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Fibroblast Growth Factor-2 Promotes Catabolism Via FGFR1-Ras-Raf-MEK1/2-ERK1/2 Axis That Coordinates With the PKCδ Pathway in Human Articular Chondrocytes

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ABSTRACT

Fibroblast growth factor 2 (FGF-2) has been found to play an anti-anabolic and/or a catabolic role in adult human articular cartilage via regulation of multiple signaling pathways. Upon FGF-2 stimulation, a molecular crosstalk between the mitogen activated protein kinase (MAPK) and protein kinase C δ (PKCδ) pathways are initiated, where PKCδ positively regulates downstream MAPK signaling. In this study, we explored the relationship between fibroblast growth factor receptor 1 (FGFR1), Ras, and PKCδ in FGF-2 signaling in human articular chondrocytes. Pathway-specific inhibition using both chemical inhibitors and siRNA targeting FGFR1 demonstrated that, upon FGF-2 stimulation, FGFR1 controlled both Ras and PKCδ activation, which converged on the Raf-MEK1/2-ERK1/2 axis. No crosstalk was observed between Ras and PKCδ. Quantitative PCR analyses revealed that both Ras and PKCδ contributed to FGF-2-mediated upregulation of MMP-13, ADAMTS5, and repression of aggrecan gene. Correspondingly, FGF-2-mediated proteoglycan loss was effectively reversed by individual pathway-specific inhibitor of Ras, PKCδ, and ERK1/2 in both 3-dimensional alginate bead culture and cartilage organ culture systems. Our findings suggest that FGFR1 interacts with FGF-2 and then activates Ras and PKCδ, which concertedly drive MAPK signaling to mediate biological effects of FGF-2. Such an integration of dual inputs constitutes a novel mechanism of FGF-2 signaling cascade in human articular chondrocytes. J. Cell. Biochem. 113: 2856–2865, 2012.

KEY WORDS: OSTEOARTHRITIS; CARTILAGE DEGENERATION; FGF-2; FGFR1; RAS; PKCδ; MAPK

Osteoarthritis (OA) is the leading cause of disability among the elderly population and a major source of health care costs in the United States [Buckwalter et al., 2004]. The OA-afflicted population was estimated to be 47.8 million in 2005, and was projected to rise to approximately 67 million by 2030, with roughly 25 million patients to report arthritis-attributable activity limitations [Hootman and Helmick, 2006]. Given the profound current and future socioeconomic impact of this disease, a thorough understanding of its pathogenesis and progression is highly desired in order to assist the development of effective therapies. To date, however, the underlying cellular molecular mechanisms of OA are still inadequately understood.

Under healthy condition, articular chondrocytes are capable of maintaining a dynamic equilibrium between synthesis and degradation of extracellular matrix (ECM) components, including type II collagen and proteoglycan including aggrecan [Goldring, 2000; Masuda et al., 2006]. By stark contrast, in degenerative state the equilibrium is disrupted, leading to progressive cartilage erosion and chondrocyte abnormalities in the lesions [Abramson and Attur, 2009]. It is now established that dysregulation and excessive production of catabolic factors, including matrix metalloproteases (MMPs), aggrecanases (ADAMTS), and destructive cytokines and growth factors in chondrocytes accounts for the degeneration of ECM [Martel-Pelletier et al., 2001; Muddasani et al., 2007; Im et al., 2007, 2008]. Among all cartilage-degrading proteases, MMP-13 and ADAMTS5 have been found to play significant roles in cartilage degradation [Billinghurst et al., 1997; Fernandes et al., 1998; Anderson et al., 2002; Le Maitre et al., 2004]. Further,

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pro-inflammatory cytokines such as interleukin-1 (IL-1) [Shinmei et al., 1988; Im et al., 2003; Le Maitre et al., 2004, 2007] and growth factors including FGF-2 [Im et al., 2007] have been implicated in upregulation of these matrix-degrading enzymes. In particular, FGF-2 has been shown to upregulate RUNX2, a key hypertrophic regulator, via PKCδ activation, which, in turn, controls type X collagen, MMP13 and ADAMTS5 at the level of transcription in articular chondrocytes [Kim et al., 2003, 2006; Kamekura et al., 2006; Tetsunaga et al., 2011]. Previously, we also demonstrated the critical role of FGF-2-mediated activation of PKCδ pathway in MMP-13 induction in adult human articular chondrocytes [Im et al., 2007].

In human articular chondrocytes, FGF-2 potently inhibits proteoglycan synthesis and accumulation induced by insulin-like growth factor 1 (IGF-1) and bone morphogenetic protein 7 (BMP7) [Loeser et al., 2005a]. Our previous studies indicated that FGF-2 level is abnormally elevated in osteoarthritic synovial fluids [Im et al., 2007], and may exacerbate cartilage degradation due to its catabolic potency ex vivo [Muddasani et al., 2007]. Specifically, MMP-13 upregulation is likely to account for FGF-2-mediated catabolic effects, considering its activities for type II collagen and aggrecan cleavage [Fosang et al., 1996; Billinghurst et al., 1997]. FGF-2-induced activation of PKCδ functions as an upstream regulator of the MAPK subgroups, including ERK, JNK, and p38, and NFκB signaling cascades, and is the only PKC isoform associated with MMP-13 induction among several PKC isoforms tested. Subsequent to their activation, MAPK and NFκB converge on transcription factor Elk-1, which directly upregulates MMP-13 transcription [Muddasani et al., 2007]. FGF-2 possesses higher affinity to fibroblast growth factor receptor 1 (FGFR1) [Zhang et al., 2006], and FGFR1 is an upstream activator of Ras in other cell types [Tsang and Dawid, 2004]. Recently, we reported that FGFR1 is the major receptor responsible for the catabolic and anti-anabolic responses to FGF-2 in human primary chondrocytes [Yan et al., 2011]. However, the potential molecular connection between FGFR1 and Ras, which may exert a specific biological impact on articular cartilage homeostasis, has not been explored in the context of FGF-2 signaling in human chondrocytes. The aim of the present study was to elucidate FGF-2-triggered upstream signaling events and their catabolic impacts on adult human articular chondrocytes in vitro and ex vivo. Given that FGF-2 activates FGFR1 in human articular chondrocytes [Muddasani et al., 2007; Yan et al., 2011], we sought to (i) determine whether FGFR1 activation induces Ras activation and the subsequent Raf-MEK1/2-ERK1/2 pathway in articular chondrocytes; (ii) determine potential signaling interplays between PKCδ and Ras; and (iii) assess the biologic effects of inhibition of specific pathways on the expression of key target genes and proteoglycan metabolism.

MATERIALS AND METHODS

MATERIALS

Human recombinant FGF-2 was purchased from the National Cancer Institute (Bethesda, MD). Human recombinant IL-1β was purchased from PeproTech (Rocky Hill, NJ). Antibodies against human phospho-Raf (Ser338), phospho-MEK1/2 (Ser217/221), phospho-ERK1/2 (Thr202/Tyr204), and ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA). Human ADAMTS5 antibody was purchased from Millipore (Billerica, MA). Antibody against human MMP-13 was provided by courtesy of Dr. Gillian Murphy (Cambridge University, UK). Pharmacological inhibitors including SU5402 (FGFR1i), Raf1 kinase inhibitor (Rafi), manumycin A (Rasi), rottlerin (PKCδi), and PD98059 (ERKi) were purchased from EMD Chemicals (Gibbstown, NJ). Stealth small interfering RNA (siRNA) targeting FGFR1 and PKCδ were purchased from Invitrogen (Carlsbad, CA). Active Ras pull-down and detection kit was purchased from Thermo Scientific (Rockford, IL).

CHONDROCYTE ISOLATION AND CULTURE

Human femoral cartilage was acquired through the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL) with approval by local ethics committee and consent from the families. Each specimen was graded for overall degenerative status based on a modified five-point scale of Collins before dissection [Muehleman et al., 1997]. For monolayer culture and treatments, healthy cartilage (grade 0 or 1, 40- to 60-year-old) was used unless otherwise indicated; for alginate bead and explant culture, moderately degenerated cartilage (grade 2 or 3, 45- to 60-year-old) was used. Cartilage was digested in pronase for 1 h, followed by overnight digestion with collagenase P as described previously [Im et al., 2003; Loeser et al., 2005a]. For monolayer culture, isolated cells were washed and suspended in culture media at 3×10^6 cells/ml, and plated onto 12-well plates at 1 ml/well. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 (1:1) containing 10% fetal bovine serum and antibiotics (complete media) for 3 days. Completed media were replaced with fresh serum-free DMEM/F-12 (1:1) 24 h before treatments, and then media were changed again with serum-free DMEM/F-12 (1:1) 2 h before experiments started. For alginate bead culture, cells were suspended in alginate at 2×10^6 cells/ml, and beads were formed in $CaCl_2$ solution, as described previously [Loeser et al., 2003]. The beads were cultured in DMEM/F-12 (1:1) supplemented with 1% mini-ITS+ premix and 0.1% ascorbic acid, at 8 beads/well in 24-well plates. For cartilage explant culture, full-thickness explants of 4 mm diameters were cut from cartilage using a biopsy punch. Explants then recovered in complete media for 2 days. Culture media were then switched to 1% mini-ITS+-supplemented media 2 days before treatments were initiated.

CHONDROCYTE STIMULATION AND IMMUNOBLOTTING

When needed, cells in monolayer were pre-incubated with individual pathway-specific inhibitor for 1 h before administering FGF-2 (100 ng/ml). After experiments were terminated, conditioned media and whole cell lysates were collected. The media were stored at 4°C with 0.1% NaN_3, and analyzed within 5 days. Cell lysates were prepared using a modified cell lysis RIPA buffer: 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.25% deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM glycerol phosphate, 1 mM $NaVO_4$, and 2 mM phenylmethylsulfonyl fluoride (Sigma, St Louis, MO). Total protein concentrations of the cell lysates were measured by a bicinchoninic acid (BCA) assay (Thermo Scientific). Equal amounts of protein were separated on 10% SDS–polyacrylamide gels, and transferred to nitrocellulose membrane for

immunoblotting analyses as described previously [Im et al., 2003]. Immunoreactivity was visualized using an ECL method (Thermo Scientific).

ACTIVE RAS PULLDOWN ASSAY

After stimulation was finished, cells were washed in cold 1× PBS twice and lysed in buffer containing 20 mM Tris (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM DTT. Lysates were clarified by centrifugation, and 500 μg total protein was incubated with 50 μg GST-Raf1-Ras-binding domain fusion protein at 4°C for 1 h, with end-over-end mixing (Millipore). Beads were collected, washed in lysis buffer, and suspended in Laemmli sample buffer, followed by boiling for 5 min. Proteins were then resolved by SDS–PAGE in 10% gels, transferred to nitrocellulose membrane, and immunoblotted as described above.

TRANSIENT TRANSFECTION OF siRNA

Nucleofection was optimized for human articular chondrocytes based on the manual of the Nucleofector™ kit (Lonza, Walkersville, MD) as described previously [Pulai et al., 2005; Loeser et al., 2005b]. Chondrocytes were cultivated for 3 days before transfection. For transfection, siRNA at a concentration of 200 nM (20 pmol/sample) was used during transfection. After 48 h, cell lysates were subjected to SDS–PAGE and immunoblotting for validation of successful knockdown. In parallel, stimulations were performed 48 h after the transfection.

TOTAL RNA EXTRACTION, cDNA SYNTHESIS, AND QUANTITATIVE PCR

Total RNA from human articular chondrocytes was isolated using Trizol reagent following the instructions provided by the manufacturer (Invitrogen). Reverse transcription (RT) was carried out with 1 μg total RNA using ThermoScript™ RT-PCR system for first strand cDNA synthesis (Invitrogen). For qPCR, cDNA was amplified using a MyiQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Relative gene expression was determined using the $\Delta\Delta C_T$ method, as detailed by manufacturer guidelines (Bio-Rad). 18S rRNA was used in reactions for normalization. The standard deviations among samples were calculated using data from at least five different donors in independent experiments. The primer sequences are summarized in Table I.

DIMETHYLMETHYLENE BLUE (DMMB) ASSAY AND DNA ASSAY

The alginate beads were processed for quantitation of proteoglycan accumulation using the DMMB binding method, as previously described [Loeser et al., 2003]. The proteoglycan levels in the cell-associated matrix were measured. DNA content in cell pellets was determined using PicoGreen (Invitrogen), as previously described [Loeser et al., 2003].

HISTOLOGY

Safranin Orange staining was carried out on full-thickness cartilage explants prepared as described above. The cartilage explants were treated with FGF-2 (100 ng/ml) or IL-1β (10 ng/ml), in the presence or absence of specific pathway inhibitors under serum-free conditions (plus mini-ITS™ + Premix). Following 11 days of incubation, all explants were fixed in 4% paraformaldehyde overnight, followed by paraffin embedding. Sections were prepared at 8 μm each. The sections were then deparaffinized, and stained with Safranin Orange dye to assess proteoglycan content in the ECM.

STATISTICAL ANALYSIS

Statistical significance was determined by one-way ANOVA followed by Sidak post-hoc test, using the SPSS17 software (IBM Corporation, Somers, NY). In Figure 6B, ANOVA followed by planned contrasts was performed. *P*-values <0.05 are considered to be statistically significant in each test.

RESULTS

SIGNALING OF Raf-MEK1/2-ERK1/2 AXIS DEPENDS ON FGF-2-MEDIATED FGFR1 AND RAS ACTIVATION IN ADULT HUMAN ARTICULAR CHONDROCYTES

We previously identified the Raf-MEK1/2-ERK1/2 signaling axis as a robust route in FGF-2-stimulated MMP-13 expression in human articular chondrocytes [Im et al., 2007]. In other cell types, FGFR1 activation has been linked to Ras activation and subsequent Raf-MEK-MAPK signal transduction [Umbhauer et al., 2000; Lunn et al., 2007]. Recently, we demonstrated that FGFR1 and 3 are two predominant FGFRs in human articular cartilage, and specific activation of FGFR1, but not FGFR3, are associated with the catabolic and anti-anabolic effects in the presence of FGF-2 in human articular chondrocytes [Yan et al., 2011]. In the present study, we wished to investigate whether the activation of FGFR1 by FGF-2 is responsible for the activation of Ras and its downstream signaling cascades Raf-MEK1/2-ERK1/2 in human articular chondrocytes. Cells in monolayer were stimulated with 100 ng/ml of FGF-2 for different durations (5 and 10 min), with or without pharmacological inhibitor of FGFR1 (SU5402). Then active Ras was pulled down. FGF-2 rapidly activated Ras within 5 min (Fig. 1A, lane

TABLE I. qPCR Primer Sequences

Gene	Primer sequence (5′ → 3′)	NCBI Gene No.	Annealing, T_m (°C)
MMP13	F: ACCCTGGAGCACTCATGTTTCCTA R: TGGCATCAAGGGATAAGGAAGGGT	NM_002427.3	60
ADAMTS5	F: CTGTGACGGCATCATTGGCTCAAA R: TTCAGGAATCCTCACCACGTCAGT	NM_007038.3	60
ACAN	F: TCTTGGAGAAGGGAGTCCAACTCT R: ACAGCTGCAGTGATGACCCTCAGA	NM_001135.3	60
18S rRNA	F: CGGCTACCACATCCAAGGAA R: GCTGGAATTACCGCGGCT	NR_003286.2	60°C

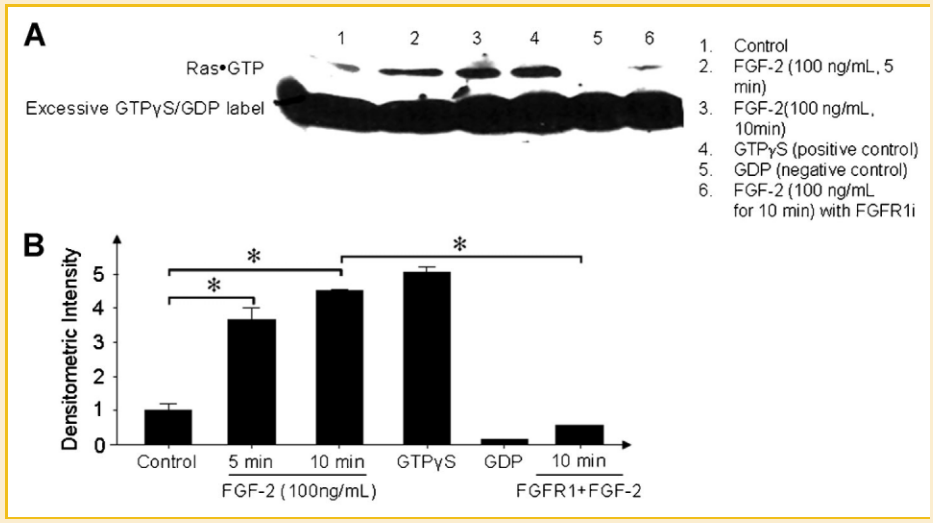

Fig. 1. FGFR1 activation results in increased Ras activity. Human articular chondrocytes in monolayer were stimulated with FGF-2 (100 ng/ml) for different periods (5 and 10 min) in the presence or absence of a FGFR1 pharmacological inhibitor SU5402 (5 μM). GTPγS and GDP were used as a positive control and a negative control, respectively. A: Cells were washed in cold PBS, and lysed in buffer containing 20 mM Tris (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM DTT. After clarification, 500 μg total protein was incubated with 50 μg GST-Raf1-Ras-binding domain fusion protein at 4°C for 1 h, with end-over-end mixing. Collected beads were washed in lysis buffer, and suspended in Laemmli sample buffer, followed by boiling for 5 min. Proteins were then resolved on a 10% SDS–PAGE gel, transferred to nitrocellulose membrane, and immunoblotted using an anti-Ras antibody. B: Densitometry of the immunoblots using the anti-Ras antibody from three independent experiments was performed. The histogram represents the amounts of active Ras·GTP (mean ± SD). The readings were normalized by the average control value to give fold changes ($^*P < 0.05$).

2), and the activity sustained longer than 10 min (Fig. 1A, lane 3). In the presence of the SU5402, however, FGF-2-induced activation of Ras was completely abolished, suggesting an essential role of FGFR1 in the induction of Ras activity by FGF-2 (Fig. 1A; lane 6). In addition, DMSO (vehicle of SU5402, 0.1% concentration) did not alter the basal or FGF-2-induced activity of Ras (data not shown). The results of Ras pull down assay were quantified by densitometric intensities and shown in Figure 1B.

Given that FGF-2 induces Ras activation via FGFR1, we studied the impact of Ras on its downstream MAPK signaling cascades. Human articular chondrocytes in monolayer were treated with FGF-2 (100 ng/ml) for 10 min, in the presence or absence of 3 μM of manumycin A, a specific pharmacological inhibitor of Ras [Hara et al., 1993]. The Ras inhibitor (Rasi) significantly diminished the activation of the Raf-MEK1/2-ERK1/2 axis, represented by their phosphorylation after FGF-2 stimulation, demonstrating an important role of Ras in MAPK signaling (Fig. 2, left panel). Total protein levels of Raf, MEK1/2 and ERK1/2 were not changed within the experimental time window (Fig. 2, right panel). Taken together, our findings suggest that FGF-2-activated Raf-MEK1/2-ERK1/2 pathway requires the signaling cue from FGFR1 and activation of its target, Ras.

FGF-2-STIMULATION OF MMP-13 AND ADAMTS5 IS VIA THE ACTIVATION OF THE Ras-Raf-MEK1/2-ERK1/2 PATHWAY IN HUMAN ARTICULAR CHONDROCYTES

We next sought to determine whether the Ras-Raf-MEK1/2-ERK1/2 signaling pathway accounts for upregulation of cartilage-degrading enzymes such as MMP-13 and ADAMTS5 after FGF-2 stimulation. Chondrocytes in monolayer were treated with FGF-2 (100 ng/ml), in the presence or absence of inhibitors of Ras (manumycin A, 3 μM), Raf inhibitor (Rafi, 50 μM), or ERK (PD98059, 50 μM) for 24 h. The conditioned media were collected for immunoblotting, and mRNA levels were quantified by qPCR analyses. Inhibition by each inhibitor of the Ras, Raf, and ERK pathway was sufficient to abrogate FGF-2-mediated upregulation of MMP-13 at the level of mRNA and corresponding protein level (Fig. 3A,B). This observation is line with our previous finding that expression of a dominant negative ERK is able to abolish FGF-2-mediated MMP-13 induction [Im et al., 2007]. Similarly, these inhibitors effectively reduced the induction of ADAMTS5 by FGF-2 at both mRNA and protein levels (Fig. 3D). This finding suggests that the FGFR1-Ras-Raf-MEK1/2-ERK1/2 axis plays a key regulatory role in controlling the production of important cartilage-degrading enzymes by FGF-2.

FGF-2-INDUCED PKCδ ACTIVATION IS FGFR1-DEPENDENT IN HUMAN ARTICULAR CHONDROCYTES

Previously, we reported that FGF-2-mediated activation of PKCδ is the rate-limiting step that controls downstream effectors, such as MAPK and NFκB, to stimulate MMP-13 production in human articular chondrocytes [Muddasani et al., 2007; Im et al., 2007]. In our current studies, we wished to determine whether the activation of PKCδ by FGF-2 is FGFR1-dependent. Human articular chondrocytes in monolayer were stimulated by FGF-2 (100 ng/ml) for 10 min in the presence or absence of SU5402 (5 μM). FGF-2 rapidly activated PKCδ, as reflected by its phosphorylation at Ser645. Such activation was significantly diminished by FGFR1 inhibition (Fig. 4A). Studies with pharmacological inhibitors were further validated by using siRNA targeting FGFR1 (siFGFR1). Primary

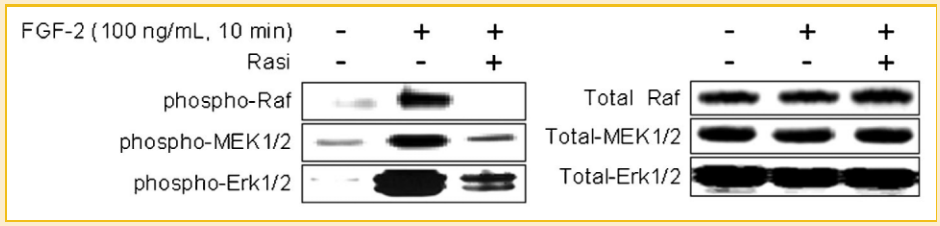

Fig. 2. Active Ras triggers MEK1/2–ERK1/2 signaling. Human articular chondrocytes in monolayer were stimulated with FGF-2 (100 ng/ml) for 10 min, in the presence or absence of a pharmacological Ras inhibitor. Then whole cell lysates were prepared. Cell lysates were prepared using modified cell lysis RIPA buffer: 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.25% deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM glycerol phosphate, 1 mM $NaVO_4$, and 2 mM phenylmethylsulfonyl fluoride. Total protein concentrations were measured by bicinchoninic acid (BCA) assay. Equal amounts of protein were resolved on 10% SDS–polyacrylamide gels, and transferred to nitrocellulose membrane for immunoblotting using anti-phospho-Raf, anti-phospho-MEK1/2, and anti-phospho-ERK1/2 antibody. Total ERK1/2 was used as a loading control. Immunoreactivity was visualized using an ECL method.

chondrocytes in monolayer were transiently transfected with siRNA using an optimized Nucleofection protocol [Im et al., 2007]. Similar to those seen in the studies with pharmacological inhibitors, FGFR1 siRNA significantly reduced FGF-2-induced PKCδ activation (Fig. 4B) suggesting that FGF-2 activates PKCδ via FGFR1. Correspondingly, FGF-2-induced activity of the Raf-MEK1/2-ERK1/2 signaling cascade was significantly reduced when FGFR1 was knocked down (Fig. 4B).

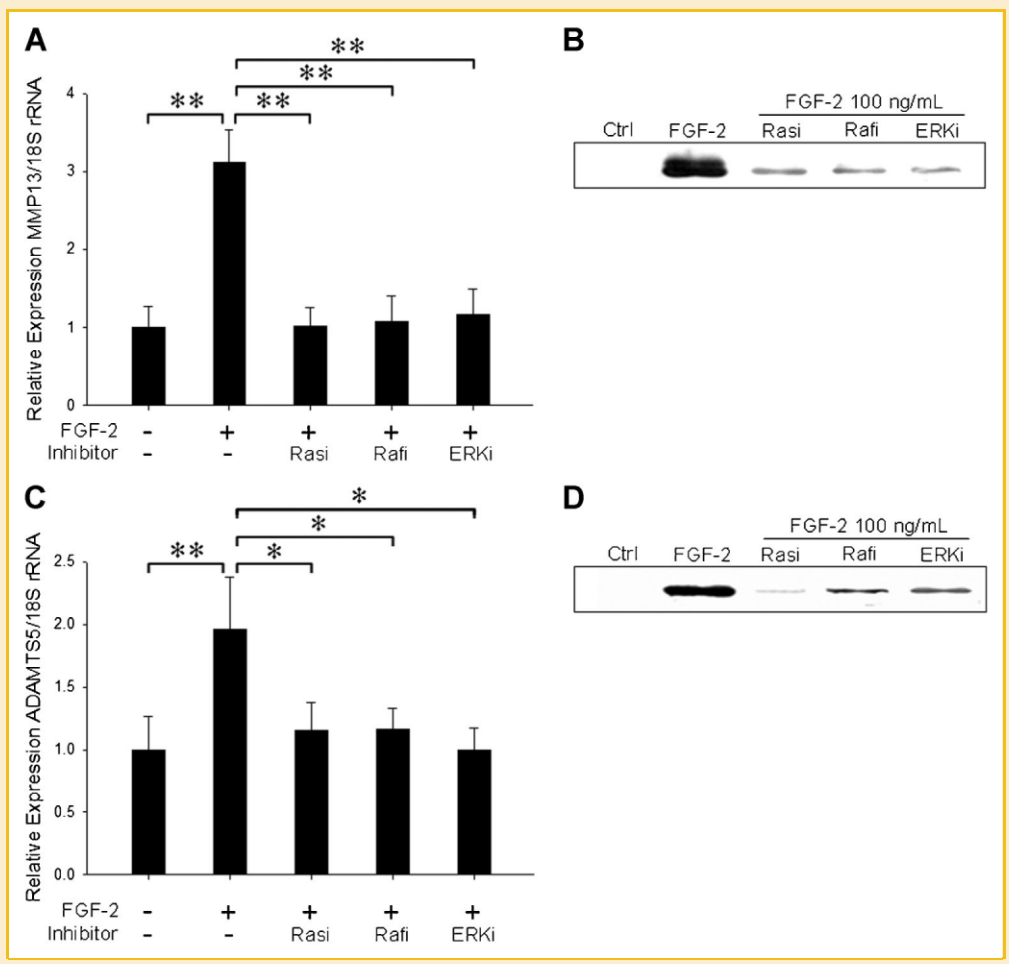

Fig. 3. Ras-MEK1/2-ERK1/2 pathway activation is required for MMP-13 and ADAMTS5 induction by FGF-2. Human articular chondrocytes in monolayer were pre-incubated with pharmacological inhibitor of FGFR1, PI3K, Ras, Raf, or ERK for 1 h, before stimulation with FGF-2 (100 ng/ml) for 24 h. A,C: Total RNA was extracted for first-strand cDNA synthesis and qPCR. 18S rRNA expression was used for normalization ($^{*}P < 0.05$; $P < 0.01$). B,D: Equal volumes of conditioned media were subjected to SDS–PAGE and immunoblotting of MMP-13 and ADAMTS5 protein. Representative blots are shown.

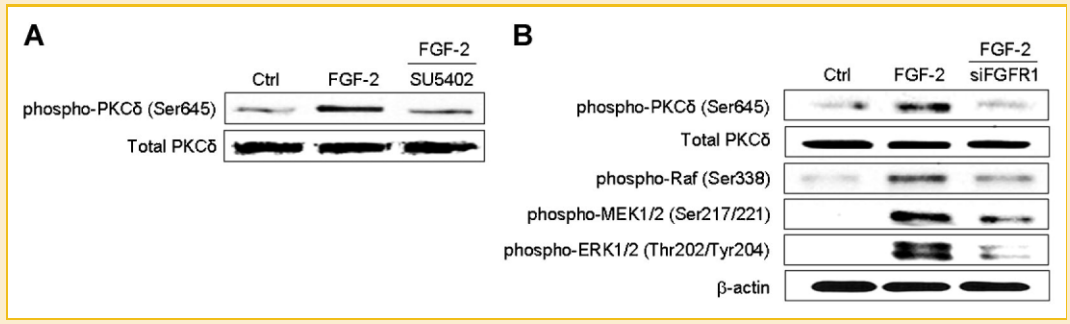

Fig. 4. FGF-2-induced PKCδ activation is FGFR1 dependent. Chondrocytes in monolayer were pre-incubated with FGFR1 inhibitor (SU5402) or transfected with FGFR1 siRNA, prior to FGF-2 stimulation (100 ng/ml, 10 min). Protein lysates were subjected to SDS–PAGE and immunoblotting of phospho-PKCδ (Ser645), phospho-Raf (Ser338), phospho-MEK1/2 (Ser217/221), and phospho-ERK1/2 (Thr202/Tyr204). Total PKCδ and β-actin blots were used as loading control. Representative blots are shown.

FGF-2-INDUCED ACTIVATION OF PKCδ IS INDEPENDENT OF RAS ACTIVATION IN ADULT HUMAN ARTICULAR CHONDROCYTES

The interaction between FGF-2 and FGFR1 triggered the activation of Ras (Figs. 1 and 2) and PKCδ (Fig. 4). Both Ras and PKCδ activated the Raf-MEK1/2-ERK1/2 axis (Figs. 1, 2, and 4) [Im et al., 2007]. Therefore, we examined potential molecular cross-talk between PKCδ and Ras in human articular chondrocytes. Chondrocytes were stimulated with FGF-2 (100 ng/ml, 10 min) in the presence or absence of a PKCδ pharmacological inhibitor (rottlerin, 4 μM). Ras pull-down assays were then performed. FGF-2 stimulation increased Ras activity by more than threefold within 10 min (Fig. 5A, lane 3), compared with control (Fig. 5A, lane 2), as represented by densitometric measurements in Figure 5B. Pre-incubation of the PKCδ inhibitor showed no effect on Ras activation (Fig. 5A; lane 6). Again, DMSO (vehicle of rottlerin, 0.1% concentration) did not change the basal or FGF-2-induced Ras activity (data not shown). Similarly, we did not observe significant changes in PKCδ activation (Ser645) in the presence of the Ras inhibitor (Fig. 5C). Our results suggest that FGF-2 ligand binds to FGFR1 and activates Ras and PKCδ pathways, and Ras and PKCδ have no direct interplay in adult human articular chondrocytes.

BLOCKADE OF RAS, PKCδ, AND MAPK PATHWAY RESCUES FGF-2-MEDIATED PROTEOGLYCAN LOSS IN ADULT HUMAN ARTICULAR CARTILAGE

Because FGF-2 induces catabolic and anti-anabolic cellular responses in articular chondrocytes, we examined the role of the Ras/PKCδ-Raf-ERK1/2 pathway in proteoglycan accumulation using an alginate bead long-term (21 days) culture system. Chondrocytes isolated from knee joints articular cartilage (grade 2 or 3, average donor age 45-year-old) in alginate beads were administered with FGF-2 (50 ng/ml), and co-incubated with or without inhibitors of Ras (manumycin A, 3 μM), PKCδ (rottlerin, 2 μM), Raf (Raf1 kinase inhibitor, 10 μM), and ERK (PD98059, 25 μM). Consistent with our previous report [Loeser et al., 2005a], FGF-2 significantly inhibited cell-associated proteoglycan deposition by ~50% (Fig. 6A, lane 2). Inhibition by each inhibitor of PKCδ, Raf, and ERK effectively reversed the inhibitory effect of FGF-2 on proteoglycan accumulation ($P < 0.01$). Blocking Ras also counteracted FGF-2-mediated proteoglycan depletion, albeit with less magnitude ($P < 0.05$) (Fig. 6A). There were no significant differences in DNA content (an established indicator of cell proliferation in chondrocyte long-term culture) or cell viability among the treated groups (data not shown).

To mechanistically understand FGF-2-mediated proteoglycan loss, we studied the effect of FGF-2 on the expression of aggrecan, the major proteoglycan in cartilage. Using chondrocytes isolated from early-stage (grade 2 or 3) as well as end-stage of OA cartilage (surgically removed from OA patients), we observed dose-dependent inhibition of aggrecan expression by FGF-2 (1, 10, 50, 100, and 200 ng/ml; Fig. 6B). Note that here we did not compare the basal mRNA levels of aggrecan between non-OA and OA chondrocytes. The cartilage destructive cytokine IL-1β (5 ng/ml) was applied in parallel as a positive control. Co-incubation with individual inhibitor of Ras, PKCδ, Raf, or ERK significantly reversed aggrecan repression by FGF-2 ($P < 0.01$; Fig. 6C). Correspondingly, PKCδ knockdown by siRNA also significantly reversed FGF-2-mediated aggrecan suppression ($P < 0.05$; Fig. 6D). These findings suggest that the Ras/PKCδ-Raf-MEK1/2-ERK1/2 axis mediates proteoglycan loss, in part via suppression of aggrecan gene expression in human articular chondrocytes.

We next sought to determine whether the effects of these inhibitors on FGF-2 also translate into cartilage organ culture (ex vivo). Full thickness cartilage explants (grades 2–3, average donor age 50-year-old) were incubated with either FGF-2 (100 ng/ml) or IL-1β (10 ng/ml) for 11 days, with or without pharmacological inhibitors of PKCδ, Raf, and ERK. Proteoglycan depletion was visualized by Safranin-O staining. Consistent with our previous report [Muddasani et al., 2007], FGF-2 elicited significant proteoglycan depletion (Fig. 6Eb) compared to control (no treatment) (Fig. 6Ea), and this effect was even more pronounced in the case of IL-1β (Fig. 6Ec). These catabolic responses were effectively abrogated by co-incubation with the inhibitors of PKCδ (Fig. 6Ed,e), Raf (Fig. 6Ef,g), and ERK (Fig. 6Eh,i). Taken together, our results indicate that FGF-2-triggered activation of PKCδ and Ras signaling cascades converges on the MAPK pathway to promote cartilage degradation, as illustrated in Figure 7.

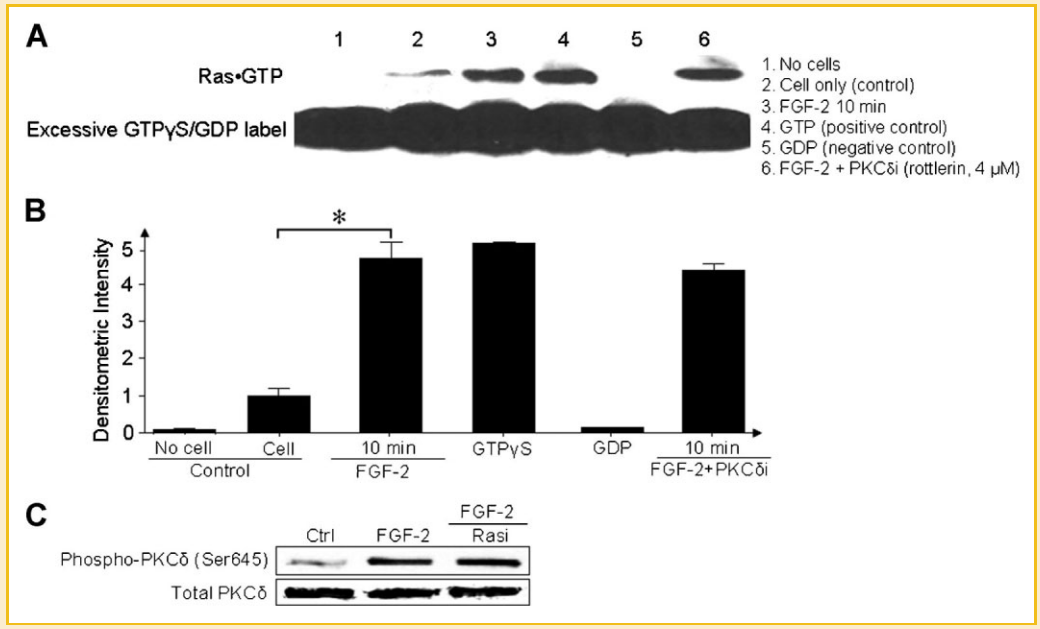

Fig. 5. PKCδ activates the MAPK pathway via Raf-MEK1/2, independent of Ras. Human articular chondrocytes in monolayer were stimulated with FGF-2 (100 ng/ml) for 10 min, in the presence or absence of a PKCδ pharmacological inhibitor rottlerin (4 μM). GTPγS and GDP were used as a positive control and a negative control in the pull-down assay, respectively. A: Cells were washed in cold PBS, and lysed in buffer containing 20 mM Tris (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM DTT. After clarification, 500 μg total protein was incubated with 50 μg GST-Raf1-Ras-binding domain fusion protein at 4 °C for 1 h, with end-over-end mixing. Collected beads were washed in lysis buffer, and suspended in Laemmli sample buffer, followed by boiling for 5 min. Proteins were then resolved on a 10% SDS–PAGE gel, transferred to nitrocellulose membrane, and immunoblotted using an anti-Ras antibody. B: Densitometry of the immunoblots using the anti-Ras antibody from three independent experiments was performed. The histogram represents the amounts of active Ras·GTP (mean ± SD). The readings were normalized by the average control value to yield fold changes ($^*P < 0.05$). C: Chondrocytes in monolayer were pre-incubated with Ras inhibitor (manumycin A) for 1 h before FGF-2 stimulation (100 ng/ml, 10 min). Protein lysates were subjected to SDS–PAGE and immunoblotting of phospho-PKCδ (Ser645). Total PKCδ blots were used as loading control.

DISCUSSION

We previously demonstrated the catabolic and anti-anabolic effects promoted by FGF-2 on adult human articular cartilage [Loeser et al., 2005a; Muddasani et al., 2007; Im et al., 2007, 2008; Ellman et al., 2008; Li et al., 2008]. Here, we revealed that the FGFR1-Ras/PKCδ-Raf-MEK1/2-ERK1/2 signaling pathway is activated after FGF-2 stimulation, which in turn mediates upregulation of matrix-degrading enzyme and downregulation of aggrecan expression. Our findings demonstrate that FGF-2 elicits PKCδ activation, which is independent of Ras activation. Importantly, both PKCδ and Ras are involved in the upregulation of catabolic enzymes, such as MMP-13 and ADAMTS5 as well as suppression of aggrecan in adult human articular chondrocytes. Blocking the PKCδ pathway appeared to elicit a more potent anti-catabolic effect than blocking the Ras pathway, highlighting its pertinent role in osteoarthritic conditions.

Binding of FGF-2 to its cognate receptor FGFR1 results in activation of multiple downstream substrates in human articular chondrocytes. These include (a) PKCδ, (b) Ras-Raf-MAPK, (c) NFκB, and (d) PI3K-Akt [Muddasani et al., 2007; Im et al., 2007; Ellman et al., 2008]. We have previously identified PKCδ as the principal rate-limiting molecule of the cellular response to FGF-2 in human articular cartilage [Im et al., 2007]. PKCδ blockade essentially prevents the activation of multiple downstream MAPK subgroups and NFκB, as well as key transcription factors, which are required for the catabolic and anti-anabolic biological actions of FGF-2 [Im et al., 2007]. Importantly, PKCδ-triggered MAPK and NFκB signaling converge on the transcription factor Elk-1 to regulate proteolytic enzyme production, such as MMP-13 in human articular chondrocytes [Muddasani et al., 2007].

The catabolic effects mediated by PKCδ are not limited in human articular cartilage. Recently, we also observed that the PKCδ pathway exerts potent catabolic and anti-anabolic effects on bovine, rabbit, and rodent intervertebral disc cartilaginous tissues in vitro, ex vivo and in vivo transgenic mouse studies [Ellman et al., 2011]. In both human articular cartilage and intervertebral disc, our previous studies suggest that PKCδ-directed activation of multiple MAPK subgroups (ERK, JNK, and p38) are required for the MMP-13 induction after stimulation with IL-1 or FGF-2 [Muddasani et al., 2007; Im et al., 2007; Ellman et al., 2008].

In the current study, we uncovered the importance of the Ras/PKCδ-Raf-MAPK pathway in FGF-2-mediated upregulation of MMP-13 and ADAMTS5 as well as suppression of aggrecan expression in human articular cartilage. Selective inhibition of ERK1/2 and p38 MAPK pathway has previously been shown to alleviate joint degeneration in an experimental OA animal model and IL-1β-induced organ culture model [Pelletier et al., 2003; Radons et al., 2006]. Therefore, specific inhibitors targeting the FGFR1-Ras/PKCδ-Raf-MEK1/2-ERK1/2 signaling pathway hold

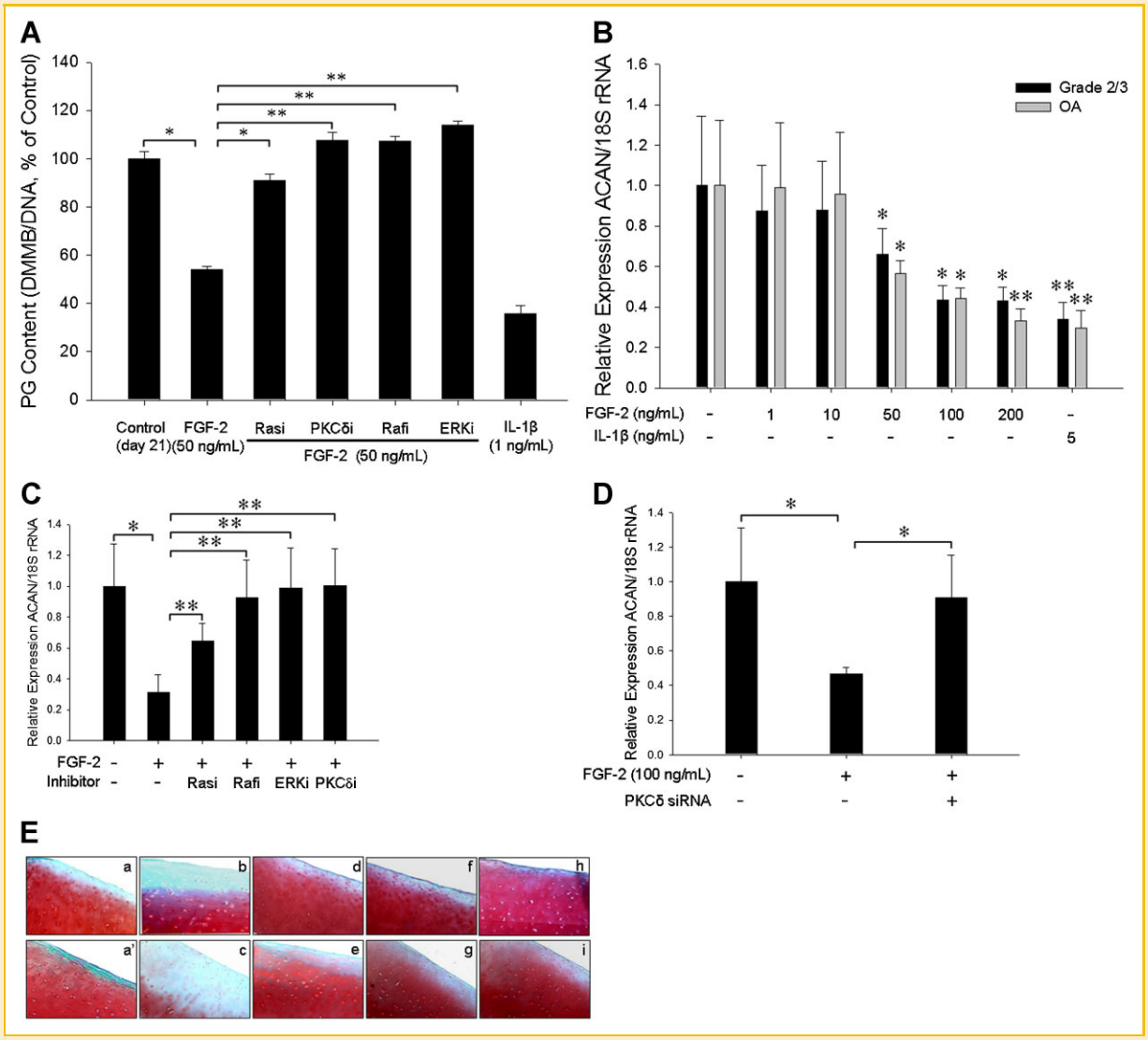

Fig. 6. Blockade of Ras, PKCδ, and MAPK pathway abolishes FGF-2-mediated proteoglycan loss in articular cartilage. A: Human articular chondrocytes in alginate beads were treated with FGF-2 (50 ng/ml), in the presence or absence of pharmacological inhibitor of Ras, PKCδ, Raf, or ERK for 21 days. IL-1β treatment was used as a positive control. The alginate beads were processed for quantitative assays using the DMMB binding method. The proteoglycan levels in the cell-associated matrix were measured. Cell numbers in pellets were determined using PicoGreen. The total proteoglycan accumulation in each treatment group was normalized using the total DNA content ($^{*}P < 0.05$; $^{**}P < 0.01$). B: Chondrocytes in monolayer (isolated from grade 2/3 and OA cartilage) were treated with different doses of FGF-2 (1, 10, 50, 100, and 200 ng/ml) for 24 h, followed by total RNA extraction and cDNA synthesis. Aggrecan mRNA expression was quantitated by qPCR. 18S rRNA was used for normalization ($^{*}P < 0.05$; $^{**}P < 0.01$). C: Chondrocytes in monolayer were pre-incubated with inhibitor of Ras, Raf, ERK, or PKCδ for 1 h, and then stimulated by FGF-2 (100 ng/ml) for 24 h. Total RNA was extracted for qPCR quantitation of aggrecan expression. 18S rRNA was chosen for normalization ($^{*}P < 0.05$; $^{**}P < 0.01$). D: Chondrocytes were first transfected with PKCδ-specific siRNA, and then treated with FGF-2 (100 ng/ml) for 24 h. Total RNA was extracted for qPCR analyses of aggrecan expression. 18S rRNA was used for normalization ($^{*}P < 0.05$). E: Full-thickness cartilage explants with 4 mm diameters in serum-free media (plus mini-ITS^{TM}+ Premix) were treated with FGF-2 (100 ng/ml) or IL-1β (10 ng/ml), in the presence or absence of pharmacological inhibitor of PKCδ, Raf, or ERK for 11 days. The explants were fixed in 4% paraformaldehyde overnight, followed by paraffin embedding. Sections were prepared at 5 μm each. The sections were then deparaffinized, and stained with Safranin O to assess gross proteoglycan content in the extracellular matrix. a,a′ Control; (b) FGF-2 (100 ng/ml); (c) IL-1β (10 ng/ml); (d) FGF-2 plus rottlerin (2 μM); (e) IL-1β plus rottlerin (2 μM); (f) FGF-2 plus Raf inhibitor (10 μM); (g) IL-1β plus Raf inhibitor (10 μM); (h) FGF-2 plus ERK inhibitor (25 μM); and (i) IL-1β plus ERK inhibitor (25 μM). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

potential in the prevention of joint disease via inhibiting matrix-degrading enzyme production and maintaining aggrecan expression. Moreover, given that inhibition of PKCδ induces a more potent anti-catabolic effect than inhibition of Ras, our data suggest that development of PKCδ-specific inhibitors bears significance for OA treatments.

We further elucidated that the FGF-2-mediated activation of PKCδ is via FGFR1, which is independent of Ras activation. Yet PKCδ and Ras combine their signaling inputs to activate Raf-MEK1/2-ERK1/2, which lead to proteolytic enzyme expression in human articular cartilage. Together with our previous findings, inhibition of PKCδ or Ras alone effectively attenuated FGF-2-induced Raf-ERK1/

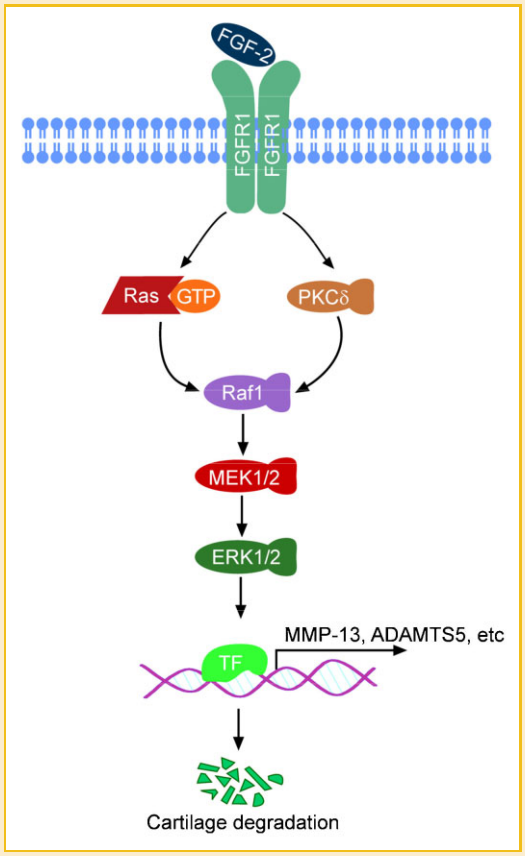

Fig. 7. Schematic model of FGF-2-triggered cartilage degeneration. FGF-2 binding to FGFR1 causes its dimerization and phosphorylation. Active FGFR1 then activates Ras and PKCδ independently, which converge on the Raf1-MEK1/2-ERK1/2 axis. As a result, downstream transcription factors (e.g., Elk-1) become active and launch the expression of target genes (e.g., MMP-13 and ADAMTS5). Production of proteolytic enzymes eventually facilitate cartilage breakdown. TF, transcription factor. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

2 signaling, downstream transcriptional responses, and proteoglycan loss [Im et al., 2007]. This suggests a possibility of combining Ras and PKCδ inhibitors in OA treatment for optimal efficacy and minimal toxicity. Our results also indicate that the Raf-ERK1/2 axis driven by both Ras- and PKCδ-mediated signals may serve as a critical regulatory component in human articular cartilage homeostasis, and hence a promising target for novel therapeutic strategies aiming at the treatment of cartilage degeneration.

We noticed that treatment of chondrocytes with FGF-2 show no significant catabolic effect on healthy and younger femurs (grade 0, <35-year-old) or healthy ankles (grade 0 or 1). For example, in a predominant fraction of cases FGF-2 (100 ng/ml) failed to repress aggrecan expression or induce cartilage-degrading enzyme expression (e.g., MMP-13, ADAMTS5) in chondrocytes isolated from grade 0 cartilage (unpublished data). By contrast, notable catabolic and anti-anabolic effects were observed when the same dose of FGF-2 was applied to damaged femoral cartilage (grade 2 or higher). These findings suggest a contextual property of FGF-2 in articular cartilage biology. Depending on the cellular status, FGF-2 may exert little or potent anti-anabolic and catabolic effects, increasing susceptibility of OA incidence after injury.

Despite the exciting therapeutic potential of pathway-specific inhibitors in degenerative joint disease treatment, it is necessary to recognize the limitations of this study before translation to a clinical setting. First, while we have defined the role of FGF-2-triggered signaling in human articular cartilage in vitro and ex vivo, the in vivo effects of anti-FGF-2 treatment on cartilage degeneration remain largely unknown. Second, further studies are warranted to identify potentially harmful side effects of pharmacological inhibitors of PKCδ, Ras, Raf, and ERK on other organs. Logically, given the avascularity nature of articular cartilage, intra-articular injection of pathway-specific inhibitors may be more feasible than systemic administration. Third, future pharmacological studies are also needed to analyze the kinetics of these inhibitors in vivo in order to optimize therapeutic doses. Finally, although we have demonstrated the potency of Rasi, PKCδi, Rafi, and ERKi in chondrocyte proteoglycan metabolism, we have not assessed their capacity to prevent and/or reverse cartilage degeneration in vivo. Therefore, further studies using an animal model are required to assess whether these inhibitors have a positive influence on cartilage regeneration and/or repair.

In summary, our results of this study revealed the complex signaling cascade initiated by FGF-2, which results in anti-anabolic and catabolic responses in adult human articular cartilage. FGF-2 binding to FGFR1 triggers the FGFR1-Ras-Raf-MEK1/2-ERK1/2 signaling pathway, as well as the FGFR1-PKCδ-Raf-MEK1/2-ERK1/2 pathway. These two pathways coordinate stimulation of matrix-degrading enzymes and depletion of proteoglycan in human articular cartilage. Activation of PKCδ by FGF-2 is independent of Ras activation, and both regulatory components are essential to the downstream biological effects. The Raf-ERK1/2 axis appears to integrate the inputs from both Ras and PKCδ, thus serving as key rate-limiting molecules in human articular cartilage degeneration. These findings provide deeper insights into the feasibility of utilizing FGF-2 pathway-specific inhibitors in prevention and/or treatment of degenerative joint and disc diseases in the future.

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