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Early postnatal ablation of the microRNA-processing enzyme, Drosha, causes chondrocyte death and impairs the structural integrity of the articular cartilage

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SUMMARY

Objective—In growth plate chondrocytes, loss of Dicer, a microRNA (miRNA)-processing enzyme, causes defects in proliferation and differentiation, leading to a lethal skeletal dysplasia. However roles of miRNAs in articular chondrocytes have not been defined *in vivo*. To investigate the role of miRNAs in articular chondrocytes and to explore the possibility of generating a novel mouse osteoarthritis (OA) model caused by intrinsic cellular dysfunction, we ablated Drosha, another essential enzyme for miRNA biogenesis, exclusively in articular chondrocytes of postnatal mice.

Design—First, to confirm that the essential role of miRNAs in skeletal development, we ablated the miRNA biogenesis pathway by deleting *Drosha* or *DGCR8* in growth plate chondrocytes. Next, to investigate the role of miRNAs in articular cartilage, we deleted *Drosha* using *Prg4*-

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Author contributions

TK conceived the project. TK, GP, and FM performed experiments and interpreted data. MC, EK, MZ and RB, generated, characterized, and provided mice. TK wrote the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2015.02.015.

CreER^{T} transgenic mice expressing a tamoxifen-activated Cre recombinase (CreER^{T}) exclusively in articular chondrocytes. Tamoxifen was injected at postnatal days, 7, 14, 21, and 28 to ablate *Drosha*.

Results—Deletion of *Drosha* or *DGCR8* in growth plate chondrocytes caused a lethal skeletal defect similar to that of *Dicer* deletion, confirming the essential role of miRNAs in normal skeletogenesis. Early postnatal *Drosha* deletion in articular chondrocytes significantly increased cell death and decreased Safranin-O staining. Mild OA-like changes, including surface erosion and cleft formation, were found in male mice at 6 months of age; however such changes in females were not observed even at 9 months of age.

Conclusions—Early postnatal *Drosha* deficiency induces articular chondrocyte death and can cause a mild OA-like pathology.

Keywords

microRNA; Drosha; Prg4; Chondrocyte; Osteoarthritis; Articular cartilage

Introduction

Osteoarthritis (OA) is the most prevalent skeletal condition in the elderly. Traditionally OA has been considered as a “wear-and tear” disease caused by cumulative mechanical stress during the aging process. However, this view of OA is changing as accumulating evidence demonstrates the involvement of other mechanisms that promote OA initiation and progression. Articular chondrocytes from old individuals show signs of age-related cellular dysfunction including telomere shortening, DNA damages, and cellular senescence-associated phenotypes[1–4]. Therefore, in addition to mechanical stress, intrinsic cellular dysfunction of articular chondrocytes likely contribute to the disease[3]. Chondrocyte dysfunction during developmental and mature stages likely impairs cartilage structure and function, whereas during degeneration stages, chondrocytes produce catabolic enzymes such as metalloproteases (MMP's) and facilitate cartilage matrix degradation. Thus, articular chondrocytes presumably play different roles depending on stages of life; however, stage-specific articular chondrocyte function has not been precisely defined.

microRNAs (miRNAs) regulate gene expression mainly at the post-transcriptional level. miRNAs are generated from long primary transcripts (pri-miRNAs) after multi-step processing; first pri-mRNAs are processed into small hairpin RNA (pre-miRNAs) by the microprocessor complex comprised of Drosha and DGCR8, and then further processed by the RNase III, Dicer. We have shown that global reduction in miRNAs by deleting *Dicer* in growth plate chondrocytes reduces cellular proliferation and accelerate chondrocyte differentiation, causing a severe skeletal growth defect and early postnatal lethality[5].We have also shown that suppression of let-7 miRNAs, the most abundant miRNAs in chondrocytes, reduces chondrocyte proliferation in the growth plate cartilage[6]. However, in articular cartilage, despite many studies have suggested possible involvement of miRNAs in physiological and pathological processes of articular chondrocytes[7], except the report that miR-140 deficiency causes OA in mice[8], the role of miRNAs *in vivo* is still largely unclear.

This is primarily due to the lack of adequate mouse tools that allow genetic manipulation in an articular chondrocyte-specific manner.

In this study, we conditionally deleted *Drosha* using *Cre* transgenic mice (*Prg4-CreER*^{T}) in which a tamoxifen-inducible Cre recombinase (CreER^T) was expressed under the endogenous promoter of the Prg4 (lubricin) gene. We show that early postnatal deletion of *Drosha* in articular chondrocytes increases cell death, decreases matrix proteoglycan content, and can cause mild OA-like structural changes. These findings suggest that miRNAs are essential for articular chondrocyte survival and structural soundness of the articular cartilage.

Methods

Mice

Floxed *Drosha*[9], floxed *DGCR8*[10], *Col2-Cre* transgenic[11] and *Prg4-CreER*^{T} knock-in mice were described[12,13]. Genotyping of *Prg4-CreER*^{T} mice was performed by PCR using the primers, Pr224 (common) 5′-TCAGGAATTCAAGCTGATTGC-3′ and Pr226 (*CreER*^{T} allele specific) 5′-AACTTGTGGCCGTTTACGTC-3′ and Pr227 (wildtype allele specific) 5′-CCTTGAGATGAAACCTGTTGAATC-3’. The *Prg4-CreER*^{T} allele and wildtype *Prg4* allele produce 420 bp- and 235 bp-long PCR amplicons, respectively. Mice used in this study were in a C57/BL6-dominant mixed background.

Tamoxifen was dissolved in sunflower oil at the concentration of 10 mg/mL. Mice were injected 0.1 mg/g BW intraperitoneally at postnatal day (P) 7, 14, 21, and 28. Mice were sacrificed at indicated ages. Tissues were fixed in 10% neutralized formalin for 2 days and decalcified in 15% Ethylenediaminetetraacetic acid (EDTA) for up to 3 weeks. Although *Prg4-CreER*^{T} allele is a functionally null allele, because heterozygous *Prg4*-null mice show normal articular cartilage[14], *Cre*-negative littermates were used as control in this study. This study was approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

Whole-mount X-gal staining

Whole-mount X-gal staining was performed as described[15]. Briefly, after sacrificing mice, limbs were dissected, and fixed in 10% formalin on ice for an hour. Specimens were washed with phosphate buffered saline (PBS) at room temperature for 30 min twice, and transferred into a staining solution containing PBS, 2 mM MgCl2, 5 mM potassium-ferricyanide, 5 mM potassium-ferrocyanide, 0.02% IGEPAL, 0.01% sodium deoxycholate, and 1 mg/mL X-gal. Specimens were incubated at room temperature rocking for 4 h to facilitate substrate penetration, then incubated at 37°C for 24 h. Specimens were rinsed with PBS, and post fixed with 10% formalin for a day, decalcified, and processed for sectioning.

qRT-PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the EvaGreen master mix reagent (Solis Biodyne) and ABI StepOne realtime PCR (Applied Biosystems) with following primers: *Drosha-L*, 5′-

GGACCATCACGAAGGACACT-3′ and *Drosha-R*, 5′-CACGGGTCTCTTGGTTTTGT-3′; *DGCR8-L*,5′-GAAACCATGGAATGGGTGACA-3′ *DGCR8-R*, 5′-TGTGGGTGCAGTAGCTGAAG-3′; *Actb-L*, 5′-GCACTGTGTTGGCATAGAGG-3′ and *Actb-R*, 5′-GTTCCGATGCCCTGAGGCTCTT-3′. miRNA quantification was performed using the MirVana mirVana qRT-PCR miRNA Detection Kit (Life Technologies). mRNA expression levels were normalized by beta actin (*Actb*) and miRNA levels were normalized by U6.

Histological analyses

Formalin-fixed mouse tissues were paraffin-processed, cut, dewaxed, and rehydrated for hematoxylin–eosin and Safranin-O staining, and other histological analyses.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the Cell death detection kit (Roche) according to the manufacturer's instruction.

BrdU labeling and staining

For BrdU labeling and detection, 0.1 mg/g BW BrdU was injected into pregnant mothers 2 h before sacrifice. Bones of embryos were dissected, fixed, processed, sectioned, and subjected to BrdU staining using the BrdU staining kit (Life technologies).

In situ hybridization

In situ hybridizationwas carried out as described[16] using probes previously described[17].

Statistical analysis

Data are expressed as mean + 95% confidence interval. Differences of two groups were tested using Student's *t* test. Statistical analysis was performed using the Prism 6 software (Graphpad).

Results

Deletion of DGCR8 or Drosha in growth plate chondrocytes causes a skeletal developmental defect

We have previously shown that deletion of *Dicer* in growth plate chondrocytes decreases cellular proliferation[5]. Since Dicer is involved in other biological functions in addition to miRNA biogenesis[18], in order to confirm that the miRNA deficiency was responsible for the skeletal defect caused by chondrocyte-specific *Dicer* deletion, we generated mice missing function of the microprocessor complex by deleting either *Drosha* or *DGCR8* in growth plate chondrocytes. Floxed *Drosha* or floxed *DGCR8* mice were crossed with Cre transgenic mice expressing Cre under the control of a mouse type II collagen promoter (*Col2-Cre*) to generate homozygous conditional knockout mice (cKO) for *Drosha* or *DGCR8*. Both *Drosha* and *DGCR8* cKO mice showed perinatal lethality unlike *Dicer* cKO mice that survive a few weeks postnatally. However, like in *Dicer* cKO mice[5], the skull shape of

Drosha and *DGCR8* cKO mice was deformed due to the reduced longitudinal growth [Fig. 1(A), (B)]. Development and mineralization of skeletal elements of these mice, including vertebrae, were poor [Fig. 1(C), (D)]. We confirmed that expression levels of several miRNAs, and *Drosha* or *DGCR8* were reduced in primary chondrocytes of *Drosha* or *DGCR8* cKO embryos [Fig. 1(E), (F)]. We also found proliferation defects in *Drosha* and *DGCR8* cKO growth plates, which was a major cellular change in *Dicer* cKO mice[5] (Fig. 2). This reduced the size of the proliferating chondrocyte domain. The reductions in size of the proliferating chondrocyte domain were best appreciated in the bidirectional growth plates in the basal skull (Fig. 3). The proliferating domain, positive for *Col2a1* and flanked by *Col10a1*-expressing hypertrophic chondrocytes, was reduced in *DGCR8* and *Drosha* cKO mice (Fig. 3), which was also observed in *Dicer* cKO mice[5]. This finding that deletion of a component of the microprocessor complex, *Drosha* or *DGCR8*, resulted in defects similar to that *Dicer* cKO mice strongly suggests that miRNA deficiency is responsible for the skeletal phenotypes of these mice.

Efficient tamoxifen-induced recombination by Prg4-CreER^T transgenic mice

Unlike growth plate chondrocytes that vigorously proliferate, differentiate, and die, articular chondrocytes proliferate slowly and persist throughout life. In order to investigate the role of miRNAs in articular chondrocytes, we deleted *Drosha* in postnatal articular chondrocytes. To target articular chondrocytes, we used *Prg4-CreER^T* transgenic mice in which tamoxifen-inducible CreER^T was expressed under the control of the endogenous promoter of the *Prg4* gene that encodes proteoglycan 4 (lubricin). Lubricin, also called superficial zone protein (SZP), is expressed in superficial zone chondrocytes of the articular cartilage but not in growth plate chondrocytes[14].

First, we tested the Cre dependent recombination efficiency after four doses of tamoxifen injection. *Prg4-CreERT* mice were crossed with R26R Cre reporter mice[19] that express LacZ upon Cre-dependent recombination. We injected 0.1 mg/g BW tamoxifen to mice at postnatal day (P) 7, P14, P21, and P28. At P30, Cre-dependent recombination was found in approximately 50% of superficial, middle and deep zone chondrocytes in the area above the tidemark [Fig. 4(A) and (A′)]. At P60, the number of LacZ-positive cells somewhat increased compared with that at P30, suggesting that *Prg4*-positive cells and their descendants still slowly proliferate during this period [Fig. 4(B)].

Postnatal articular chondrocyte-specific Drosha deletion can cause mild OA-like changes

With this injection regimen, we administered tamoxifen to *Prg4-CreER^T:Drosha$^{fl/fl}$* (cKO) mice and sex-matched control littermates. Mice were sacrificed at 2, 4, 6, and 9 months of age. At 2 month of age, we found a patchy reduction in Safranin O staining in the tibial articular cartilage of cKO mice, which was exacerbated at 4 month of age [Fig. 5(A)]. The structural integrity of the articular cartilage was relatively well preserved at 4 months of age. At 6 months of age, damages of the articular surface and cartilage matrix, including surface erosion and clefts, started appearing in male cKO mice [Fig. 5(A), (B)]. We did not observe overt morphological changes in synovial tissues (Supplementary Fig. 1). In female mice, these changes except the reduced Safranin O staining were not observed at 6 and 9 months of age (Supplementary Fig. 2). In order to investigate the mechanism for the reduced

proteoglycan content in cKO cartilage, we evaluated expression of ADAMTS5, a major aggrecanase encoded by *Adamts5*. We did not find overt upregulation in ADAMTS5 in 2 month-old cKO cartilage (Supplementary Fig. 3(B)). In addition, we quantified gene expression of aggrecan (*Acan*), decorine (Dcn), *Sox9*, and *Adamts5* in primary rib chondrocytes isolated from *Col2-Cre:Drosha*$^{fl/fl}$ cKO embryos. We found significant reductions in *Acan* and *Sox9*, whereas *Adamts5* expression was unaffected in cKO chondrocytes (Supplementary Fig. 3(B)). These results suggest that the reduced proteoglycan content in cKO mice is caused by the reduction in proteoglycan production due to chondrocyte dysfunction rather than increased catabolism.

Drosha deletion induces cell death of articular chondrocytes

To investigate the mechanisms, we performed the TUNEL assay to assess cell death. In control articular cartilage, cell death were detected only in calcified zone chondrocytes below the tidemark, whereas *Drosha* cKO showed significant numbers of TUNEL-positive cells in the superficial and middle zones above the tidemark at 2 and 4 months of age [Fig. 6(A), (B)]. We did not find BrdU positive articular chondrocytes either in 4 month-old control or cKO mice after 3 weeks of daily BrdU injection, confirming the very low proliferation rate of articular chondrocytes (data not shown). Therefore, the major consequence of postnatal *Drosha* deletion in articular chondrocytes appears to be cell death unlike in developing growth plates in which *Drosha* deficiency causes a proliferation defect. We did not observe upregulation of matrix metalloproteinase-13(MMP-13) or discoidin domain-containing receptor 2 (DDR-2), ones of markers often upregulated in OA[20] (Supplementary Fig. 4).

Discussion

In this study, we demonstrate that ablation of microprocessor function via genetic deletion of *Drosha* or *DGCR8* in growth plate chondrocytes causes a skeletal defect similar to that of *Dicer*-deficiency. This suggests that miRNA deficiency is responsible for the reduction in growth plate chondrocyte proliferation and skeletal defects in these mutant mice.

Although qualitatively similar, *Dicer* deletion shows somewhat milder abnormalities compared with deletion of *Drosha* or *DGCR8*. The precise reason for the differences in phenotypical severity among these models is unknown. Other than the trivial possibilities, such as different efficiencies in gene deletion or miRNA elimination, since both *Dicer* and *Drosha* are known to acts on non-miRNA substrates[21,22], it is also formally possible that loss of additional functions other than miRNA biogenesis that these miRNA processing enzymes possess might contribute to the difference in the skeletal defect severity of these mice. Nevertheless, the finding that these mice show qualitatively similar abnormalities strongly suggests that miRNA deficiency is the primary cause for the skeletal defects observed in these mice.

Tissue-specific genetic manipulation in postnatal articular chondrocytes has been difficult due to the lack of adequate genetic tools. While *Gdf5-Cre* transgenic mice has been used to delete genes exclusively in articular chondrocytes and synovial tissues[23], its expression is limited to embryonic stages. Therefore, it was not possible to manipulate genes postnatally

in an articular chondrocyte-specific manner using this Cre line. Unlike *Gdf5*, *Prg4* expression increases in postnatal stages probably because *Prg4* is induced by various physiological stimuli[13]. Thus far, the *Prg4-CreER*^{T} mouse is the only Cre line that allows gene deletion in postnatal articular chondrocytes but not in growth plate chondrocytes. With the tamoxifen injection regimen used in this study, we demonstrate that the *Prg4-CreER*^{T} transgenic line is a useful tool to manipulate genes in postnatal articular chondrocytes with a reasonable efficiency.

We have demonstrated that early postnatal deletion of *Drosha* in *Prg4*-expressing cells and their descendants causes cell death, reduces the proteoglycan content in articular cartilage, and can induce OA-like changes. While *Drosha* deletion in growth plate chondrocytes primarily caused a proliferation defect, it resulted in cell death in articular chondrocytes. This disparity in consequences of *Drosha* deletion is likely reflecting the difference in the speed of cellular turnover between these two cartilage tissues. Growth plate chondrocytes proliferate vigorously, differentiate, die, and are constantly replaced, whereas articular chondrocytes proliferate slowly and stay in the tissue permanently. Since cell death appears to be a relatively late consequence of *Drosha* deletion, cell death may be difficult to observe in the growth plate whose turnover is rapid whereas proliferation defects may not be easy to detect in slowly proliferating articular chondrocytes.

The reduction in proteoglycan content was the most noticeable change whereas structural changes such as cleft formation were relatively mild and only observed at later stages in male mice. This is in line with the previous findings that male mice tend to develop OA with greater severities and frequency than females in various OA models[24–28]. Although precise mechanisms are unknown, differences in sex hormones[26], and bone and joint structures[27] have been proposed to play roles in the sex-dependent difference in OA.

Even with the substantial cell death and the markedly reduced proteoglycan content, the cartilage structure was relatively well preserved in our model. The degrees of OA-like changes assessed by the semi-quantitative Ostaoarthritis Research Society International (OARSI) mouse OA scoring system[29] were grade 1–2 (average 1.67) in the 6 month-old male cKO mice that showed most severe changes in this study, whereas other mice showed only a reduction in Safranin O staining (grade 0.5). A previous study demonstrated that postnatal deletion of *Sox9* in cartilage caused a dramatic reduction in proteoglycan content in the articular cartilage without overt osteoarthritic changes[30]. This suggests that articular chondrocyte dysfunction does not significantly affect the maintenance of the articular cartilage structure once the tissue is established, which may explain why we observed relatively mild changes in the articular cartilage structure despite the substantial cell death caused by *Drosha* deficiency. In our study, *Drosha* was disrupted in early postnatal stages when mouse skeleton were still rapidly growing. Although we did not find overt morphological abnormalities in articular cartilage development, it is possible that chondrocyte dysfunction caused by *Drosha*-deficiency during developmental stages compromises the quality and/or quantity of matrix proteins, and might cause cartilage matrix fragility, leading to mild OA-like changes at later stages in some mice. Applying greater mechanical stresses, such as surgical OA induction and aging, may reveal the possible matrix fragility of Drosha-deficient cartilage.

In summary, this study demonstrates that *Drosha* is necessary for postnatal articular chondrocyte survival and maintenance of normal proteoglycan content in the cartilage. Because *Drosha* is necessary for biogenesis of most miRNAs, this effect is likely mediated by global deficiency of miRNAs; understanding specific roles of individual miRNAs in articular chondrocytes *in vivo* is an important future research agenda. Although the cartilage structure was relatively well preserved, mild OA-like changes can develop in some *Drosha* cKO mice in 6 months of age. Thus, inducing articular chondrocyte dysfunction at early postnatal stages via *Drosha* deletion may provide a novel mouse OA model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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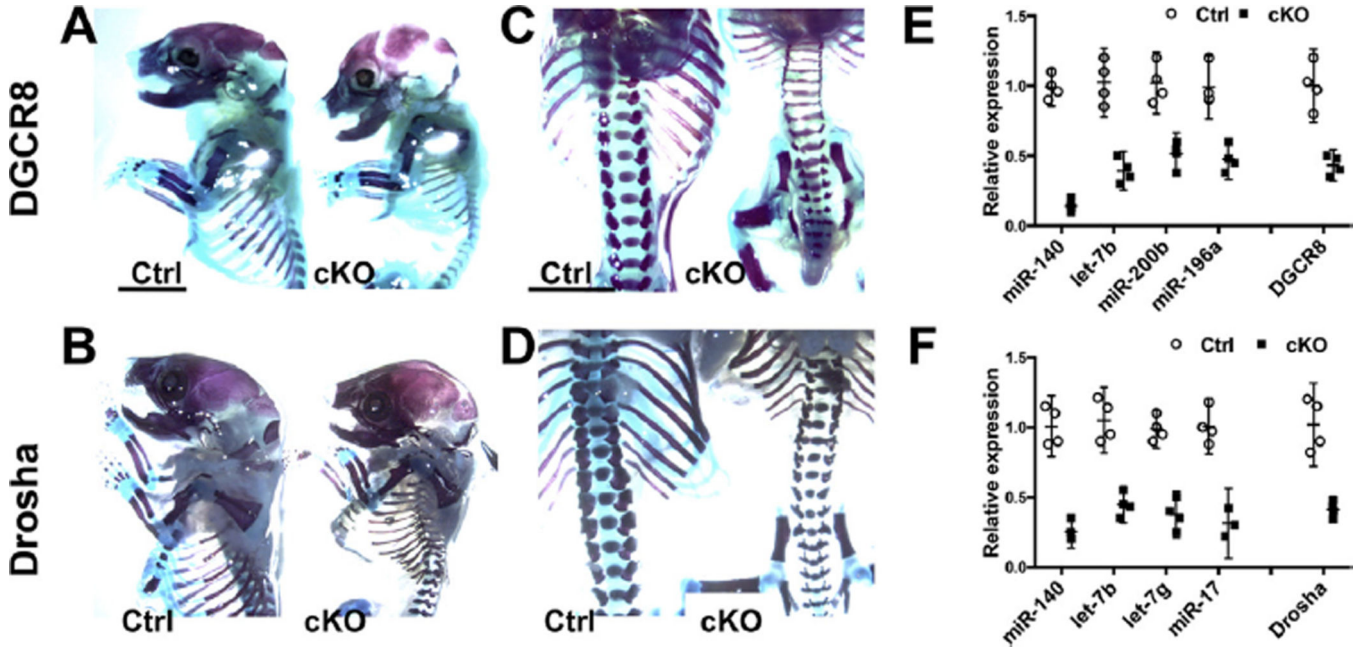

Fig. 1.

Chondrocyte-specific deletion of a microprocessor component, *Drosha* or *DGCR8*, causes lethal skeletal dysplasia. *Drosha* or *DGCR8* was deleted in growth plate chondrocytes using *Col2-Cre* transgenic mice. Skeletal preparations of cKO mice cKO of *DGCR8* (A, C), and *Drosha* (B, D) mice at E18.5. *DGCR8* and *Drosha* cKO mice show deformed skulls and poor skeletal growth compared with Cre-negative control littermates (Ctrl). Reductions in expression of indicated miRNAs and genes in primary rib chondrocytes in *DGCR8* (E) and *Drosha* (F) cKO mice. Scale bars, 0.5 mm (A, B) and 0.25 mm (C, D). All miRNAs and genes showed significant reductions in cKO mice. ($n = 4$, $P < 0.001$).

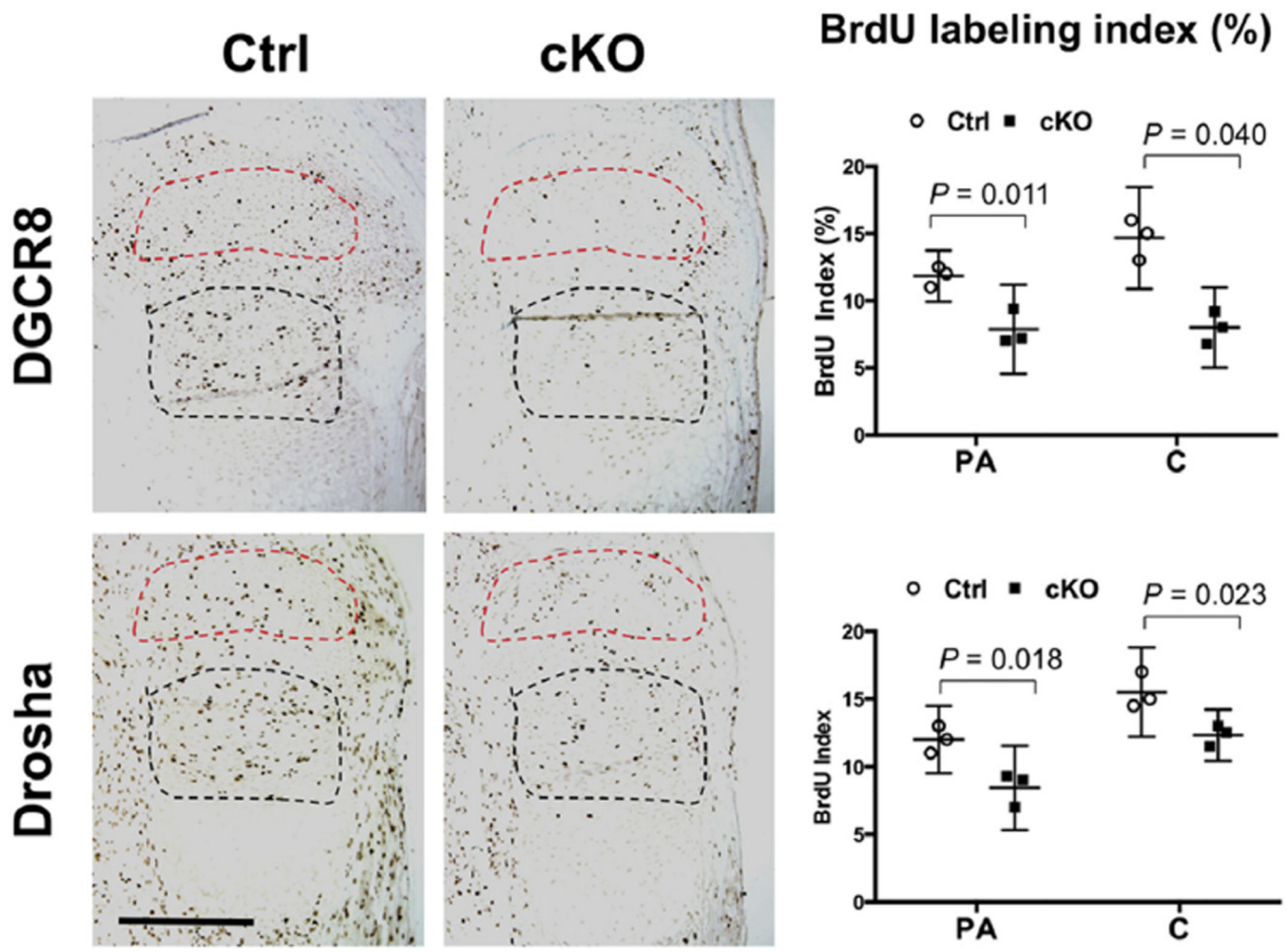

Fig. 2.

Reduced chondrocyte proliferation of *DGCR8* and *Drosha* cKO growth plates. BrdU positive cells and total nuclei of periarticular chondrocytes (PA; indicated by red dotted lines) and columnar proliferating chondrocytes (C; indicated by black dotted lines) were counted on the proximal tibial growth plate of E17.5 embryos of control (Ctrl) and cKO mice. The BrdU labeling index was calculated by dividing the number of BrdU-positive nuclei by the total number of nuclei. Three mice each genotype were analyzed ($n = 3$).

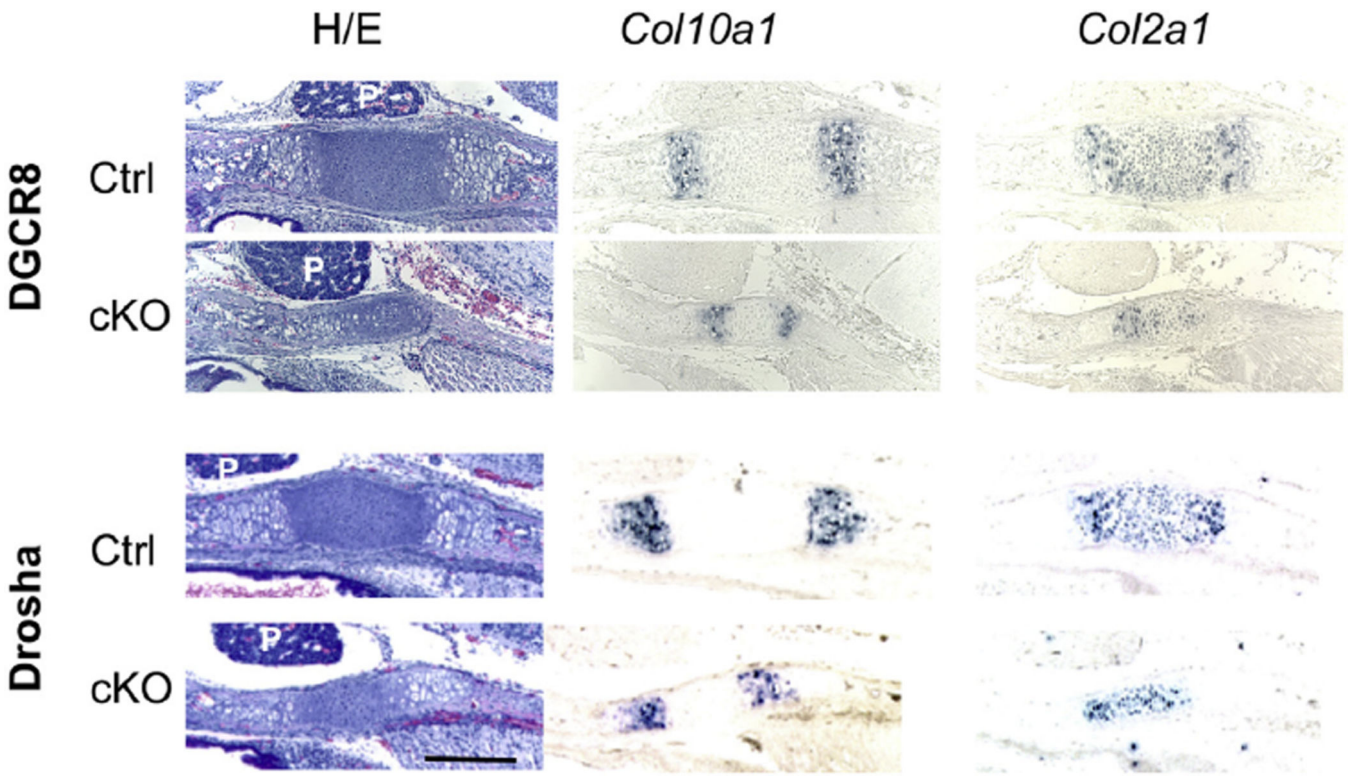

Fig. 3.

Reduced proliferating chondrocytes of the basiosphenoidal/basioccipital growth plate in the basal skull in *DGCR8* and *Drosha* cKO mice. Sagital sections of the basal skull of E18.5 embryos were subjected to H/E staining and *in situ* hybridization for *Col10a1* and *Col2a1*. P, pituitary gland. Scale bar, 100 μm.

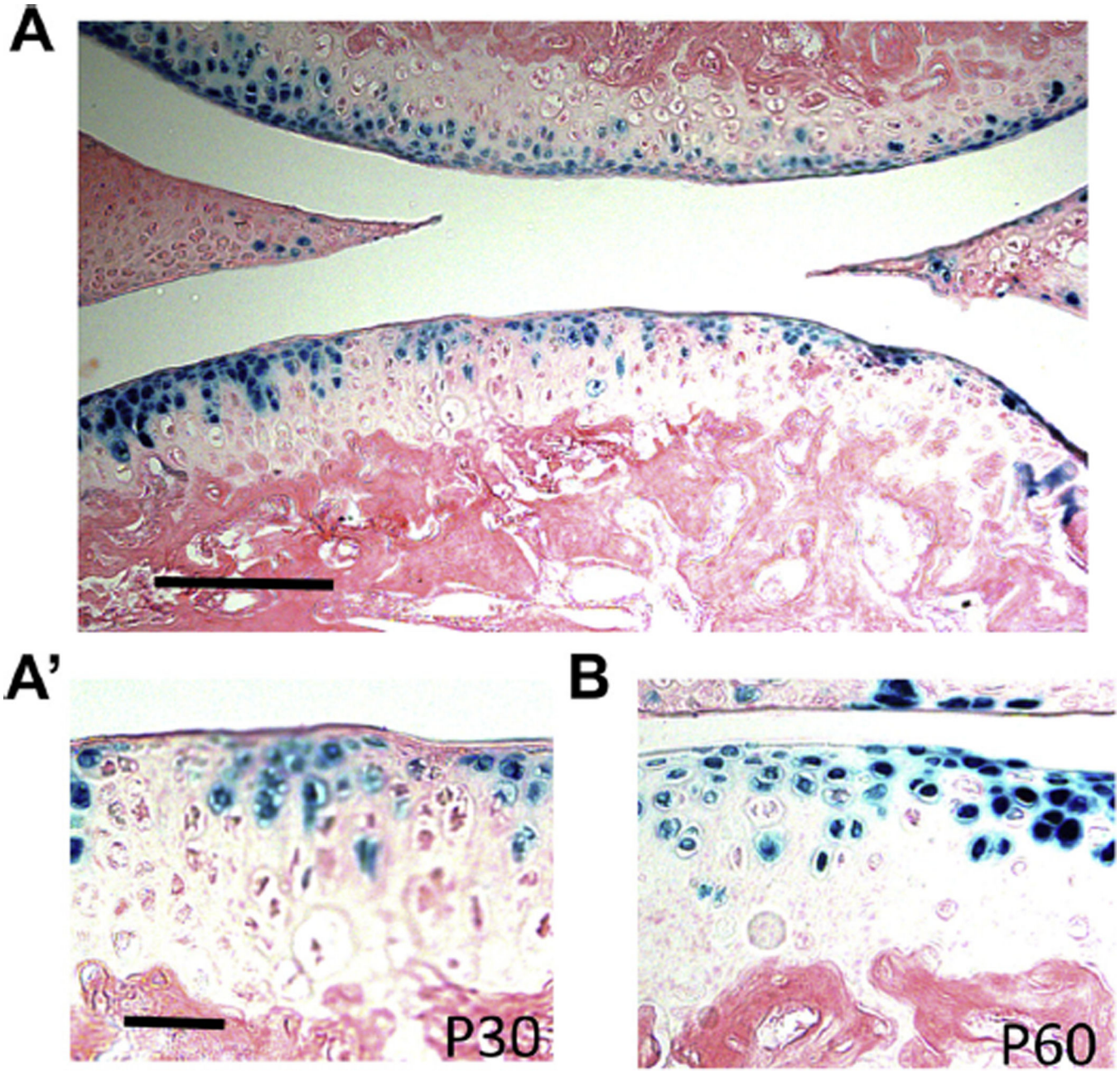

Fig. 4.

Efficient postnatal tamoxifen-induced, *Prg4CreER*^{T}-dependent DNA recombination in articular chondrocytes. Transgene mice doubly positive for the *Prg4-CreER*^{T} and *R26R* Cre reporter alleles were injected with tamoxifen at P7, 14, 21, and 28. Whole mount X-gal staining shows X-gal-positive cells in superficial and middle zone chondrocytes at P30 (A and A′) and P60 (B).

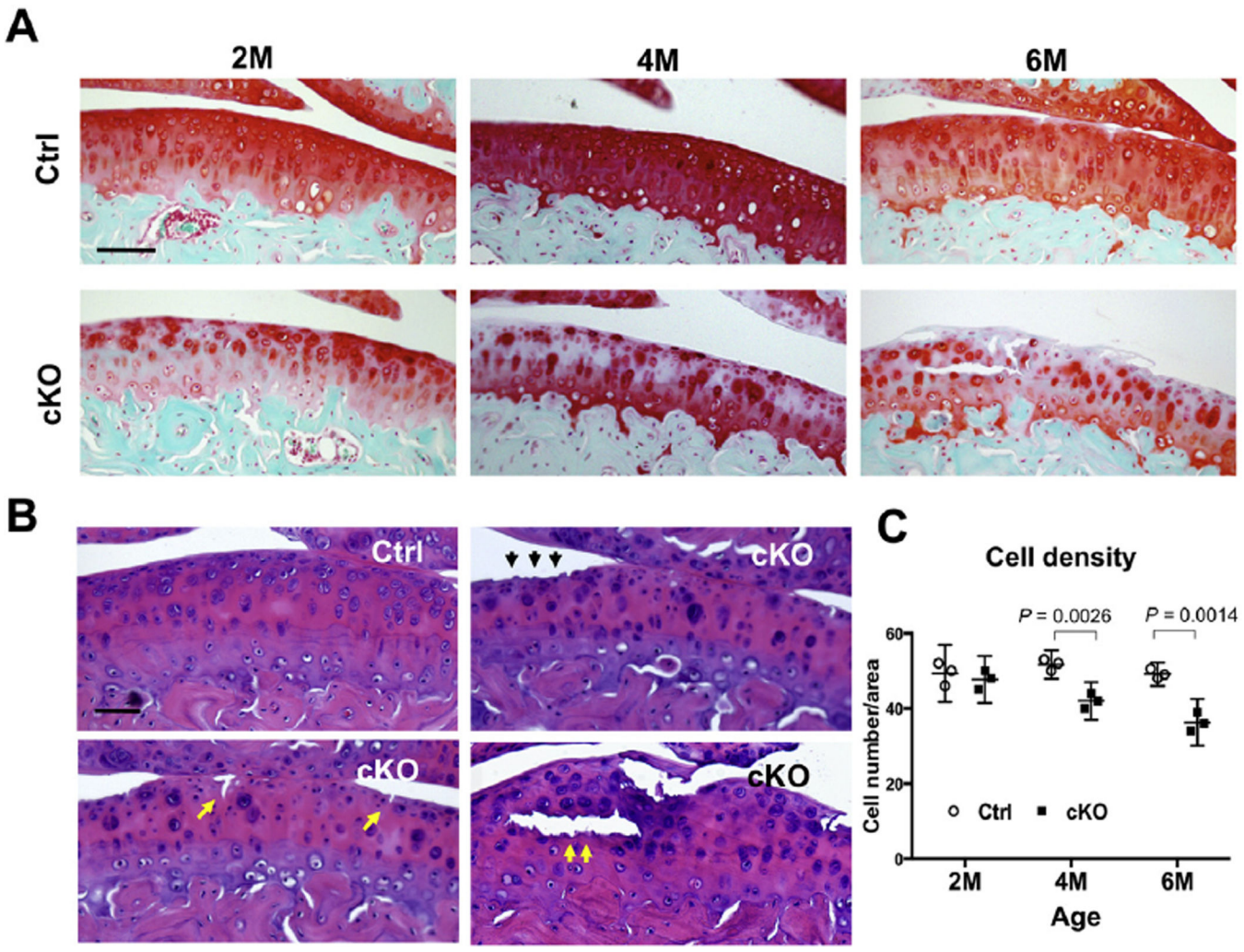

Fig. 5.

Histological changes caused by postnatal deletion of *Drosha* in articular chondrocytes. (A) Safranin O staining of the articular cartilage of the proximal tibia of control (Ctrl) and *Drosha* cKO male mice. Sagital sections of the medial tibial plateau at indicated ages are shown. Reduced Safranin O staining is noted at 2 months, while the structural integrity of the cartilage tissue is relatively well preserved until 4 months. (B) H/E stained section of 6-month old male cKO articular cartilage. Mildly reduced cellularity, surface erosion (black arrows), vertical (single yellow arrows) and horizontal clefts (double yellow arrow) are present. These structural changes are minimal in female cKO mice (Supplementary Fig. 1). More than two cKO mice with each sex were analyzed at each time point. (C) Reduced cellularity in *Drosha*-deficient cartilage. Cell numbers per an arbitrarily defined area above the tidemark of the tibial plateau were counted in mice at indicated ages. (n = 3).

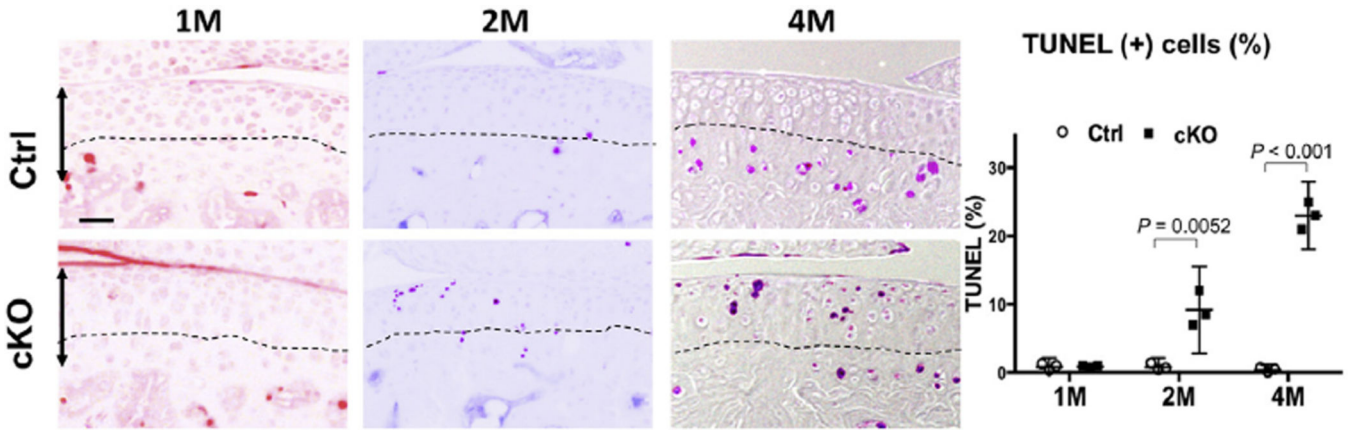

Fig. 6.

Increased cell death in the superficial and mid zones in *Drosha* cKO cartilage at 4 months old. Both control (Ctrl) and cKO mice show TUNEL-positive cells in the calcified zone below the tidemark (dotted lines), whereas cKO mice show a significant increase in number of TUNEL-positive cells in the superficial, middle, and deep zones above the tidemark at 4 and 6 months. TUNEL-positive cells above the tidemark were counted, normalized by the total number of cells, and compared. Double arrows indicate the articular cartilage. Scale bar, 50 μm. (n = 3).