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 ng/ml transforming growth factor-β1 (TGF-β1) with or without 100 ng/ml bone morphogenetic protein-2 (BMP-2), 100 ng/ml growth differentiation factor-5 (GDF-5) or 100 ng/ml 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
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 cell expansion. 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/flaxx solution (Wako) and passaged at a density of 1 × 10⁴ cells/cm² in growth medium consisting of DMEM, 10% FBS, 1% antibiotics, and 10 ng/ml 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 × 10⁴ cells/well to form spheroids. For chondrogenic differentiation, spheroids were cultured in 200 μl of chondrogenic induction medium consisting of DMEM, 4.5 mg/ml 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 μg/ml ascorbic acid-2-phosphate (Sigma), 0.1 μM dexamethasone (Sigma), 40 μg/ml L-proline (Peptide Institute Inc., Osaka, Japan), and 10 ng/ml recombinant human TGF-β1 (Peprotech). To investigate the effect of growth factors on chondrogenesis, 100 ng/ml human recombinant BMP-2 (Shenandoah Biotechnology, Warwick, PA, U.S.A.), 100 ng/ml human recombinant GDF-5 (ProSpec-Tany TechnoGene, Rehovot, Israel), or 100 ng/ml 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+1, 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% CO₂ 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 μg/ml papain at 65°C for 4 hr. DNA content was measured by Hoechst 33258 dye (Dojindo Molecular Technologies, Kumamoto,
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Table 1. Primers used for semi-quantitative RT-PCR

| Genes   | Sequence (5′-3′)       |
|---------|------------------------|
| SOX9    | Forward: AAGCTCTGGAGGGTCTGAAA  
|         | Reverse: ACTTTGAAATCGCGGTGGTCTT |
| ACAN    | Forward: CCTCATGTCATCTGCTGTTG  
|         | Reverse: CAGGGTGGGTTCGTTATGAGA |
| COL2    | Forward: CCGAACTCCACAAACAAACA  
|         | Reverse: AGGCACATTCACTGCAAGCACCC |
| COL1    | Forward: GTGACACACCCACCTCAAGAC  
|         | Reverse: TTCCAGTGGAGTGGCAGACATC  
| COL10   | Forward: TTCCAGGAGAGCGAGGCAATC  
|         | Reverse: TTTCAGTGGCCTCGTGTC |
| GAPDH   | Forward: TGACACCCACTCTTCCACCTTC  
|         | Reverse: CGGTTGTGTCAGCAAATTCA |

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).
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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 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.

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,
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

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 ($P<0.05$).
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 ng/ml) [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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REFERENCES

1. Ayerst, B. I., Smith, R. A. A., Nurcombe, V., Day, A. J., Merry, C. L. R. and Cool, S. M. 2017. Growth differentiation factor 5-mediated enhancement of chondrocyte phenotype is inhibited by heparin: implications for the use of heparin in the clinic and in tissue engineering applications. *Tissue Eng. Part A*, 23: 275–292. [Medline] [CrossRef]

2. Bartuneck, J., Croissant, J. D., Wijns, W., Gofflot, S., de Lavareille, A., Vanderheyden, M., Kaluzhny, Y., Mazouz, N., Willemsen, P., Penicka, M., Mathieu, M., Homsy, C., De Bruyne, B., McIntee, K., Lee, I. W. and Heyndrickx, G. R. 2007. Pretreatment of adult bone marrow mesenchymal stem cells with cardiacmyogenic growth factors and repair of the chronically infarcted myocardium. *Am. J. Physiol. Heart Circ. Physiol.* 292: H1095–H1104. [Medline] [CrossRef]

3. Becerra, J., Andrades, J. A., Guerado, E., Zamora-Nava, P., Lopez-Puertas, J. M. and Reddi, A. H. 2010. Articular cartilage: structure and regeneration. *Tissue Eng. Part B Rev.* 16: 617–627. [Medline] [CrossRef]

4. Bertolo, A., Schlaeffi, P., Malonzo-Marty, C., Baur, M., Pützel, T., Steffen, F. and Stoyanov, J. 2015. Comparative characterization of canine and human mesenchymal stem cells derived from bone marrow. *Int. J. Stem Cell Res. Ther.* 2: 1–7.

5. Caplan, A. I. 2017. Mesenchymal stem cells: Time to change the name! *Stem Cells Transl. Med.* 6: 1445–1451. [Medline] [CrossRef]

6. Caron, M. J. J., Emans, P. J., Cremers, A., Surtel, D. A. M., Coolsen, M. M. E., van Rhijn, L. W. and Welting, T. J. M. 2013. Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. *Osteoarthritis Cartilage* 21: 604–613. [Medline] [CrossRef]

7. Cheng, S., Lou, J., Wright, N. M., Lou, C., Avello, L. V. and Riew, K. D. 2001. In vitro and in vivo induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene. *Calcif. Tissue Int.* 68: 87–94.

8. Cheng, T., Yang, C., Weber, N., Kim, H. T. and Kuo, A. C. 2012. Fibroblast growth factor 2 enhances the kinetics of mesenchymal stem cell chondrogenesis. *Biochem. Biophys. Res. Commun.* 426: 544–550. [Medline] [CrossRef]

9. Coleman, C. M., Vaughan, E. E., Bowe, D. C., Mooney, E., Howard, L. and Barry, F. 2013. Growth differentiation factor-5 enhances in vitro mesenchymal stromal cell chondrogenesis and hypertrophy. *Stem Cells Dev.* 22: 1968–1976. [Medline] [CrossRef]

10. Danišović, L., Varga, I. and Polák, S. 2012. Growth factors and chondrogenic differentiation of mesenchymal stem cells. *Tissue Cell* 44: 69–73. [Medline] [CrossRef]

11. Francis-West, P. H., Abdelfattah, A., Chen, P., Allen, C., Parish, J., Ladher, R., Allen, S., MacPherson, S., Luyten, F. P. and Archer, C. W. 1999. Mechanisms of GDF-5 action during skeletal development. *Development* 126: 1305–1315. [Medline]

12. Goldring, M. B., Tsuchimochi, K. and Ijiri, K. 2006. The control of chondrogenesis. *J. Cell. Biochem.* 97: 33–44. [Medline] [CrossRef]

13. Gow, D. J., Sester, D. P. and Hume, D. A. 2010. CSF-1, IGF-1, and the control of postnatal growth and development. *J. Leukoc. Biol.* 88: 475–481.

doi: 10.1292/jvms.18-0551
14. Handorf, A. M. and Li, W. J. 2011. Fibroblast growth factor-2 primes human mesenchymal stem cells for enhanced chondrogenesis. PLoS One 6: e22887. [Medline] [CrossRef]

15. Hata, K., Takahata, Y., Murakami, T. and Nishimura, R. 2017. Transcriptional Network Controlling Endochondral Ossification. J. Bone Metab. 24: 75–82. [Medline] [CrossRef]

16. Indrawattana, N., Chen, G., Tadokoro, M., Shann, L. H., Ogushi, H., Tateishi, T., Tanaka, J. and Bunyaratvej, A. 2004. Growth factor combination for chondrogenic induction from human mesenchymal stem cell. Biochem. Biophys. Res. Comman. 320: 914–919. [Medline] [CrossRef]

17. Innes, J. E., Gordon, C., Vaughan-Thomas, A., Rhodes, N. P. and Clegg, P. D. 2013. Evaluation of cartilage, synovium and adipose tissue as cellular sources for osteochondral repair. Vet. J. 197: 619–624. [Medline] [CrossRef]

18. Johnstone, B., Hering, T. M., Caplan, A. I., Goldberg, V. M. and Yoo, J. U. 1998. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp. Cell Res. 238: 265–272. [Medline] [CrossRef]

19. Kanda, Y. 2013. Investigation of the freely available easy-to-use software ‘EZR’ for medical statistics. Bone Marrow Transplant. 48: 452–458. [Medline] [CrossRef]

20. Kisiel, A. H., McDuffee, L. A., Masaud, E., Bailey, T. R., Esparza Gonzalez, B. P. and Nino-Fong, R. 2012. Isolation, characterization, and in vitro proliferation of canine mesenchymal stem cells derived from bone marrow, adipose tissue, muscle, and periosteum. Am. J. Vet. Res. 73: 1305–1317. [Medline] [CrossRef]

21. Kuroda, Y. and Dezawa, M. 2014. Mesenchymal stem cells and their subpopulation, pluripotent muse cells, in basic research and regenerative medicine. Anat. Rec. (Hoboken) 297: 98–110. [Medline] [CrossRef]

22. Lin, H. Y., Fujita, N., Endo, K., Morita, M., Takeda, T. and Nishimura, R. 2017. Canine mesenchymal stem cells adhering to adipocytes in bone marrow. Stem Cells Dev. 26: 431–440. [Medline] [CrossRef]

23. Michelsen, J. 2013. Canine elbow dysplasia: aetiopathogenesis and current treatment recommendations. Vet. J. 196: 12–19. [Medline] [CrossRef]

24. Michelsen, J., Vassall, J. D., Baird, A., Guillemin, R. and Oci, L. 1986. Basic fibroblast growth factor induces angiogenesis in vitro. Proc. Natl. Acad. Sci. U.S.A. 83: 7297–7301. [Medline] [CrossRef]

25. Murphy, M. K., Huey, D. J., Hu, J. C. and Athanasiou, K. A. 2015. TGF-β1, GDF-5, and BMP-2 stimulation induces chondrogenesis in expanded human articular chondrocytes and marrow-derived stromal cells. Stem Cells 33: 762–773. [Medline] [CrossRef]

26. Nilsson, A., Isgaard, J., Lindahl, A., Dahlström, A., Skottner, A. and Isaksson, O. G. 1986. Regulation by growth hormone of number of chondrocytes containing IGF-1 in rat growth plate. Science 233: 571–574. [Medline] [CrossRef]

27. Pizzute, T., Li, J., Zhang, Y., Davis, M. E. and Pei, M. 2016. Fibroblast Growth Factor Ligand Dependent Proliferation and Chondrogenic Differentiation of Synovium-Derived Stem Cells and Concomitant Adaptation of Wnt/Mitogen-Activated Protein Kinase Signals. Tissue Eng. Part A 22: 1036–1046. [CrossRef]

28. Puetzer, J. L., Petitte, J. N. and Loboa, E. G. 2010. Comparative review of growth factors for induction of three-dimensional in vitro chondrogenesis in human mesenchymal stem cells isolated from bone marrow and adipose tissue. Tissue Eng. Part B Rev. 16: 435–444. [Medline] [CrossRef]

29. Russell, K. A., Chow, N. H. C., Du kokf, D., Gibson, T. W. G., LaMarre, J., Betts, D. H. and Koch, T. G. 2016. Characterization and immunomodulatory effects of canine adipose tissue- and bone marrow-derived mesenchymal stem cells. PLoS One 11: e0167442. [Medline] [CrossRef]

30. Russell, K. A., Gibson, T. W. G., Chong, A., Co, C. and Koch, T. G. 2015. Canine platelet lysate is inferior to fetal bovine serum for the isolation and propagation of canine adipose tissue- and bone marrow-derived mesenchymal stem cells. PLoS One 10: e0136621. [Medline] [CrossRef]

31. Ryche, J. K. 2010. Diagnosis and treatment of osteoarthritis. Top. Companion Anim. Med. 25: 20–25. [Medline] [CrossRef]

32. Sakimura, K., Matsumoto, T., Miyamoto, C., Osaki, M. and Shindo, H. 2006. Effects of insulin-like growth factor I on transforming growth factor beta1 induced chondrogenesis of synovium-derived mesenchymal stem cells cultured in a polyglycolic acid scaffold. Cells Tissues Organs (Print) 183: 55–61. [Medline] [CrossRef]

33. Sanderson, R. O., Beata, C., Flipo, R. M., Genevois, J. P., Macias, C., Tacke, S., Vezzoni, A. and Innes, J. F. 2009. Systematic review of the management of canine osteoarthritis. Vet. Rec. 164: 418–424. [Medline] [CrossRef]

34. Schmidt, M. B., Chen, E. H. and Lynch, S. E. 2006. A review of the effects of insulin-like growth factor and platelet derived growth factor on in vivo cartilage healing and repair. Osteoarthritis Cartilage 14: 403–412. [Medline] [CrossRef]

35. Schmitt, B., Ringé, J., Häupl, T., Notter, M., Manz, R., Burmester, G. R., Sittigering, M. and Kaps, C. 2003. BMP2 initiates chondrogenic lineage development of adult human mesenchymal stem cells in high-density culture. Differentiation 71: 567–577. [Medline] [CrossRef]

36. Schneider, P. R. A., Buhrmann, C., Mobasher, A., Matis, U. and Shahkami, M. 2011. Three-dimensional high-density co-culture with primary tenocytes induces tenogenic differentiation in mesenchymal stem cells. J. Orthop. Res. 29: 1351–1360. [Medline] [CrossRef]

37. Sekiya, I., Larson, B. L., Vuoristo, J. T., Reger, R. L. and Prockop, D. J. 2005. Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res. 320: 269–276. [Medline] [CrossRef]

38. Shintani, N. and Hunziker, E. B. 2007. Chondrogenic differentiation of bovine synovium: bone morphogenetic proteins 2 and 7 and transforming growth factor β1 induce the formation of different types of cartilaginous tissue. Arthritis Rheum. 56: 1869–1879. [Medline] [CrossRef]

39. Solchaga, L. A., Penick, K., Porter, J. D., Goldberg, V. M., Caplan, A. I. and Welter, J. F. 2005. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. J. Cell. Physiol. 203: 398–409. [Medline] [CrossRef]

40. Spina, J., Warnock, J., Duesterdieck-Zellmer, K., Baltzer, W., Ott, J. and Bay, B. 2014. Comparison of growth factor treatments on the fibrochondrogenic potential of canine fibroblast-like synoviocytes for meniscal tissue engineering. Vet. Surg. 43: 750–760. [Medline] [CrossRef]

41. Storm, E. E. and Kingsley, D. M. 1999. GDF5 coordinates bone and joint formation during digit development. Dev. Biol. 209: 11–27. [Medline] [CrossRef]

42. Sun, J., Li, J., Li, C. and Yu, Y. 2015. Role of bone morphogenetic protein-2 in osteogenic differentiation of mesenchymal stem cells. Mol. Med. Rep. 12: 4230–4237. [Medline] [CrossRef]

43. van der Kraam, P. M., Blaney Davidson, E. N. and van den Berg, W. B. 2010. Bone morphogenetic proteins and articular cartilage: To serve and protect or a wolf in sheep clothing? J. Osteoarthritis Cartilage 18: 735–741. [Medline] [CrossRef]

44. Wang, W., Rigueur, D. and Lyons, K. M. 2014. TGFβ1 signaling in cartilage development and maintenance. Birth Defects Res. C Embryo Today 102: 37–51. [Medline] [CrossRef]

45. Webster, R. A., Blaber, S. P., Herbert, B. R., Wilkins, M. R. and Vesey, G. 2012. The role of mesenchymal stem cells in veterinary therapeutics - a review. N. Z. Vet. J. 60: 265–272. [Medline] [CrossRef]

46. Ytrehus, B., Carlson, C. S. and Ekman, S. 2007. Etiology and pathogenesis of osteochondrosis. Vet. Pathol. 44: 429–448. [Medline] [CrossRef]