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Instructions for use

Coculture of equine mesenchymal stem cells and mature equine articular chondrocytes results in improved chondrogenic differentiation of the stem cells

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Abstract

Bone marrow derived mesenchymal stem cells (MSCs) can be used to repair articular cartilage defects, these cells should be properly stimulated so that they could differentiate morphologically and hold cellular synthetic features closer to maturely differentiated chondrocytes. It is well known that tissue specific environment plays an important role in cell fate determination. Once improved isolation, proliferation and differentiation protocols have been developed, the likelihood of spontaneous differentiation of MSCs into divergent lineages will be reduced, thus increasing their value for cartilage repair. The purpose of this study was to improve chondrogenic differentiation of equine MSCs using coculture with mature equine articular chondrocytes (ACs), along with the determination of the effect of adding transforming growth factor (TGF) β1 in the pellet culture system. Following confirmation of multilineage (adipogenic, osteogenic and chondrogenic) differentiation, isolated MSCs, ACs and coculture of both cell types were transferred into pellet culture system in a DMEM-based medium supplemented with or without TGFβ1. Chondrogenic differentiation was evaluated histologically and the relative mRNA expressions of collagen type 1 α1 (*COL1A1*), collagen type 2 α1(*COL2A1*), aggrecan (*ACAN*) and SRY-box 9 (*SOX9*) were estimated by quantitative RT-PCR. Cocultured cells showed diffuse distribution of extracellular matrix (ECM), whereas in chondrocyte pellets it was more localized to central regions. Expression of *COL2A1*, *ACAN* and *SOX9* genes were higher in cocultured pellets when compared to MSCs and ACs-composed pellets. Addition of TGFβ1 in chondrogenic differentiating medium did not consistently amplify expression of the above mentioned genes. Differentiation of equine MSCs was enhanced by coculturing in association with mature ACs, improving expression of cartilage-specific genes and producing a more homogeneous production of ECM within the newly formed cocultured cartilage. The use of the coculture system could possibly enhance the capacity of MSC-derived chondrocytes to build up stable articular cartilage-like constructs, which could play an important role in articular cartilage repair and regeneration.

Key words: chondrocyte, coculture, differentiation, horse, mesenchymal stem cell.

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Introduction

Many attempts have been made to restore defects of articular cartilage but the results have been largely unsatisfactory, reflecting the poor healing capacity of cartilage. This is partially due to its avascular nature, allied to the fact that the resident chondrocytes fail to mount an effective repair process and the cartilage appears unable to recruit local sources of progenitor cells from either articular surface[8] or from the synovial lining of the joint cavity[10]. Current investigations of cartilage repair have focused on cell transplantation with or without supporting scaffolds[5]. One of the limitations in cell transplantation to repair cartilage defects is the acquisition of a sufficient number of autologous chondrocytes, along with concerns being raised over the maintenance of chondrocyte phenotype in monolayer culture *in situ*, the leaking of chondrocytes from the primary site, and uneven distribution in the three-dimensional space.

Mesenchymal stem cells (MSCs) have been suggested as a cell source for the repair of cartilage lesions, as they are undifferentiated pluripotent cells capable of differentiating into many cell types[4]. In order to be adequate as a therapeutic option these cells should be properly stimulated so that they could reach the end point differentiated status of mature chondrocytes.

Differentiation of MSCs *in vitro* into chondrocytes was first achieved for equine cells in monolayer culture, using supplemented medium[9]. Microenvironment influences stem cells in order to choose a differentiation pathway, and in the case of chondrogenesis, one of the main determining factors is the cell density circumstances[22]. Even mature articular chondrocytes (ACs) once in monolayer culture dedifferentiate during their proliferation[14,15]. Thus, cell density is one of the critical requirements for stabilizing the chondrocyte phenotype. High density cultures promote chondrogenic differentiation since they support cell-to-cell interaction[19,28,30]. Critical factors in chondrogenic differentiation of MSCs *in vitro* appear to be not only the high initial cell density but also the exposure of the cells to signals from glucocorticoids and members of the transforming growth factor (TGF) β family, which can closely mimic the cell lineage developmental process[7,11,12,17,25].

When considering stem cells for cartilage repair, it is imperative to develop well-defined and efficient protocols for directing stem cell differentiation into the chondrogenic lineage *in vitro* in order to improve integration within recipient cartilaginous tissues. Improving isolation, proliferation and differentiation protocols will also reduce the likelihood of spontaneous differentiation of stem cells into divergent lineages. Only recently coculture of MSCs and other stem/progenitor cells with mature cells of tissues has gained attention and is being used increasingly to drive their differentiation towards required lineages. Coculture with mature chondrocytes to promote chondrogenic differentiation in stem cells have been investigated in human embryonic stem cells[2,23], human mesenchymal stem cells[1,21], mouse embryonic stem cells[26] and in porcine mesenchymal stem cells[29] but not yet been performed in equine mesenchymal stem cells.

In this study, we hypothesized that equine MSCs and equine ACs cultured in the same microenvironment under high density conditions would promote MSCs to differentiate into a morphological and cellular synthetic features close to maturely differentiated chondrocytes, which is the goal of a good differentiation system. We also investigated whether the addition of TGFβ1 would improve the differentiation potential of either cell type.

Materials and Methods

Harvest and isolation of equine MSCs and articular chondrocytes: We adhered to the

guidelines reported in the "Guide for the Care and Use of Animals" of the Graduate School of Veterinary Medicine, Hokkaido University. Bone marrow was obtained from the sternebra of a clinically normal 8 year-old and a 6 year-old Thoroughbred (males). The animals were kept under sedation (5 μg/kg medetomidine) and local anesthesia (20 ml 2% lidocaine). The marrow was drawn into 25 ml syringes containing 1,000 units of heparin following aseptic preparation of the harvesting sites and infiltration with local anesthetic. One volume of bone marrow sample was mixed with two volumes of phosphate-buffered saline (PBS), and the mixture was centrifuged at $300 \times g$ for 10 minutes. The supernatant was discarded, and the pellet was washed two more times with Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL, Grand Island, NY, USA). After determination of the cell viability and the number using trypan blue staining, $5.0 \times 10^4/cm^2$ nucleated cells were plated in T-75 culture flasks in DMEM (low glucose) containing: penicillin G 100 U/ml, streptomycin 100 μg/ml, HEPES 2.4 mg/ml, $NaHCO_3$ 3.7 mg/ml, and 10% fetal bovine serum. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 4 days of culturing, the nonadherent cells were removed by changing the culture media. Following the initial 4 days, medium was changed every 2 to 3 days. On days 12 to 14, cells were detached (0.25% trypsin/ 0.1% EDTA) and replated according to the standard cultural technique at a 1 : 3 or 1 : 4 dilution (1st passage). In order to characterize harvested cells as stem/ multipotential cells, multilineage differentiation was performed. When cells began to reach near confluent stage, they were trypsinized and initiated stimulation to differentiate into adipogenic and osteogenic lineage in monolayer culture. As for chondrogenic lineage, cells were used for the preparation of pellet culture, carried along with stimulating medium.

Chondrocytes were isolated from articular cartilage fragments harvested from femoropatellar joint of a 3 month-old male foal. Briefly, cartilage pieces were digested with 0.1 mg/ml of collagenase (Wako Pure Chemical, Osaka, Japan) in DMEM for 12 hr at 37°C in a humidified atmosphere containing 5% CO_2. Isolated primary chondrocytes were cultured in monolayer manner under same conditions as MSC, until used for pellet culture.

Adipogenic and osteogenic assays: In order to characterize the isolated cells as stem/progenitor cells, multilineage differentiation was performed. For adipogenic differentiation, cells from 1st to 4th passages were plated at a density of $2.0–4.0 \times 10^3$ cells/cm^2 in 6-well plates and cultured in control medium until the cells cultured reached confluence. At this point, adipogenic differentiation[18] was stimulated by culturing the cells with adipogenesis-inducing medium containing DMEM (high glucose, 4.5 g/l), 1 μM dexamethasone, 0.5 mM indomethacin, 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxantine (all from Sigma, St. Louis, MO, USA), 10% FBS for 3 days, and for 3 more days in adipogenesis-maintenance medium containing DMEM (high glucose, 4.5 g/l), 10 μg/ml insulin, and 10% FBS. This stimulation was twice repeated (total 18 days treatment). The cells in control group were cultured in control medium (DMEM high glucose). The level of differentiation was followed by histological examination, visualizing lipid vacuoles with Oil red O staining, and the expression of the adipogenic-specific gene peroxisome proliferator-activated receptors γ 2 (*PPARG2*) on day 21 of differentiation.

Osteogenic differentiation was induced in the cells from early passages (1–4) using osteogenic medium[21] from the first day of cell culture in T-60 culture dishes, in a density of 5.0×10^4 cells/cm^2. The control group was cultured in control medium alone, whereas the inducing group was cultured in osteogenic medium containing 100 nM dexamethasone, 10 mM sodium β-glycerophosphate, and 0.05 mM

ascorbic acid (all from Sigma). The cells were cultured for 24 days with medium being changed every 3 days. On day 24, cells from control and osteogenesis group were exposed to 10 nM 1,25-dihydroxyvitamin D3 (Vitamin D; Biomol Research Laboratory, Plymouth Meeting, PA, USA) for more 3 days. The level of osteogenic differentiation was evaluated by visualization of alkaline phosphatase (ALP) activity and by the demonstration of mineralized materials in the cell culture by using von Kossa staining. To analyze osteogenic gene expression, osteocalcin (*BGLAP*) and collagen type I (*COL1A1*), total RNA was harvested on day 27 at the completion of the differentiation protocol.

Induction of chondrogenesis through high density pellet culture and coculture of MSCs with ACs: Equine MSCs from 2nd passage and equine ACs were trypsinized from previous monolayer culture flasks and suspended in complete chondrogenic differentiating media consisting of DMEM (high glucose); insulin-transferrin-selenious acid (ITS) 6.25 μg/ml; bovine serum albumin 1.25 mg/ml; proline 0.35 mM, pyruvate 1 mM, ascorbate 2-phosphate 50 μg/ml and dexamethasone 100 nM. Aliquots of 1.0×10^6 cells in 1 ml of defined media were spun down at $400 \times g$ for 8 minutes. Pellets were cultivated at 37°C in a humidified atmosphere including 5% CO_2 for 20 days. Cells were divided into 6 experimental groups, according to cell content and the presence of TGFβ1 (0 and 10 ng/ml) in the medium: stem cell alone (S), coculture of stem cells and chondrocytes in a 1 : 1 ratio (M) and chondrocytes alone (C); along with TGFβ1 supplemented cells: ST, MT and CT (Fig. 1). Cocultures were done at a ratio of 1 : 1.

Chondrogenic differentiation was evaluated by histochemical identification of extracellular matrix (ECM) production yielded by differentiated chondrocytes, aggrecan was visualized by alcian blue staining (pH 2.5). The expression of chondrogenic-specific genes, collagen type II (*COL2A1*), aggrecan (*ACAN*) and Sox 9 (*SOX9*),

as well as collagen type I (*COL1A1*), which is expressed by undifferentiated cells, were analyzed on day 21 of differentiation.

Measurement of mRNA level by qualitative and quantitative PCR method: For evaluating the changes of gene expression in each of the differentiating assays, reverse-transcription polymerase chain reactions (RT-PCR) were carried out on all three lineages (adipogenesis, osteogenesis and chondrogenesis), and PCR products were identified by electrophoresis. Quantitative RT-PCR (qRT-PCR) was also performed to identify differences in the genes expression within the 6 experimental cartilage constructs obtained from pellet cultures. Total RNA of pellets and monolayer cultured cells was extracted using Trizol (Invitrogen, Lie Technologies, Carlsbad, CA, USA) according to the manufactures protocol. First strand complementary DNA (cDNA) was synthesized from 300 ng of total RNA by using M-MLV reverse transcriptase (Invitrogen) with oligo (dT) 20 as a primer in 20 μl reaction mixture. PCR was carried out in a 20-μl final volume containing PCR buffer, 2.5 mM dNTP mixture, 0.3 μM each of the relevant equine-specific primers (Table 1), and *Taq* polymerase
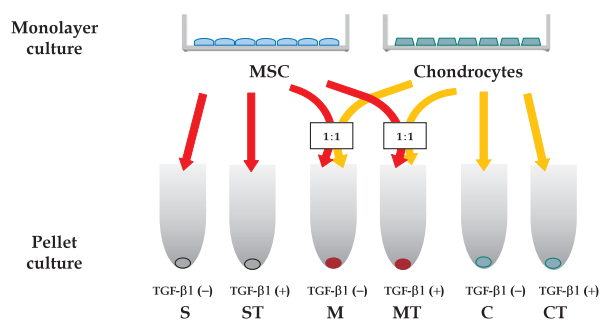

Fig. 1. Scheme of the chondrogenic differentiation in pellet culture. Chondrogenic differentiation was carried out in 6 experimental groups of pellet culture, being divided according to cell content and presence of TGFβ1. Stem cells alone (S), stem cells + TGFβ1 (ST), coculture of stem cells and chondrocytes (M), coculture of stem cells and chondrocytes + TGFβ1 (MT), chondrocytes alone (C), chondrocytes + TGFβ1 (CT). Cocultured pellets were cultured in 1 : 1 ratio of each cell type.

(1.25 units/tube; Takara Biomedicals, Otsu, Japan). Thirty five cycles of thermal cycling were carried out at 94°C for 30 s, 55–60°C for 30 s, and 74°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was the housekeeping gene for chondrogenesis and osteogenesis assay, while actin, beta (*ACTB*) was used for adipogenesis assay. The amount of cDNA was measured by qPCR analysis by using Light Cycler System (Roche, Mannheim, Germany). The amount of PCR product was estimated by measurement of the intensity of fluorescence of SYBR Green I interacting with PCR product. The mRNA expression level of target genes was normalized by mRNA level of *GAPDH*. The quality of the PCR products was checked by melting curve analysis and electrophoresis.

Histological analysis of the pellets: Deposition of minerals in the osteogenic cultures was demonstrated by von Kossa staining. Cell cultures were rinsed twice in PBS, fixed with 4% paraformaldehyde for 15 min, and rinsed three times in distilled water. The cells were stained with 2% silver nitrate in a dark environment for 10 min followed by washes and exposure to light for another 10 min. ALP activity was visualized in glutaraldehyde-fixed cells by the azo-dye method. Briefly, cells were fixed by glutaraldehyde for 5 min, incubated with AS-BI phosphate solution and Fast blue BB salt for 2 hr, washed in water and counterstained with 1% Safranin O for 2 min.

Accumulation of lipids vacuoles was visualized by Oil red O staining. In brief, 10% formalin fixed cells were stained with 0.3% Oil red O for 20 min, rinsed with water, and counterstained with hematoxylin.

Pellets were harvested after 21 days of culture and fixed with 20% buffered paraformaldehyde for 3 hr at room temperature; they were embedded in 0.5% agarose gel, serially dehydrated, infiltrated, paraffin embedded and sectioned at 4-μm thickness for hematoxylin-eosin (HE) and alcian blue (pH 2.5) stains evaluation. Histochemical stains were performed according to standardize protocols for HE and alcian blue.

Table 1. Summary of gene-specific primer pairs used in PCR

Name	Symbol	Primer nucleotide sequence	Accession number	Position	Product size (bp)
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Forward 5' CCAGAACATCATCCCTGCTT Reverse 5' CGTATTTGGCAGCTTTCTCC	AF157626	478–635	158
Actin beta	*ACTB*	Forward 5' CGACATCCGTAAGGACCTGT Reverse 5' GTGGACAATGAGGCCAGAAT	NM001081838	861–1052	192
Collagen type 1 alpha 1	*COL1A1*	Forward 5' AGCCAGCAAGATCGAGAACAT Reverse 5' CGTCTCCATGTTGCAGAAGA	XM001499586	3461–3631	171
Collagen type 2 alpha 1	*COL2A1*	Forward 5' AGCAGGAATTTGGTGTGGAC Reverse 5' TCTGCCCAGTTCAGGTCTCT	U62528	4325–4548	224
Aggrecan	*ACAN*	Forward 5' GCCTCAGGACTTCCAGAAA Reverse 5' CACTGGACTCAACAAGCTG	XM001917528	5773–5902	128
Osteocalcin (bone gamma-carboxyglutamate protein)	*BGLAP*	Forward 5' GTGCAGAGTCTGGCAGAGGT Reverse 5' TCGTCACAGTCTGGGTTGAG	XM001915727	80–245	166
SRY (sex determining region Y)-box 9	*SOX9*	Forward 5' GGGCTGCCTAAATTGTGTGT Reverse 5' AGCAACCCTCAAACTCTCCA	XM001498424	2807–2974	168
Peroxisome proliferator-activated receptor gamma 2	*PPARG2*	Forward 5' TCGCCTTCTTGTTCGTCTTT Reverse 5' ATCTACTGTCTGGGGACCTT	XM001499929	1723–1897	175

Results

Cell culture

MSCs were isolated from equine bone marrow based on their property of attaching to plastic culture flasks and soon assumed a bipolar to polygonal fibroblast-like shape. During monolayer culturing by standard cell culture technique, an active proliferation potential was observed up to passage ten or eleven, and from then on isolated MSCs entered senescence with low propagation ability.

Adipogenic differentiation

After 21 days of differentiation, adipogenesis was identified as cells were transformed from a fibroblastic phenotype into oval shaped cells, followed by formation of lipid vacuoles confirmed by Oil red O staining (Fig. 2a, 2b). The expression of the adipose-specific gene *PPARG2* was also confirmed by PCR (Fig. 2c). In contrast, cells in the control group maintained their fibroblastic shape with no cells containing lipid droplets by Oil red O, and gene analysis by RT-PCR could not identify expression of *PPARG2*.

Osteogenic differentiation

Gradually cell morphology changed from fibroblastic shape to a cuboidal form when treated with osteogenic medium. Cell colonies were found to be positive for ALP activity (Fig. 3b) and mineralized nodules could be demonstrated through von Kossa staining (Fig. 3a), by the end of the differentiation period of 27 days. Gene expression of *COL1A1* (Fig. 3c) and *BGLAP* (Fig. 3d), a typical marker of the osteoblastic phenotype, was detected in the differentiated cells. The cells cultured in control medium did not show such characteristics.

Chondrogenic differentiation

Defined pellets formed within 24 hr of culture in all groups (S, M and C). They all assumed spherical shapes and floated in the culture medium inside the culture tubes. Pellets generally grew bigger with time, becoming harder and smoother in appearance. However, pellets formed by only MSCs did not show any obvious increase in their size. Chondrogenic differentiation was observed at different levels in all cartilage constructs obtained from pellet
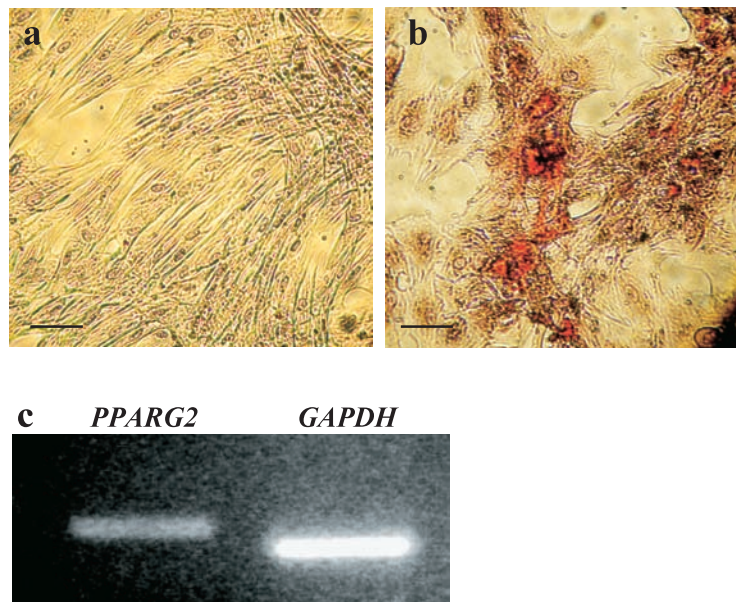

Fig. 2. Adipogenic differentiation. Adipogenesis is demonstrated by Oil red O staining and gene expression. Presence of lipid vacuoles are visualized after 21 days of differentiation (b), contrasting to control (a). Scale bars 400 µm (a) and 450 µm (b). *PPARG2* was detected in induced cells by PCR (c).
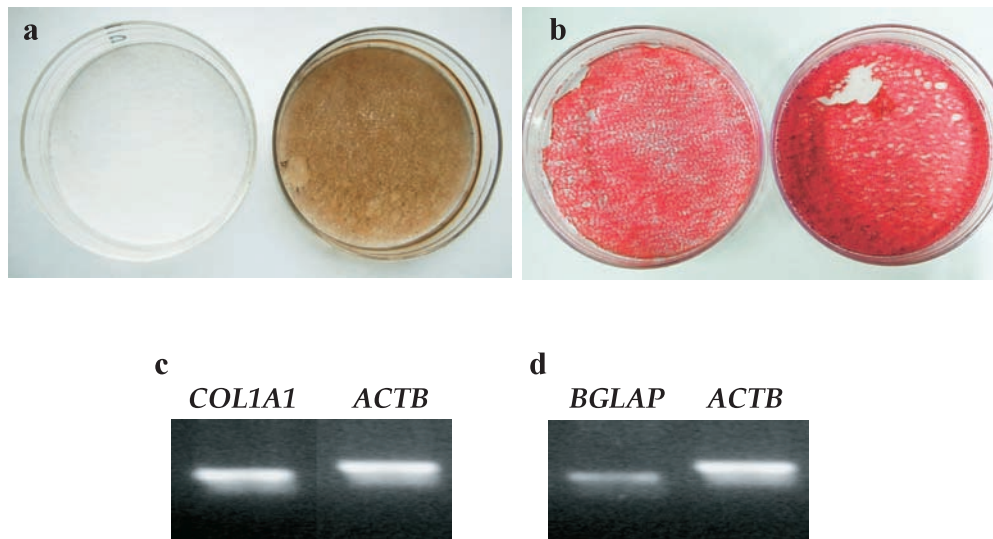
Fig. 3. Osteogenic differentiation. Osteogenesis is demonstrated by the deposition of calcium nodules using von Kossa staining (a), ALP staining (b) and gene expression of *COL1A1* (c) and *BGLAP* by PCR (d). In a and b, the left panel plates are showing the control group.
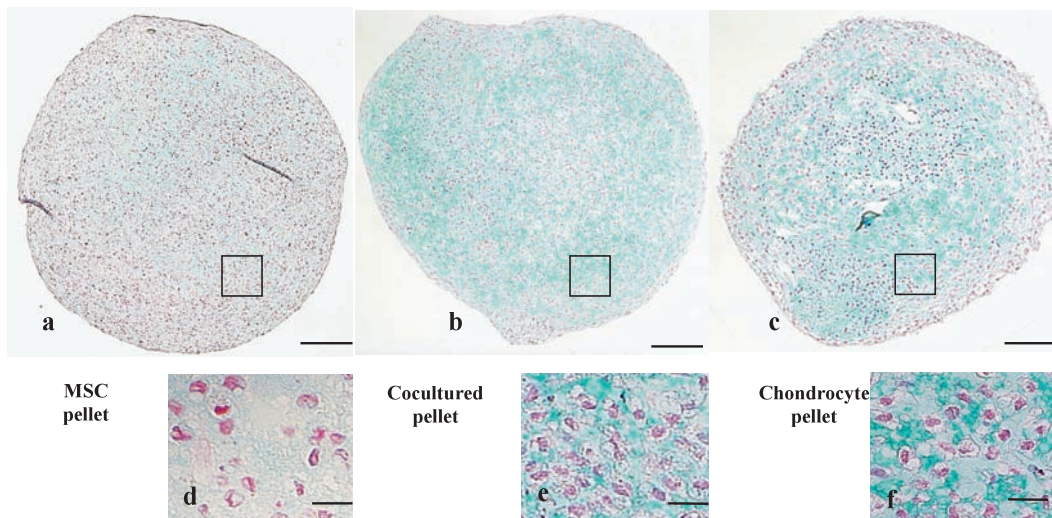

Fig. 4. Cartilage matrix formation in pellet cultures. Proteoglycan deposition was assessed with Alcian blue staining. Micromass cultures were composed of equine bone marrow MSCs (a), coculture of MSCs and ACs (b), and only by ACs (c). Scale bars 200 μm (a, b, c) and 400 μm (d,e,f). Chondrogenic differentiation is observed in all pellets, where chondrocyte morphology was present (d, e, f), but the production of cartilage ECM is evident mainly in cocultured (e) and chondrocyte (f) pellets.

cultures. MSC-only pellets (S) weakly stained to alcian blue, showing reduced production of cartilage-specific matrix (Fig. 4a). Cocultured pellets (M) expressed a more homogeneous staining through out the pellet, with a more even distribution of cartilage ECM (Fig. 4b). Chondrocyte-only pellets (C) showed intense matrix staining although it was more localized to central sections of the constructs (Fig. 4c). It was possible to observe a clear chondrocyte phenotype with cell lacunae formation, particularly in coculture with chondrocyte pellets. All pellets could express, to some extent, genes conditioned to chondrogenic differentiation (Fig. 5), apart from MSCs only composed pellets (S and ST) which demonstrated low level expression of

ACAN and *SOX9* through PCR. The addition of TGFβ1 did not consistently enhance chondrogenesis, which was observed by qRT-PCR evaluation of chondrocyte-specific gene expression of pellets treated and not treated with TGFβ1. Cocultured pellets (M) had a lower expression of *COL1A1* and higher expression of *COL2A1*, *ACAN* and *SOX9* when compared to pellets composed of only MSC (S), as well as pellets formed by chondrocytes (C).

Discussion

Several attempts have been made to achieve regeneration of articular cartilage defects, since such tissue has poor ability to self repair[11,13]. Research is increasingly focused on tissue regeneration by the application of relevant precursor or multipotent stem cells[21]. Mesenchymal stem cells isolated from bone marrow are multipotent cells capable of differentiating into many cell types and maybe suitable for cartilage repair[4]. Chondrocytes are separated by a rich ECM in the normal cartilageneous tissue, making them lose their

cell-to-cell interaction. However, when these cells are extracted from the cartilage they still retain the ability to form functional gap junctions in culture[8]. There is a possibility that isolated chondrocytes give or receive signals to or from MSCs. If there are positive effects on each cell type following mixing of the MSCs with mature chondrocytes, a coculture system has potential to be a useful approach to cartilage tissue engineering. The main advantage with this system is that it allows intimate contact between the 2 cell types, possibly resulting in a more efficient transduction of molecular signals that induce chondrogenesis. In this study, the effects of direct coculture of equine MSCs and adult ACs in a high density pellet culture system were investigated, along with the influence of TGFβ1 as a chondrogenesis inductive factor.

TGFβ1 is a multifunctional polypeptide that regulates a variety of biological functions including cell proliferation, differentiation, and extracellular synthesis[16,20]. Upregulation of sulfated glucosaminoglycan and collagen biosynthesis promoted by TGFβ1 had been demonstrated *in vitro* in chondrocytes[6]. When considering MSCs, the use of TGFβ1 as a stand
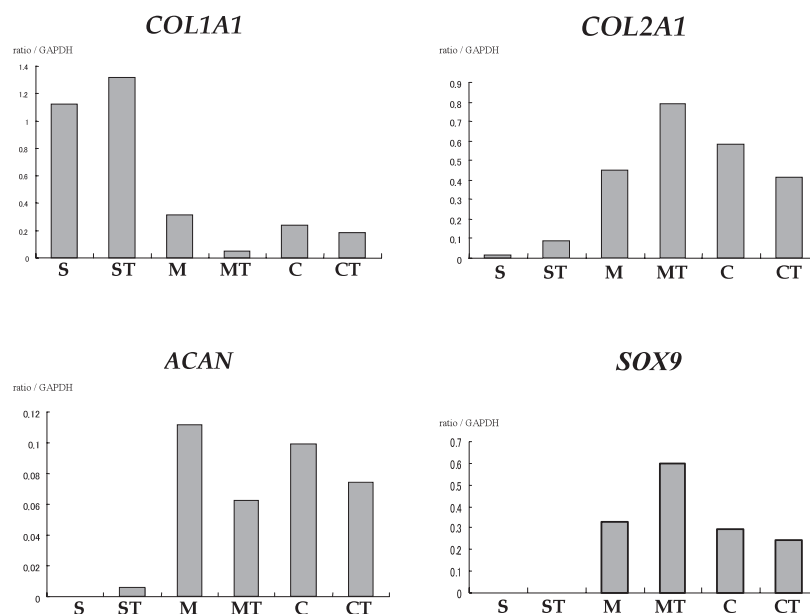

Fig. 5. Qualitative RT-PCR comparing pellet cultures. Pellets were composed by MSCs (S), coculture (M) and ACs (C). The performance of TGF- β1 (T) treated cells was also evaluated (ST, MT, CT).

alone supplement to enhance chondrogenic differentiation had a limited value, although the addition of other growth factors as adjuvants, such as insuline-like growth factor (IGF) 1, yielded improved production of cartilageneous ECM[24]. Previous reports showed that partial chondrogenic differentiation was observed as well in TGFβ1-treated MSCs monolayer cultures, once the production of *ACAN* and *COL2A1* was only slightly enhanced[25]. Im *et al.*[11] compared the effect of TGFs in MSCs undergoing monolayer culture to the ones in pellet culture system, where a reduction in cell number was observed in monolayer cultured MSCs, as opposed to the results in pellet culture where chondrocyte-like differentiation of cells was induced with an increase in *ACAN* and *COL2A1* production. Further, the differentiation was enhanced with the addition of IGF-1 in the differentiating media. Spontaneous chondrogenic differentiation of MSCs, independent of exogenous bioactive substances, in a pellet culture system have been also reported, confirming the importance of culture conditions prior to chondrogenesis[3]. In the present study, the addition of TGFβ1 did not show consistent enhancement of the expression of cartilage-specific genes by cells of any lineage kept in pellet culture system. In our case, the microenvironment of high density culture was already satisfactory to initiate chondrogenic differentiation of the cells, which was significantly intensified by the intimate contact between MSCs and mature chondrocytes promoted by cocultured pellet system. It has been previously reported that TGFβ1 did not optimize in any way MSCs to undergo chondrogenesis, for which they demonstrated spontaneous ability[3], which was consistent with our findings.

Mouse embryonic stem cells (ES) were sorted as fetal liver kinase (Flk) 1 positive cells on flow cytometry[27]. Flk-1 is a receptor tyrosine kinase for vascular endothelial growth factor expressed by several progenitor cells. Following mixture of the sorter ES with chondrocytes isolated from porcine joints, cells were seeded onto polyglycolic acid (PGA) disks and implanted subcutaneously into nude mice. Flk-1 positive cells formed pure cartilages after 4 weeks of co-transplantation with mature chondrocytes, whereas Flk-1 positive cells alone could not form cartilage in the subcutaneous environment, indicating that the chondrogenic differentiation of those sorted ES requires the signals provided by mature chondrocytes[26]. Zhou *et al.*[29] evaluated as well the potential of chondrogenesis in a micromass coculture of porcine MSC and bovine chondrocyte seeded onto PGA scaffold. Their results were similar to the ones that were revealed in our study, where constructs formed only by MSC shrunk gradually, although histology showed a small amount of cartilage formation. However, constructs formed by cocultured cells, as well as by chondrocytes only, formed mature cartilage with high contents of *COL2A1* and *ACAN*. In our study, cocultured pellets expressed similar concentrations of *ACAN* and a greater expression of *COL2A1* and *SOX9* when compared to chondrocyte only-formed pellets. The effects of coculture in pellet mass culture has also been demonstrated by Bigdeli *et al.*[2], where it is showed that coculture of human ES and chondrocytes resulted in increased production of ECM and differentiated as a more homogeneous cell population. Vats *et al.*[23] demonstrated that indirect coculture of human ES and human chondrocytes separated using cell inserts displayed an increased accumulation of sulfated glucosaminoglycans as well as expression of *SOX9* and *COL2A1*.

This current study demonstrates that coculture of equine MSCs with chondrocytes in a pellet mass culture had a profound effect on their differentiation potential. The cellular morphology in the pellet was similar to that of ACs cultured in pellet mass culture. The cocultured MSCs showed an improved ability to form cartilage construct by the presence of cartilage-specific genes and homogeneous distribution of ECM production throughout the construct. In this study, the coculture in a high-

density pellet culture appeared to have a more positive effect directing chondrogenic differentiation than the addition of TGFβ1.

Despite this significantly increased chondrogenic potential detected after coculture, there is a need to further investigate the factors important for chondrogenesis of MSCs resulting in further improvement of their matrix-forming ability. The results from this part of the study confirmed the potential of the culture microenvironment to influence equine MSC differentiation towards the chondrogenic lineage. Significant improvement in differentiation was observed in coculture with mature equine chondrocytes. Taken together, these results showed the importance of cell microenvironment for improving MSC cell fate determination into chondrogenic features and can lead to the development of an effective stem cell therapy for equine joint/cartilage injuries in the near future.

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