

Identification of a *Prg4*-Expressing Articular Cartilage Progenitor Cell Population in Mice

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Objective. To generate knockin mice that express a tamoxifen-inducible Cre recombinase from the *Prg4* locus (*Prg4*^{GFPCreERT2} mice) and to use these animals to fate-map the progeny of *Prg4*-positive articular cartilage cells at various ages.

Methods. We crossed *Prg4*^{GFPCreERT2} mice with *Rosa26*^{loxlacZ} or *Rosa26*^{mTmG} reporter strains, administered tamoxifen to the double heterozygous offspring at different ages, and assayed Cre-mediated recombination by histochemistry and/or fluorescence microscopy.

Results. In 1-month-old mice, the expression of the *Prg4*^{GFPCreERT2} allele mirrored the expression of endogenous *Prg4* and, when tamoxifen was administered for 10 days, caused Cre-mediated recombination

in ~70% of the superficial-most chondrocytes. *Prg4*^{GFPCreERT2}-expressing cells were mostly confined to the top 3 cell layers of the articular cartilage in 1-month-old mice, but descendants of these cells were located in deeper regions of the articular cartilage in aged mice. On embryonic day 17.5, *Prg4*^{GFPCreERT2}-expressing cells were largely restricted to the superficial-most cell layer of the forming joint, yet at ~1 year, the progeny of these cells spanned the depth of the articular cartilage.

Conclusion. Our results suggest that *Prg4*-expressing cells located at the joint surface in the embryo serve as a progenitor population for all deeper layers of the mature articular cartilage. Also, our findings indicate that *Prg4*^{GFPCreERT2} is expressed by superficial chondrocytes in young mice, but expands into deeper regions of the articular cartilage as the animals age. The *Prg4*^{GFPCreERT2} allele should be a useful tool for inducing efficient Cre-mediated recombination of loxP-flanked alleles at sites of *Prg4* expression.

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While much is known about the mechanisms that regulate the formation and maturation of growth plate cartilage (for review, see refs. 1 and 2), much less is known about the development of articular cartilage (for review, see refs. 3 and 4). In contrast to growth plate chondrocytes, which establish a transient cartilage template that is replaced by bone (i.e., endochondral ossification), articular chondrocytes establish a permanent cartilage tissue. Growth plate and articular chondrocytes arise from distinct progenitor populations, such that articular chondrocytes share a common origin with synovial cells that line the joint cavity (5,6). In humans (and other large mammals), adult articular cartilage has been divided into 4 zones based on histologic features. The nonmineralized articular cartilage consists of a superficial zone, composed of flattened chondrocytes; a middle zone, composed of relatively small

round chondrocytes; and a deep zone, which contains larger round chondrocytes arranged in a columnar fashion. Beneath these top 3 zones lies the mineralized articular cartilage (which is separated from the non-mineralized cartilage by the tidemark), and deeper still, the subchondral bone.

Both the identity of the cells that give rise to the articular cartilage and the mechanism by which the articular cartilage grows are incompletely understood. Two growth mechanisms seem plausible: interstitial and appositional. For interstitial growth, a precursor cell population would give rise to distinct types of articular chondrocytes (i.e., superficial, middle, and deep), and these chondrocyte subtypes would populate their corresponding layers. For appositional growth, a precursor population would first give rise to cells in the superficial layer, which subsequently would undergo a maturation process to populate the other cartilage zones.

Prior studies have attempted to delineate the mechanism of cartilage growth using metabolic labeling. Tritiated thymidine incorporation in the rabbit knee joint demonstrated 2 proliferative regions, one within the superficial zone of the articular cartilage and the other within the subchondral plate (7). Hunziker and colleagues noted that the superficial articular cartilage cells in the medial femoral condyle of 2-month-old NZW rabbits proliferated more slowly than cells within the transitional and upper radial zones of this tissue (8). Bromodeoxyuridine (BrdU) labeling in the opossum knee joint demonstrated that cells in both the superficial and middle layers incorporate BrdU (9). Pulse-chase experiments in mice have demonstrated that the superficial-most articular chondrocytes in newborn animals retain a pulse of BrdU/5-ethynyl-2'-deoxyuridine (EdU) for up to 6 weeks, suggesting that these cells may constitute a slowly cycling stem cell population (10,11). Indeed, it has been proposed that the superficial cells of the articular cartilage may comprise a progenitor/stem cell population since only cells from this layer can give rise to colonies with high colony-forming efficiencies in a Notch-dependent manner, and show phenotypic plasticity when injected into the chick limb bud (12). However, none of these studies was able to trace the fate of superficial zone chondrocytes by lineage labeling in articular cartilage.

Lubricin, encoded by the *Prg4* locus, is abundantly expressed by superficial zone chondrocytes and synoviocytes (13). Individuals with a genetic deficiency of *PRG4* have camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome (13). Patients with CACP have normal-appearing joints at birth, but with

advancing age develop joint failure associated with noninflammatory synovial hyperplasia and subintimal fibrosis of the synovial capsule (14). While *Prg4*^{-/-} mice similarly display significant joint abnormalities, heterozygous *Prg4* mutant (*Prg4*^{+/-}) mice appear normal (15). Herein, we describe a mouse strain that has a chimeric green fluorescent protein (GFP)-tamoxifen-inducible Cre recombinase knocked into the endogenous *Prg4* locus (*Prg4*^{GFPcreERT2}). We demonstrate that *GFPcreERT2* expression mirrors endogenous *Prg4* expression in this strain, and we use this strain to identify and lineage-trace descendants of *Prg4*^{GFPcreERT2}-expressing cells (and by extrapolation, *Prg4*-expressing cells) in articular cartilage. We report that young mice express *Prg4*^{GFPcreERT2} in cells located near the cartilage surface, and that these cells serve as progenitors for cells located in both the superficial and deeper regions of the articular cartilage in older mice. We also found that *Prg4*^{GFPcreERT2} is expressed by superficial articular chondrocytes in young mice, but expands into deeper regions of the articular cartilage as the animals age.

MATERIALS AND METHODS

Mouse strains. *Generation of Prg4*^{GFPcreERT2} mice. We designed a targeting vector (Figure 1A) that would insert a GFPcreERT2 cassette and a PGKneo cassette (16) into the translation initiation codon site within exon 2 of the *Prg4* locus. The targeting vector carried the GFPcreERT2 cassette followed by a PGKneo cassette flanked by F1p recombinase target (FRT) sites, which were bordered by ~2 kb of homologous *Prg4* locus sequence on both ends. *Prg4*-containing BAC clone RP23-147F24 (BACPAC Resources) was used to amplify the 2-kb 5' homology and the 1.8-kb 3' homology targeting arms by polymerase chain reaction (PCR). We flanked the PGKneo sequence with FRT sites in order to remove this cassette after correct targeting was achieved. PGK-DTA (encoding diphtheria toxin to select against nonhomologous recombination) was inserted downstream of the 3' homologous arm. We performed homologous recombination in the 129 embryonic stem (ES) cell line and chose a correctly targeted clone (see Supplementary Figure 1A, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>) to generate mice with the *Prg4*^{GFPcreERT2-PGKneo} allele. Targeting in ES cells was assayed by PCR analysis, using primers amplifying either 5' or 3' correctly targeted arms, followed by either *Eco* RI or *Sac* I restriction digestion, respectively, of the PCR-generated fragments to ensure specificity of amplification. Correctly targeted ES cells were injected into mouse blastocysts to eventually generate a line of mice containing *Prg4*^{GFPcreERT2-PGKneo}. We excised the PGKneo cassette using a F1p recombinase-expressing mouse (*ACT-FLPe*) (17) to produce mice with the *Prg4*^{GFPcreERT2} allele alone. In subsequent crosses we distinguished the wild-type (*Prg4*⁺) and knockin (*Prg4*^{GFPcreERT2}) alleles using PCR (see Supplementary Figure 1B, available on

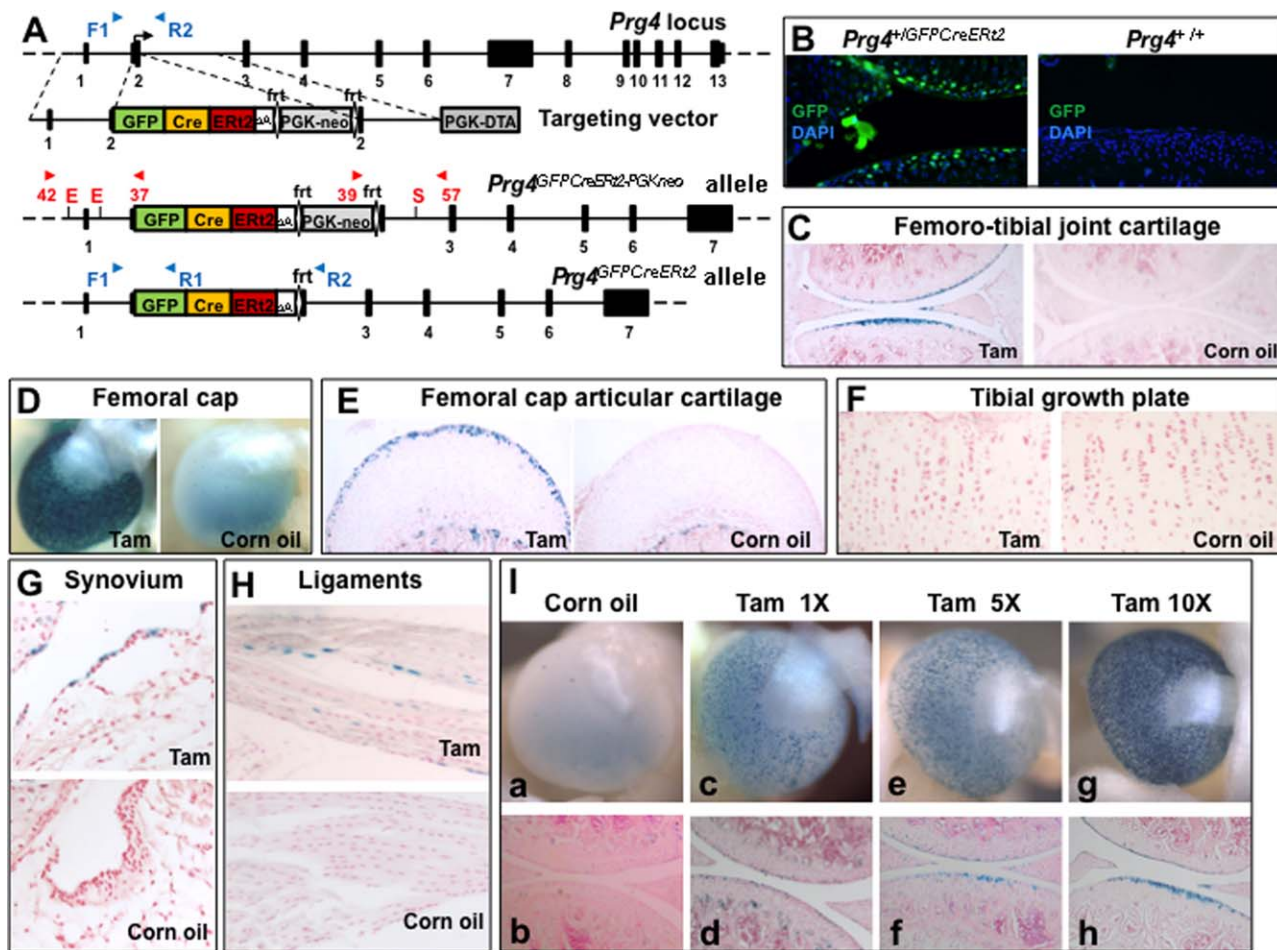


Figure 1. *Prg4*^{GFP-Cre-ER²} drives robust recombination in superficial articular chondrocytes in 1-month-old mice. **A**, Schematic diagram of exon-intron structure of the wild-type *Prg4* allele (not drawn to scale), the targeting vector, and the *Prg4* knockin allele prior to and after excision of the PGK-neo cassette. **B**, Photomicrographs depicting immunofluorescence detection of GFP-Cre-ER² protein using a fluorescence-labeled anti-green fluorescent protein (anti-GFP) antibody in the knee joints of 1-month-old *Prg4*^{GFP-Cre-ER²} and *Prg4*^{+/+} mice. **C–H**, X-Gal-stained knee joints (**C**), femoral heads (**D**), femoral head sections (**E**), tibial growth plates (**F**), synovial tissues (**G**), and ligaments (**H**) obtained on postnatal day 34 from *Prg4*^{GFP-Cre-ER²}; *Rosa26*^{+/floxlacZ} mice that had been given daily intraperitoneal injections of either tamoxifen (Tam) or vehicle (corn oil) from postnatal day 21 to postnatal day 31. **I**, Whole mounts of femoral heads (**a**, **c**, **e**, and **g**) and sections of the knee joints (**b**, **d**, **f**, and **h**) of *Prg4*^{GFP-Cre-ER²}; *Rosa26*^{+/floxlacZ} mice that had been given either corn oil (**a** and **b**) or a 1-, 5-, or 10-day course of tamoxifen (**c–h**). The mice were killed on postnatal day 34 (3 days after the last injection), followed by whole-mount X-Gal staining of their femoral heads and knee joints. Original magnification $\times 200$ in **B**, $\times 40$ in **C**, **E**, and **I** parts **b**, **d**, **f**, and **h**; $\times 16$ in **D** and **I** parts **a**, **c**, **e**, and **g**; $\times 100$ in **F–H**.

the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>). The primers used were TCAGGAATCAAGCTGATTGC (F1), AACTGTGGCCGTTTACGTC (R1), and CCTTGAGATGAAACCTGTTGAATC (R2). Primer pair F1/R1 produces a 337-bp amplicon from the *Prg4*^{GFP-Cre-ER²} allele, and primer pair F1/R2 produces a 258-bp amplicon from the *Prg4*⁺ allele. The *Prg4*^{GFP-Cre-ER²} mice were maintained on a mixed genetic background (i.e., 129/Sv \times C57BL/6) and donated to The Jackson Laboratory for distribution (catalog no. 022757).

Mouse reporter strains. *Rosa26*^{floxlacZ} mice (18) (catalog no. 003309) and *Rosa26*^{mTmG} mice (19) (catalog no. 007576) were obtained from The Jackson Laboratory. *Tg(Foxa3-cre);Rosa26*^{+/floxlacZ} mice were generated by crossing *Tg(Foxa3-cre)* mice (20) with homozygous

Rosa26^{floxlacZ/floxlacZ} mice. We induced Cre recombinase activity in postnatal mice with the *Prg4*^{GFP-Cre-ER²} allele by administering intraperitoneal (IP) injections of tamoxifen (100 mg/kg/dose) diluted in corn oil (10 mg/ml). Injection of corn oil alone was used as a negative control. We induced Cre recombinase activity in embryonic day 17.5 fetuses by giving a single IP injection of 4 mg tamoxifen in corn oil to the dam. We studied a minimum of 3 mice per genotype, treatment, and age group. *Osx-Cre* (21) and *Coll-Cre-ER²-Cre* alleles have been described previously (22). *Rosa26*^{+/floxtdTomato} (23) was used as a re-combination reporter allele. Two-month-old *Coll-Cre-ER²-Cre; Rosa26*^{+/floxtdTomato} mice were given a single injection of tamoxifen and harvested 1 week after the injection. *Osx-Cre; Rosa26*^{+/floxtdTomato} mice were harvested at 2 months of

age, and processed for frozen sectioning. EdU (50 mg/kg body weight; Invitrogen) was administered either as a single IP injection to the dam for embryonic labeling, or daily (by IP injection) for 10 consecutive days to 1-month-old mice.

Immunofluorescence and fluorescence microscopy.

Anti-GFP rabbit IgG antibody fraction (Alexa Fluor 555 conjugate; Invitrogen), anti-aggrecan antibody (AB1031; Millipore), anti- α -smooth muscle actin (Anti-Actin, α -Smooth Muscle; Sigma), anti-CD31 (CD31/platelet endothelial cell adhesion molecule 1; Invitrogen), and anti-albumin (Abcam) antibodies were used to detect their cognate proteins.

RESULTS

Generation and characterization of $Prg4^{+/GFPCreERt2}$

mice. We chose to insert a GFPCreERt2 cassette into the translation initiation site of the endogenous *Prg4* locus (located in exon 2) to ensure that expression of this construct reflects the endogenous expression of the *Prg4* gene (Figure 1A) (see Supplementary Figures 1A and B, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>). $Prg4^{+/GFPCreERt2}$ mice are viable, fertile, and lack the joint contractures, gait abnormalities, protein deposition on the articular cartilage, and synovial hyperplasia that are apparent in lubricin-knockout animals (15). Like the joints of $Prg4^{-/-}$ mice (15), those of 9-month-old homozygous $Prg4^{GFPCreERt2/GFPCreERt2}$ mice lacked the superficial-most layer of articular chondrocytes, exhibited a proteinaceous deposit on top of their articular cartilage (see Supplementary Figure 1C, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>), and displayed synovial hyperplasia (data not shown). GFPCreERt2 expression in superficial zone chondrocytes from ~1-month-old $Prg4^{+/GFPCreERt2}$ mice was detected by using an anti-GFP antibody (Figure 1B), but not by direct GFP fluorescence of the fusion protein (see Supplementary Figure 2A, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>), suggesting that GFPCreERt2 was expressed in superficial zone articular chondrocytes at a relatively low level. Consistent with this notion, RNA sequencing analysis of articular cartilage revealed that *GFPCreERt2* expression was 12- to 50-fold lower than endogenous *Prg4* expression (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>).

To assay recombination driven by the $Prg4^{GFPCreERt2}$ allele, we crossed $Prg4^{+/GFPCreERt2}$ mice

with $Rosa26^{loxlacZ/floxlacZ}$ mice and analyzed β -galactosidase expression in the knee and hip joints of the double heterozygous offspring (i.e., $Prg4^{+/GFPCreERt2};Rosa26^{+/floxlacZ}$) treated with tamoxifen. Expression of β -galactosidase was detected in femorotibial cartilage (Figure 1C), femoral cap cartilage (Figures 1D and E), synovial fibroblasts (Figure 1G), and the ligaments of the knee (Figure 1H) in $Prg4^{+/GFPCreERt2};Rosa26^{+/floxlacZ}$ mice injected with tamoxifen. The pattern of expression observed reflects one previously described for endogenous *Prg4* expression using in situ hybridization (15) and the expression pattern observed in mice containing a β -galactosidase-expressing *Prg4* gene trap, $Prg4^{+/lacZgenetrap}$ (Figure 2W) (Warman ML, et al: unpublished observations).

We further assessed the extent of $Prg4^{GFPCreERt2}$ -driven recombination in the articular cartilage by treating $Prg4^{+/GFPCreERt2};Rosa26^{+/floxlacZ}$ mice with either a 1-, 5-, or 10-day course of tamoxifen. Mice were killed on postnatal day 34, 3 days after the last injection. Whole-mount X-Gal staining of the femoral heads and knee joints from these mice demonstrated that after 10 days of tamoxifen injection, there were high rates of Cre-mediated recombination in chondrocytes that were located in the superficial region of the articular cartilage (Figure 1I, parts c–h). In contrast, when we administered only vehicle (i.e., corn oil), very few cells (~5 cells per the entire surface of the femoral cap) displayed evidence of Cre-mediated recombination (Figure 1I, parts a and b). Consistent with previous reports that *Prg4*/lubricin is expressed in articular cartilage but not in growth plate cartilage (15), we observed no evidence of Cre-mediated recombination in growth plate cartilage in tamoxifen-treated $Prg4^{+/GFPCreERt2};Rosa26^{+/floxlacZ}$ mice (Figure 1F).

$Prg4^{GFPCreERt2}$ expression by superficial articular chondrocytes in young mice, but expansion into deeper regions of the articular cartilage as the mice age. We evaluated the expression of *Prg4* in the knee articular cartilage of mice at differing ages by pulsing $Prg4^{+/GFPCreERt2};Rosa26^{+/floxlacZ}$ embryos on day 17.5 and postnatal mice at either 1, 3, 6, or 18 months of age with tamoxifen. When embryos were given a single pulse of tamoxifen on embryonic day 17.5 and harvested on postnatal day 0, β -galactosidase-expressing cells were restricted to the superficial cell layers of the developing joint (Figures 2A–C). Mice given 10 days of tamoxifen treatment, beginning on postnatal day 21, and then killed (on postnatal day 34) 3 days after the last injection, displayed a majority of β -galactosidase-expressing cells in the 3 most superficial cell layers of the articular cartilage (Figures 2D–F). Interestingly,

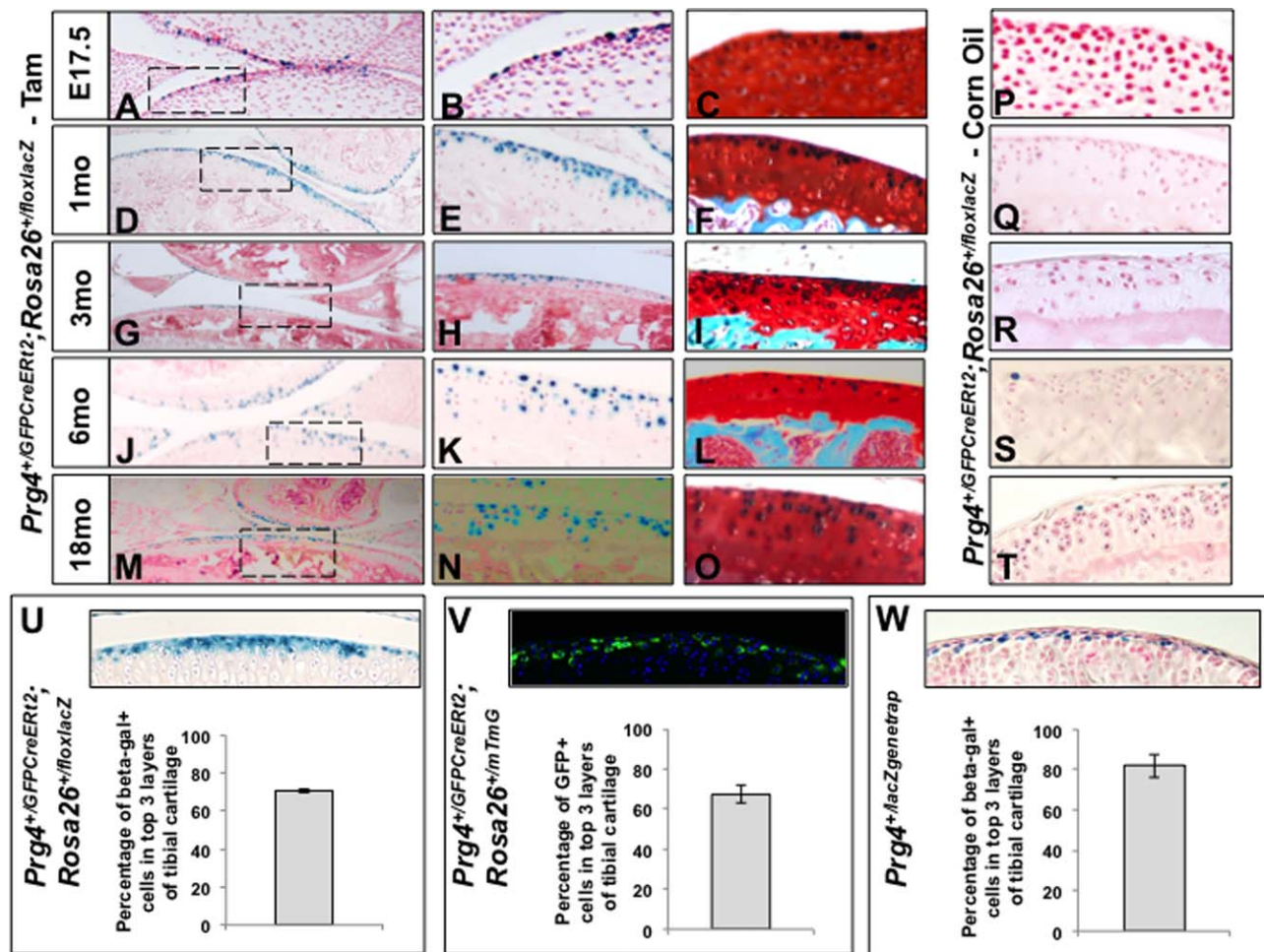


Figure 2. *Prg4*^{GFPcreERt2} is initially expressed by superficial zone chondrocytes in mice and is additionally expressed by deeper-zone chondrocytes as the mice age. **A–O**, Photomicrographs of X-Gal-stained knee joint cartilage from either postnatal day 0 (**A–C**), 1-month-old (**D–F**), 3-month-old (**G–I**), 6-month-old (**J–L**), or 18-month-old (**M–O**) *Prg4*^{GFPcreERt2};*Rosa26*^{+/floxlacZ} mice that had received either a single pulse (on embryonic day 17.5 [E17.5]) (**A–C**) or 10 consecutive days of injections (**D–O**) of tamoxifen (Tam) and were killed 3 days after the last injection. **A**, **D**, **G**, **J**, and **M** show X-Gal–fast red staining. **B**, **E**, **H**, **K**, and **N** show higher-magnification images of the boxed areas in **A**, **D**, **G**, **J**, and **M**, respectively. **C**, **F**, **I**, **L**, and **O** show high-magnification images following X-Gal–Safranin O–fast green staining. Knee joint tibial articular cartilage is displayed either on the bottom of the image or by itself in each panel. Original magnification $\times 100$ in **A–C**; $\times 40$ in **D–O**. **P–T**, Photomicrographs of X-Gal-stained knee joint tibial articular cartilage from postnatal day 0 (**P**), 1-month-old (**Q**), 3-month-old (**R**), 6-month-old (**S**), or 18-month-old (**T**) *Prg4*^{GFPcreERt2};*Rosa26*^{+/floxlacZ} mice treated with corn oil. Original magnification $\times 100$ in **P**; $\times 40$ in **Q–T**. **U** and **V**, X-Gal staining of knee joint cartilage from 3 postnatal day 34 *Prg4*^{GFPcreERt2};*Rosa26*^{+/floxlacZ} mice (**U**) and fluorescence microscopy of knee joint cartilage from 3 postnatal day 34 *Prg4*^{GFPcreERt2};*Rosa26*^{+/mTmG} mice (**V**) that were each given daily intraperitoneal injections of tamoxifen from postnatal day 21 to postnatal day 31. Top, Tibial cartilage from one such mouse of each genotype is shown. Bottom, Bars show the mean \pm SEM percentage of X-Gal-stained cells (**U**) or green fluorescent protein (GFP) fluorescing cells (**V**) within the 3 most superficial cell layers. Original magnification $\times 40$ in **U**; $\times 200$ in **V**. **W**, X-Gal staining of knee joint cartilage from a postnatal day 34 *Prg4*^{+/lacZgenetrap} mouse. Top, Tibial cartilage from one mouse is shown. Bottom, Bars show the mean \pm SEM percentage of β -galactosidase-expressing cells within the 3 most superficial cell layers of tibial articular cartilage. Original magnification $\times 200$.

3-month-old, 6-month-old, and 18-month-old *Prg4*^{+/GFPcreERt2};*Rosa26*^{+/floxlacZ} mice that were given tamoxifen for 10 days (and killed 3 days after the last injection) had β -galactosidase expression in deeper layers of the articular cartilage (Figures 2G–O). In contrast, in control *Prg4*^{+/GFPcreERt2};*Rosa26*^{+/floxlacZ} mice (that were injected with corn oil) very few cells displayed

evidence of Cre-mediated recombination, with the oldest (18-month-old) mice showing the highest basal level of spontaneous recombination restricted to occasional superficial chondrocytes in their articular cartilage (Figures 2P–T). Consistent with these findings, we observed that a 15-month-old *Prg4*^{+/lacZgenetrap} mouse similarly displayed β -galactosidase expression in both

superficial and deeper regions of the femoral head articular cartilage, extending nearly to the subchondral bone (see Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at <http://online.library.wiley.com/doi/10.1002/art.39030/abstract>). Taken together, these findings indicate that expression of *Prp4*, which initially is restricted to the most superficial cells in the articular cartilage, extends into deeper cells within this tissue as mice age.

Next, we determined the frequency of recombination driven by the *Prp4^{GFP-CreERT2}* allele in the tibial articular cartilage of 1-month-old mice, using either *Rosa26^{+/-loxlacZ}* or *Rosa26^{+/-mTmG}* reporter alleles. Postnatal day 21 mice were injected with tamoxifen for 10

consecutive days, killed on postnatal day 34 (3 days after the last injection), and the number of recombined cells in the 3 most superficial cell layers of the tibial articular cartilage was counted (Figures 2U and V). Recombination occurred in 70% and 67% of the 3 most superficial cell layers of the tibial articular cartilage in tamoxifen-treated *Prp4^{+/-GFP-CreERT2};Rosa26^{+/loxlacZ}* and *Prp4^{+/-GFP-CreERT2};Rosa26^{mTmG}* mice, respectively (Figures 2U and V). To estimate the percentage of cells that normally express *Prp4* in these top 3 cell layers, we used the β -galactosidase-expressing *Prp4* gene-trap mouse strain (*Prp4^{+/-lacZ}genetrap*); this *Prp4* gene-trap strain displayed detectable β -galactosidase expression in 82% of the chondrocytes located in the top 3 cell

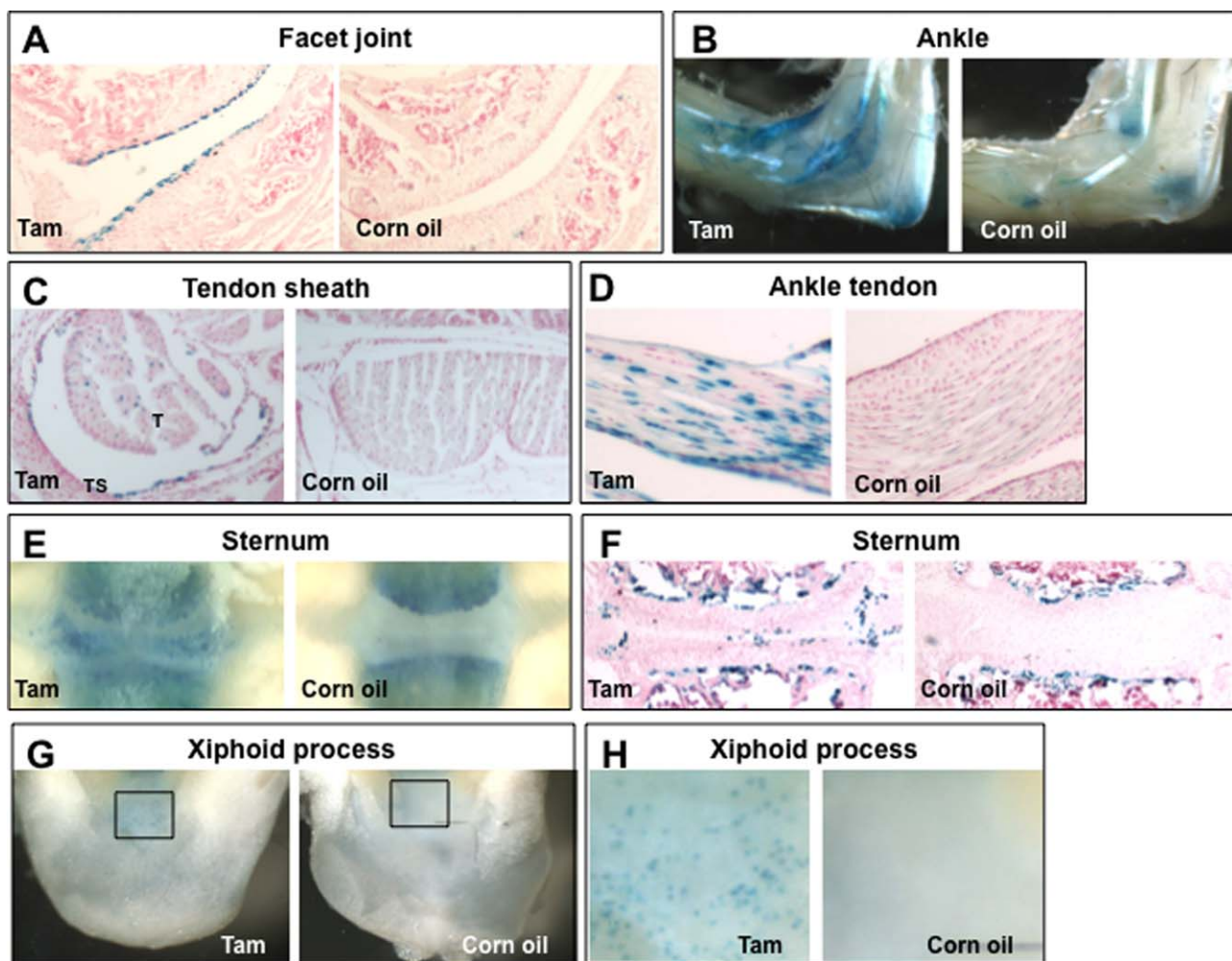


Figure 3. *Prp4^{GFP-CreERT2}* is expressed in several cell types in 1-month-old mice. Photomicrographs and photographs of X-Gal-stained tissue samples that were harvested from postnatal day 34 *Prp4^{+/-GFP-CreERT2};Rosa26^{+/-loxlacZ}* mice that had been given daily intraperitoneal injections of either tamoxifen (Tam) or vehicle (corn oil) from postnatal day 21 to postnatal day 31 are shown. Sections displayed in A–F were counterstained with fast red. Note that *Prp4^{+/-GFP-CreERT2}*-mediated recombination is observed in vertebral facet joint chondrocytes (A), ankle tendons (B and D), tendon sheaths (C), sternal chondrocytes (E and F), and the xiphoid process (G and H). H shows higher-magnification views of the boxed areas in G. T = tendon; TS = tendon sheath. Original magnification $\times 100$ in A, C, and D; $\times 16$ in B; $\times 40$ in E–H.

layers of the articular cartilage (Figure 2W). Taken together, these data suggest that the effective recombination efficiency of the *Prg4*^{GFP^{CreERT2}} allele (following 10 days of tamoxifen injection) is ~85% in *Prg4*-expressing articular chondrocytes.

***Prg4*^{GFP^{CreERT2}} drives recombination outside of articular cartilage.** Extraarticular findings in patients with CACP syndrome, including pericarditis and intervertebral disc degeneration, suggested that *PRG4* would be expressed at sites other than diarthrodial joints (14). This was confirmed by multitissue Northern blot analysis (13) and other tissue-specific gene expression studies (24). Therefore, we looked for other sites of tamoxifen-induced *Prg4*^{GFP^{CreERT2}}-mediated recombination. In the skeletal system, we found recombination in tendons, tendon sheaths, vertebral facet joints, and sternal chondrocytes (Figure 3) (see Supplementary Figure 4, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>). We also observed recombination in the heart and liver (see Supplementary Figures 4 and 5, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>), consistent with prior findings that endogenous *Prg4* is expressed in the liver (13). In this tissue, tamoxifen-induced *Prg4*^{GFP^{CreERT2}} activity was detected in ~5% of the hepatocytes, but not in Kupffer, endothelial, or Ito cells (see Supplementary Figure 5, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>).

***Prg4*-expressing cells in both fetal and postnatal day 21 mice are progenitors for chondrocytes in deeper zones of adult articular cartilage.** We performed lineage tracing of *Prg4*-expressing cells in fetal (approximate embryonic day 17.5) *Prg4*^{+ /GFP^{CreERT2}}; *Rosa26*^{+ /flox^{lacZ}} mice by giving a single dose of tamoxifen to the dam. The dam's offspring were killed on either postnatal day 0, postnatal day 14, or at 2 months or 12 months of age, and the distribution of β -galactosidase-expressing chondrocytes in the femorotibial joint was determined. We observed that β -galactosidase expression, which is specifically restricted to the superficial-most layer of the developing knee joint in postnatal day 0 mice (Figures 2A–C and Figures 4A and B), expands into deeper layers of the knee joint in postnatal day 14 mice (Figures 4C and D), extends throughout the depth of the articular cartilage in mice older than age 2 months (Figures 4E and F), and beyond the tidemark in 12-month-old mice (Figures 4G and H). Of note, in all postnatal mice, the progeny of the recombined cells were present in both

the superficial and deeper regions of the articular cartilage (Figures 4C–H). In addition, “columnar clones” of β -galactosidase-expressing chondrocytes that extended through the entire depth of the articular cartilage (extending from the nonmineralized region into the mineralized matrix) were apparent in both 2-month-old and 12-month-old mice (Figures 4E–H). In some cases, β -galactosidase-expressing cells were also retained in the most superficial layer of such “columnar clones,” suggesting that the clonal progenitors gave rise to progeny in both the superficial cell layer and in deeper regions of the developing articular cartilage.

These findings suggest that a radial expansion of *Prg4*-expressing cells in the mouse articular cartilage takes place by 2 months of age to give rise to cells spanning the entire depth of this tissue. Indeed, a single pulse of EdU (on embryonic day 17.5) was incorporated into proliferating cells in both the surface and deeper layers of the forming joint on postnatal day 0 (Figure 4I). In contrast, postnatal EdU administration (by sequential injections of EdU from postnatal days 21–31) indicated that cellular proliferation in the articular cartilage was principally restricted to cells at or near the articular surface on postnatal day 34 (Figures 4J and K), consistent with the notion that cells in the superficial region of the articular cartilage proliferate to give rise to cells that come to lie in deeper regions of this tissue. Importantly, β -galactosidase-expressing chondrocytes in 2-month-old *Prg4*^{+ /GFP^{CreERT2}}; *Rosa26*^{+ /flox^{lacZ}} mice (which had received tamoxifen on embryonic day 17.5) were embedded throughout the aggrecan-positive articular cartilage matrix (Figure 4L) and extended well into the hypertrophic region of this tissue (marked by *Osx1-Cre* and aggrecan expression) (Figure 4M) but not into the underlying subchondral bone (marked by *Coll-CreERT2* expression) (Figure 4N). Taken together, these findings indicate that *Prg4*-expressing cells that are located in the superficial-most layer of the developing joints in the day 17.5 mouse embryo give rise to deeper layers of articular chondrocytes, but not to subchondral osteocytes in the adult joint.

We also performed lineage tracing in postnatal day 21 mice by treating them for 10 consecutive days with either tamoxifen or vehicle alone and then analyzing their femorotibial joints either on postnatal day 34 (i.e., at 1 month) or at 6 months or 18 months of age. Confirming what we had previously observed (Figures 2D–F), *Prg4*^{GFP^{CreERT2}}-mediated recombination occurred in cells closest to the articular surface in 1-month-old mice (Figures 5A and B). However, at 6 and 18 months of age, increasing numbers of recombined chondrocytes expressing β -galactosidase were present in

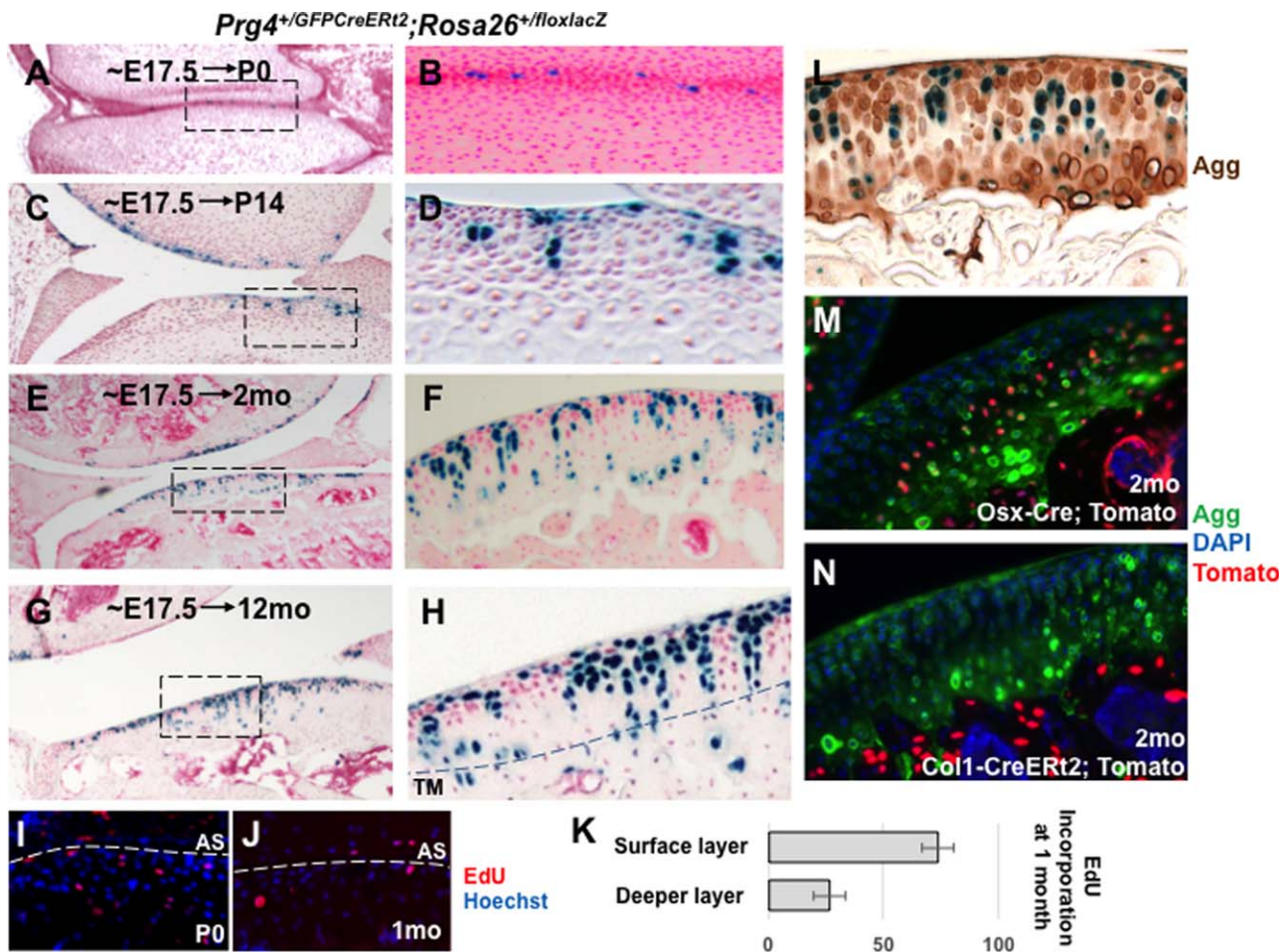


Figure 4. *Prg4*-expressing cells in the superficial-most cell layer of the forming joint in day 17.5 mouse embryos (E17.5) are progenitors for all zones of adult articular cartilage. Pregnant dams were pulsed with tamoxifen at approximately embryonic day 17.5, and their *Prg4*^{+/GFP*CreERT2*};*Rosa26*^{+/flox*lacZ*} progeny were harvested at either postnatal day 0 (P0) (A and B), postnatal day 14 (C and D), 2 months of age (E and F), or 12 months of age (G and H). A, C, E, and G show photomicrographs of X-Gal-fast red-stained joints. B, D, F, and H show higher-magnification views of the boxed areas in A, C, E, and G, respectively. Knee joint tibial articular cartilage is displayed either on the bottom of the image or by itself in each panel. The tidemark (TM) was identified as a line of discontinuity in the intensity of fast red staining of the extracellular matrix and is indicated by a broken line. I and J, Incorporation of 5-ethynyl-2'-deoxyuridine (EdU) into the knee joint cartilage in postnatal day 0 (I) and 1-month-old (J) mice. The location of the articular surface (AS) is delineated by the broken line. K, Quantitation of the percentage of EdU-positive cells in either the superficial or deeper layers of the articular cartilage in 1-month-old mice injected for 10 days with EdU (as shown in J). Bars show the mean \pm SD. L, Immunodetection of aggrecan (Agg) in 2-month-old *Prg4*^{+/GFP*CreERT2*};*Rosa26*^{+/flox*lacZ*} mice pulsed with tamoxifen at approximately embryonic day 17.5. Lineage-traced cells are blue and remain within the articular cartilage, as indicated by the aggrecan immunostaining. M and N, Expression of tdTomato and aggrecan in the knee joints of 2-month-old *Osx1-Cre*;*Rosa26*^{+/flox*lacZ*} mice (M) and *Col1-CreERT2-Cre*;*Rosa26*^{+/flox*lacZ*} mice that had been administered tamoxifen (N) and killed 7 days later. Original magnification $\times 100$ in A–D and I; $\times 40$ in E–H and J; $\times 200$ in L–N.

both the superficial and deeper regions of the articular cartilage (Figures 5C–F), but rarely in the mineralized matrix (i.e., below the tidemark). The percentage of β -galactosidase-expressing cells in the thickest region of the tibial articular cartilage to lie above the tidemark significantly increased between 1 and 18 months of age in *Prg4*^{+/GFP*CreERT2*};*Rosa26*^{+/flox*lacZ*} mice that had been

injected with tamoxifen from postnatal day 21 to postnatal day 30 (Figure 5G), suggesting that at least some of the *Prg4*^{GFP*CreERT2*} lineage-labeled cells were proliferating during this time. Notably, the greatest depth of *Prg4*^{GFP*CreERT2*} lineage-labeled cells in older mice was observed in the thickest region of the nonmineralized articular cartilage (usually near the middle of the

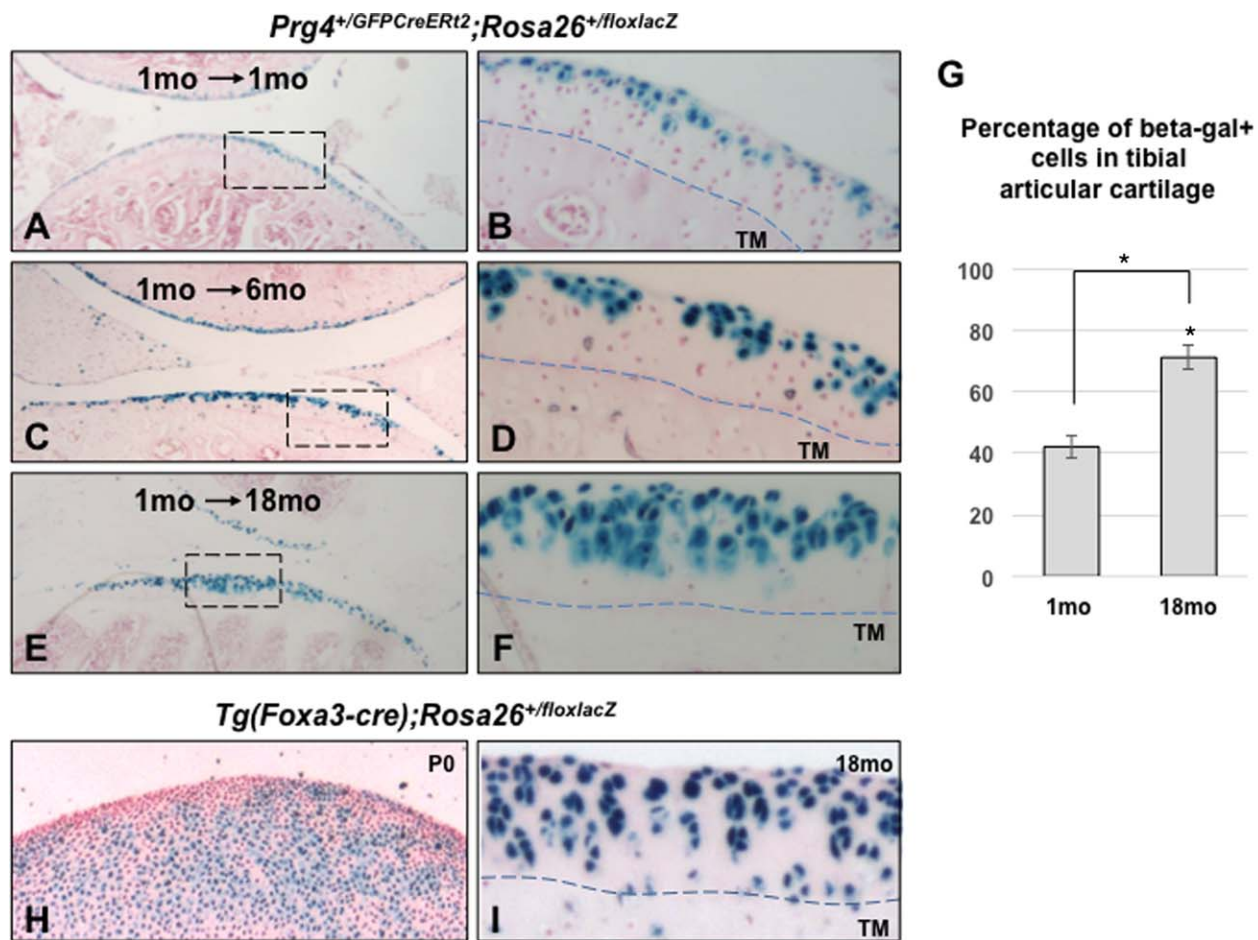


Figure 5. *Prg4*-expressing cells in 1-month-old mice are progenitors for both superficial and deeper regions of the adult articular cartilage. **A–F**, Photomicrographs of knee joint cartilage from ~1-month-old (**A** and **B**), 6-month-old (**C** and **D**), and 18-month-old (**E** and **F**) *Prg4⁺/GFPCreERT2; Rosa26⁺/floxlacZ* mice that had been given daily intraperitoneal (IP) injections of tamoxifen from postnatal day 21 to postnatal day 31. **A**, **C**, and **E** show X-Gal-fast red staining. **B**, **D**, and **F** show higher-magnification views of the boxed areas in **A**, **C**, and **E**, respectively. Original magnification $\times 100$ in **A** and **B**; $\times 40$ in **C–F**. **G**, Quantitation of the percentage of cells expressing β -galactosidase located in the thickest region of the tibial articular cartilage (above the tidemark [TM]) in 1-month-old or 18-month-old *Prg4⁺/GFPCreERT2; Rosa26⁺/floxlacZ* mice that had been given daily IP injections of tamoxifen from postnatal day 21 to postnatal day 31. Bars show the mean \pm SD. * = $P < 0.05$ by Student's *t*-test. **H** and **I**, Postnatal day 0 (**H**) or 18-month-old (**I**) *Tg(Foxa3-cre); Rosa26⁺/floxlacZ* mouse knee articular cartilage stained with X-Gal-fast red. The tidemark was identified as a line of discontinuity in the intensity of fast red staining of the extracellular matrix, and is indicated by the broken line. Original magnification $\times 100$ in **H**; $\times 40$ in **I**.

condyle), the same region of the articular cartilage in which we previously observed the greatest amount of *Prg4* induction in response to wheel running (25).

A 1-day pulse of tamoxifen to fetal (approximate embryonic day 17.5) *Prg4⁺/GFPCreERT2; Rosa26⁺/floxlacZ* mice yielded “columnar clones” of β -galactosidase-expressing chondrocytes that extended the entire depth of the articular cartilage in 12-month-old mice, even extending below the tidemark in some cases (Figures 4G and H). In contrast, a 10-day pulse of tamoxifen to 1-month-old *Prg4⁺/GFPCreERT2; Rosa26⁺/floxlacZ* mice resulted in β -galactosidase-expressing chondrocytes that extended to maximally two-thirds the depth of the

articular cartilage in 18-month-old mice, and were not found below the tidemark (Figures 5E and F). Taken together, these findings imply that the deepest cells of the articular cartilage that lie adjacent to the tidemark are descendants of *Prg4*-expressing cells that were lineage labeled on embryonic day 17.5, but are not descendants of *Prg4*-expressing cells that were lineage labeled at 1 month of age. In comparison, 18-month-old *Tg(Foxa3-cre); Rosa26⁺/floxlacZ* mice (20) displayed β -galactosidase-expressing chondrocytes throughout the full depth of their articular cartilage (Figure 5I). On postnatal day 0, *Tg(Foxa3-cre); Rosa26⁺/floxlacZ* mice expressed β -galactosidase in all chondrocyte zones of

the growth plate (26) and throughout the entire epiphyseal cartilage (Figure 5H). In summary, these results indicate that the earliest-born cells (which are descendants of cells expressing *Prg4* on embryonic day 17.5 but not on postnatal day 30) give rise to the deepest layer of the articular cartilage, and thus suggest that articular cartilage grows in an “appositional” manner (much like the growth plate) with a progenitor population confined to the superficial region of the developing articular cartilage.

DISCUSSION

We generated knockin mice that express a tamoxifen-inducible Cre recombinase from the *Prg4* locus (*Prg4^{GFP-CreERT2}*). In the knee joint, *Prg4^{GFP-CreERT2}* is expressed by cells at the surface of the developing joint in embryonic day 17.5 mice and by superficial articular chondrocytes in 1-month-old mice. Therefore, we used the *Prg4^{GFP-CreERT2}* allele along with the Cre-inducible reporter gene, *Rosa26^{flloxlacZ}*, to fate-map the descendants of *Prg4*-expressing cells in both fetal and 1-month-old mice. We found that *Prg4*-expressing cells that were lineage labeled at the surface of the developing joint in day 17.5 mouse embryos gave rise to columns of β -galactosidase-expressing cells that in some cases extended through the entire depth of the articular cartilage in 2-month-old mice, and to just below the tidemark in 12-month-old mice. In some instances, these columns of β -galactosidase-expressing cells (that had been lineage labeled by *Prg4^{GFP-CreERT2}* expression in day 17.5 embryos) extended from only the middle of the nonmineralized matrix to the surface, or clusters of β -galactosidase-expressing cells were restricted to either the deep or superficial regions of the articular cartilage. We interpret these findings to indicate that *Prg4*-expressing cells in the developing joint of the day 17.5 embryo give rise to chondrocytes in all regions of the articular cartilage, and that the embryonic day 17.5 lineage-labeled descendants of these *Prg4*-expressing cells display differing amounts of subsequent cell division.

Prg4-expressing chondrocytes in 1-month-old mice also produced descendants in deeper regions of the articular cartilage. However, in this case the lineage-labeled descendants of *Prg4*-expressing cells (which were labeled at 1 month of age) extended from the articular surface to maximally two-thirds the depth of the articular cartilage in 18-month-old mice and never reached the tidemark. These findings indicate that the deepest cells of the articular cartilage that lie adjacent to the tidemark are descendants of *Prg4*-

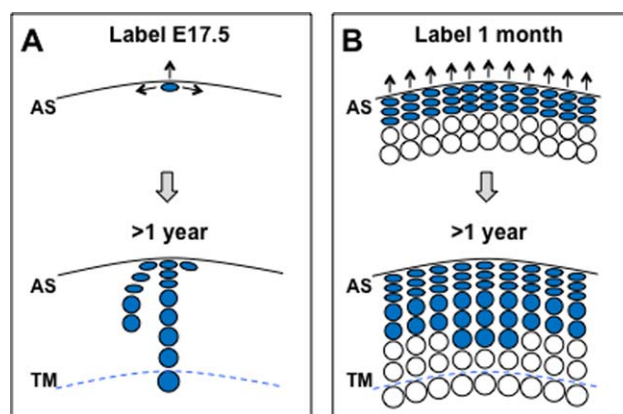


Figure 6. Model depicting the growth of articular cartilage based on *Prg4^{GFP-CreERT2}* fate-mapping. Growth occurs appositionally toward the articular surface (AS) and also laterally in the superficial zone. **A**, The progeny of embryonic day 17.5 (E17.5) *Prg4^{GFP-CreERT2}*-expressing cells (shown in blue) are present in all articular cartilage layers and can in some cases extend as columnar clones from the superficial zone to the calcified cartilage beneath the tidemark (TM) in adult mice. **B**, The progeny of *Prg4^{GFP-CreERT2}*-expressing cells in 1-month-old mice (shown in blue) extend from the superficial zone to maximally two-thirds the depth of the articular cartilage. These findings indicate that the deepest cells of the articular cartilage that lie adjacent to the tidemark are descendants of *Prg4^{GFP-CreERT2}*-expressing cells that were lineage labeled on embryonic day 17.5 but are not descendants of *Prg4^{GFP-CreERT2}*-expressing cells that were lineage labeled at 1 month of age. Thus, our findings suggest that the deeper layers of the articular cartilage are “born” first.

expressing cells that were lineage labeled on embryonic day 17.5 but are not descendants of *Prg4*-expressing cells that were lineage labeled at 1 month of age. Thus, our findings suggest that the deeper layers of the articular cartilage are “born” first. We interpret these observations to indicate that a significant component of articular cartilage growth is appositional, with *Prg4*-expressing progenitor cells first giving rise to cells that will eventually come to lie deep within this tissue, and subsequently giving rise to cells that will eventually come to lie in more superficial regions of the articular cartilage (see Figure 6).

Interestingly, we noted that *Prg4*-expressing cells that were lineage labeled in 1-month-old *Prg4^{+/GFP-CreERT2};Rosa26^{+/flloxlacZ}* mice gave rise to β -galactosidase-expressing cells in the meniscus, synovium, and patella (see Supplementary Figure 6, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39030/abstract>), even though most β -galactosidase-expressing superficial chondrocytes had been lost in these older mice. The presence of β -galactosidase-expressing

synoviocytes in 18-month-old mice suggests either that some *Prg4*^{GFP*CreERT2*}-expressing cells in 1-month-old mice have stem cell-like features that continue to generate synoviocytes or that some synoviocytes live for a very long time. Importantly, only a small fraction of synovial lining cells (~5%) were β -galactosidase positive in these mice, implying that most synoviocyte stem cells were not expressing *Prg4*^{GFP*CreERT2*} at 1 month of age.

We noted that although only a relatively small number of recombined cells were located at the surface of the developing joint in *Prg4*^{+/*GFP*CreERT2**}; *Rosa26*^{+/*lloxlacZ*} mice that had been injected with tamoxifen on embryonic day 17.5 and killed on postnatal day 0, the number of recombined cells (located in both the superficial and deeper regions of the knee joint) in mice killed at 12 months of age increased by several fold. This increased number of recombined progeny cells in both the superficial and deeper layers of the articular cartilage in postnatal mice suggests that superficial progenitors in the embryo both self-renew (in the superficial zone) and give rise to cells located in deeper regions of the adult articular cartilage. In addition, because proliferation mostly occurs in relatively superficial articular cartilage cells in 1-month-old mice, it seems likely that superficial zone articular cartilage cells comprise a progenitor cell population for this tissue, in both prenatal and young mice. Indeed, cells in the superficial zone of articular cartilage have previously been demonstrated to be label-retaining cells (10,11), suggesting that these cells may constitute a slowly cycling stem cell population.

Our findings do not rule out the possibility that the *Prg4*-expressing population (on either embryonic day 17.5 or postnatal day 30) is heterogeneous, with only a subset of these cells serving as progenitors for the expansion of the articular cartilage. Notably, these findings are consistent with prior observations, which have indicated that while transient epiphyseal chondrocytes (which are subsequently replaced by bone) are derived from matrilin 1-expressing progenitors, adult articular cartilage derives from progenitors that have never expressed matrilin 1 (6).

Interestingly, the greatest apparent expansion of *Prg4*-expressing progenitors in the knee joint (on either embryonic day 17.5 or postnatal day 30) occurred in the tibial articular cartilage, near the middle region of the condyles. This is the same region of the articular surface in which we previously observed the greatest amount of *Prg4* induction in response to wheel running (25). In addition to inducing *Prg4* expression, wheel running also promotes proliferation of *Prg4*-expressing

cells in the articular cartilage (25). Thus, it seems plausible that the greatest amount of cell proliferation in *Prg4*-expressing articular cartilage progenitors occurs in the regions of this tissue that experience the highest levels of mechanical loading, and thus lie in the central domains of the condyles.

Inducing *Prg4*^{GFP*CreERT2*}-mediated recombination in older mice (i.e., at 3, 6, or 18 months of age) revealed *Prg4*^{GFP*CreERT2*} expression in both superficial and deeper-zone chondrocytes. This finding was confirmed in *Prg4*^{+/*lacZ*genetrap**} mice, which similarly displayed β -galactosidase expression uniquely in the superficial zone of the articular cartilage at 1 month of age, but in both the superficial and deeper regions of femoral head articular cartilage at 15 months of age. While both *Prg4*^{GFP*CreERT2*} and *Prg4*^{+/*lacZ*genetrap**} transcripts lack exons 3–13 of native *Prg4* (and thus may display differential messenger RNA stability compared to the native transcript), our findings suggest that expression of the *Prg4* locus, which initially is restricted to the most superficial cells in the articular cartilage, is extended into deeper cells within this tissue as mice age. Thus, in contrast to either embryonic day 17.5 or 1-month-old mice, in which *Prg4*^{GFP*CreERT2*}-expressing cells are mostly restricted to the top layers of the articular cartilage, in older mice, deeper-zone chondrocytes (in addition to superficial zone chondrocytes) are capable of expressing *Prg4*.

It seems unlikely that the age-dependent expansion of *Prg4* expression into deeper regions of the articular cartilage in both *Prg4*^{+/*GFP*CreERT2**} and *Prg4*^{+/*lacZ*genetrap**} mice is due to haploinsufficiency of *Prg4* in these animals, since the articular cartilage of these heterozygous mice displays neither age-dependent fibrillation nor cartilage degradation. In addition, families carrying CACP syndrome mutations in *PRG4* do not show an increased incidence of osteoarthritis among individuals who are heterozygous for mutations in this gene. Taken together, our findings indicate that chondrocytes that lie in deeper regions of the articular cartilage both originate from precursors that had initially expressed *Prg4* within the superficial zone and become capable of re-expressing *Prg4* in response to aging.

As anticipated from the clinical consequences of genetic *PRG4* deficiency, *Prg4*^{GFP*CreERT2*} expression was not limited to articular chondrocytes. We also observed expression in other tissues previously reported to express *Prg4*, including synovium, tendon, ligament, heart, and liver. In addition, we observed scattered *Prg4*^{GFP*CreERT2*}-expressing cells in other sites, including sternal cartilage, the xiphoid process, and the proximal part of the aorta. Importantly, we never observed

Prg4^{GFP*CreERT2*}-mediated recombination in growth plate chondrocytes, which distinguishes *Prg4*^{GFP*CreERT2*} mice from both *Col2*^{*CreERT2*} and *Agg*^{*CreERT2*} mice (27,28), which express *CreERT2* in both growth plate and articular cartilage. Ten consecutive days of tamoxifen treatment of 1-month-old *Prg4*^{GFP*CreERT2*/+} mice is capable of inducing recombination of either a *Rosa26*^{*loxlacZ*} or a *Rosa26*^{*mTmG*} allele in ~70% of the top 3 layers of the articular cartilage. We think that the relatively low level of expression of the *Prg4*^{GFP*CreERT2*} allele (~12- to 50-fold lower than the level of endogenous *Prg4*) may account for the need for multiple tamoxifen injections to induce maximal levels of recombination in the articular cartilage.

Unlike the *Gdf5*^{*Cre*} allele, which can drive recombination in articular cartilage progenitors but not in the growth plate in the embryo (5,29), the *Prg4*^{+/*GFP*CreERT2**} mouse strain uniquely drives recombination specifically in both *adult* articular cartilage and in synovial cells, and thus can be used to determine the effect of altering the expression of a loxP-flanked gene in these tissues in adult animals, without affecting that function of the gene in growth plate cartilage. However, because recombination driven by the *Prg4*^{GFP*CreERT2*} allele is only transiently restricted to the superficial zone of articular cartilage (in animals postnatal day 30 or younger) and displays age-dependent expansion into deeper zones of the articular cartilage, the localization of genetically altered cells driven by this *Cre* driver changes as animals age.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Warman and Lassar had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Kozhemyakina, Zhang, Kobayashi, Warman, Lassar.

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Analysis and interpretation of data. Kozhemyakina, Zhang, Ionescu, Ayturk, Ono, Kronenberg, Warman, Lassar.

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