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Mechanical motion promotes expression of Prg4 in articular cartilage via multiple CREB-dependent, fluid flow shear stress-induced signaling pathways

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Lubricin is a secreted proteoglycan encoded by the Prg4 locus that is abundantly expressed by superficial zone articular chondrocytes and has been noted to both be sensitive to mechanical loading and protect against the development of osteoarthritis. In this study, we document that running induces maximal expression of Prg4 in the superficial zone of knee joint articular cartilage in a COX-2-dependent fashion, which correlates with augmented levels of phospho-S133 CREB and increased nuclear localization of CREB-regulated transcriptional coactivators (CRTCs) in this tissue. Furthermore, we found that fluid flow shear stress (FFSS) increases secretion of extracellular PGE2, PTHrP, and ATP (by epiphyseal chondrocytes), which together engage both PKA- and Ca++-regulated signaling pathways that work in combination to promote CREB-dependent induction of Prg4, specifically in superficial zone articular chondrocytes. Because running and FFSS both boost Prg4 expression in a COX-2-dependent fashion, our results suggest that mechanical motion may induce Prg4 expression in the superficial zone of articular cartilage by engaging the same signaling pathways activated in vitro by FFSS that promote CREB-dependent gene expression in this tissue.

[Keywords: CREB; PGE2; PTHrP; Prg4/lubricin; articular cartilage; extracellular ATP]

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While a great deal is known about the signals that regulate the formation and maturation of growth plate cartilage [for review, see Lefebvre and Bhattaram 2010], considerably less is known about the signals that regulate these processes in articular cartilage [for review, see Pacifici et al. 2005]. Growth plate and articular chondrocytes arise from distinct progenitor populations such that articular chondrocytes share a common origin with synovial cells that line the joint cavity [Koyama et al. 2008]. The superficial-most layer of articular cartilage is distinguished from deeper layers of articular cartilage (and from cartilage cells in the growth plate) by expression of lubricin. Lubricin is a secreted proteoglycan encoded by the Prg4 locus that is highly expressed by both superficial zone articular chondrocytes and synoviocytes [Marcelino et al. 1999]. Prg4 also encodes the related gene products superficial zone protein (SZP), megakaryocyte-stimulating factor [MSF], and hemangiopoietin [HAPO], representing alternative spliced transcripts and/or post-translationally modified proteins [for review, see Rhee et al. 2005]. Individuals with two mutant alleles of Prg4 have the camptodactyly-arthropathy-coxa vara pericarditis syndrome [CACP] [Marcelino et al. 1999] and display non-inflammatory synoviocyte hyperplasia and subintimal fibrosis of the synovial capsule [Bahabri et al. 1998]. Mice lacking Prg4 [Prg4−/−] appear normal at birth but subsequently display synovial hyperplasia, subintimal fibrosis, and an abnormal articular cartilage surface [Rhee et al. 2005]. In contrast, heterozygous Prg4 mutant [Prg4+/−]...
mice do not display these joint pathologies, consistent with the recessive nature of CACP (Rhee et al. 2005).

Col2-driven overexpression of lubricin in the articular cartilage has recently been shown to protect this tissue from degradation following surgically induced joint destabilization [Ruan et al. 2013], suggesting that lubricin may somehow counter the signaling pathways that lead to cartilage destruction. Hence, an understanding of the signals that regulate lubricin expression in the articular cartilage could potentially provide a therapeutic opportunity to modulate expression of this gene and thus stem the degradation of articular cartilage observed during osteoarthritis. While Wnt/β-catenin signaling has been found to be essential for expression of Prg4/lubricin during early development [Koyama et al. 2008; Yasuhara et al. 2011], the signals that maintain the expression of this locus in adult articular cartilage remain obscure. Studies have pointed to a role for both TGF-β signaling [Lee et al. 2008] and mechanical loading [Nugent et al. 2006a,b, Jones et al. 2009] in the maintenance of Prg4/ lubricin expression in either newborn or adult articular cartilage. In this study, we both document that voluntary wheel running induces the expression of the Prg4 locus in the superficial zone of knee joint articular cartilage and identify multiple signaling pathways downstream from fluid flow shear stress (FFSS) that promote the expression of Prg4 in this tissue in a CREB-dependent manner.

Results

Wheel running increases recombination driven by the Prg4GFPCreERt2 allele

We recently developed a strain of knock-in mice that express a tamoxifen-inducible Cre recombinase from the Prg4 locus [Prg4GFPCreERt2] [E Kozhemyakina, M Zhang, A lonescu, N Ono, A Kobayashi, H Kronenberg, ML Warman, and AB Lassar, in prep.]. To examine whether voluntary wheel running could affect the expression of Prg4 in knee joints, we employed 3-mo-old Prg4GFPCreERt2+/+; Rosa26floxlacZ/+ mice, which were housed individually either with or without access to a running wheel. After 3 wk of such housing, the animals were injected with tamoxifen for seven consecutive days and subsequently maintained either with or without their running wheels [experimental time course outlined in Fig. 1A]. Three days following the last tamoxifen injection, the animals were sacrificed, and expression of β-galactosidase in their knee joints was determined by X-gal staining [Fig. 1A–C]. Interestingly, we observed a near doubling in the number of cells expressing β-galactosidase in the knee joints of animals that had run [quantitated in Fig. 1C], with the greatest increase observed in the tibial plateau [Fig. 1B]. These results indicate that running animals display higher levels of Prg4GFPCreERt2-driven recombination in knee joint articular cartilage, suggesting that signaling pathways downstream from mechanical motion may increase the expression of this locus.

An increased number of β-galactosidase-expressing cells in the knees of Prg4GFPCreERt2+/+; Rosa26floxlacZ/+ mice that had run could reflect an increased level of expression from the Prg4 locus and/or increased proliferation of Prg4-expressing cells induced by running. To address the first possibility, we evaluated β-galactosidase expression in GFP-expressing cells by immunocytochemistry [which is more quantitative than X-gal staining] in the knees of Prg4GFPCreERt2+/+; Rosa26floxlacZ/+ animals that had been housed either with or without a running wheel. We observed both an increase in the number of GFP-expressing cells in the knees of animals that had run [Fig. 1D, middle panels] and markedly greater β-galactosidase fluorescence in the superficial-most cell layer of the tibial articular cartilage in running animals [Fig. 1D, bottom panels]. Increased β-galactosidase expression per cell in the superficial-most layer of tibial articular cartilage suggests that running increased the expression of the Prg4GFPCreERt2 locus in these cells, thus leading to earlier recombination of the Rosa26floxlacZ allele after initiation of tamoxifen administration. Consistent with this notion, we observed that running animals expressed greater levels of Prg4 mRNA in their articular cartilage, as assayed by RT-qPCR [Fig. 1E].

To examine whether wheel running increased the proliferation of pre-existing Prg4-expressing cells, we administered tamoxifen to Prg4GFPCreERt2+/+; Rosa26floxlacZ/+ mice for 7 d and then housed these animals for 31 d in either the absence or presence of a running wheel. Ten days prior to sacrifice [i.e., at 21 d with/without a running wheel], the animals were given seven consecutive daily pulses of Edu to label newly replicated DNA [experimental time course outlined in Supplemental Fig. 1A]. Most importantly, this regimen of tamoxifen administration prior to running did not significantly increase the number of β-galactosidase-positive cells in the articular cartilage [Supplemental Fig. 1B,C], indicating that the apparent increase in Prg4 expression following 1 mo of running [documented in Fig. 1A–E] reflected augmented expression of this locus in a greater number of articular cartilage cells rather than an expansion of pre-existing Prg4-expressing cells. Interestingly, however, running did increase a relatively small number of Edu-positive cells [Supplemental Fig. 1D, E] in both Prg4-expressing and -nonexpressing articular cartilage cells [Supplemental Fig. 1F], suggesting that mechanical motion does increase cellular proliferation in articular cartilage to a small extent.

FFSS promotes the secretion of a signaling molecule that in turn induces Prg4 expression in chondrocytes

Because running increased the expression of Prg4 in superficial zone chondrocytes, we explored whether FFSS could similarly induce expression of this gene. FFSS has been shown to transduce mechanical motion in osteocytes via both connexin and pannexin channels, which release both ATP and PGE2 into the extracellular space [Bao et al. 2004; Cherian et al. 2005; Li et al. 2005; Batra et al. 2012]. These signaling molecules in turn interact
Running and FFSS induce Prg4 expression

Figure 1. Wheel running increases Prg4 expression.  [A] Three-month-old female Prg4GFPCreERt2/+; Rosa26loxlacZ/+ mice were housed in either the absence or presence of a running wheel for 31 d. The animals were injected with tamoxifen daily from day 22 to 28 and sacrificed for X-gal staining at day 31.  [B] Whole-mount X-gal staining of the knee joints taken from either corn-oil-injected (TAM −/−) or tamoxifen-injected (TAM +/+; Rosa26loxlacZ−/−) mice that were housed either without (Control) or with (Running) a running wheel.  [C] Sections of the knee joints depicted in B (n = 6 running or 6 control mice). For each mouse, the total number of β-galactosidase-expressing cells in five sections of the lateral condyles and five sections of the medial condyles in both knees was quantitated.  [D] The knees of either control or running Prg4GFPCreERt2/+; Rosa26loxlacZ/+ mice (treated as outlined in A) were sectioned and immunostained with both anti-GFP and anti-β-galactosidase antibodies.  [E] Endogenous expression of Prg4 relative to β-actin was determined by RT-qPCR.  [F] Whole-mount X-gal staining of the knee joints of running Prg4GFPCreERt2/+; Rosa26loxlacZ−/− mice administered either a control gavage solution (Running) or such a solution containing Celecoxib (Running & Celecoxib).  [G] The knees of either six control or six Celecoxib-treated Prg4GFPCreERt2/+; Rosa26loxlacZ−/− mice (all housed with running wheels) were stained with X-gal and sectioned. The total number of β-galactosidase-expressing cells in five sections of the lateral condyles and five sections of the medial condyles was quantitated and normalized to the number of rotations of the running wheels (n = 6 control mice, n = 6 Celecoxib-treated mice). Throughout this and the following figures, significance was calculated using Student’s t-test. (*) P < 0.05; (**) P < 0.01. Error bar indicates standard error of the mean.

with their cognate receptors to alter gene expression in these cells. To examine whether mechanical motion/FFSS may act to similarly regulate Prg4 expression in articular cartilage, epiphyseal chondrocytes were isolated from 5-d-old Prg4GFPCreERt2/+; Rosa26loxlacZ−/− animals and cultured in tissue culture dishes on either a static surface or a rotary shaker, which delivered a steady laminar, nonpulsatile shear stress of ∼7.6 dyn/cm². The cells were harvested after exposure to either static conditions or FFSS for either 2, 4, 8, or 24 h (outlined in Fig. 2A). We monitored expression of both the Prg4GFPCreERt2 and the Prg4WT alleles by assaying expression of either GFP or Prg4, respectively, by RT-qPCR. We found that FFSS robustly induced the expression of both Prg4 alleles [Fig. 2B] and similarly induced both the secretion of Prg4 protein into the cell culture medium and the intracellular accumulation of GFP [Fig. 2C]. We also found that FFSS induced the expression of Prg4 in chondrocytes isolated from the superficial zone, but not the middle zone, of newborn bovine articular cartilage [Fig. 2D], indicating that the ability of FFSS conditions to induce the expression of Prg4 is regionally restricted in immature bovine articular cartilage. Consistent with this notion, we observed that, when cultured in the presence of tamoxifen, FFSS induced Prg4GFPCreERt2-driven recombination of the Rosa26loxlacZ allele in only a few percent (i.e., ∼3%) of epiphyseal chondrocytes that had been isolated from 5-d-old Prg4GFPCreERt2/+; Rosa26loxlacZ−/− animals [Supplemental Fig. 2]. Because articular cartilage precursors that will go on to express Prg4 are only a small percentage of epiphyseal chondrocytes at this stage of development [E Kozhemyakina, M Zhang, A Ionescu, N Ono, A Kobayashi, H Kronenberg, ML Warman, and AB Lassar, in prep.], it seems likely that superficial zone articular cartilage progenitors may be uniquely competent to express Prg4 in response to FFSS.

To determine whether FFSS induced the secretion of a signaling molecule that in turn induced Prg4 expression, we isolated conditioned medium from murine epiphyseal chondrocytes cultured under either static conditions (i.e., control-conditioned medium) or on a rotary shaker (i.e., FFSS-conditioned medium). The two conditioned mediums were then applied to epiphyseal chondrocytes cultured under static conditions (outlined in Fig. 2E). We found that, while FFSS-conditioned medium robustly induced the expression of endogenous Prg4...
in chondrocytes cultured under static conditions, control-conditioned medium failed to elicit this effect [Fig. 2F]. Thus, FFSS promotes the secretion of a signaling molecule that in turn induces Prg4 expression.

**FFSS promotes the secretion of PGE2 by chondrocytes, which is necessary for maximal expression of Prg4**

Because FFSS induces the secretion of PGE2 in osteocytes [Cherian et al. 2005; Li et al. 2005], we examined whether such is the case in chondrocytes. Indeed, we found that administration of FFSS to epiphyseal chondrocytes increased the expression of PGE2 in the medium [Fig. 3A]. Most importantly, depletion of >90% of PGE2 from FFSS-conditioned medium attenuated the ability of this medium to induce the expression of Prg4 in static chondrocytes by ~50% [Fig. 3B–D]. Thus, PGE2 is likely to be one component in FFSS that induces the expression of Prg4. Indeed, administration of PGE2 to chondrocytes cultured under static conditions was able to induce the expression of Prg4 but to a lesser extent than FFSS culture conditions [Fig. 3E]. Hence, PGE2 works synergistically with other components secreted into FFSS-conditioned medium to induce Prg4 expression. PGE2 has four distinct G-protein-coupled receptors: EP1 is coupled to G<sub>a11</sub>[α], EP2 and EP4 are coupled to G<sub>a</sub>[α], and EP3 is coupled to G<sub>a</sub>[α] [for review, see Sugimoto and Narumiya 2007]. We found that, while administration of the EP2 agonist Butaprost could also induce the expression of Prg4 in static epiphyseal chondrocytes, administration of the EP4 agonist CAY10598 did not elicit this effect [Fig. 3E]. Consistent with the notion that PGE2/EP2 signaling is necessary for maximal induction of Prg4 in response to FFSS, we observed that treatment of epiphyseal chondrocytes with either the COX-2 inhibitor Celecoxib (which blocks PGE2 synthesis) or the EP1/2 antagonist AH6809 blocked the induction of Prg4 by FFSS in these cells by ~50% [Fig. 3F]. In contrast, administration of either an EP1 antagonist (SC19220) or an EP4 antagonist (L161982) did not block the ability of FFSS to induce Prg4 expression [Fig. 3F]. Thus, FFSS-induced secretion of PGE2 and its paracrine interaction with EP2 account for ~50% of FFSS-induced expression of Prg4 expression in chondrocytes.

**Running induces maximal Prg4 expression in the knee joint via a COX-2-dependent pathway**

In addition to increasing secretion of PGE2 into the medium [Fig. 3A], FFSS also significantly increased [by sixfold] the expression of Ptgs2 [Supplemental Fig. 3A], which encodes COX-2, a critical enzyme controlling synthesis of PGE2. Interestingly, wheel running similarly...
increased the accumulation of PGE2 in the superficial zone of knee joint articular cartilage (Supplemental Fig. 3B, red arrows). To examine whether PGE2 synthesis is necessary for wheel running to induce the expression of Prg4 in knee joints, we housed 3-mo-old Prg4GFPCreER\textsubscript{2}/\textsuperscript{+}; Rosa26floxlacZ/\textsuperscript{+} mice in the presence of running wheels (as described above) and administered either a control gavage solution or such a solution containing Celecoxib, a COX-2 antagonist. Celecoxib administration markedly suppressed Prg4GFPCreER\textsubscript{2}-mediated recombination to \( \sim 65\% \) of that observed in control running animals (Fig. 1F,G), suggesting that mechanical motion boosts maximal Prg4 expression in articular chondrocytes via a COX-2-dependent pathway.

**FFSS enhances the expression of PTHrP, which also promotes the expression of Prg4 in epiphyseal chondrocytes**

Interestingly, we observed that FFSS administration to epiphyseal chondrocytes increased the expression of PTHrP (Fig. 3G), which signals via the G\( \alpha \text{q} \)-coupled PTH/PTHrP receptor PTh1r [for review, see Sakamoto et al. 2005]. To investigate whether PTHrP could similarly induce the expression of Prg4 in chondrocytes, we transfected static epiphyseal chondrocytes with either an empty expression vehicle (RCAS) or this expression vehicle encoding PTHrP (RCAS-PTHrP). We observed that overexpression of PTHrP induced Prg4 expression in static epiphyseal chondrocytes and, conversely, that knockdown of PTHrP with siRNA attenuated the ability of FFSS to induce Prg4 expression in these cells by \( \sim 30\% \) (Fig. 3H). Taken together, our findings suggest that FFSS promotes expression of Prg4 in chondrocytes via induction of both PGE2 and PTHrP secretion.

**FFSS promotes secretion of extracellular ATP, which is also necessary to induce maximal expression of Prg4 expression in articular chondrocytes**

As discussed above, FFSS has been shown to promote the secretion of both ATP and PGE2 in osteocytes [Bao et al. 2004; Cherian et al. 2005; Li et al. 2005; Batra et al. 2012]. Interestingly, we found that FFSS similarly induced
epiphyseal chondrocytes to secrete ATP into their medium (Fig. 4A). ATP has many types of extracellular receptors, including both ion channels (P2X receptors) and those coupled to G protein signaling (P2Y receptors). Because the P2X7 receptor for ATP has previously been demonstrated to play a role in both sensing mechanical loading in bones and transducing FFSS signaling in osteocytes (Bao et al. 2004; Li et al. 2005; Thi et al. 2012), we evaluated its role in promoting the expression of Prg4 in chondrocytes in response to FFSS. Administration of either ATP (1 mM) or BzATP (100 mM) to static chondrocytes induces secretion of PGE2 into the medium. Induction of Prg4 expression in epiphyseal chondrocytes by extracellular ATP was attenuated by the EP1/2 antagonist AH6809 (1 mM). FFSS-mediated induction of Prg4 in epiphyseal chondrocytes was assayed in the presence of either dominant-negative (DN) forms of either Gq/11 or Gq/11, siRNAs directed against either GAPDH (control) or P2Y2, or the calcineurin antagonist FK506. Addition of the Ca²⁺ chelator BAPTA attenuated induction of Prg4 by FFSS, and, conversely, addition of the Ca²⁺ ionophore A23187 induced the expression of Prg4 in static chondrocytes. Throughout this figure, Prg4 expression was assayed relative to β-actin by RT-qPCR. (* P < 0.05; ** P < 0.01. Error bar indicates standard error of the mean.

Figure 4. FFSS induces expression of Prg4 in articular chondrocytes via both ATP and PGE2 signaling pathways. (A) Concentration of ATP secreted into conditioned medium harvested from epiphyseal chondrocytes cultured under either FFSS or static conditions for the indicated time periods. (B) Administration of the P2X7 antagonists A438079 (1 and 10 μM) or Brilliant Blue-G (BBG; 1 and 10 μM) to epiphyseal chondrocytes significantly decreased induction of Prg4 by FFSS. (C) FFSS-mediated induction of Prg4 expression in epiphyseal chondrocytes isolated from either wild-type Balb/c mice (with P451 in P2X7), wild-type C57Bl/6J mice (with P451L in P2X7), or P2X7⁻/⁻ mice (in a C57Bl/6J background). (D) Increasing amounts of ATP (0.01, 0.1, 1, and 10 μM) were administered to static chondrocytes in either the absence or presence of PGE2 (0.1 μM). (E) Increasing amounts (1, 10, and 100 μM) of the P2X7 agonist BzATP robustly induce the expression of Prg4 in static chondrocytes. (F) Administration of either ATP (1 mM) or BzATP (100 μM) to static chondrocytes induces secretion of PGE2 into the medium. Induction of Prg4 expression in epiphyseal chondrocytes by extracellular ATP was attenuated by the EP1/2 antagonist AH6809 (1 μM). (H) FFSS-mediated induction of Prg4 in epiphyseal chondrocytes was assayed in the presence of either dominant-negative (DN) forms of either Gq/11 or Gq/11, siRNAs directed against either GAPDH (control) or P2Y2, or the calcineurin antagonist FK506. (I) Addition of the Ca²⁺ chelator BAPTA attenuated induction of Prg4 by FFSS, and, conversely, addition of the Ca²⁺ ionophore A23187 induced the expression of Prg4 in static chondrocytes. Throughout this figure, Prg4 expression was assayed relative to β-actin by RT-qPCR. (* P < 0.05; ** P < 0.01. Error bar indicates standard error of the mean.)
by extracellular ATP is attenuated by 50% in the presence of the EP1/2 antagonist AH6809 [Fig. 4G], again suggesting that ATP and PGE2 work in combination to induce expression of Prg4. Most importantly, just like FFSS administration, either exogenous PGE2 or ATP can induce Prg4 expression only in superficial zone [as opposed to middle zone] bovine articular chondrocytes [Fig. 2D], attesting to the unique competence of superficial zone articular chondrocytes to express this gene in response to PGE2 and/or ATP signaling pathways.

**Ca**++ signaling is critical for maximal expression of Prg4

Both the ligand-gated P2X7 ATP receptor and the Gq[alpha]/G11[alpha]-coupled P2Y2 ATP receptor have been demonstrated to be expressed in human primary chondrocytes [Millward-Sadler et al. 2004; Knight et al. 2009] and, upon ATP binding, to induce increased levels of intracellular Ca**++** [Millward-Sadler et al. 2004; Grol et al. 2012]. Thus, we investigated whether expression of dominant-negative (DN) forms of either Gq[alpha] or G11[alpha] or siRNA knockdown of P2Y2 would affect FFSS-mediated induction of Prg4. Indeed, we found that expression of either DN-Gq11[alpha] or siRNA against P2Y2 attenuated FFSS-induced Prg4 expression by ~35% [Fig. 4H]. Consistent with the idea that FFSS induces Prg4 expression in a Ca**++**-dependent fashion, we observed that addition of the Ca**++** chelator BAPTA to the cell culture medium significantly attenuated induction of Prg4 by FFSS [Fig. 4I] and, conversely, that addition of the Ca**++** ionophore A23187 robustly induced the expression of Prg4 in static chondrocytes [Fig. 4I]. In addition, we observed that inhibition of the calcium-regulated phosphatase calcineurin by administration of FK506, attenuated FFSS-induced Prg4 expression by ~40% [Fig. 4H], indicating that calcineurin activity is critical for maximal induction of Prg4 by FFSS.

Knockdown of pannexin 1 attenuates the ability of FFSS to induce expression of Prg4

Mechanical loading/FFSS signal transduction in osteocytes is mediated by ATP/PGE2 release from the cell via either the pannexin 1 channel [which is coupled to the P2X7 ATP receptor] [Bao et al. 2004; Li et al. 2005; Thi et al. 2012] or the connexin 43 hemichannel [Cherian et al. 2005]. Because either extracellular ATP or PGE2 is capable of inducing Prg4 expression in epiphyseal chondrocytes [Figs. 3, 4], we investigated whether FFSS-mediated induction of Prg4 expression in these cells similarly relied on pannexin 1. Indeed, we found that siRNA-mediated knockdown of mouse pannexin 1 significantly attenuated induction of Prg4 expression in murine epiphyseal chondrocytes by FFSS [Supplemental Fig. 4A], which was rescued by overexpression of human pannexin 1 [Supplemental Fig. 4E]. Consistent with prior work in osteoblasts, we observed that siRNA-mediated knockdown of pannexin 1 reduced the secretion of both ATP and PGE2 into the medium by chondrocytes that had been cultured under FFSS conditions [Supplemental Fig. 4B,C]. In addition, we observed that siRNA-mediated knockdown of connexin 43 could also attenuate FFSS-induced Prg4 expression and decreased secretion of both ATP and PGE2 in epiphyseal chondrocytes [data not shown]. Together, these results support the notion that induction of Prg4 expression in chondrocytes in response to FFSS is mediated via ATP/PGE2 release from the cell by the pannexin 1 channel and/or the connexin 43 hemichannel.

**FFSS engages a PKA/CREB signaling pathway to promote Prg4 expression**

In osteoblasts, administration of FFSS can induce signaling pathways that stabilize beta-catenin and thereby promote the activity of Lef/TCF transcription factors [Xia et al. 2010] and/or induce the activity of protein kinase A [Cherian et al. 2003]. Notably, while culture of epiphyseal chondrocytes under FFSS conditions resulted in increased activation of either TOP-luciferase [a Lef/TCF reporter] [Fig. 5A] or CRE-luciferase [a CREB reporter] [Fig. 5B], administration of either exogenous PGE2 or ATP induced expression of only the CRE-luciferase reporter [Fig. 5A,B]. Thus, extracellular PGE2 and ATP activate signaling pathways that specifically promote the activity of CREB transcription factors in chondrocytes. To evaluate whether activation of PKA was sufficient to induce the expression of Prg4, we either treated epiphyseal chondrocytes with forskolin [which increases the activity of adenylate cyclase] or transfected these cells with an activated form of PKA. We observed that activation of either PKA or adenylate cyclase was sufficient to induce the expression of Prg4 in epiphyseal chondrocytes cultured under static conditions [Fig. 5C]. To examine whether PKA signaling is necessary for FFSS to enhance Prg4 expression in epiphyseal chondrocytes, we examined whether administration of the PKA antagonist H89 would affect Prg4 induction by FFSS. Indeed, we found that H89 administration significantly attenuated the ability of FFSS to induce Prg4 expression by ~60% [Fig. 5D]. One potential pathway for PKA signaling to induce gene transcription is via PKA-mediated phosphorylation of the transcription factor CREB [on Ser133]. To examine whether CREB activity is necessary for FFSS to induce Prg4 expression, we transfected epiphyseal chondrocytes with an expression vehicle encoding ACREB, a dominant-negative form of CREB that has been previously used to block CREB activity both in vitro [Ahn et al. 1998] and in vivo [Long et al. 2001]. Indeed, transfection of an expression vehicle encoding ACREB attenuated the ability of FFSS to induce Prg4 expression [Fig. 5D]. Consistent with a role for CREB in promoting the induction of Prg4, we found that FFSS increased steady-state levels of both total and phospho-Ser133 CREB in epiphyseal chondrocytes [Fig. 5E] and also increased binding of phospho-Ser133 CREB to chromatin encompassing either of two CREB-binding sites [both of which contain the CRE consensus core-binding sequence CGTCA] located either upstream of the transcriptional start site (TSS) of Prg4 [i.e., CRE1, at ~4503 base pair [bp] relative to the TSS] or in the first intron of this gene [i.e., CRE2, at +679 bp relative to the TSS] [Fig. 5F]. In addition,
we found that FFSS could induce the expression [in transfected epiphyseal chondrocytes] of a luciferase reporter containing the Prg4 basal promoter driven by either reiterated consensus CRE-binding sites (CRE-firefly luciferase) or mutated CRE-binding sites (FOP-firefly luciferase) plus SV40 Renilla luciferase. The chondrocytes were cultured in the presence of either FFSS, exogenous PGE2 (0.01, 0.1, and 1 μM), ATP (0.1 and 1 mM), the Wnt agonist BIO (10 μM), or forskolin (FSK, 10 μM). The ratio of TOP/FOP luciferase activity (normalized to that of SV40 Renilla luciferase) is shown for each treatment regimen. (B) Epiphyseal chondrocytes were cotransfected with a luciferase reporter driven by reiterated consensus CRE-binding sites (CRE-firefly luciferase) plus SV40 Renilla luciferase. The chondrocytes were cultured in the presence of either FFSS, exogenous PGE2 (0.01, 0.1, and 1 μM), ATP (0.1 and 1 mM), the Wnt agonist BIO (10 μM), or FSK (1 μM). Relative luciferase activity (RLA; normalized to that of SV40 Renilla luciferase) is shown for each treatment regimen. (C) Transfection of epiphyseal chondrocytes [cultured under static conditions] with an expression vehicle encoding an activated form of PKA or treatment with FSK was sufficient to induce expression of Prg4. Prg4 expression was assayed relative to β-actin by RT-qPCR. (D) Administration of the PKA antagonist H89 or transfection with an expression vehicle encoding ACREB attenuated the ability of FFSS to induce Prg4 expression in epiphyseal chondrocytes. Prg4 expression was assayed relative to β-actin by RT-qPCR. (E) FFSS increases steady levels of both total and phospho-S133 CREB in epiphyseal chondrocytes. (F) FFSS increases binding of phospho-CREB to two conserved CRE-binding sites (CRE1 and CRE2) that surround the TSS of Prg4 in epiphyseal chondrocytes, as detected by chromatin immunoprecipitation. (G) Epiphyseal chondrocytes were cotransfected with a firefly luciferase reporter driven by the Prg4 basal promoter plus four repeats of Prg4 sequences encompassing either wild-type or mutant CRE1- or CRE2-binding sites plus SV40-Renilla luciferase. The cells were cultured under either static or FFSS conditions, and RLA is displayed. In both cases, mutation of the CRE consensus core-binding site completely eliminated induction of these reporters by FFSS. Throughout this figure: (*) P < 0.05, (**) P < 0.01. Error bar indicates standard error of the mean.

Either FFSS or running induces both an increase of phospho-S133 CREB and nuclear translocation of the CREB-regulated transcriptional coactivators 1/2 (CRTC1/2) in chondrocytes

In addition to P300/CREB, CREB interacts with another coactivator family, termed either transducers of regulated CREB activity [TORCs] or CRTCs [for review, see Altarejos and Montminy 2011]. The CRTC family consists of three members [CRTC1, CRTC2, and CRTC3]. Nuclear translocation and subsequent interaction of the CRTC proteins with CREB is blocked by phosphorylation of CRTC proteins by salt-inducible kinases 1/2 (SIK1/2), which phosphorylate CRTC proteins [on S151 in CRTC1 and on S171 in CRTC2] to generate an essential 14-3-3-binding site, thus inducing interaction of these proteins with cytoplasmic 14-3-3 proteins [for review, see Altarejos and Montminy 2011]. SIK-mediated phosphorylation of CRTC proteins is regulated by either CaMK 1/IV, which can phosphorylate SIK2, resulting in subsequent SIK2 ubiquitination and proteolysis [Sasaki et al. 2011], or PKA-mediated phosphorylation of SIK family members, which blocks the activity of these proteins [for review, see Altarejos and Montminy 2011]. In addition, the Ca**+-inducible phosphatase calcineurin can also remove the
SIK2 phosphorylation mark from CRTC proteins (for review, see Altarejos and Montminy 2011) and thereby promote the nuclear translocation of CRTC proteins and their subsequent interaction with CREB. Thus, either increased intracellular Ca$^{2+}$/activated calmodulin/CaMK or elevated PKA signaling blocks CRTC protein phosphorylation by SIK2 and renders CRTC1/2 proteins able to translocate into the nucleus and interact with CREB (schematically outlined in Fig. 7, below).

Because we found that FFSS promotes the induction of Prg4 expression in epiphyseal chondrocytes by inducing the activity of CREB, we therefore evaluated whether FFSS could induce dephosphorylation of phospho-S151 in CRTC1 and thereby induce nuclear translocation of this CREB coactivator. Indeed, we observed that FFSS administration to epiphyseal chondrocytes did indeed promote dephosphorylation of phospho-S151 in CRTC1 [Fig. 6A] and concomitantly induced a striking translocation of predominantly cytoplasmic CRTC1 [in static epiphyseal chondrocytes] into the nucleus [in FFSS-treated cells] [Fig. 6B]. Interestingly, application of extracellular ATP to static chondrocytes similarly induced nuclear translocation of CRTC1 [Fig. 6B]. Like CRTC1, nuclear translocation of CRTC2 was similarly promoted in epiphyseal chondrocytes by administration of either extracellular ATP or FFSS culture conditions [Supplemental Fig. 6]. In addition to promoting nuclear translocation of CRTC1/2, FFSS also induced the accumulation of phospho-S133 CREB into the nucleus of cultured epiphyseal chondrocytes [Fig. 6C].

To investigate whether mechanical motion in vivo similarly promoted accumulation of nuclear phospho-S133 CREB or nuclear translocation of CRTC1, we evaluated the expression [and localization] of both phospho-S133 CREB and CRTC1 in knee joint articular cartilage of mice that had been housed for 1 mo in either the absence or presence of a running wheel. We observed that wheel running induced a striking increase in phospho-S133 CREB levels in tibial articular cartilage in the knee [Fig. 6D]. Because PKA-mediated phosphorylation of CREB on Ser133 increases CREB interaction with P300/CBP [for review, see Altarejos and Montminy 2011], these findings suggest that wheel running promotes interaction of CREB with P300/CBP in articular chondrocytes. CRTC1 was predominantly located in the cytoplasm in tibial articular chondrocytes of mice housed in the absence of a running wheel, as evidenced by crescents of CRTC1 immunofluorescence in the cytoplasm surrounding DAPI-stained nuclei [Fig. 6E, left panels]. In contrast, in tibial articular chondrocytes of mice housed for 1 mo with a running wheel, CRTC1 levels were increased, and CRTC1 was evident in both the cytoplasm and the nucleus [i.e., overlapping with DAPI staining] [Fig. 6E, right panels]. Together, these findings indicate that running induces both an increase of phospho-S133 CREB and increased nuclear localization of CRTC1 in articular chondrocytes.

**Discussion**

Wheel running induces Prg4 expression via PGE2 signaling

Lifelong voluntary wheel running has been found to have a protective effect against the progression of osteoarthritis in mice that are heterozygous for mutations in the Col2a1 gene [Lapvetelainen et al. 2001]. Because Prg4...
expression in the articular cartilage both has been noted to be sensitive to mechanical loading [Nugent et al. 2006a,b, Jones et al. 2009] and was recently found to protect against the development of osteoarthritis [Ruan et al. 2013], we wondered whether voluntary wheel running might induce the expression of Prg4 in articular cartilage and thereby slow the progression of osteoarthritis. By employing Prg4<sup>GFP</sup>CreER<sup>2</sup>/+; Rosa26<sup>xfloXlacZ</sup>/4 mice, we found that voluntary wheel running (i.e., exercise) does indeed increase expression of the Prg4 locus, most significantly in the superficial-most cells in tibial articular cartilage. In addition, we also observed a slight increase in EdU incorporation in the articular cartilage following 1 mo of wheel running, but this augmented level of cell proliferation was not sufficient to significantly increase the pre-existing Prg4-expressing cell population. Running induced an increased accumulation of PGE2 in the superficial zone of knee articular cartilage, which correlated with significantly increased levels of both phospho-S133 CREB and nuclear-localized CRTC1 in this tissue. Most notably, the increased expression of Prg4 in articular cartilage induced by wheel running was significantly attenuated by administration of the COX-2 inhibitor Celecoxib, suggesting that PGE2 signaling is necessary for maximal expression of Prg4 in the articular cartilage following exercise. Muscular motion has previously been noted to be necessary for appropriate joint formation by promoting contraction-dependent activation of β-catenin in the developing joint [Kahn et al. 2009]. Seeing that activation of G<sub>α</sub>[α]-coupled receptors can stabilize β-catenin and thereby enhance Wnt signaling [Castellone et al. 2005; Ke et al. 2012], it will be interesting to determine whether the effects of muscular motion on early joint formation is also mediated by the induction of ligands such as PGE2 and/or PTHrP [discussed below], both of which signal via G<sub>α</sub>[α]-coupled receptors and are induced by mechanical motion in articular cartilage [Chen et al. 2008; this study].

**FFSS induces Prg4 expression via PGE2/PTHrP/ extracellular ATP/CREB signaling pathways**

FFSS has been shown to transduce mechanical motion in osteocytes via both connexin and pannexin channels, which release both ATP and PGE2 into the extracellular space that in turn interact with their cognate receptors to alter gene expression in these cells [Bao et al. 2004; Cherian et al. 2005; Li et al. 2005; Batra et al. 2012]. Indeed, mice engineered to lack the P2X7 ATP receptor display a striking reduction in the rate of periosteal bone formation [Ke et al. 2003], and administration of COX-2 inhibitors has been demonstrated to block mechanical load-induced increase in bone formation [Forwood 1996; Li et al. 2002]. Thus, mechanical loading induces bone formation via both ATP and PGE2 signaling pathways. In the case of PGE2, this pathway has been demonstrated to negatively regulate osteoblast-specific expression of sclerostin [Galea et al. 2011], which is an inhibitor of bone growth. In chondrocytes, we found that FFSS increases the expression of both Ptgs2 (which encodes the PGE2 biosynthetic protein COX-2) and PTHrP, promotes the secretion of both extracellular ATP and PGE2, and induces the expression of Prg4, which is specifically expressed in superficial zone articular chondrocytes. Indeed, we observed that administration of either FFSS, PGE2, or extracellular ATP was only able to induce the expression of Prg4 in superficial, but not deeper, zone bovine articular chondrocytes and in only 3% of epiphysial chondrocytes that had been isolated from 5-d-old Prg4<sup>GFP</sup>CreER<sup>2</sup>/+; Rosa26<sup>xfloXlacZ</sup> animals, thus suggesting that superficial zone articular chondrocytes (or their progenitors) express a competence factor necessary for expression of Prg4 in response to these signals.

It seems likely that both the ligand-gated P2X7 ATP receptor and the G<sub>α</sub>[α]/G<sub>11</sub>[α]-coupled P2Y2 ATP receptor play a role in FFSS-mediated induction of Prg4 expression, as knockout/knockdown of either receptor (in epiphysial chondrocytes) attenuates this response to FFSS. In addition, FFSS apparently induces maximal Prg4 expression in a Ca<sup>2+</sup>-dependent fashion, as we observed that addition of the Ca<sup>2+</sup> chelator BAPTA to the cell culture medium significantly attenuated induction of Prg4 by FFSS and, conversely, that addition of the Ca<sup>2+</sup> ionophore A23187 robustly induced the expression of Prg4 in static chondrocytes. Together, our findings suggest that FFSS-induced secretion of PGE2, PTHrP, and extracellular ATP works in synergy to promote Prg4 expression in superficial zone articular chondrocytes by engaging both PKA and Ca<sup>2+</sup> signaling pathways that increase levels of phospho-S133 CREB and promote dephosphorylation and nuclear accumulation of CRTC family members [summarized in Fig. 7]. In addition, we observed that FFSS increased binding of phospho-S133 CREB to two CRE-binding sites located in the regulatory regions of the Prg4 gene and noted that FFSS could induce the expression of a luciferase reporter driven by the Prg4 basal promoter plus four repeats of the Prg4 regulatory sequences encoding either of these CRE-binding sites. In both cases, mutation of the CRE consensus core-binding site completely eliminated induction of these reporters by FFSS. Most importantly, expression of a dominant-negative form of CREB (ACREB) [Ahn et al. 1998; Long et al. 2001] attenuated the induction of Prg4 by FFSS. Taken together, our findings strongly support the notion that either mechanical motion (i.e., wheel running) or FFSS induces the expression of Prg4 in superficial zone articular chondrocytes by promoting CREB-dependent induction of this gene.

While we observed that administration of FFSS of ~7.6 dyn/cm<sup>2</sup> can induce the expression of Prg4 in either a subpopulation of murine epiphysial chondrocytes or bovine superficial articular chondrocytes in vitro, it is unclear how this level of shear force compares with that experienced by articular chondrocytes in response to mechanical loading in vivo. On the other hand, because either administration of FFSS in vitro or wheel running both induce secretion of PGE2 and nuclear accumulation of phospho-CREB and CRTC1 and promote COX-2-dependent expression of Prg4 in articular chondrocytes, it seems likely that administration of FFSS in vivo or wheel
running induces these mechano-stimulated responses via similar signaling pathways.

The chondro-protective activities of either PTh1r signaling or wheel running may be due to the induced expression of Prg4

In addition to PGE2/EP2 signaling, Prg4 expression in the articular cartilage may also be induced via the PTH/PTHrP receptor PTh1r, which, like EP2, is also coupled to Gs, (for review, see Sakamoto et al. 2005). PThrP is known to be expressed in articular cartilage (Chen et al. 2008), and its expression has been shown to be induced in articular chondrocytes by joint loading (Chen et al. 2008) or FFSS administration to epiphyseal chondrocytes (Supplemental Fig. 3G), thus supporting a role for the PTh1 receptor (along with the PGE2 receptor EP2) in promoting the expression of Prg4 in response to wheel running/FFSS. Consistent with this idea, we observed that overexpression of PThrP induced Prg4 expression in static epiphyseal chondrocytes and, conversely, that knockdown of PThrP with siRNA attenuated the ability of FFSS to induce Prg4 expression in chondrocytes. Interestingly, conditional deletion of PThrPflox/flox from articular cartilage (as driven by GDF5-Cre) has been noted to cause increased cartilage degradation following surgically induced joint destabilization (Macica et al. 2011). In contrast, forced expression of transgenic Prg4 in articular cartilage (Ruan et al. 2013) and systemic administration of recombinant human PTH[1-34] (teriparatide) (Sampson et al. 2011) both significantly attenuated cartilage degeneration in vivo and FFSS applied to articular cartilage in vitro increase extracellular levels of PGE2, PTHrP, and ATP in this tissue. PGE2 and ATP escape from the cell via the P2X7-coupled pannexin 1 channel. PGE2 and PTHrP work via the Gs-coupled EP2 receptor and the PTH1 receptor, respectively, to activate PKA and induce phosphorylation of CREB-S133 (for review, see Altarejos and Montminy 2011). Extracellular ATP works via both the P2X7 (Grol et al. 2012) and the P2Y2 receptors to elevate intracellular Ca++, which in turn induces de-phosphorylation and nuclear translocation of CRTC family members CRTC1/2. Phospho-S133 CREB works in combination with nuclear CRTC1/2 to induce expression of Prg4 in articular chondrocytes.

Running and FFSS induce Prg4 expression in articular cartilage (which is itself chondro-protective in murine osteoarthritis models) (Ruan et al. 2013).

Materials and methods

Mouse strains, wheel-running regimen, tamoxifen and Celecoxib treatment, and EdU injection

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Harvard Medical School. Prg4GFPCreERt2+/Rosa26floxlacZ/+; Rosa26floxlacZ/+ mice (either 3-mo-old females [used in Fig. 1] or 2-mo-old males [used in Supplemental Fig. 1]) (E Kozhemyakina, M Zhang, A Ionescu, N Ono, A Kobayashi, H Kronenberg, ML Warman, and AB Lassar, in prep.) were housed individually either with or without a running wheel (9.5-in diameter) connected to a digital counter (Mini Mitter) for 31 d prior to sacrifice. Tamoxifen (2 g/kg body weight; Sigma) was administered daily by intraperitoneal [i.p.] injection for either seven or 10 consecutive days as indicated in the figure legends. EdU (50 mg/kg body weight; Invitrogen) was administered daily by i.p. injection for seven consecutive days (Supplemental Fig. 1). Celecoxib (50 mg/kg body weight; LC Laboratories) was administered daily by oral gavage for 31 consecutive days (Fig. 1F,G).

Isolation of mouse epiphyseal chondrocytes and bovine articular chondrocytes

Mouse epiphyseal chondrocytes were isolated from both the femoral heads and femoral and tibial condyles of 5-d-old mice as previously described (Goss et al. 2008). Mouse epiphyseal chondrocytes were isolated from either Prg4GFPCreERt2+/Rosa26floxlacZ/+ (C57BL/6 background), C57BL/6 wild-type, or BALB/c wild-type mice. Unless otherwise mentioned, cell culture experiments were performed with epiphyseal chondrocytes isolated from 5-d-old Prg4GFPCreERt2+/ mice. Bovine superficial zone and deeper zone articular chondrocytes were isolated from 1- to 2-wk-old bovine calves as previously described (Kisiday et al. 2005).
Culture of chondrocytes under static or FFSS conditions

Chondrocytes (mouse epiphyseal or bovine articular) were seeded at 2 × 10^5 cells per square centimeter into regular six-well tissue culture plates (culture plate diameter = 3.48 cm) (Corning) and cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 [DMEM/F-12] containing 10% Nu-Serum IV (BD Bioscience) and penicillin/streptomycin (culture medium) [1 mM] in a humidified air for 48 h (until cells were subsequently cultured under either static or FFSS conditions). Thirty minutes prior to exposure to subsequent static or FFSS conditions, medium was very gently changed (with 4 mL of new culture medium). For cultures exposed to culture dishes were placed onto an orbital shaker (with a 0.5-cm amplitude) for 4 mL of new culture medium. For cultures exposed to culture dishes were placed onto an orbital shaker (with a 0.5-cm amplitude) for

\[ \text{frequency of rotation (rotations per second)} = \frac{\text{angle of gyration of the shaker (in centimeters), \( \eta \) is the density of the medium (in grams per milliliter), \( \eta = 7.5 \times 10^{-4} \text{ dyn/cm}^3 \) at 37\(^\circ\)C, and \( f \) is the frequency of rotation (rotations per second)} \]

\[ [2 \pi f] \]

\[ (2 \pi f)^3, \]

\[ \text{where } \alpha \text{ is the radius of gyration of the shaker (in centimeters), } \rho \text{ is the density of the medium (in grams per milliliter), } \eta = 7.5 \times 10^{-4} \text{ dyn/cm}^3 \text{ at } 37\(^\circ\)C, \]

\[ \text{and } f \text{ is the frequency of rotation (rotations per second)} \]

\[ \text{(Pearce et al. 1996).} \]

Chondrocyte cultures in dishes left undisturbed in a similar 37\(^\circ\)C environment were used as static (no flow) controls. Small molecules used for cell culture included PGE2, Butaprost, ilar 37 and a steady laminar, nonpulsatile shear stress of 7.6 dyn/cm^2 at 37\(^\circ\)C, which exposed the chondrocytes to a steady laminar, nonpulsatile shear stress of 7.6 dyn/cm^2 at 37\(^\circ\)C, dish can be calculated as

\[ \text{frequency of rotation (rotations per second)} = \frac{\text{angle of gyration of the shaker (in centimeters), } \rho \text{ is the density of the medium (in grams per milliliter), } \eta = 7.5 \times 10^{-4} \text{ dyn/cm}^3 \text{ at } 37\(^\circ\)C, and } f \text{ is the frequency of rotation (rotations per second)} \]

\[ [2 \pi f] \]

\[ (2 \pi f)^3, \]

\[ \text{where } \alpha \text{ is the radius of gyration of the shaker (in centimeters), } \rho \text{ is the density of the medium (in grams per milliliter), } \eta = 7.5 \times 10^{-4} \text{ dyn/cm}^3 \text{ at } 37\(^\circ\)C, \]

\[ \text{and } f \text{ is the frequency of rotation (rotations per second)} \]

\[ \text{(Pearce et al. 1996).} \]

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Running and FFSS induce Prg4 expression