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Comparison of the effect of growth factors on chondrogenesis of canine mesenchymal stem cells

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ABSTRACT. Mesenchymal stem cells (MSCs) are proposed to be useful in cartilage regenerative medicine, however, canine MSCs have been reported to show poor chondrogenic capacity. Therefore, optimal conditions for chondrogenic differentiation should be determined by mimicking the developmental process. We have previously established novel and superior canine MSCs named bone marrow peri-adipocyte cells (BM-PACs) and the objective of this study was to evaluate the effects of growth factors required for *in vivo* chondrogenesis using canine BM-PACs. Spheroids of BM-PACs were cultured in chondrogenic medium containing 10 *n*g/m*l* transforming growth factor-β1 (TGF-β1) with or without 100 *n*g/m*l* bone morphogenetic protein-2 (BMP-2), 100 *n*g/m*l* growth differentiation factor-5 (GDF-5) or 100 *n*g/m*l* insulin-like growth factor-1 (IGF-1). Chondrogenic differentiation was evaluated by the quantification of glycosaminoglycan and Safranin O staining for proteoglycan production. The expression of cartilage matrix or hypertrophic gene/protein was also evaluated by qPCR and immunohistochemistry. Spheroids in all groups were strongly stained with Safranin O. Although BMP-2 significantly increased glycosaminoglycan production, Safranin O-negative outer layer was formed and the mRNA expression of COL10 relating to cartilage hypertrophy was also significantly upregulated ($P<0.05$). GDF-5 promoted the production of glycosaminoglycan and type II collagen without increasing COL10 mRNA expression. The supplementation of IGF-1 did not significantly affect cartilaginous and hypertrophic differentiation. Our results indicate that GDF-5 is a useful growth factor for the generation of articular cartilage from canine MSCs.

KEY WORDS: canine, cartilage, chondrogenesis, growth factor, mesenchymal stem cell

Articular cartilage contains abundant extracellular matrix (ECM), rich in glycosaminoglycans (GAGs), proteoglycans, and type II collagen [3]. Articular cartilage injury is common in dogs, because young and old dogs often suffer from joint diseases such as hip dysplasia, osteochondritis dissecans, and rupture of the cranial cruciate ligament [24, 32, 47]. Dogs suffering from pain or lameness caused by injured cartilages are traditionally treated with administration of analgesics such as non-steroidal anti-inflammatory drugs, or debridement of necrotic tissues including subchondral bone. However, regardless of these treatments, severely injured cartilage never regenerates because of blood supply privation in the articular cartilage, which ultimately results in secondary osteoarthritis and causes progressive joint degeneration and persistent joint pain [34]. Therefore, a new and radical therapeutic strategy to regenerate cartilage tissue is required.

Mesenchymal stem cells (MSCs) are immature cells that can be easily isolated from somatic tissues such as bone marrow, adipose, and synovial tissues [21]. MSCs have high proliferative capability and multipotency to differentiate into osteoblasts, adipocytes and chondrocytes [5]. MSCs are also known to differentiate into chondrocytes in aggregation culture, mimicking the process of cartilage development. Therefore, MSCs hold promise for practical application to cartilage regenerative medicine in dogs [18, 46]. However, previous studies in canine MSCs have failed to demonstrate sufficient chondrogenic potential with abundant ECMs such as proteoglycans and type II collagen [4, 17, 20, 30, 31]. We previously established novel methods of culturing canine MSCs named bone marrow peri-adipocyte cells (BM-PACs), isolated from cells adhering to mature adipocytes in bone marrow [22]. BM-PACs showed superior proliferative capability and multipotency compared to those of conventional bone marrow-derived MSCs and can be a promising cell source for regenerative therapy in dogs. Although the potential of robust chondrogenesis is still unclear, the investigation of optimal conditions for the chondrogenic differentiation using canine BM-PACs

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is useful to mimic the chondrogenic process of MSCs.

In the early stage of cartilage development, condensed mesenchymal cells rapidly proliferate and differentiate into chondrocytes, which subsequently mature into articular chondrocytes [15]. However, a portion of chondrocytes separately undergo hypertrophic differentiation with losing their cartilaginous phenotype, ultimately inducing vascularization and bone formation [15]. To effectively maximize the chondrogenic potential of canine MSCs, it is necessary to explore the chondrogenic conditions that mimic the environment of cartilage development to avoid hypertrophic and osteogenic differentiation.

During *in vivo* cartilage development, many growth factors controlling the formation of cartilage and bone are reported to be secreted, such as transforming growth factor-β1 (TGF-β1), bone morphogenetic protein-2 (BMP-2), growth differentiation factor-5 (GDF-5), and insulin-like growth factor-1 (IGF-1) [12]. TGF-β1 is a key regulator of cartilage development from early mesenchymal condensation to terminal differentiation [45]. In 1998, Johnstone *et al.* first reported that TGF-β1 was essential for *in vitro* cartilage generation from rabbit MSCs in a three-dimensional culture system [18]. Both BMP-2 and GDF-5 belong to the TGF-β superfamily. Although BMP-2 stimulates the proliferation and matrix production of chondroprogenitors in early chondrogenesis, it also strongly stimulates hypertrophic differentiation and osteogenesis [44]. Interestingly, GDF-5, also known as cartilage-derived morphogenetic protein-1, induces the formation of mesenchymal condensation [11]. In later development stages, GDF-5 is reported to be expressed in the joint interzone and control joint formation [42]. IGF-1 is a regulator of proliferation and differentiation in many types of cells and is known to promote cell division of chondrocytes at various stages [27]. Therefore, these growth factors have been reported to promote *in vitro* chondrogenic differentiation of human MSCs in combination with TGF-β1 [9, 10, 29, 33, 38], and similar chondrogenic effects can be expected in canine MSCs. Hence, more effective chondrogenic conditions can be established to generate high-quality cartilages by evaluating the effect of these growth factors on canine MSC chondrogenesis.

The purpose of this study was to investigate the optimal growth factor conditions for canine MSCs to enhance the synthesis of articular cartilage ECMs without inducing hypertrophic differentiation. We supplemented BMP-2, GDF-5, and IGF-1 to chondrogenic induction medium containing TGF-β1 and evaluated their effects on the chondrogenesis of BM-PACs.

MATERIALS AND METHODS

Animals

Bone marrow samples were harvested from six healthy young beagles (3 males and 3 females, aged between 8 and 13 months) under general anesthesia, induced with propofol, and maintained with isoflurane (2.0%) in oxygen. All animal experiments were approved by the Animal Care Committee of the Graduate School of Agricultural and Life Sciences at the University of Tokyo (the approval number P15-30).

Isolation and expansion of canine BM-PACs

Canine BM-PACs were cultured according to a previously described method [22] with modifications in the composition of the growth medium after ceiling culture. Briefly, bone marrow aspirated from the proximal humerus was subjected to density gradient centrifugation with Ficoll-Paque (GE Healthcare, Little Chalfont, U.K.). After centrifugation, the top adipose layer containing mature adipocytes was transferred into a new tube and washed twice with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin/amphotericin-B (Wako, Tokyo, Japan). The mature adipocytes, to which BM-PACs adhered, were then placed in 25-cm^2 flasks completely filled with DMEM supplemented with 20% FBS and 1% antibiotics and subjected to ceiling culture at 37°C in a humidified atmosphere containing 5% CO_2. The ceiling culture was maintained for 5–7 days without replacing the medium until the cells reached 100% confluence. The confluent cells were detached with 0.25% trypsin/1 mM ethylenediaminetetraacetic acid solution (Wako) and passaged at a density of 1×10^4 cells/cm^2 in growth medium consisting of DMEM, 10% FBS, 1% antibiotics, and 10 *n*g/m*l* human recombinant basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ, U.S.A.).

Chondrogenic differentiation

Cells at passage 1 were seeded in low-adhesion 96-multiwell plates (Sumitomo Bakelite, Tokyo) at a density of 3×10^4 cells/well to form spheroids. For chondrogenic differentiation, spheroids were cultured in 200 *μl* of chondrogenic induction medium consisting of DMEM, 4.5 mg/m*l* D-(+)-glucose (Sigma, St. Louis, MO, U.S.A.), 1% ITS liquid media supplement (Sigma), 1% linoleic acid-albumin from bovine serum albumin (Sigma), 50 *μ*g/m*l* ascorbic acid-2-phosphate (Sigma), 0.1 *μ*M dexamethasone (Sigma), 40 *μ*g/m*l* L-proline (Peptide Institute Inc., Osaka, Japan), and 10 *n*g/m*l* recombinant human TGF-β1 (Peprotech). To investigate the effect of growth factors on chondrogenesis, 100 *n*g/m*l* human recombinant BMP-2 (Shenandoah Biotechnology, Warwick, PA, U.S.A.), 100 *n*g/m*l* human recombinant GDF-5 (ProSpec-Tany TechnoGene, Rehovot, Israel), or 100 *n*g/m*l* human recombinant IGF-1 (R&D Systems, Minneapolis, MN, U.S.A.) was added to chondrogenic induction medium. Groups cultured with BMP-2, GDF-5, and IGF-1 were referred to as T+B, T+G, and T+I, respectively. As a control, spheroids were cultured in chondrogenic induction medium (T group). Spheroids were cultured for 14 days at 37°C in an atmosphere containing 5% CO_2 and the medium was changed twice a week. The diameter of the spheroids was continuously measured by microscopic observation.

Biochemical analysis

For quantification of GAGs and DNA, spheroids were collected 7 and 14 days after chondrogenic induction and digested with 100 *μ*g/m*l* papain at 65°C for 4 hr. DNA content was measured by Hoechst 33258 dye (Dojindo Molecular Technologies, Kumamoto,

Japan). Fluorescence intensity was measured using the multilabel counter ARVO MX (Perkin Elmer, Waltham, MA, U.S.A.) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Calf thymus DNA (Sigma) was used for the generation of a standard curve. GAG content was then quantified by a Blyscan Kit (Biocolor, Westbury, NY, U.S.A.) according to the manufacturer's instructions. The optical density was measured at 630 nm and total GAG content was calculated. For comparison of GAG production, GAG content was normalized to DNA content (GAG/DNA). Each experiment was performed in duplicate.

Table 1. Primers used for semi-quantitative RT-PCR

Genes		Sequence (5′-3′)
SOX9	Forward	AAGCTCTGGAGGCTGCTGAA
	Reverse	ACTTGTAATCCGGGTGGTCTTT
ACAN	Forward	CCTACGATGTCTACTGCTATGTGG
	Reverse	CAGGGTGGCGTTATGAGATTC
COL2	Forward	CCCGAACCCACAAACAACA
	Reverse	AGCCATTCAGTGCAGAGCC
COL1	Forward	GTAGACACCACCCTCAAGAGC
	Reverse	TTCCAGTCGGAGTGGCACATC
COL10	Forward	TTCCAGGACAGCCAGGCATCA
	Reverse	TTCCCAGTGCCTTCTGGTCC
GAPDH	Forward	TGACACCCACTCTTCCACCTTC
	Reverse	CGGTTGCTGTAGCCAAATTCA

Histology and immunohistochemistry

Spheroids were fixed with 10% formalin neutral buffer solution (Wako), dehydrated, and embedded in paraffin. Specimens were cut into 4-μm sections. Safranin O staining was performed for the detection of proteoglycans. For immunohistochemistry of type II, I, and X collagen, sections were treated with 50 μg/m*l* proteinase K (Promega, Madison, WI, U.S.A.) for 10 min at room temperature. For type II collagen, an additional antigen retrieval step was carried out using 25 mg/m*l* hyaluronidase (Sigma) for 2 hr at 37°C. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min. After washing with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the sections were blocked with TBS-T containing 10% normal goat serum (Sigma) for 30 min at room temperature, and then incubated with rabbit anti-type II collagen antibody (1:200; LSL, Tokyo, Japan), mouse anti-type I collagen antibody (1:1,000; Abcam, Cambridge, U.K.), and mouse anti-type X collagen antibody (1:1,000; Sigma) at 4°C overnight. The slides were washed with TBS-T and incubated with horseradish peroxidase-labeled polymer (Dako, Tokyo, Japan) for 1 hr at room temperature. Finally, diaminobenzidine substrate (Dako) was placed on the slides and all slides were counterstained with hematoxylin.

Real-time polymerase chain reaction (RT-PCR)

After 14 days of chondrogenic induction, the spheroids were homogenized in TRI Reagent (Cosmo Bio, Tokyo, Japan) and total RNA was obtained. Complementary DNA was synthesized with ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). The real-time fluorescence intensity of the SYBR Green dye (Thunderbird SYBR qPCR Mix, Toyobo) was monitored with the Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, U.S.A.). The mRNA expression of chondrogenic marker genes (SOX9, ACAN, and COL2), the fibrocartilage marker gene COL1, and the hypertrophic chondrocyte marker gene COL10 was evaluated. The primers used for PCR are listed in Table 1. All reactions were performed as follows: 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. The mRNA expression levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In addition, the ratio of COL2 to COL1 (COL2/COL1) was calculated to evaluate differentiation into hyaline cartilage. Each experiment was performed in triplicate.

Statistical analysis

All data were expressed as the mean ± standard deviation. Comparisons were made using one-way analysis of variance followed by Tukey's multiple comparisons test between multiple unpaired groups. Statistical significance was defined as $P<0.05$. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [19].

RESULTS

Spheroid diameter

Spheroids in all groups maintained their size from day 7 through to day 14, and the spheroid diameter in the T+B group was significantly larger than that in the other groups ($P<0.05$, Fig. 1).

Quantification of DNA and GAGs

Chondrogenically induced spheroids were digested and the DNA and GAG contents in the spheroids were measured. The DNA content in the spheroids in the T+B group was significantly higher than those in the other groups after 7 and 14 days of chondrogenic induction ($P<0.05$, Fig. 2A). The GAG content was significantly higher in the T+B group than those in the T and T+I groups ($P<0.05$, Fig. 2B). Treatment with GDF-5 increased GAG deposition, but not significantly. To correct for cell number in the spheroids, GAG content was divided by DNA content. After 7 days of chondrogenic induction, there was no significant differences in GAG/DNA among the four groups. However, GAG/DNA was significantly higher in the T+B and T+G groups than those in the other groups on day 14 (Fig. 2C).

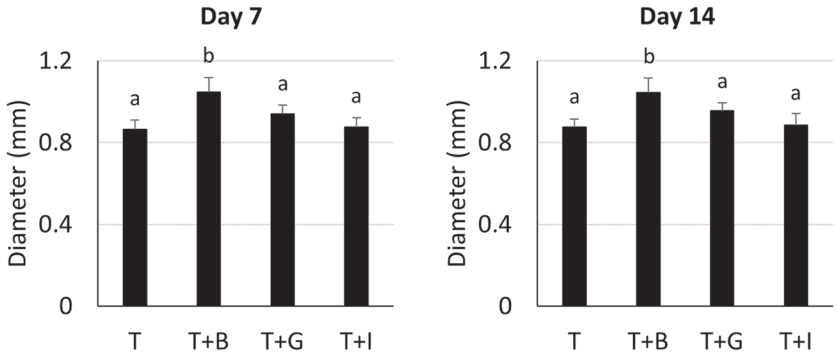

Fig. 1. The diameter of spheroids after 7 and 14 days of chondrogenic differentiation under the presence of bone morphogenetic protein-2 (BMP-2) (B), growth differentiation factor-5 (GDF-5) (G), or insulin-like growth factor-1 (IGF-1) (I) combined with transforming growth factor-β1 (TGF-β1) (T). Spheroid diameter in the T+B group was significantly larger than those in the other groups. Groups indicated by different letters show significant differences (P<0.05).

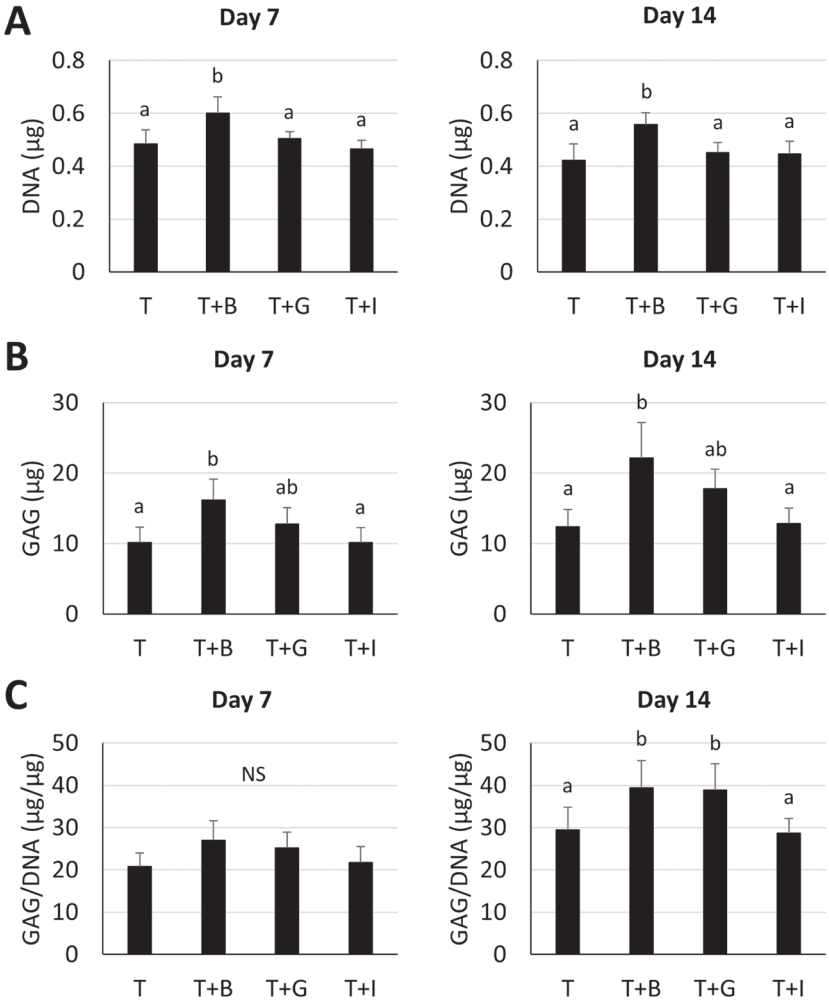

Fig. 2. Quantification of DNA and glycosaminoglycan (GAG) content after 7 and 14 days of chondrogenic induction under the presence of bone morphogenetic protein-2 (BMP-2) (B), growth differentiation factor-5 (GDF-5) (G), or insulin-like growth factor-1 (IGF-1) (I) combined with transforming growth factor-β1 (TGF-β1) (T). (A) The DNA content of spheroids in the T+B group was significantly higher than those in the other groups. (B) Total GAG content in spheroids was significantly higher in the T+B group than in the other groups. (C) To evaluate the efficiency of GAG production, the ratio of GAG to DNA was calculated. GAG/DNA was significantly increased in the T+B and T+G groups on day 14. Groups indicated by different letters show significant differences (P<0.05).

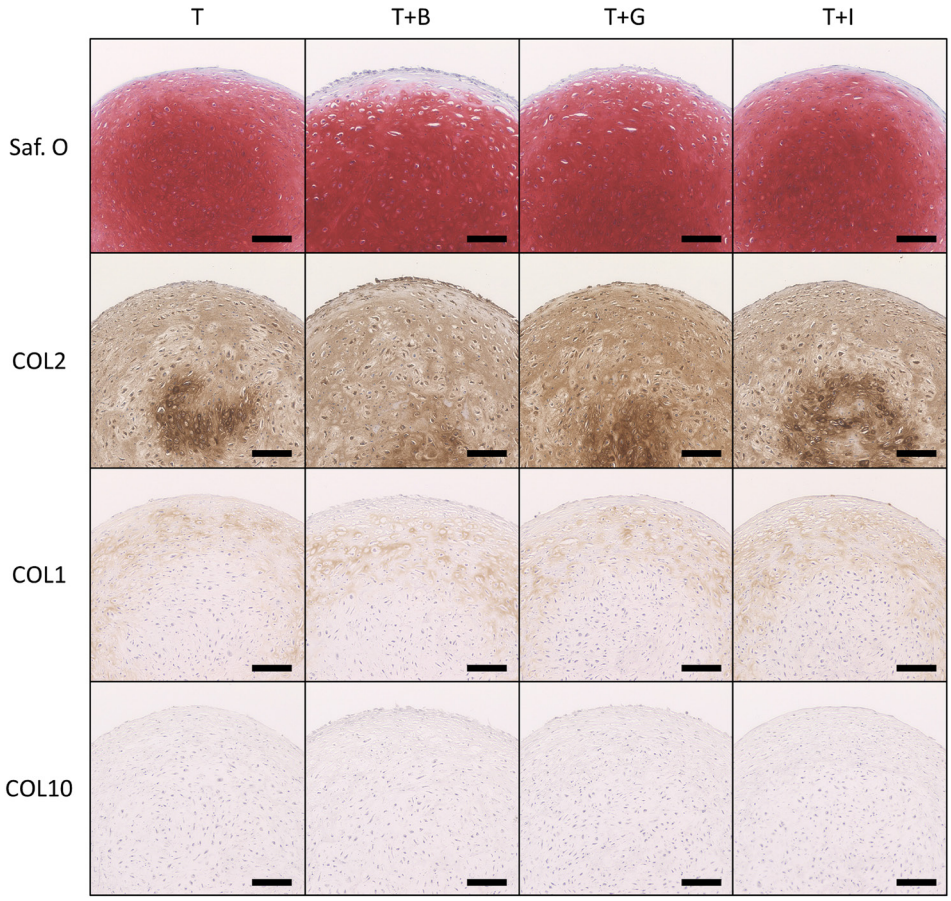

Fig. 3. Safranin O staining and immunohistochemistry for type II, I, and X collagen after 14 days of chondrogenic induction under the presence of bone morphogenetic protein-2 (BMP-2) (B), growth differentiation factor-5 (GDF-5) (G), or insulin-like growth factor-1 (IGF-1) (I) combined with transforming growth factor-β1 (TGF-β1) (T). Spheroids in all groups were strongly stained with Safranin O, and stronger staining was observed in the center of the spheroids. In the T+B group, a Safranin O-negative outer layer was formed. All spheroids expressed type II collagen, especially in the center of the spheroids, and the T+G group showed higher expression of type II collagen. The outer fibroblastic layer of the spheroids in the T+B group showed slight expression of type II collagen. Type I collagen was apparently detected on the outer surface of spheroids in all groups. No type X collagen expression was detected in any group. All scale bars indicate 100 μm.

Histological evaluation

After 14 days of chondrogenic induction, Safranin O staining and immunohistochemistry for type II, I, and X collagen were performed to evaluate the deposition of cartilage matrix (Fig. 3). Spheroids in all groups were strongly stained with Safranin O, and the center of the spheroids revealed stronger staining. However, there was no apparent difference in the intensity of Safranin O staining among all groups. In the T+B group, a thicker Safranin O-negative outer layer was formed and consisted of fibroblast-like cells. Immunohistochemistry revealed that all spheroids expressed type II collagen, especially in the center. The outer fibroblastic layer of the spheroids in the T+B group was slightly positive for type II collagen immunostaining. In the T+G group, the spheroids showed higher expression of type II collagen. Type I collagen, which is a marker of fibrocartilages, was apparently detected on the outer surface of the spheroids in all groups. Type X collagen, a marker of hypertrophic chondrocytes, was not detected in any group.

Gene expression analysis

To assess the expression of chondrogenic genes, cultured spheroids were collected on day 14 (Fig. 4). The expression of chondrogenic genes (SOX9, ACAN, and COL2) and the fibrocartilage gene COL1 did not show remarkable differences in the different chondrogenic media. The ratio of COL2 to COL1 (COL2/COL1) was higher in the T+G group, but not significantly (P=0.14). Additionally, BMP-2 treatment significantly increased the expression of COL10, a hypertrophic gene (P<0.05). The expression of COL10 was upregulated in the T+G group, but not significantly.

DISCUSSION

Spheroids of BM-PACs were cultured in chondrogenic induction medium supplemented with BMP-2, GDF-5, or IGF-1 to evaluate the potential of these growth factors for enhancing chondrogenesis. BMP-2 increased the spheroid diameter, DNA content,

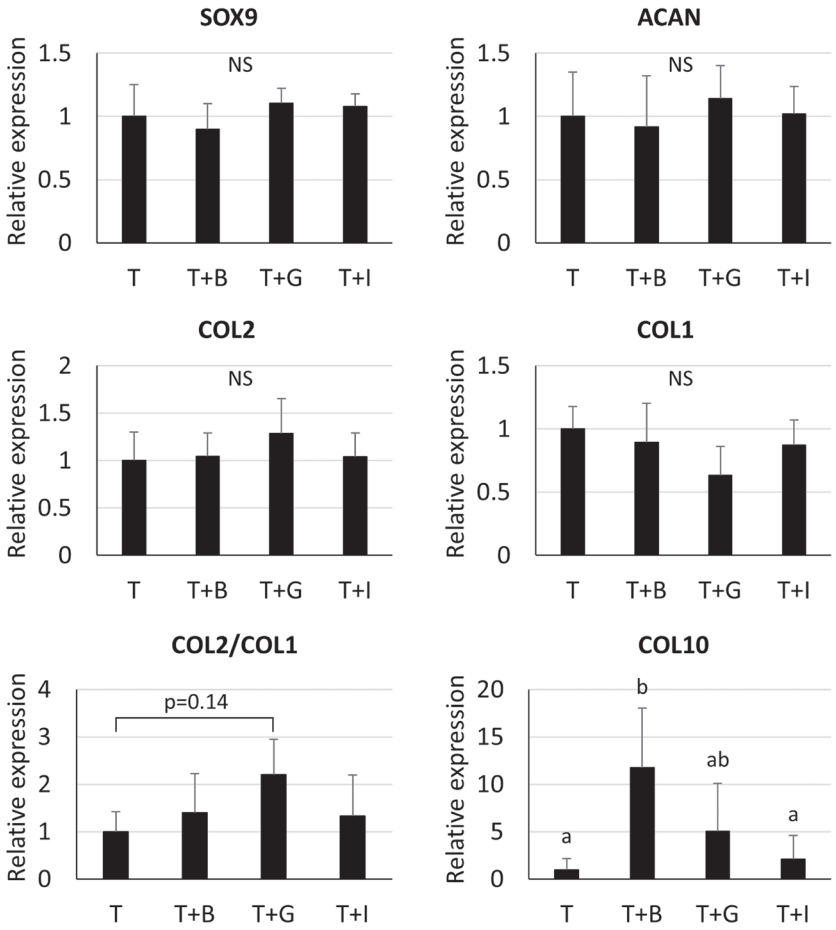

Fig. 4. Gene expression analysis after 14 days of chondrogenic induction under the presence of bone morphogenetic protein-2 (BMP-2) (B), growth differentiation factor-5 (GDF-5) (G), or insulin-like growth factor-1 (IGF-1) (I) combined with transforming growth factor-β1 (TGF-β1) (T). There was no apparent change in the mRNA expression of SOX9, ACAN, COL2, and COL1. The ratio of COL2 to COL1 (COL2/COL1) was higher in the T+G group, but not significantly. The expression of COL10 was significantly increased in the T+B group. The expression of COL10 was upregulated in the T+G group, but not significantly. Groups indicated by different letters show significant differences ($P<0.05$).

and GAG/DNA ratio. These results suggested that BMP-2 enhanced the proliferation of BM-PACs as well as GAG production per cell. Consistent with our results, it has been previously reported that BMP-2 promotes cell proliferation and cartilaginous matrix production during chondrogenesis of human MSCs [26, 36, 38, 39]. In contrast, supplementation of GDF-5 increased only GAG/DNA. IGF-1 had no significant effect on GAG or DNA content. Based on the results in this study, it is proposed that unlike BMP-2, GDF-5 stimulates only GAG production in chondrogenically differentiated cells, without promoting growth activity.

As shown in the histological evaluation, all spheroids demonstrated evidence of chondrogenic differentiation with sufficient expression of proteoglycans and type II collagen. However, a difference was observed in the outer layer of the spheroids. The outer layer of the spheroids in the T+B group showed negative Safranin O staining and slight immunostaining for type II collagen. While BMP-2 is known to be a strong inducer of chondrogenesis, it has also been demonstrated that *in vitro* osteogenic differentiation of MSCs is promoted by BMP-2 treatment [43]. Cheng *et al.* have reported that forced expression of BMP-2 in rabbit MSCs induced *in vivo* ectopic bone tissue formation [7]. Furthermore, in this study, BMP-2 significantly increased the mRNA expression of COL10. This result is consistent with a previous study, which showed that the expression of type X collagen was upregulated by BMP-2 during chondrogenesis of human bone marrow-derived MSCs and rabbit periosteal explants [6, 38]. Therefore, it is suggested that cells in the outer layer of the spheroids were losing their cartilaginous phenotype, and contributed to further endochondral ossification. Accordingly, BMP-2 possibly induced hypertrophic differentiation during chondrogenesis of canine BM-PACs. Nevertheless, immunohistochemistry revealed the absence of type X collagen expression in the present study. Sekiya *et al.* demonstrated that the mRNA expression of COL10 was already increased before the histochemical detection of hypertrophy during *in vitro* chondrogenesis of human MSCs [38]. Although there is a possibility that immunohistochemistry could not detect the slight protein expression of type X collagen, the observation for a longer period should be performed to evaluate hypertrophic differentiation. In contrast, GDF-5 increased the expression of type II collagen and the index of hyaline cartilage genes (COL2/COL1) without significantly upregulating COL10 mRNA expression. These results are in agreement with a previous study by Ayerst *et al.*, which demonstrated that the combination of TGF-β1 and GDF-5 enhanced chondrogenic gene expression without stimulating type X collagen expression during

the chondrogenesis of human bone marrow-derived MSCs [1]. Although further studies are required to evaluate the long-term effect of GDF-5 on hypertrophic differentiation, our results demonstrated that GDF-5 promoted hyaline cartilage differentiation of canine BM-PACs without inducing hypertrophy and could be useful for articular cartilage regeneration.

Some studies have shown that IGF-1 has additive effects on TGF-β-induced human MSC chondrogenesis through the regulation of proliferation, apoptosis, and cartilage matrix production even under the presence of insulin [16, 33]. In this study, supplementation of IGF-1 at the same concentration as these previous studies increased neither GAG production nor the expression of hypertrophic genes in canine BM-PACs. IGF-1 is a polypeptide with a very similar amino acid sequence to that of insulin and regulates cartilage homeostasis and ECM synthesis via its receptor, IGF-1R [13, 35]. Longobardi *et al.* demonstrated the chondroinductive effect of IGF-1 in mouse bone marrow-derived MSCs under the absence of insulin because insulin has low binding affinity to IGF-1R [23]. Since it has been reported that canine MSCs react with recombinant human IGF-1 at much lower concentration (2–5 *n*g/m*l*) [2, 37], there is a possibility that the effect of IGF-1 was masked by the presence of insulin during chondrogenic differentiation of canine BM-PACs. To clarify the effect of IGF-1 on canine MSC chondrogenesis, chondrogenic assay at different concentrations of IGF-1 or under an insulin-free condition should be demonstrated.

The induction medium containing TGF-β1 alone successfully induced cartilaginous spheroids with the expression of proteoglycans and type II collagen. In this study, we routinely supplemented bFGF to the growth medium of BM-PACs before chondrogenic induction. bFGF, also known as FGF-2, is a growth factor that stimulates cell division and vascularization [25]. Previous studies have indicated that bFGF supplementation to growth medium enhanced the proliferation and chondrogenic potential of human MSCs [8, 14, 28, 40]. Although the effect and underlying mechanism of bFGF preconditioning is unclear in canine MSCs, it is suggested that bFGF contributed to the robust chondrogenesis of canine BM-PACs.

Despite the poor chondrogenic capacity of canine MSCs, so far, only a few studies have investigated the effect of growth factors on canine MSC chondrogenesis [30, 41]. Russel *et al.* observed no evidence of cartilage matrix deposition in canine adipose tissue-derived MSCs, even when BMP-2 was added to chondrogenic induction medium containing TGF-β3 [30]. Since this study is the first report to evaluate the effect of GDF-5 and its chondrogenic effect was confirmed only in BM-PACs, further studies should be conducted to elucidate whether GDF-5 promotes chondrogenesis of other types of canine MSCs.

In conclusion, BMP-2 stimulated cell proliferation and GAG production in spheroids of canine MSCs during chondrogenic differentiation induced by TGF-β, but upregulation of the type X collagen gene indicated that BMP-2 simultaneously promoted hypertrophic differentiation. Moreover, the combination of TGF-β1 and GDF-5 increased the deposition of glycosaminoglycan and tended to increase type II collagen expression without inducing hypertrophic differentiation. These findings indicate that, when combined with TGF-β1, GDF-5 is more useful growth factor to allow *in vitro* articular cartilage generation from canine MSCs compared with BMP-2 and IGF-1 and will accelerate research on canine cartilage regeneration and disease treatment.

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