博士論文（要約）

Fabrication of hyaline cartilage using mesenchymal stem cells for canine articular cartilage regeneration

（犬の関節軟骨再生を目指した間葉系幹細胞からの硝子軟骨作製法の検討）

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General Introduction

1. Pathology and treatments of articular cartilage injury in dogs

Articular cartilage consists of chondrocytes and abundant extracellular matrix (ECM) such as glycosaminoglycan, proteoglycan, type II collagen, and hyaluronic acid.[1] These ECMs retain water in the tissue, which leads to the specific viscoelastic property of articular cartilage. Articular cartilage injury is common in both young and old dogs because they are often affected by joint diseases such as hip and elbow dysplasia, osteochondritis dissecans, and rupture of the cranial cruciate ligament.[2–5] Damaged articular cartilage never regenerates spontaneously due to its avascular structure. Dogs suffering from cartilage injury show lameness caused by joint pain and swelling. The administration of non-steroidal anti-inflammatory drugs or analgesics is the most common treatment to manage these symptoms. The debridement of damaged cartilage to the depth of bleeding subchondral bone is one of the surgical treatments for focal cartilage defects. This allows stem cells in bone marrow to migrate to the defect site, but the defect is probably healed with fibrocartilage which is inferior to hyaline cartilage in lubricity and viscoelasticity. Therefore, regardless of these treatments, severely damaged articular cartilage never regenerates to functional cartilage resulting in secondary osteoarthritis and progressive joint degeneration.[6] Hence, a novel therapeutic strategy should be required to provide radical treatment for articular cartilage injury in dogs.

Recently, regenerative therapy using mesenchymal stem cells (MSCs) can be a promising candidate for articular cartilage reconstruction.

2. MSCs as a cell source for articular cartilage regeneration in dogs

MSCs are immature cells which can be isolated from adult somatic tissues such as bone marrow, fat tissue and synovium.[7] In a living body, MSCs have significant roles in tissue repair and homeostasis. MSCs have high proliferative ability and multipotency for mesodermal lineages including bone and cartilage.[8] Additionally, when compared with pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), MSCs have less tumorgenicity and ethical problems. Lower cost for preparation can promote availability and versatility of MSCs in veterinary medicine. For these reasons, MSCs are expected to be the most useful cell source for cartilage regenerative therapy in dogs.

In the early stage of *in vivo* cartilage development, mesenchymal cells form cell aggregate called "mesenchymal condensation." After that, cells differentiate into chondrocytes and rapidly proliferate to form hyaline cartilage tissue.[9] During maturation, a portion of chondrocytes continues to produce hyaline cartilage-specific ECMs, which contributes to the formation of articular cartilage. Another portion of

chondrocytes separately undergoes hypertrophy and apoptosis, which finally leads to vascularization and replacement of cartilage by bone.[9,10] Therefore, *in vitro* chondrogenic differentiation of MSCs has been performed in three-dimensional (3D) culture system to recapitulate mesenchymal condensation. In 1998, Johnstone et al. first reported that transforming growth factor-β1 (TGF-β1) was essential for *in vitro* cartilage generation from rabbit MSCs in a 3D pellet culture system.[11] Although many studies have tried TGF-β1-induced chondrogenesis of canine MSCs for several decades, *in vitro* chondrogenic ability of canine MSCs have been very limited and generation of clinically applicable hyaline cartilage tissue consisting of abundant ECM such as proteoglycan and type II collagen is still challenging.[12–16] Since chondrogenic differentiation of MSCs is modulated by a variety of growth and environmental factors. Thus, it is necessary to explore optimal factors to improve the chondrogenic differentiation of canine MSCs for articular cartilage regeneration.

3. Factors affecting chondrogenesis of MSCs

3.1. Fetal bovine serum (FBS) concentration in chondrogenic medium

FBS is widely used as a medium supplement to help cell growth and differentiation because it contains various growth factors, cytokines, and hormones.

Although FBS promotes proliferation and ECM production of chondrocytes, it has been reported that adding FBS to chondrogenic induction medium inhibits MSC chondrogenesis and increases apoptosis.[17,18] Conversely, Diekman et al. reported that 10% FBS promoted the chondrogenic differentiation of human adipose tissue-derived MSCs, while the opposite effect was observed on human BMMSCs.[19] Therefore, the effect of FBS on chondrogenesis is expected to vary widely depending on cell types. To our knowledge, no study has investigated the impact of FBS in chondrogenic induction medium for canine MSCs, and it remains unclear at what concentration FBS should be added.

3.2. Fibroblast growth factor-2 (FGF-2) preconditioning

FGF-2 is a member of the FGF family and plays an important role in embryogenesis, angiogenesis, wound healing and cell proliferation.[20] FGF-2 has been reported to be necessary for self-renewing and maintenance of stemness in ESCs and iPSCs.[20–22] It is also suggested that FGF-2 preserve the multipotency of MSCs.[23,24] Many studies have demonstrated that FGF-2 treatment during expansion culture promotes cell proliferation and subsequent chondrogenesis of human MSCs.[25–28] The similar effect on cell proliferation is observed as well as in canine bone marrow-derived MSCs

(BMMSCs).[29] However, it is not well documented whether FGF-2 preconditioning affects the chondrogenic differentiation of canine MSCs.

3.3 Other growth factors

All the process of *in vivo* cartilage development is known to be strictly controlled by many types of growth factors[30], such as morphogenetic protein-2 (BMP-2) and growth differentiation factor-5 (GDF-5) which are the member of TGF-β superfamily. BMP-2 activates the expression of SOX9, which is a master regulator essential for chondrogenesis and stimulates the proliferation and ECM production of chondroprogenitors.[31,32] However, it also strongly stimulates hypertrophic differentiation and osteogenesis.[33] GDF-5, also known as cartilage-derived morphogenetic protein-1, induces the formation of mesenchymal condensation. In later development stages, GDF-5 is reported to be expressed in the joint interzone and control joint formation.[34] Insulin-like growth factor-1 (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.[35] These three growth factors are also reported to improve *in vitro* human MSC chondrogenesis in combination with TGF-β1. [31,36–39] Since similar chondrogenic effects can be expected in canine MSCs, more effective chondrogenic

conditions can be established to generate ECM-rich hyaline cartilage tissue.

3.4 Oxygen concentration

Generally, cells are cultured under normoxic condition (20%), however, it is known that physiological oxygen level is lower than 20%, even in lung or blood (up to 14%).[40] In particular, oxygen level in articular cartilage is much lower (1-6%) compared with other organs due to avascularity.[6,41,42] Under hypoxic condition, hypoxia-inducible factor-1α (HIF-1α) transfers into the nucleus and activates the transcription of genes involved in cell proliferation, viability, and differentiation.[43,44] It has been demonstrated that hypoxia induces upregulation of SOX9, which is a critical chondrogenic gene, through HIF-1α translocation into nucleus.[45–48] Additionally, Amarilio et al. demonstrated that HIF-1α knockout during skeletogenesis induced cartilage deformation.[49] Therefore, oxygen tension is considered to be one of the most crucial environmental factors to determine the chondrogenic fate of MSCs. Previous studies have demonstrated that exposure to hypoxia during expansion or differentiation phase results in enhancing chondrogenesis of human MSCs.[50–54] Considering these reports, chondrogenic differentiation of canine MSCs is also expected to be improved by hypoxic conditions.

4. Strategy of regenerative therapy for articular cartilage

To date, regenerative approaches for canine articular cartilage injury have been exclusively focused on intra-articular injection of MSCs. Many researchers have performed intra-articular injection of MSCs on experimentally-induced osteoarthritis model or dogs suffering from spontaneous osteoarthritis. However, some studies have shown no difference between experimental and control joint,[55,56] and others have proved only short-term effects such as delayed progression of osteoarthritis, relieved lameness, and pain.[57–63] One of the reasons for few effects is probably that injected cells do not engraft in injured cartilage, but in synovium and meniscus.[57,59,64] Therefore, intra-articular injection of MSCs seems to be a symptomatic treatment and thus other radical approaches are necessary.

Tissue engineering approach for tissue regeneration has made rapid progress from the 1990s.[65] In 1997, Cao et al. have suggested that appropriate combination of cells, growth factors, and scaffolds can possibly provide a variety of 3D tissue or organs for transplantation.[66] Thereafter, many researchers have focused on the development of a novel tissue engineering technology to fabricate large and functional 3D tissue *in vitro*. The general approach for tissue engineering is seeding cells onto 3D shaped scaffolds, such as natural or artificially synthetic polymers, ceramics, and decellularized matrix.

In addition, bioprinting has emerged as a useful scaffold-based strategy for fabrication of 3D tissue with more precisely arranged structure.[67–70] In particular, many researchers have focused on cartilage tissue engineering because the 3D environment is very important for chondrogenesis as mentioned above. A number of studies have proved the effectiveness of cartilage tissue using scaffolds on human chondral defects.[71] However, the scaffolds used for 3D tissue construction contain foreign materials which have been reported to have a possibility to induce foreign body reaction after implantation.[72–77] To solve this problem, it is expected to develop a novel tissue engineering technology which can fabricate 3D tissue constructs without foreign materials.

Recently, a bio 3D printer, which enables to fabricate 3D tissue constructs without any artificial scaffolds by stabbing spheroids with fine needle arrays according to pre-designed form, has been developed.[78] After spheroid fusion is achieved, the construct can be pulled out from the needles as a self-sustained scaffold-free tissue in the desired form. This platform technology has been already applied for regeneration of blood vessels, liver, trachea, peripheral nerve and urinary bladder.[79–84] Thus, it is expected that this technology is applicable to *ex vivo* cartilage fabrication for canine articular cartilage regeneration.

5. Purpose of the study

From mentioned above, I set a goal of this study to establish a culture method for inducing hyaline cartilage from canine MSCs and fabricate 3D hyaline cartilage constructs with a novel bio 3D printer. In *Chapter 1*, I explored the optimal medium condition for chondrogenic differentiation of canine MSCs. Firstly, I investigated the effect of FBS concentration in chondrogenic induction medium and FGF-2 preconditioning on canine MSC chondrogenesis. Next, I explored the optimal growth factor that further enhances chondrogenesis of canine MSCs. In *Chapter 2*, I investigated how hypoxic condition regulates the hyaline cartilage differentiation of canine MSCs. Lastly, in *Chapter 3*, I tried to fabricate hyaline cartilage constructs using canine MSC spheroids using a bio 3D printer and evaluate its effects on cartilage regeneration in a canine chondral defect model.

Chapter 1

The optimal medium condition for chondrogenesis of canine MSCs

Chapter 1-1

The effect of FGF-2 and serum on chondrogenesis of canine MSCs

Introduction

Articular cartilage consists of water, chondrocytes and abundant extracellular matrix (ECM) including proteoglycan, type II collagen and hyaluronic acid.[85] Once articular cartilage is severely damaged, it has little self-repair or regenerative capacity due to its avascular structure, causing persistent joint pain. Autologous chondrocyte transplantation is one solution for cartilage defects in humans.[86] However, there are some complications with this treatment, including donor site morbidity and hypertrophy of the transplant.[87,88] Moreover, harvested chondrocytes have limited proliferation ability and undergo dedifferentiation which results in the loss of their original phenotype, transforming into fibrocartilage.[89–91] Therefore, a new cell source is required to regenerate articular cartilage.

Mesenchymal stem cells (MSCs) are easily isolated from adult somatic tissues such as bone marrow and fat with minimally invasive procedures and low cost. MSCs show high proliferative ability and can differentiate into several mesenchymal lineages including adipocytes, osteoblasts, and chondrocytes. Therefore, MSCs are expected to be a clinically useful cell source for cartilage regeneration in veterinary medicine. Many researchers have tried to isolate and chondrogenically differentiate canine MSCs. Unfortunately, most reports have shown that canine MSCs have a poor chondrogenic

capacity and produce only small amounts of glycosaminoglycan in a pellet culture.[12–16] Thus, further studies should be conducted to verify the critical factors for the optimal chondrogenic condition of canine MSCs.

Fetal bovine serum (FBS) is widely used as a medium supplement to aid cell proliferation and differentiation because it contains several types of growth factors, including both cytokines and hormones. Previous evidence suggests that adding FBS to chondrogenic induction medium inhibits MSC chondrogenesis and increases apoptosis.[17,18] Conversely, Diekman et al. reported that 10% FBS promoted the chondrogenic differentiation of human adipose tissue-derived MSCs, whilst the opposite was observed for human bone marrow MSCs (BMMSCs).[19] To our knowledge, no study has investigated the impact of FBS in chondrogenic induction medium for canine MSCs, and it remains unclear at what concentration FBS should be added.

Fibroblast growth factor-2 (FGF-2), also known as basic FGF, is a member of the FGF family and is a strong mitogen for several cell types. FGF-2 also plays an important role in embryogenesis, cell differentiation, and maintenance of stemness in pluripotent stem cells.[20] Previous studies have indicated that FGF-2 promoted the proliferation of human MSCs in monolayer culture and subsequent chondrogenic differentiation.[25–28] However, it is not understood if FGF-2 preconditioning affects the chondrogenic

differentiation of canine MSCs.

Novel canine MSCs, known as bone marrow peri-adipocyte cells (BM-PACs), derived from cells adhering to adipocytes in bone marrow have previously developed.[92] Since BM-PACs showed superior proliferation and differentiation ability compared to that of BMMSCs derived from bone marrow mononuclear cells, the higher chondrogenic potential could be expected under optimal conditions. In this study, we aimed to establish the optimal conditions for canine BM-PAC chondrogenesis for two approaches: FGF-2 preconditioning and FBS concentration in a chondrogenic medium. First, canine BM-PACs were expanded with or without FGF-2 and their proliferation ability and undifferentiated state were evaluated. Subsequently, these cells were cultured in chondrogenic medium containing 0%, 1%, and 10% FBS to determine the effect of FBS concentration on chondrogenic differentiation of canine MSCs.

Materials & Methods

Animals

Six healthy young beagles, (four male and two female), were used in this study to obtain bone marrow. Bone marrow samples were harvested under general anesthesia. Anesthesia was 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).

Culture of canine BM-PACs

Canine BM-PACs were isolated according to our previously published method.[92] Briefly, bone marrow was aspirated from the proximal humerus using a sterilized 15-gauge bone marrow biopsy needle (Angiotech Pharmaceuticals, Inc., Vancouver, Canada). After density gradient centrifugation with Ficoll-Paque (GE Healthcare, Little Chalfont, UK), the top adipose layer containing mature adipocytes was collected and washed with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Lot No. 1526235, Gibco/Invitrogen) and 1% penicillin-streptomycin/amphotericin-B (Wako). Following a second wash, the

adipose layer was placed in 25 cm^2 flasks filled with DMEM supplemented with 20% FBS and 1% antibiotics. Cells were then subjected to ceiling culture at 37 °C in a humidified atmosphere containing 5% CO_2 for seven days. If cells did not grow to 80% confluence within 7 days, the flask was inverted and the culture was maintained with medium containing 10% FBS.

Cell proliferation assay

Passage 0 BM-PACs were plated at a density of 1×10^4 cells/cm^2 and expanded in growth medium supplemented with or without 10 ng/mL of human recombinant FGF-2 (Peprotech, Rocky Hill, NJ). The confluent cells were detached with 0.25% trypsin/1 mM EDTA solution (Wako) and the number of viable cells was counted using a trypan blue exclusion test. Doubling time (DT) was calculated according to the following formula: $DT = T * \log 2 / (\log Nt - \log N0)$, where T is the incubation period (days), and N0 and Nt indicate the initial seeding cell number and final harvesting cell number, respectively.

Chondrogenic induction in spheroid culture

Cells expanded with or without FGF-2 were plated in low-adhesive 96-multiwell

plates (Sumitomo Bakelite, Tokyo) to achieve spheroid formation at a density of 3×10^4 cells per well. For chondrogenic differentiation, spheroids were cultured in chondrogenic induction medium consisting of DMEM, 4.5 mg/mL D-(+)-glucose (Sigma, St. Louis, MO, USA), 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 transforming growth factor-β1 (Peprotech). To evaluate the effect of serum on chondrogenesis, 0%, 1% or 10% FBS was added to chondrogenic induction medium. Chondrogenic induction was maintained for 14 days at 37 °C under 5% CO_2, and the medium was changed twice a week. The diameter of two spheroids was measured in each individual case by repeated microscopic observations at day 7 and 14, and their mean diameter was calculated. An experimental scheme is shown in Fig. 1-1.

DNA and glycosaminoglycan contents

After 7 and 14 days of chondrogenic induction, four spheroids in each group were digested with 100 μg/mL papain at 65 °C for 4 hours. Total spheroid DNA content was determined with Hoechst 33258 dye (Dojindo Molecular Technologies, Kumamoto, Japan). Fluorescence intensity was measured using the multilabel counter ARVO MX

(Perkin Elmer, Waltham, MA) at an excitation wavelength of 355 nm and an emission of 460 nm. A standard curve was generated by serial dilution of calf thymus DNA (Sigma). Total spheroid glycosaminoglycan (GAG) was then quantified using a Blyscan Kit (Biocolor, Westbury, NY) according to the manufacturer's instructions. The optical density at 630 nm was read using a microplate reader (BioRad, Hercules, CA) and total GAG content was normalized to total DNA content. Each experiment was done in duplicate.

Histological staining and immunohistochemistry

After 14 days of chondrogenic induction, spheroids were fixed with 10% formalin neutral buffer solution (Wako) and embedded in paraffin. Specimens were cut into 4-μm sections. To detect proteoglycan, sections were stained with Safranin O/Fast Green. Immunohistochemistry was done to assess the expression of collagen type II, I and X. Antigen retrieval was carried out with 50 μg/mL proteinase K (Promega, Madison, WI) for 10 min at room temperature. For collagen type II, the additional antigen retrieval step was done using 25 mg/mL hyaluronidase (Sigma) for 2 hours at 37 °C. The sections were then incubated with 0.3% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity and washed with Tris-buffered saline with 0.1% Tween-

20 (TBS-T). After blocking with TBS-T containing 10% normal goat serum (NGS, Sigma) for 30 min at room temperature, the sections were incubated with rabbit anti-bovine type II collagen antibody (1:200, LB-1297; LSL, Tokyo, Japan), mouse anti-bovine type I collagen antibody (1:1000, ab6308; Abcam, Cambridge, UK) and mouse anti-porcine type X collagen antibody (1:1000, C7974; Sigma) at 4 °C overnight. Slides were washed with TBS-T three times for 5 min each and incubated with HRP labeled polymer (K4001 and K4003, Dako, Tokyo) for 1 hour at room temperature. Finally, sections were washed with TBS-T three times for 5 min each and DAB substrate (Dako) was applied. All slides were counterstained with hematoxylin.

Quantitative real-time RT-PCR

Total RNA was extracted with TRI Reagent (Cosmo Bio, Tokyo, Japan) from cells treated with or without FGF-2 for two days, and spheroids after 14 days of chondrogenic induction. Complementary DNA was synthesized with ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Real-time quantitative PCR was done using real-time monitoring of SYBR Green dye (Thunderbird SYBR qPCR Mix, Toyobo) fluorescence increase on the Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA). The mRNA expression of the chondrogenic marker gene (SOX9) and

pluripotent marker genes (SOX2 and Oct4) was evaluated in cells treated with or without FGF-2. The expression levels of mRNA were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The mRNA expression of SOX9, ACAN, COL2, COL1, COL10, and HPRT1 was evaluated in chondrogenic spheroids. HPRT1 was used as an endogenous control gene. The primers used for PCR are listed in Table 1-1. The PCR cycling conditions used were 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each experiment was done in triplicate.

Statistical analysis

All data were expressed as mean ± standard deviation. Comparisons were made using the Student's *t*-test between two unpaired groups. One-way analysis of variance followed by Tukey's multiple comparisons test was used to detect any statistical differences between multiple unpaired groups. Statistical significance was accepted at $p < 0.05$. 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).[93]

Results

The effect of FGF-2 on BM-PACs in monolayer expansion

BM-PACs expanded with FGF-2 for two days became smaller and more spindle-shaped than those expanded without FGF-2 (Fig. 1-2A). Additionally, BM-PACs expanded with FGF-2 reached confluent in a shorter time than cells expanded without FGF-2. The doubling time of BM-PACs expanded with FGF-2 was significantly shorter ($p < 0.0001$) than that of cells expanded without FGF-2 (Fig. 1-2B). The expression of chondrogenic and immature markers was compared after two days of FGF-2 stimulation. There was no significant difference in the mRNA expression of SOX9 and Oct4 between the two groups (Fig. 1-2C). However, significantly higher expression of SOX2 mRNA was observed in BM-PACs expanded with FGF-2. ($p < 0.05$)

Spheroid diameter

After 7 and 14 days of chondrogenic induction, there was no significant difference in the diameter of spheroids amongst the FGF-2(-) groups (Fig. 1-3). However, the diameter of spheroids in FGF-2(+) groups became larger depending on the FBS concentration in the chondrogenic induction medium. Additionally, a higher concentration of FBS increased the diameter of spheroids in FGF-2(+) groups from day

7 to day 14.

Biochemical analysis

The DNA content of spheroids in FGF-2(-) groups was stable throughout chondrogenic differentiation regardless of FBS concentration. In contrast, DNA content in FGF-2(+) groups increased in an FBS concentration-dependent manner (Fig. 1-4A). Spheroids in FGF-2(+) groups showed significantly higher GAG deposition ($p < 0.05$) than those in FGF-2(-) groups after 7 and 14 days of chondrogenic differentiation (Fig. 1-4B). To evaluate the efficiency of GAG production, total GAG content was normalized to total DNA content (GAG/DNA). Lower FBS concentration in chondrogenic medium tended to increase GAG/DNA ratio and the highest mean value was observed in the FGF-2(+)-0% FBS group (Fig. 1-4C).

Histology

Safranin O staining was done to evaluate the deposition of proteoglycan in chondrogenic spheroids (Fig. 1-5). In FGF-2(-) groups, spheroids showed stronger staining when cultured in lower concentrations of FBS. However, all spheroids in FGF-2(+) groups were strongly stained with Safranin O, even when cultured with 10% FBS.

The central region of spheroids showed stronger staining compared to the peripheral region. Higher concentrations of FBS formed a thicker Safranin O-negative layer consisting of fibroblast-like cells on the outer surface of spheroids. Immunohistochemistry results after 14 days of chondrogenic induction are shown in Fig. 1-6. The expression of type II collagen was detected in all culture conditions and there was a tendency toward greater type II collagen expression in spheroids cultured with lower concentrations of FBS. Additionally, the central region of spheroids tended to express more type II collagen compared to the peripheral region. In FGF-2(-) groups, type I collagen, a marker expressed by fibrocartilage, was homogenously detected over the entire spheroid. In FGF-2(+) groups, type I collagen distribution was significant in the middle to the outer surface of the spheroid, and type I collagen accumulation was not apparent in the center of spheroids. The expression of type X collagen, a marker for hypertrophic cartilage, was not observed for any conditions.

Quantitative RT-PCR

Spheroids cultured in chondrogenic induction medium containing 0, 1 or 10% FBS were collected at day 14 to do quantitative RT-PCR. These results are shown in Fig. 1-7. The expression of the chondrogenic genes, SOX9, ACAN and COL2, was significantly

upregulated in the FGF-2(+)-0% FBS group ($p < 0.05$). Lower FBS concentration in chondrogenic medium tended to increase the expression of chondrogenic genes. There were no remarkable changes in the expression of COL1 and COL10 amongst the groups.

Discussion

In this study, BM-PACs became smaller and proliferated rapidly when treated with FGF-2 during monolayer expansion. This result is consistent with those in a previous study, which showed that FGF-2 enhanced the mitotic potential of human and canine BMMSCs.[28,29] Following treatment with FGF-2, there was no change in the expression of chondrogenic genes, suggesting that the stimulation with FGF-2 did not lead to cell differentiation into a chondrogenic lineage at this point. Additionally, I showed that the mRNA expression of SOX2 was significantly higher in cells treated with FGF-2. SOX2 is one of the transcription factors essential for the maintenance of the undifferentiated state of embryonic stem cells and induced pluripotent stem cells. Yoon et al. demonstrated that SOX2 was also important for the maintenance of proliferation and multipotency in human BMMSCs.[94] Moreover, it has been reported that FGF-2 upregulated the expression of SOX2 in human apical papilla-derived MSCs, resulting in enhanced proliferation ability and differentiation potential.[95] However, the expression of Oct4 was not changed by treatment with FGF-2. SOX2 and Oct4 are generally thought to work together to sustain an undifferentiated state. However, a previous report has indicated that Oct4 is not required for self-renewal and multipotency in mouse BMMSCs.[96] As mentioned above, it is suggested that FGF-2 stimulation plays an

important role in SOX2-dependent enhancement of canine BM-PAC proliferation while maintaining an undifferentiated state.

Higher concentrations of FBS in the chondrogenic medium significantly decreased GAG/DNA ratio in FGF-2(-) groups. FBS is known to contain several growth factors including transforming growth factor beta (TGF-ß), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF).[97] Amongst these, EGF and VEGF have been reported to inhibit chondrogenesis of MSCs.[98,99] Based on these reports, it is assumed that these FBS growth factors had an inhibitory effect on chondrogenic differentiation of canine BM-PACs in this study. Although FBS has lot-to-lot variations, there is a possibility that some lots of FBS contain a small amount of growth factors which inhibit chondrogenesis. Therefore, FBS should not be added to the induction medium in chondrogenesis of canine MSCs. In contrast, the GAG/DNA ratio of spheroids in FGF-2(+) groups was not significantly decreased by FBS addition. Therefore, I hypothesize that FGF-2 preconditioning makes spheroids impervious to the inhibitory effect of FBS during chondrogenic differentiation. Moreover, spheroids in FGF-2(+) groups became larger and contained more GAG and DNA than those in FGF-2(-) groups. These findings indicate that cells treated with FGF-2 proliferated in spheroids while maintaining chondrogenic capacity, resulting in increased spheroid size.

Consistent with biochemical analysis, spheroids in FGF-2(-) groups had stronger Safranin O staining at lower concentrations of FBS, and all spheroids had strong Safranin O staining in FGF-2(+) groups. However, fibroblast-like cells were observed on the outer surface of spheroids and a thicker Safranin O-negative layer was formed in an FBS concentration-dependent manner. Generally, chondrogenically induced MSC pellets form a superficial cell layer consisting of fibroblast-like cells expressing type I collagen, even under the absence of FBS.[100] Similarly, the fibroblastic layer which showed slight type I collagen expression and no cartilage matrix was also observed in 0% FBS groups. It is thought that cells on the outer surface of spheroids are prevented from differentiating into chondrocytes due to direct exposure to inhibitory factors in FBS. Histological staining showed that FGF-2(+) groups produced more proteoglycan and less type I collagen compared with that in FGF-2(-) groups. A previous study on human BMMSCs also demonstrated that FGF-2 preconditioning enhanced GAG production and suppressed the expression of type I collagen.[101] Ito et al. also reported that FGF-2 preconditioning inactivated both insulin-like growth factor-I and TGF-β pathways that were spontaneously upregulated by long-term culture, and enhanced the chondrogenic potential of human MSCs.[102] Although the underlying mechanism remains unknown, FGF-2 treatment is considered useful for the production of hyaline-like cartilage tissue

using MSCs. Consistent with the previous studies,[103–105] the central region of spheroids tended to express more cartilaginous ECMs. This is probably because spheroids have the gradient of oxygen concentration in their microenvironment and cells in the center are exposed to a hypoxic condition which enhances chondrogenesis.[54,106–108] Immunostaining detected no expression of type X collagen. Hypertrophic chondrocytes are known to contribute to the formation of bone tissue *in vitro* and *in vivo*.[109] Therefore, avoiding the hypertrophy of cartilage tissue is an important issue for cartilage regeneration. However, the expression of COL10 mRNA was observed in all groups. Sekiya et al. have shown that the mRNA expression of COL10 is present before the findings of significant hypertrophy and increased over time during *in vitro* chondrogenic differentiation of human MSCs.[110] Since there is a possibility that immunostaining failed to detect slight expression of type X collagen, further study will be needed to observe the long-term outcome. Finally, we did quantitative real-time PCR after chondrogenic induction for 14 days. The mRNA expression of chondrogenic genes was significantly upregulated in the FGF-2(+)-0% FBS group. These results are consistent with biochemical analysis and histological evaluation. In summary, the combination of FGF-2 preconditioning and serum-free chondrogenic induction medium is an efficient method for chondrogenesis in canine BM-PACs.

To our knowledge, this is the first report that has allowed efficient chondrogenic differentiation of canine MSCs and the generation of ECM-rich cartilage tissue with strong Safranin O staining. Reich et al. reported the chondrogenic induction of canine adipose-derived MSCs using a chondrogenic medium containing 1% FBS, however down-regulation of SOX9 expression and poor GAG deposition was observed.[111] In the context of our results, this was probably due to the addition of FBS into the chondrogenic medium. Further, it was reported that chondrogenesis of canine fat, synovium, and bone marrow-derived MSCs was unsuccessful even after induction with serum-free chondrogenic medium.[12,16] It is thought that FGF-2 preconditioning may improve chondrogenesis of these types of canine MSCs. Even though the results of our study may be applicable only to BM-PACs, further studies should be designed to elucidate whether FBS and FGF-2 affect the chondrogenic differentiation of canine MSCs derived from other tissues. Nevertheless, BM-PACs could be a promising cell source for canine cartilage regenerative medicine.

In conclusion, FGF-2 enhanced the proliferative ability and mRNA expression of SOX2 in canine BM-PACs. During chondrogenic induction, cells preconditioned with FGF-2 could produce abundant cartilage matrix with low type I collagen expression. Additionally, higher FBS concentrations inhibited chondrogenic differentiation,

particularly at the outer surface of spheroids. The combination of FGF-2 preconditioning and serum-free chondrogenic induction medium efficiently promoted chondrogenesis of canine BM-PACs. These results will be useful for the abundant production of high-quality cartilage tissue from canine MSCs and contribute to the development of research for cartilage injury or joint diseases in dogs.

Table 1-1. Primers used for quantitative real-time PCR

Genes		Sequence (5'-3')
SOX9	F	AAGCTCTGGAGGCTGCTGAA
	R	ACTTGTAATCCGGGTGGTCTTT
Oct4	F	GCAGTGACTATTCGCAACGA
	R	ATTTGAATGCATGGGAGAGC
SOX2	F	AGTCTCCAAGCGACGAAAAA
	R	GCAAGAAGCCTCTCCTTGAA
ACAN	F	CCTACGATGTCTACTGCTATGTGG
	R	CAGGGTGGCGTTATGAGATTC
COL2	F	CCCGAACCCACAAACAACA
	R	AGCCATTCAGTGCAGAGCC
COL1	F	GTAGACACCACCCTCAAGAGC
	R	TTCCAGTCGGAGTGGCACATC
COL10	F	TTCCAGGACAGCCAGGCATCA
	R	TTCCCAGTGCCTTCTGGTCC
GAPDH	F	TGACACCCACTCTTCCACCTTC
	R	CGGTTGCTGTAGCCAAATTCA
HPRT1	F	GCCTTCTGCAGGAGAACCTC
	R	ATCACTAATCACGACGCTGGG

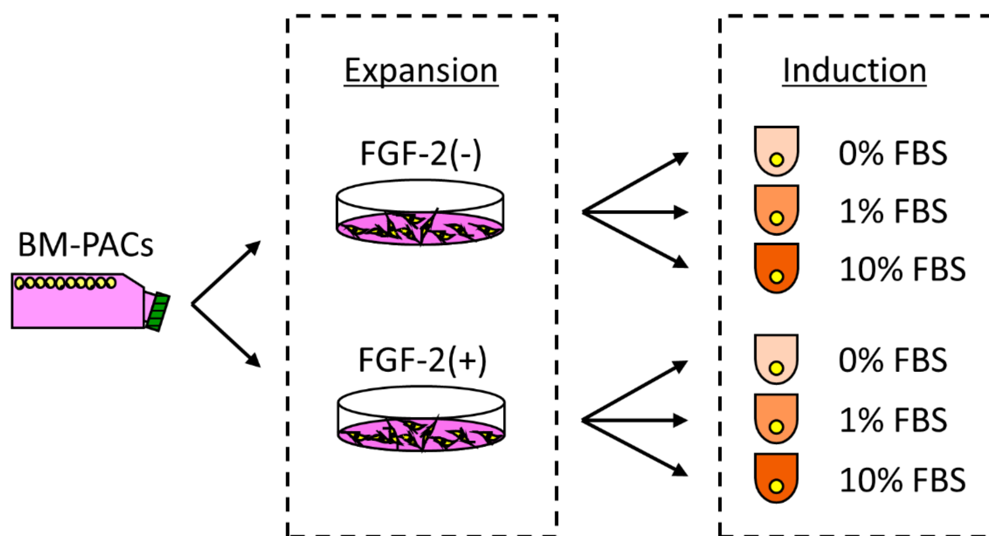

FIG. 1-1. Schematic representation of the experiment. P0 BM-PACs were passaged and expanded with or without FGF-2 in monolayer culture. Subsequently, cells were subjected to suspension culture to form spheroids in chondrogenic differentiation media containing 0, 1, or 10% FBS. BM-PACs, bone marrow peri-adipocyte cells; FGF-2, fibroblast growth factor-2; FBS, fetal bovine serum.

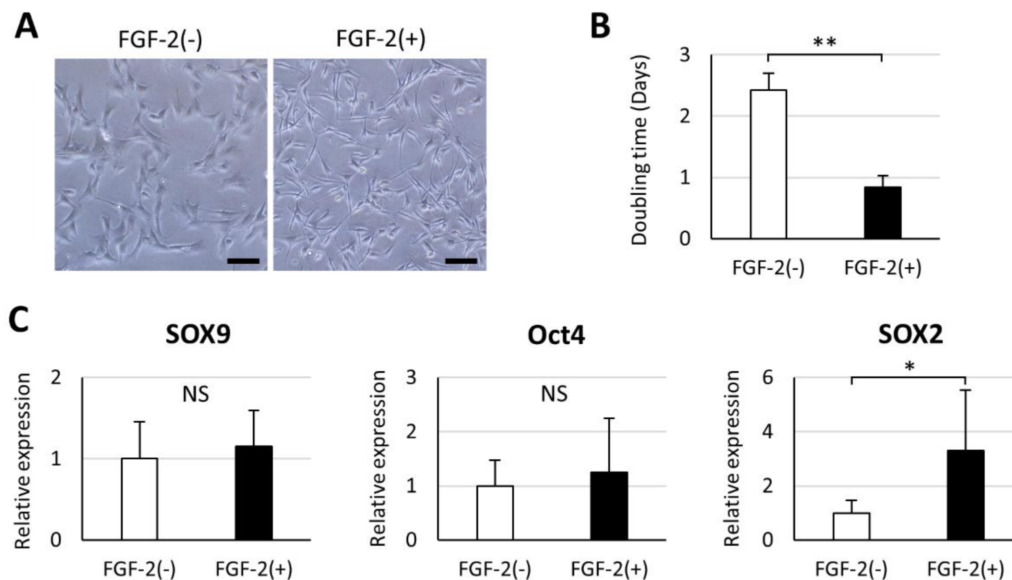

FIG. 1-2. The effect of FGF-2 on BM-PACs in monolayer expansion. **(A)** Cell morphology after two days of FGF-2 stimulation was observed using phase microscopy. Cells treated with FGF-2 displayed smaller and spindle-shaped morphology. Scale bars indicate 200 μm. **(B)** Population doubling times were calculated when cells were confluent. Significantly shorter doubling time was observed in the FGF-2(+) group (**$p < 0.0001$). **(C)** The mRNA expression of chondrogenic and immature genes was assessed by quantitative PCR at day 2. The expression of SOX2 mRNA in the FGF-2(+) group was significantly higher than that in FGF-2(-) group (*$p < 0.05$). BM-PACs, bone marrow peri-adipocyte cells; FGF-2, fibroblast growth factor-2; NS, no significance.

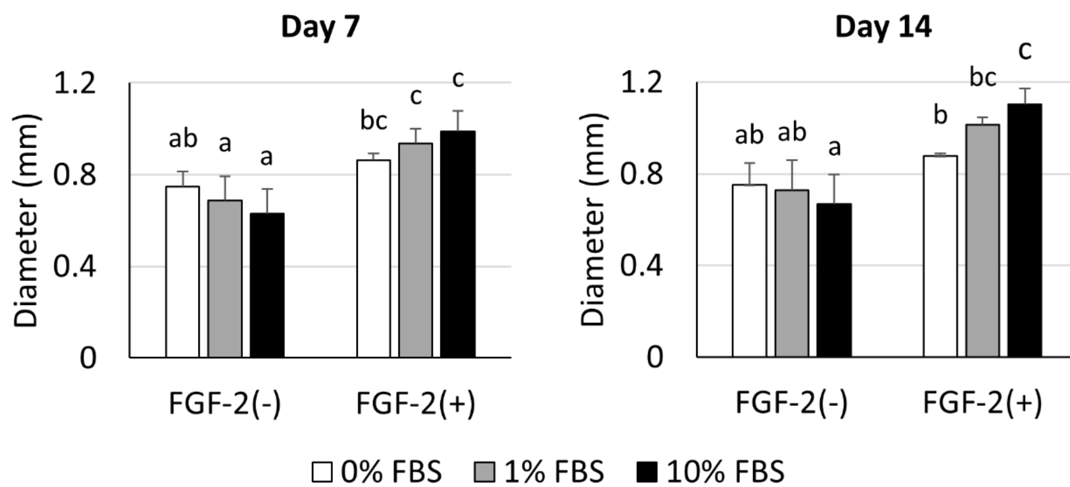

FIG. 1-3. The diameter of spheroids after 7 and 14 days of chondrogenic differentiation. No significant difference was recorded for spheroid diameter amongst FGF-2(-) groups. Spheroids in FGF-2(+) groups became larger depending upon FBS concentration. All groups not sharing common letters are significantly different ($p < 0.05$). FGF-2, fibroblast growth factor-2; FBS, fetal bovine serum.

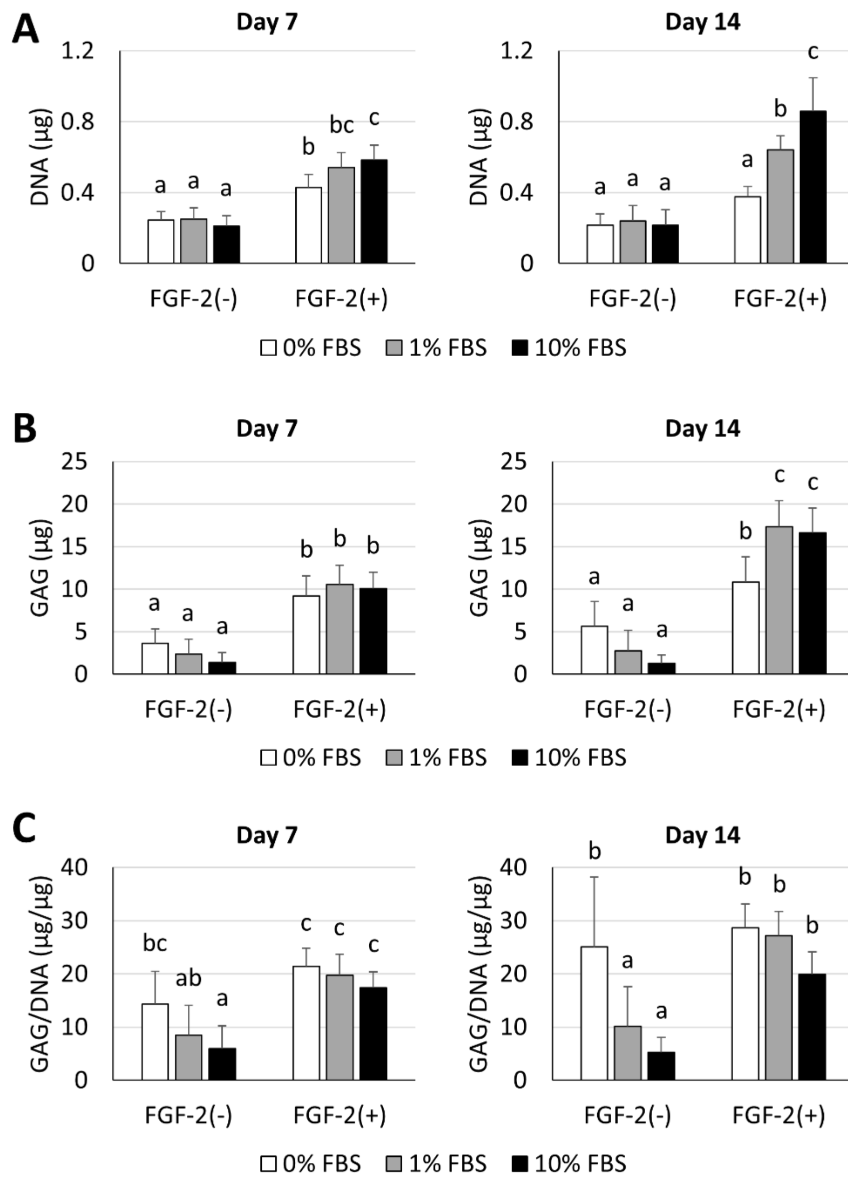

FIG. 1-4. Biochemical analysis of DNA and GAG at day 7 and 14. (**A**) DNA contents of spheroids were measured with Hoechst 33258 dye. The DNA contents of spheroids in FGF-2(+) groups increased in an FBS concentration-dependent manner, whilst the DNA content in FGF-2(-) groups was stable throughout chondrogenic induction regardless of FBS concentration. (**B**) Total spheroid GAG contents in FGF-2(+) groups were significantly higher than those in FGF-2(-) groups after 7 and 14 days of chondrogenic differentiation. (**C**) A lower FBS concentration in the chondrogenic medium tended to increase GAG/DNA ratio. All groups not sharing common letters are significantly different ($p < 0.05$). GAG, glycosaminoglycan; FGF-2, fibroblast growth factor-2; FBS, fetal bovine serum.

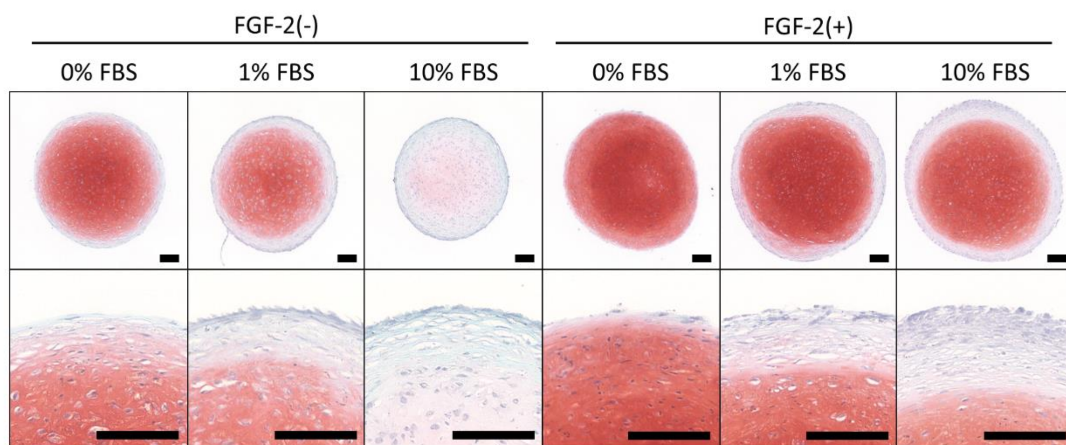

FIG. 1-5. Safranin O staining of spheroids after 14 days of chondrogenic differentiation. In FGF-2(-) groups, spheroids cultured with a lower concentration of FBS showed stronger staining than those cultured at higher concentrations. All spheroids showed strong staining with Safranin O in FGF-2(+) groups. Safranin O-negative outer layers consisted of fibroblast-like cells. Higher concentrations of FBS formed a thicker Safranin O-negative layer. All scale bars indicate 100 µm. FGF-2, fibroblast growth factor-2; FBS, fetal bovine serum.

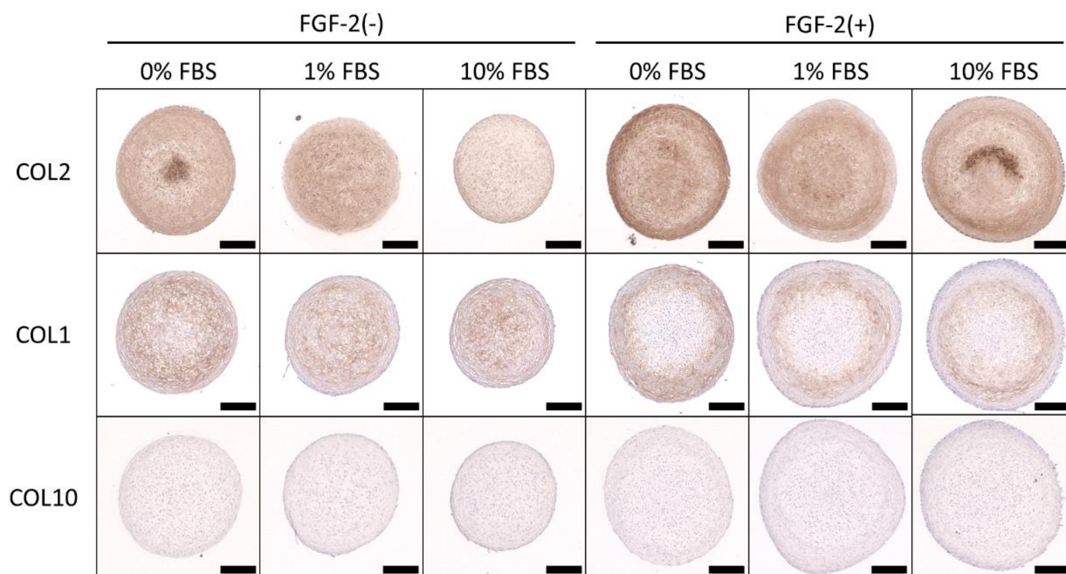

FIG. 1-6. Immunohistochemistry for collagen type II, I, and X of spheroids at day 14. Spheroids cultured with a lower concentration of FBS tended to express more type II collagen. Type I collagen was uniformly detected over the spheroid in FGF-2(-) groups, while in FGF-2(+) groups, its distribution was more pronounced in the middle to the outer surface. Type X collagen was not detected under any conditions. All scale bars indicate 200 μm. FGF-2, fibroblast growth factor-2; FBS, fetal bovine serum.

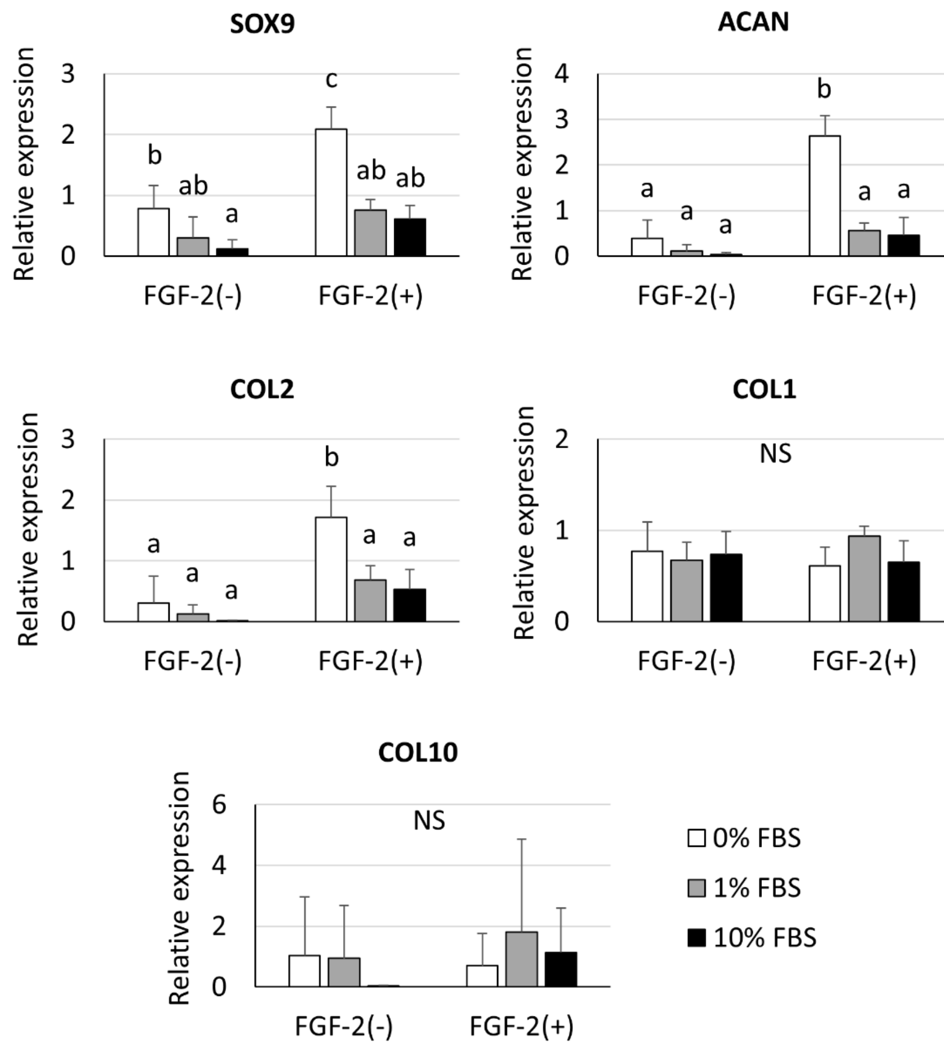

FIG. 1-7. Quantitative RT-PCR was done to assess mRNA expression levels in chondrogenic spheroids. The expression of SOX9, ACAN and COL2 were significantly upregulated in FGF-2(+)-0% FBS group. There were no remarkable expression changes observed for COL1 or COL10. All groups not sharing common letters are significantly different ($p < 0.05$). FGF-2, fibroblast growth factor-2; FBS, fetal bovine serum; NS, no significance.

Chapter 1-2

Comparison of the effect of growth factors on chondrogenesis of canine MSCs

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。3 年以内に出版予定。

Chapter 2

Role of hypoxic conditions in hyaline cartilage differentiation of canine MSCs

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Chapter 3

Fabrication of canine hyaline cartilage construct and its therapeutic effect on cartilage defect model

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。3 年以内に出版予定。

Summary and conclusion

Although articular cartilage injury is common pathology in dogs, severely injured articular cartilage never regenerates spontaneously due to its avascularity. The conventional treatments lead to the formation of fibrocartilage instead of hyaline cartilage finally resulting in secondary osteoarthritis (OA) progression. Therefore, a radical treatment such as regenerative medicine is necessary for articular cartilage injury in dogs. Mesenchymal stem cells (MSCs) are the promising cell source for articular cartilage regeneration because they have a multipotency for mesodermal cells including chondrocytes with lower cost. However, the generation of three-dimensional (3D) tissue with pure hyaline cartilage from canine MSCs has been still challenging. Recently, a novel bio 3D printer using spheroids of cells as a building block has been developed. Therefore, it is expected that canine hyaline cartilage constructs can be fabricated with this technology if canine MSCs could differentiate into hyaline cartilage. The objective of this study was to establish a culture method of hyaline cartilage differentiation of canine MSCs and to fabricate hyaline cartilage constructs with a bio 3D printer.

In *Chapter 1*, the optimal cell culture condition for chondrogenic differentiation of canine bone marrow peri-adipocyte cells (BM-PACs), which is a novel canine MSCs isolated from bone marrow, was explored. Since fetal bovine serum (FBS) concentration in chondrogenic medium and fibroblast growth factor-2 (FGF-2) preconditioning have

been reported to affect chondrogenesis of human MSCs, I firstly evaluated their effects on chondrogenesis of BM-PACs. When serum-free chondrogenic medium and FGF-2 preconditioning were combined, BM-PACs efficiently underwent chondrogenic differentiation to produce hyaline cartilage extracellular matrices (ECMs) with low expression of type I collagen which is a fibrocartilage marker. Then, I compared the effect of bone morphogenetic protein-2 (BMP-2), growth differentiation factor-5 (GDF-5), and insulin-like growth factor-1 (IGF-1) on chondrogenesis of BM-PACs because these growth factors control *in vivo* cartilage development. I demonstrated that GDF-5 increased the deposition of hyaline cartilage ECMs without inducing hypertrophic differentiation while BMP-2 enhanced the expression of the hypertrophic gene. Based on the results obtained in *Chapter 1*, I could establish the optimal medium condition for chondrogenesis of canine MSCs, but there remained slight fibrocartilage mixture in the generated cartilage.

To overcome this problem, I focused on hypoxic conditions. Hypoxic conditions have been reported to play a pivotal role in chondrogenic fate determination of MSCs via nuclear translocation of hypoxia-inducible factor-1α (HIF-1α). Therefore, in *Chapter 2*, I investigated how hypoxic condition regulates the hyaline cartilage differentiation of canine BM-PACs. The results showed that HIF-1α translocated into the nucleus and BM-

PACs differentiated into hyaline cartilage consisting of abundant hyaline cartilage matrix and little type I collagen when cultured under hypoxic condition during the chondrogenic phase. Moreover, I successfully demonstrated that BM-PACs exposed to hypoxic condition only in early chondrogenic phase retained hyaline cartilaginous phenotypes even after the culture was switched to normoxia; that is, the cells were committed to differentiate into hyaline cartilage at the early stage of chondrogenesis. These results indicated that hyaline-fated cartilage could be generated from canine MSCs under the hypoxic condition and expected to retain ECM components even after transplantation.

Lastly, in *Chapter 3*, I aimed to fabricate canine hyaline cartilage construct with a spheroid-based bio 3D printer and evaluated its therapeutic effects on canine chondral defect model. I presented that spheroid-spheroid contact promoted chondrogenesis of BM-PACs and 3D hyaline cartilage constructs could be fabricated under hypoxic condition. Then, autologously fabricated constructs were transplanted into chondral defects created in the weight-bearing area of canine medial femoral condyles. Although individual differences were observed, this pilot study suggested that canine cartilage defects could be repaired with hyaline cartilage instead of fibrocartilage by the transplantation of hyaline cartilage constructs.

In conclusion, I established the optimal medium condition to allow robust generation of hyaline-like cartilage from canine MSCs which have been reported to have limited chondrogenic potential. Moreover, I found that canine MSCs could be committed to differentiate into more pure hyaline cartilage by hypoxic culture during early chondrogenesis. Combining these culture methods and 3D bioprinting technology, I fabricated canine hyaline cartilage constructs successfully from canine MSCs. These results provide a potential to accelerate the development of novel cartilage regenerative therapies and *ex vivo* model of cartilage diseases in dogs. Finally, I designed a pilot study with a small sample size to assess the feasibility of hyaline cartilage constructs in canine chondral defects. Although further modifications are required, the transplantation of hyaline cartilage constructs was expected to be a radical therapeutic strategy for canine cartilage injury.

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