

# MT1-MMP–dependent, apoptotic remodeling of unmineralized cartilage: a critical process in skeletal growth

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Skeletal tissues develop either by intramembranous ossification, where bone is formed within a soft connective tissue, or by endochondral ossification. The latter proceeds via cartilage anlagen, which through hypertrophy, mineralization, and partial resorption ultimately provides scaffolding for bone formation. Here, we describe a novel and essential mechanism governing remodeling of unmineralized cartilage anlagen into membranous bone, as well as tendons and ligaments. Membrane-type 1 matrix metalloproteinase (MT1-MMP)–dependent dissolution of unmineralized cartilages, coupled with apoptosis of non-hypertrophic chondrocytes, mediates remodeling of these

cartilages into other tissues. The MT1-MMP deficiency disrupts this process and uncouples apoptotic demise of chondrocytes and cartilage degradation, resulting in the persistence of “ghost” cartilages with adverse effects on skeletal integrity. Some cells entrapped in these ghost cartilages escape apoptosis, maintain DNA synthesis, and assume phenotypes normally found in the tissues replacing unmineralized cartilages. The coordinated apoptosis and matrix metalloproteinase-directed cartilage dissolution is akin to metamorphosis and may thus represent its evolutionary legacy in mammals.

## Introduction

In all tissues, remodeling of extracellular matrix components is critical for development, growth, and homeostasis. With their broad substrate specificity, the matrix metalloproteinases (MMPs) figure prominently as potential molecular pruning tools of the extracellular environment (Massova et al., 1998; Vu and Werb, 2000; Brinckerhoff and Matrisian, 2002). The role of MMPs has been extensively investigated in a variety of developmental and pathophysiological conditions, and several members of this proteinase family have been demonstrated as essential in that capacity (Chin and Werb, 1997; Vu and Werb, 2000; Colnot and Helms, 2001; Brinckerhoff and Matrisian, 2002; Egeblad and Werb, 2002; Galis and Khatri, 2002; Loftus et al., 2002; Seiki, 2002).

The skeleton is particularly rich in extracellular matrix, and remodeling is central to skeletal physiology throughout life. Although some bones develop directly from a soft connective tissue via intramembranous ossification, the majority of skeletal segments develop through endochondral ossification. In this process, embryonic cartilage anlagen grow and mineralize in a highly coordinated fashion before the resultant mineralized cartilage is finally resorbed by osteoclasts and replaced by newly formed bone (de Crombrughe et al., 2001; Karsenty and Wagner, 2002). Chondrocytes progress through a precisely regulated and well-characterized sequence of cell proliferation and differentiation events. Cell division in the epiphyses is followed by maturation and hypertrophy, with a switch from type II to type X collagen expression (Chan and Jacenko, 1998). Hypertrophic chondrocytes, in turn, promote the mineralization of the surrounding extracellular matrix (Karsenty and Wagner, 2002), which later undergoes resorption by osteoclasts (chondro-

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Abbreviations used in this paper: MC, Meckel's cartilage; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 MMP.

clasts) (Birkedal-Hansen, 1995; Roodman, 1999; Teitelbaum, 2000; Vaananen et al., 2000). At the same time, hypertrophic chondrocytes also undergo apoptosis (Gerber et al., 1999; Cancedda et al., 2000; Karsenty and Wagner, 2002). Next, bone is deposited around the remnants of mineralized cartilage (cartilage “cores”) by newly differentiated osteoblasts. The resulting mineralized chondro-osseous structure (called the primary spongiosa) is further resorbed by osteoclasts and replaced by secondary bone. So, endochondral bone formation inevitably and unfailingly proceeds through a stage of cartilage maturation, culminating in oblique mineralization before the mineralized cartilage is remodeled into bone. Remodeling of existing bone into newer bone takes place throughout life (Dempster, 2002) and ensures mineral homeostasis and optimal mechanical properties of the skeleton. It is accomplished through the coordinated action of bone-resorbing osteoclasts and bone-forming osteoblasts.

Both endochondral ossification and postnatal bone remodeling, the two best-known and most widely investigated mechanisms of tissue remodeling operating in the skeleton, share a common paradigm where a preexisting mineralized matrix is replaced by a newer mineralized matrix. This is the case in endochondral ossification, which is crucial to embryonic development, and postnatally, where bone turnover is necessary for growth, mineral homeostasis, and maintenance of skeletal mass.

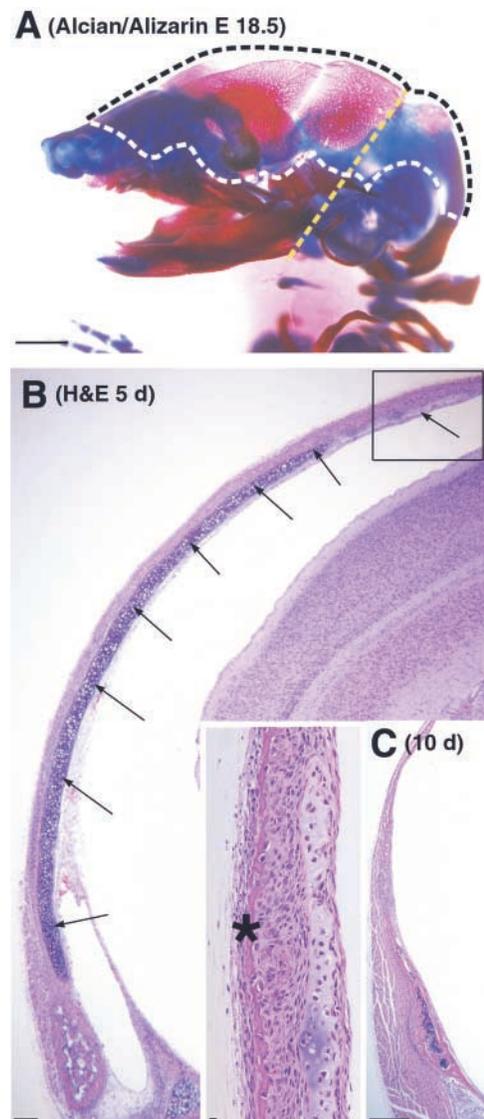
Here, we describe a novel mechanism of tissue remodeling in the postnatal mouse skeleton and define its cellular and molecular determinants. We show that cartilage remodels into bone in the postnatal skeleton at multiple sites via a nonendochondral mechanism; i.e., without progression through the sequence of matrix mineralization and osteoclastic removal as described in previous paragraphs. The process involves coordinated dissolution of the unmineralized cartilaginous matrix by a process that is absolutely dependent on the membrane-type 1 MMP (MT1-MMP) and timely apoptosis of nonhypertrophic chondrocytes. The same mechanism of MT1-MMP-dependent removal of unmineralized cartilage also allows remodeling of cartilage into ligament at specific sites. Disruption of this mechanism in MT1-MMP-deficient mice results in severe skeletal pathology, highlighting the general significance of remodeling of unmineralized cartilage into other tissues for skeletal growth and integrity.

## Results

### Unmineralized cartilages of the cranial vault

We have previously shown that MT1-MMP-deficient mice develop an abnormal head shape during the first days of postnatal life as a result of persistence of embryonic cartilages at the sites of the formation of calvarial bones (Holmbeck et al., 1999). Because formation of calvarial bones is not thought to follow the endochondral paradigm, the very existence of calvarial cartilages is commonly overlooked, and their function and fate in cranial morphogenesis have remained elusive (Kaufman, 1995; Chen et al., 1998).

We undertook a detailed analysis of the skull formation in wild-type mice ranging from 14.5 d postcoitum to 10 d



**Figure 1. Morphology of cartilage anlagen and membranous bones in the wild-type mouse calvarium.** (A) Gross morphology of the skull of an E18.5-d-old mouse embryo stained with alizarin red/alcian blue. The dotted line marks the region of membranous ossification. Note the extensive cartilaginous anlagen in the region where parietal and interparietal bones develop. The yellow dotted line indicates the plane of sectioning used in preparing histological material, as shown in B. (B) Section through the skull of a 5-d-old mouse, demonstrating the parietal cartilage (arrows) and the leading edge of the developing parietal bone (inset, asterisk). The inset shows a higher magnification of the area boxed in B. Note that parietal bone grows outside of the parietal cartilage rudiment. The cartilage matrix is not stained by hematoxylin (has lost the basophilia that indicates a normal proteoglycan content) in the region where membranous ossification is in progress. (C) Low-power magnification of parietal bone from 10-d-old wild-type mouse demonstrating complete removal of the parietal cartilage. Bars: (A) 1 mm; (B) 100  $\mu$ m; (B, inset) 10  $\mu$ m; (C) 333  $\mu$ m.

postnatal using whole-mount alizarin red/alcian blue staining and histology. Embryonic cranial cartilages were prominent at midgestation and grew substantially in the latter part of embryogenesis, reaching their most conspicuous expanse at day 17.5 after coitus. At this point, cranial bones were

well developed, and cartilages and cognate membranous bones were easily visualized by staining with alizarin red and alcian blue (Fig. 1 A, 18.5 d after coitus). The development of membranous bone initially coincided with the presence of cognate cartilage structures, but these cartilages gradually disappeared as ossification proceeded. The disappearance or removal of these cartilages started in the anterior apical region of the cranium and proceeded gradually towards the basal posterior part of the skull where the last cartilage tissue disappeared around day 10 after birth (Fig. 1 C). Both macroscopic inspection and cross sectioning of the parietal bone (Fig. 1 B) revealed that bone and cognate cartilages were spatially separated by a cell-rich layer, whereas endochondral ossification takes place within the cartilage scaffold.

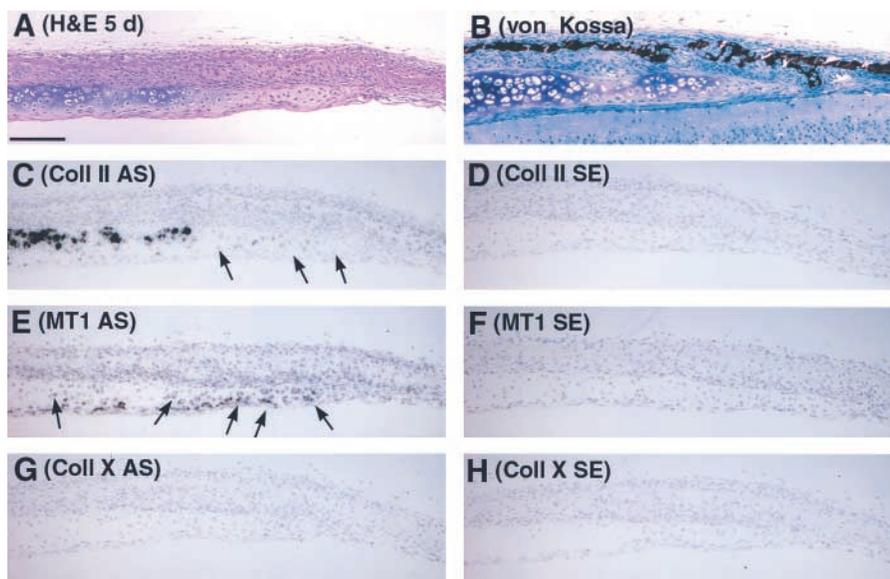
### A different mechanism of cartilage remodeling

Removal of calvarial cartilage anlagen, which was complete by day 10, did not involve the conventional sequence of chondrocyte hypertrophy, matrix mineralization, and osteoclastic resorption that characterizes endochondral bone formation. Before the disappearance of the parietal cartilage, von Kossa staining demonstrated that the entire anlage remained unmineralized (in sharp contrast to the overlying, forming parietal bone; Fig. 2 B), and TRAP cytochemistry failed to reveal osteoclasts over its surfaces (unpublished data). Most of the cartilage appeared as typical hyaline cartilage (Fig. 1 B and Fig. 2 A), with chondrocytes residing in a proteoglycan-rich, basophilic matrix and expressing abundant type II collagen mRNA (Fig. 2 C). In contrast, the apical portions of the parietal cartilage could be easily distinguished from the rest of the anlage based on the loss of proteoglycan (reflected in the loss of basophilia and alcianophilia) and variable degrees of matrix dissolution (Fig. 1 B and Fig. 2 A). In this region, neither type II collagen mRNA (Fig. 2 C) nor type X collagen mRNA (Fig. 2 G) was expressed. Thus, chondrocytes initially existed

in a steady state characterized by type II collagen expression, without ever entering a stage of hypertrophy or type X collagen expression. However, these chondrocytes eventually began to express MT1-MMP mRNA in a highly spatially restricted pattern (Fig. 2 E and Fig. 3 F). TUNEL staining demonstrated frequent apoptotic chondrocytes in the same region (Fig. 3, B and D). Thus, expression of MT1-MMP in specific regions of the parietal cartilage coincided spatially with cessation of type II collagen expression, with gradual disappearance (regression) of the matrix, and with apoptotic demise of chondrocytes. These changes stand in sharp contrast to the sequence of events that characterize the fate of cartilage during endochondral ossification in long bones, and define a precise and highly regulated program for the removal of unmineralized cartilage anlagen during the organogenesis of the cranial vault.

### MT1-MMP is necessary for dissolution of unmineralized cartilage

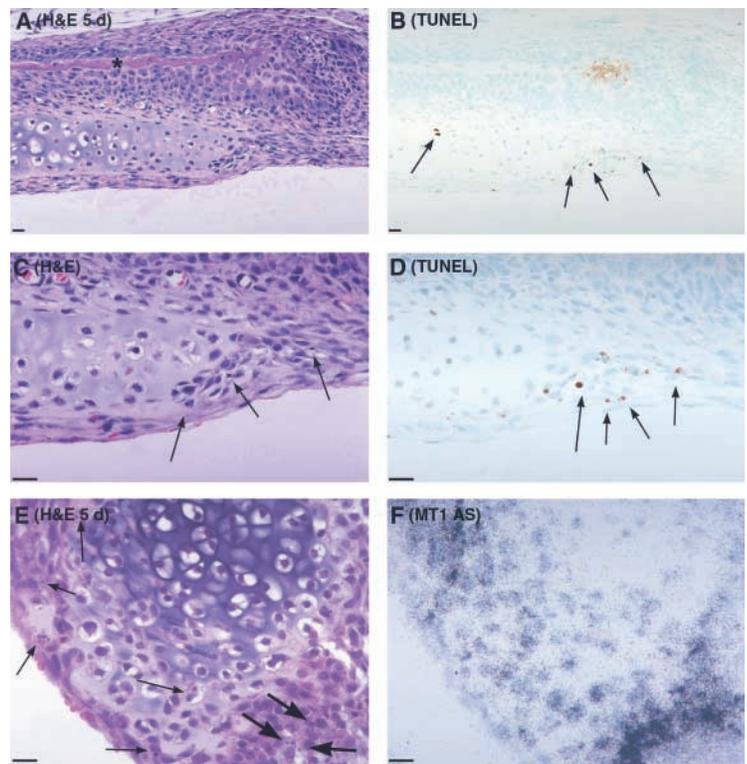
In MT1-MMP-deficient mice, vestiges of the embryonic cranial cartilages that precede the membranous bone persisted on the inner aspect of the skull up to (at least) 62 d of age, whereas no trace of them could be found in age-matched littermate controls (Fig. 4, compare A, D, and G with B, E, and H). The parietal bones of wild-type mice at 24 d and thereafter developed contiguous inner and outer bony plates (Fig. 4, A, D, and G). In MT1-MMP-deficient mice, only the outer bony plate formed, whereas the inner plate was replaced by the remnants of the parietal cartilage anlage (Fig. 4, B, E, and H). At 62 d, the mutant mice showed dramatic osteoclastic resorption of the outer bony plate, whereas the cartilage vestige occupying the position of the missing, inner bony plate remained undegraded (Fig. 4 H). Complete depletion of proteoglycan (as assessed by alcian and basophilic staining, not depicted) and empty chondrocyte lacunae were consistently



**Figure 2. Nonendochondral fate of calvarial cartilages.** Sections of calvarial cartilages from 5-d-old wild-type mice. (A) Hematoxylin and eosin (H&E) staining; (B) von Kossa staining; (C, E, and G) in situ hybridization with antisense probes specific for Type II collagen (C); MT1-MMP (E); and Type X collagen (G) mRNAs. (D, F, and H) Control hybridization with sense probes Type II collagen (D), MT1-MMP (F), and Type X collagen (H). All sections, except the one shown in B, are serial sections from the same sample. Expression of type II collagen mRNA (C) is observed in the basophilic region of the cartilage, but has ceased in the apical portion (arrows), which is devoid of proteoglycan and appears nonbasophilic in H&E-stained sections (A). In the same location, expression of type X collagen cannot be detected (G), and von Kossa staining is negative for mineral in the cartilage (B), indicating that chondrocytes do not progress to hypertrophy and

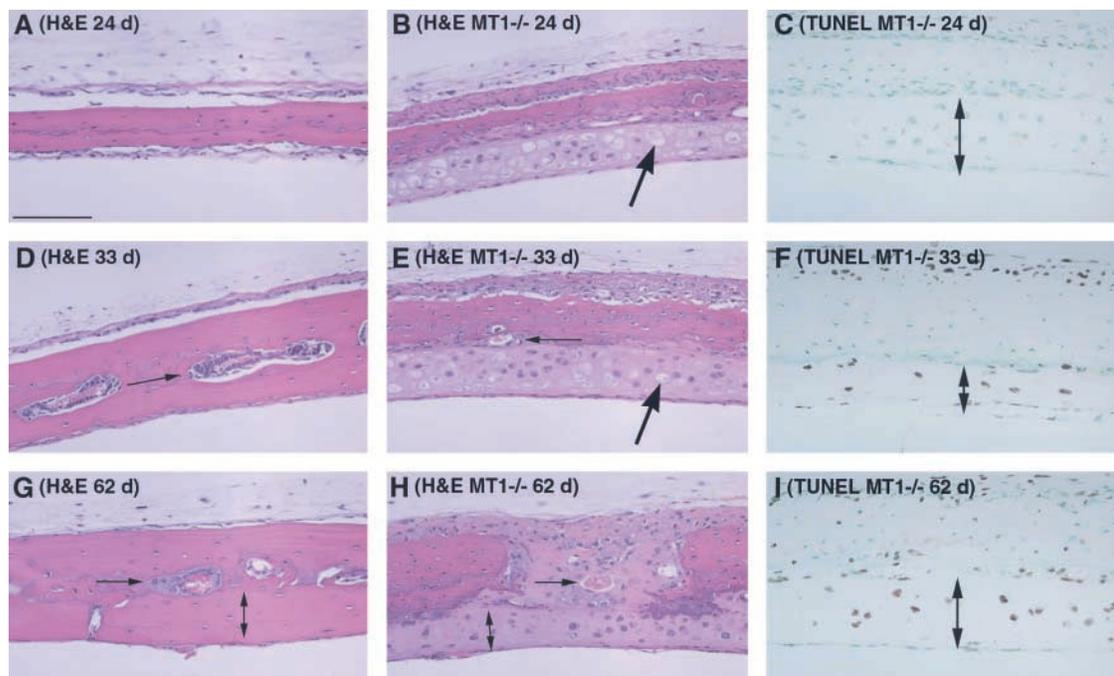
matrix mineralization. (E) MT1-MMP is particularly strongly expressed in this region (arrows), but not in those chondrocytes that reside in the basophilic area of the cartilage and express type II collagen. Bar, 100  $\mu$ m.

**Figure 3. Apoptosis of nonhypertrophic chondrocytes in calvarial cartilages coincides with MT1-MMP expression.** Sections from 5-d-old wild-type mouse. (A, C, and E) Hematoxylin and eosin (H&E) staining. (B and D) TUNEL staining. (F) In situ hybridization specific for MT1-MMP mRNA. (A) Section demonstrating regression of the calvarial cartilage proximate to the emerging parietal bone (asterisk). (B–D) Apoptotic bodies and TUNEL-positive nuclei in the chondrocytes of the regressing calvarial cartilages (arrows). (E and F) Detail of similar process from other areas demonstrating multiple apoptotic bodies (E, arrows), and serial section (F) demonstrating robust expression of MT1-MMP mRNA in cells undergoing apoptosis. Note that apoptotic chondrocytes and sites of MT1-MMP expression specifically correspond to nonbasophilic areas of cartilage. Bars, 10  $\mu$ m.

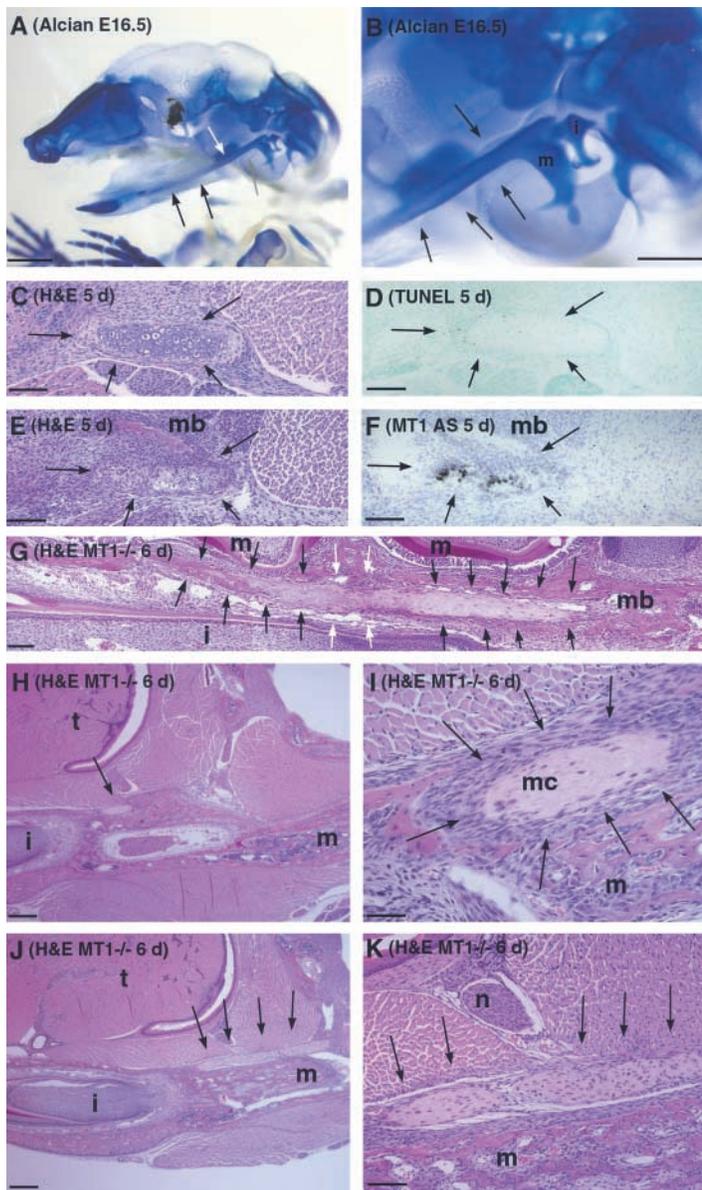


observed in the cartilage vestiges and attested to their inefficient removal. TUNEL staining demonstrated that the remaining chondrocytes underwent apoptosis in increasing

numbers (Fig. 4, C, F, and I). Besides appearing at a much later time than in the wild-type animal, apoptotic calvarial chondrocytes were randomly distributed within the cartilage



**Figure 4. Persistence of ghost calvarial cartilages and delayed apoptosis of resident chondrocytes in MT1-MMP-deficient mice.** Inability to remove calvarial cartilages leaves permanent vestiges thereof in MT1-MMP-deficient mice. In MT1-MMP-deficient mice, these structures (C, F, H, and I, double-headed arrow) precisely replace the inner plate of parietal bones (G, double-headed arrow); i.e., the bone located on the inner side of the vascular and marrow spaces (D, E, G, and H, horizontal arrows). Apoptotic demise of resident chondrocytes is markedly delayed, but progressively increases with age, and continues up to 62 d after birth; i.e., 52 d after the complete removal of the entire cartilage in wild-type mice (C, F, and I). Apoptotic chondrocytes leave behind empty chondrocytic lacunae (B and E, large arrows). Removal of the matrix does not occur, and a characteristic ghost cartilage morphology results from the combination of loss of basophilia, partial loss of chondrocytes, and retention of the matrix scaffold. (A–C) 24 d; (D–F) 33 d; (G–I) 62 d. Bar, 100  $\mu$ m.



**Figure 5. The nonendochondral fate of Meckel's cartilage (MC) is MT1-MMP dependent.** (A–F) Wild-type mouse. (G–K) MT1-MMP-deficient mice. In wild-type mice (A and B), MC is conspicuous at E16.5. The rostral portion of MC (A, black arrows) undergoes hypertrophy and endochondral ossification, while the midportion (A, white arrow) is replaced by membranous bone, and its posterior portion (A, gray arrow) by the sphenomandibular ligament (detailed view of MC [arrows], malleus [m], and incus [i] shown in B). Both processes are completed in the wild-type mouse by day 5, when only small residual portions of MC can be observed after thorough sectioning of the mandible (C, arrows). The nonbasophilic peripheral portions of these islands contain multiple apoptotic chondrocytes (D, arrows). MT1-MMP mRNA (E and F, arrows) is expressed in chondrocytes residing in the dissolving matrix next to the mandibular bone (E and F, mb). Extensive remnants of MC are retained in 6-d-old MT1-MMP-deficient mice (G–K, black and white arrows), and older. MC remnants have lost basophilia (proteoglycan content), and significant numbers of chondrocytes, thus appearing as a ghost cartilage with multiple empty chondrocyte lacunae, best seen in I (mc). (G) Sagittal section of the hemimandible demonstrating molars (m), MC (black and white arrows), and mandibular bone (mb). (H–K) Coronal sections through the mouse skull. I and K are higher magnification views of MC remnants shown in H and J. t, tongue; i, lower incisor; m, mandible; mc, Meckel's cartilage; n, nerve. (A–B) Whole-mount alcian blue staining. (C, E, G–K) Hematoxylin and eosin (H&E)-stained sections. (D) TUNEL staining. (F) In situ hybridization for MT1-MMP. Bars: (A) 1 mm; (B) 0.5 mm; (C–G, and K) 100  $\mu$ m; (I) 40  $\mu$ m; (H and J) 200  $\mu$ m.

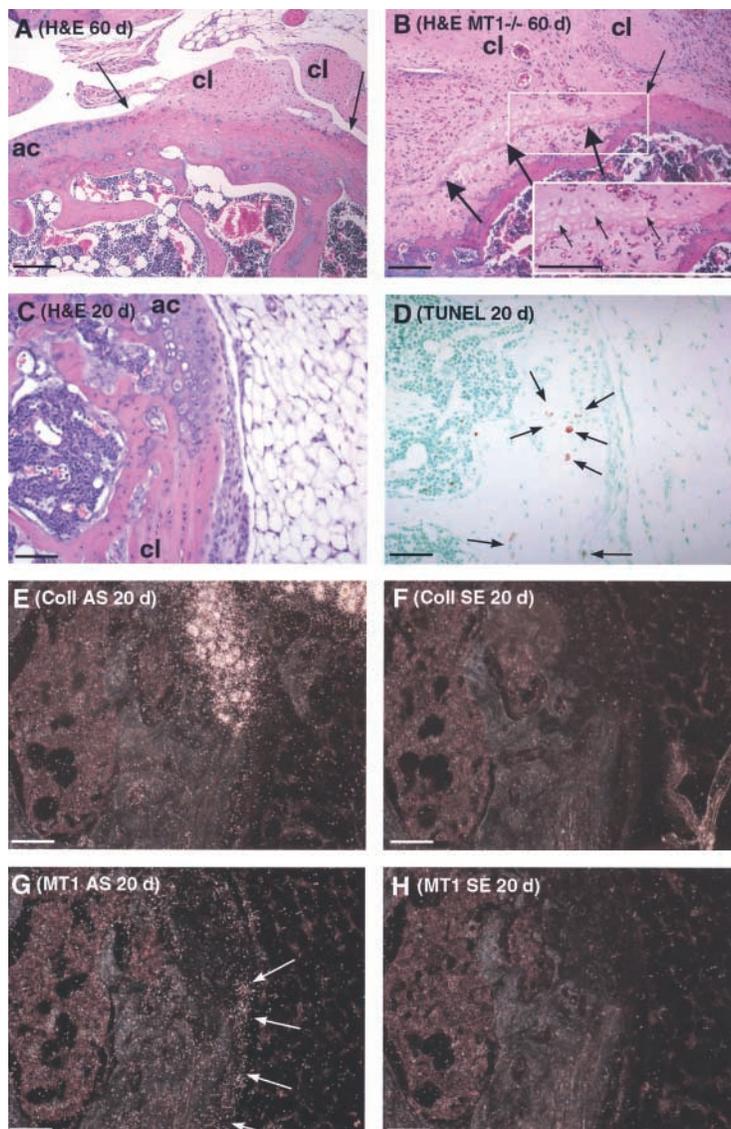
vestiges of MT1-MMP mice. The empty chondrocyte lacunae, evidence of prior and ongoing chondrocyte demise, the proteoglycan depletion, and persistence of extracellular matrix combined with the delayed apoptosis to give the vestiges the appearance of “ghost” cartilages, a hallmark of MT1-MMP deficiency. We concluded from these observations that removal of calvarial cartilages in wild-type mice is accomplished by a coordinated process of MT1-MMP-dependent degradation of the collagenous scaffold, coupled with apoptosis of nonhypertrophic chondrocytes. In MT1-MMP-deficient mice, impairment of this process leads to the persistence of ghost cartilages in the calvarium, which serve as anatomical landmarks of the specific sites where the cartilage scaffold is removed in the wild-type animals.

#### Fate of Meckel's cartilage (MC) is MT1-MMP dependent

We asked whether the same set of events and tissue changes encountered in the removal of calvarial cartilages could be identified elsewhere in the mouse skull. MC, a branchial

arch derivative known to give rise to the mandibular bone, the sphenomandibular ligament, and the malleus, is a cartilaginous anlage destined for complete replacement by bone and ligament via a complex set of events (Muhlhauser, 1986). The rostral portion of MC goes through the conventional sequence of hypertrophy, mineralization, and endochondral replacement by bone, thus giving rise to the rostral portion of the mandible (Fig. 5 A, black arrows). In contrast, the posterior part of MC does not undergo endochondral ossification and turns into both fibrous (sphenomandibular ligament) and bone (mandible and malleus) tissues via other, and as of yet undefined, remodeling events (Fig. 5 B). MC is fully conspicuous at day 16.5 in the mouse (Fig. 5, A and B), and its remodeling into definitive structures is completed around day 5. Careful analysis of serial cranial sections of day 5 wild-type mice showed that the rostral part of MC was fully replaced by bone, whereas portions of the posterior segment were still present (Fig. 5 C, arrows). The same changes that were observed in the disappearing calvarial cartilages of wild-type mice were also seen in certain re-

**Figure 6. MT1-MMP and apoptosis-dependent remodeling of cartilage at interfaces with other connective tissues.** (A and B) 60-d-old wild-type mouse and MT1-MMP-deficient littermates, respectively. (A) Demonstrates the normal morphology at the site of insertion of the anterior cruciate ligament (cl) in the tibia. Note the direct transitions between articular cartilage (ac), bone, and ligament; boundary indicated by arrows. (B) Demonstrates the severe disease observed in MT1-MMP-deficient mice at transition points (magnified in inset, defined by white frame). Note the large portion of ghost cartilage (large arrows and inset) and the extensive adhesive and fibrotic changes in the surrounding tissues. Small arrow indicates the compromised transition point. In the inset, small arrows indicate the multiple empty chondrocyte lacunae of the ghost cartilage. (C–H) Serial sections through the insertion of cruciate ligaments (cl) in the knee joint of 20-d-old wild-type mouse. (C) Hematoxylin and eosin (H&E)-stained section demonstrating the direct transition between articular cartilage (ac) and cruciate ligament (cl). (D) TUNEL staining demonstrating apoptotic cells at the transition point (arrows). E and G, expression of type II collagen and MT1-MMP mRNA, respectively. Type II collagen is abundantly expressed in articular chondrocytes. In contrast, expression of MT1-MMP is most intense in the transition point and areas below (G, arrows). F and H are sense probes for type II collagen and MT1-MMP, respectively. Bars, 50  $\mu$ m.



gions of MC. In MCs of wild-type mice at day 5 (i.e., before completion of its removal), highly localized and sharply defined zones of loss of matrix basophilia (Fig. 5, C and E) coincided spatially with apoptosis of resident chondrocytes (Fig. 5 D) and expression of MT1-MMP (Fig. 5 F).

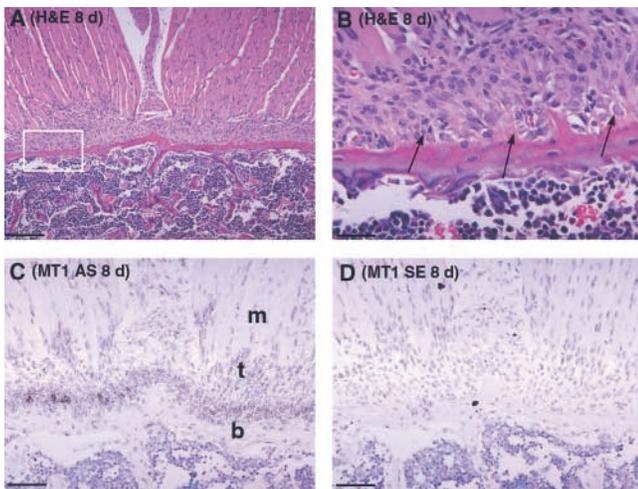
Extensive ghosts of MC were found in MT1-MMP-deficient mice at day 5 and beyond (Fig. 5, G–K). Complete proteoglycan depletion, numerous empty chondrocyte lacunae, and apoptosis of residual chondrocytes were observed in MC vestiges, as also seen in calvarial cartilages. We concluded from these data that the mechanism governing remodeling of the posterior portion of MC into bone and ligament was impaired in MT1-MMP-deficient mice, leading to the same type of tissue changes as those observed in calvarial cartilages.

#### Interfaces between different connective tissues rely on MT1-MMP-dependent remodeling

Having identified a seemingly unique mode of cartilage dissolution in two different locations of the skull, we probed whether this mechanism might be used at other sites in the postnatal skeleton. We previously reported that MT1-

MMP-deficient mice develop severe arthropathy (Holmbeck et al., 1999). Joints in these animals show conspicuous ghost cartilages at sites of transition between different articular tissues; e.g., at sites of ligament insertion into cartilage and bone (Fig. 6 B). Analysis of corresponding locations in wild-type mice (Fig. 6, A and C) demonstrated a seamless transition from articular cartilage to ligament with loss of proteoglycan (Fig. 6 C) and cessation of type II collagen expression (Fig. 6 E). TUNEL staining revealed that the transition also involved apoptotic demise of cells and coincident expression of MT1-MMP (Fig. 6, D and G). These findings suggested that the matrix remodeling at the ligament–cartilage interface was governed by the same process responsible for matrix remodeling in the embryonic cartilage of the cranial vault and in MC.

Analysis of other connective tissue interfaces (outside of joints) demonstrated that MT1-MMP is specifically expressed in these locations as well. MT1-MMP mRNA expression was restricted to the muscle–tendon and tendon–bone transitions in the basisphenoid region of wild-type mice (Fig. 7). Notably, MT1-MMP was expressed at sites



**Figure 7. MT1-MMP expression at tendon bone interface in 8-d-old wild-type mouse.** (A and B) Hematoxylin and eosin (H&E) stain. (C and D) In situ hybridization specific for MT1-MMP mRNA. (C) Antisense probe; (D) sense control. MT1-MMP is highly expressed in the transition between muscle (m), tendon (t), and bone (b) (A; and detailed view in B of area boxed in A). Note the characteristic Sharpey's fibers (B, arrows). Bars: (A) 100  $\mu$ m; (B) 25  $\mu$ m; (C and D) 40  $\mu$ m.

where Sharpey's fibers, characteristic of tendon–bone interfaces, are formed (Fig. 7, A and C). In mutant littermates, the corresponding sites showed severe fibrosis and scarring indicative of deficient matrix turnover (unpublished data).

### MT1-MMP–dependent remodeling is necessary for long bone growth

The indication that MT1-MMP was essential for maintenance of tissue interfaces led us to analyze in detail if our findings could also account for the lack of long bone growth observed in MT1-MMP–deficient mice. Longitudinal bone growth involves two distinct but tightly coordinated ossification processes of the physes, both of which are equally important for growth of long bone. One is the endochondral ossification at the growth plate, and the other is the intramembranous bone formation at the perichondrium. Sections of epiphyses of growing wild-type mice clearly demonstrated a zone of direct transition between unmineralized cartilage and the leading edge of periosteal (perichondral) ossification. The zone of unmineralized cartilage merging with the growing periosteum was located outside of the zone of chondrocyte proliferation of the growth plate and is known as the groove of Ranvier (Ranvier, 1873; Shapiro et al., 1977; Fig. 8 A, small black arrows). Proteoglycan depletion, cessation of type II collagen expression (Fig. 8 B), lack of type X collagen expression (not depicted), highly focused MT1-MMP expression (Fig. 8 D), and chondrocyte apoptosis (Fig. 8 G) were prominent features of this transition zone. Analysis of the same region in MT1-MMP–deficient mice revealed a much-enlarged area of cartilaginous tissue where the basophilia was lost and many chondrocyte lacunae appeared empty (ghost cartilage; Fig. 8 H, black arrows). The size of this ghost cartilage increased with age (Fig. 8, J and K), and at 60 d large portions of devitalized, unmineral-

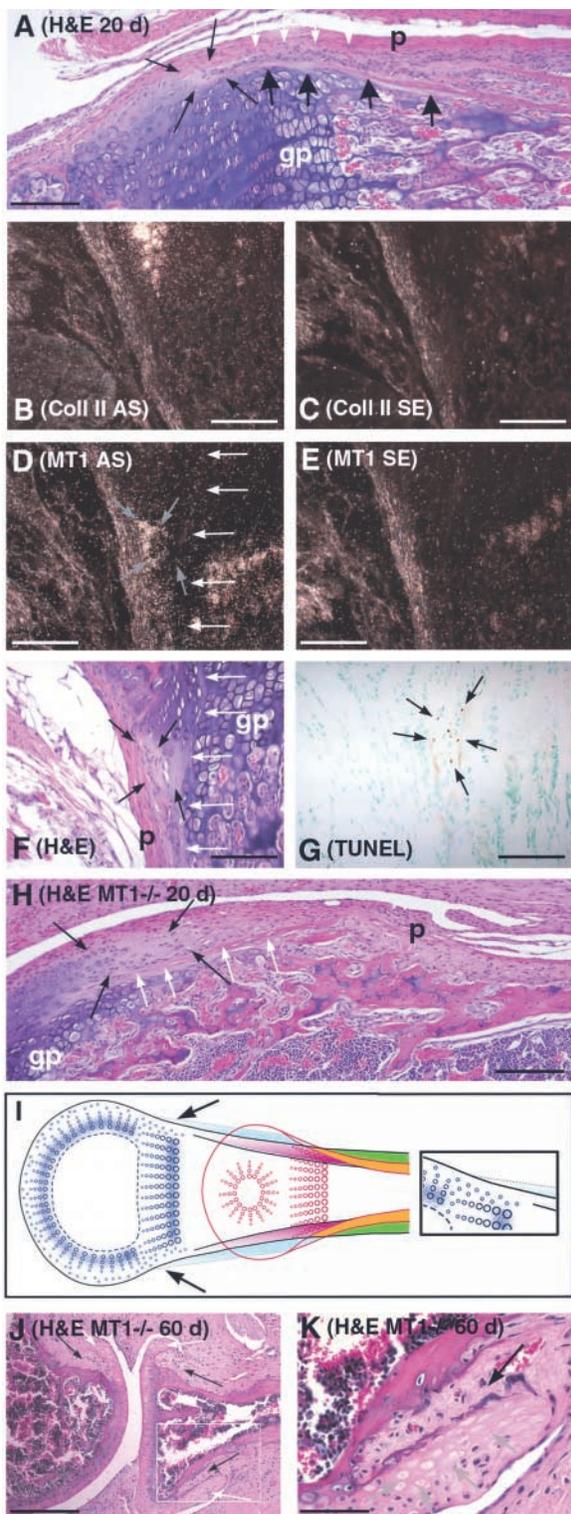
ized articular cartilage were located at the lateral edges of the joint surfaces. Tidemarks (i.e., lines separating unmineralized articular cartilage from deeper-lying mineralized articular cartilage) could be traced from articular surfaces into the ghost cartilages in which they demarcated the unresorbed, unmineralized matrix from the underlying, actively resorbed, mineralized articular cartilage (Fig. 8 K, black arrow). We concluded that growth of cortical bone requires an MT1-MMP–dependent program for remodeling of unmineralized epiphyseal cartilage, along with endochondral ossification of the growth plate. As at other skeletal sites, this program involves apoptosis of nonhypertrophic chondrocytes and spatially restricted, MT1-MMP–dependent matrix degradation. Impaired remodeling of unmineralized cartilage into cortical bone in MT1-MMP–deficient mice results in the retention of unmineralized portions of articular cartilages normally destined for MT1-MMP–dependent resorption during growth.

### Remodeling of connective tissues and cell fate

We found that extensive MT1-MMP–dependent remodeling of unmineralized cartilage into other connective tissues involves apoptotic demise of resident cells. Whether all cells in the cartilage destined for remodeling undergo apoptosis, or some survive and acquire the phenotype of the definitive tissue in that location, remains a pertinent question in the context of our findings. The cartilage ghosts and individual chondrocytes trapped therein are frozen in time and retain their matrix in MT1-MMP–deficient mice. This provides the unique opportunity to ask whether any of the chondrocytes immobilized in the undegraded calvarial cartilage were destined for a fate other than apoptotic demise. BrdU labeling showed that some chondrocytes retained within ghost cartilage did remain capable of DNA synthesis at 33 d (Fig. 9 B); i.e., 23 d later than the time of complete clearance of the corresponding anlage in the wild-type mice. Moreover, direct evidence of mitosis was easily detected, and many chondrocytic lacunae appeared to contain two or more nuclei at 33 d and later time points (Fig. 9, A, C, and D). Additionally, numerous chondrocytes in the ghost cartilage, although remaining silent for type II and type X collagen expression, started to express type I collagen and osteocalcin mRNA (Fig. 9, E and G), which are characteristic of mature bone-forming cells. Furthermore, mineralization of the extracellular matrix was observed in the ghost cartilage at later time points (49 d; Fig. 9 I). This demonstrated that a subset of chondrocytes belonging to transient cartilaginous anlagen escaped apoptotic demise in MT1-MMP–deficient mice, proliferated, and switched to an osteogenic-like phenotype.

### Discussion

Analysis of the phenotype of MT1-MMP–deficient mice has revealed a novel mechanism for cartilage remodeling into other connective tissues that operates in the postnatal skeleton of the mouse. Previously, analysis of the MT1-MMP–deficient mice led us to recognize that membranous bone formation in the calvarium proceeds through transient cartilage models, which are remodeled by a nonendochondral

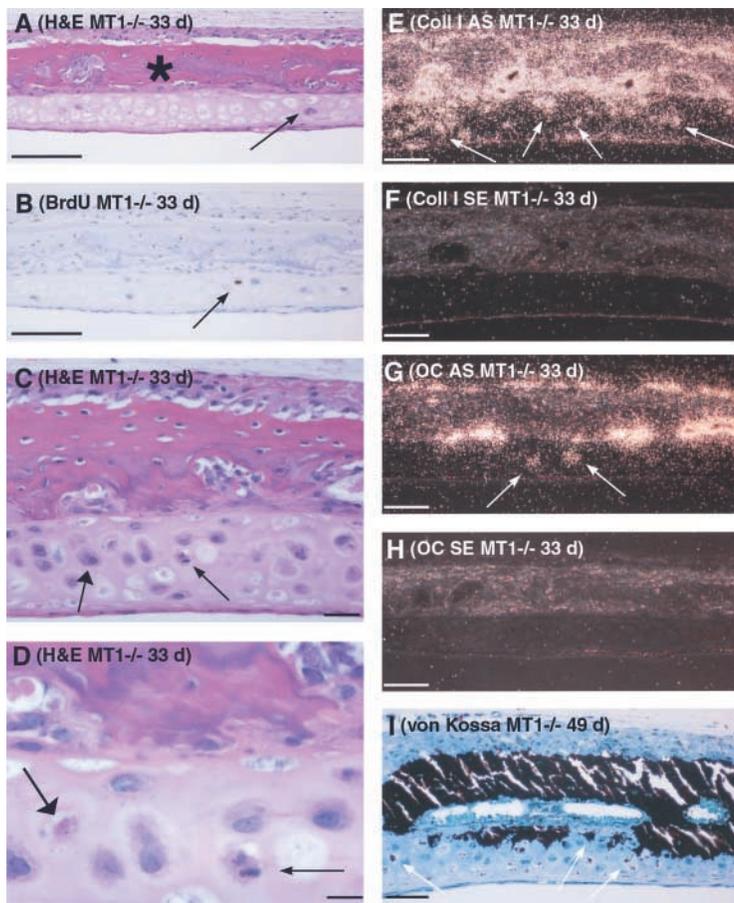


**Figure 8. MT1-MMP and apoptosis-dependent remodeling of cartilage in long bone growth.** (A) Hematoxylin and eosin (H&E)-stained section of femur from a 20-d-old wild-type mouse; small black arrows indicate point of matrix remodeling. Large black arrows indicate boundary of mineralized cartilage; white arrows indicate proliferating osteogenic cells. gp, growth plate; p, periosteum. (B–G) Serial sections from the same animal hybridized with anti-sense probe for type II collagen (B), antisense probe for MT1-MMP (D), sense probe for type II collagen (C), and sense probe for MT1-MMP (E). (F) H&E; (G) TUNEL stain. Coincident loss of proteoglycan (F, arrows), chondrocyte apoptosis (G, arrows), expression of

mechanism. Detailed characterization of this process has now identified a set of highly coordinated events that together define a developmental program distinct from endochondral ossification. In endochondral ossification, chondrocytes mature to hypertrophy (marked by type X collagen expression) and direct the mineralization of the extracellular matrix, which is later removed by osteoclasts. In contrast, the developmental program leading to removal of transient calvarial cartilage anlagen entails spatially restricted expression of MT1-MMP, lack of expression of collagen type X, and cessation of type II collagen expression in chondrocytes. Concurrently, the extracellular matrix is degraded, and the majority of nonhypertrophic chondrocytes undergo apoptosis. This process is blocked in MT1-MMP-deficient mice, highlighting its strict dependence on MT1-MMP. The lack of degradation of the anlagen caused by MT1-MMP deficiency results in the persistence of distinct, devitalized, ghost cartilages, which retain the shape and anatomical position of the original cartilages, but are noted for a wealth of empty chondrocyte lacunae, and a complete loss of proteoglycan content. Chondrocytes retained therein mostly undergo a delayed apoptotic fate, from which some cells escape.

Analysis of the rest of the postnatal skeleton in the MT1-MMP-deficient mice and wild-type littermates allowed us to recognize that the same mechanism of nonendochondral remodeling also operates in MC. The complex development of this branchial arch derivative and the nonendochondral fate of its posterior portion have long attracted attention. However, the mechanisms governing the nonendochondral ossification of the posterior part of the mandible (and its histological details) originating from MC have remained elu-

MT1-MMP (D, gray arrows), and cessation of type II collagen expression are observed at the transition point between unmineralized epiphyseal cartilage and the leading edge of periosteal ossification. (D, F, and H, white arrows) Border with mineralized cartilage. (H) Femur from 20-d-old MT1-MMP-deficient littermate stained with H&E. Note the presence of ghost cartilage at the junction of epiphyseal cartilage and periosteum (black arrows). (I) Schematic representation of endochondral and nonendochondral remodeling during long bone growth adapted from Gray's Anatomy (Warwick and Williams, 1973). In the smaller bone, cortex is shown in orange. During long bone growth, unmineralized cartilage (unshaded small blue circles and inset) is remodeled directly into bone (pink) continuous with existing bone (green). The expanse of the cartilage matrix remodeled directly into bone during growth from a small bone (red) to a larger bone (black) is indicated by dotted lines and turquoise shading. Outside of the growth plate, arrows identify the position of direct remodeling of unmineralized cartilage into an osteogenic tissue at the leading edge of periosteal ossification (regions known as the groove of Ranvier, inset). Nonhypertrophic chondrocytes are depicted as small blue circles. Hypertrophic chondrocytes and mineralized cartilage is depicted as larger circles shaded blue. (J and K) Long-term consequences of MT1-MMP deficiency for cartilage remodeling. Elbow joint of a 60-d-old MT1-MMP-deficient mouse. Both radius and humerus show extensive areas of unremodeled ghost cartilage (white arrows) that are continuous with articular surfaces and undermined by (osteoclastic) resorption of the underlying, mineralized subarticular cartilage (black arrows). (K) Detail of the area boxed in J. Resorption of mineralized subarticular cartilage occurs immediately underneath the tidemark (black arrow), and the unremodeled articular ghost cartilage displays numerous empty chondrocyte lacunae (gray arrows). Bars: (A–H) 100  $\mu$ m; (J) 200  $\mu$ m; (K) 27  $\mu$ m.



**Figure 9. Cell fate in ghost cartilages.** (A–H) Calvarial sections from 33-d-old MT1-MMP-deficient mouse. (A, C, and D) Hematoxylin and eosin (H&E) staining; (B) BrdU labeling. (E) In situ hybridization with antisense probe detecting type I collagen mRNA. (F) Sense probe for type I collagen. (G) In situ hybridization with antisense probe specific for osteocalcin mRNA. (H) Sense probe for osteocalcin. (I) von Kossa-stained calvarial section from a 49-d-old MT1-MMP-deficient mouse. (A) 23 d after the time of complete removal of parietal cartilages in wild-type mice, the skull bone (asterisk) in mutant animals is still lined with cartilage. Some chondrocytes in ghost parietal cartilages (MT1-MMP-deficient mouse) have escaped apoptosis (A, arrow), and remained capable of DNA synthesis as shown by BrdU labeling (B, arrow). Some chondrocytes undergo mitosis (C and D, small arrows), leading to formation of pairs of chondrocytes in a single lacuna (C, large arrow). Other chondrocytes undergo apoptosis (D, large arrow). Besides resuming cell division, chondrocytes immobilized in ghost cartilages turn on the expression of type I collagen mRNA (E, arrows), and osteocalcin, a marker of late osteogenic differentiation (G, arrows). (I) At 49 d, extensive ectopic mineralization of the matrix has appeared in the ghost cartilage of MT1-MMP-deficient mice; some cells, presumably apoptotic, mineralize themselves (I, arrows). Bars: (A and B) 100  $\mu$ m; (C) 25  $\mu$ m; (D) 10  $\mu$ m; (E–I) 50  $\mu$ m.

sive. We have now shown that this process, like the ossification of significant portions of the calvarium, is MT1-MMP dependent, and involves dissolution of the unmineralized matrix and coordinated chondrocyte apoptosis. It must be noted that the posterior portion of MC also gives rise to a major ligamentous structure, the sphenomandibular ligament, and to the malleus. Hence, MT1-MMP-dependent degradation of the caudal portion of MC is related to its replacement by two different connective tissues, namely ligament and bone.

A similar mechanism of unmineralized cartilage remodeling operates in the appendicular skeleton. Longitudinal growth of long bones involves not only precisely coordinated growth and differentiation of chondrocytes in the growth plate and remodeling of metaphyseal mineralized cartilage into bone but also involves the coordinated remodeling of unmineralized cartilage into cortical bone. Likewise, growth of joint structures necessitates the simultaneous remodeling of ligament insertion sites and of transition points from articular cartilage and bony structures. Notably, the severe arthropathy that develops in MT1-MMP-deficient mice with age specifically affects these transition points, where severe scarring, excess osteoclasia, and persistence of dead cartilage are observed (Holmbeck et al., 1999). The MT1-MMP-deficient mouse phenotype highlights the physiological significance of MT1-MMP-dependent processes in multiple sites. More importantly, however, the present data establish that the same physiological mechanism of tissue remodeling op-

erates in the remodeling of calvarium, MC, and limb bone, where it ensures harmonic growth and structural integrity.

Timely apoptosis of hypertrophic chondrocytes during endochondral bone formation is an integral and essential part of the endochondral ossification program (Vu et al., 1998). Our data demonstrate that apoptosis of nonhypertrophic chondrocytes is also essential for the novel cartilage remodeling and developmental program we have described here. It is of note that this process involves neither mineralization nor osteoclastic removal of cartilage matrix, in sharp contrast to apoptosis of hypertrophic chondrocytes in the growth plate. Instead, it does involve timely degradation of unmineralized cartilage scaffold. In MT1-MMP-deficient mice, where this process is blocked, apoptosis of chondrocytes in the ghost cartilages is significantly delayed. A related observation has been made in endochondral ossification in the growth plate. Inhibition of angiogenesis results in delayed osteoclastic removal of the matrix surrounding the hypertrophic chondrocytes and, at the same time, in delayed apoptosis of these cells (Gerber et al., 1999). Based on these observations, it is possible that survival of chondrocytes, either hypertrophic or nonhypertrophic, is dependent on integrity of the surrounding matrix. Conversely, chondrocyte apoptosis might be triggered by degradation of the mineralized extracellular matrix by osteoclasts in hypertrophic cartilage, and by MT1-MMP-mediated degradation of unmineralized matrix in the nonhypertrophic cartilage. Anoikis, the variant of apoptosis induced by loss of matrix anchorage

in epithelial cell types (Frisch and Sreaton, 2001), may thus be operating in cartilage and other connective tissues as well.

In general terms, regression of a temporary rudiment and its replacement by a definitive organ in nonmammalian species fits the definition of metamorphosis (Tata, 1993). Metamorphosis in lower vertebrates shares many similarities with developmental events spanning late fetal and early postnatal life in mammals (Tata, 1993). Increasing evidence links MMPs to metamorphosis both in vertebrates (Hanken and Summers, 1988; Berry et al., 1998a,b) and *Drosophila melanogaster*, in which MMP function is indispensable in metamorphosis rather than during larval development (Page-McCaw et al., 2003). Coordinated MMP-mediated matrix degradation and apoptosis are the effectors of intestinal metamorphosis and many other metamorphic events in *Anura* (Damjanovski et al., 1999; Ishizuya-Oka et al., 2000; Jung et al., 2002). While bordering on violating orthodoxy in the use of biological terminology, we propose that the type of tissue remodeling we have identified in the mouse represents a correlate in mammals of metamorphic events in lower species. A direct link between thyroid hormone signaling, MMPs, and metamorphosis in vertebrates is well documented (Shi and Ishizuya-Oka, 2001). Interestingly, some phenotypic traits of MT1-MMP-deficient mice (Holmbeck et al., 1999) mimic human cretinism and hypothyroidism (McLean and Podell, 1995) on the one hand, and some traits of thyroid hormone receptor knockout models on the other hand (Gothe et al., 1999). Likewise, an important role for nuclear receptor signaling in regulation of MT1-MMP and other MMPs has been suggested (Jimenez et al., 2001).

Bone remodeling is frequently considered in the context of skeletal physiology and disease. Here, we seek to highlight the significance of remodeling across different connective tissues, including bone, for skeletal physiology. Not only do developmental events, such as MC differentiation into ligament and bone, depend on efficient remodeling of one connective tissue into another, but also longitudinal bone growth and joint homeostasis demonstrate a requirement for this type of remodeling. One question raised by these observations is whether any degree of single cell phenotypic plasticity is involved in transconnective tissue remodeling. Direct phenotypic conversion of (some) chondrocytes into osteoblast-like cells has been suggested by several investigators (Gentili et al., 1993; Galotto et al., 1994; Riminucci et al., 1998). The persistence of ghost cartilage in MT1-MMP-deficient mice enabled us to investigate the ultimate fate of chondrocytes, which remained immobilized in their embryonic location. We have shown that escape from apoptosis, initiation of cell division, and switch to an osteogenic phenotype does occur in some of these chondrocytes. One might infer that in MT1-MMP-deficient mice, these events provide a "snapshot" of remodeling in motion, which in wild-type animals escapes ordinary histological detection due to the rapid restructuring of the target tissues. In this process, MT1-MMP might be involved in regulating connective tissue cell fate through proteolytic activity, whereby matrix is not only removed but also restructured, to cue resident cells onto their path of either death or differentiation.

In conclusion, apoptotic MT1-MMP-dependent (metamorphic) remodeling of unmineralized cartilage underlies

the normal development and growth of different connective tissues. These tissues include ligaments, as in the case of the sphenomandibular ligament and the cruciate ligaments. This process is essential in bone, as in the case of calvarial bones, periosteal bone at Ranvier's groove, and in the mandible. Together, these findings represent a novel, generalized mechanism of tissue remodeling that is essential for tissue integrity.

## Materials and methods

### Generation of MT1-MMP-deficient mice

All animals used in this study were maintained under protocols approved by the National Institute of Dental and Craniofacial Research (NIDCR) Animal Care and Use Committee. Mice deficient for MT1-MMP were generated as described previously (Holmbeck et al., 1999).

### Tissue processing, in situ hybridization, and cytochemistry

For histological analysis, tissues were harvested from 129 Rej/NIH black Swiss MT1-MMP mutant animals and littermate controls, fixed overnight at RT in 4% formaldehyde in PBS, washed briefly in PBS, decalcified in PBS/0.25 M EDTA at RT, embedded in paraffin, and sectioned at 6  $\mu$ m. Slides were either processed for hematoxylin/eosin staining or hybridized with <sup>32</sup>P-labeled riboprobes transcribed with either T3 or T7 RNA polymerase as described previously (Blavier et al., 2001). MT1-MMP mRNA was detected with a probe corresponding to nucleotides 291–902 of the mouse cDNA (GenBank/EMBL/DBJ accession no. X83536) cloned into pBluescript. Osteocalcin was detected using a probe corresponding to nucleotides 3–337 of the mouse osteocalcin cDNA cloned into pCRII TOPO. Type II collagen was detected using a 1.1-kb cDNA probe (Watanabe and Yamada, 1999). Type I collagen was detected using a 2-kb rat cDNA probe (provided by Y. Yamada, NIDCR, Bethesda, MD), and type X collagen was detected using a probe spanning nucleotides 1854–2491 of the mouse cDNA (Kong et al., 1993). BrdU labeling and detection was performed as described previously (Vu et al., 1998). Cells undergoing apoptosis were detected using the Apoptag in situ kit (Intergen). Additional tissues were embedded undecalcified in glycol methacrylate at a low temperature or in methyl methacrylate. 2–4- $\mu$ m-thick sections were reacted for tartrate resistant acid phosphatase or AP, as described previously (Bianco et al., 1984). Whole-mount stain of crania with alcian blue/alizarin red was performed as described previously (Kaufman, 1995).

### Photomicrograph acquisition

Photomicrographs were captured on a microscope (model Optiphot-2; Nikon) equipped with Plan Apo 2/0.08, 10/0.45, 20/0.75, and 40/0.95 objectives using a camera (model DMC 2; Polaroid) and software (Polaroid), and assembled using Adobe Photoshop 5.0.

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