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

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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  $\beta 1$  and  $\beta 2$ , 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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tured in monolayer manner, which suggests a possible function in maintaining the terminal stage of differentiation. This data extends our knowledge about the unusual species-specific bovine MSC chondrogenesis, allowing us to define the phenotype of the differentiated cells. Furthermore, this study contributes to our understanding of known chondrogenic-growth factors in autocrine and/or paracrine manner playing a role in the spontaneous differentiation.

**Key Words :** bovine, chondrocyte, chondrogenesis, mesenchymal stem cell, pellet culture.

### 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*)  $\beta 1$ ,  $\beta 2$  and  $\beta 3$ , 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*  $\beta 1$  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 ( $5 \times 10^4 / \text{cm}^2$ ) were plated in T-75 culture flasks with DMEM (low glucose) containing penicillin G 100 U/ml, streptomycin 100  $\mu\text{g}/\text{ml}$ , amphotericin B 0.25  $\mu\text{g}/\text{ml}$ , HEPES 2.4 mg/ml,  $\text{NaHCO}_3$  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%  $\text{CO}_2$  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  $\mu\text{m}$  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  $\mu\text{g}/\text{ml}$ , transferrin 6.25  $\mu\text{g}/\text{ml}$ , selenious acid 6.25  $\mu\text{g}/\text{ml}$ , bovine serum albumin 1.25 mg/ml, pyruvate 1 mM, linoleic acid 5.35  $\mu\text{g}/\text{ml}$  and ascorbate 2-phosphate 50  $\mu\text{g}/\text{ml}$  (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%  $\text{CO}_2$  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 ( $5 \times 10^3$  cells/ $\text{cm}^2$ ) 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  $\mu\text{m}$  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 0.3% H<sub>2</sub>O<sub>2</sub> 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% H<sub>2</sub>O<sub>2</sub>.

*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)<sub>20</sub> 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 MgCl<sub>2</sub>, 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

Table 1. Gene expression markers used in quantitative “Real Time” reverse transcriptase polymerase chain reaction (qRT-PCR).

Gene	Primer nucleotide sequence	Product size (bp)	Accession Number
<i>GAPDH</i>	Forward 5' CCT TCA TTG ACC TTC ACT ACA TGG TCT A Reverse 5' TGG AAG ATG GTG ATG GCC TTT CCA TTG	127	U85042
<i>Sox9</i>	Forward 5' CAT GAA GAT GAC CGA CGA G Reverse 5' CGT CTT CTC CGT GTC GGA	118	AF278703
<i>Coll 1</i>	Forward 5' TGC TGG CCA ACC ATG CCT CT Reverse 5' CGA CAT CAT TGG ATC CTT GCA G	120	AB008683
<i>Coll 2</i>	Forward 5' ATC CAT TGC AAA CCC AAA GG Reverse 5' CCA GTT CAG GTC TCT TAG AG	147	X02420
<i>Coll 9</i>	Forward 5' CCA ATG GGC TGC CTG A Reverse 5' TGC CAC TGG GAA TCG AA	232	AF419343
<i>Coll 10</i>	Forward 5' CAT GCT GCC ACA AAC AGC Reverse 5' TGG ATG GTG GGC CTT TTA	110	X53556
<i>COMP</i>	Forward 5' TTC GGA ACG CAC TGT GG Reverse 5' TGC AGG AAC CAG CGG TA	112	X74326
<i>Big</i>	Forward 5' TCT GCT GCG GTT GTG G Reverse 5' GCC CCA CCT GCA CAC T	158	NM178318
<i>TGF <math>\beta</math>1</i>	Forward 5' GGT GCC CGA ACC TGT G Reverse 5' CAG CCG GTT GCT GAG G	130	M36271
<i>TGF <math>\beta</math>2</i>	Forward 5' TCC AAC CCA GCG CTA CA Reverse 5' AAG TGC AGC AGG GAC AGT G	155	M19154
<i>FGF 2</i>	Forward 5' GCA TTC ACG CTG GCA AA Reverse 5' TTC GGC AAC AGC ACA CC	107	M13440
<i>PTH-rP</i>	Forward 5' GCC CGG CAA GAA AAA GA Reverse 5' GCG ATG TTG CGG AGA TG	146	AB097837
<i>IGF 1</i>	Forward 5' TGC TCT CCA GTT CGT GTG C Reverse 5' AGA GGC GCG CAG TAC ATC T	156	X15726
<i>BMP 6</i>	Forward 5' CCC TTC ATG GTG GCT TTC T Reverse 5' CTT GGG TGC AAT GAT CCA G	234	NM001718
<i>Ihh</i>	Forward 5' CCC GCT CAC CTG CTC TT Reverse 5' AGC AGG ATG CCA CCA CA	226	L38517

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



Figure 1 . Morphology of the pellets at day 21 (white arrow). The pellet size is approximately 4 mm in diameter.

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 (*big*) and cartilage oligomeric matrix protein (*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-fold) up-regulated in the pellets compare to the monolayer, and was expressed constantly at low level in freshly iso-

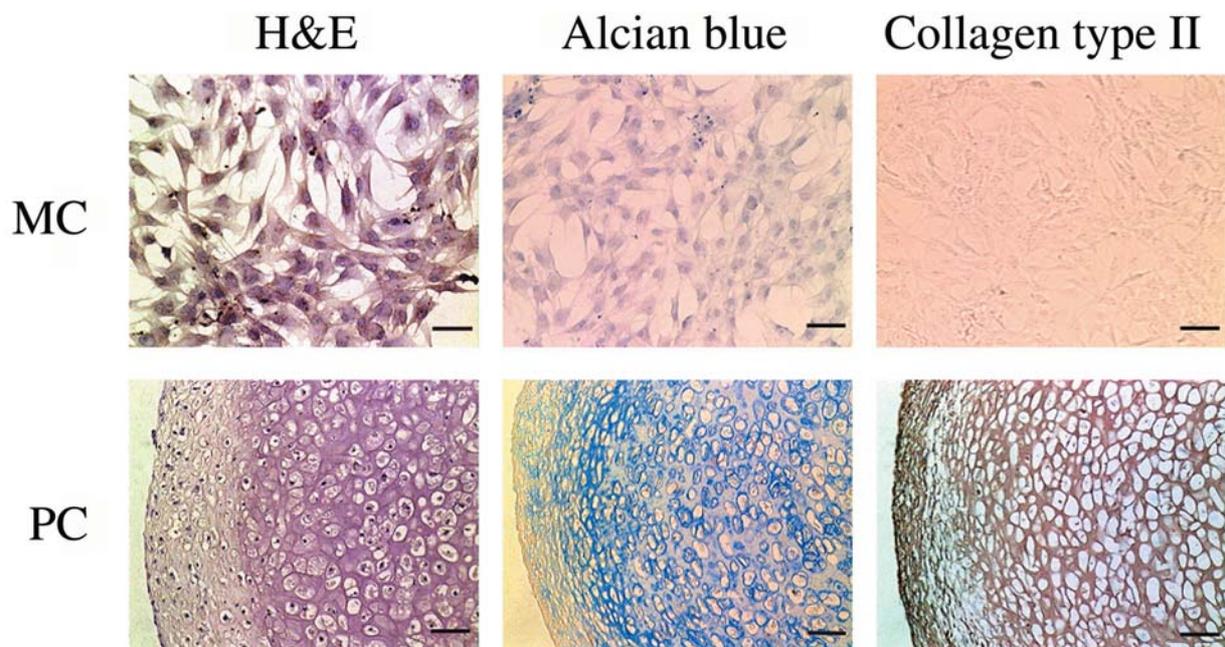
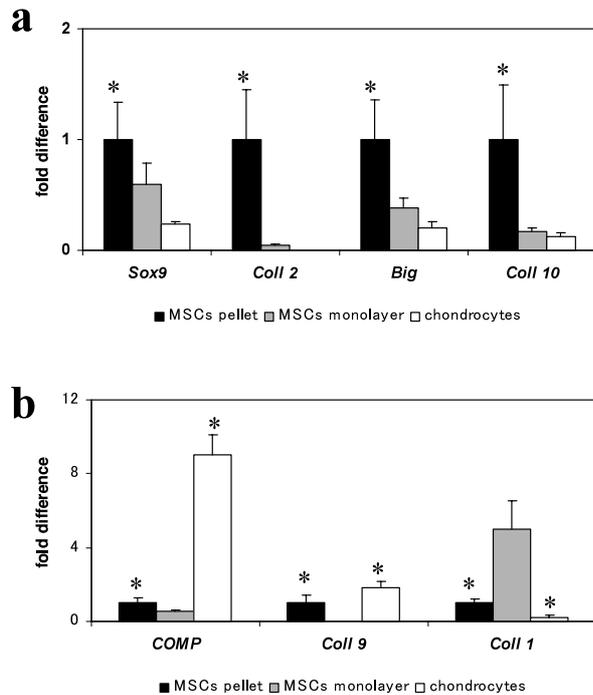


Figure 2 . Histological and immunohistological analyzes of mesenchymal stem cells (MSCs) cultured for 21 days in monolayer and pellet culture. Tissue of pellet culture shows typical characteristics of chondrocytes and cartilage.

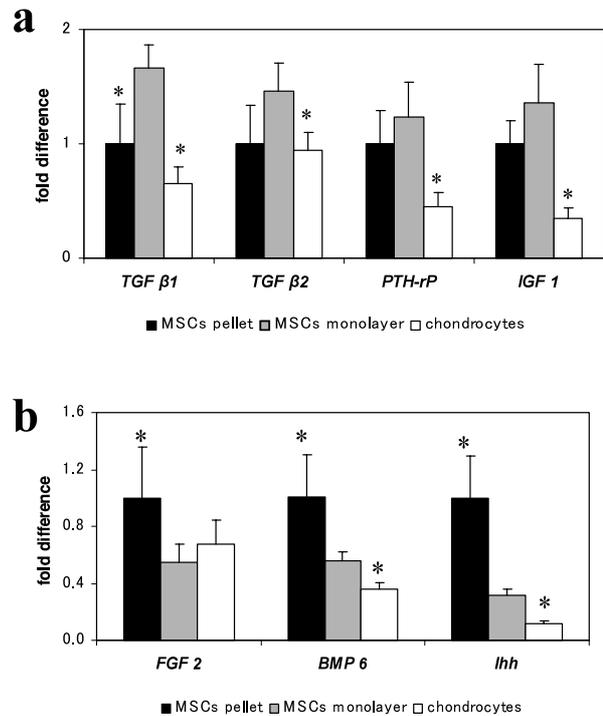
MC : Monolayer culture ; PC : Pellet culture ; H&E : Hematoxylin and eosin staining ; Alcian blue : Alcian blue staining ; Collagen type II : Immunohistochemical staining for collagen type II. Scale bar 100  $\mu$ m.



Figures 3a and 3b. Quantitative analyses of the chondrocytes-specific genes expression in MSCs from monolayer and pellet culture on day 21 and freshly isolated chondrocytes (cultured over night). The expression level was normalized with respect to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The expression level of the genes in pellets was represented as 1. Data were represented as mean  $\pm$  standard deviation (SD).

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



Figures 4a and 4b. Quantitative analyses of the chondrogenic growth factor genes expression in MSCs from monolayer and pellet culture on day 21 and freshly isolated chondrocytes (cultured over night). The expression level was normalized with respect to the expression of *GAPDH* mRNA. The expression level of the genes in pellets was represented as 1. Data were represented as mean  $\pm$  SD.

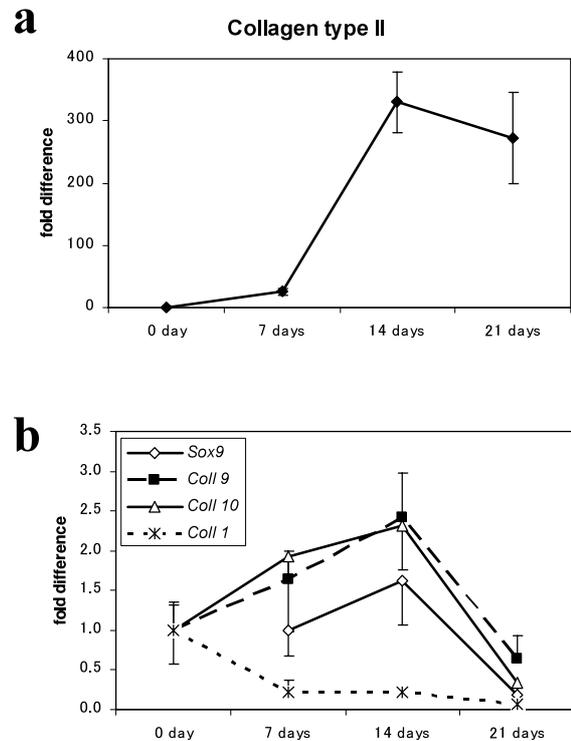
stage of differentiation the most of the analyzed growth factors (*TGF  $\beta$ 1* and  $\beta$ 2, *PTH-rP* and *IGF 1*) were down-regulated in pellets compared to monolayer (Fig. 4b). Among them, *TGF  $\beta$ 1* was only significantly ( $p < 0.5$ ) 1.6-fold lower expressed in pellet than in monolayer (Fig. 4b). Growth factors that were determined to be significantly up-regulated, more than 2-fold, in pellet than 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  $\beta$ 1* and  $\beta$ 2 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*.

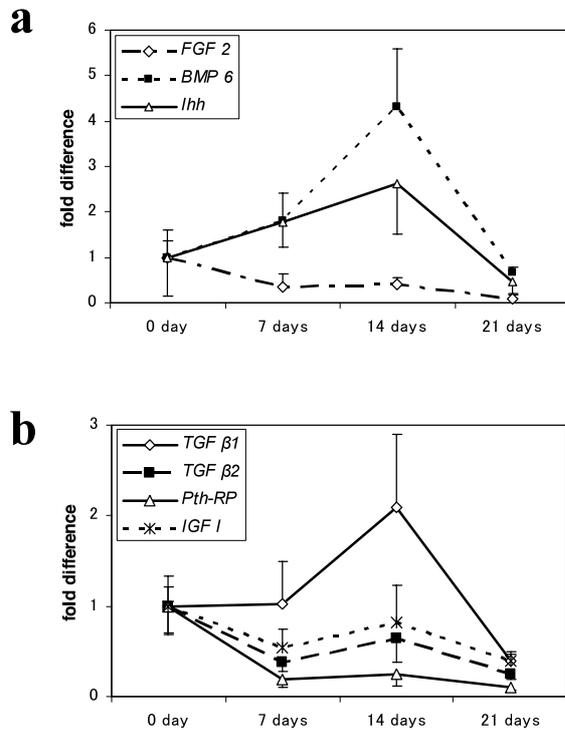
### Discussion

Present work is extended study of our previous reported research about spontaneous chondrogenic differentiation of bovine MSCs



Figures 5a and 5b. Quantitative analyses of the chondrocytes-specific genes expression in MSCs cultured for 21 days in pellet culture system. The expression level was normalized with respect to the expression of GAPDH mRNA. The expression level of the genes on day 0 was represented as 1. Sox9 is not expressing at day 0. Data were represented as mean  $\pm$  SD.

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  $\beta$ 1* and  $\beta$ 2, *BMP 6*, *FGF 2*, *PTH-rP*, *Ihh* and *IGF 1*, were



Figures 6a and 6b. Quantitative analyses of the chondrogenic growth factor genes expression in MSCs cultured for 21 days in pellet culture system. The expression level was normalized with respect to the expression of GAPDH mRNA. The expression level of the genes on day 0 was represented as 1. Data were represented as mean  $\pm$  SD.

up-regulated during differentiation, reaching maximum expression level after 14 days culturing. In the cells which underwent chondrogenesis, expression of *TGF  $\beta$  1* and  *$\beta$  2*, *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  $\beta$*  [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 de-differentiation 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  $\beta$* , *FGF 2*, *IGF 1*, *BMP*, *PTHrP* and *Ihh* are known to influence discrete steps in chondrogenesis pathway [24, 32]. Members of *TGF  $\beta$*  super-family have been shown to play a major role in bone and cartilage development. Especially, the action of *TGF  $\beta 1$*  as potent promoters of the MSC chondrogenic differentiation have been well documented elsewhere [1, 18]. Our results, at transcriptional level, demonstrated that *TGF  $\beta 1$*  and  *$\beta 2$*  in autocrine and/or paracrine manner might have influence on bovine MSC chondrogenesis. Pretreatment of

MSCs with *TGF  $\beta 1$*  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  $\beta 1$*  and  *$\beta 2$* , 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 through 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 [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 through 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  $\beta 3$*  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 periarticular 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  $\beta$ 1* and  *$\beta$ 2*, *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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