Excessive mechanical loading promotes osteoarthritis through the gremlin-1–NF-κB pathway

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Exposure of articular cartilage to excessive mechanical loading is deeply involved in the pathogenesis of osteoarthritis. Here, we identify gremlin-1 as a mechanical loading-inducible factor in chondrocytes, detected at high levels in middle and deep layers of cartilage after cyclic strain or hydrostatic pressure loading. Gremlin-1 activates nuclear factor-κB signalling, leading to subsequent induction of catabolic enzymes. In mice intra-articular administration of gremlin-1 antibody or chondrocyte-specific deletion of Gremlin-1 decelerates osteoarthritis development, while intra-articular administration of recombinant gremlin-1 exacerbates this process. Furthermore, ras-related C3 botulinum toxin substrate 1 activation induced by mechanical loading enhances reactive oxygen species (ROS) production. Amongst ROS-activating transcription factors, RelA/p65 induces Gremlin-1 transcription, which antagonizes induction of anabolic genes such as Sox9, Col2a1, and Acan by bone morphogenetic proteins. Thus, gremlin-1 plays essential roles in cartilage degeneration by excessive mechanical loading.
Osteoarthritis (OA) is the most prevalent joint disorder occurring with articular cartilage degradation. Since the establishment of experimental mouse models with surgically induced knee joint instability, a large number of studies have revealed the major molecules or signalling pathways responsible for OA, such as a disintegrin-like and metallopeptidase with a thrombospondin type 1 motif 5 (Adams5), matrix metalloproteinase-13 (Mmp13), hedgehog signalling, syndecan-4, Wnt signalling, and hypoxia-inducible factor 2-alpha (HIF-2α)14–16. In particular, Mmp13 is responsible for degradation of type 2 collagen (Col2a1), a major matrix protein component of articular cartilage, and plays essential roles in OA development6,10.

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein complex plays essential roles in various biological processes including cell survival, proliferation, differentiation, apoptosis, aging, inflammation, and immune responses14–16. It consists of v-rel reticuloendotheliosis viral oncogene homologue A (RelA, also known as p65), RelB, Rel, p105/p50, and p100/p52. These proteins form heterodimers to function as transcriptional activators. Inhibitors of NF-κB (IκkB) proteins, including IκBα, IκBβ, IκBγ, IκBe, IκBC, and Bcl-3, bind and sequester NF-κB family members within the cytoplasm17. In response to one of several signals, activation of IκkB kinases (IKKs) results in phosphorylated IκB proteins, which causes their degradation to enable free NF-κB complexes to translocate from the cytoplasm into the nucleus where they trigger target gene transactivation18. NF-κB signalling, which is widely involved in OA pathophysiology through various effects, is activated in osteoarthritic chondrocytes during aging and inflammation19. NF-κB signalling is essential to induce various inflammation-related factors, including Mmp proteins, inducible nitric oxide synthase (iNOS), interleukin 1 beta (IL-1β), tumour necrosis factor alpha (TNF-α), and HIF-2α. HIF-2α further induces various catabolic enzymes and OA-related genes6,8,9. Recently, we showed that NF-κB signalling regulates articular cartilage homeostasis and degeneration in a biphasic manner20. Although NF-κB signalling is inactivated in normal articular chondrocytes, a small amount of intranuclear RelA is required for transcriptional induction of anti-apoptotic genes that are indispensable for chondrocyte survival20. Phosphorylated IκBα and increased intranuclear RelA accompany cartilage degeneration, leading to induction of catabolic and inflammatory molecules and acceleration of OA development20.

In addition to molecular biology research, clinical and epimiologic studies previously revealed various factors to be associated with OA pathogenesis, including aging, obesity, joint instability, trauma, and joint inflammation. Excessive mechanical loading is regarded as the essence of several of these factors. A previous in vitro experiment using cell-stretcher systems showed induction of MMP13 by excessive mechanical loading21. NF-κB signalling is also regulated by mechanical loading19,22. However, molecular mechanisms underlying cartilage degeneration by excessive mechanical loading remain unknown.

Here, we describe a signalling pathway linking excessive mechanical loading to cartilage degeneration. We perform a screen for genes altered by mechanical loading, and focus on the NF-κB-related gene Gremlin-1 among the mechanical stress-inducible candidates. We examine its expression in articular cartilage, roles in in vitro and in vivo OA development, and further upstream and downstream pathways connecting excessive mechanical loading to cartilage degeneration.

**Results**

**Excessive stress loading induces gremlin-1 in chondrocytes.** We first examined a time-course of Mmp13 mRNA levels in mouse primary chondrocytes after 0.5 Hz, 10% cyclic tensile strain loading for 30 min. Mmp13 mRNA expression was slightly increased 1 h after loading, peaked by 12–24 h, and declined thereafter to baseline by 72 h after loading (Fig. 1a). To identify target genes mediating Mmp13 induction by stress loading, we performed microarray analysis using mRNA samples of chondrocytes before and 24 h after loading. Abundantly expressed genes upregulated or downregulated more than two-fold are shown in Supplementary Tables 1 and 2. Among upregulated and downregulated NF-κB-related genes identified by gene ontology analyses (Supplementary Tables 3 and 4), we focused on gremlin-1 (Greml1), which showed abundant expression and was most highly upregulated by mechanical loading in the group (Supplementary Table 3). Gremlin-1 is a secreted protein and known bone morphogenetic protein (BMP) antagonist13. mRNA and protein levels of gremlin-1, as determined by real-time RT-qPCR and immunocytochemistry, increased after loading (Fig. 1b, c).

Next, we examined Gremlin-1 mRNA expression in mouse femoral heads under cyclic hydrostatic pressure loading by the original, developed system (Supplementary Fig. 1a–e). Although Gremlin-1 mRNA levels were unchanged by 5-MPa loading, they were significantly increased by 20 MPa (Fig. 1d). Immunofluorescence determined that gremlin-1 protein was highly increased within the deep zone of the femoral head after 20 MPa loading (Fig. 1e). We next examined localization of gremlin-1 protein in the articular cartilage of a knee OA mouse model, which involves surgical resection of the medial meniscus and medial collateral ligament23. In normal mouse articular cartilage, gremlin-1 protein was predominantly localized within the deep layer (Fig. 1f). Two weeks after surgical induction, gremlin-1 was highly detected in and around deep layer chondrocytes (Fig. 1f). Its expression was slightly increased in the superficial zone after 4 weeks (Fig. 1f). We confirmed increased gremlin-1 protein levels in mouse primary chondrocytes, femoral heads, and articual cartilage using immunohistochemistry with a second primary antibody, and observed decreased protein levels using siRNA against gremlin-1 as a negative control (Supplementary Fig. 2a–d). We further examined gremlin-1 expression in human articular cartilage surgical specimens. Similar to the expression pattern in mouse cartilage, gremlin-1 protein was localized within the middle and deep layers of mildly degenerated human cartilage in the lateral tibial compartment (Supplementary Fig. 3). Gremlin-1 was intensively detected in the clustered chondrocytes in the superficial zone in severe human OA cartilage of the medial tibial compartment, as well as in and around deep layer chondrocytes (Supplementary Fig. 3).

**Gremlin-1 exerts catabolic effects in vitro.** To investigate the effects of gremlin-1, which is increased by excessive mechanical loading and/or during OA development, we administered recombinant human gremlin-1 (rhGREM1) protein to cultured chondrocytes. In mouse primary chondrocytes, catabolic marker genes such as Mmp13 and Adams5 were increased, while Sox9 (a master transcription factor for chondrocytes) and cartilage matrix genes Col2a1 and Acan were decreased by rhGREM1 in a dose-dependent manner, and time-dependent manner up to 24 h (Fig. 2a, and Supplementary Fig. 4). Similar results were obtained using rhGREM1 manufactured by a second company (Supplementary Fig. 5). Type 10 collagen (Col10a1), a hypertrophic chondrocyte marker, was not changed by rhGREM1 (Supplementary Fig. 6). In ex vivo cultures of femoral head cartilage from wild-type (WT) mice, release of proteoglycans into the medium was increased by rhGREM1 treatment in a dose-dependent manner (Fig. 2b), while mRNA levels of marker genes within femoral heads were altered similarly to cultured chondrocytes (Fig. 2c).
Gremlin-1 exerts catabolic effects on mouse cartilage. We next examined the effects of gremlin-1 in vivo. The surgical OA model was prepared by injecting rhGREM1 into the knee joints of WT C57BL/6J male mice at 8 weeks of age, twice a week. Eight weeks after surgical induction, OA development was significantly enhanced by intra-articular administration of rhGREM1 (Fig. 3a). Chondrocyte apoptosis determined by TUNEL staining was unchanged by rhGREM1 treatment (Fig. 3b). Mmp13, Adamts5, IκBα, phosphorylated IκBα, and HIF-2α were highly detected in the remnant cartilage of rhGREM1-treated groups (Fig. 3c).

Moreover, intra-articular injection of rhGREM1-induced OA development even in normal mouse knee joints (Supplementary Fig. 7).

In a contrasting approach, we used an antibody against gremlin-1. To confirm the neutralizing effect of the commercially obtained antibody, we treated mouse primary chondrocytes with rhGREM1 and gremlin-1 antibody. rhGREM1-induced increases in Mmp13 and Adamts5 were significantly reduced by antibody treatment (Supplementary Fig. 8). We then injected the antibody into the knee joints of OA model mice twice a week. Eight weeks after surgery, OA progression was significantly suppressed by antibody injection (Fig. 3d). Moreover, chondrocyte apoptosis, expression of catabolic marker proteins, and phosphorylated IκBα were also decreased (Fig. 3e, f).

Conditional knockout of gremlin-1 suppresses OA development. To reveal the physiological roles of gremlin-1 in articular cartilage, we performed in vivo loss-of-function analyses using transgenic mice in which Cre recombinase was fused to a mutated ligand-binding domain of the human oestrogen receptor driven by the Col2a1 promoter (Col2a1-CreERT2) to knockout gremlin-1 in adult articular cartilage after skeletal growth. Thus, administration of the oestrogen antagonist tamoxifen resulted in translocation of the fusion protein into nuclei causing gene targeting. We generated tamoxifen-inducible chondrocyte-specific homozygous gremlin-1 knockout mice by mating Col2a1-CreERT2 mice with Grem1^{0/0} mice (Col2a1-CreERT2;Grem1^{0/0}). Col2a1-CreERT2;
Grem1 (cKO) mice developed normally and displayed no skeletal abnormalities compared with Grem1 (Cntl) littermates (Supplementary Fig. 9). Tamoxifen was injected into 7-week-old cKO and Cntl littermates daily for 5 days, and the surgical OA model was induced 2 days after the last injection. Eight weeks after surgical induction, OA development was significantly inhibited in cKO knee joints compared with Cntl joints (Fig. 4a). Chondrocyte apoptosis and expression of catabolic enzymes HIF-2α and phosphorylated IκBα were suppressed by gremlin-1 knockout (Fig. 4b, c).

We further examined OA development with aging. Tamoxifen was injected into cKO and Cntl littermates daily for 5 days at 8 weeks, 6 months, and 12 months. At 18 months of age, cKO mice were significantly resistant to cartilage degeneration compared with control littermates (Fig. 4d), and showed no skeletal abnormalities (Supplementary Fig. 9). Chondrocyte apoptosis and expression of catabolic enzymes HIF-2α and phosphorylated IκBα were suppressed by gremlin-1 knockout, similar to results observed in the surgical OA model (Fig. 4e, f). Efficient knockout of gremlin-1 in articular chondrocytes was confirmed at both mRNA and protein levels (Fig. 4c, f, Supplementary Figs. 10, and 11).

**Gremlin-1 activates NF-κB signaling.** We further analysed molecular mechanisms underlying the catabolic effects of gremlin-1 in articular cartilage by performing a luciferase assay using reporter vectors containing response elements for representative signalling pathways. Among them, the NF-κB reporter vector showed the highest transactivity induced by rhGREM1 (Fig. 5a). When various amounts of rhGREM1 were applied to cells transfected with the NF-κB reporter vector, transactivity was increased in a dose-dependent manner (Fig. 5b). In addition to rhGREM1, we next treated mouse femoral head cartilage...
with the IKK inhibitor BMS-345541. Increased release of proteoglycans into the medium by rhGREM1 treatment was significantly inhibited by BMS-345541 to a level similar to control (Fig. 5c). Femoral heads from RelA\textsuperscript{fl/fl} (RelA-Cntl) and Col2a1-Cre;RelA\textsuperscript{fl/fl} (RelA-cKO) mice were also treated with rhGREM1. Release of proteoglycans was enhanced by rhGREM1 in RelA-Cntl femoral heads; however, it was not enhanced in RelA-cKO femoral heads (Fig. 5d). Moreover, rhGREM1-induced increases in Mmp13 and Adamts5 were not observed in RelA-cKO femoral heads, and decreases in anabolic marker genes including Sox9, Col2a1, and Acan were not rescued (Fig. 5e). These data indicate that gremlin-1 induces catabolic enzymes through NF-κB signalling, but does not suppress anabolic genes in the same pathway.
We next examined which molecule functions as a receptor for gremlin-1 to stimulate NF-κB signalling. Among several candidates from previous reports, we focused on vascular endothelial growth factor receptor 2 (VEGFR2), which is known to bind to gremlin-1. Using the selective VEGFR2 inhibitor SU5416 in combination with rhGREM1, we found that increased Mmp13 and Adamts5 by rhGREM1 were suppressed by SU5416 in a dose-dependent manner (Supplementary Fig. 12a). Moreover, SU5416 did not affect expression of Sox9, Col2a1, or Acan, similar to RelA-cKO (Fig. 5e, Supplementary Fig. 12a). The rate of p-VEGFR2-positive cells increased 10 min after rhGREM1 treatment, while that of VEGFR2-positive cells was unaltered (Supplementary Fig. 12b). mRNA levels of VEGF family members were not upregulated by rhGREM1 treatment in mouse primary chondrocytes (Supplementary Fig. 13).

Gremlin-1 antagonizes the anabolic effects of BMPs. Gremlin-1 treatment increased the expression of catabolic enzymes Mmp13 and Adamts5, while decreasing the expression of cartilage matrix genes Col2a1 and Acan (Fig. 2a). Among these alterations, inhibition of NF-κB signalling almost completely suppressed the increase of catabolic enzymes by gremlin-1 (Fig. 5c–e), but it did not affect observed decreases of catabolic factors (Fig. 5e).

We next examined antagonistic effects between gremlin-1 and BMPs. In mouse primary chondrocytes, rhBMP-2, rhBMP-4, and rhBMP-7 enhanced the expression of anabolic markers (Fig. 6). Anabolic effects elicited by rhBMPs were diminished by rhGREM1 treatment (Fig. 6).

**Discussion**

The present study identified gremlin-1 as a key factor mediating cartilage degeneration by excessive mechanical loading. Gremlin-1 activates NF-κB signalling, which results in induction of catabolic enzymes such as Mmp13 and Adamts5. The Rac1–ROS–NF-κB axis plays an essential role in gremlin-1 induction by excessive mechanical loading. Genetic deletion or neutralization of gremlin-1 by intra-articular injection of an antibody suppressed OA development in mice.

Gremlin-1 is a well-known secreted BMP antagonist that was formerly identified as a regulator of limb formation. Gremlin-1 exerts potent inhibitory action via binding to and forming heterodimers with BMP-2, BMP-4, and BMP-7. Binding to selective BMPs prevents ligand–receptor interactions and subsequent downstream signalling. Indeed, gremlin-1 regulates development of limb, lung, kidney and retina through BMP inhibition. In addition to its inhibitory effect on BMP, gremlin-1 exerts direct effects on cell function via
BMP-independent mechanisms. For example, VEGFR2 was identified as a novel receptor for gremlin-1. The gremlin-1–VEGFR2 axis exerts a proinflammatory effect, which is mediated by activation of NF-κB signalling. In addition to its roles in the development of various organs, gremlin-1 has been implicated in many diseases and pathogenic mechanisms, such as heart, lung and liver fibrosis, as well as osteogenesis, angiogenesis and cancer—some of which may be mediated by this axis.

Considering that the induction of catabolic factors by gremlin-1 was diminished by inhibition of VEGFR2 or NF-κB in the present data, the axis is also responsible for cartilage degeneration by gremlin-1. However, we cannot exclude the possibility that carrier proteins in rhGREM1 solution may exert some catabolic effects in intra-articular injection experiments.
Fig. 4 Regulation of osteoarthritis development by gremlin-1. a Safranin O staining and OARSI scores of mouse knee joints of Grem1fl/fl (Cntl) and Col2a1-CreERT2;Grem1fl/fl (cKO) mice 8 weeks after surgery. Tamoxifen induction was performed at 7 weeks. Both experimental groups consist of n = 9 biologically independent animals. Inset boxes indicate regions of immunofluorescence in (c). Scale bars, 100 μm. b TUNEL staining and rate of TUNEL-positive cells in mouse knee joints of Cntl and cKO mice 8 weeks after surgery. Nuclei were stained with DAPI (blue). Scale bars, 50 μm. The percentage of positive cells in the immunofluorescence is shown below. n = 5 biologically independent experiments. c Safranin O staining and immunofluorescence of Mmp13, Adamts5, IκBα, phosphorylated IκBα (dual Ser32/36), HIF-2α, and gremlin-1 proteins in mouse knee joints of Cntl and cKO mice 8 weeks after surgery. Scale bars, 50 μm. The percentage of positive cells in the immunofluorescence is shown below. n = 5 biologically independent experiments. d Safranin O staining and OARSI scores of mouse knee joints of Cntl and cKO mice at 18 months of age. Tamoxifen induction was performed for 5 days at 8 weeks, 6 months, and 12 months. Both experimental groups consist of 5 biologically independent experiments. Inset boxes indicate regions of immunofluorescence in (e). Scale bars, 100 μm. e TUNEL staining and rate of TUNEL-positive cells in mouse knee joints of Cntl and cKO mice at 18 months of age. Tamoxifen induction was performed at 7 weeks. Both experimental groups consist of 5 biologically independent experiments. (Student’s unpaired two-tailed t-test)

Fig. 5 NF-κB signalling mediates the catabolic effects of gremlin-1. a Luciferase assay for screening of downstream signalling pathways of gremlin-1 using reporter vectors containing a response element for each pathway or transcription factor. *P < 0.05 versus each vehicle control (one-way ANOVA test). b Activities of luciferase reporter vectors containing an NF-κB motif with rhGREM1 treatment. *P < 0.05 versus 0 (vehicle) (one-way ANOVA test). c Amount of proteoglycan released into culture medium from wild-type mouse femoral heads cultured with or without rhGREM1 (10 μg/mL) and IKK inhibitor BMS-345541 (5 μM) for 3 days. #P < 0.005 (one-way ANOVA test). d Amount of proteoglycan released into culture medium from RelAΔfl/Δfl (Rela–Cntl) and Col2a1-Cre/RelAΔfl/Δfl (Rela–cKO) m femoral heads cultured with or without 10 μg/mL rhGREM1, #P < 0.001 (one-way ANOVA test). e mRNA levels of marker genes in RelA–Cntl and RelA–cKO femoral heads cultured with or without rhGREM1. ##P < 0.001 (one-way ANOVA test). All data are expressed as mean ± SD of biologically independent three samples per group.

In contrast to these catabolic effects, downregulation of cartilage matrix gene expression by gremlin-1 was not dependent on the NF-κB pathway. Moreover, the present in vitro data revealed that gremlin-1 suppressed induction of Sox9, Col2a1, and Acan by rhBMPs (Fig. 6). BMPs play essential roles in bone and cartilage formation, and have been extensively used in cartilage regenerative research. Exogenous BMP-2 or BMP-4 enhance chondrocyte differentiation of stem cells and cartilage matrix production by chondrocytes. BMP-7 also exhibits chondrogenic effects and the ability to repair cartilage and suppress
BMP-2, which is highly expressed in the hypertrophic zone, promotes hypertrophic differentiation of chondrocytes in the proliferative zone. BMP-4 expression is very low in the growth plate, while BMP-7 is more highly expressed in the hypertrophic zone than in the hypertrophic zone. Hypertrophic differentiation is involved in OA development through downregulation of anabolic genes by antagonizing BMPs.

BMPs are also involved in hypertrophic differentiation during endochondral ossification. BMP-2, which is highly expressed in the hypertrophic zone, promotes hypertrophic differentiation of chondrocytes in the proliferative zone. BMP-4 expression is very low in the growth plate, while BMP-7 is more highly expressed in the hypertrophic zone than in the hypertrophic zone. Hypertrophic differentiation is involved in OA development through downregulation of anabolic genes by antagonizing BMPs.

The Rho family of small GTPases, such as Rac, Cdc42, and RhoG, are master regulators of many cellular activities. Conformationally regulated by the binding of GTP and GDP, GTPases actively function with their downstream target proteins when bound to GTP. Among various types of stimulants, mechanical loading activates Rac1 through effects on the actin cytoskeleton. Rac1 binds the complex of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes to enhance ROS production in a variety of cell types in response to mechanical loading. Activation of the Rac1-ROS pathway enhances transcription of several molecules, such as NF-κB, HIF-1α, c-Jun, and Nrf2. Amongst upregulated transcription factors, RelA strongly induced gremlin-1 expression. Luciferase assays revealed functional NF-κB consensus motifs within the proximal 5′-flanking region of the GREM1 gene. In turn, gremlin-1 exerts catabolic effects through the NF-κB pathway. In fact, Mmp13 mRNA levels are slightly and transiently increased one hour after stress loading, and peak by 12–24 h after loading. Early and late increases of Mmp13 mRNA may result from activation of NF-κB signalling by Rac1-ROS and gremlin-1, respectively. Thus, iterated intensive mechanical loading causes gremlin-1 protein to excessively accumulate within the deep layer of articular cartilage, where it may function to amplify mechanical stress-induced NF-κB activation. However, it is difficult to discern the entire hierarchical relationship between these molecules in one experimental system. The hypothesis shown in Fig. 8e should be examined by following studies in the future.

In conclusion, we found that induction of gremlin-1 by excessive mechanical loading occurs via the Rac1-ROS-RelA/p65 pathway.
axis, which accelerates OA development mainly through the NF-κB pathway. Our findings contribute to understanding of the pathophysiology of various diseases in which mechanical loading, gremlin-1, and NF-κB are involved, including OA.

Methods

Cell cultures. Mouse chondrogenic ATDC5 cells (RIKEN Cell Bank) were grown and maintained in Dulbecco’s Modified Eagle’s Media with F-12 supplement (DMEM/F-12, 1:1) with 5% fetal bovine serum (FBS). Primary articular chondrocytes were isolated from 6-day-old C57BL/6j mice according to a standard protocol using collagenase I[21]. Primary chondrocytes were cultured in DMEM with 10% FBS. The medium was changed every 3 days. For most experiments, primary cells were transferred to serum-free DMEM for 24 h before being exposed to stimuli. In some experiments, cells were pretreated with recombinant human gremlin-1 (rhGREM1, #120-42, PeproTech for Figs. 2, 3, 5, 6, Supplementary Figs. 4, 6–8, 12, and 13; Selleck Chemicals), recombinant human BMP-4 (rhBMP4, #120-05, PeproTech), recombinant human BMP-7 (rhBMP7, #120-03, PeproTech), I KK inhibitor BMS-345541 (B9935, Sigma), selective VEGFR2 inhibitor SU5416 (ab145056, Abcam), FAK inhibitor PF-573228 (S0213, Selleck Chemicals), ROCK inhibitor Y-27632 (257-00511, WAKO), Rac1 inhibitor NSC23766 (SML9952, Sigma) and/or EHT1864 (S7482, Selleck Chemicals). For gene silencing experiments, siRNA against mouse gremlin-1 (#1320001, Thermo Fisher Scientific) or negative control (#12935300, Thermo Fisher Scientific) were transfected into mouse primary chondrocytes using Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. Cells were harvested 24 h after transfection.

Animals. All animal experiments were authorized by the Animal Care and Use Committee of The University of Tokyo. We have complied with all relevant ethical regulations. In each experiment, we compared the genotypes of littermates maintained in a C57BL/6j background. Col2a1-CreERT mice[2] and RelAfl/fl mice[3] were generously provided by Professor Fanxin Long (Washington University, St. Louis) and Professor Roland M. Schmid (Technical University of Munich), respectively. To generate Col2a1-Cre; RelAfl/fl and Col2a1-CreERT;RelAfl/fl mice, RelAfl/fl mice were mated with Col2a1-Cre or Col2a1-CreERT mice to obtain Col2a1-Cre;RelAfl/fl and Col2a1-CreERT;RelAfl/fl mice, respectively, which were then mated with RelAfl/fl mice. Col2a1-CreERT mice and Gremlin-1fl/fl mice were generated as previously described[5]. To generate Col2a1-CreERT;Gremlin-1fl/fl mice, Gremlin-1fl/fl mice were mated with Col2a1-CreERT mice to obtain Col2a1-CreERT;Grem1fl/fl mice, which were then mated with Gremlin-1fl/fl mice. To confirm knockout efficiency, we directly isolated mRNA from the articular cartilage of 16-week-old Grem1fl/fl and Col2a1-CreERT;Grem1fl/fl animals that had received tamoxifen administration at 7 weeks of age. For animal studies, no randomization was used.

Human samples. We obtained samples of human articular cartilage undergoing total knee arthroplasty. Written informed consent was obtained from all individuals, and approval was provided by the ethics committee of the University of Tokyo. We have complied with all relevant ethical regulations.

Cyclical tensile strain loading of mouse primary chondrocytes. Mouse primary chondrocytes were seeded into silicon stretch chambers coated with fibronectin at a density of 1 x 10⁶ cells/chamber; each chamber had a culture surface of 2 x 2 cm. After 48 h, cyclical tensile strain (0.5 Hz, 10% elongation) was applied for 30 min using an STB-140 mechanical stretch system (STREX, Osaka, Japan) in a CO₂ incubator[21]. Control cells were seeded onto the same chambers and cultured without cyclical tensile strain.

Cyclical hydrostatic pressure loading of mouse femoral head explants. The cyclical hydrostatic pressure loading system was developed by STREX. Femoral heads obtained from 3-week-old mice were put into the chamber, which consisted of gas permeable membranes with a metallic frame (Supplementary Fig. 1a); each chamber was filled with DMEM/F-12 (1:1) containing 5% FBS and set into a cylindrical container (Supplementary Fig. 1a-c). Cyclical hydrostatic compression pressure was applied by the pumping machine connected to the cylindrical container (Supplementary Fig. 1a-c). We employed 5 and 20 MPa for normal and excessive stress loading, respectively, because maximum pressure to human knee cartilage ranges from 3 to 9 MPa during a normal gait[23-24]. We applied 5 or...
20 MPa hydrostatic pressure to mouse femoral heads for 30 min at a frequency of 0.1 Hz. Control femoral heads were put into the same chambers and cultured without cyclic hydrostatic pressure.

**qRT-PCR.** Total RNA was purified with an RNeasy Mini Kit (Qiagen). Microarray experiments were performed using SurePrint G3 Mouse Gene Expression 8 × 60 K (Agilent Technologies). Arrays were then scanned with an Agilent DNA Microarray Scanner (G2565CA), and acquired array images were analyzed by Agilent Feature Extraction software (version 10.10.1.1).

**Microarray analysis.** Total RNA was isolated from mouse primary chondrocytes before and 24 h after cyclic tensile strain loading using an RNeasy Mini Kit (Qiagen). Microarray experiments were performed using SurePrint G3 Mouse Gene Expression 8 × 60 K (Agilent Technologies). Arrays were then scanned with an Agilent DNA Microarray Scanner (G2565CA), and acquired array images were analyzed by Agilent Feature Extraction software (version 10.10.1.1).

**Histological analyses.** Tissue samples were fixed in 4% paraformaldehyde buffered with phosphate-buffered saline (PBS, pH 7.4) at 4 °C for 1 day. Specimens were decalcified with 10% EDTA (pH 7.4) at 4 °C for 2 weeks, embedded in paraffin, and 4-µm thick sagittal sections were cut from specimens. Safranin O staining was performed according to standard protocols. For immunohistochemistry, sections were incubated with antibodies against gremlin-1 (1:250; LS-C125371, LSBio for Fig.1c; 1:250, AF956, Santa Cruz Biotechnology), p-IκBα (1:100; sc-101713, Santa Cruz Biotechnology), and/or HIF-2α (1:200; NB100-122, Novus Biologicals). For immunofluorescence, a CSA II Biotin-Free Catalyzed Amplification System (Agilent Technologies) and Hoechst 33258 (Agilent Technologies) counterstain were used. TdT-mediated dUTP nick end labelling (TUNEL) staining was performed with an In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions. Histological analyses were performed at least three times using 2-3 mice per group or genotype for confirmation of results. Images were visualized under a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan). Counts of immunofluorescence-positive cells were calculated in five independent squares (100 × 100 µm) of articular cartilage harvested from the mouse medial tibial plateau, using BZ analyser software (Keyence). The same parameters were used for all acquisitions, and representative pictures are shown in the figures.

**Immunocytochemistry.** Cells were fixed with 4% paraformaldehyde buffered with PBS (pH 7.4). After incubation for 10 min in PBS containing 0.1% Triton X-100 (Sigma Aldrich), cells were rinsed in PBS, and treated with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich). Samples were stained using primary antibodies against Grem1 (1:500, LS-C125371, LSBio for Fig. 1a; c and Additional file 2a), VEGFR2 (1:500, 55B11, Cell Signaling Technology for Supplementary Fig. 12b) and p-VEGFR2 (1:500, Tyr951, #2471, Cell Signaling Technology for Supplementary Fig. 12b) for 24 h in PBS containing 1% BSA at 4 °C, followed by AlexaFluor 488-conjugated mouse and rabbit secondary antibodies (1:500, Invitrogen) for 1 h. Slides were then treated with Vectashield Mounting Medium with DAPI (H-1200, Vector Laboratories) and Hoechst 33258 (Agilent Technologies) counterstain were used. Immunocytochemistry was performed with 2-3 mice per group or genotype for confirmation of results. Images were visualized under a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan). Counts of immunofluorescence-positive cells were calculated in five independent squares (100 × 100 µm) using BZ analyser software (Keyence).
and Greml1−/− mice daily for 5 days. A surgical procedure was then performed to establish an experimental OA model in 8-week-old male mice. Under general anesthesia, resection of the medial collateral ligament and medial meniscus was performed using a surgical microscope. Mice were analysed 8 weeks after surgery. We also analysed OA development with aging using 18-month-old mice bred under physiological conditions. For the aging experiments, tamofoxifen induction was performed for 5 days at 8 weeks, 6 months, and 12 months. All mice were maintained under the same conditions (three mice per condition), and OA severity was quantified by the Osteoarthritis Research Society International (OARSI) system, which was assessed by two observers blinded to the experimental groups.

Intra-articular administration of rhGREM1 or gremlin-1-neutralizing antibody. We performed intra-articular administration of rhGREM1 or gremlin-1-neutralizing antibody twice a week for 8 weeks after OA surgery, or normal mice without surgery. For each administration, we injected 10 µL of 10 µg/mL rhGREM1 solution diluted in PBS, 10 µL of 10 µg/mL gremlin-1-neutralizing antibody (LS-Cl25371, LSbio) solution diluted in PBS, or 10 µL of PBS as a vehicle control.

Proteoglycan release assay. Proteoglycan release from mouse femoral head explants was analysed according to the standard protocol. Briefly, femoral heads were harvested from 3-week-old WT or RelAfl/fl and Col2a1-Cre-RelAfl/fl mice, and cultured for 3 days with or without rhGREM1 (10 µg/mL) in DMEM containing 1% penicillin/streptomycin. Proteoglycan content within the medium was measured as sulfated glycosaminoglycan using a colorimetric assay using dimethylmethylene blue.

Luciferase assay. Reporter constructs containing the human GREM1 proximal promoter region were prepared by PCR amplification of a human genomic DNA template, which were cloned into the pGL4.10[luc2] vector (Promega). Deletion and mutation constructs were created by PCR. Transfection of ATDC5 cells was performed in triplicate in 48-well plates using Lipofectamine 2000 transfection reagent (Thermo-Fisher). The reaction mixture consisted of Lipofectamine 2000 with 400 ng of plasmid DNA, 200 ng of pGL reporter vector, 200 ng of effector vector, and 4 ng of pRL-TK internal control vector (Promega) per well. For screening of downstream gremlin-1 signalling, we purchased pGL4.49[luc2P/TCF-LEF RE/Hygro], pGL4.48[luc2P/CRE/Hygro], pGL4.44[luc2P/API RE/Hygro], pGL4.42 [luc2P/PIRE/Hygro], pGL4.39[luc2P/ATFF6 RE/Hygro], pGL4.38[luc2P/p53 RE/Hygro], pGL4.33[luc2P/SRE/Hygro], pGL4.32[luc2P/NF-κB RE/Hygro], and pGL4.29[luc2P/CRE/Hygro] vectors (Promega). Transfections were performed as described above except with PBS and rhGREM1 (1 µg/mL) in lieu of effector vectors. To evaluate the dose-response of NF-κB activity, we used 0.1, 1, and 10 µg/mL of rhGREM1. Luciferase assays were performed with a PicoGene Dual Sea-Passy Luminescence Kit (Toyo Ink) using a GloMax 96 Microplate Luminometer (Promega). Data are shown as the ratio of firefly activity to Renilla activity.

Rac1 activation assay. To evaluate Rac1 activity, a Rac1 Pull-down Activation Assay Biochem Kit (Cytoskeleton) was employed. Mouse primary chondrocytes were plated at a density of 1 × 10^6 cells in 2 x 2 cm stretch chambers. After cyclic tensile strain (0.5 Hz, 10% elongation) was applied for 30 min. The resulting pull-down product was then immunoblotted with Rac1 antibody according to the manufacturer's instructions. His-tagged Rac1 protein in the kit was used for control. The membranes were incubated with a horseradish peroxidase-conjugated antibody (Promega), and visualized with ECL prime (GE Healthcare) and an AE-6981 Light Capture II (ATTO, Tokyo, Japan). Control cells were seeded onto identical chambers and cultured without cyclic tensile strain. Original images of the assay were shown in Supplementary Fig. 15.

Construction of expression vectors. Full-length human or mouse cDNA sequences of transcription factors were PCR-amplified and cloned into pCMV-HA or pShuttle vector (Clontech). We generated adenovirus vectors using an Adeno-X expression system (Clontech) and verified all vectors by DNA sequencing.

Fluorescence detection of ROS. Intracellular ROS accumulation was evaluated by fluorescent microscopy using a Total ROS Detection Kit (ENZ-51010, Enzo Life Sciences). Mouse primary chondrocytes were plated in 2 x 2 cm stretch chambers and cyclic tensile stress (0.5 Hz, 10% elongation) was applied for 30 min. Cells were stained with 1 µM of ROS-sensitive fluorescent dye at 37 °C for 30 min, and then washed once with 1x wash buffer. After washing, cells were immediately overlaid with a coverslip and observed under a BZ-X710 fluorescence microscope (Keyence). Fluorescence signals were subsequently detected with excitation at 490 nm and emission at 525 nm for detection of ROS.

Statistical analyses. JMP Pro 13 was used for statistical analysis. Values are expressed as mean ± standard deviation. For comparisons between two groups, Student's unpaired two-tailed t-test was applied. For multiple comparisons, statistical analysis was performed by a two-way repeated measures for variance (ANOVA) test for Fig. 1a and a one-way ANOVA followed by Tukey–Kramer honest significant difference comparison. Sample sizes were chosen based on prior literature using similar methods and reporting moderate effect size. P values < 0.05 were considered significant.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The raw microarray data are deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession no. GSE103159. All other data supporting the findings of this study are available within the article and the Supplementary Information file, or are available from the corresponding author on reasonable request.

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