Original article

Basic fibroblast growth factor promotes meniscus regeneration through the cultivation of synovial mesenchymal stem cells via the CXCL6–CXCR2 pathway

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Running title

BFGF-cultured SMSC boost meniscus regrowth

Abstract

Objective: To investigate the efficacy of basic fibroblast growth factor (bFGF) in promoting meniscus regeneration by cultivating synovial mesenchymal stem cells (SMSCs) and to validate the underlying mechanisms.

Methods: Human SMSCs were collected from patients with osteoarthritis. Eight-week-old nude rats underwent hemi-meniscectomy, and SMSCs in pellet form, either with or without bFGF (1.0×10^6 cells per pellet), were implanted at the site of meniscus defects. Rats were divided into the control (no transplantation), FGF (−) (pellet without bFGF), and FGF (+) (pellet with bFGF) groups. Different examinations, including assessment of the regenerated meniscus area, histological scoring of the regenerated meniscus and cartilage, meniscus indentation test, and immunohistochemistry analysis, were performed at 4 and 8 weeks after surgery.

Results: Transplanted SMSCs adhered to the regenerative meniscus. Compared with the control group, the FGF (+) group had larger regenerated meniscus areas, superior histological scores of the meniscus and cartilage, and better meniscus mechanical properties. RNA sequencing of SMSCs revealed that the gene expression of chemokines that bind to CXCR2 was upregulated by bFGF. Furthermore, conditioned medium derived from SMSCs cultivated with bFGF exhibited enhanced cell migration, proliferation, and chondrogenic differentiation, which were specifically inhibited by CXCR2 or CXCL6 inhibitors.

Conclusion: SMSCs cultured with bFGF promoted the expression of CXCL6.

This mechanism may enhance cell migration, proliferation, and chondrogenic differentiation, thereby resulting in superior meniscus regeneration and cartilage preservation.

Keywords

synovial mesenchymal stem cells, basic fibroblast growth factor, meniscus regeneration, CXCR2, CXCL6

Introduction

The meniscus plays an important role in the knee as a shock absorber, load distributor, knee stabilizer, lubricator, and proprioceptor[1,2]. Meniscus injury is associated with an increased risk of knee osteoarthritis (OA)[3,4]. However, due to its hypocellularity and hypovascularity, the self-healing capability of the damaged meniscus is highly limited[5,6]. Although there are different methods for treating damaged meniscus, they remain suboptimal[7].

Mesenchymal stem cells (MSCs) have self-renewal and multilineage differentiation capability[7,8]. and they are used as a therapeutic option for meniscus regeneration in animal models. For example, the transplantation of synovial MSC aggregates[9] and the intra-articular injection of human bone marrow MSCs contribute to meniscal repair and attenuation of cartilage degeneration in rats[10]. Among the various sources of MSCs, synovial MSCs (SMSCs) have superior potential for chondrogenic differentiation and proliferation[7,8]. Hence, they are a promising cell source of meniscus regeneration[7,11]. However several issues, such as limited cell proliferation and non-uniformity of cartilage regeneration, should be overcome[12].

Different methods can enhance the tissue-regenerative function of SMSCs[13,14]. Basic fibroblast growth factor (bFGF) binds to FGF receptors, and they have diverse biological functions, such as anabolic and concomitant catabolic effects, on cartilage maintenance[15,16]. BFGF promotes the proliferation and chondrogenic differentiation of MSCs in vitro[13,14]. A previous study reported that SMSCs cultured with bFGF promoted cartilage regeneration in vitro and in vivo. However, the detailed mechanisms remain unclear[17].

We hypothesized that SMSCs cultured with bFGF can promote meniscal

regeneration, a phenomenon that has not yet been reported to the best of our knowledge. The current study aimed to investigate the promotion of meniscus regeneration by SMSCs cultured with bFGF and to validate the underlying mechanism.

Materials and methods

All experiments were approved by the Ethical Review Board of Osaka University Hospital (number: 15409-7, date: April 12, 2016) and the Animal Experimental Committee of Osaka University (number: 29-024-018, date: June 27, 2017).

Preparation of synovial MSC

Human synovium was collected from five patients with OA (four women and one man; mean age: 76 [range: 70–88] years; demographics of each patient are shown in Supplementary table 1) during total knee arthroplasty. SMSCs were isolated from the synovium and cultured, as described in a previous study[18]. We mainly isolated macroscopically hyper-vascular synovium from the supra patella during total knee arthroplasty. SMSCs were separated into the FGF (−) and FGF (+) groups. The SMSCs of the FGF (−) group were cultured in high-glucose DMEM containing 10% FBS and 1% antibiotic–antimitotic (Sigma-Aldrich) and growth medium (GM). The SMSCs of the FGF (+) group were cultured in GM with 5 ng/mL of bFGF (Wako, Osaka, Japan) at 37°C with humidified 5% CO_2[17]. SMSCs were expanded and used for experiments in vivo and in vitro at passages 3–5.

Rat meniscal defect model

The SMSCs were cultured in GM (without FGF; FGF [−] or with FGF; FGF [+]) for

7 days prior to implantation. Next, 1×10^6 SMSCs harvested from passage 3 were centrifuged in 96-well ultra-low attachment plates (Costar, NY, the USA, Cat#7007), and surgery was performed on the following day. Eight-week-old male immunocompromised rats (F344/NJcl-rnu/rnu; CLEA Japan, Fujinomiya, Japan) were anesthetized via the intraperitoneal injection of 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol. The right knee joint was surgically accessed via a medial parapatellar incision. The patella was laterally dislocated to identify the medial meniscus, which was then resected at the level of the medial collateral ligament[9]. The control group was created by applying fibrin glue (Beriplast, CSL Behring, King of Prussia, Pennsylvania, the USA) to the defect site of the meniscus. In the FGF (-) group and FGF (+) group, two SMSC pellets (5.0×10^5 cells per pellet) were placed on the sites of the meniscus defects and secured in place with two drops of fibrin glue (Supplementary Fig. 1). Patellar dislocation was subsequently reduced, and the joint capsule and skin were sutured in separate layers. Thereafter, the rats were immobilized in full knee extension with fiberglass casts at 4 and 8 weeks[19], and the casts were replaced every week. The rats were sacrificed at 4 (n = 8) or 8 (n = 7–8) weeks for analysis. The sample size was determined and evaluated according to the previous study[9].

Macroscopic observation

The meniscus was meticulously dissected from the femoral and tibial condyles at 4 and 8 weeks after surgery. Macroscopic images were captured with Nikon AZ 100 (Nikon, Tokyo, Japan). Quantification of the regenerated meniscus size was performed using Image J (National Institutes of Health)[9].

Histological examination

The rat meniscus and proximal tibia in 4% paraformaldehyde were fixed, dehydrated, and decalcified in 10% ethylenediaminetetraacetic acid. The samples were then embedded in paraffin wax and cut into 5-μm-thick sections for Safranin O staining. These sections were subsequently visualized using APERIO CS2 (Leica, Tokyo, Japan). The regenerated meniscus was evaluated using the modified Pauli's score, which ranges from 0 to 21 (Supplementary Table 2)[20,21]. The assessment of cartilage degeneration of the medial tibia plateau was conducted using the Osteoarthritis Research Society International (OARSI) score, which ranges from 0 to 24[22].

Immunohistochemistry

Paraffin-embedded sections were used for immunochemistry, as described in a previous study [17]. The antibodies used in the experiments, along with their concentrations, are described in Supplementary Table 3.

DAB-stained area overlap ratio (%)

We used paraffin-embedded sections to evaluate the differentiation of transplanted SMSC pellet into chondrocyte-like cells. The sections of the regenerated meniscus stained for type II collagen and human vimentin were utilized, and the DAB-stained area was quantified using Image J (National Institutes of Health), as described in a previous study[23].

Meniscus indentation test

Eight-week samples of regenerated meniscus were utilized in the meniscus indentation test. The whole meniscus tissue was placed at the base of the testing apparatus

(ElectroForce5500, TA Instruments, Newcastle, DE, the USA) via a custom-fabricated jig assembled with a load cell. An indenter with a diameter of 1 mm was applied to the meniscus at the mid-substance and anterior horn and at the mid-point of these two points (with a total of 3 points) at a rate of 0.06 mm/s, up to a depth of 0.03 mm from the meniscus surface. Translation and compression forces were recorded during the examination, and the tangent modulus was calculated[24]. Three individual samples from three different donors were used distinct from the one used for tissue sectioning.

RNA sequencing

SMSCs were seeded in six-well plates at a density of 1.0×10^5 cells/well and incubated with GM with bFGF (bFGF; FGF (+)) or without bFGF (FGF (−)) for 7 days. Subsequently, the cells were subjected to RNA sequencing. RNA libraries for sequencing were prepared using the TruSeq Stranded Total RNA kit (Illumina, San Diego, CA, the USA). Whole transcriptome sequencing was performed using the RNA samples with the Illumina NovaSeq 6000 platform in a 101-bp single-end mode. The sequenced reads were mapped to the human reference genome sequence (hg19) using TopHat version 2.0.13 combined with Bowtie2 version 2.2.3 and SAM tools version 1.0. The number of fragments per kilobase of exon per million mapped fragments was calculated using Cufflinks version 2.2.1. These data were subsequently analyzed using iDEP 96[25].

Real-time quantitative polymerase chain reaction

RNA was extracted from cultured cells using the RNeasy Mini Kit (QIAGEN, Dusseldorf, Germany) and subsequently converted to cDNA via the ReverTra Ace qPCR Master Mix (TOYOBO, Osaka, Japan). Gene expression was quantified via real-time

PCR, with SYBR green master mix (Applied Biosystems, Foster City, CA, the USA) on the Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, the USA). Target gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase, and the fold changes were calculated relative to the control group using the $2^{-\Delta\Delta}$ Ct method. Supplementary Table 4 depicts the primer sequences.

Conditioned medium (CM) preparation

CM was prepared, as described in a previous study[26]. In particular, SMSCs were seeded at a density of 1.0×10^5 cells/well in six-well plates and cultured in GM without bFGF (CM [FGF−]) or with bFGF (CM [FGF+]) for 24 h. The medium was subsequently changed to serum-free medium or medium containing 1% FBS and cultured for additional 48 h. The supernatant was collected and centrifuged at 1700 × g for 5 min at 4°C to eliminate cellular debris. The supernatant was then filtered via a 0.22-μm filter (EMD Millipore, Billerica, MA, the USA) and stored at −80°C until utilized for experiments.

Cell migration assay

The effects of CM on the migratory capacity of SMSCs were assessed using the transwell migration assay (Costar, NY, the USA, Cat#3422). The upper compartment of the assay was populated with 5×10^4 SMSCs/100 μl serum-free medium. The lower compartments were each filled with 500 μl of CM (control; 0% FBS + DMEM, CM [FGF−], CM [FGF+]). The cells were then incubated for 12 h at 37°C. Upon completion of the incubation period, the upper compartment was fixed with 4% paraformaldehyde for 5 min, stained with 0.5% crystal violet for 10 min, and subsequently washed three times with PBS. The upper surface of the transwell membrane was then swabbed with a

cotton applicator to remove any residual cells. Subsequently, the number of migrated cells was quantified via visual examination under 100x magnification using a TE2000-U microscope (Nikon, Tokyo, Japan) in five randomly selected fields.

Meniscus explant for avascular healing via SMSC recruitment

Human meniscus tissue was isolated from patients undergoing total knee arthroplasty and was subsequently cut into a width of 1 cm. In addition, longitudinal incisions with a total thickness of 5 mm were made in the inner zone of the meniscus[27]. The SMSC pellet (5×10^5 cells per pellet) was then transplanted onto the sites of meniscus defects. The meniscus explants were subsequently cocultured with monolayer-cultured human SMSCs (passage 5) for 1 week. The migrating SMSCs within the meniscus were labeled using SCT108 (Merck, Darmstadt, Germany) for detection. Frozen sections were prepared, as described in a previous study, to quantify the number of migrated SMSCs[28]. The samples were evaluated using FV3000 (Olympus, TOKYO, Japan) at a 20× magnification and were excited using the 488- and 640-nm lasers. The images were subsequently processed using CellSens (Olympus, TOKYO, Japan).

Cell proliferation assay

SMSCs were seeded in a 96-well plate at a density of 2.0×10^4 cells/well. The cells were treated with each CM (control: 1% FBS + DMEM, CM [FGF-], and CM [FGF+]). The medium was changed daily. The cell proliferation assay was used on days 0, 1, 3, and 5 with Cell Count Reagent SF (Nacalai Tesque, Inc., Kyoto, Japan), according to the manufacturer's instructions. The SF reagent was added to each well, and the plate was incubated at 37°C for 2 h. Absorbances were then measured at a wavelength of 450 nm

using Microplate Reader (Multiskan GO; Thermo Fischer Scientific, Vantaa, Finland).

Western blot analysis

SMSCs were seeded in a 10-cm plate at a density of 1.0×10^6 cells/plate. The cells were cultured in a serum-free medium (0% FBS + DMEM) for 24 h and then exchanged with each conditioned medium (control: 0% FBS + DMEM, CM [FGF-], and CM [FGF+]) for 15 min. The total cell lysate was separated with RIPA buffer (Thermo Fischer Scientific, Vantaa, Finland). Western blot (WB) analysis was conducted, as described in a previous study[17]. The antibodies used in the experiments, along with their concentrations, are described in Supplementary Table 5.

Chondrogenic differentiation

To cultivate three-dimensional pellets, 2×10^5 SMSCs sourced from passage 3 were centrifuged within a 15-mL polypropylene tube and cultured in GM. On the following day, the medium was replaced with chondrogenic medium (0% FBS + DMEM or CM [FGF-] or CM [FGF+]), which included 1% ITS + premix (Corning, NY, the USA), 50 μg/mL L-ascorbic acid 2-phophate (Sigma-Aldrich), 40 μg/mL L-proline (Sigma-Aldrich), and 10 ng/mL TGF-β3 (PeproTech, Rocky Hill, NJ, the USA). The medium was replaced twice per week, and the cells were maintained at 37°C with humidified 5% CO_2. The pellets were cultured for 2 weeks and subsequently embedded in paraffin wax. Paraffin-embedded pellets were cut with a thickness of 5 μm and subsequently subjected to Safranin O staining. As described in a previous study, the staining intensity was quantified using Image J[29].

Inhibition of CXCR2, ERK pathways, and CXCLs

We used the inhibitors of CXCR2, ERK pathways, and CXCLs in the migration assays, cell proliferation assays, qPCR, WB analysis, and chondrogenic differentiation experiments to investigate the effects of CM to elucidate its underlying mechanism. Supplementary Table 6 shows the aforementioned inhibitors.

Enzyme-linked immunosorbent assay (ELISA)

Using conditioned medium (FBS 0% and FBS 1%), Quantikine Colormetric sandwich ELISA for CXCL6 (R&D systems, MN, USA) was conducted following the manufacturer's instructions.

Statistical analysis

Data were analyzed with GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, the USA)[29]. In vitro, one-way analysis of variance with the Tukey post-hoc test was used to differentiate between each group. In vivo, differences between three groups were assessed using the Kruskal-Wallis test. A P value of < 0.05 was considered statistically significant. Data were presented as means with 95% confidence intervals.

Results

The transplantation of SMSC pellet significantly promoted meniscus regeneration and prevented cartilage degeneration of the medial tibia plateau in a rat model

At 4 weeks after surgery, the areas of the regenerated meniscus in the FGF (−) and FGF (+) groups were significantly larger than that of the control group. At 8 weeks, the regenerated meniscus area in the FGF (+) group was larger than that of the control group

(Figs. 1A, B).

Regarding cartilage injuries, the control group had a thinner cartilage layer and poorer Safranin O stainability at 4 weeks after surgery. These factors further caused severe cartilage injuries at 8 weeks after surgery. Conversely, the FGF (+) group had a thick cartilage layer and good Safranin O stainability at 4 and 8 weeks after surgery (Fig. 1C). The OARSI scores of the FGF (+) group was better than that of the control group at 4 weeks, and the OARSI scores of the FGF (+) group showed a tendency to be better compared to that of the control group at 8 weeks (Fig. 1D). The corresponding donor and transplanted animal pairs, along with the associated regenerative meniscus area, modified Pauli's score, and OARSI score are shown in Supplementary Table 7.

We utilized ElectroForce5500 to evaluate the tangent modulus of the regenerated meniscus using the indentation test (Fig. 1E). The tangent modulus of the control group was lower than that of the sham group. Conversely, the tangent modulus of the FGF (+) group was significantly higher than that of the control group (Fig. 1F). Regarding the regenerated meniscus (Fig. 2A), the stainability of Safranin O and type II collagen in the FGF (+) group was higher than that in the other groups. In addition, the FGF (+) group exhibited hyaline cartilage-like repair with chondrocyte-like round-shaped cells in the lacuna. Subsequently, we evaluated the expression of the specific markers of MSCs (CD44, CD73, and CD90)[30] to confirm the role of SMSCs in meniscus regeneration. As a positive control, human SMSC pellets exhibited robust expression of CD44, CD73, and CD90 (Supplementary Fig. 2). The, normal meniscus presented with minimum expression of these MSC markers. Meanwhile, and the regenerated meniscus had abundant expression. Hence, SMSCs had an important role in meniscus regeneration.

Compared with the control group at 4 and 8 weeks, the FGF (+) group had higher modified Pauli's score (Figs. 2B, C).

In the immunohistochemical analysis, the FGF (−) and FGF (+) meniscus samples had both human vimentin- and type II collagen-positive regions, which indicated a successful engraftment of SMSCs and chondrogenic regeneration (data not shown). Subsequently, we assessed the localization of type II collagen- and human vimentin-positive regions to investigate whether transplanted SMSCs may differentiate directly into chondrocyte-like cells, which can produce type II collagen. However, these regions were found to be quite different from one another (Fig. 2D). This was quantitively confirmed by the overlap ratio of type II collagen- and human vimentin-positive regions (Fig. 2E). Therefore, the implanted human SMSCs were successfully engrafted in the meniscus defect area. However, they may not have undergone direct differentiation into chondrocyte-like cells.

RNA sequence

Most variable genes were calculated and divided into four clusters using iDEP 96 (Fig 3A). Genes that were upregulated in SMSCs with bFGF were present in cluster B. Based on the immunohistochemical analysis results of the regenerated meniscus (Fig. 3B), we focused on the pathway of cell chemotaxis. Subsequently, using the Kyoto Encyclopedia of Genes and Genomes, we detected the pathway of cytokine–cytokine receptor interaction. Ultimately, the gene of CXCLs, which bind to CXCR2, was found to be upregulated in SMSCs with bFGF (Fig. 3C).

BFGF promoted the gene expression of chemokines in SMSCs

qPCR was conducted to evaluate gene expression in SMSCs. The cells were seeded in six-well plates at 1.0×10^5 cells/well and cultured in GM without bFGF (FGF− group) or with bFGF (FGF+ group) for 7 days. Thereafter, the total RNA was extracted from SMSCs. The expression levels of CXCLs that bind to CXCR2 in the FGF+ group were higher than those in the FGF− group at each time point (Fig. 3D). Immunostaining using an anti-CXCR2 antibody revealed that CXCR2 was expressed abundantly in the synovium, meniscus, and cartilage (Fig. 3E).

The conditioned medium of SMSCs cultured with bFGF promoted cell migration, and this mechanism was attenuated by the CXCL6 inhibitor

The transwell migration assay was used with the conditioned medium (FGF (−) or FGF (+)) of SMSCs to investigate the effect of SMSC migration (Fig. 4A). The CM (FGF (+)) significantly enhanced the migration of SMSCs, and this effect was significantly suppressed by the use of SB265610 and PD98059 (Figs. 4B, C). Among CXCLs binding to CXCR2, the gene expression of CXCL6 was highest in SMSCs (Fig. 4D). We subsequently used different CXCL inhibitors to investigate the most effective chemokine that bind to CXCR2 in cell migration. Results revealed that among the CXCL inhibitors, a CXCL6 inhibitor was the most effective in suppressing cell migration (Fig. 4E). SMSCs were treated with each CM for 24 h, and RNA was extracted. The gene expression of CXCL6 was significantly upregulated in the CM (FGF−) and further in the CM (FGF (+)). SB265610 and PD98059 decreased these effects with or without bFGF (Fig. 4F).

The SMSC pellet cultured with bFGF promoted the migration of SMSCs in meniscus explants

A meniscus explant examination was conducted to investigate the effect of SMSC migration using SMSC pellet (without FGF; FGF [-] or with FGF; FGF [+]) (Fig. 4G).

The FGF (+) group exhibited a significantly higher number of migrated SMSCs than that of the control group (Figs. 4H, I).

The conditioned medium of SMSCs cultured with bFGF promoted the gene expression of CXCL6 in SMSCs and induced the phosphorylation of ERK and Akt

Based on the cell proliferation assay of SMSCs, the CM (FGF (+)) group showed a significant increase compared with the control group on day 5 (Fig. 5A). The use of SB265610, PD98059, and MAB333 (a CXCL6 inhibitor) inhibited proliferation on day 5 (Fig. 5B). WB analysis revealed that CM (FGF (-)) and CM (FGF (+)) induced the phosphorylation of ERK and Akt in SMSCs and that PD98059 suppressed this phosphorylation (Fig. 5C).

The conditioned medium of SMSCs cultured with bFGF promoted the chondrogenic differentiation of SMSC pellet

In a 2-week chondrogenic medium culture, the CM (FGF (+)) group had a higher degree of Safranin O stainability than the other groups (Figs. 5D, E). The use of SB265610, PD98059, and MAB333 significantly reduced the Safranin O-stained area (Figs. 5D, E). Hence, CXCL6 produced by SMSCs may enhance the chondrogenic differentiation of SMSCs (Fig. 6).

The conditioned medium of SMSCs cultured with bFGF contained higher levels of

CXCL6.

The CM (FGF (+)) group exhibited higher levels of CXCL6 compared to the other groups in the presence of 1% FBS (Supplementary Fig. 3).

CXCL6 was found to be expressed in the regenerated rat meniscus.

To investigate the involvement of CXCL6 in meniscus regeneration, immunostaining was conducted on the regenerated meniscus. The expression of CXCL6 was observed in the regenerated meniscus, confirming their presence not only in implanted human cells but also in host rat cells. Furthermore, the proportion of CXCL6-positive cells was higher in the FGF (+) group compared to the control group (Supplementary Fig. 4).

Discussion

To the best of our knowledge, the current study first evaluated the regeneration effects and detailed mechanism of SMSCs cultured with bFGF.

SMSCs are a promising cell source for meniscus regeneration, and regenerated meniscus cells were mainly derived from the surrounding synovial tissue [27,31]. A previous study has shown that the transplantation of SMSC aggregates can promote meniscus regeneration and prevent cartilage degeneration[9]. Further, intra-articular-injected SMSCs contributed to meniscal repair and loss of cartilage degeneration[32] in animal models. The therapeutic potential of SMSCs in meniscus regeneration is discussed from various aspects, which include direct differentiation of different cell lineages, secretion of growth factor, cell–cell interactions, and release of extracellular vesicle[10,33–39]. However, SMSCs have limitations. That is, they have limited cell proliferation and non-uniformity of cartilage regeneration[12]. Therefore, various methods that can enhance the regenerative

function of SMSCs have been investigated. Some studies have reported that growth factors can promote the proliferation and chondrogenic differentiation of SMSCs[13,14].

Recently, bFGF has attracted attention in cartilage regeneration research in vitro[13,14]. However, to date, there are no reports on the use of SMSCs cultured with bFGF for meniscus regeneration in vivo. Immunohistochemistry results revealed that meniscus regeneration by SMSC transplantation might be correlated to indirect factors, such as humoral factors and chemokines. The conditioned media of MSC include a wide variety of secreted growth factors, chemokines, and hormones with immunomodulatory, angiogenic, anti-apoptotic, and extracellular vesicle (EV)-mediated functions[40]. RNA sequencing revealed that chemokines that bind to CXCR2 were mainly upregulated by bFGF in SMSCs. CXCR2 is the primary G protein-coupled receptor of ELR-CXC chemokines. Further, it is expressed in different cell types, such as neutrophils, monocytes, eosinophils, and endothelial[41], chondrocyte[42], and synovial[43] cells. The binding of various chemokines, such as CXCLs, to CXCR2 activates multiple G protein-coupled receptor signaling cascades, including the ERK, PI3K/Akt, MAPK, and STAT3 pathways. These pathways promote cell migration, proliferation, and cytokine and chemokine production and form a positive loop to enhance the function of CXCR2[44–47].

According to the migration assay of SMSCs, CXCL6 is the most important chemokine in SMSC migration. CXCL6 is a well-known chemoattractant for neutrophils[48], which binds to CXCR1 and CXCR2. CXCR2 has a higher affinity than CXCR1 for chemokines, and it plays a unique role in cell chemotaxis[49]. CXCL6 is contained within the CM from MSCs[50]. Furthermore, recent studies have shown that EVs from SMSCs upregulated the gene expression of CXCL5/6 in chondrocyte cells and promoted the regeneration of meniscus via CXCR2 signaling[37]. Moreover, they activate

CXCL6-CXCR2 signaling in chondrocytes, leading to the maintenance of cartilage homeostasis[42]. These results are in accordance with our findings. However, the aforementioned report requires weekly intra-articular injection of EVs[37], which may need additional effort or cost in refining EVs compared with our method. Therefore, CXCL6 can be an essential factor for meniscus and cartilage regeneration.

The limitations of this study include the following: First, human SMSCs were used. Thus, an in vivo xenograft model using immunodeficient rats must be adopted. Second, we examined a small animal model over a brief duration. Hence, a large animal model with a longer duration should be utilized. Third, knockout or transgenic animals were not used in the evaluation of genetic effects. Fourth, insufficient investigation has been conducted regarding the duration of the knee immobilization and the impairments caused immobilization.

The current study summarized the proposed mechanism of meniscus regeneration (Fig. 6). The transplantation of SMSC pellets cultured with bFGF accelerated the secretion of CXCL6, which binds to CXCR2 and activates the downstream of the ERK and Akt pathways. Thus, cell migration from surrounding synovial tissue, cell proliferation, and chondrogenic differentiation may be promoted, thereby improving meniscus regeneration. The transplantation of SMSCs cultured with bFGF can be a simple and effective treatment option for promoting meniscus regeneration.

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CONTRIBUTIONS OF AUTHORS

AG and KE take responsibility for the integrity of the work as a whole, from inception to the completed manuscript.

Conception and design: AG, YE, MH, SY, GO, and KE.

Analysis and interpretation of the data, statistical expertise:

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AG, YE, MH, SY, GO, AM, KT, TM, YF, TK, MT, TI, TT, TK, NO, SOyama, SOkada, KN, and KE.

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COMPETING INTEREST STATEMENT

No conflicts of interest were declared.

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FIGURE LEGENDS

Figure 1

The transplantation of synovial mesenchymal stem cells (SMSC) pellet promoted meniscus regeneration and mitigated cartilage degeneration. (A) Macroscopic findings of the meniscus at 4 and 8 weeks. Control (left), fibroblast growth factor (FGF (−)) (center), and FGF (+) (right). The yellow dotted line indicates the regenerated meniscus, with a scale bar of 1 mm. (B) The regenerative meniscus area at 4 and 8 weeks (n = 7–8 for each group). (C) Macroscopic findings of the tibial plateau 4 and 8 weeks after surgery. Control (left), FGF (−) (center), and FGF (+) (right), with a scale bar of 600 μm. (D) Osteoarthritis Research Society International scores of the cartilage (n = 7–8 for each group). (E) A schematic representation of the meniscus indentation test. (F) The tangent modulus of the regenerated meniscus (n = 8 per each group). Data were presented as means with 95% confidence intervals. A P value according to Kruskal-Wallis test.

Figure 2

Histological evaluation of regenerative meniscus in rats. (A) Representative sections of the normal and regenerated meniscus at 4 and 8 weeks. Control (left), fibroblast growth factor (FGF (−)) (center), FGF (+) (right). (B, C) Modified Pauli's score of the regenerative meniscus at 4 and 8 weeks (n = 7−8 per each group). (D) Representative immunohistochemical staining for type II collagen and human vimentin at 8 weeks. (E) Overlap of type II collagen with human vimentin-stained area (n = 7−8 for each group). Data were presented as means with 95% confidence intervals. A P value according to Kruskal-Wallis test.

Figure 3

Effects of basic fibroblast growth factor (bFGF) on gene expression in human synovial mesenchymal stem cells (SMSCs). (A) The most variable genes were calculated and clustered into four groups using iDEP96. (B) The enriched pathways for cluster B were identified. (C) Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed the enriched genes, which were indicated in red. (D) Gene expression of chemokines that bind to CXCR2 was assessed in SMSC culture using growth medium with bFGF (FGF (+)) and without bFGF (FGF (−)) (n = 3). (E) Immunohistochemical analysis of CXCR2 was performed on rat knee samples. A higher magnification focused on the synovium (with a scale bar of 200 μm) and the meniscus (with a scale bar of 200 μm).

Figure 4

Effects of conditioned medium (CM) and synovial mesenchymal stem cell (SMSC) pellet on SMSC migration. (A) Schematic representation of the migration assay of SMSCs. (B) Representative magnification of SMSC migration in response to CM, with a scale bar of 100 μm. (C) Number of migrated SMSCs in response to CM and the effects of SB25610 and PD98059 (n = 3). (D) Fold change in the gene expression of each CXCL versus that of CXCL5 in SMSCs (n = 4). (E) Effect of each CXCL inhibitor on SMSC migration was evaluated (n = 3). (F) The relative gene expression of CXCL6 and the effects of SB265610 and PD98059 were quantified (n = 4). (G) Schematic representation of the SMSC migration assay in meniscus explant culture. (H) Representative sections of SMSC migration into human meniscus tears, with control (left), FGF (−) (center), and

FGF (+) (right), with a scale bar of 50 μm. (I) Number of migrated SMSCs per 0.1 mm^2 (n = 5 for each group). Data were presented as means with 95% confidence intervals. (C), (D), (E), (F) A P value according to one-way analysis of variance using the Tukey post-hoc test. (I) A P value according to Kruskal-Wallis test.

Figure 5

Effects of conditioned medium (CM) on the proliferation and chondrogenic differentiation of synovial mesenchymal stem cell (SMSCs). (A) The cell proliferation assay was performed with CM, with absorbance being proportional to the cell number. (B) The effects of SB265610, PD98059, and MAB333 on cell proliferation on day 5 (n = 5). (C) Effects of CM and PD98059 on the phosphorylation of ERK and Akt in SMSCs. (D) Representative sections of human SMSC pellets at 2 weeks were examined using different chondrogenic CM. (E) Effects of CM and SB265610, PD98059, and MAB333 on the Safranin O-stained area of SMSC pellets (n = 3). Data were presented as means with 95% confidence intervals. ¶ control vs FGF (+) =0.096. A P value according to one-way analysis of variance using the Tukey post-hoc test.

Figure 6

Graphical abstract of this study. Transplantation of synovial mesenchymal stem cell (SMSCs) in pellet form, cultured with bFGF, enhances the secretion of CXCLs that bind to CXCR2. This might lead to the phosphorylation of ERK and Akt in host SMSCs, thereby promoting cell migration, proliferation, and chondrogenic differentiation. These mechanisms may regenerate the meniscus, enhance its mechanical property, and prevent cartilage degeneration.

Supplementary Figure 1

Menisectomy and transplantation of the rat knee.

Details of the animal experiments conducted in this study.

Supplementary Figure 2

Histological analysis of human synovial mesenchymal stem cell (SMSCs) in pellet form

A representative magnification of the human SMSC pellet stained using CD44, CD73, and CD90 antibodies.

Supplementary Figure 3

Quantitative assessment of CXCL6 in the conditioned medium.

CXCL6 levels in the conditioned medium (0% FBS and 1% FBS) used in this study were measured using ELISA. Data were presented as means with 95% confidence intervals. A P value according to one-way analysis of variance using the Tukey post-hoc test.

Supplementary Figure 4

Histological evaluation of regenerative meniscus in rats (CXCL6).

Representative sections of the normal and regenerated meniscus at 8 weeks. Control (left), without bFGF (FGF (−)) (center), and with bFGF (FGF (+)) (right). CXCL6 positive cell ratio was evaluated. A P value according to Kruskal-Wallis test.

Figure 1

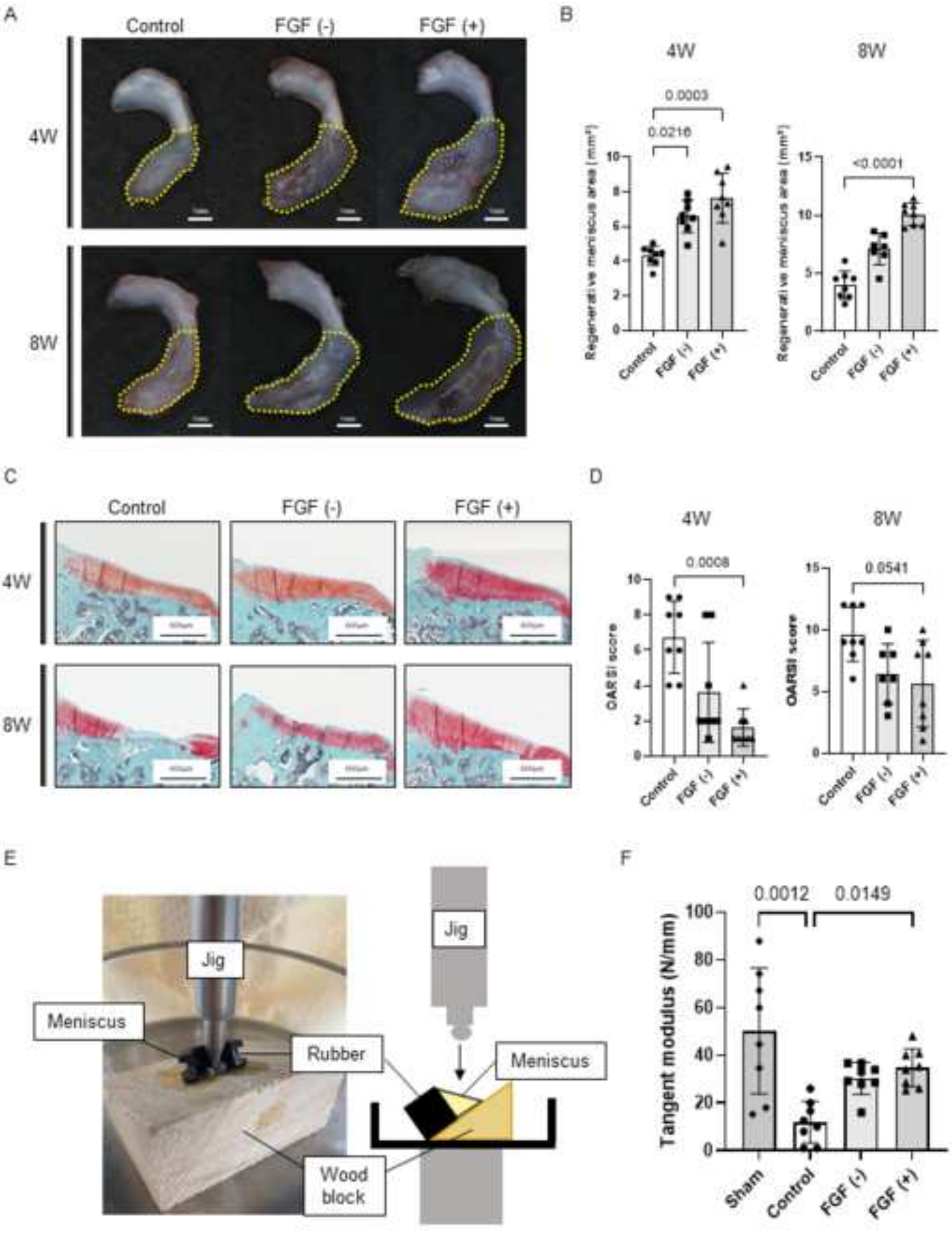
A
Control
FGF (-)
FGF (+)
4W
8W
B
4W
8W
0.0003
0.0216
<0.0001
Regenerative meniscus area (mm²)
Control
FGF (-)
FGF (+)
C
Control
FGF (-)
FGF (+)
4W
8W
D
4W
8W
0.0008
0.0541
OARSI score
Control
FGF (-)
FGF (+)
E
Jig
Meniscus
Rubber
Wood block
F
0.0012
0.0149
Tangent modulus (N/mm)
Sham
Control
FGF (-)
FGF (+)

Figure 2

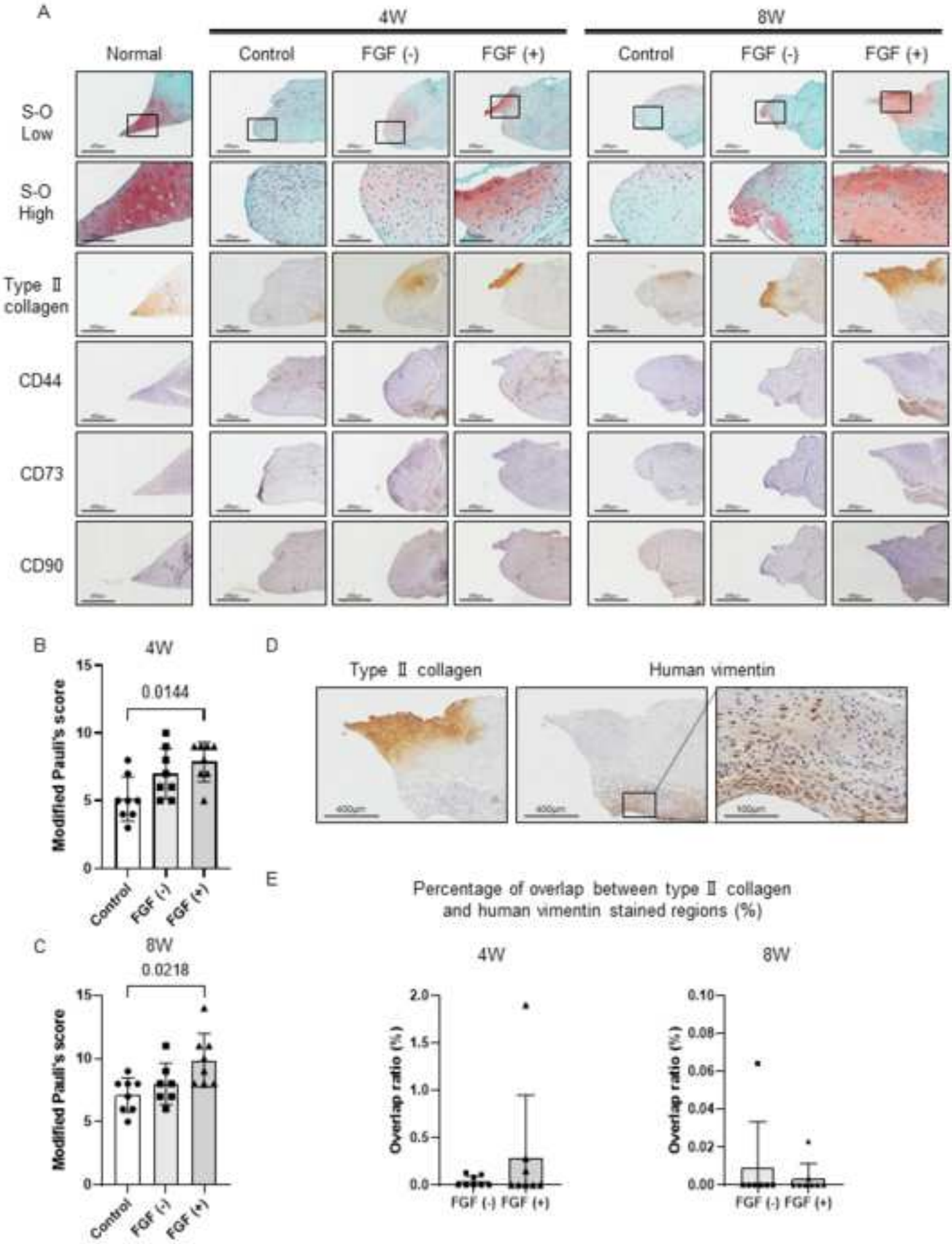
A
4W
8W
Normal
Control
FGF (-)
FGF (+)
Control
FGF (-)
FGF (+)
S-O Low
S-O High
Type Ⅱ collagen
CD44
CD73
CD90
B
4W
0.0144
Modified Pauli's score
Control
FGF (-)
FGF (+)
C
8W
0.0218
Modified Pauli's score
Control
FGF (-)
FGF (+)
D
Type Ⅱ collagen
Human vimentin
E
Percentage of overlap between type Ⅱ collagen and human vimentin stained regions (%)
4W
8W
Overlap ratio (%)
FGF (-) FGF (+)
Overlap ratio (%)
FGF (-) FGF (+)

Figure 3

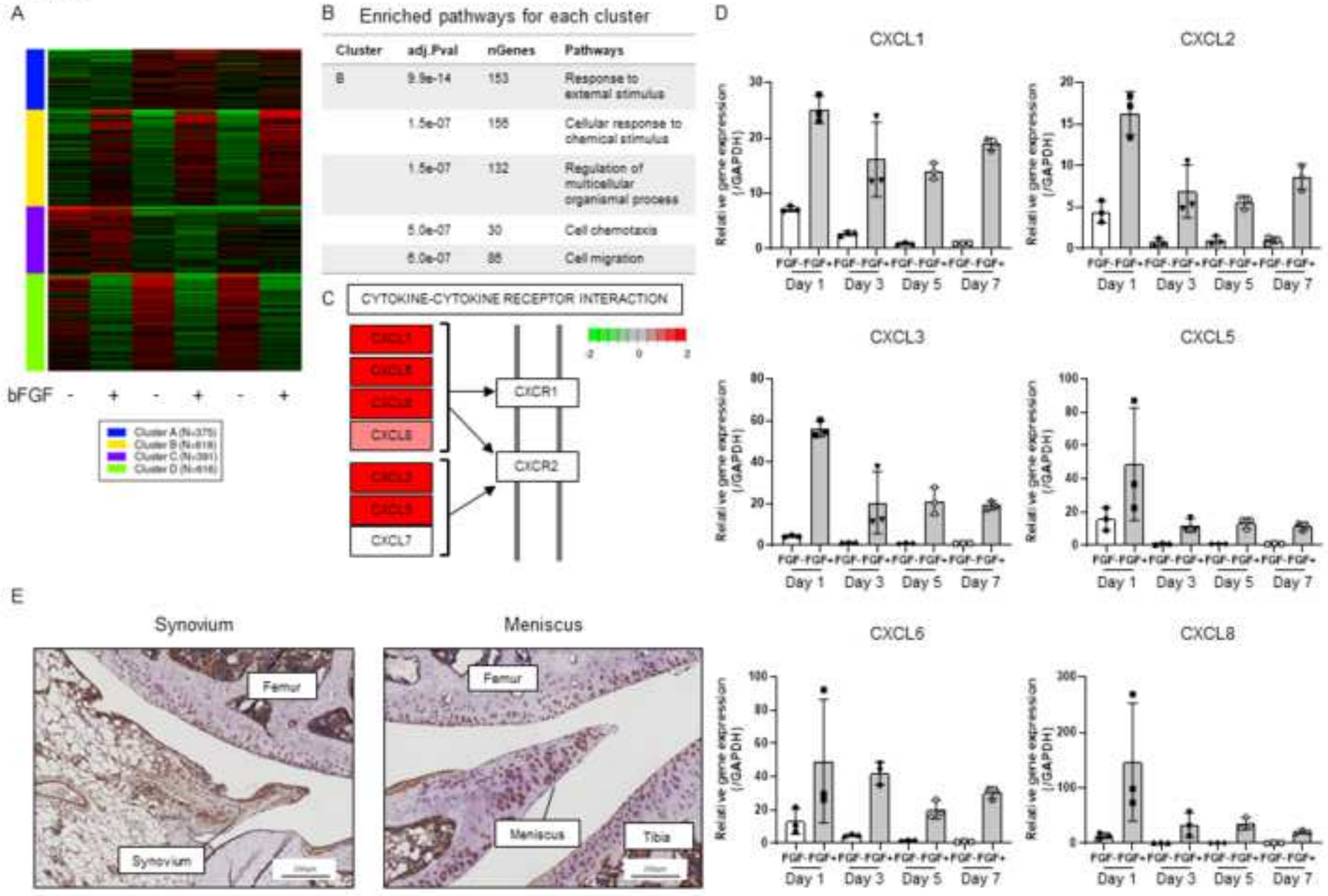
A
bFGF - + - + - +
Cluster A (N=375)
Cluster B (N=818)
Cluster C (N=391)
Cluster D (N=616)
B Enriched pathways for each cluster
Cluster | adj.Pval | nGenes | Pathways
B | 9.9e-14 | 153 | Response to external stimulus
1.5e-07 | 155 | Cellular response to chemical stimulus
1.5e-07 | 132 | Regulation of multicellular organismal process
5.0e-07 | 30 | Cell chemotaxis
6.0e-07 | 86 | Cell migration
C
CYTOKINE-CYTOKINE RECEPTOR INTERACTION
CXCL1
CXCL2
CXCL3
CXCL5
CXCL6
CXCL7
CXCL8
CXCR1
CXCR2
D
CXCL1
CXCL2
CXCL3
CXCL5
CXCL6
CXCL8
Relative gene expression (/GAPDH)
FGF- FGF+ FGF- FGF+ FGF- FGF+ FGF- FGF+
Day 1 Day 3 Day 5 Day 7
E
Synovium
Meniscus
Femur
Synovium
Femur
Meniscus
Tibia

Figure 4

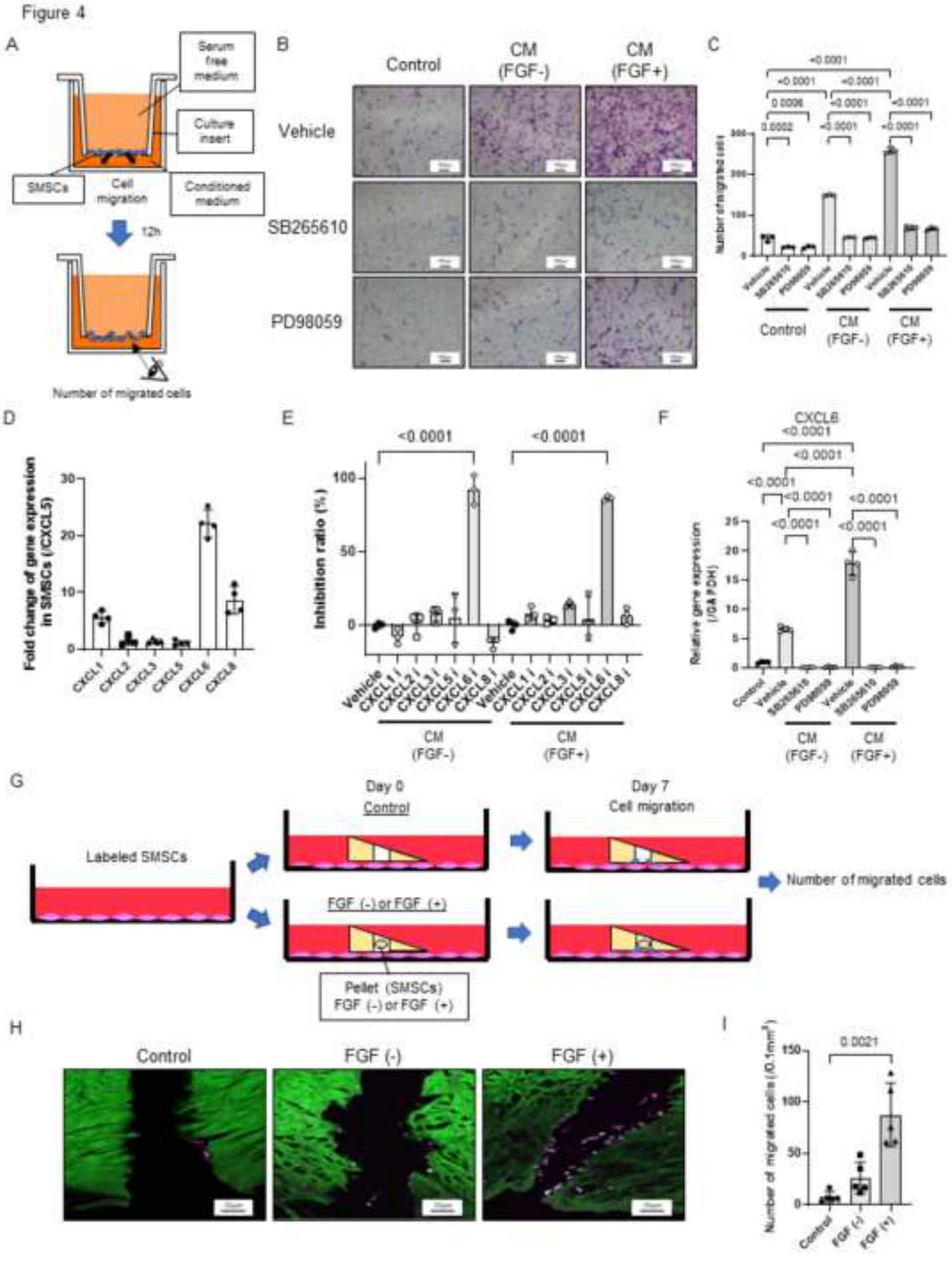
A
Serum free medium
Culture insert
SMSCs
Cell migration
Conditioned medium
12h
Number of migrated cells
B
Control
CM (FGF-)
CM (FGF+)
Vehicle
SB265610
PD98059
C
Control
CM (FGF-)
CM (FGF+)
D
Fold change of gene expression in SMSCs (/CXCL5)
CXCL1
CXCL2
CXCL3
CXCL5
CXCL6
CXCL8
E
Inhibition ratio (%)
<0.0001
<0.0001
CM (FGF-)
CM (FGF+)
F
CXCL8
Relative gene expression (/GAPDH)
CM (FGF-)
CM (FGF+)
G
Day 0
Control
Day 7
Cell migration
Labeled SMSCs
FGF (-) or FGF (+)
Pellet (SMSCs) FGF (-) or FGF (+)
Number of migrated cells
H
Control
FGF (-)
FGF (+)
I
Number of migrated cells (/0.1mm²)
0.0021
Control
FGF (-)
FGF (+)

Figure 5

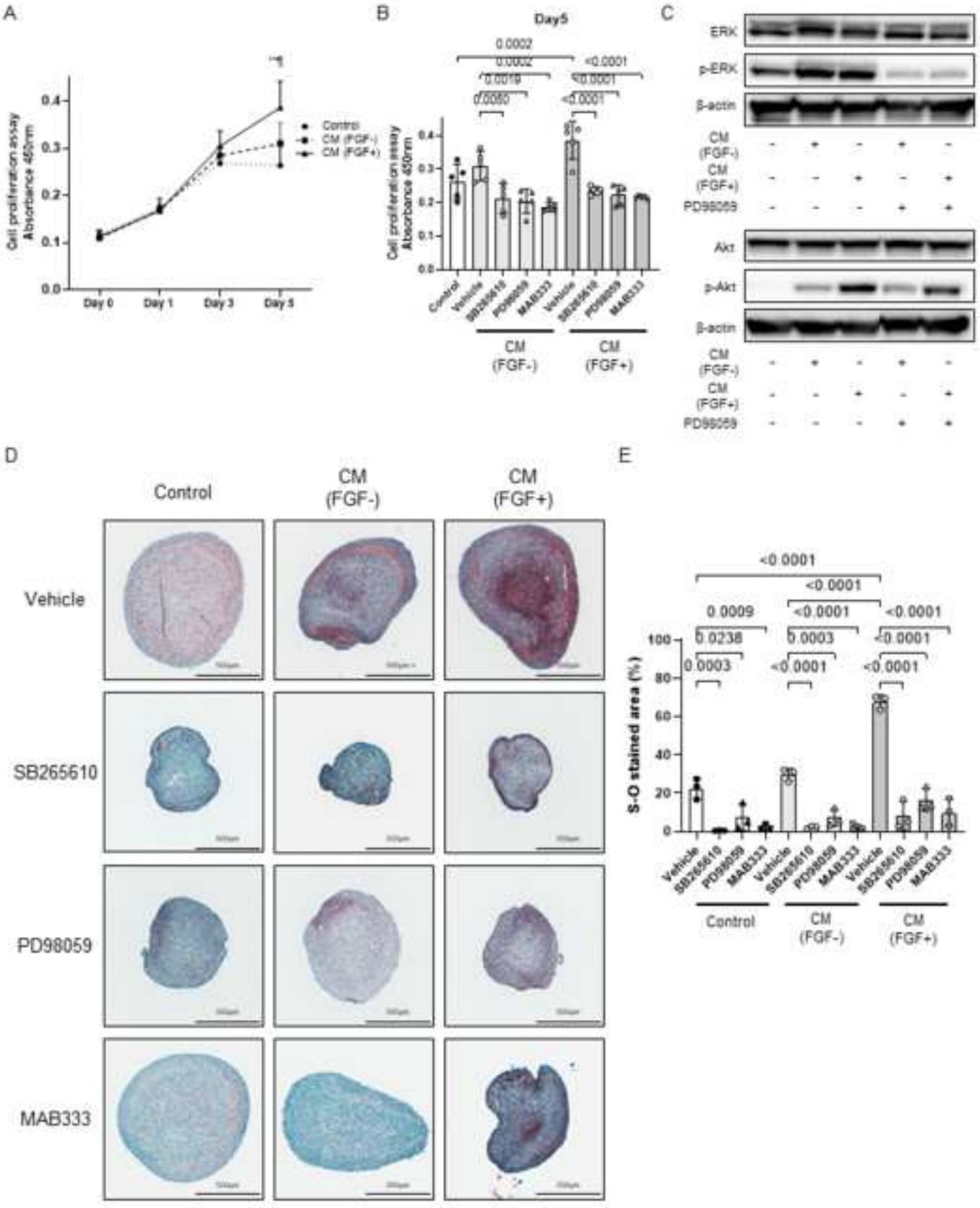
Figure 5
A
Cell proliferation assay Absorbance 450nm
Control
CM (FGF-)
CM (FGF+)
Day 0
Day 1
Day 3
Day 5
B
Day5
Cell proliferation assay Absorbance 450nm
0.0002
0.0002
<0.0001
0.0019
<0.0001
0.0050
<0.0001
Control
Vehicle
SB265610
PD98059
MAB333
CM (FGF-)
CM (FGF+)
C
ERK
p-ERK
β-actin
CM (FGF-)
CM (FGF+)
PD98059
Akt
p-Akt
β-actin
D
Control
CM (FGF-)
CM (FGF+)
Vehicle
SB265610
PD98059
MAB333
E
S-O stained area (%)
<0.0001
<0.0001
0.0009
<0.0001
<0.0001
0.0238
0.0003
<0.0001
0.0003
<0.0001
<0.0001
Control
CM (FGF-)
CM (FGF+)

Figure 6

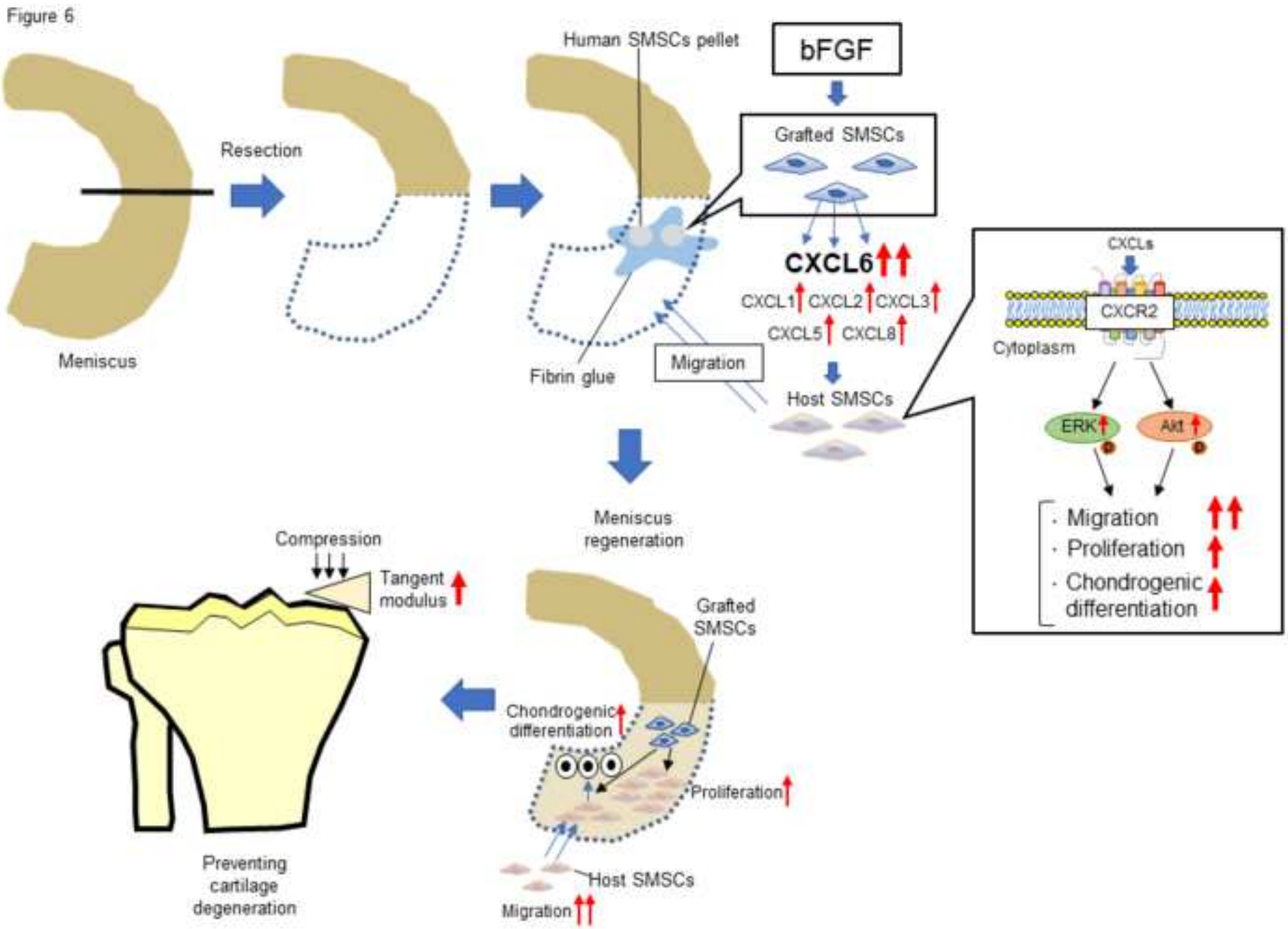
Figure 6
Meniscus
Resection
Human SMSCs pellet
Fibrin glue
bFGF
Grafted SMSCs
CXCL6
CXCL1 CXCL2 CXCL3
CXCL5 CXCL8
Migration
Host SMSCs
CXCLs
CXCR2
Cytoplasm
ERK
Akt
Migration
Proliferation
Chondrogenic differentiation
Meniscus regeneration
Compression
Tangent modulus
Preventing cartilage degeneration
Grafted SMSCs
Chondrogenic differentiation
Proliferation
Host SMSCs
Migration

Supplemental method and table

Supplemental Figure 1

Supplemental Figure 2

Supplemental Figure 3

Supplemental Figure 4