Regulation of \textit{Gdf5} expression in joint remodelling, repair and osteoarthritis

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\textit{Growth and Differentiation Factor 5 (GDF5)} is a key risk locus for osteoarthritis (OA). However, little is known regarding regulation of \textit{Gdf5} expression following joint tissue damage. Here, we employed \textit{Gdf5-LacZ} reporter mouse lines to assess the spatiotemporal activity of \textit{Gdf5} regulatory sequences in experimental OA following destabilisation of the medial meniscus (DMM) and after acute cartilage injury and repair. \textit{Gdf5} expression was upregulated in articular cartilage post-DMM, and was increased in human OA cartilage as determined by immunohistochemistry and microarray analysis. \textit{Gdf5} expression was also upregulated during cartilage repair in mice and was switched on in injured synovium in prospective areas of cartilage formation, where it inversely correlated with expression of the transcriptional co-factor \textit{Yes-associated protein} (Yap). Indeed, overexpression of Yap suppressed \textit{Gdf5} expression in chondroprogenitors \textit{in vitro}. \textit{Gdf5} expression in both mouse injury models required regulatory sequence downstream of \textit{Gdf5} coding exons. Our findings suggest that \textit{Gdf5} upregulation in articular cartilage and synovium is a generic response to knee injury that is dependent on downstream regulatory sequence and in progenitors is associated with chondrogenic specification. We propose a role for \textit{Gdf5} in tissue remodelling and repair after injury, which may partly underpin its association with OA risk.

Growth and Differentiation Factor 5 (GDF5) is a major risk locus for osteoarthritis (OA), the most common joint disease characterised by progressive loss of articular cartilage, remodelling of subchondral bone, chondro-osteophyte formation and synovitis. Common variants spanning a large 130 kb interval confer risk of hip and knee OA\textsuperscript{1–3}. A well-studied SNP is located in the 5' UTR of the \textit{GDF5} gene (rs143383), with the OA susceptibility allele resulting in decreased \textit{GDF5} expression\textsuperscript{2,4–6}.

\textit{Gdf5} plays important roles during joint formation. It is one of the earliest genes expressed in the embryonic joint interzone\textsuperscript{7–10}, fated to give rise to joint tissues including articular cartilage, synovium, menisci, and ligaments\textsuperscript{11,12}. \textit{Gdf5}-expressing progenitors are continuously recruited into joint interzones throughout development\textsuperscript{13} and their progeny retain skeletal joint stem/progenitor activity in adulthood\textsuperscript{14}. Following injury to the joint surface, \textit{Gdf5}-lineage mesenchymal stromal/stem cells (MSCs) proliferate to underpin synovial hyperplasia and migrate to the site of injury, through the activity of the transcriptional co-factor \textit{Yes-associated protein} (Yap), where they repair cartilage\textsuperscript{14}.

Loss-of-function mutations in \textit{GDF5} have been linked to congenital disorders including Hunter-Thompson syndrome\textsuperscript{15}, brachydactyly type C\textsuperscript{16}, and DuPan syndrome\textsuperscript{17}. These syndromes are partly phenocopied in \textit{brachypodism} (bp) mice, which harbour \textit{Gdf5} coding mutations\textsuperscript{7}. Homozygous \textit{bp} mice have dysmorphic knees lacking cruciate ligaments\textsuperscript{18,19}. Heterozygous \textit{bp} mice, which model human \textit{GDF5} variants that cause decreased
EDTA in PBS. Samples were embedded and sectioned as described\(^14\). Sections were stained with Nuclear Fast Red.

**Methods**

**Mice.** All methods were carried out in accordance with relevant guidelines and regulations. All animal experimental protocols were approved by the UK Home Office and the Animal Welfare and Ethical Review Committee of the University of Aberdeen. Two Gdf5 BAC transgenic mouse lines were used\(^{18,22,23}\). They both harbour a BAC transgene containing mouse Gdf5 with an IRES-LacZ cassette in the 3' UTR. Gdf5UP-LacZ mice contain a modified BAC extending 110 kb upstream to 30 kb downstream of Gdf5 coding exons, which includes a conserved regulatory region adjacent to the promoter upstream of the Gdf5 coding exons. Gdf5DOWN-LacZ mice contain a modified BAC extending a further 109 kb downstream, which includes additional regulatory regions downstream of the Gdf5 coding exons. Both lines were maintained as heterozygotes on a FVB background. Gdf5-CreER mice\(^{13}\) were provided by Dr. Elazar Zelzer (Weizmann Institute of Science, Israel) and crossed with Cre-inducible tdTomato (tdTom) reporter mice (Jackson Laboratory; B6.Cg-Gt(Rosa)26Sortm14(CAG-tdTom)Hze/J)\(^{25}\). Mice were group-housed in conventional cages on a 12:12 light-dark cycle, in a temperature-controlled room with water and food ad libitum and environmental enrichment provided. Tamoxifen (Sigma) dissolved in corn oil was administered by gavage at 6 weeks of age (180 mg/kg daily for 5 days), or to the pregnant dam at E11.5 (120 mg/kg), E13.5 (160 mg/ml) and E15.5 (160 mg/ml), and embryos were collected following euthanasia of the pregnant dam at E19.0.

**Surgical procedures.** Male mice, 11–12 weeks old, underwent surgical unilateral destabilisation of the medial meniscus (DMM) on the left knee\(^{26}\) while the right knee served as internal control, and mice were euthanised 2 or 8 weeks later. Female mice, 9–11 weeks old, underwent surgery to induce unilateral joint surface injury by medial parapatellar arthroscopy as previously described\(^{41}\), and were euthanised 6–7 days or 4 weeks later. For all surgeries, isoflurane inhalation anaesthesia was used, and mice received a subcutaneous injection of 0.1 mg/kg Vetyergesic (containing 0.3 mg/ml Buprenorphine) on the day of surgery and the following day. Mice were kept group-housed.

**X-gal staining.** Whole-mount staining with X-gal to detect β-galactosidase (β-gal) activity was performed as described\(^{37}\), with modifications. Limbs were fixed in 4% PFA for 2 h at 4 °C, washed 3x in wash buffer (0.1 M potassium ferricyanide and 20 mM Tris buffer, pH 7.4) for 6 days at room temperature, then washed 3x in PBS. X-gal staining was carried out using a TRAP staining kit (Sigma). Immunohistochemistry was performed as described\(^{28,29}\) and environmental enrichment provided. Tamoxifen (Sigma) dissolved in corn oil was administered by gavage at 6 weeks of age (180 mg/kg daily for 5 days), or to the pregnant dam at E11.5 (120 mg/kg), E13.5 (160 mg/ml) and E15.5 (160 mg/ml), and embryos were collected following euthanasia of the pregnant dam at E19.0.

**Histology and immunohistochemistry.** Samples were fixed in 4% PFA at 4 °C and decalcified in 10% EDTA in PBS. Samples were embedded and sectioned as described\(^{41}\). Sections were stained with Nuclear Fast Red (Vector Laboratories, UK) to stain nuclei, or with safranin-O (Sigma) to stain glycosaminoglycans in the cartilage matrix red, with fast green (Sigma) counterstain, following standard protocols. TRAP staining to detect osteoclasts was carried out using a TRAP staining kit (Sigma). Immunohistochemistry was performed as described\(^{28,29}\) and environmental enrichment provided. Tamoxifen (Sigma) dissolved in corn oil was administered by gavage at 6 weeks of age (180 mg/kg daily for 5 days), or to the pregnant dam at E11.5 (120 mg/kg), E13.5 (160 mg/ml) and E15.5 (160 mg/ml), and embryos were collected following euthanasia of the pregnant dam at E19.0.

**Quantification of X-gal staining.** Colour deconvolution was applied to images of X-gal-stained sections to remove the Nuclear Fast Red counterstaining using Imagel with Fiji package and Colour Deconvolution Plugin (Dr. Gabriel Landini, University of Birmingham, UK) based on published methods\(^{37}\). All images were acquired with the same magnification, resolution and light settings. The number, size and staining intensity of

**Human tissue collection.** All human cartilage samples were obtained after informed consent and in accordance with the relevant guidelines and regulations, with approval from the NHS Grampian Biorepository Tissue Bank Committee. OA samples were obtained from five patients (47 to 79 years old, all female) undergoing knee arthroplasty. Normal samples were obtained from five joints (two knee joints, 1st metatarsal phalangeal joint, ankle joint, talo-calcaneal joint) donated by three patients (40 to 59 years old, two males, one female) undergoing excision or amputation surgery for tumours unrelated to the joint sampled.
X-gal-stained chondrocytes in the tibial cartilage was then determined by creating a binary image using thresholding and watersheding, and analysing particles by redirecting measurements to matching greyscale images. Four sections per sample were analysed. Total X-gal staining was calculated by multiplying the number and staining intensity of X-gal-stained chondrocytes.

**Primary cell isolation and in vitro chondrogenesis.** Cells were isolated from Gdf5 BAC mouse knees as described. Chondrogenesis was induced in high-cell density pellet culture (2.5–3 × 10⁵ cells) with 10 ng/ml TGFβ3 (Gibco) or 300 ng/ml BMP-2 (Prospect) for 21 days, as described. Pellets were fixed in 4% PFA for 15 min, X-gal-stained for 4 h and post-fixed for 15 min, cryoprocessed, sectioned and stained with Toluidine Blue or Nuclear Fast Red.

**Overexpression and knockdown experiments.** C3H10T1/2 cells (American Type Culture Collection, USA) were retrovirally transduced to express wildtype or constitutively active YAP1, as described. Cells were seeded in monolayer (15,000/cm²), transduced the next day, and RNA extracted 2 days later. Alternatively, transduced cells were seeded in high-cell density micromass culture (4 × 10⁶ cells) in chemically-defined serum-free medium (high-glucose DMEM with glutamine, supplemented with 50 μg/ml ascorbic acid, 1 mg/ml recombinant human insulin, 0.55 μg/ml transferrin, 0.5 μg/ml sodium selenite, 50 μg/ml BSA and 470 μg/ml linoleic acid) and the next day RNA was extracted. For knockdown experiments, cells were seeded at 42,000/cm² and transfected the next day with DsiRNA (Supplementary Table 2) (Integrated DNA Technologies, USA) using Mirus TransIT-X2 reagent (Mirus Bio LLC, USA). The following day, cells were seeded in micromass culture (2.5–3 × 10⁵ cells) and cultured under chondrogenic conditions by treatment with 300 ng/ml BMP-2, as described. After 4 days, RNA was extracted for analysis of gene expression.

**Gene expression analysis.** Total RNA was extracted using TRIzol reagent (Invitrogen, Paisley, UK) according to standard protocols, and RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Labtech, Uckfield, UK). cDNA was synthesised using random hexamer primers and SuperScript Reverse Transcriptase (Invitrogen), according to manufacturer’s instructions. Quantitative PCR (qPCR) was performed with a Roche LightCycler 480 using SYBR Green Master (Roche), according to standard protocols. Expression of genes of interest was normalised to expression of Hprt1. Primer sequences are listed in Supplementary Table 3.

**Statistical analysis.** Microarray data were analysed using Bioconductor (Affy package for pre-processing and normalization and Limma for statistical comparison of expression levels using a false-discovery-rate of 5%). Principal component analysis was performed using the prcomp package in R. All other data were analysed using GraphPad Prism v5 and SigmaPlot v13. A p-value ≤ 0.05 was considered statistically significant. For comparison of two groups, one-tailed or two-way ANOVA with Holm-Sidak post-test was used. Data following a lognormal distribution were log-transformed for statistical testing. N-numbers and data points on graphs represent individual mice, patients, or in vitro experiments, with horizontal lines indicating mean.

**Results Gdf5 expression in OA.** To investigate Gdf5 expression in experimentally induced OA, we used two Gdf5-LacZ reporter mouse lines. Gdf5UP-LacZ mice contain a BAC extending 110 kb upstream to 30 kb downstream of Gdf5 coding exons, which includes a conserved regulatory region adjacent to the promoter upstream of the Gdf5 coding exons. Gdf5DOWN-LacZ mice contain a BAC extending a further 109 kb downstream, which includes additional regulatory regions downstream of the Gdf5 coding exons that are not present in the Gdf5UP-LacZ BAC. Both BACs were modified to contain an IRES-LacZ cassette in the 3′UTR of the Gdf5 gene, thus LacZ expression is indicative of the activity of the Gdf5 regulatory regions contained within the BAC. While both mouse lines express LacZ in the knee during development, only Gdf5DOWN-LacZ mice express LacZ in the knee in adulthood (Supplementary Fig. 1). The Gdf5DOWN-LacZ BAC is also able to rescue the knock phenotype in bp mice, indicating it contains the regulatory regions necessary for adequate expression in the knee. Here, we found that the LacZ expression pattern in Gdf5DOWN-LacZ adult knees resembled the tdTom labelling pattern in knees from adult mice with a knock-in of CreER at the endogenous Gdf5 locus crossed with Cre-inducible tdTom reporter mice shortly after tamoxifen induction (Supplementary Fig. 2A,B). tdTom labelling was sparse, likely due to inefficient Cre-recombination as observed in embryos (Supplementary Fig. 2C–E). Nonetheless, these data support LacZ expression in knees from adult Gdf5DOWN-LacZ mice as reflecting transcriptional activity of endogenous Gdf5.

We analysed LacZ expression in the knees of Gdf5-LacZ mice after DMM (Fig. 1A,B). In Gdf5DOWN-LacZ mice, increased LacZ expression was observed in medial compartment articular cartilage at 2 weeks, particularly in areas with early signs of damage, as shown by loss of Safranin O staining which stains proteoglycans in the cartilage extracellular matrix (Fig. 1A). Quantification showed an increase in both the number of LacZ-expressing chondrocytes and average X-gal staining intensity per chondrocyte (Fig. 1C), resulting in a significantly higher overall LacZ-expression in the medial tibial plateau cartilage in DMM knees. At 8 weeks after DMM, LacZ expression persisted in articular cartilage of Gdf5DOWN-LacZ mice but was less pronounced and undetectable in areas of severe damage (Fig. 1A). In Gdf5UP-LacZ mice, no LacZ expression was detectable in the cartilage at either time-point (Fig. 1A). These data indicate that Gdf5 downstream regulatory elements are activated in articular chondrocytes in the early phase of OA.

LacZ expression was also detected in the medial synovium of Gdf5DOWN-LacZ mice at 2 weeks post-DMM and remained detectable at 8 weeks, specifically in ectopic chondrocytes and surrounding fibroblast-like cells (Fig. 2A). In addition, LacZ was expressed in chondrocytes at 2 weeks post-DMM but was no longer detectable.
in mature osteophytes at 8 weeks (Fig. 2B). LacZ expression was not detected in knees from Gdf5UP-LacZ mice at either time-point (Fig. 2B). We infer that Gdf5 is expressed in areas of forming ectopic cartilage during OA.

For clinical relevance, we analysed data from published microarrays of human cartilage from knees of normal donors and OA patients 33. GDF5 expression was upregulated in the cartilage of OA patients (Fig. 3A), along-side increased expression of cartilage degrading proteins known to be upregulated in OA (MMP13, ADAMTS5) (Fig. 3B). GDF5 expression correlated with expression of SOX11 and WNT9A (Fig. 3B,C), known upstream regulators of Gdf5 expression during development34–36, indicating these factors may also modulate GDF5 expression in human articular cartilage during OA. Immunohistochemistry for GDF5 on articular cartilage samples from a distinct cohort of OA patients and controls confirmed GDF5 was upregulated in OA cartilage (Fig. 3D and Supplementary Fig. 3).

Gdf5 expression following joint surface injury. To investigate Gdf5 expression during cartilage repair, we analysed LacZ expression in the Gdf5-LacZ transgenic mice 4 weeks after joint surface injury. In Gdf5DOWN-LacZ mice, chondrocytes in the repair tissue strongly expressed LacZ. We also detected prominent LacZ expression in chondrocytes in the native cartilage immediately adjacent to the repair tissue (Fig. 4A). In contrast, no staining was observed in repaired cartilage in Gdf5UP-LacZ mice (Fig. 4A). In support of these findings, while undetectable in monolayer culture, LacZ expression was detected in MSCs isolated from the knees of Gdf5DOWN-LacZ mice following chondrogenic differentiation in pellet culture, but not in chondrogenic pellets of Gdf5UP-LacZ MSCs (Fig. 4B). These data indicate upregulation of Gdf5 expression, mediated by downstream regulatory regions, during articular cartilage repair.

Since LacZ was switched on in Gdf5DOWN-LacZ MSCs during chondrogenesis, we next analysed the synovium, which contains stem/progenitor cells that can undergo chondrogenic differentiation following injury and are postulated to repair injured cartilage14,28,37. LacZ was not detectable in synovium during homeostasis in either
model (Supplementary Fig. 1). One week after joint surface injury, the synovium was hyperplastic, as expected28,38. In the synovium on the lateral side of the knee, not incised during surgery, \( \text{LacZ} \) remained undetectable in both mouse lines at both time-points (Fig. 5A and not shown), indicating that \( \text{Gdf5} \) expression is not switched on in synovium in response to cartilage injury. However, in synovium on the medial side, which was incised during surgery, small clusters of \( \text{LacZ} \)-expressing cells with a fibroblast-like morphology were detected in \( \text{Gdf5DOWN-LacZ} \) mice (Fig. 5B), and such cells persisted at 4 weeks after injury (Fig. 5C). They were predominantly localized near surgical sutures, where fibroblast-like cells that stained strongly for \( \beta \)-gal were observed around small clusters of \( \text{LacZ} \)-expressing chondrocytes embedded in a matrix containing collagen type II (Fig. 5D). Thus, as in DMM mice, \( \text{Gdf5} \) expression is upregulated in synovium in areas of prospective cartilage formation, suggesting a role for \( \text{Gdf5} \) in chondrogenic specification and differentiation.

Yap suppresses \( \text{Gdf5} \) expression in chondroprogenitors. We previously reported that Yap is upregulated in synovium after joint surface injury and is required for the local expansion of \( \text{Gdf5} \)-lineage MSCs and their recruitment to the cartilage defect44, whereas Yap prevents chondrogenic differentiation32. Here, we compared expression of \( \text{LacZ} \) and Yap in \( \text{Gdf5DOWN-LacZ} \) mouse knees after joint surface injury and observed areas in synovium where Yap and \( \text{LacZ} \) showed an inverse expression pattern, with cells that expressed \( \text{LacZ} \) showing diminished Yap compared to surrounding cells (Fig. 5E). We hypothesized that high Yap activity during cell proliferation inhibits chondrogenic differentiation, as reported42, by actively suppressing chondrogenic factors including \( \text{Gdf5} \). Hence, we determined the effect of overexpression of Yap on \( \text{Gdf5} \) expression in high-cell-density cultures using murine C3H10T1/2 MSCs. After one day of high-cell-density micromass culture, \( \text{Gdf5} \) expression was upregulated approximately 20-fold when compared to cells in monolayer (Fig. 6A), as previously reported with human synovial MSCs39. Strikingly, overexpression of YAP1 prevented the upregulation of \( \text{Gdf5} \) in micromass (Fig. 6A). In contrast, YAP1 overexpression failed to prevent the upregulation of \( \text{Wnt9a} \), known to be upstream of \( \text{Gdf5} \), even when cells were transduced to express constitutively active YAP1S127A (Fig. 6B). Conversely, knockdown of Yap in C3H10T1/2 MSCs in micromass increased \( \text{Gdf5} \) expression, an effect that was synergistically enhanced with concomitant knockdown of the paralog of Yap, Transcriptional Co-Activator with
PDZ binding motif (Taz) (Fig. 6C–E). Wnt9a expression was not similarly modulated by Yap and Taz knockdown (Fig. 6F). Altogether, these data identify Yap as a negative regulator of Gdf5 expression in chondrogenic MSCs, and indicate that Yap acts downstream of Wnt9a, possibly by directly modulating the activity of one or more transcription factors acting on Gdf5 cis-regulatory elements.

**Discussion**

Allelic variants at the GDF5 locus have been linked to OA risk, suggesting GDF5 plays important roles in joint maintenance throughout life. Expression of Gdf5 in adult articular cartilage has been reported in mice and humans, with upregulation in OA. Little was known regarding Gdf5 expression in response to acute joint surface defects, which can progress to OA in the absence of repair, or during the different stages of OA. Here,
we show Gdf5 expression in remodelling joint tissues, using two BAC LacZ reporter mouse strains harbouring distinct yet partially overlapping regions of the Gdf5 locus\textsuperscript{19,22}. After joint surface injury, Gdf5 was highly expressed in chondrocytes both inside the newly formed cartilage repair tissue and in the adjacent stressed cartilage. Similarly, Gdf5 was upregulated in cartilage during early-stage OA, particularly in areas of initial damage.

**Figure 4.** LacZ expression is upregulated during cartilage repair and *in vitro* chondrogenesis. (A) Areas of healed cartilage (dashed line) in the patellar groove of the femur of Gdf5DOWN-LacZ (n = 4/10) and Gdf5UP-LacZ (n = 3/10) mice, with LacZ-expressing chondrocytes (blue, arrows) detected in Gdf5DOWN-LacZ mice 4 weeks post-injury. LacZ, whole-mount X-gal staining to detect LacZ expression; Safo & FG, Safranin O and Fast Green counterstaining; Fast Red, Nuclear Fast Red counterstaining. Scale bars, 100 μm. (B) Histological sections of chondrogenic cell pellets. Synovial cells were isolated from Gdf5DOWN-LacZ and Gdf5UP-LacZ mice and treated *in vitro* for 21 days with TGFβ (10 ng/ml) or BMP-2 (300 ng/ml) to induce chondrogenesis, followed by X-gal staining to detect LacZ expression. Tol blue, Toluidine blue metachromatic staining indicates deposition of cartilage proteoglycans; Fast Red, Nuclear Fast Red counterstaining. LacZ-expressing chondrocytes (blue, arrows) were observed in Gdf5DOWN-LacZ cell pellets, but not Gdf5UP-LacZ pellets, under both culture conditions. Scale bars, 100 μm.
Figure 5. Gdf5 is switched on in areas undergoing ectopic cartilage formation in synovium. LacZ expression in lateral (A) and medial synovium (B–E) from Gdf5DOWN-LacZ mice 1 week (A,B) or 4 weeks (C–E) after joint surface injury (n = 4 for both timepoints). (A) LacZ expression was not detected in synovium (S) on the lateral side. (B) Clusters of LacZ-expressing fibroblast-like cells (blue, arrows) were found in the medial synovium, near the site of surgical incision. (C) LacZ expression in medial synovium persisted at 4 weeks after injury, particularly near surgical sutures (asterisk). Dotted line indicates area shown in (D) in a consecutive section. (D) IHC staining for Collagen type II (Col2; light brown) revealing LacZ-expressing chondrocytes (blue, arrowheads) embedded in a cartilage matrix surrounded by LacZ-expressing fibroblast-like cells (blue, arrows). (E) IHC staining for Yap showing LacZ-expressing cells (blue) with little or no Yap interspersed between Yap-expressing cells (light brown) that did not detectably express LacZ. LacZ, whole-mount X-gal staining to detect LacZ expression; Fast Red, Nuclear Fast Red counterstaining. Scale bars, 100 μm.
and was detected in forming chondrophytes. Given the known chondrogenic activity of Gdf5\textsuperscript{12,43}, our findings implicate a role for Gdf5 in new cartilage formation following injurious events in adulthood, possibly representing an attempt to repair joint damage. During late-stage OA, areas of advanced cartilage damage displayed markedly reduced LacZ staining, in line with previous studies reporting decreased Gdf5 expression in extensively damaged cartilage in mice with inflammatory or degenerative arthritis\textsuperscript{34,40}. These data support a role for Gdf5 in the maintenance and repair of articular cartilage in adult life, and provide a rationale for the administration of exogenous Gdf5 to aid cartilage repair in OA treatment\textsuperscript{44}.

We show that Gdf5 expression after injury and during OA is dependent on DNA sequence more than 30 kb downstream from the Gdf5 coding region. This downstream sequence contains joint-specific regulatory elements\textsuperscript{22}, and is both capable of, and necessary for, rescuing the bp knee phenotype in mice\textsuperscript{19,22–24}. Importantly, it harbours many common risk variants for OA, of which several reside in known enhancers. Our findings indicate that such downstream variants may confer OA risk partly through modulating Gdf5 expression in the adult knee in response to injurious events, thereby impacting on joint maintenance and reparative processes. They further indicate that the effect of a human variant such as the rs143383 SNP in the 5'UTR\textsuperscript{2,4–6} is likely to be dependent on cis-acting variants present in downstream cis-regulatory elements that are critical to drive adequate expression of Gdf5. Whether downstream regulatory elements involved in repair are different from those involved in OA development remains to be determined.

The identification of molecules that regulate Gdf5 expression will provide critical insights into joint formation, maintenance and disease. We have unveiled a regulatory mechanism, to our knowledge hitherto unreported, that links Yap activity to Gdf5 expression. Undetectable in quiescent synovium, Gdf5 was switched on in activated chondroprogenitors in synovium following injury, concomitant with Yap downregulation. In chondrogenic

Figure 6. Yap suppresses Gdf5 expression. (A,B) C3H10T1/2 cells were transduced with retrovirus encoding YAP1 or constitutively active YAP1\textsuperscript{S127A}, or with empty vector (Control), and cultured in standard monolayer (\(n = 5\) experiments), or in high-density micromass for 1 day (\(n = 4\) experiments; data from separate experiments). (C–F) C3H10T1/2 cells were transected with DsiRNA to knock down Yap, or Yap and Taz, or with mismatch DsiRNA (Control), and cultured for 4 days in chondrogenic micromass culture. Gdf5 (A,E), Wnt9A (B,F), Yap (C) and Taz (D) expression were determined by quantitative RT-PCR. All data were normalised to expression of Hprt1, and are shown relative to expression of the gene of interest in non-transduced cells in monolayer (A,B), or in micromass (C–F). *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\), based on two-way ANOVA with Holm-Sidak post-test for pairwise comparisons (A,B) or one-way ANOVA with Holm-Sidak post-test for comparisons against control (C–E).
MSCs, Yap suppressed expression of Gdf5 but not Wnt9a, known to induce Gdf5 expression. Our data indicate that Yap negatively regulates Gdf5 expression, possibly downstream of Wnt9a, and we propose that Yap needs to be down-regulated to enable Gdf5 expression to prime progenitors towards chondrogenesis. Indeed, Yap prevents MSC chondrogenic differentiation in vitro. Candidate transcription factors that could partner with Yap to regulate Gdf5 include Sox11, reported to directly regulate Gdf5 expression and found here to correlate with GDF5 expression in human OA cartilage, and ZEB1, since ZEB1 binding sites are present in the enhancer upstream of the Gdf5 promoter region and a direct interaction between ZEB1 and Yap has been reported.

In conclusion, Gdf5 is upregulated in stressed cartilage, switched on in chondroprogenitors and expressed in newly forming cartilage during tissue remodelling following knee injury. This is dependent on activity of downstream regulatory sequence and occurs irrespective of whether the injury is acute or the result of chronic joint instability, indicating that Gdf5 modulation is not linked to a specific injurious event. An understanding of the regulation of Gdf5 in the context of remodelling, repair and OA pathogenesis will have important implications for joint surface regenerative therapies and OA treatment.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
A.J.R. and C.D.B. conceived and oversaw the project. K.K., A.J.R. and C.D.B. designed experiments and performed and analysed experiments. H.W., A.J.R. and K.K. performed mouse surgeries. F.C., K.K. and A.J.R. performed and analysed in vitro experiments. A.H.K.R. collected human tissues. K.A.H. advised on institutional affiliations. K.K., A.J.R. and C.D.B. provided and analysed microarray data. K.K., A.J.R. and C.D.B. wrote the manuscript, with input from T.D.C. All authors critically reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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