Mesenchyme-specific Knockout of ESET Histone Methyltransferase Causes Ectopic Hypertrophy and Terminal Differentiation of Articular Chondrocytes*

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The exact molecular mechanisms governing articular chondrocytes remain unknown in skeletal biology. In this study, we have found that ESET (an ERG-associated protein with a SET domain, also called SETDB1) histone methyltransferase is expressed in articular cartilage. To test whether ESET regulates articular chondrocytes, we carried out mesenchyme-specific deletion of the ESET gene in mice. ESET knock-out did not affect generation of articular chondrocytes during embryonic development. Two weeks after birth, there was minimal qualitative difference at the knee joints between wild-type and ESET knock-out animals. At 1 month, ectopic hypertrophy, proliferation, and apoptosis of articular chondrocytes were seen in the articular cartilage of ESET-null animals. At 3 months, additional signs of terminal differentiation such as increased alkaline phosphatase activity and an elevated level of matrix metalloproteinase (MMP)-13 were found in ESET-null cartilage. Staining for type II collagen and proteoglycan revealed that cartilage degeneration became progressively worse from 2 weeks to 12 months at the knee joints of ESET knockout mutants. Analysis of over 14 pairs of age- and sex-matched wild-type and knock-out mice indicated that the articular chondrocyte phenotype in ESET-null mutants is 100% penetrant. Our results demonstrate that expression of ESET plays an essential role in the maintenance of articular cartilage by preventing articular chondrocytes from terminal differentiation and may have implications in joint diseases such as osteoarthritis.

Significance: Learning regulatory mechanisms of articular chondrocytes is critical to the understanding of joint diseases.

Article cartilage lines the joints and makes low friction and painless movement possible throughout life. Articular cartilage consists of a sparse population of articular chondrocytes that are responsible for the synthesis of an extracellular matrix consisting of collagens, proteoglycans, and noncollagenous proteins. Failure of articular chondrocytes to maintain the integrity of articular cartilage and/or to repair its damage is a common feature in joint diseases such as osteoarthritis.

All chondrocytes are derived from mesenchymal stem cells during skeletogenesis and joint formation and invariably follow two separate developmental paths; growth plate chondrocytes first appear during embryonic development and display a transient phenotype characterized by rapid proliferation and terminal differentiation, whereas articular chondrocytes are found only at the joint, are slow in cellular turnover, and are phenotypically stable. One possible mechanism for the development of osteoarthritis is the failure of articular chondrocytes to maintain their latent phenotype. In such a model, articular chondrocytes become mitotically active, proliferate, and express proteins such as type-X collagen, alkaline phosphatase, and matrix metalloproteinase-13 (MMP-13), proteins that are indicative of chondrocyte terminal differentiation. This is a process reminiscent of growth plate chondrocytes during endochondral ossification (1, 2). It should be pointed out, however, that osteoarthritis-like changes in joints may also occur without evidence of chondrocyte hypertrophy but instead are caused by inflammatory insults, increases in metabolic stress, and reactive oxygen species or, merely the accumulated effects of cell death (3, 4).

At the present time, a major unanswered question in skeletal development is the regulatory differences between articular and growth plate chondrocytes. Although certain regulatory factors of growth plate chondrocytes are also found in articular chondrocytes, it is not clear whether a common mechanism exists to control these two distinct chondrocyte populations.

3 The abbreviations used are: MMP, matrix metalloproteinase; ALP, alkaline phosphatase; ERG, ERG-related gene; ETS, erythroblast transformation-specific; PCNA, proliferating cell nuclear antigen; CKO, conditional knock-out; E, embryonic day.
We recently demonstrated that overexpression of the ESET histone methyltransferase protein (together with histone deacetylase HDAC4) leads to repression of Runx2-mediated gene transcription, that the endogenous ESET protein level is transiently up-regulated in prehypertrophic chondrocytes within the growth plate to function as a "gate keeper" in allowing entry into terminal differentiation, and that ESET knock-out in mesenchymal cells results in premature hypertrophy of growth plate chondrocytes during skeletal development (5). To investigate whether ESET is also essential for the maintenance of articular chondrocytes phenotype, in this study, we have evaluated the knee joints of more than 14 pairs of age- and sex-matched wild-type and ESET-null mice at different time points after birth. Here we report that ESET protein is expressed in articular chondrocytes and that mesenchyme-specific deletion of the ESET gene is associated with ectopic hypertrophy of articular chondrocytes, degeneration of articular cartilage, and other overt signs of terminal differentiation.

**EXPERIMENTAL PROCEDURES**

**Generation of Conditional ESET-null Mice**—All animal studies were carried out in the C57BL/6 genetic background. Mating between ESET(exons 15&16)Flox/WT and ESET(exons 15&16)Flox/WT;Prx1-Cre gave rise to offspring with various genotypes at the expected Mendelian ratio. Newborn pups were kept at five or fewer per litter (by removing the healthy littermates) to increase survival of the knock-out mutants. Weaned mutant mice were fed with normal diet plus soft main-
defects in other organ systems, thus enabling us to investigate the effects of ESET knock-out on articular chondrocytes.

Because the knee represents a major weight-bearing joint covered on both ends by articular cartilage, in this study, we have focused on the effects of ESET knock-out on the knee joint. To examine ESET expression in articular cartilage, we carried out immunohistostaining of knee sections using an anti-ESET antibody. As shown in Fig. 1b, ESET protein is found in chondrocytes within the articular cartilage in wild-type mice. In (exons 15&16)CKO/CKO mutant mice, ESET protein is evidently eliminated from chondrocytes within the articular cartilage. ESET-positive staining is present in the medullary cavities of both wild type and (exons 15&16)CKO/CKO mutants.

Two weeks after birth, the knee joints in wild-type mice did not exhibit much phenotypic difference when examined by histological staining (Fig. 1c). Although the mutant mice lacked epiphyseal plates that are responsible for postnatal growth of the long bones, the gross morphology and thickness of articular cartilage covering the femoral condyles and tibial plateau in (exons 15&16)CKO/CKO mice were similar to those of the wild-type mice.

ESET Knock-out Does Not Affect Generation of Articular Chondrocytes—It is possible that mesenchyme-specific knock-out of ESET could block the normal generation of articular chondrocytes during embryonic development. ESET-null mice thus may lack articular cartilage, and chondrocytes seen at the joints of (exons 15&16)CKO/CKO mutants could actually be derived from epiphyseal chondrocytes that now extend to the joint surface. To rule out such a possibility, we performed immunohistostaining for matrilin-1, a matrix protein that is known to be excluded from articular cartilage (9). As shown in Fig. 2a, although epiphyseal chondrocytes stained strongly for matrilin-1 in articular cartilage, a thin layer of cartilage near the joint surface is indeed negative for matrilin-1 in wild-type embryos. Similar exclusion of matrilin-1 from articular cartilage near the joint surface is also evident in ESET-null embryos.

To further confirm that formation of articular chondrocytes is not affected by ESET knock-out during joint development, we examined expression of ERG, an ETS family transcription factor whose mRNA has been reported to accumulate at the forming synovial joint but absent from growth plate cartilage (10, 11). As shown in Fig. 2b, immunohistostaining with an anti-ERG antibody confirmed expression of ERG protein at the developing knee joint in both wild type and (exons

FIGURE 1. ESET genomic structure and expression in articular chondrocytes. a, diagrams of ESET protein domains and the floxed ESET allele. a. a., amino acids. b, ESET protein expression at the knee joint was assessed by an anti-ESET antibody in wild-type and ESET-null mice. DAPI counterstaining of nuclei was used for tissue outline. Arrows indicate strong staining for ESET protein in subchondral bone marrow cells. c, coronal sections of 2-week-old knee joints were stained by H&E for cell morphology and by Safranin O for proteoglycans (red). Scale bar: 400 μm.

FIGURE 2. Characterization of articular chondrocytes in ESET-null embryos. a, sagittal sections of knee joints from E18.5 embryos were stained by H&E or with an anti-matrilin-1 antibody. DAPI counterstaining of nuclei was used for tissue outline. The images were merged to show the absence of matrilin-1 in articular cartilage. b, sagittal sections of developing knee from E15.5 embryos were stained by H&E or with a rabbit antibody to show similar ERG expression within future joints in both wild-type and ESET-null embryos. A total of three wild-type and three knock-out embryos were examined, and results from a typical experiment are shown. Genotypes of the embryos are indicated on the left side. Scale bars: 400 μm (a), 200 μm (b).
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A new study has provided evidence that ESET deletion does not impair generation of articular chondrocytes. These findings suggest that articular chondrocytes are not directly dependent on ESET for their development.

**Figure 3. Articular chondrocyte hypertrophy and proliferation in 1-month-old ESET-null mice.**

- **a.** 1-month-old knee joints were stained by H&E and Safranin O for detailed morphological analysis.
- **b.** 1-month-old knee sections were stained with an anti-type II collagen antibody. The type II collagen network was stained red, and nuclei were stained blue by DAPI.
- **c.** Type X collagen antibody specifically stains hypertrophic chondrocytes (red) in 1-month-old knee sections. Anti-PCNA antibody detects cells positive for the proliferation marker in 1-month-old knee sections. Arrows in a–d indicate locations of hypertrophic or proliferative chondrocytes near the superficial zone in ESET-null mice. A total of three wild-type and five knock-out mice were examined, and images from representative experiments are shown. Genotypes of the mice are indicated on the top. Scale bar: 50 μm.

**ESET Knock-out Causes Apoptosis in Articular Chondrocytes**

Chondrocyte hypertrophy is known to be associated with programmed cell death. To show apoptosis among articular chondrocytes in ESET mutants at 1 month of age, we performed staining with an antibody against active caspase 3, an early apoptosis marker that is activated in apoptotic cells both by the extrinsic (death ligand) and by the intrinsic (mitochondrial) pathways. As confirmed in Fig. 4a, increased staining for active caspase 3 was seen throughout the articular cartilage of 1-month-old ESET knock-out mice (n = 3 for wild type, n = 5 for knock-out). To further confirm that increased staining for active caspase 3 correlates with DNA fragmentation (the last phase of apoptosis) in articular chondrocytes, we performed TUNEL assay on the knee sections of ESET-null mutants. Although we did not find any TUNEL assay-positive articular chondrocytes at the knee joints of 1-month-old wild-type animals, positive TUNEL assay staining was readily detectable at the knee joints of 1-month-old ESET knock-out mice (Fig. 4b). These results indicate that expression of ESET protein is essential to the blockage of apoptosis that would otherwise take place even in the articular chondrocytes of young adult animals.

**ESET Knock-out Accelerates Degeneration of Articular Cartilage**

Analysis of the ESET-null mice at 3 months demonstrated a marked decrease in articular cartilage thickness as indicated by decreased type-II collagen staining (Fig. 5a). A merged image with DAPI allows for the visualization of type II collagen staining in relationship to the articular chondrocytes. When measured in multiple animals (n = 3 each for wild type and knock-out), the average cartilage thickness...
at the lateral condyle in (exons 15&16)CKO/CKO mice was about 40% of that found in sex- and age-matched wild-type animals. Additionally at this age, as shown in Fig. 5b, the superficial zones of the articular cartilage in ESET-null mice were no longer stained positive by Safranin O, an indication that portions of the joint surface had lost their proteoglycan content.

MMP-13 is a marker for terminal differentiation and the most active collagenase against type II collagen, the main component of articular cartilage (18). During embryogenesis, MMP-13 is expressed by hypertrophic chondrocytes at the epiphyseal plate, and its proteolytic activity is responsible for reorganization of the cartilaginous analgen, which allows growth and ossification (19). When assessed for MMP-13 in the ESET-null mice, as shown in Fig. 5c, MMP-13 protein was also detected in the superficial zone of ESET-null mice but not in wild-type controls. d, alkaline phosphatase activity was nearly undetectable in wild-type articular cartilage, but ESET-null chondrocytes close to the articular surface all stained positive for alkaline phosphatase activity. Lower panels represent enlargements of the selected areas in the top panels. A total of three wild-type and three knock-out mice were examined, and representative experiments are shown. Scale bar: 400 μm.
Because (exons 15&16)\textsuperscript{CKO/CKO} mice can live for more than 1 year, we also examined the knee joints in 1-year-old mice. Although wild-type mice showed normal thickness of articular cartilage at this age, degradation of articular cartilage in 12-month-old ESET-null mice was even more severe. It was noticed that cartilage at the joint in 12-month-old ESET-null mice was mostly surrounded by type II collagen-negative cells and that there was very little cartilage left to cover the joint surface. On average, the thickness of cartilage remnant at the lateral condyle in 12-month-old (exons 15&16)\textsuperscript{CKO/CKO} mice was less than 20% of that found in sex- and age-matched wild-type animals (n = 5 each for wild-type and knock-out mice). This confirms the notion that loss of type II collagen and proteoglycans seen at 3 months in ESET-null mice is progressive (Fig. 6, a and b).

**DISCUSSION**

We have provided evidence that mesenchyme-specific ESET knock-out has little effect on embryonic development of articular chondrocytes. In the absence of ESET histone methyltransferase, however, adult articular chondrocytes fail to maintain their phenotype, which predisposes the articular cartilage to degeneration. It appears that both the loss of articular cartilage cellularity (due to chondrocyte hypertrophy followed by apoptosis) and an increase in collagenase activity (MMP-13) are responsible for degeneration of articular cartilage in ESET-null adult mice.

In this study, we have demonstrated that ESET is expressed in articular cartilage, that mesenchyme-specific knock-out of ESET causes ectopic hypertrophy and apoptosis in articular chondrocytes, and that terminal differentiation of articular chondrocytes predisposed articular cartilage in the knock-out animals to degeneration. It should be pointed out that we have analyzed more than 14 pairs of age- and sex-matched wild-type and (exons 15&16)\textsuperscript{CKO/CKO} mice (with ages ranging from 2 weeks to 12 months), and all ESET mutant mice exhibited similar defects described here. The articular chondrocyte phenotype found in ESET knockouts is therefore 100% penetrant. Our findings support the notion that a similar mechanism controls the differentiation of both growth plate chondrocytes and articular chondrocytes through sharing a common set of regulatory factors such as ESET.

In normal growth plate development, chondrocytes at various differentiation stages are all organized in specific zones, and chondrocytes mature in an orderly sequence. In this study, we observed hypertrophy of articular chondrocytes taking place in 1-month-old mutants but not in 3-month-old mutants. Also, only a fraction of articular chondrocytes appear to be hypertrophic at 1 month in these knockouts. Why did hypertrophy not occur in all of the articular chondrocytes in a synchronized manner? The answer could be that articular chondrocytes are not uniform in behavior. In addition to being subdivided into three histological zones (superficial zone at the surface, transitional zone in the middle, and deep zone near the calcified cartilage), articular chondrocytes within the same zone may also exhibit individual differences due to changes in microenvironment such as topographical variations (20–22). It appears that by 3 months, articular chondrocytes that are susceptible to hypertrophy in the face of ESET knock-out are long gone, and the effects of ESET deletion on the remaining articular chondrocytes are manifested not through hypertrophy but through other changes such as elevated ALP and MMP-13 expression. Despite this heterogeneity in chondrocyte responses, one constant observation remains that ESET-null articular cartilage is defective and invariably undergoes premature degeneration in all mutant animals.

ESET belongs to a group of proteins (Suv39h, G9a, GLP, and ESET) that are responsible for specific methylation of H3-K9 (23). As a major epigenetic marker, H3-K9 methylation is known as a “histone code” for gene silencing. Interestingly, a recent study showed that H3-K9 methylation in the promoter region of NFAT1, an essential transcriptional regulator of articular cartilage homeostasis, determines its age-dependent expression in articular chondrocytes (24). In addition, transgenic studies have shown that ERG protein (with which ESET is known to associate) regulates the developmental behavior of most epiphyseal chondrocytes and helps them acquire a permanent articular chondrocyte phenotype in embryos (11).

What could be the underlying mechanism responsible for such an accelerated degeneration of articular cartilage in adult ESET-null mice? We noticed that the activities of both ALP and MMP-13 are elevated in ESET-null articular cartilage, and both genes are well known downstream targets of Runx2 protein (25–27). In our recent studies to understand the molecular
mechanisms governing ESET regulation of growth plate chondrocytes, we have found that overexpressed ESET protein (together with HDAC4) physically interacts with and functionally inhibits Runx2 and that repression of Runx2-mediated reporter gene transactivation by co-transfected ESET is dependent on its intrinsic H3-K9 methyltransferase activity (5). Because Runx2 is a hypertrophy-promoting transcription factor that is normally repressed in articular chondrocytes (28), we believe that ESET is also an essential component of a protein complex that represses Runx2 activity in articular chondrocytes and that specific knock-out of ESET in articular chondrocytes results in de-repression of Runx2 activity and ectopic expression of Runx2 target genes such as type X collagen and MMP-13 described in this study. This aberrant gene expression program in turn promotes hypertrophy and terminal differentiation of articular chondrocytes, leading to eventual destruction of articular cartilage at the joint.

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