# Combined use of bFGF and GDF-5 enhances the healing of medial collateral ligament injury

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ABSTRACT

Basic fibroblast growth factor (bFGF) and growth and differentiation factor (GDF)-5 stimulate the healing of medial collateral ligament (MCL) injury. However, the effect of isolated and combined use of bFGF/GDF-5 remains still unclear. We investigated cellular proliferation and migration responding to bFGF/GDF-5 using rabbit MCL fibroblasts. Rabbit MCL injury was treated by bFGF and/or GDF-5 with peptide hydrogels. Gene expression and deposition of collagens in healing tissues were evaluated. bFGF/GDF-5 treatment additively enhanced cell proliferation and migration. bFGF/GDF-5 hydrogels stimulated Col1a1 expression without increasing Col3a1 expression. Combined use of bFGF/GDF-5 stimulated type I collagen deposition and the reorganization of fiber alignment, and induced better morphology of fibroblasts in healing MCLs. Our study indicates that combined use of bFGF/GDF-5 might enhance MCL healing by increasing proliferation and migration of MCL fibroblasts, and by regulating collagen synthesis and connective fiber alignment.

**Keywords:** medial collateral ligament; bFGF; GDF-5; hydrogel scaffold; ligament healing

## Introduction

Medial collateral ligament (MCL) injury frequently occurs in sports and trauma. Severe MCL injury results in the loss of joint function, which can lead to the poor performance in sports activities and the early development of osteoarthritis [1]. Experimental and clinical studies have revealed that MCL injury can heal spontaneously by conservative treatments. [1-3] However, connective fiber structure, biochemical composition, and biomechanical property of healed MCL are quite different from normal ligament [1].

Several growth factors have been used in the application of tissue engineering to stimulate the proliferation and differentiation of mesenchymal cells [4-6]. Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), growth and differentiation factor (GDF), insulin-like growth factor, platelet-derived growth factor (PDGF), and transforming growth factor (TGF)-β have been reported to enhance the cellular responses of MCL cells [1,7,8]. The proliferation of ligament cells is stimulated by bFGF, EGF, and PDGF [9]. On the other hand, the syntheses of extracellular matrix (ECM) such as type I and III collagen are increased by TGF-β1 and GDF-5 treatments [7,10,11]. The TGF-β superfamily is a multifunctional growth factor, that include TGF-βs, GDFs, and bone morphogenetic proteins, for many cellular processes such as proliferation and differentiation [12,13]. We have previously demonstrated that bFGF acts as the major stimulator in cellular proliferation and migration of MCL fibroblasts [14]. The migration of MCL cells is also activated by GDF-5 treatment in vitro. In addition, bFGF and GDF-5 individually increase the expression of α1(I) collagen gene (Col1a1), a major ECM component of ligament, in MCL cells [14]. The purpose of this study is to compare the availability of isolated and combined use of bFGF and GDF-5 for the healing and remodeling of injured MCL.

The paracrine communication among cells, growth factors, mechanical stresses, and bridging scaffolds is important for tissue regeneration and repairing process. The ideal

scaffold for ligament tissue engineering should be biodegradable, exhibit sufficient mechanical strength, and promote the formation of ligamentous tissue. Several authors have reported about potential ligament scaffolds using collagen, silk, biodegradable polymers, and composite materials [15-19]. As a major component of ligament, collagen scaffolds are temporally highlighted. However, their mechanical properties diminish rapidly with time even after cross-linking treatment. Furthermore, the profound immunogenic reactions also make a limitation in the clinical application of collagen scaffolds [8]. Silk has been studied as a candidate for ligament scaffold because of its biocompatibility, slow degradability, and remarkable mechanical capability [15,18]. On the other hand, the polymeric scaffolds using poly-glycolic acids and poly-lactic acids show excellent mechanical strength. However, their hydrophobicities prevent cellular adhesion, proliferation, and subsequent maturing steps [17]. In the present study, we used peptide hydrogels, that potentiate providing a delivery of growth factors at injured tissue [20], as a self-assembling biodegradable scaffold to promote the healing of MCL injury.

## Materials and methods

*Cells and cell culture:* The Institutional Review Board approval was obtained before beginning all animal studies. Ligament fibroblasts were isolated from MCL of 10-week-old Japanese white rabbits (n = 4) (Shimizu Laboratory Supplies) with collagenase (Sigma-Aldrich) treatments as described [21]. Cells between passage 3 and 6 were used for experiments. Four different MCL fibroblasts were prepared and used for the following assays.

*Cell proliferation assay:* Cell proliferation assays were performed as described previously [22]. To investigate the effect of bFGF and/or GDF-5, MCL cells were seeded as $1 \times 10^4$ cells/well onto 96-well plates. A serum-free DMEM containing 0.1% BSA (Invitrogen) was

used as a control. Recombinant human bFGF (kindly provided by Kaken Pharmaceutical Co.) and recombinant human GDF-5 (BioVision) were used. These growth factors were added into serum-free DMEM at indicated concentrations (n = 5). Cells were incubated for 48 h prior to addition of WST-1 (Roche). The optical density (OD) was measured by using a Model 550 microplate reader (Bio-Rad). The data obtained by subtraction of readings at 630 nm from those at 450 nm were used for evaluation. The mean value derived from 5 wells was evaluated. The assays were run in triplicate for each sample.

*Cell migration assay:* Cell migration assays were performed in a modified Boyden chamber (48-well chemotaxis chamber AP48, Neuro Probe) as described previously [23]. Collagen-coated polycarbonate membrane (8 μm pore, Neuro Probe) was placed over the bottom chamber filled with a medium containing growth factors (bFGF, GDF-5, and bFGF/GDF-5) at indicated concentrations (n = 4). MCL cells (1 x $10^4$ cells) were seeded in the upper chamber of each well. The assembled chamber was incubated for 8 h to allow cells for migration through the membrane. Non-migrating cells on the upper surface of the membrane were removed by scraping and the membrane was stained with Diff-Quik (Sysmex). The total number of cells with nuclei that migrated per well was counted as described [23]. The mean value derived from 4 wells was evaluated, and the assays were performed in triplicate for each sample.

*MCL injury model and growth factor treatment:* Thirty female Japanese white rabbits with a body weight of 2.0-2.5 kg were used for this study. All experiments were performed under the approval of the Institutional Review Board. Following anesthesia, 3 cm skin incision was made in the medial side of the left knee, and the left MCL (width, approximately 4 mm) was exposed. A Penrose drain was placed underneath the MCL. Then, a full thickness partial excision of MCL was created by 3 mm diameter biopsy punch [24,25]. These excisions were

performed at proximal and distal one-third of MCL for RNA and histological analyses. The right MCL was used as a negative control. No treatment was applied in a group of partial excision control (n = 3). The defect was filled with 20 μl of bioabsorbable peptide hydrogels (PuraMatrix, BD) as a group of scaffold alone (n = 3). This 16 amino acid peptide (RADARADARADARADA) self-assembles into a nanofiber network. MCL treatments were performed using hydrogels contained 10 μg of each growth factor (bFGF, GDF-5, and bFGF/GDF-5; n = 3). After surgery, the animal was housed in a cage without knee immobilization until the time of evaluation.

*Histological analysis:* Animals were sacrificed at 2 and 4 weeks after surgery. MCL samples were fixed by 10% formalin solution and embedded in paraffin blocks. Coronal sections of partially excised MCL lesions (6 μm thicknesses) were assessed by hematoxylin-eosin (HE) and Masson trichrome staining as described [12,23]. The organization of fibrous connective tissue at excised lesion was evaluated using fiber alignment score [26]. The appearance of cell shape was also assessed using morphology of fibroblasts score [26]. Histological finding was assessed by 5 independent investigators.

*Quantitative real-time PCR analysis:* MCL samples at 2 and 4 weeks after surgery were harvested for RT-PCR analyses. Total RNA was isolated using ISOGEN reagent (Nippon Gene). RNA samples were reverse-transcribed with ReverTra Ace (Toyobo) to cDNAs. PCR amplification was performed in the presence of 10 pmol of each specific primer. The primer sets for rabbit Col1a1, Col3a1, glyceraldehyde-3-phosphate dehydrogenase (G3pdh) were used [14,27]. The cycle number was selected in the linear part of the amplification curve. Quantitative real-time PCR analyses were performed using Mx3000P instrument (Stratagene) and Brilliant II Fast SYBR Green QPCR Master Mix (Stratagene) as described [28]. Amplification of G3pdh was used for normalization. The final expression value was calculated in dividing each expression level of partial excision control. These

experiments were assessed in triplicate.

*Immunohistochemical analysis:* MCL sections were also assessed by immunohistochemical analyses using anti-type I (Merck) and anti-type III (Monosan) collagen antibodies as described [21]. To quantify signal density, images were analyzed by Image J 1.31 as described [29]. The mean value derived from 5 different images was evaluated.

*Statistical analysis:* Data were expressed as means SD. Differences among groups were compared by using the Mann-Whitney *U*-test. Statistical significance was established at $p < 0.05$.

## Results

### *bFGF/GDF-5 treatment increases the cellular proliferation and migration of MCL fibroblasts*

The proliferation of bFGF- and bFGF/GDF-5-treated cells were increased up to 1.7- and 2.1-fold levels of control, respectively (Fig. 1A, 100 ng/ml). GDF-5 single treatment did not influence MCL cell proliferation (Fig. 1A, GDF-5). Combined use of bFGF/GDF-5 induced similar increase of MCL cellular proliferation in comparison with bFGF alone (Fig. 1A). MCL cell migration responding to bFGF and GDF-5 was increased to 2- and 2.5-fold levels of control, respectively (Fig. 1B, 100 ng/ml). In addition, bFGF and GDF-5 cooperatively stimulated the migration of MCL cells (Fig. 1B, bFGF/GDF-5). These results prompted us to investigate the effect of bFGF/GDF-5 combined treatment in a ligament injury model.

### *bFGF/GDF-5 hydrogels stimulate the healing of MCL injury*

In rabbit MCL injury model, the combined treatment using bFGF/GDF-5 and hydrogels stimulated the healing of injured tissues. Remodeling of fiber alignment was improved in bFGF/GDF-5-hydrogel group (n = 3) at 4 weeks (Fig. 2, A and B). Fiber alignment score was higher in bFGF/GDF-5 hydrogels than in the other groups (Fig. 2D). Cellular morphology of bFGF/GDF-5-hydrogel group showed a fibroblast-like shape in repaired tissues (Fig. 2, A and B, 4 weeks). The evaluation score for the morphology of fibroblasts was higher in bFGF/GDF-5 hydrogels than in the other groups (Fig. 2F).

*Combined therapy using bFGF/GDF-5 hydrogels stimulates Col1a1 expression and type I collagen synthesis without increasing type III collagen deposition during ligament healing*

Combined treatment of bFGF/GDF-5 hydrogels increased Col1a1 expression in healing tissue at 2 weeks after MCL injury (Fig. 3A). However, GDF-5 hydrogels did not enhance the Col1a1 expression (Fig. 3, A and B). High amount of type I collagen synthesis was observed in bFGF/GDF-5-hydrogel group at 4 weeks after surgery (Fig. 4, A and D, bFGF/GDF-5). The alignment of type I collagen fibers was well-organized in bFGF/GDF-5-hydrogel group (Fig. 4A, 4 weeks). On the other hand, Col3a1 expression was stimulated by bFGF hydrogels at 2 weeks (Fig. 3C). bFGF hydrogels induced type III collagen deposition at 4 weeks (Fig. 4, B and F). GDF-5 hydrogels prevented the increase of Col3a1 expression in healing tissues (Fig. 3C). The deposition of type III collagen was poorly detected in GDF-5- and bFGF/GDF-5-hydrogel groups (Fig. 4, B, E, and F).

## Discussion

Growth factors and scaffolds that activate cellular proliferation and migration of ligament fibroblasts could potentially induce sufficient ECM synthesis and early ligament healing. bFGF-impregnated pellet using hydroxyethyl methacrylate stimulates initial

remodeling process with a decreased number of cells and better orientation of the collagen fibers in a canine anterior cruciate ligament (ACL) injury model [30]. A poly-L-lactic acid braid scaffold combined with bFGF and a gelatin hydrogel results in enhanced mechanical strength and collagen production of regenerated ACL tissue in a rabbit model [3]. The potential of synergistic effect of growth factors has also been investigated. A combination of TGF-β1 and PDGF does not enhance the structural property of healing MCL compared to the use of PDGF alone [31]. TGF-β2/PDGF treatment has no significant effect compared to TGF-β2 single treatment [32]. On the other hand, combined local application of TGF-β1 and EGF improves the structural property of tendon graft in an ACL reconstruction model [33]. Although TGF-β1 and GDF-5 synergistically stimulate the proliferation of bone marrow-derived stem cells on a braided poly(lactic-co-glycolic acid) scaffold, synergistic increase of collagen synthesis is not observed by the combination of TGF-β1/GDF-5 [34]. In the present study, we demonstrated that the combined use of bFGF and GDF-5 enhanced the well-organized deposition of type I collagen in injured MCL in vivo (Figs. 2-4). These findings suggest that the combined treatment using bFGF, GDF-5, and hydrogels may be a strong candidate to induce the optimum healing after ligament injury. This study includes several limitations such as MCL injury model, in vivo sample number, and lack of biomechanical analysis. To investigate ligament healing using animal models, reproducible injury must be created. Complete rupture model of MCL may represent sever MCL injury. However, scar formation at injured MCL is unstable and experimental assessment of repairing tissue is difficult. Although our partial excision model might not represent clinical MCL injury, ligament defect was prepared under certain conditions. Further investigations including biomechanical analysis will be required to understand the synergistic effect of bFGF and GDF-5 in ligament healing.

Type I collagen is the predominant genetic type of collagen found in normal ligaments in a ratio of 3:1 with type III collagen [35]. In the process of ligament healing, the

deposition of type III collagen typically precedes that of type I collagen, and then type III collagen is replaced by the stiffer ECM molecule, type I collagen [36,37]. The early production of type III collagen might be important in establishing the initial wound structure and in providing a basic lattice for subsequent healing events [38]. However, type I collagen synthesis does not start after type III collagen deposition, but begins shortly after ligament injury [39]. It is probable that type III collagen might be predominantly synthesized in immature repairing tissues during ligament healing. To reduce undesirable ECM molecules in repairing tissue might potentiate inducing successful ligament healing. Antisense gene therapy against Decorin increases the diameters of collagen fibers and the tensile strength of healing ligament in a rabbit MCL injury model [40]. Suppression of Col3a1 expression using antisense oligonucleotides might be useful approach in the treatment of ligament injuries [19]. In this study, both the gene expression and protein deposition of type I collagen were increased by the combined treatment of bFGF and GDF-5 in the healing tissue after MCL injury (Figs. 3 and 4). On the other hand, the expression and deposition of type III collagen were stimulated in bFGF-hydrogel group, but not in bFGF/GDF-5-hydrogel group (Figs. 3C and 4). GDF-5 deficiency strongly increases Col3a1 expression in the infarct zone of heart after myocardial infarction using GDF-5 knockout mice [41]. The brachypodism mice that carry a functional null mutation of GDF-5 gene exhibit the hypoplasia of condyles and intra-articular ligaments in the knee joint, and result in anterior dislocation of the knee [42]. In addition, GDF-5 treatment reduces the expression of type III procollagen [11]. These findings suggest that GDF-5 might have a key role in the healing and development of ligaments by regulating the synthesis of type III collagen.

In conclusion, our present study indicates that the combined use of bFGF, GDF-5, and hydrogels might enhance the healing of MCL injury by increasing the proliferation and migration of ligament fibroblasts, and by regulating the collagen synthesis and connective fiber alignment.

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Figure legends

**Fig. 1. bFGF/GDF-5-combined treatment increases MCL cellular proliferation and migration.**

(A) MCL cell proliferation was increased up to 1.7- and 2.1-fold levels of control with bFGF and bFGF/GDF-5 treatments, respectively (100 ng/ml). Significant difference was observed between bFGF/GDF-5-treated cells and untreated cells. GDF-5 single treatment did not stimulate MCL cell proliferation in this assay. Open, light-gray, dark-gray, and filled bars indicate the index of OD (450-630 nm) at growth factor concentrations of 0, 1, 10, and 100 ng/ml, respectively. Each datum is the mean of five different wells. * $p < 0.05$.

(B) bFGF and GDF-5 enhanced MCL cell migration to 2- and 2.5-fold levels of control, respectively (100 ng/ml). Combined treatment of bFGF/GDF-5 increased the migration of MCL cells to a 4-fold level of control (100 ng/ml). Open, light-gray, dark-gray, and filled bars indicate the number of migrated cells in the presence of growth factors at concentrations of 0, 1, 10, and 100 ng/ml, respectively. Each datum is the mean of four different wells. * $p < 0.05$.

**Fig. 2. bFGF/GDF-5 hydrogels promote the healing of rabbit MCL injury.**

(A, HE stain; B, Masson trichrome stain) Partial excision group showed irregular pattern of fiber alignment in healing tissues even at 4 weeks after surgery. Disorganized fibrous connective tissue was observed in hydrogel control group even at 4 weeks (A and B). On the other hand, bFGF and bFGF/GDF-5 hydrogels stimulated the reorganization of fiber alignment at 4 weeks. Bars, 25 μm.

(C-F) The remodeling of injured MCL was stimulated by bFGF/GDF-5 hydrogels. Irregularity of fiber alignment was observed in partial excision group. Hydrogel control group also showed disorganized fiber alignment after MCL injury (C and D). The treatment with bFGF hydrogels increased fiber alignment score of damaged MCL (C and D). Further

improvement of fiber alignment was observed by the combined treatment of bFGF/GDF-5 hydrogels at 4 weeks (D). The morphology of fibroblasts score was increased in bFGF/GDF-5-hydrogel group at 2 and 4 weeks after surgery (E and F). Open, dotted, diagonal-hatched, gray, and hatched gray bars indicate the score in the groups of no treatment, hydrogels alone, bFGF, GDF-5, and bFGF/GDF-5 hydrogels after partial excision of MCL, respectively. * $p < 0.05$.

**Fig. 3. bFGF/GDF-5 hydrogels stimulate Col1a1 expression and prevent the increase of Col3a1 expression during ligament healing.**

Col1a1 expression was increased up to 2.2- and 3.0-fold levels of control by bFGF and bFGF/GDF-5 hydrogels at 2 weeks after MCL injury, respectively (A). No significant difference in Col1a1 expression was observed among 5 groups at 4 weeks (B). Col3a1 expression was stimulated up to a 2.8-times higher level of control by bFGF hydrogels at 2 weeks (C). Administration of GDF-5 prevented the increase of Col3a1 expression (C, GDF-5 and bFGF/GDF-5). No significant increase of Col3a1 was observed among each group at 4 weeks (D). Open, dotted, diagonal-hatched, gray, and hatched gray bars indicate relative collagen expressions in the groups of no treatment, hydrogels alone, bFGF, GDF-5, and bFGF/GDF-5 hydrogels, respectively. * $p < 0.05$.

**Fig. 4. bFGF and GDF-5 cooperatively regulate collagen synthesis during the remodeling process of ligament injury.**

(A, anti-type I collagen antibody; B, anti-type III collagen antibody) Immunohistochemical analyses revealed that type I collagen synthesis was increased in bFGF-containing hydrogels at 2 and 4 weeks after surgery (A, bFGF and bFGF/GDF-5). The fiber alignment of type I collagen was well-organized in bFGF/GDF-5-hydrogel group at 4 weeks (A). The deposition of type III collagen was increased in bFGF-hydrogel group (B, bFGF). The synthesis of type

III collagen was maintained at a low level in GDF-5-containing group (B, GDF-5 and bFGF/GDF-5). Bars, 25 μm.

(C-F) The signal density of immunohistochemical staining was evaluated. Type I collagen deposition was increased up to a 5.3-fold level of partial excision control by bFGF/GDF-5 hydrogels at 4weeks (D). However, type III collagen deposition was inhibited by bFGF/GDF-5-combined treatment (E and F). On the other hand, bFGF hydrogels stimulated the deposition of both type I and III collagens in repairing tissue at 4 weeks after MCL injury (E and F). Open, dotted, diagonal-hatched, gray, and hatched gray bars indicate each signal density in the groups of no treatment, hydrogels alone, bFGF, GDF-5, and bFGF/GDF-5 hydrogels, respectively. * $p < 0.05$.

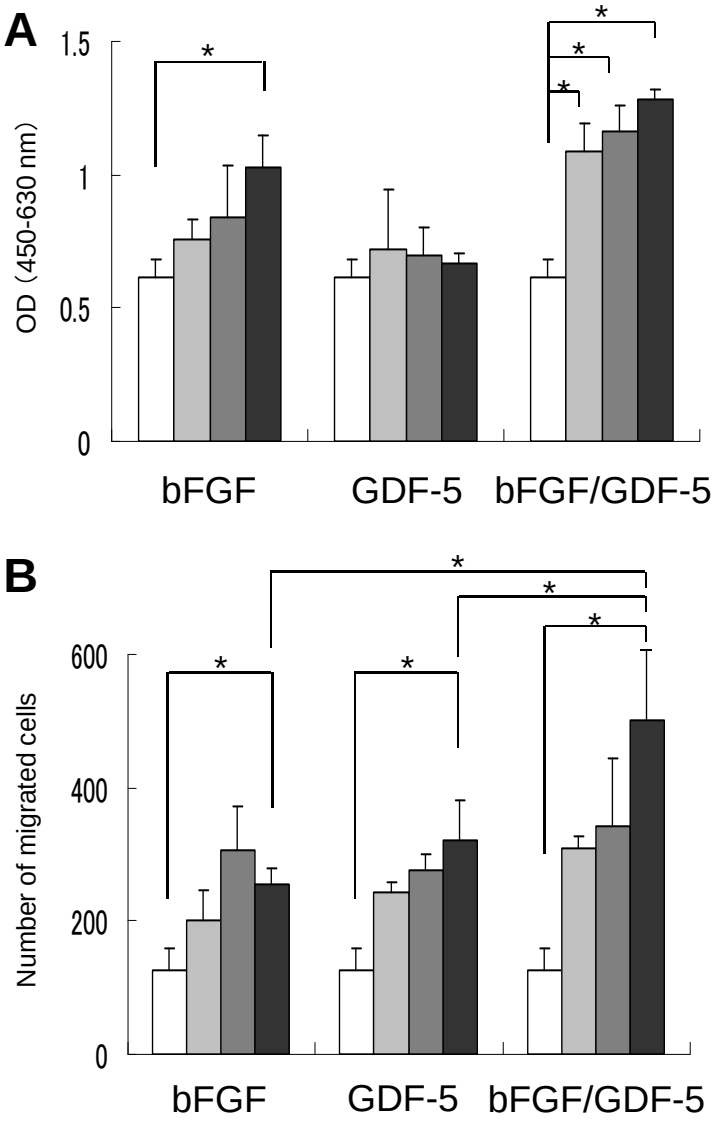


Figure 1

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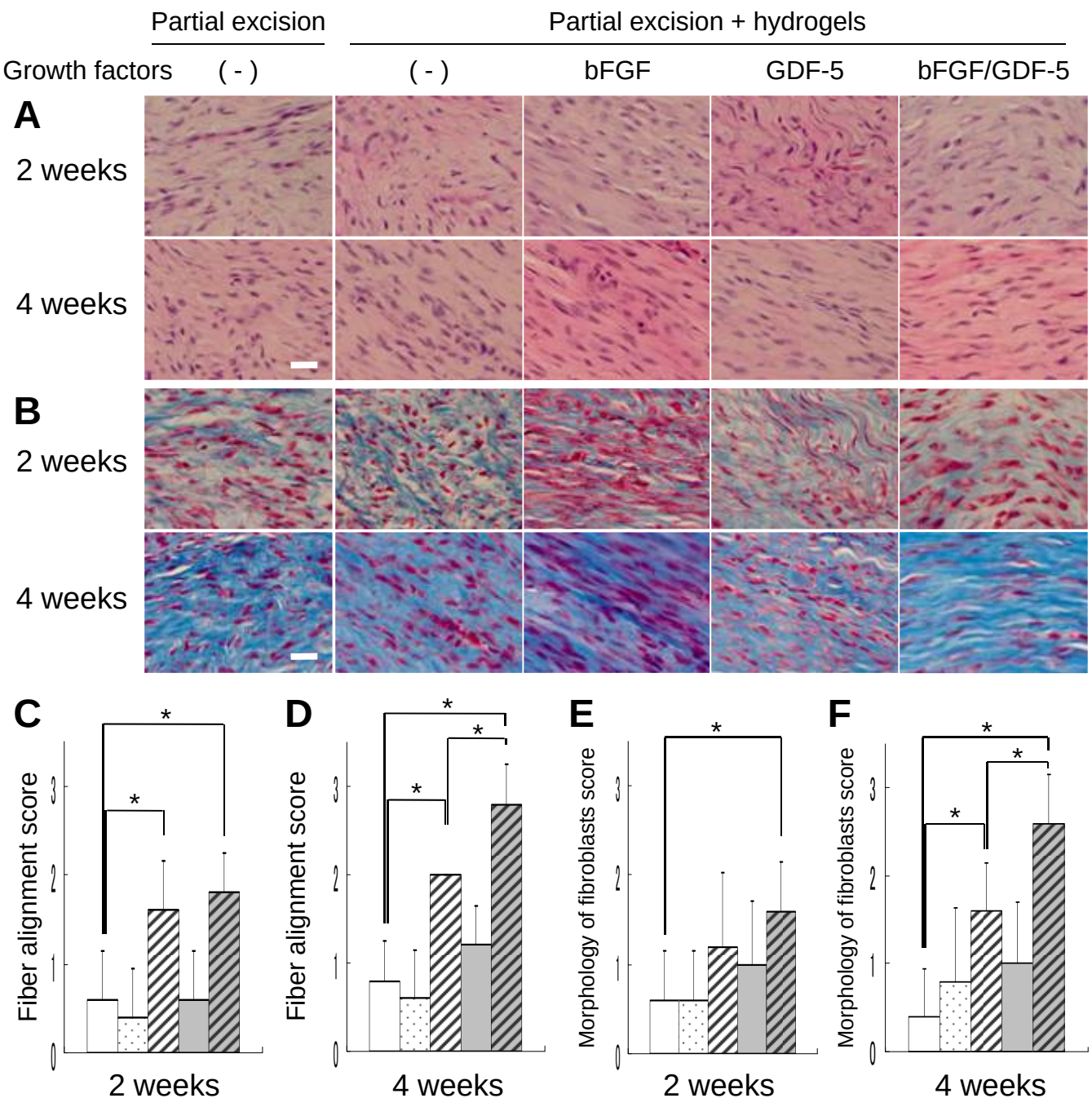


Figure 2

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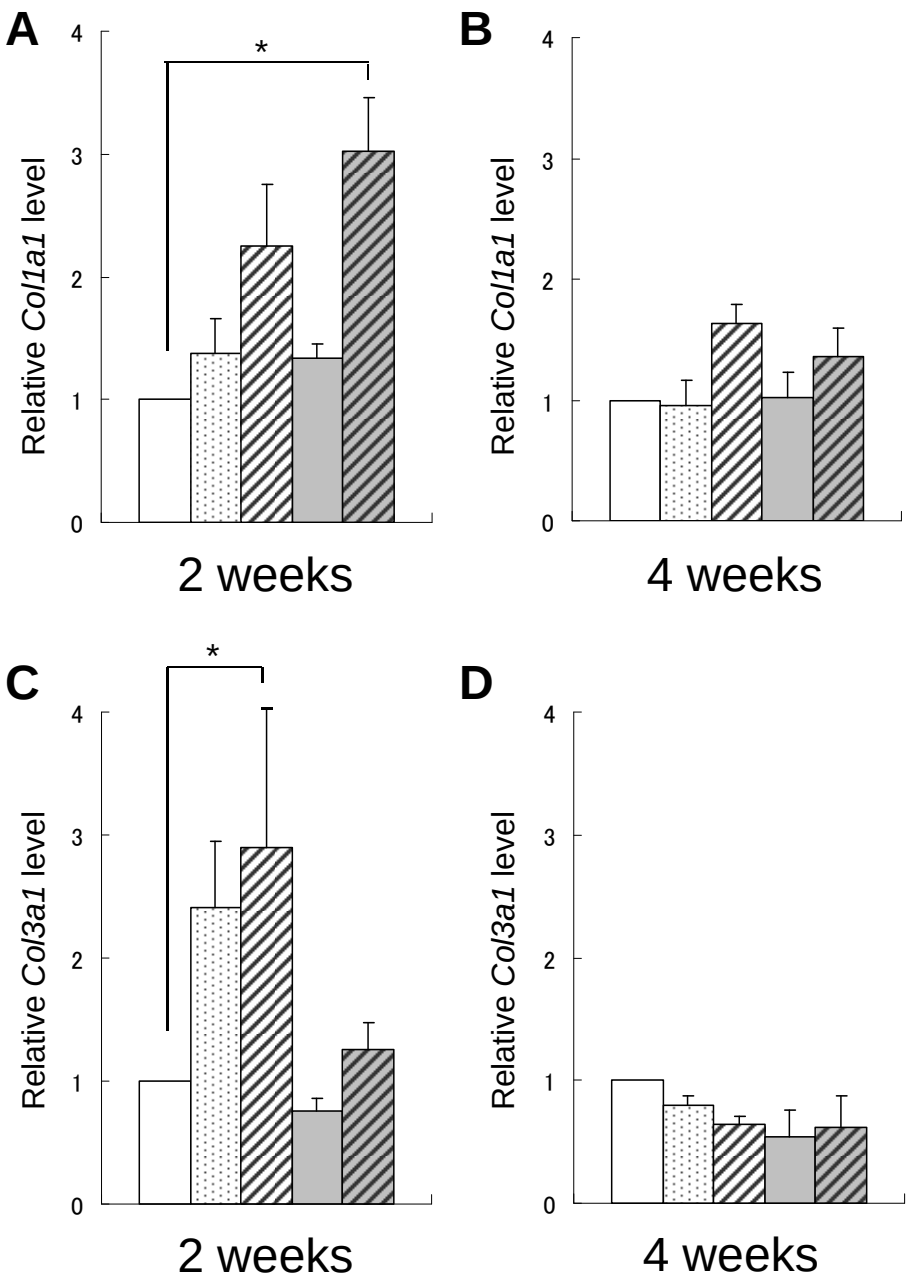


Figure 3

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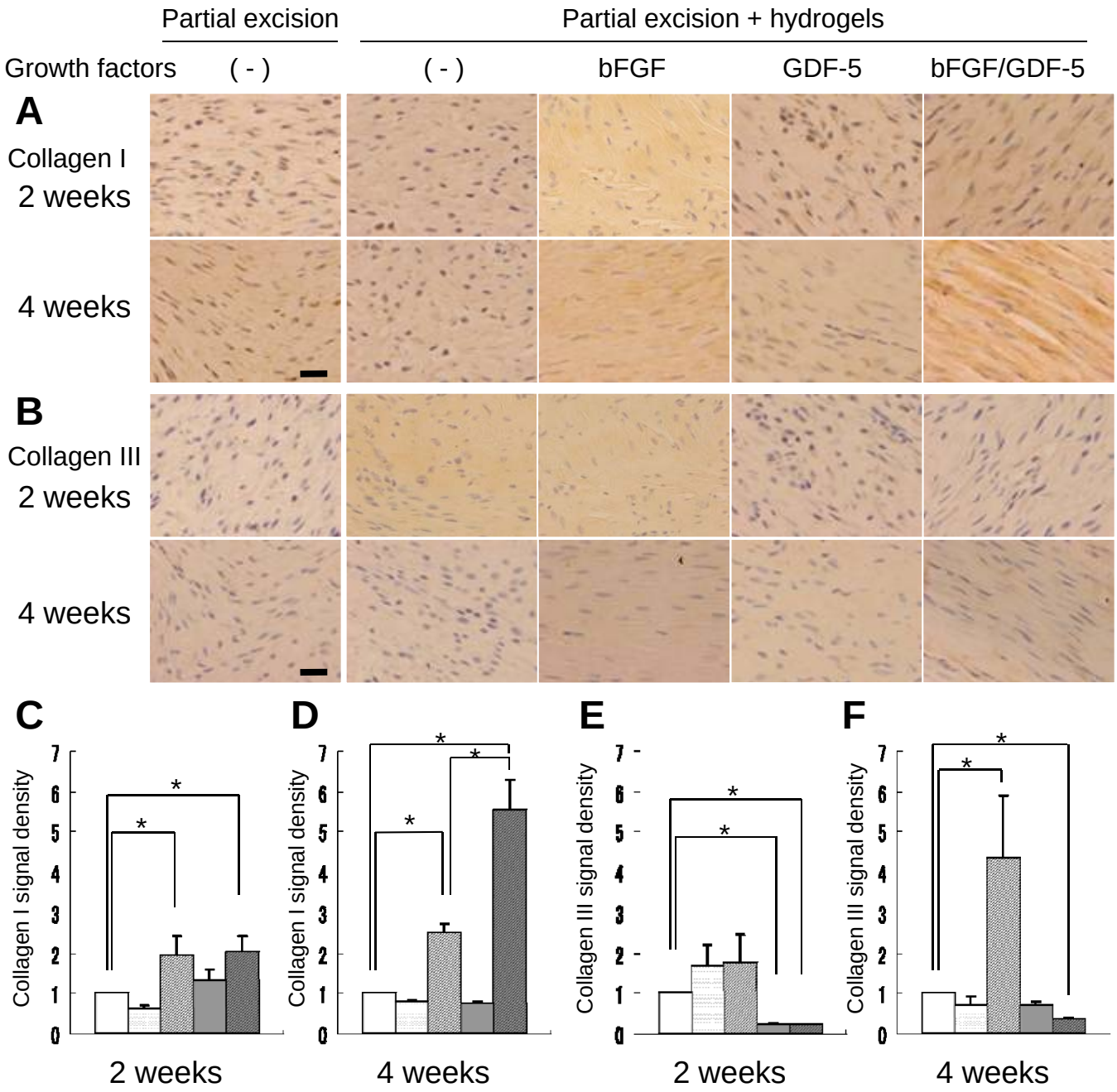


Figure 4

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