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Species-Specific Biological Effects of FGF-2 in Articular Cartilage: Implication for Distinct Roles Within the FGF Receptor Family

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ABSTRACT

Existing literature demonstrates that fibroblast growth factor-2 (FGF-2) exerts opposing, contradictory biological effects on cartilage homeostasis in different species. In human articular cartilage, FGF-2 plays a catabolic and anti-anabolic role in cartilage homeostasis, driving homeostasis toward degeneration and osteoarthritis (OA). In murine joints, however, FGF-2 has been identified as an anabolic mediator as ablation of the FGF-2 gene demonstrated increased susceptibility to OA. There have been no previous studies specifically addressing species-specific differences in FGF-2-mediated biological effects. In this study, we provide a mechanistic understanding by which FGF-2 exerts contradictory biological effects in human versus murine tissues. Using human articular cartilage (ex vivo) and a medial meniscal destabilization (DMM) animal model (in vivo), species-specific expression patterns of FGFR receptors (FGFRs) are elucidated between human and murine articular cartilage. In the murine OA model followed by intra-articular injection of FGF-2, we further correlate FGFR profiles to changes in behavioral pain perception, proteoglycan content in articular cartilage, and production of inflammatory (CD11b) and angiogenic (VEGF) mediators in synovium lining cells. Our results suggest that the fundamental differences in cellular responses between human and murine tissues may be secondary to distinctive expression patterns of FGFRs that eventually

Abbreviations used: OA, osteoarthritis; FGF-2, fibroblast growth factor 2; FGFR, FGF receptor; MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with a thrombospondin type 1 motif; VEGF, vascular endothelial growth factor; BMP-7, bone morphogenetic protein-7 (otherwise known as osteogenic protein-1).

Xin Li and Michael Ellman contributed equally to the study.

The authors declare that they have no competing interests.

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determine biological outcomes in the presence of FGF-2. The complex interplay of FGFRs and the downstream signaling cascades induced by FGF-2 in human cartilage should add caution to the use of this particular growth factor for biological therapy in the future. J. Cell. Biochem. 113: 2532–2542, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: FIBROBLAST GROWTH FACTOR-2; ARTICULAR CARTILAGE; OSTEOARTHRITIS; FGFR

Osteoarthritis (OA) is the leading cause of disability among the elderly population and affects millions of people worldwide [Buckwalter et al., 2004]. Although the pathogenesis of OA is not yet fully understood, it is a progressive degenerative joint disease characterized by the breakdown of cartilage extracellular matrix (ECM). Under normal conditions, articular chondrocytes maintain a dynamic equilibrium between synthesis and degradation of ECM components, including type II collagen fibrils that form a network surrounding large, hydrated aggregates of the proteoglycan (PG) aggrecan. This unique structure allows healthy cartilage to function as a shock absorber and withstand compressive loads [Nakata et al., 1993]. In OA, however, there is a disruption of matrix equilibrium leading to progressive loss of cartilage tissue and clonal expansion of cells in the depleted regions. Chondrocyte metabolism is unbalanced due to excessive production of catabolic factors, including matrix metalloproteinases (MMPs), aggrecanases (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, aka ADAMTS), and other pro-inflammatory cytokines and growth factors released by chondrocytes that aid in the destruction of PGs in the ECM [Martel-Pelletier et al., 2001; Im et al., 2007ab; Muddasani et al., 2007].

Fibroblast growth factor-2 (FGF-2, also known as basic FGF) is a member of a large FGF family of structurally related proteins that modulates the growth, differentiation, migration, and survival of a wide variety of cell types [Bikfalvi et al., 1997; Ellman et al., 2008]. The mitogenic capacity of FGF-2 in growth plate cartilage [O'Keefe et al., 1994; Rosselot et al., 1994] and adult articular cartilage [Osborn et al., 1989] is well reported in the literature. However, studies on FGF-2 from a variety of species have yielded inconsistent and often contradictory results with regards to its anabolic and/or catabolic role in articular cartilage homeostasis [Ellman et al., 2008].

Several authors have suggested a chondroprotective role of FGF-2 in cartilage metabolism and suggested its use for cartilage regeneration and repair [Cuevas et al., 1988; Cucchiarini et al., 2005; Hiraide et al., 2005; Inoue et al., 2006; Kaul et al., 2006; Deng et al., 2007; Schmal et al., 2007; Stewart et al., 2007b; Sawaji et al., 2008; Chia et al., 2009]. For example, FGF-2 is released after injury or mechanical compression of the ECM and has been found to mediate beneficial, protective effects in murine joints [Vincent et al., 2002; Chia et al., 2009]. Mice lacking FGF-2 (FGF-2 null mice) developed accelerated OA (with age and following surgical joint destabilization), and this was reversed by delivery of subcutaneous recombinant FGF-2 [Chia et al., 2009]. In the same model, FGF-2 null mice demonstrated a super-induction of ADAMTS-5 mRNA, suggesting that FGF-2 normally suppresses aggrecanase activity, consistent with previous findings [Sawaji et al., 2008]. Others have studied the use of FGF-2 in scaffold models of cartilage regeneration with promising results in a variety of species [Inoue et al., 2006; Deng et al., 2007; Stewart et al., 2007b].

In contrast, our group and others have demonstrated a catabolic and/or anti-anabolic role of FGF-2 in articular cartilage homeostasis [Loeser et al., 2005; Im et al., 2007b, 2008; Muddasani et al., 2007; Ellman et al., 2008; Yan et al., 2011]. In human adult articular cartilage, FGF-2 is pathologically associated with joint destruction via upregulation of MMPs and aggrecanases, as well as stimulation of reactive oxygen species such as nitric oxide and superoxide anion, which may accelerate cartilage degradation [Wang et al., 2004; Ellman et al., 2008; Im et al., 2008]. In both articular chondrocytes and meniscal chondrocytes, FGF-2 alters the ratio between type II and type I collagen, potentially inducing the formation of fibrocartilage, a defective substitute for healthy hyaline cartilage [Schmal et al., 2007; Stewart et al., 2007b]. Further, in our laboratory, FGF-2 was found to inhibit the synergistic anabolic effects of IGF-1 and BMP-7 via protein kinase C δ (PKCδ)-dependent activation of multiple MAP kinases (ERK1/2, p38 and JNK) in human adult articular chondrocytes [Loeser et al., 2005; Im et al., 2007b]. FGF-2 also activates the NF κB pathway, which converges with the MAP kinase pathway to activate the transcription factor Elk-1, stimulating MMP-13 transcription [Muddasani et al., 2007].

Recently, Yan et al. [2011] proposed that the conflicting roles of FGF-2 may be dependent upon differences in the balance of FGF receptors (FGFRs) within the tissue of interest. Extracellular signals from FGF-2 are transduced into the cell through members of four structurally related, high affinity FGF receptors, FGFR1–4 [Jaye et al., 1992; Johnson and Williams, 1993; Mohammadi et al., 1997; Ornitz, 2000]. Previously, we reported that human knee joint articular cartilage predominantly expresses FGFR1 and FGFR3 with negligible or undetectable levels of FGFR2 and FGFR4, as assessed by flow cytometry, western blotting, immunoprecipitation, and real-time PCR experiments [Yan et al., 2011]. Of these two receptors, FGFR1 has been shown to be the FGFR responsible for many of the negative biological consequences after stimulation with FGF-2, including upregulation of MMP-13 and inhibition of PG accumulation [Im et al., 2007b]. Indeed, in degenerative cartilage, there is an upregulation of FGFR1 compared to FGFR3 [Im et al., 2007b], suggesting a predominant role of the FGF-2-FGFR1 pathway in cartilage degeneration. FGFR3, on the other hand, plays a dominant anabolic role after ligand binding by FGF-18, demonstrated by potent stimulation of PG synthesis [Davidson et al., 2005]. These findings reveal important opposing effects of FGFR1 and FGFR3 in human articular cartilage.

Interestingly, the effects of FGF-2 on articular cartilage may be species-dependent. For example, in mice cartilage, FGF-2 induces

overall anabolic effects [Chia et al., 2009], whereas its role in human articular cartilage appears to be catabolic and anti-anabolic [Im et al., 2007b, 2008; Muddasani et al., 2007; Ellman et al., 2008; Yan et al., 2011]. To date, however, the reasons for species-specific differences of FGF-2 remain unknown. Based on Yan's recent work [Yan et al., 2011], it is plausible that species-specific differences (i.e., mice vs. human cartilage) after stimulation with FGF-2 may potentially be accounted for by varying expression levels of the FGFRs in each species, which regulate the end effects of FGF-2 stimulation. To our knowledge, studies evaluating species-specific FGFR profiles have yet to be published.

In the current study, we sought to provide a better understanding of why FGF-2 exerts contradictory biological effects between human and murine tissues in the laboratory. Using ex vivo and in vivo techniques, we examined the expression patterns of FGFR1–4 in human compared to murine articular cartilage in an effort to clarify the complex interplay of FGF-2-mediated effects in ECM homeostasis. The findings were correlated with symptomatology in mice, helping to uncover whether the effects of FGF-2 mediate pain perception in vivo. The findings of this study add to the growing controversy over the potential clinical use of FGF-2 as a biological treatment for OA.

MATERIALS AND METHODS

ACQUISITION OF ADULT HUMAN ARTICULAR CARTILAGE TISSUES

Adult human knee cartilage tissues were obtained within 24 h of death from donors (age 40–65, mean 52) via the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL), with approval by the local ethics committee and informed consent obtained from the families (ORA#:L03090306). Surgically removed cartilage from OA patients (age ranging from 40 to 65) were obtained from the Orthopedic Tissue and Implant Repository Study with consent from the patients. Prior to dissection, each specimen was graded for overall degenerative changes based on the modified 5-point scale of Collins [Muehleman et al., 1997]. Human tissues were handled strictly according to the guidelines of the Human Investigation Committee of Rush University Medical Center.

MATERIALS

Human recombinant FGF-2 was purchased from National Cancer Institute (Bethesda, MD). Human recombinant FGF18 and BMP-7 were purchased from PeproTech (Rocky Hill, NJ). Antibodies against human Flg (FGFR1), Bek (FGFR2), FGFR3 and FGFR4 were purchased from Santa Cruz (Santa Cruz, CA). Secondary antibodies against rabbit IgG and non-enzymatic cell dissociation buffer were purchased from Life Technologies (Carlsbad, CA). Antibodies were purchased for immunohistochemistry, including CD11b, (Santa Cruz), FGFR1,2,3,4 (Santa Cruz), and VEGF (Abcam, MA).

FLOW CYTOMETRY ANALYSIS

Human articular chondrocytes isolated from adult knee joints (Collins grade 1–3, average age 52) were cultured in monolayer and maintained in complete media for 1–2 days before initiation of experiments. Cells were detached from the substrate by the addition of non-enzymatic dissociation buffer (Life Technologies), blocked by goat serum, incubated with primary antibodies against each FGFR isoform (Santa Cruz), and incubated with a FITC-tagged secondary antibody (Life Technologies). Isotype IgG was used in parallel to evaluate background fluorescence. Samples were measured using a FACS Canto II flow cytometer, and data were analyzed by FACS DIVA software (BD Flow Cytometry Systems, San Jose, CA). Fluorescence histogram plots were used to compare mean fluorescence intensities of different samples.

SURGICAL INDUCTION OF KNEE OA AND GROWTH FACTOR INJECTION

OA was induced using a medial meniscal destabilization (DMM) model, as previously described [Inglis et al., 2008], using >6-month-old adult male mice (IACUC approval #11-054). Briefly, mice were anesthetized with 1.5% isoflurane in oxygen and the left knee was shaven and prepared for aseptic surgery. A medial para-patellar arthrotomy was performed to expose the anterior medial menisco-tibial ligament, which was elevated with a microprobe and severed using curved dissecting forceps. Complete disruption of the ligament was confirmed visually by the ability to manually displace the medial meniscus with fine forceps. The patella was repositioned, and the skin incision closed with 7-0 polypropylene sutures. Sham surgery was performed on the left knee of a separate group of mice and consisted of skin incision and medial capsulotomy only, followed by skin closure as described above.

Two weeks after DMM surgery, mice were briefly anesthetized for intra-articular microinjection with a 30-G needle of FGF-2 (2.5 μg in 5 μl per knee joint) or saline (vehicle, 5 μl), mixed with fibrinogen solution (1.5–3 μg/μl), followed by injection of 1 μl of thrombin (0.2 unit) to convert the fibrinogen into fibrin (enzymatic reaction takes <10–20 s). Weekly behavioral mechanical pain assessments were performed for development of OA-induced pain using the Chaplan method [Chaplan et al., 1994]. The animals were sacrificed 5 weeks following surgery, which represents the early-phase of OA (3 weeks after FGF-2/fibrinogen/thrombin injection). A schematic diagram of the experimental process is illustrated in Figure 1.

BEHAVIORAL PAIN TESTS

Symptomatic OA was assessed by weekly behavioral pain tests using von Frey filaments (mechanical allodynia), as described previously [Im et al., 2010]. Briefly, after mice were allowed to acclimate for 30 min on a wire mesh grid, a calibrated set of von Frey filaments (0.02–5.5 g range, Stoelting) were applied below the plantar hind paw to determine the 50% force withdrawal threshold using an iterative method. The filament was applied to the skin with enough pressure to buckle, and was maintained for up to 6 s. A brisk lifting of the foot was recorded as a positive response. If no response was observed, the filament with the next highest force was applied, while the filament with the next lowest force was applied upon a positive response.

HISTOLOGICAL, IMMUNOHISTOCHEMICAL ASSESSMENTS

Following sacrifice, each knee was dissected aseptically and fixed in 4% paraformaldehyde, with decalcification in EDTA, which was changed every 5 days. The decalcified knee was then transected in the mid-sagittal plane and paraffin-embedded. Serial knee sections

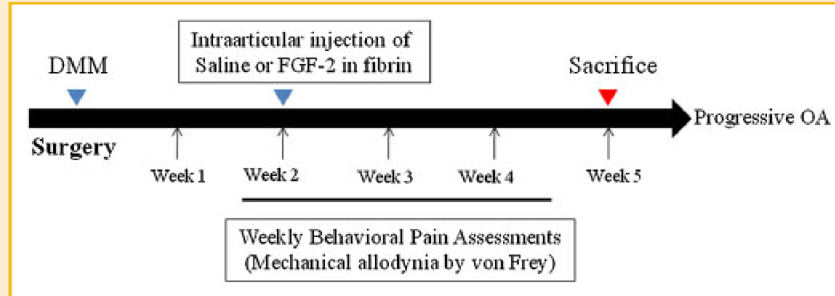

Fig. 1. Schematic diagram of experimental time schedule.

of exact 5-μm thickness from the middle part of the knee were obtained to prepare slides. Full-thickness of human articular cartilages was paraffin-embedded followed by serial sections (5-μm thickness) for histological analyses.

Safranin-Orange (Safranin-O) staining was performed using full thickness of cartilage slices prepared from human knee joint tissues (Collins grads 1–3, average age 52) and a 4-mm diameter puncher as previously described [Muddasani et al., 2007]. Human articular cartilage explants were cultured in 1 ml of Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% fetal bovine serum. Following a 2-day recovery period, the cartilage explants were treated with or without FGF-2 (10, 50, and 100 ng/ml) under serum-free conditions (mini-ITS). Following 14 days of incubation, the explants were fixed with 4% paraformaldehyde overnight, embedded in paraffin, and 8 μM sections were prepared. The paraffin sections were deparaffinized and stained with Safranin-O staining to assess matrix PG content. Incubation of adult human articular cartilage explants with anabolic growth factors such as FGF-18 or BMP-7 (each 100 ng/ml) was conducted in parallel for comparison purposes. An unblinded investigator grouped the slides and randomly numbered them; these groups were then graded by two different blinded investigators (H-JI and XL). A relative grade was assigned from 0 to 4, where 0 signifies no staining (PG loss) and 4 signifies the most intense stain (normal cartilage) based on Safranin-O. Two independent examinations were performed, and the repeatability of grading on the two occasions was determined using Cohen kappa statistics.

For immunostaining, sections were incubated with 20 μg/ml proteinase K for 30 min at 37°C for antigen retrieval. Sections were then incubated for 30 min in a blocking solution containing 0.03% H_2O_2 in PBS, followed by a 30-min incubation in a blocking solution containing 0.1% horse serum/1% bovine serum albumin (BSA) in PBS. Then, the sections were incubated overnight at 4°C in a solution of the primary antibody diluted in PBS/0.1% BSA. The sections were thoroughly washed three times for 10 min each in PBS, followed by incubation for 1 h in horse anti-rabbit/mouse biotinylated universal secondary antibody (R.T.U. Vectastain Kit, Vector Laboratories). The sections were incubated with the VECTASTAIN R.T.U. Elite ABC reagent (Vector Laboratories), followed by emersion for 4 min in a solution of the substrate diaminobenzidine (DAB) (Vector Laboratories), and carefully monitored for the development of the opaque reaction product.

STATISTICAL ANALYSIS

Statistical significance was determined by Student's *t*-test, or one-way repeated measures ANOVA followed by Sidak post hoc testing using the SPSS 17 program. *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

FGF-2 MEDIATES PROTEOGLYCAN LOSS IN A DOSE-DEPENDENT MANNER IN HUMAN KNEE JOINT ARTICULAR CARTILAGE

Given that human knee joint articular cartilage predominantly expresses FGFR1 and FGFR3 with negligible or undetectable levels of FGFR2 and FGFR4 [Yan et al., 2011], and that FGFR1 is specifically responsible for FGF-2-mediated catabolic and anti-anabolic biological responses using in vitro techniques [Im et al., 2007b; Ellman et al., 2008; Yan et al., 2011], we first sought to confirm that FGF-2 indeed mediates PG loss in human adult articular cartilage using an ex vivo organ culture model. Full-thickness human knee joint articular cartilage samples of identical size (Collin's grade 1 or 3) were incubated in the presence of increasing doses of FGF-2 (10, 50, and 100 ng/ml) in order to reveal the presence or absence of a dose-dependent response on ECM degradation. Indeed, FGF-2 stimulation of human knee joint cartilage samples significantly induces PG depletion in a dose-dependent fashion (Fig. 2a–d). Treatments with the well-known cartilage anabolic growth factors BMP7 (100 ng/ml) and FGF-18 (100 ng/ml), which activates FGFR3 [Yan et al., 2011], were included in parallel for comparison purposes (Fig. 2e,f).

INTRA-ARTICULAR INJECTION WITH FGF-2 REVERSES PROGRESSIVE CARTILAGE DEGRADATION IN A MURINE OA MODEL IN VIVO

Previous and current studies using adult human articular cartilage samples in vitro and ex vivo demonstrate the catabolic, pathological role of FGF-2 in cartilage homeostasis [Loeser et al., 2005; Im et al., 2007b, 2008; Muddasani et al., 2007; Ellman et al., 2008; Yan et al., 2011]. However, the literature also suggests an anabolic role of FGF-2 in animal models such as murine cartilage in vitro [Chia et al., 2009; Vincent, 2011]. Therefore, we wished to compare our initial findings from human cartilage (Fig. 2) to a murine animal OA model in vivo. As described above, OA was generated in murine knee joints using a DMM model, which induces a slow-onset of arthritis similar

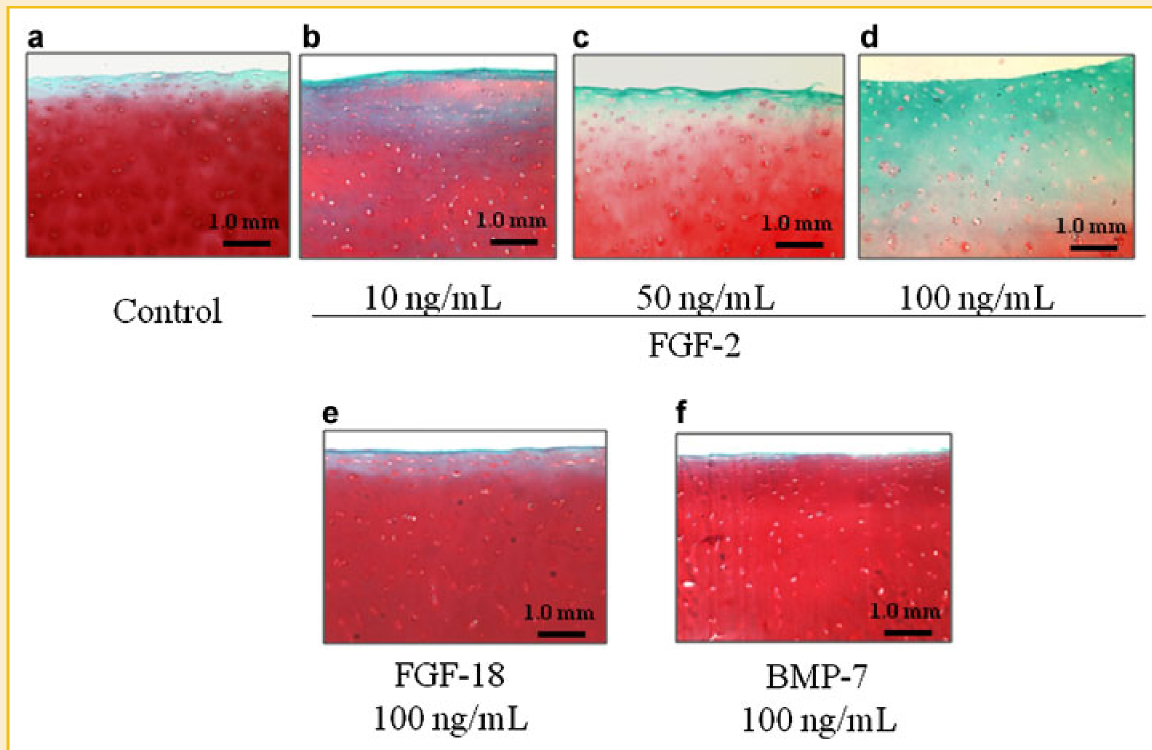

Fig. 2. Histological assessment of FGF-2 effects on adult human articular cartilage. Safranin-O staining was performed using full thickness cartilage explants prepared from human knee joint tissues. The explants were cultured in the presence or absence of varied concentrations of FGF-2 (10, 50, and 100 ng/ml) in mini-ITS. Following 14 days of incubation, the explants were fixed with 4% paraformaldehyde overnight and embedded in paraffin, and 8 μM sections were prepared for histological assessment using Safranin-O staining to assess matrix proteoglycan loss. Representative histological results from seven different donors (n = 7) are shown (Collins grade 1–3, average age 52 years old). Anabolic growth factors such as FGF-18 and BMP-7 (each 100 ng/ml concentration) are included in the explant culture experiments as a positive control.

to that seen in human OA. Our preliminary test results revealed that fibrinogen solution at a concentration of 1.5–3 μg/μl rapidly (within 10 s) forms a fibrin clot following co-injection with 1 μl thrombin (0.2 units) in vivo (data not shown). Therefore, at week 2 post-surgery, 5 μl of fibrinogen solution (1.5 μg/μl) containing FGF-2 (2.5 μg per knee joint) was injected into select murine knee joints, followed by injection of 1 μl thrombin to form a fibrin clot (Fig. 1). At 5 weeks post-surgery, which correlates with the previously defined time period for development of early-stage OA (34), animals were euthanized to harvest the knee joints for histological analyses. Results from our in vivo OA murine animal studies are quite striking. We found contradictory results compared to those obtained using human knee joint tissues. In the murine model, rather than accelerating cartilage degradation after initial injury, intra-articular injection with FGF-2 arrests and reverses the progression of OA, as reflected by restoration of PG content close to the sham control level (Fig. 3a–c).

EXPRESSION PATTERN AND REGULATION OF THE FGFR SUBTYPES ARE DISTINCTIVE IN A SPECIES-DEPENDENT MANNER

Given the above results, we next sought to understand why FGF-2 exerts opposing biological effects between human tissues ex vivo and murine tissues in vivo. Because the biological responses of FGF-2 are mediated through FGFRs, we first determined the basal expression patterns of FGFR family members (FGFR1–4) in human versus murine knee joint articular cartilage. Normal human knee joint articular chondrocytes (Collin's grade 0 or 1, average age 52.5 years, n = 4) were subjected to flow cytometric analyses using anti-FGFR1–4 antibodies. Similar to our previous findings [Yan et al., 2011], results consistently reveal that FGFR3 and FGFR1 are the two most prominent receptors at the cell surface of normal human articular chondrocytes (Fig. 4a). On the other hand, in OA knee joint cartilage, FGFR3 level is markedly decreased compared to normal articular cartilage assessed by immunohistochemistry using anti-FGFR3 antibody (Fig. 4b).

Next, we determined basal expression patterns of FGFR subtype family members in murine knee joints by immunohistochemistry using anti-FGFR1–4 antibodies. Histometric analyses were performed, and the percentage of immuno-positive chondrocytes was calculated under a fixed measuring frame (400 μm × 140 μm). In contrast to human articular chondrocytes, in murine knee articular cartilage, FGFR2 and 4 are the two most prominent receptors, followed by FGFR1 and then FGFR3 (Fig. 5a–d).

Finally, we previously reported that the level of FGFR3 is markedly reduced in human OA cartilage compared to normal cartilage, and FGF-2 negatively regulates FGFR3 by inhibiting the expression of this receptor at a transcriptional level [Yan et al., 2011]. In our murine in vivo studies here, induction of OA slightly reduces the levels of all FGFR subtypes, with the exception of FGFR3, which demonstrates no significant change after induction of

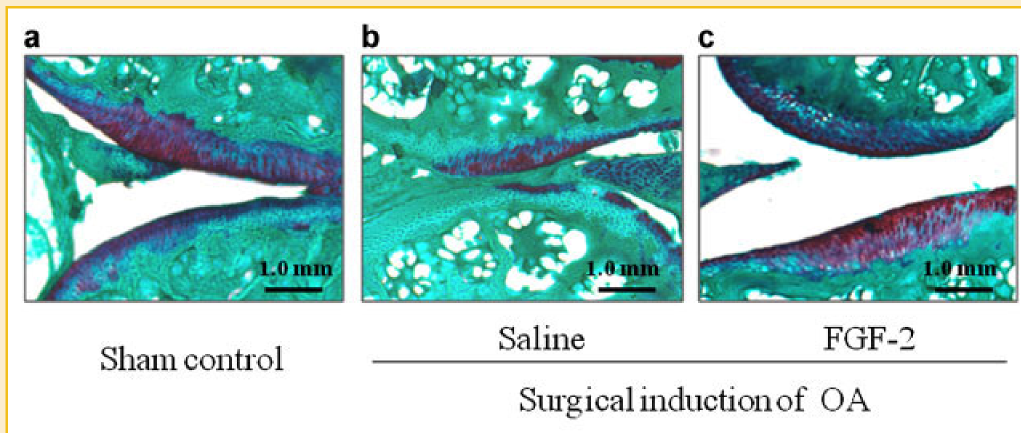

Fig. 3. Histological assessments of a single intra-articular injection of FGF-2 in fibrinogen solution. Two weeks after OA induction by DMM, mice were administered a single 5 μl intra-articular injection of (c) FGF-2 (2.5 μg per knee joint) or (b) saline solution mixed with fibrinogen solution (1.5 μg/ml) in 1:1 (v/v), followed by 0.2 unit (1 μl) of thrombin (total 6 μl per knee joint). Mice were euthanized at week 5 post-surgery for Safranin-O staining to assess PG depletion by comparing to the sham surgery control (intra-articular injection of saline, fibrinogen, thrombin) (a) (original magnification 40×, n = 4 for each group)

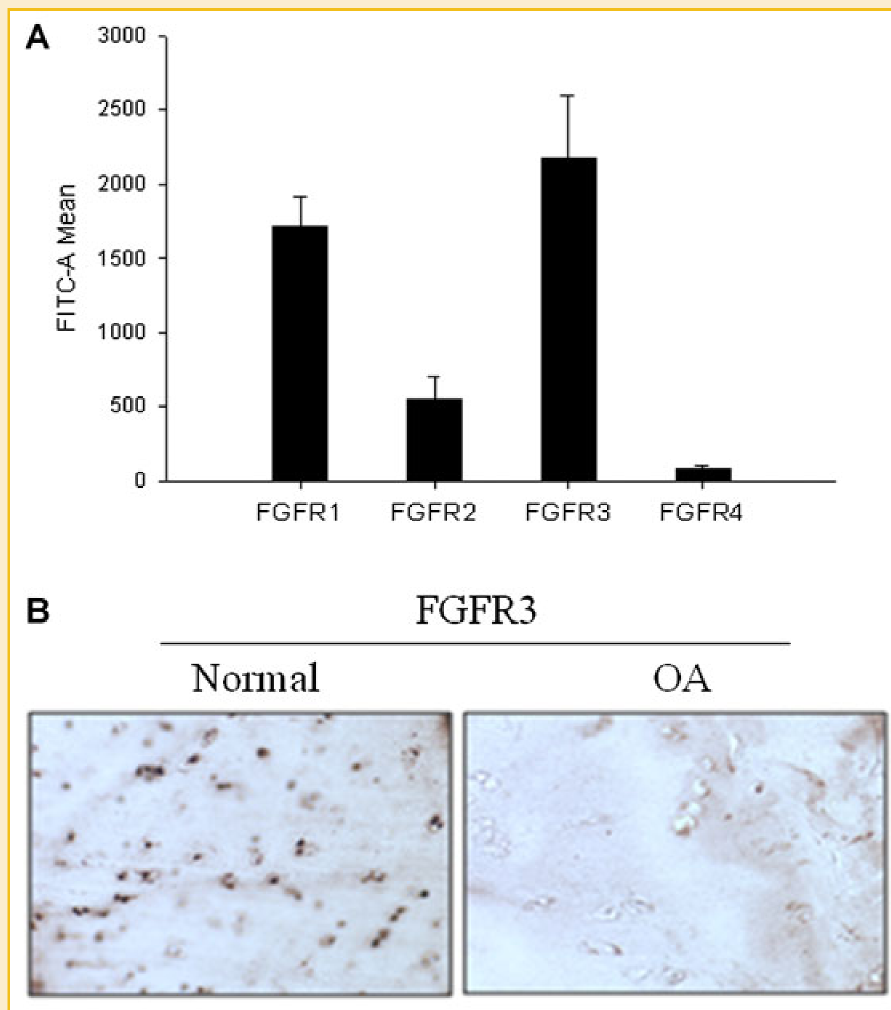

Fig. 4. Expression profiles of FGFR in human articular knee joint cartilage. a: Human articular chondrocytes isolated from adult human knee joints were subjected to flow cytometry analysis using a FACS Canto II flow cytometer and FACS DIVA software for data analyses. Primary human chondrocytes were incubated with primary antibodies against each FGFR isoform followed by incubation with a FITC-tagged secondary antibody. Isotype IgG was used in parallel to evaluate background fluorescence. Fluorescence histogram plots were prepared by comparing mean fluorescence intensities of different samples (n = 4). B: Level of FGFR3 expression is compared between normal knee joint cartilage (grade 0 or 1) and OA cartilage (surgically removed) using anti-FGFR3 antibody by immunohistochemistry. The results represent three donors for age- and gender-matched human adult cartilage samples (n = 3).

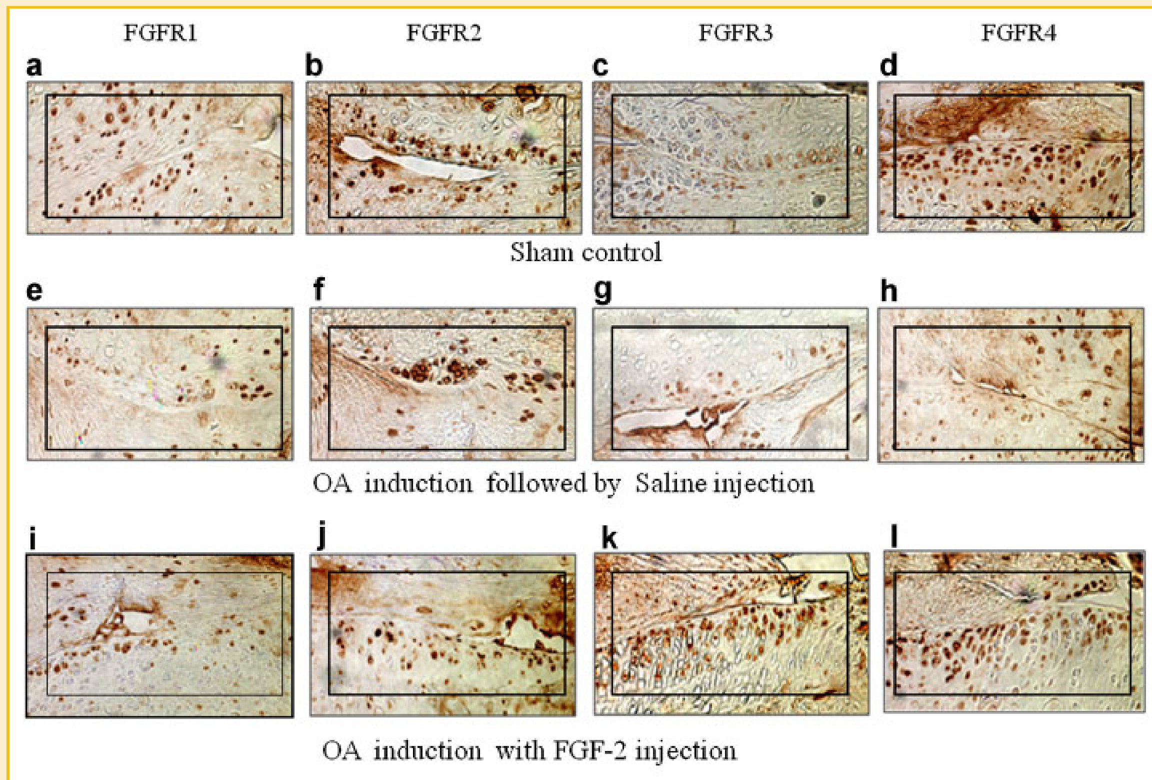

Fig. 5. FGFR expression profiles in knee joints from OA animal model. Five weeks after DMM surgery (3 weeks after intra-articular injection of FGF-2 or saline), animals were euthanized for immunohistological assessments for profiling FGFR family members (FGFR1–FGFR4). Sections were processed to incubate with primary antibodies of FGFR1 (a,e,i), FGFR2 (b,f,j), FGFR3 (c,g,k) or FGFR4 (d,h,l) followed by visualization using Vectastain Kit and Nikon SMZ1000. For histometric analysis, we selected superficial zone layers of tibia/femur sections of knee joints as indicated by squares. The % of immuno-positive chondrocytes was calculated under a fixed measuring frame (400 μm $\times$ 140 μm) (immune-positive cell number/total cell number $\times$ 100) (n = 4).

OA in the murine model (Fig. 5e–h). Importantly, the level of FGFR3 is markedly upregulated after intra-articular injection with FGF-2 (Fig. 5k compared to sham control (c) or OA induction followed by saline injection (g)), while other FGFR subtypes remain unchanged (Fig. 5i,j,l). Our findings suggest that these differential expression profiles of FGFRs and distinctive regulatory pattern of FGFR3 may partly explain the contradictory biological responses to FGF-2 between human and murine cartilage.

FGF-2-MEDIATED PREVENTION OF CARTILAGE DEGRADATION IN MICE HAS NO SIGNIFICANT EFFECT ON SYMPTOMATIC JOINT PAIN RELIEF

Because intra-articular injection of FGF-2 significantly reverses DMM-induced progression of OA in vivo, we sought to determine whether injection with FGF-2 alleviates OA-induced pain in the same murine model. We attempted to correlate cartilage regeneration induced by FGF-2 with reduced pain responses, as determined by weekly behavioral tests. Early pain events were observed by mechanical allodynia (decreased withdrawal force threshold) representing development of OA-associated pain at 2 weeks post-surgery, as previously described [Im et al., 2010]. Surprisingly, our behavioral pain assessments reveal that the FGF-2-mediated reversal of cartilage degradation in an OA animal model is not significantly associated with reduced OA symptoms (Fig. 6). While the pain threshold was increased for the FGF-2 injection group at 3 weeks post-surgery (1-week post-injection), there was no significant difference between the FGF-2 and saline injection groups at 4 weeks post-surgery (2 weeks post-injection).

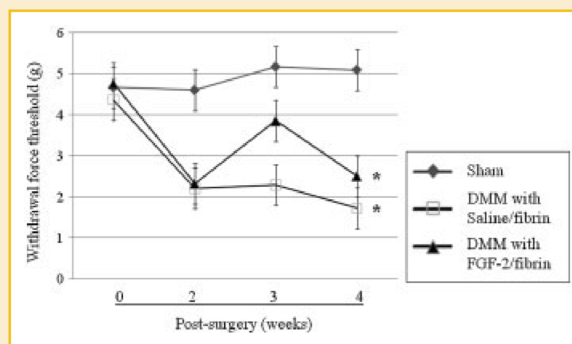

Fig. 6. Behavioral pain assessment in the DMM-induced OA model. A mouse model of joint pain was generated by an established surgical approach (DMM). Mechanical allodynia (von Frey) was assessed by 50% force withdrawal threshold, in grams (g). Following DMM, there was a decrease in force threshold compared to sham animals, but no difference between FGF-2 and saline injected DMM mice. $^{*}P < 0.05$.

INTRA-ARTICULAR INJECTION WITH FGF-2 PROMOTES INFLAMMATION AND ANGIOGENESIS IN MURINE OA MODEL IN VIVO

Angiogenesis, inflammation, and innervation are highly interconnected and recognized as potential sources of pain induced by OA [Bonnet and Walsh, 2005; Ashraf et al., 2011]. Accumulated evidence also supports the pathophysiological roles of synovial angiogenesis in arthritic joints [Brahn et al., 2008; Szekanecz et al., 2010]. Angiogenesis may introduce sensory nerves into the synovium and/or aneural cartilage, while inflammation can sensitize nerves already present within the joint [Brahn et al., 2008; Szekanecz et al., 2010]. Therefore, to explore the possible explanation why FGF-2-induced cartilage regeneration has no significant effect on pain relief, we performed immunohistochemistry experiments using antibodies targeting CD11b and VEGF (well-known markers for inflammation and angiogenesis, respectively) within synovium lining area (Fig. 7a). Joint tissues from the sham control with saline injection group hardly show immunoreactivity to CD11b, as shown in Figure 7b. The DMM surgery group followed by saline injection slightly increases the inflammatory response as reflected by mild immunoreactivity to CD11b in the synovial area (Fig. 7c), which is significantly increased by intra-articular injection with FGF-2 (Fig. 7d) compared to sham control or DMM surgery followed by saline injection group (Fig. 7b,c, respectively). Similarly, FGF-2 injection markedly increases VEGF production in synovial lining cells (Fig. 8d) compared to either sham control (Fig. 7b) or surgical control (DMM surgery followed by saline/fibrin gel injection, Fig. 7c). These findings reveal that, despite its regenerative effects on cartilage deposition, FGF-2 induces pro-inflammatory and angiogenic mediators in vivo, potentially sensitizing the joint to pain associated with OA.

DISCUSSION

The current study is the first to provide a biochemical explanation for the conflicting effects induced by FGF-2 on articular cartilage of two different species, human and mice. The fundamental differences in the cellular responses between human and murine tissues may be attributed to distinctive expression patterns of FGFRs that eventually determine the biological outcomes in the presence of FGF-2.

Existing literature demonstrates conflicting roles of FGF-2 on cartilage homeostasis. Several groups have demonstrated an anabolic role of FGF-2 and suggested its use for cartilage regeneration and repair [Cuevas et al., 1988; Thompson et al., 1991; Cucchiarini et al., 2005; Hiraide et al., 2005; Inoue et al., 2006; Kaul et al., 2006; Deng et al., 2007; Schmal et al., 2007; Tsai et al., 2007; Stewart et al., 2007b]. More recently, Vincent and colleagues demonstrated that FGF-2 protects against joint degeneration [Vincent et al., 2002; Chia et al., 2009]. In their studies, FGF-2 null mice developed accelerated OA following surgical destabilization and exhibited super-induction of matrix-degrading enzymes compared to control, and this was reversed by subcutaneous delivery of recombinant FGF-2 [Chia et al., 2009]. On the other hand, studies from our laboratory [Loeser et al., 2005; Muddasani et al., 2007; Yan et al., 2011] and others [Tchetina et al., 2005; Stewart et al., 2007a] revealed that, at least in human articular cartilage, FGF-2 is pathologically associated with joint destruction due to (i) upregulation of MMPs and aggrecanases via PKCδ-dependent MAPK and NF κB pathways; (ii) suppression of PG accumulation; (iii) stimulation of reactive oxygen species such as nitric oxide and superoxide anion; (iv) clustering of cells with little surrounding ECM resulting in the formation of fibrocartilage, a poor substitute for

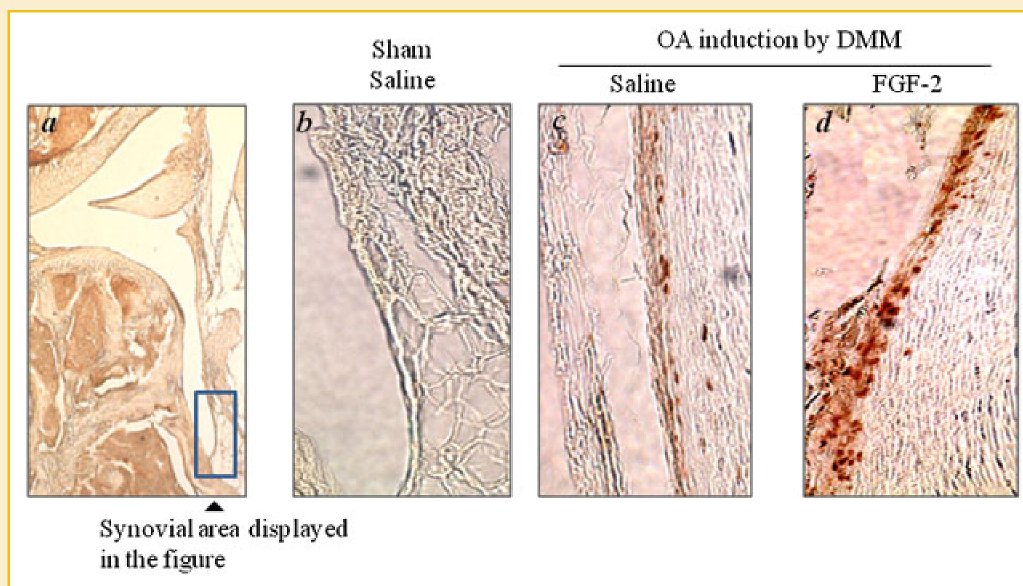

Fig. 7. Immunohistological assessments for inflammation within synovial lining in the DMM-induced murine OA model (a). Knee joint tissue sections from sham (b), saline (c) and FGF-2 injected mice (d) were stained with anti-CD11b antibody (1:50 dilution). Intra-articular single injection of saline, fibrinogen, thrombin was performed on the left knee joint (n = 4 for each group).

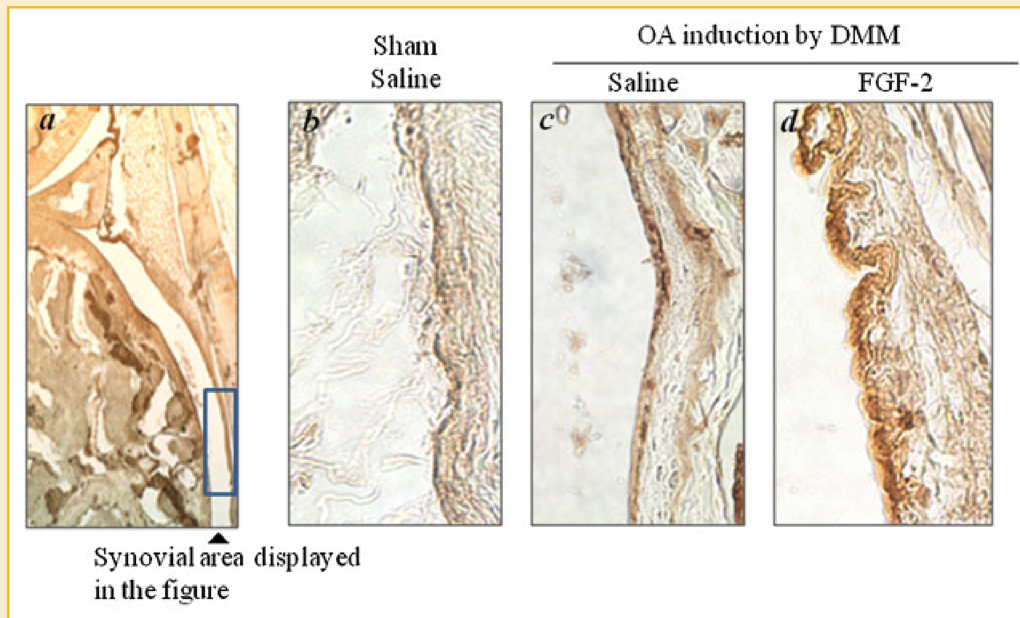

Fig. 8. Immunohistological assessments for angiogenesis within synovial lining in the DMM-induced murine OA model (a). Knee joint tissue sections from sham (b), saline (c) and FGF-2 injected mice (d) were stained with anti-VEGF antibody (1:100 dilution). Intra-articular single injection of saline, fibrinogen, thrombin was performed on the left knee joint (n = 4 for each group).

hyaline cartilage; and (v) potent antagonism against well-known cartilage anabolic factors, such as insulin-like growth factor-1 (IGF-1) and BMP7 [Qu et al., 1995; Loeser et al., 2005; Tchetina et al., 2005; Im et al., 2007b; Muddasani et al., 2007].

In normal human articular cartilage, FGFR1 and FGFR3 are predominantly expressed, with negligible or undetectable levels of FGFR2 and FGFR4 [Yan et al., 2011]. In degenerative human cartilage such as OA cartilage, however, FGFR3 expression level is markedly reduced, and this event is closely linked with enhanced catabolic signaling in the presence of FGF-2. The degrees of the induction of cartilage degrading enzyme production (MMPs, ADAMTS) and the suppression of PG accumulation and synthesis in degenerative cartilage after FGF-2 stimulation correlate with the reduced FGFR3 expression [Im et al., 2007b]. Recently, Yan et al. [2011] suggested that the conflicting roles of FGF-2 in cartilage may be dependent upon differences in the balance of FGFR family members (ratio of FGFR1:FGFR3) within the tissue of interest. It was proposed that although FGF-2 interacts with both FGFR1 and FGFR3, FGFR1 is principally responsible for FGF-2-induced catabolic activities in human articular chondrocytes while FGFR3 exerts chondroprotective effects. For example, a well-known cartilage anabolic growth factor, FGF-18 specifically activates FGFR3-dependent signaling pathway in articular cartilage, which promotes cartilage anabolism [Davidson et al., 2005]. These findings revealed important opposing effects of FGFR1 and FGFR3 in human articular cartilage.

By contrast, in murine articular cartilage, FGFR2 and FGFR4 are predominantly expressed, and FGF-2 exerts anabolic effects via reversal of cartilage degeneration in vivo. At present, the precise roles of FGFR2 and FGFR4 are not clearly understood. However, our findings suggest a potential role of FGFR2 and FGFR4 in murine cartilage homeostasis in response to FGF-2 stimulation. It is quite striking observing opposite effects of FGF-2 on FGFR3 expression in human and murine articular cartilage. FGF-2 highly upregulates FGFR3 in murine chondrocytes (current study), whereas FGF-2 significantly downregulates FGFR3 in human articular chondrocytes [Yan et al., 2011]. Thus, in mice, FGFR3 may also play an anabolic role in articular cartilage after injection with FGF-2.

One of the long-standing and fundamental questions in OA research is the apparent lack of correlation between the severity of pain and the severity of joint disease radiographically or histologically. For decades, researchers have been attempting to uncover factors that connect pain with cartilage degeneration. Our behavioral assessments in an OA murine model demonstrate that FGF-2-mediated ECM formation is not closely connected with alleviation of OA pain symptoms. Despite the capacity of FGF-2 to induce murine cartilage regeneration, mice injected with FGF-2 did not experience significant alleviation of pain compared to control (injection with saline alone).

Given these findings, we examined the effect of FGF-2 on two processes, inflammation and angiogenesis within the synovial lining area, in an attempt to provide an explanation for persistence of pain symptoms independent of FGF-2-mediated ECM formation. Angiogenesis, inflammation, and innervation are highly interconnected and recognized as potential sources of pain induced by OA, and recent evidence supports the pathophysiological roles of synovial angiogenesis in arthritic joints [Bonnet and Walsh, 2005; Brahn et al., 2008; Szekanecz et al., 2010; Ashraf et al., 2011]. Stimulation of inflammatory and angiogenic mediators promotes vascular ingrowth, neural ingrowth, and sensory innervation, potentially resulting in indirect sources for pain generation in OA [Bonnet and Walsh, 2005]. Indeed, our findings suggest that FGF-2

stimulates both inflammatory and angiogenic effectors in synovium as shown by increased immunoreactivity to CD11b and VEGF, which are well-known markers of inflammation and angiogenesis, respectively [Dayer, 2002]. This serves as one plausible explanation for pain induction independent of cartilage regeneration. These findings are also corroborated by a previous study, in which co-presence of angiogenic and inflammatory mediators was increased in OA synovium compared to normal synovium in human joint tissues in vitro and rat joint tissues in vivo [Im et al., 2010].

There are several limitations of this study. First, because the majority of our studies are based on histological analyses of articular cartilage, further detailed analyses are necessary for other structural components of the joint, including synovium, ligament and subchondral bone. Second, the relative abundance of FGFRs in articular cartilage is dynamic in nature and may change depending on the given state of the cartilage at any given time [Vincent, 2011]. Third, our behavioral pain assessments are limited to 4- to 5-week spans after surgery, which mimics early-stage OA conditions in the presence of FGF-2. It is therefore necessary to reassess these findings at a later time point to explore whether the cartilage regeneration by FGF-2 in a mouse model, indeed, correlates with alleviation of pain in a more severe stage of OA. Finally, we compared human cartilage using an ex vivo organ culture system to murine studies using in vivo techniques, making comparison between the two difficult in terms of data interpretation.

In summary, this study adds to the growing controversy over the use of FGF-2 as a biological treatment strategy for OA. Based on our findings, we believe that precise characterization of the biological roles of each FGFR in adult human articular cartilage provides critical information for OA therapy at the receptor level. For example, further studies are indeed warranted to elucidate the therapeutic potential of FGFR3 agonists, as well as the role, if any, of FGFR2 and FGFR4, in human articular cartilage. The complex interplay of FGFRs and the downstream signaling cascades induced by FGF-2 in human cartilage should add caution to the use of this particular growth factor for biological therapy in the future.

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