b). *Coll 2, 9 and 10* showed similar gene expression pattern of *sox9* (Fig. 5) ; for example, *coll 2* has the minimal basal, barely detected, expression level at day 0, to the maximum of 330-fold higher at day 14, and finally to end at day 21 with 272-fold increased expression compared with the day 0 (Fig. 5a) . Opposite of that, *coll 1* from day 0 to day 21 continuously decreased, and reached 14-fold lower expression than at the beginning of the culture (Fig. 5b).

All analyzed chondrogenic factors during differentiation demonstrated similar expression profile, which increased from day 7 to day 14, then decreased until day 21, reaching the minimal detected level. Among the growth factors, *BMP 6, TGF β1 and β2* increased for approximately 2-fold from day 7 to day 14 (Fig. 6). When expression patterns of the chondrocytes specific genes and growth factors were compared could be notice that they were synchronized ; on day 14 growth factors had the higher expression pick which was followed with the maximum expression level of *sox9, coll 2, 9 and 10*.

**Discussion**

Present work is extended study of our previous reported research about spontaneous chondrogenic differentiation of bovine MSCs in pellet cultural system without addition of any bioactive stimulants [5]. Chondrocytes-specific gene expression profile, and histological and immunohistochemical findings , clearly demonstrated that bovine MSCs underwent through entire process of chondrogenesis. Furthermore, during MSCs chondrogenic differentiation we analyzed gene expression of the known growth factors that participate in chondrogenesis in other species, in purpose to determinate which of them may take place in the mechanism of bovine MSC chondrogenesis. All analyzed well documented chondrogenic factors, *TGF β1 and β2, BMP 6, FGF 2, PTH-rP, Ihh and IGF 1*, were
up-regulated during differentiation, reaching maximum expression level after 14 days culturing. In the cells which underwent chondrogenesis, expression of TGF β1 and β2, PTH-rP and IGF 1 dramatically decreased, and it was under the expression level of undifferentiated cells, suggesting that probably they do not have some remarkable influence in the terminal stage of differentiation. On other hand, FGF 2, Ihh and BMP 6, were found to be up regulated compared with the undifferentiated cells. These facts lead us to conclusions that they may have some influence in maintaining phenotype of the mature or prehypertrophic chondrocytes.

Sox9 is a member of the Sox (Sry-type HMG box) genes family and has been shown to be expressed predominantly in mesenchymal condensation and cartilage and play a key role in chondrogenesis [3,34]. It regulates expression of the gene encoding coll 2, 9 and 11, and enhances agg gene promoter/enhancer activity [2,27]. Sox9 expression starts in mesenchymal progenitor cells, reaches high level in differentiated chondrocytes and finish with complete down-regulation in hypertrophic chondrocytes [36]. It was demonstrated that over expression of this gene in mouse MSC can enhance chondrogenesis, and that cell mediated sox9 gene therapy could be treatment for articular cartilage regeneration [31]. Bovine MSCs in undifferentiated stage (monolayer culture) did not or barely expressed sox9, but when cells were cultured in condensate condition, its expression dramatically increased. During culture time, sox9 expression pattern was synchronized with the expression of other chondrocytes-specific genes, suggesting possible trigger-function in their transcription and strong expression-interactions among them.

To demonstrate MSCs differentiation, it is necessary to evaluate the presents of the specific lineage markers. Expression of coll 2 is a typical phenotype of articular chondrocytes, as well as in vitro aggregate culture of stem cells undergoing chondrogenesis in response to TGF β [25]. In bovine MSCs, up-regulated expression of coll 2 mRNA was detected just after few days of pellet culture (data not shown), and its transcription dramatically increased during the following period. Detecting increased transcription of the other cartilage-characteristic components big, COMP and coll 9 supported the evidence of chondrogenesis. COMP is one of major non-collagenous proteins in the cartilage [14], which interacts with coll 1, 2 and 9, and plays a role in fibril formation and maintenance of the extracellular collagen network [30].
Zaucke et al., demonstrated that COMP and coll 9 are even more sensitive markers for dedifferentiation of chondrocytes than coll 2 [35].

Hypertrophic chondrocytes expressed high level of alkaline phosphatase, coll 10 and diminish level coll 2, and are predisposed to undergo apoptotic cell death for new bone formation (endochondral ossification). On the other hand, coll 10 is also detected in articular cartilage in the superficial zone [26], in vitro cultured chondrocytes [23] or in the undifferentiated MSCs [1]. We also found coll 10 mRNA in the freshly isolated bovine chondrocytes and in undifferentiated MSCs cultured in monolayer in both serum free and FBS supplemented medium. Cells morphology in the centre of the pellet and the increased expression of coll 10 and Ihh suggested that some of the cells reached prehypertrophic or hypertrophic stage. However, sox9, coll 2 and other chondrogenic markers were highly expressed, suggesting that the generated tissue had more characteristics of hyaline cartilage than of hyperopic zone of endochondral plate. Fact more that the cell differentiation did not turn in osteogenesis it was the low, and with the tendency of decreasing, coll 1 expression (Fig. 3b), and the absent of osteocalcin mRNA (data not shown).

Several growth factors, including TGF β, FGF 2, IGF 1, BMP, PTHrP and Ihh are known to influence discrete steps in chondrogenesis pathway [24, 32]. Members of TGF β super-family have been shown to play a major role in bone and cartilage development. Especially, the action of TGF β1 as potent promoters of the MSC chondrogenic differentiation have been well documented elsewhere [1, 18]. Our results, at transcriptional level, demonstrated that TGF β1 and β2 in autocrine and/or paracrine manner might have influence on bovine MSC chondrogenesis. Pretreatment of MSCs with TGF β1 followed by exposure of cells to IGF 1 in 3-D culture significantly increased the formation of chondrocytes markers, agg and coll 2 mRNA [33]. Our results showed that IGF 1 has synchronized expression pattern with TGF β1 and β2, and suggest that there function in the process of differentiation interface. FGF 2 has important roles in various processes in embryogenesis. Its expression was detected in differentiated chondrocytes [17], and was demonstrated that has potential to inhibit differentiation of chondrocytes into hypertrophic chondrocytes in vivo [20]. It was reported that the chondrogenic effect of FGF is accomplished by regulation of sox9 expression trough mitogen-activated protein kinase pathway [22]. We detected slight change of FGF 2 expression from day 7 to day 14, but when the expression level in the differentiated cells (day 21) was compared to that in undifferentiated cells, we found significant difference between them. In the growth plate of endochondral bones, FGF 2 and PTH-rP stimulate resting chondrocytes to proliferate and suppress terminal differentiation of hypertrophic chondrocytes in vivo [16]. It was reported that PTH-rP mediates sox9 expression and negatively regulate chondrocytes maturation. Furthermore, PTH-rP in combination with Ihh regulates chondrocytes differentiation thought establishment of a negative feedback mechanism, whereby Ihh and PTH-rP can together suppress hypertrophy [29, 32].

The family of BMPs is known to induce and/or support differentiation of mesenchymal cells into chondrocytes and osteoblast lineage [4, 11]. It was reported that BMP 6 alone cannot induce chondrogenesis, but in combination with TGF β3 and dexamethasone can significantly stimulate synthesis of cartilage-characteristic ECM, consequently leading to increase size and weight of the pel-
lets [15, 28]. BMP 6 is expressed in hypertrophic chondrocytes, and in autocrine manner has influence in the late stage of chondrocytes maturation [8, 19] by enhancing the expression of all hypertrophic chondrocytes markers, including coll 10 and decreasing the proliferation rate. During endochondral bone formation, low expression of PTH-rP supports BMP 6 expression in growth plate chondrocytes, inducing chondrocytes terminal differentiation and expression of Ihh. Then Ihh stimulates PTH-rP expression in the periartricular region leading to reduction in BMP 6 and inhibition of terminal differentiation [12]. The same effect of BMP 6 on chondrocytes maturation was noticed in our experiment. The low basal expression level of PTH-rP resulted in increased expression of BMP 6, and subsequently induced coll 10 and Ihh transcription.

In conclusion, by following the gene expression, we demonstrated that bovine MSCs under condensate culture condition spontaneously underwent complete chondrogenesis. During this process the well-known chondrogenic factors TGF β1 and β2, BMP 6, Ihh, PTH-rP and FGF 2 in autocrine and/or paracrine manner had remarkable influence. The present data will provide useful information concerning the role of growth factors in defining the unique process of bovine MSC chondrogenesis.

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