Identification of the skeletal progenitor cells forming osteophytes in osteoarthritis

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ABSTRACT

Objectives. Osteophytes are highly prevalent in osteoarthritis (OA) and are associated with pain and functional disability. These pathological outgrowths of cartilage and bone typically form at the junction of articular cartilage, periosteum and synovium. The aim of this study was to identify the cells forming osteophytes in OA.

Methods. Fluorescent genetic cell-labelling and tracing mouse models were induced with tamoxifen to switch on reporter expression, as appropriate, followed by surgery to induce destabilization of the medial meniscus (DMM). Contributions of fluorescently labelled cells to osteophytes after 2 or 8 weeks, and their molecular identity, were analysed by histology, immunofluorescence staining, and in situ hybridisation. \textit{Pdgfra-H2BGFP} mice and \textit{Pdgfra-CreER} mice crossed with multi-colour \textit{Confetti} reporter mice were used for identification and clonal tracing of mesenchymal progenitors. Mice carrying \textit{Col2-CreER}, \textit{Nes-CreER}, \textit{LepR-Cre}, \textit{Grem1-CreER}, \textit{Gdf5-Cre}, \textit{Sox9-CreER} or \textit{Prg4-CreER} were crossed with \textit{tdTomato} reporter mice to lineage-trace chondrocytes and stem/progenitor cell sub-populations.

Results. Articular chondrocytes or skeletal stem cells identified by \textit{Nes}, \textit{LepR}, or \textit{Grem1} expression did not give rise to osteophytes. Instead, osteophytes derived from \textit{Pdgfra}-expressing stem/progenitor cells in periosteum and synovium that are descendants from the \textit{Gdf5}-expressing embryonic joint interzone. Further, we show that \textit{Sox9}-expressing progenitors in periosteum supplied hybrid skeletal cells to the early osteophyte, while \textit{Prg4}-expressing progenitors from synovial lining contributed to cartilage capping the osteophyte, but not to bone.

Conclusion. Our findings reveal distinct periosteal and synovial skeletal progenitors that cooperate to form osteophytes in OA. These cell populations could be targeted in disease modification for treatment of OA.

Keywords: Osteoarthritis, osteophytes, stem cells, synovium, periosteum
INTRODUCTION

A characteristic feature of osteoarthritis (OA) is the formation of osteophytes, which are osteo-cartilaginous outgrowths that typically form at the joint margins, in the region where the synovium attaches to the edge of the articular cartilage and merges with the periosteum. Osteophytes are established through growth of an initial cartilage template that is at least partially replaced with bone containing marrow cavities.[1,2] At later stages, the bone is typically covered by a cartilage cap that can merge with the articular cartilage.[1,2] Despite their high prevalence, the cell populations giving rise to osteophytes in OA remain to be defined.

Several skeletal stem/progenitor cell (SSC) populations have been identified which vary in their ability to form cartilage and bone during skeletal development, maintenance and repair. These include perivascular cells marked by expression of Pdgfrα and Sca1, Nestin (Nes), or leptin receptor (Lepr), and Gremlin-1 (Grem1)-expressing cells.[3–9] In addition, during bone regeneration, Sox9-expressing progenitors in periosteum initiate cartilage callus formation by giving rise to cells that co-express chondrocyte and osteoblast markers.[10,11] Recently, we used a Gdf5-Cre model that is active in the embryonic knee joint interzone, but not in the adult normal or OA knee,[12–14] to show that Gdf5-lineage descendants include SSCs in the adult with ability to repair a focal cartilage defect.[15,16] The adult Gdf5 lineage includes Prg4-expressing progenitors in the superficial zone of articular cartilage and synovial lining.[15,17–19]

Here, we show that osteophytes derive from Pdgfra-expressing Gdf5-lineage cells. These include Sox9-expressing progenitors in periosteum that give rise to hybrid skeletal cells that molecularly resemble those observed during bone repair, and Prg4-expressing cells from the synovial lining that supply chondrocytes to the outer cartilage layer but do not contribute to bone. Thus, our data define the progenitor cell subsets contributing to osteophyte formation in experimental OA.
METHODS

Mice

All animal experimental protocols were approved by the UK Home Office and the Animal Welfare and Ethical Review Committees of the University of Aberdeen and University of Cambridge, the University of Southern California Institutional Animal Care and Use Committee, the Murdoch Children's Research Institute Animal Ethics Committee, or the Animal Use Committee for University Hospital Münster. We used Col2-CreER,[20] Pdgfra-CreER,[21] Nes-CreER,[22] LepR-Cre,[23] Grem1-CreER,[9] Gdf5-Cre,[16] Prg4-GFP-CreER,[18] and Sox9-CreER mice,[24] Cre-inducible TdTomato,[25] Confetti,[26] and mTmG reporter mice,[27] and Pdgfra-H2BGFP[28] and Nes-GFP[29] mice (see Supplementary Table 1). Wild-type SWR/J mice were used for in situ hybridisation experiments. Mice were maintained on a 12:12 light-dark cycle, in a temperature-controlled room, with water and food ad libitum.

Tamoxifen administration and surgery

Administration of tamoxifen, dissolved in corn oil, was optimised for each mouse strain based on published studies[9,11,18,30,31] and pilot experiments to achieve efficient labelling of intended target cells while minimising impact on animal welfare (see Supplementary Table 2 for details). Adult male mice underwent surgical unilateral destabilisation of the medial meniscus (DMM) through resection of the medial menisco-tibial ligament,[32] with the contralateral knee serving as unoperated or sham-operated control (see Supplementary Table 2 or figure legends for age at surgery). Mice were anaesthetised with ketamine (50 mg/kg) and medetomidine (0.67 mg/kg) with atipamezole (1 mg/kg) post-operatively, ketamine (90 mg/kg) and xylazine (10 mg/kg), or isoflurane with 0.1 mg/kg buprenorphine subcutaneously for analgesia. Proliferating cells were labelled by subcutaneous injection of 2 mg bromodeoxyuridine (BrdU) immediately after surgery followed by 1
mg/ml BrdU in drinking water for 2 weeks. Mice were euthanised for analysis 1, 2 or 8 weeks after surgery. Experimenters were not blinded.

**Histology and immunohistochemistry**

Tissues were fixed in 4% paraformaldehyde and decalcified in 4-10% EDTA in PBS or 33% (v/v) formic acid with 13.5% (w/v) trisodium citrate dihydrate. Samples were embedded in paraffin or frozen in OCT and sectioned. Sections were stained with safranin-O (Sigma), with or without fast green (Sigma). Fluorescent proteins were either detected by their native fluorescence in cryosections, or via immunofluorescence staining on paraffin sections,[33,34] using antibodies listed in Supplementary Table 3. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies), Hoechst, or TO-PRO-3 (ThermoFisher).

**Imaging**

Images were acquired using a Zeiss Axioscan Z1 slide scanner, Axioskop 40 with Progress XT Core 5 camera, Axio Imager 2.0 and Axio Observer Z1 with AxioCam MRm and AxioCam MrC, 710 META Laser-Scanning Confocal Microscope, or Nikon AZ100 Microscope with a Nikon Digital sight DS-Fi1 camera. Image analysis was performed using ZEN (Zeiss), ImageJ and QuPath softwares.[35] All analyses were performed on a minimum of 3 sections per sample. Percentages of labelled cells were calculated from the summed cell counts of all sections analysed for each sample, with marrow spaces excluded.

**RNA In situ hybridisation**

Fluorescence RNA in situ hybridisation was performed on 7-mm paraffin sections as described.[36] RNA probes[11] were generated following kit instructions (Sigma-Aldrich: 11277073910 and 11685619910) and were detected with Anti-Digoxigenin-POD (Sigma-Aldrich: 11207733910) and
Anti-Fluorescein-POD (Sigma-Aldrich: 11426346910). For double-fluorescence in situ hybridisation, the TSA Cyanine 3 and Fluorescein system from Perkin Elmer was used as directed (NEL753001KT).

**Cell isolation and flow cytometry**

Cells were isolated from mouse knees as described,[15] and stained with fixable viability dye eFluor780 (eBiosciences, Hatfield, UK) and antibodies listed in Supplementary Table 4. Data were acquired on a BD Fortessa flow cytometer and analysed using FlowJo v10 software (Ashland, OR, USA). Unstained and single-labelled cells or antibody-labelled CompBeads (BD Biosciences) were used to set compensation. Debris and doublets were excluded based on Forward and Side Scatter parameters, dead cells were excluded based on viability dye staining, and gates were applied based on Fluorescence-Minus-One controls (Supplementary Fig. 1).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism v5. Graphs show data points of individual mice, with mean ± 95% confidence interval (CI). N-numbers indicate number of mice.
RESULTS

Osteophytes derive from \textit{Pdgfra}-expressing progenitors in periosteum and synovium

In the DMM model of OA in mice, osteophytes form at the joint margins, often merging with the articular cartilage, similar to human OA.[1,2] We combined the DMM model with genetic cell-labelling and tracing models, to unravel the cell populations giving rise to osteophytes. \textit{Pdgfra} is broadly expressed by mesenchymal stem and progenitor cells. Using \textit{Pdgfra-H2B\textsubscript{GFP}} mice to identify and trace \textit{Pdgfra}-expressing cells by long-lived GFP expression, we observed GFP+ cells throughout synovium and periosteum of the adult knee (Fig. 1a). In parallel, we tested whether pre-existing articular chondrocytes contribute to osteophyte using \textit{Col2-CreER;ROSA26:loxP-STOP-loxP-TdTomato (Tom)} mice treated with tamoxifen at 2 weeks of age, when articular chondrocytes still express high levels of \textit{Col2a1} and are efficiently labelled (Fig. 1b).[30] Two weeks after DMM in tamoxifen-induced \textit{Pdgfra-H2B\textsubscript{GFP};Col2-CreER;Tom} mice, GFP+ chondrocytes were present throughout the osteophyte and were negative for Tom (Fig. 1c). Furthermore, there was negligible contribution from Tom+ pre-existing chondrocytes to mature osteophytes at 8 weeks post-DMM (Fig. 1d). These findings indicate that osteophytes do not develop from articular cartilage but derive from \textit{Pdgfra}-expressing stem/progenitor cells.

Next, we induced adult \textit{Pdgfra-CreER;Confetti} mice with tamoxifen to trace individual \textit{Pdgfra}-expressing cells through stochastic expression of membrane-bound CFP, nuclear GFP, cytoplasmic YFP, or cytoplasmic RFP. Negligible fluorescence was detected in the absence of tamoxifen. In unoperated knees of tamoxifen-induced mice, fluorescently labelled cells were observed in periosteum and synovium (Fig. 1e). Consistent with previous studies utilising the \textit{R26-Confetti} reporter,[37,38] GFP was rarely detected and omitted from analysis. Two weeks after DMM, 23.9% (95% CI [17.3%, 30.6%], n=4) of cells in the osteophyte were labelled with either CFP, YFP or RFP (Fig. 1f,g). This may reflect the low sensitivity of the \textit{R26-Confetti} reporter to Cre-mediated
recombination,[17] and aided the identification of distinct clonal cell populations. Intriguingly, clusters of identically coloured chondrocytes, likely derived from individual stem/progenitor cells, were observed in the deep periosteum at the bone surface, while distinct clusters of chondrocyte-like and fibroblast-like cells were found in the overlying synovial tissue (Fig. 1f). These data indicate that \textit{Pdgfra}-expressing stem/progenitor cells clonally expand and give rise to chondrocytes that form osteophytes in experimental OA, and suggest a dual contribution from cells in periosteum and synovium.

**SSCs marked by expression of \textit{Nes}, \textit{LepR}, or \textit{Grem1} do not give rise to osteophytes**

Since SSC populations marked by expression of \textit{Nes}, \textit{LepR}, or \textit{Grem1} express \textit{Pdgfra},[3,7–9] we investigated whether they contribute to osteophyte formation. Cells marked by \textit{Nes}-GFP or \textit{Nes-CreER};\textit{Tom} expression remained confined to vascular niches in synovium, periosteum and bone marrow, likely including pericytes and endothelial cells,[8,9,39] with no detectable contribution to either cartilage or bone of the osteophyte at 2 or 8 weeks post-DMM (Fig. 2a–c). We next analysed \textit{LepR-Cre};\textit{Tom} mice, since \textit{LepR}-traced cells have been reported to make significant contributions to adult bone turnover and repair following fracture.[8] \textit{LepR}-traced cells were present in synovium and periosteum but negligible contribution to osteophytes was observed (Fig. 2d,e). We also used \textit{Grem1-CreER};\textit{Tom} mice to trace \textit{Grem1}-expressing SSCs, as they are distinct from \textit{Nes}-GFP+ cells and contribute to fracture repair.[9] Following tamoxifen induction at 7 weeks of age, we found no contribution of \textit{Grem1}-traced cells to osteophytes at 2 weeks post-DMM (Fig. 2f). These data show that \textit{Nes-}, \textit{LepR-}, and \textit{Grem1}-expressing SSCs do not form osteophytes.

**Osteophytes arise from adult progeny of the \textit{Gdf5}-expressing embryonic joint interzone**

Cells in adult knees that are traced from \textit{Gdf5}-expressing joint interzone cells in the embryo are present in synovium and adjacent periosteum (Fig. 3a), and include SSCs.[15] Analysis of cells
isolated from knees of Pdgfra-H2BGFP;Gdf5-Cre;Tom mice (Fig. 3b) revealed that the vast majority of Tom+ Gdf5-lineage cells express Pdgfra-H2BGFP (93.6%, 95% CI [87.6%, 99.6%], n=9), with Gdf5-lineage cells constituting approximately one-third of all Pdgfra-H2BGFP+ cells (Fig. 3c,d). Both Pdgfra-expressing Gdf5-lineage cells (GFP+Tom+) and other Pdgfra-expressing cells (GFP+Tom-) expressed, to varying degrees, the mesenchymal stromal cell and fibroblast markers podoplanin (Pdpn/Gp38), CD90, CD73, CD51 and CD105, while neither population included haematopoietic cells (CD45+), endothelial cells (CD31+), or erythrocytes (Ter-119+) (Fig. 3e-h, Supplementary Fig. 1). These findings indicate that adult Gdf5-lineage cells are a subset of Pdgfra-expressing cells that may form osteophytes.

We thus induced DMM in Gdf5-Cre;Tom mice, followed by 2 weeks of BrdU administration to label proliferating cells. At 2 weeks post-DMM, Gdf5-lineage cells had extensively proliferated and expanded (Fig. 4a,b), and they were major contributors to Col2-expressing chondrocytes in the osteophytes (Fig. 4b). Tom+ Gdf5-lineage cells constituted 82.5% (95% CI [65.1%, 99.8%], n=4), and BrdU+ cells constituted 82.4% (95% CI [69.9%, 94.8%], n=4), of cells within osteophytes at 2 weeks post-DMM (Fig. 4c). Tom+ Gdf5-lineage cells remained abundant in mature osteophytes at 8 weeks post-DMM (Fig. 4d). They included 87.7% (95% CI [80.0%, 95.4%], n=7) of cells in the cartilage cap and 70.8% (95% CI [63.6%, 78.1%], n=7) of osteocytes in the bone (Fig. 4e), as well as bone lining cells at endosteal surfaces (Fig. 4d). These data indicate that the Pdgfra-expressing progenitors that form osteophytes are largely contained within the joint-resident Gdf5-lineage population, which undergo extensive proliferation to supply cells that form both cartilage and bone.

**Sox9-expressing progenitors give rise to hybrid skeletal cells to initiate osteophytes**

Next, we sought to refine which progenitor populations contribute to osteophytes. Clonal tracing of Pdgfra-expressing cells had indicated a possible dual origin from periosteum and synovium (Fig. 1f). Sox9-expressing progenitors in adult periosteum supply skeletal cells to the callus during femoral
fracture repair and large-scale rib regeneration.[10,11] We therefore performed lineage tracing of adult Sox9-expressing cells in experimental OA by treating Sox9-CreER;Tom mice with tamoxifen prior to DMM surgery. Mice not treated with tamoxifen showed absence of Tom expression. In knees of tamoxifen-treated uninjured mice, Tom+ cells were detected in periosteum (Fig. 5a). At 2 weeks post-DMM, we observed Tom+ Sox9-traced chondrocytes throughout the early osteophyte (Fig. 5b), thus identifying Sox9-expressing progenitors as important contributors of osteophytes.

During regeneration of the adult mouse rib bone, Sox9-expressing periosteal cells form a callus composed of hybrid skeletal cells; these “hybrid” cells are characterised by strong co-expression of chondrocyte and osteoblast genes.[11] Similarly, Col2a1-expressing cells in the forming osteophyte co-expressed the osteoblast and hypertrophic chondrocyte marker Ocn as early as 1 week post-DMM (Fig. 5c). In contrast to growth plate chondrocytes, they also co-expressed Col1a1 (Fig. 5c), which was particularly apparent in the large osteophytes that typically form on the Col2-CreER background (Supplementary Fig. 2). At 2 weeks post-DMM, Col2a1-expressing chondrocytes were also positive for the osteoblast and hypertrophic chondrocyte marker Spp1, and the hypertrophic chondrocyte marker Col10a1 (Fig. 5d). We confirmed in the Sox9-CreER;Tom model that Sox9-traced cells are the source of at least some hybrid cells, based on co-localization of Tom with Col2a1 and Col1a1 mRNA expression (Fig. 5e). These findings indicate that the early osteophyte is composed of Sox9-derived hybrid skeletal cells similar to those described for rib bone regeneration.

**Prg4+ progenitors contribute to cartilage but not to bone in osteophytes**

The Gdf5 lineage includes Prg4-expressing cells in the synovial lining, which expand in response to acute focal cartilage injury.[17] We therefore investigated whether Prg4-expressing cells contribute to osteophyte formation. We first used Prg4-CreER;ROSA26:loxP-membrane-Tomato-loxP-membrane-GFP (mTmG) mice induced with tamoxifen at 7 weeks of age. No GFP was detected in mice not treated with tamoxifen. At 2 weeks post-DMM, we observed expansion of GFP+ Prg4-
traced cells in synovium, and Prg4-traced cells were found in the outer region of the early osteophyte, while minimal contributions of these cells to the deeper hybrid skeletal cells, expressing Col2a1 and Col1a1, was observed (Fig. 6a,b). To confirm these findings and determine the role of Prg4-traced cells at later stages of osteophyte formation, we performed similar Prg4-tracing experiments using the Cre-inducible Tom reporter model, and extended analysis to 8 weeks post-DMM (Fig. 6c-h). Osteophytes in Prg4-CreER;Tom mice at 2 weeks post-DMM were typically more advanced than those observed in the Prg4-CreER;mTmG model, with a layer of cartilage surrounding a hypertrophic centre undergoing remodelling to bone (Fig. 6d). As well as expanding in synovium, Tom+ Prg4-traced cells constituted 41.9% (95% CI [27.7%, 56.1%], n=8) of chondrocytes embedded in a cartilage matrix immunostaining for Col2 (Fig. 6d,e). Consistent with the data in the Prg4-CreER;mTmG model (Fig. 6b), they were predominantly found in the outer region, with some contribution to Col10-expressing hypertrophic chondrocytes in deeper regions of the osteophyte (Fig. 6f). At 8 weeks post-DMM, Tom+ Prg4-traced cells persisted in the cartilage cap covering the mature osteophyte but were barely detected in bone (Fig. 6g,h). Thus, Prg4-lineage cells from the overlying synovial tissue supply chondrocytes to the forming osteophyte, and while they persist in the cartilage cap of the mature osteophyte, they make negligible contributions to bone.
DISCUSSION

Osteophytes are a key feature of OA, and their occurrence is a criterion for imaging-based diagnosis of OA.[40] In peripheral joint OA, osteophytes are associated with pain, knee structural progression and incidence of joint replacement.[41-43] Nonetheless, research in OA pathogenesis has largely focused on mechanisms of articular cartilage breakdown, while the extensive joint remodelling events have been considered secondary. Osteophytes, however, are not always linked to severity of articular cartilage loss.[44] Understanding the biology of osteophyte formation will provide critical insights in the mechanisms underlying the structural derangements that occur in OA joints.

We show that osteophytes primarily arise from descendants of Gdf5-expressing embryonic joint interzone cells that reside in the adult knee. Together with our previous data showing that Gdf5-lineage cells underpin synovial hyperplasia and cartilage repair after injury,[15] these findings point to a central role of Gdf5-lineage cells in the maintenance, repair, and remodelling of adult joint tissues. Although osteophytes typically develop close to articular cartilage, Col2a1-expressing chondrocytes from articular cartilage did not give rise to osteophytes. Instead, we show that osteophytes originate at least in part from a population of Sox9-expressing progenitors in periosteum, with progeny of Prg4-expressing synovial-lining cells supplying chondrocytes to the cartilage but not osteoblast-lineage cells that form the bone of the osteophyte.

Several SSC populations have been implicated in bone fracture repair, including SSCs identified by expression of Nes, LepR, or Grem1.[4,8,9,45] We observed negligible contribution of these SSC populations to osteophytes in OA. Yet intriguingly, our data indicate that the initial stages of osteophyte formation are similar to endochondral bone repair in the mouse femur,[10] rib,[11] and zebrafish lower jaw.[36] During bone repair, Sox9-expressing cells in periosteum supply chondrocytes and osteoblasts, and help to orchestrate callus formation.[10,11] Furthermore, the
early callus of the regenerating rib includes Sox9-lineage cells with a hybrid chondrocyte/osteoblast identity,[11] similar to what we observed in the early stages of osteophyte formation.

Until this study, it was not known whether the osteophyte derives from a single progenitor population, or whether multiple progenitor populations cooperate to form the different tissue layers of the osteophyte. Our data indicate that within the Gdf5-lineage population that forms all parts of the osteophyte, periosteal Sox9-expressing progenitors give rise to the deeper hybrid cells that form the ossifying cartilage template, while synovial Prg4-expressing cells supply chondrocytes but make negligible contributions to osteoblast-lineage cells. Recently, it was shown that joint development occurs through a continuous influx of new cells into the Gdf5-expressing joint interzone and flanking regions, with cells being temporally specified to contribute differentially to the multiple tissues of the joint.[17,46] Our data show that lineage fate determination persists in the adult joint between sub-populations of Gdf5-lineage cells and suggest the co-existence of distinct progenitor cell subsets with restricted differentiation potential that may have become imprinted through development.

Human osteophytes in OA hip and knee joints share similar pathological features to those seen in mice, with endochondral bone covered by a cartilage cap that merges with or overgrows the articular cartilage.[2] Notably, our molecular phenotype data in mouse is consistent with observations in human, where an overlap of Col1 and Col2 expression is found in the early stages of osteophyte formation, and chondrocytes in the osteophyte express osteocalcin.[2,47] Adult human synovium and periosteum are known to contain mesenchymal progenitors,[48,49] which may retain the ability to orchestrate an aberrant joint morphogenetic process.[15] Various molecular factors and pathways have been implicated in osteophyte formation, including TGFβ and BMP signalling[1]. Here, we propose a model whereby joint-resident progenitor cell subsets in periosteum and synovium that ontogenetically derive from the embryonic joint interzone respond to such signals and cooperate to form osteophytes in OA (Figure 7). Our data define progenitor cell subsets that could be targeted as part of disease modification strategies for treatment of OA.
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AUTHOR CONTRIBUTIONS
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COMPETING INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY

All data supporting the findings of this study are available within the Article and its Supplementary Information files, or are available from the corresponding authors upon reasonable request.
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FIGURE LEGENDS

Figure 1. **Pdgfra**-lineage progenitors, not articular chondrocytes, clonally expand to form osteophytes.

(a) GFP expression (green) by cells in periosteum and synovium of the knee of a 15-week-old mouse carrying the *Pdgfra-H2BGFP* transgene (n=2). (b) Eight-week-old *Col2-CreER;Tom* mice induced with tamoxifen at 2 weeks of age (n=8, 2 experiments, 7-8 weeks old). Note Tom-labelled cells (red) in articular and growth plate cartilage of the knee. (c) *Pdgfra-H2BGFP;Col2-CreER; Tom* mice induced with tamoxifen at 2 weeks of age and analysed 2 weeks after DMM (n=4, plus n=3 *Col2-CreER;Tom* only). Note *Pdgfra*-expressing cells (green) in osteophyte that are negative for Tom (red). (d) Tom expression (red) in *Col2-CreER;Tom* mice induced with tamoxifen at 2 weeks of age and analysed 8 weeks after DMM (n=6). (e-g) *Pdgfra-CreER;Confetti* mice were induced with tamoxifen from 11-12 weeks of age, followed by DMM surgery and analysis 2 weeks later (n=4). (e) CFP (blue), YFP (yellow) and RFP (red) expression in contralateral knee serving as internal control. Arrows indicate labelled cells along periosteal surface. (f) CFP (blue), YFP (yellow) and RFP (red) expression in osteophyte of destabilised knee. Arrows indicate monochromatic chondrocyte clusters within periosteum, arrowheads indicate distinct monochromatic clusters of chondrocyte-like and fibroblast-like cells in overlying synovium. (g) Percentage of cells in osteophytes labelled with each of the fluorescent proteins, and total percentage of cells labelled (mean ± 95% CI, n=4). Fluorescence microscopy images in (a,b,d) show nuclear counterstain in blue. Dashed white lines in (c,d,f) indicate boundary between osteophyte and edge of tibia. Brightfield images of near-adjacent sections stained with Safranin O and Fast Green are shown on the left in (c-f). A: Articular cartilage, G: Growth plate, PS: Periosteum and Synovium junction, S: Synovium. Scale bars in all panels indicate 100 µm.
Figure 2. Perivascular and Grem1-expressing skeletal stem cells do not contribute to osteophyte formation.

(a-c) Nes-CreER;Tom mice, some also carrying Nes-GFP, were induced with tamoxifen neonatally. (a) Nes-traced cells (red) and Nes-GFP+ cells (green) in knee from 13-week-old unoperated mouse (n=3, 2 experiments, 6-13 weeks old). (b) Nes-traced cells (red, n=6) and Nes-GFP+ cells (green, n=3) in knee 2 weeks post-DMM. Arrows indicate labelled cells around blood vessels in synovium. (c) Nes-traced cells (red) in knee 8 weeks post-DMM (n=3). Arrows indicate labelled cells associated with bone marrow vasculature within osteophyte. (d,e) LepR-Cre;Tom mice underwent DMM surgery at 12 weeks and were analysed 8 weeks later. (d) LepR-traced cells (red) in uninjured contralateral knee serving as internal control (n=3). Arrows indicate labelled cells in synovium and periosteum. (e) LepR-traced cells (red) in destabilised knee (n=4). Arrows indicate labelled cells in synovium. (f) Grem1-CreER;Tom mice were induced with tamoxifen at 7 weeks of age and left unoperated (n=2) or analysed 2 weeks after DMM (n=3). Arrow indicates osteophyte. Fluorescence microscopy images in all panels show nuclear counterstain in blue. Dashed white lines in (b,c,e) indicate boundary between osteophyte and edge of tibia. Brightfield images of near-adjacent sections stained with Safranin O and Fast Green are shown on the left in (a-e). A: Articular cartilage, G: Growth plate, PS: Periosteum and Synovium junction, S: Synovium. Scale bars in all panels indicate 100 µm.

Figure 3. Gdf5-lineage cells are a subset of Pdgfra-expressing cells in the adult knee.

(a) Tom+ Gdf5-lineage cells (red) in 14-week-old Gdf5-Cre;Tom mouse knee. Nuclear counterstain is shown in blue. A: Articular cartilage, PS: Periosteum and Synovium junction, S: Synovium. Scale bar indicates 100 µm. (b) Knee of 11-week-old Pdgfra-H2BGFP;Gdf5-Cre;Tom mouse showing Tom (red; Gdf5-lineage cells) and GFP expression (green; Pdgfra-expressing cells) (n=3). A: Articular cartilage, G: Growth plate, PS: Periosteum and Synovium junction, S: Synovium. Scale bar indicates 100 µm. (c-h) Freshly isolated cells from knees of Pdgfra-H2BGFP;Gdf5-Cre;Tom mice (7-10 weeks old) were
analysed by flow cytometry. See Supplementary Fig. 1 for gating strategies and FMO controls. (c) Representative flow plot showing Tom and GFP expression by single viable cells (n=9, 4 experiments). (d) Percentage of single viable cells that expressed one or both fluorescent labels (mean ± 95% CI, n=9, 4 experiments). (e-h) Phenotypic analysis detecting a range of mesenchymal and fibroblast (Gp38, CD90, CD73, CD51, and CD105), haematopoietic (CD45), endothelial (CD31), or erythrocyte (Ter-119) markers. (e) Representative flow plots showing expression of Tom and the indicated markers within single viable GFP+ cells (n=4-5 for each marker, 4 experiments). (f-h) Percentage of single viable cells that express the indicated markers within (f) GFP+Tom+ (Gdf5-lineage cells), (g) GFP+Tom- (other Pdgfra-expressing cells), and (h) GFP-Tom- cell populations (mean ± 95% CI, n=4-5 for each marker, 4 experiments).

Figure 4. Joint-resident SSCs within the Gdf5-lineage form osteophytes.
(a-c) Adult Gdf5-Cre;Tom mice underwent surgery at 9 weeks to induce DMM in one knee, with contralateral knee sham-operated, and BrdU administered from surgery until end of experiment 2 weeks later. (a) Tom+ Gdf5-lineage cells (red) and BrdU-labelled cells (green) in sham-operated knee (n=4). Arrows indicate Tom+ cells along the periosteal surface with incorporated proliferation label. (b) Tom+ Gdf5-lineage cells (red) and BrdU-labelled cells (green) in destabilised knee (n=4). Note Tom+ cells with incorporated proliferation label throughout the osteophyte. Co-staining for Tom (red) with Col2 (green) to reveal cartilage matrix surrounding Tom+ cells is shown on the far right (image from different mouse). (c) Percentage of cells in osteophytes that are Tom+ Gdf5-lineage cells, and percentage of cells in osteophytes that have incorporated the BrdU proliferation label (mean ± 95% CI, n=4). (d,e) Gdf5-Cre;Tom mice underwent DMM surgery at 9-14 weeks and were analysed 8 weeks after DMM. (d) Tom+ Gdf5-lineage cells (red) in mature osteophyte (n=7). Arrows indicate Tom+ bone lining cells along endosteal surfaces and arrowheads indicate Tom+ osteocytes embedded within bone of the osteophyte. Enlarged image on right shows Tom+ chondrocytes in the
cartilage cap. (e) Percentage of cells in osteophytes that are Tom+ Gdf5-lineage cells (mean ± 95% CI, n=7), divided into the cartilage cap region, and osteocytes within bone. Fluorescence microscopy images in all panels show nuclear counterstain in blue. Dashed white lines in (b,d) indicate boundary between osteophyte and edge of tibia. Brightfield images of near-adjacent sections stained with Safranin O and Fast Green are shown on the left. Boxed regions indicate areas shown at higher magnification on the right. A: Articular cartilage, G: Growth plate, PS: Periosteum and Synovium junction, S: Synovium. Scale bars in all panels indicate 100 µm.

Figure 5. Sox9-expressing progenitors give rise to hybrid cells in the early osteophyte.

(a,b,e) Sox9-CreER;Tom mice were induced with tamoxifen at 7 weeks of age. (a) Tom+ Sox9-traced cells (red) in articular cartilage (A), growth plate (G), and scattered within periosteum (P) of knee from 9-week-old uninjured mouse (n=3). Boxed region on left indicates area shown at higher magnification on the right (different tissue sections are shown). (b) Tom+ Sox9-traced cells (red) throughout osteophyte (outlined with dashed white line on far right) at 2 weeks post-DMM (n=3). Brightfield image of near-adjacent section stained with Safranin O and Fast Green is shown on the left. Boxed region is shown at higher magnification on the far right. A: Articular cartilage, G: growth plate. (c,d) Double fluorescence in situ hybridisation in wild-type mouse knees at 1 week (c) or 2 weeks post-DMM (d) for indicated mRNA targets. Note co-expression of Col2a1 (red) with Col1a1, Ocn, Spp1, or Col10a1 (green) in the early osteophyte, and absence of Col1a1 in the tibial growth plate (G). Merged and individual channel images of the boxed osteophytes are shown to the right. n=3 for each probe combination. (e) Co-detection of Tom with Col2a1 and Col1a1 mRNA in osteophyte of Sox9-CreER;Tom mouse (n=3). Individual and merged channel images are shown. Note Tom+ Sox9-traced cells (magenta) co-expressing Col2a1 (green) and Col1a1 (red) in outlined area. Fluorescence microscopy images in all panels show nuclear counterstain in blue. Scale bars indicate 100 µm in (a,b) and 200 µm in (c-e).
Figure 6. Contribution of Prg4-expressing progenitors to osteophytes.

(a,b) *Prg4-CreER;tmTOR* mice were induced with tamoxifen at 7 weeks of age, followed by surgery to induce DMM in one knee, with contralateral knee sham-operated. *Prg4*-traced cells were detected with anti-GFP antibody (green), and *Col1a1* or *Col2a1* mRNA expression by fluorescence in situ hybridisation (red). (a) GFP+ *Prg4*-traced cells at articular surface and in synovial lining in control sham-operated knee (n=3). Note membrane localisation of GFP, indicating successful *tmTOR* conversion. (b) GFP+ *Prg4*-traced cells at 2 weeks post-DMM. Note expansion in synovium but minimal contribution to hybrid cells that express *Col1a1* and *Col2a1* in the early osteophyte (n=3). Boxes indicate magnified images to the right, shown as merged and individual channels. Arrowheads indicate rare *Prg4*-traced cells expressing *Col2a1*. (c-h) *Prg4-CreER;Tom* mice were induced with tamoxifen at 8 weeks of age. (c) Tom+ *Prg4*-traced cells (red) in synovial lining and superficial zone of articular cartilage in 10-week-old uninjured mouse (n=7, 3 experiments). Green: Col2 immunostaining (n=3). (d) Tom+ *Prg4*-traced cells (red) in osteophyte at 2 weeks post-DMM (n=8, 2 experiments). Note Tom+ cells in Col2+ (green) cartilage matrix and overlying synovial tissue. (e) Percentage of cells that expressed Tom at 2 weeks post-DMM in Col2+ cartilage matrix or Col2- tissue of the osteophyte (mean ± 95% CI, n=8, 2 experiments). (f) Tom+ *Prg4*-traced cells (red) in Col10+ (green) hypertrophic cartilage of osteophyte 2 weeks post-DMM, indicated by arrows (n=4). (g) Tom+ *Prg4*-traced cells (red) in osteophyte at 8 weeks post-DMM (n=7, 2 experiments). Green: Col2 immunostaining. (h) Percentage of cells that expressed Tom at 8 weeks post-DMM in Col2+ cartilage matrix or Col2- tissue of the osteophyte cap, or among osteocytes in the osteophyte bone (mean ± 95% CI, n=7, 2 experiments). Fluorescence microscopy images show nuclear counterstain in blue. Brightfield images of Safranin-O-stained near-adjacent sections are shown on the left in (d,g). Dashed white lines indicate boundary between osteophyte and edge of tibia in (d,f,g). A: Articular cartilage, G: Growth plate, PS: Periosteum and Synovium junction, S: Synovium. Scale bars indicate 200 µm in (a,b) and 100 µm in (c,d,f,g).
Our data show that Pdgfra+ Gdf5-lineage progenitors, which in the normal joint are present at the junction of periosteum and synovium near the articular cartilage, are activated in OA to form both the cartilage and bone of the osteophyte. They include Prg4-expressing progenitors (orange) residing in synovial lining and Sox9-expressing progenitors (green) in the underlying periosteum. During the early stage of osteophyte formation, Sox9-expressing progenitors in periosteum give rise to hybrid skeletal cells that form a transient cartilage template which is remodelled to bone. Progeny of Prg4-expressing progenitors are recruited to the forming osteophyte and supply chondrocytes to the cartilage, but they make negligible contributions to osteoblast-lineage cells that form the bone.

A: Articular cartilage, M: meniscus.
**Nes-GFP; Nestin-CreER; Tom**

Neonatal induction

**LepR-Cre; Tom**

Control

**Nestin-CreER; Tom**, neonatal induction

**Grem1-CreER; Tom**, induction at 7 w

8 w DMM

2 w DMM
Gdf5-Cre; Tom, BrdU for 2 w after DMM

C

Labelled cells (%)

100 90 80 70 60 50 40 30 20 10 0

Tom BrdU

Gdf5-Cre; Tom

8 w DMM

Cap Bone

Tom+ cells (%)

0 20 40 60 80 100

Cap Bone
Supplementary Figure 1. Flow cytometry analysis of adult Gdf5-lineage and Pdgfra-expressing cells. (a) Gating strategy to identify GFP-Tom-, GFP+Tom- and GFP+Tom+ cells within single live cells freshly isolated from knees of adult Pdgfra-H2BGFP; Gdf5-Cre; Tom mice (n=9, 3 experiments). Erythrocytes and debris were gated out based on Forward and Side Scatter profile. Doublets and aggregates were excluded based on Forward Scatter parameters. Dead cells were excluded based on viability dye staining. (b) Gates for detection of cell surface markers were set using fluorescence-minus-one (FMO) controls. (c) Representative flow cytometry plots for the analysis of cell surface markers (n=4-5 for each marker, pooled data from 3 experiments).
Supplementary Figure 2. Hybrid cells in early osteophytes of *Col2-CreER* animals. Detection of *Col1a1* (a) and *Col2a1* (b) in early osteophyte (arrow) at 1 week post-DMM by fluorescence in situ hybridisations on adjacent sections of knee from 9-week-old *Col2-CreER; mTmG* mouse (n=2). Note also expression of *Col2a1* but not *Col1a1* in chondrocytes within the tibial growth plate (G). Nuclear counterstain is shown in blue. A: Articular cartilage. Scale bar indicates 100 µm.
Supplementary Table 1. Transgenic mouse lines.

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Supplementary Table 2. Tamoxifen administration and age at surgery.

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**Supplementary Table 3. Antibodies for immunohistochemistry.**

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**Supplementary Table 4. Antibodies for flow cytometry.**

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