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# Galectin-3 Is a Downstream Regulator of Matrix Metalloproteinase-9 Function during Endochondral Bone Formation

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**Endochondral bone formation is characterized by the progressive replacement of a cartilage anlagen by bone at the growth plate with a tight balance between the rates of chondrocyte proliferation, differentiation, and cell death. Deficiency of matrix metalloproteinase-9 (MMP-9) leads to an accumulation of late hypertrophic chondrocytes. We found that galectin-3, an in vitro substrate of MMP-9, accumulates in the late hypertrophic chondrocytes and their surrounding extracellular matrix in the expanded hypertrophic cartilage zone. Treatment of wild-type embryonic metatarsals in culture with full-length galectin-3, but not galectin-3 cleaved by MMP-9, mimicked the embryonic phenotype of *Mmp-9* null mice, with an increased hypertrophic zone and decreased osteoclast recruitment. These results indicate that extracellular galectin-3 could be an endogenous substrate of MMP-9 that acts downstream to regulate hypertrophic chondrocyte death and osteoclast recruitment during endochondral bone formation. Thus, the disruption of growth plate homeostasis in *Mmp-9* null mice links galectin-3 and MMP-9 in the regulation of the clearance of late chondrocytes through regulation of their terminal differentiation.**

## INTRODUCTION

Normal skeletal development depends on the tightly regulated differentiation of chondrocytes and strict coordination between synthesis and degradation of the extracellular matrix (ECM). Most bones in the vertebrate skeleton form by the process of endochondral ossification, in which a cartilage template is replaced by bone (for review see Karsenty and Wagner, 2002; Kronenberg, 2003). This process occurs separately at two different sites in long bones, with the primary site arising in the center of the shaft (diaphysis) and the secondary sites in the ends of the bones (epiphyses). Diaphyseal differentiation of chondrocytes leads to the formation of two symmetrical structures, the growth plates, composed of columns of differentiating chondrocytes, which progress gradually from the proliferating to the hypertrophic state. These growth plates maintain their size and overall structure throughout long bone growth and are pushed apart while

mineralized bone is formed between them, causing overall bone elongation. This developmental process depends on the integration of cartilage matrix remodeling, vascular invasion of hypertrophic cartilage and trabecular bone formation. Differentiation of chondrocytes is accompanied by changes in expression of ECM macromolecules in the growth plate. Chondrocytes express collagen type II (*Col2*) during proliferation and maturation, but primarily express collagen type X (*Col10*) when they differentiate to the hypertrophic stage. The specialized matrix surrounding the hypertrophic chondrocytes (HC) becomes calcified; HC then undergo programmed cell death and the resulting empty lacunae are invaded by capillaries. Concomitantly, the cartilage matrix is partially degraded and osteoblasts colonize this template and synthesize trabecular bone (Karsenty and Wagner, 2002; Kronenberg, 2003).

Multiple signaling molecules regulate proliferation and maturation of chondrocytes. These include Indian hedgehog (Ihh), parathyroid hormone (PTH), and parathyroid hormone-related protein (PTHrP) and their receptors fibroblast growth factor (FGF) FGF18 and its receptor FGFR3, vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF), bone morphogenetic proteins (BMPs), and Wnt proteins (reviewed in Karsenty and Wagner, 2002; Kronenberg, 2003). In addition, the transcription factors Sox9 and Runx2/Cbfa1 play major roles in this process.

In contrast, much less is known concerning the late differentiation of HC and their terminal differentiation or programmed cell death. The protease inhibitor cystatin 10 (Koshizuka *et al.*, 2003) and the transcription factor c-maf (MacLean *et al.*, 2003) have been shown to play a role in the last steps of chondrocyte differentiation. Particularly compelling evidence has been obtained from transgenic mice

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Abbreviations used: Col2, collagen type II; Col10, collagen type X; CTGF, connective tissue growth factor; ECM, extracellular matrix; HC, hypertrophic chondrocyte(s); MMP, matrix metalloproteinase; PECAM, platelet endothelial cell adhesion molecule; TRAP, tartrate-resistant acidic phosphatase; TG2, tissue transglutaminase; MT1-MMP, membrane type 1 matrix metalloproteinase; VEGF, vascular endothelial growth factor.

deficient for matrix metalloproteinases (MMPs). In *Mmp-9* null mice, HC express *Col10* and differentiate relatively normally as matrix mineralization occurs, but their disappearance is delayed and the hypertrophic area expands dramatically during the rapid growth phase of postnatal skeletal development in these animals (Vu *et al.*, 1998). Deficiency in MMP-9 decreases the rate of HC programmed cell death, trabecular bone formation, and initial vascular recruitment (Vu *et al.*, 1998). MMP-13 (also called collagenase-3) also regulates the late differentiation of HC (Stickens *et al.*, 2004; Wu *et al.*, 2002).

Galectin-3 is expressed in skeletal tissues, including notochord, developing cartilage and bone (Fowles *et al.*, 1995) and osteoblasts (Aubin *et al.*, 1996). It is an excellent substrate of MMP-9 *in vitro* (Ochieng *et al.*, 1994). Galectin-3, which was first identified as macrophage marker 2 protein (Ho and Springer, 1982), belongs to the protein family of  $\beta$ -galactoside-specific lectins. It lacks a signal sequence and, as such, is present intracellularly, but is also secreted by a nonclassical pathway (Sato *et al.*, 1993). Depending on cell type it can be localized in ECM, on the cell surface, in the cytoplasm or nucleus (Barondes *et al.*, 1994). Multiple studies have underlined the extracellular functions of galectin-3 and assigned it as a new matricellular protein (for review, see Ochieng *et al.*, 2004). Galectin-3 has been implicated in a variety of cellular functions, notably, differentiation, RNA splicing and cell adhesion, depending on its subcellular localization. These functions depend on interaction of galectin-3 with various ligands, including glycosylated ECM molecules such as laminin or fibronectin and hensin advanced glycation end products (AGE), integrins, and intracellular proteins such as Bcl-2 (Yang *et al.*, 1996). Expression of galectin-3 is under the control of the transcription factor Runx2/Cbfa1 (Stock *et al.*, 2003), a key regulator of osteoblast differentiation and chondrocyte maturation. Interestingly, *Galectin-3* null mice exhibit a growth plate phenotype with precocious HC apoptosis uncoupled from the other features of endochondral ossification, namely ECM degradation, vascular invasion and trabecular bone formation (Colnot *et al.*, 2001).

In this study we took advantage of *Mmp-9* null mice to evaluate the mechanisms regulating terminal differentiation of HC, during the critical phase of rapid bone growth in young mice. We have identified galectin-3 as a physiologically relevant endogenous substrate of MMP-9 and a downstream regulator required during endochondral ossification.

## MATERIALS AND METHODS

### Animal Studies

The generation of *Mmp-9* null mice has been described previously (Vu *et al.*, 1998). All the animals used in this study were maintained under protocols approved by the University of California San Francisco Animal Care and Facilities Committee.

### Histological Analysis and Immunohistochemistry

For histological analysis, metatarsals were dissected from 2-wk-old mutant animals and littermate controls; embryonic humeri were dissected from mutant animals and littermate controls at embryonic day (E)15.0 and E16.0. Tissues were fixed in 4% paraformaldehyde at 4°C overnight. The tissues were then washed in phosphate-buffered saline (PBS) and decalcified in 0.5 M EDTA (pH 7.4) for 7 d (2-wk-old metatarsals) or 1 d (E16.0 humeri) at 4°C before processing and embedding in paraffin.

For semithin sections, samples were fixed, decalcified, dehydrated, and embedded in Embed 812 (Electron Microscopy Sciences, Fort Washington, PA). Semithin sections (1  $\mu$ m) were stained with Toluidine blue (Sigma, St. Louis, MO).

Paraffin sections (5  $\mu$ m) were stained with Safranin O and Fast Green or Alcian blue and Nuclear fast red (Sigma). For TRAP staining, sections were deparaffinized and rehydrated and stained using a leukocyte acid phosphatase kit and Fast Red Violet as a substrate (Sigma) at 37°C for 1 h. The sections were then washed in distilled water and counterstained with Methyl Green (Sigma). Mononucleated and multinucleated TRAP-positive cells were counted on a minimum of six serial sections chosen among the most median part of six different metatarsals for WT and *Mmp-9* null animals at 40 $\times$  magnification and expressed as total number of TRAP-positive cells along the chondro-osseous junction.

For galectin-3 immunostaining, sections were deparaffinized and washed in PBS; endogenous peroxidase activity was quenched in 0.3% methanol in H<sub>2</sub>O<sub>2</sub> for 30 min and washed in PBS, and then sections were heated in citrate buffer before the initial blocking step done in PBS + 3% bovine serum albumin (BSA) for 30 min. Sections were washed in PBS and blocked in PBS + 5% goat serum for 1 h. The sections were then incubated with a rat monoclonal anti-mouse galectin-3 antibody described previously (Colnot *et al.*, 1999) and diluted in PBS containing 1 mg/ml BSA at 4°C overnight. After primary antibody incubation, the slides were washed in PBS, blocked again in PBS plus 5% goat serum for 30 min, and then incubated with a biotinylated goat anti-rat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 in PBS for 1 h, washed, and then incubated with Vector Elite ABC reagent (Vector Laboratory, Burlingame, CA) for 1 h, washed, developed with DAB substrate, and counterstained with Methyl Green (Sigma). Galectin-3-positive cells were counted on a minimum of six serial sections chosen among the most median part of six different metatarsals for wild-type (WT) and *Mmp-9* null animals at 40 $\times$  magnification and expressed as total number of galectin-3-positive cells along chondro-osseous junction or percentage of galectin-3-positive HC.

For PECAM immunostaining, sections were deparaffinized and washed in PBS; endogenous peroxidase activity was quenched in 0.3% methanol in H<sub>2</sub>O<sub>2</sub> for 30 min and washed in PBS, and then sections were treated with Ficin (Zymed Laboratories, South San Francisco, CA) for 15 min at ambient temperature. Blocking treatment was done as described, and the sections were then incubated with a rat monoclonal anti-mouse CD31(PECAM) antibody (clone MEC 13.3, PharMingen, San Diego, CA) at dilution 1:100 in PBS + 1 mg/ml BSA at 4°C overnight. Secondary antibody incubation and revelation were done as described above.

### In Situ Hybridization

Plasmids were linearized with the appropriate restriction enzymes to transcribe either sense or antisense <sup>35</sup>S-labeled riboprobes (*Col2* and *Col10*: Albrecht *et al.*, 1997; *Mmp9*, *Mmp13*, *Mit1-Mmp*, and *Op*: Colnot and Helms, 2001; and *Gal3* probes Colnot *et al.*, 2001) were described previously.

Slides were deparaffinized, treated with proteinase K (20  $\mu$ g/ml) for 5 min at ambient temperature, and hybridized with <sup>35</sup>S-labeled antisense riboprobes in hybridization buffer (50% deionized formamide, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5 mg/ml yeast tRNA, 10% dextran sulfate, and 1 $\times$  Denhardt's) in a humidified chamber at 55°C for embryonic tissues and at 45°C for postnatal tissues, overnight. After hybridization, slides were treated with RNase A, washed to a final stringency of 50% formamide, 2 $\times$  SSC at 60 or 50°C, dipped in emulsion, exposed for 1–2 wk, developed, and counterstained with DAPI (Colnot *et al.*, 2003; Ferguson *et al.*, 1999).

### Protein Extraction and Western Blotting

Hypertrophic cartilage area of growth plates were dissected from *Mmp-9* null animals and littermate controls from 2-wk-old mice and frozen immediately in liquid nitrogen. Tissues were reduced to powder under nitrogen using a very small pestle and mortar and then tissues were homogenized using an Ultra Turrax homogenizer. Total proteins were extracted in RIPA buffer (20 mM Tris, pH 7.2, 10 mM EDTA, 0.3 M NaCl, 0.1% Triton X-100, 0.05% Tween-20) with protease inhibitors (5  $\mu$ g/ml aprotinin, benzamide, 5  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml pepstatin; Sigma) overnight at 4°C. After centrifugation extracts were quantified by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). For protein profile analysis, 5  $\mu$ g of total protein (corresponding approximately to the equivalent of proteins extracted from 2 growth plates) was loaded per lane on 4–10% gradient gel run in 4-morpholinopropanesulphonic acid (MOPS) running buffer (Novex Invitrogen, Carlsbad, CA) under reducing conditions; the gels were fixed and stained with a Fast Silver kit (Geno Technology, St. Louis, MO).

For Western blotting analysis 40  $\mu$ g total protein was loaded per lane on 10% Bis-Tris gel run in MOPS running buffer under reducing (for galectin-3, CTGF, transglutaminase or collagen type II) or nonreducing (for VEGF) conditions. After transfer to PVDF membrane (Bio-Rad, Hercules, CA), blotting was performed with a goat polyclonal anti-mouse VEGF antibody that preferentially recognizes VEGF dimers (R&D Systems, Minneapolis, MN), a mouse monoclonal anti-galectin-3 antibody clone A3/A12 (Research Diagnostics, Flanders, NJ), a rabbit polyclonal anti-transglutaminase II antibody or a mouse monoclonal anti-collagen type II antibody (NeoMarkers, Fremont, CA), and a rabbit polyclonal anti-CTGF antibody (Torrey Pines Biolabs, San Diego, CA) diluted according to the manufacturer's recommendations. After incubation with horseradish peroxidase-conjugated secondary antibody, goat polyclonal anti-rabbit, sheep polyclonal anti-mouse (Amersham Biosciences, Buckinghamshire, England), rabbit polyclonal anti-goat (Sigma) diluted 1/10,000, blots were developed with ECL (Amersham Biosciences,

Buckinghamshire, England). Equal loading was verified on a gel by Coomassie blue staining.

### Embryonic Metatarsal Cultures

Metatarsals from hind limbs of E16.5 mice were dissected and cultured as described previously (Blavier and Delaisse, 1995). In brief, the three middle metatarsals from each hind limb of E16.5 mice were carefully dissected and cultured for 4 d on Millicell culture plate inserts in 700  $\mu$ l BGJb medium (Life Technologies Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum. Recombinant human galectin-3 was purified as previously described (Massa *et al.*, 1993) by bacterial expression and lactose affinity chromatography followed by dialysis against PBS with 1 mM EDTA. Cleaved galectin-3 was obtained after digestion with active MMP-9 as described previously (Ochieng *et al.*, 1994) and digestion was verified by running samples on 12% SDS polyacrylamide gels and silver-stained. Cleaved or uncleaved proteins were added to a final concentration of 0.1  $\mu$ M. Medium was changed daily. After 4 d of culture, metatarsals were fixed and processed for paraffin sections. Safranin O and TRAP staining were done as described above. Paraffin sections stained with Safranin O were photographed under microscope at a magnification of 10 $\times$  and size of the growth plate was measured on a minimum of three serial sections chosen among the most median part of several different metatarsals for WT and mutant animals as indicated.

### Statistical Analysis

The quantitative data were compared between the means of two groups using unpaired Student's *t* tests. Results are expressed as mean plus or minus SEM and significant differences are indicated as *P* values. GraphPad calculator was used to perform the tests.

### Image Acquisition

Photomicrographs were acquired with a microscope Leica DMR (Deerfield, IL) equipped with HC Plan Fluotar 5/0.15; 10/0.30; 20/0.50 or Plan Apo 40/0.75; 100/1.40 coupled to a Leica DC500 digital camera using Leica firecam 1.2.0 software. Images were assembled using Adobe Photoshop software version 6.0.1 (San Jose, CA). In situ hybridization images were taken under dark-field and image analysis was performed as previously described (Ferguson *et al.*, 1999).

## RESULTS

### Absence of MMP-9 Causes an Expanded Zone of Late Differentiated HC

The program of differentiation from proliferating and maturing chondrocytes to HC is characterized by expression of *Col10* mRNA (Chan and Jacenko, 1998). As HC further differentiate into late HC, they express *Mmp-13* and *Osteopontin* (*Op*; Inada *et al.*, 1999; D'Angelo *et al.*, 2000), and their surrounding matrix undergoes mineralization. To investigate the molecular defects leading to the expansion of the HC zone in *Mmp-9* null mice, we characterized the differentiation state of the HC in the expanded hypertrophic cartilage zone by analyzing the expression of HC markers by in situ hybridization on sections from metatarsals of 2-wk-old mice. The zone of *Col10* expression dramatically expanded in *Mmp-9* null compared with WT mice, whereas *Col2* expression was unchanged (Figure 1A), indicating that differentiated HC accumulate in the growth plates of *Mmp-9* null mice. Moreover, the expanded zones of expression of *Mmp-13* and *Op* showed that late HC persist. In *Mmp-9* null mice several rows of HC just above the chondro-osseous junction expressed *Mmp-13* and *Op* (Figure 1B, c–f); in WT mice these late markers were detected in very few cells in the last rows because of a rapid exit of these chondrocytes from the growth plate (Figure 1B, a and b). These observations indicate that, although chondrocytes can undergo late hypertrophy in *Mmp-9* null mice, late HC accumulate, due to a defect in their programmed cell death and their rate of exit from the growth plate.

To obtain a more detailed view of the organization of the cells at the chondro-osseous junction, we stained semithin tissue sections from growth plates embedded in plastic with Toluidine blue. In WT mice the chondro-osseous junction was characterized by a row of empty lacunae, whereas in

*Mmp-9* null mice very few lacunae were empty and the HC appeared very enlarged and fully expanded in the lacunae (Figure 2, A and B). Moreover, mononuclear cells and capillaries were distant from the last intact transverse septa in WT, but were stacked directly under the last row of HC in *Mmp-9* null mice (Figure 2B, asterisk). Alcian blue staining indicated a decrease in proteoglycan content at the chondro-osseous junction concomitant with a decrease in trabecular bone in *Mmp-9* null mice (Figure 2, C and D). Capillaries exhibited relatively normal morphology and platelet endothelial cell adhesion molecule (PECAM) staining showed no major differences in vascular recruitment at the chondro-osseous junction between *Mmp-9* null and WT mice (Figure 2, E and F).

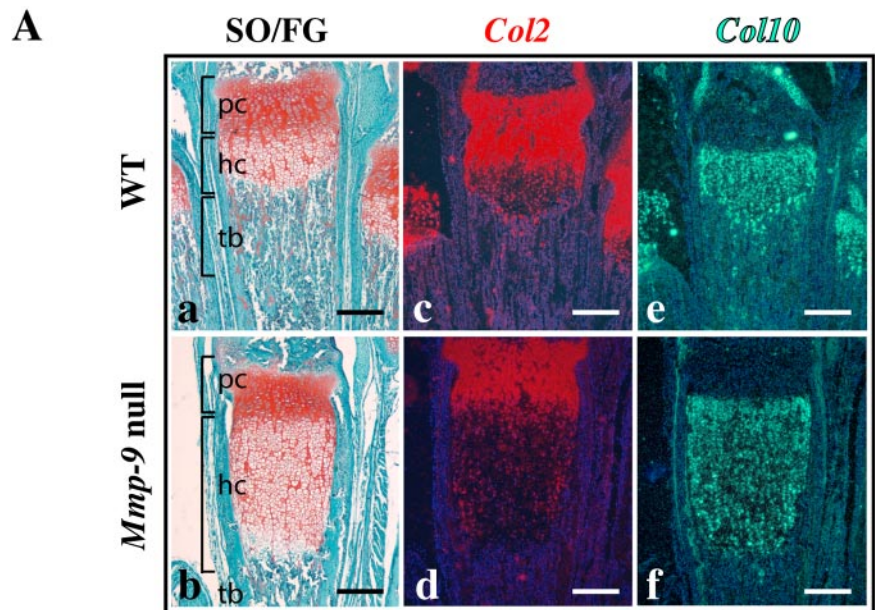
In contrast, the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts increased from  $3.3 \pm 0.5$  cells/section in WT mice to  $10.5 \pm 1.5$  cells/section at the chondro-osseous junction of *Mmp-9* null mice ( $p \leq 0.005$ ;  $n = 6$ ; Figure 2, G–I). Multinucleated cells in this region also showed a modest difference, with an average of  $6.4 \pm 0.5$  cells/section in *Mmp-9* null mice versus  $2.6 \pm 0.4$  cells/section in WT mice ( $p \leq 0.005$ ;  $n = 6$ ). These results suggest increased recruitment of mononucleated osteoclast precursors and multinucleated osteoclasts in *Mmp-9* mice.

Taken together, these data indicate that the extended hypertrophic zone in *Mmp-9* null mice at this specific developmental stage results from the accumulation of both HC and late HC. Capillaries are present at the ossification front, but their progression into the hypertrophic cartilage could be delayed. Moreover, the defective disappearance of HC, likely due to a defect in their programmed cell death, is coupled with an increase in osteoclast precursors recruited at the chondro-osseous junction.

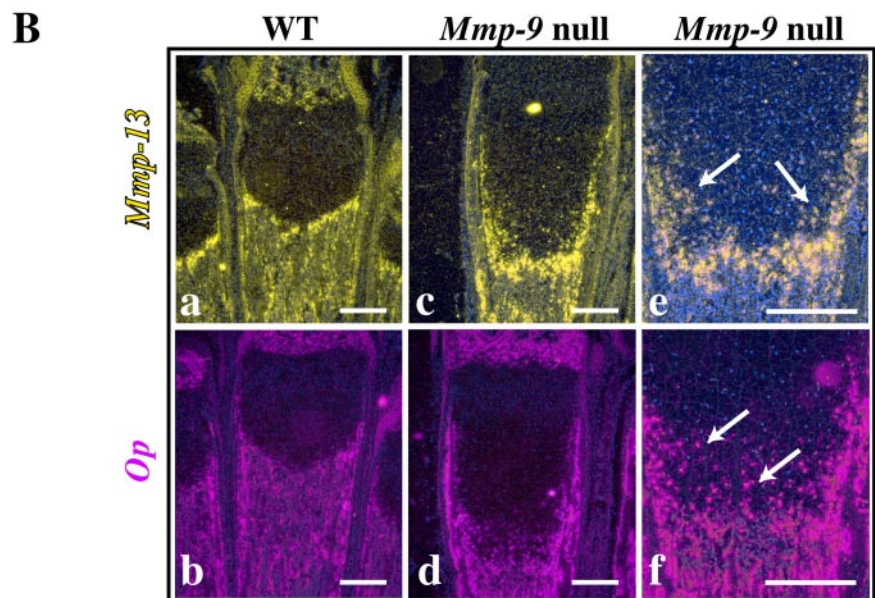
### Protein Profile Analyses of Hypertrophic Cartilage Reveals Galectin-3 as an In Vivo Target of MMP-9

To gain an understanding of the molecular basis for the dysregulated hypertrophic differentiation in *Mmp-9* null mice, we compared profiles of proteins extracted from HC zones (including the primary ossification front) of WT and *Mmp-9* null HC by SDS-PAGE. We obtained reproducible patterns showing striking differences between genotypes. Several proteins were present at higher levels in *Mmp-9* null mice (Figure 3A). Although some proteins were down-regulated with deficiency of MMP-9, we focused on the up-regulated ones, because we were most particularly interested in elucidating the in vivo substrates of MMP-9. To identify these proteins we screened several specific candidates involved in HC differentiation and endochondral bone formation. Western blotting for tissue transglutaminase II (TG2) detected two isoforms, but showed no difference between WT and *Mmp-9* null samples. Similarly, we observed no differences for either connective tissue growth factor (CTGF) or collagen type II  $\alpha 1$  chain (Col2 $\alpha 1$ ; Figure 3B).

Interestingly, VEGF and galectin-3 increased in *Mmp-9* null samples (Figure 3B). For VEGF, both major isoforms, VEGF<sub>164</sub> and VEGF<sub>120</sub>, increased. We were unable to cleave these VEGF forms with MMP-9, suggesting that they are not direct in vivo substrates. In contrast, galectin-3, a known substrate of MMP-9 in vitro (Ochieng *et al.*, 1994), was particularly enriched in our samples. Moreover, we observed more uncleaved monomeric and dimeric forms of galectin-3 in *Mmp-9* null samples compared with WT. The dimeric forms, which were stable to reducing conditions, are likely to be extracellular galectin-3 cross-linked by TG2. Because the antibody used recognizes an epitope in NH<sub>2</sub> domain of galectin-3, which contains a cleavage site for MMP-9, we



**Figure 1.** Accumulation of late terminally differentiated hypertrophic chondrocytes in *Mmp-9* null mice. Serial sections of growth plates from 2-wk-old *MMP-9* null and WT mice. (A) Safranin O/Fast Green (SO/FG) staining (a and b) and in situ hybridization with antisense probes for *collagen type II* (*Col2*; c and d) and *collagen type X* (*Col10*; e and f) show an increase in the hypertrophic chondrocyte zone (hc) but not the proliferating chondrocyte zone (pc) in *MMP-9* null mice compared with WT. (B) In situ hybridization with antisense probes for late hypertrophic markers, *Mmp-13* (a and c) and *Osteopontin* (*Op*; b and d) show the accumulation of late hypertrophic chondrocytes in *MMP-9* null mice. (e and f) Higher magnification of *Mmp-13* and *Op* expression pattern in *MMP-9* null mice. Note that the accumulation of hypertrophic chondrocytes expressing these markers (arrows) in several rows at the chondro-osseous junction. pc, proliferating chondrocytes; hc, hypertrophic chondrocytes; tb, trabecular bone. Scale bars, 250  $\mu$ m.



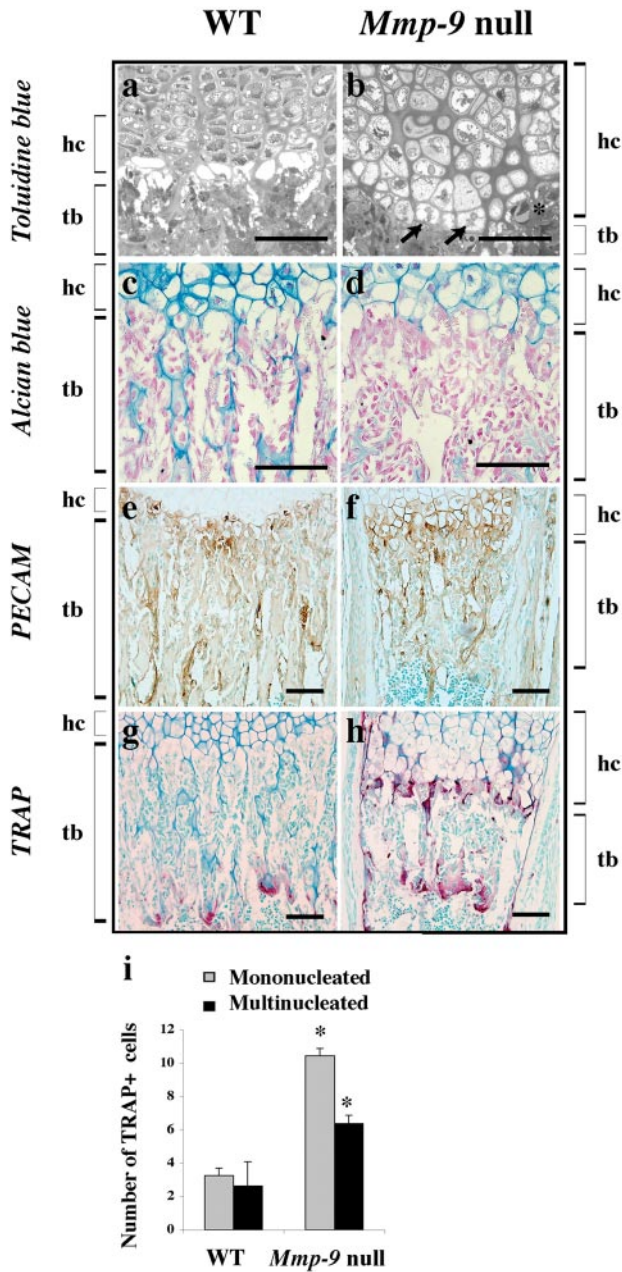
were able to detect only uncleaved galectin-3. These results show that uncleaved galectin-3 accumulates in the HC zone and primary ossification front of mice deficient for MMP-9 and strongly suggest that galectin-3 is a downstream regulator of MMP-9 action in vivo during endochondral ossification.

#### *Galectin-3 Expression Is Disturbed during the Initial Stage of Endochondral Bone Formation in Mmp-9 Null Mice*

The increase in galectin-3 observed in *Mmp-9* null hypertrophic cartilage could be due to an increase in *galectin-3* mRNA expression resulting in increased synthesis or an accumulation in galectin-3 protein due to a decrease in its

degradation. We first analyzed the *galectin-3* mRNA expression pattern in embryonic bones of WT and *Mmp-9* null mice relative to other markers of chondrocyte differentiation by in situ hybridization. At embryonic day 15.0 (E15.0), the majority of WT humeri already had primary ossification centers, whereas no ossification centers were observed in *Mmp-9* null humeri (Figure 4A, a and b). The expression of *Col2* was similar in WT and *Mmp-9* null mice (Figure 4A, e and f); however, the zone of *Col10* expression expanded in *Mmp-9* null embryonic bones, reflecting the expanded HC zone in these animals (Figure 4A, g and h).

*Mmp-13* and *Op* are expressed by hypertrophic chondrocytes and osteoblasts (Tuckermann *et al.*, 2000; McLean *et al.*, 2003) and *Mt1-Mmp* is expressed by osteoclasts (Sato *et al.*,



**Figure 2.** Altered organization of the chondro-osseous junction in *Mmp-9* null mice. Histological analysis of the chondro-osseous junction. (a and b) Representative pictures of semithin sections stained with Toluidine blue showing the abnormal survival of the last row of hypertrophic chondrocyte (arrows) in *Mmp-9* null mice compared with WT. A capillary in close contact with last transverse septa is marked by an asterisk (\*). (c–h) Longitudinal sections through the central part of growth plates. (c and d) Alcian blue staining; note the decrease in staining in *Mmp-9* null mice in the last row of hypertrophic chondrocytes and trabeculae. (e and f) PECAM staining showing no major differences in growth plate vascularization between *Mmp-9* null and WT mice. (g and h) TRAP staining, note the increase in TRAP-positive cells recruited at the chondro-osseous junction in *Mmp-9* null mice. hc, hypertrophic chondrocytes; tb, trabecular bone. Scale bars, 125  $\mu$ m. (i) Quantification of mononuclear and multinucleated TRAP-positive cells recruited at the chondro-osseous junction, showing an increase in *Mmp-9* null mice (n = 6 different metatarsals; \*p  $\leq$  0.005).

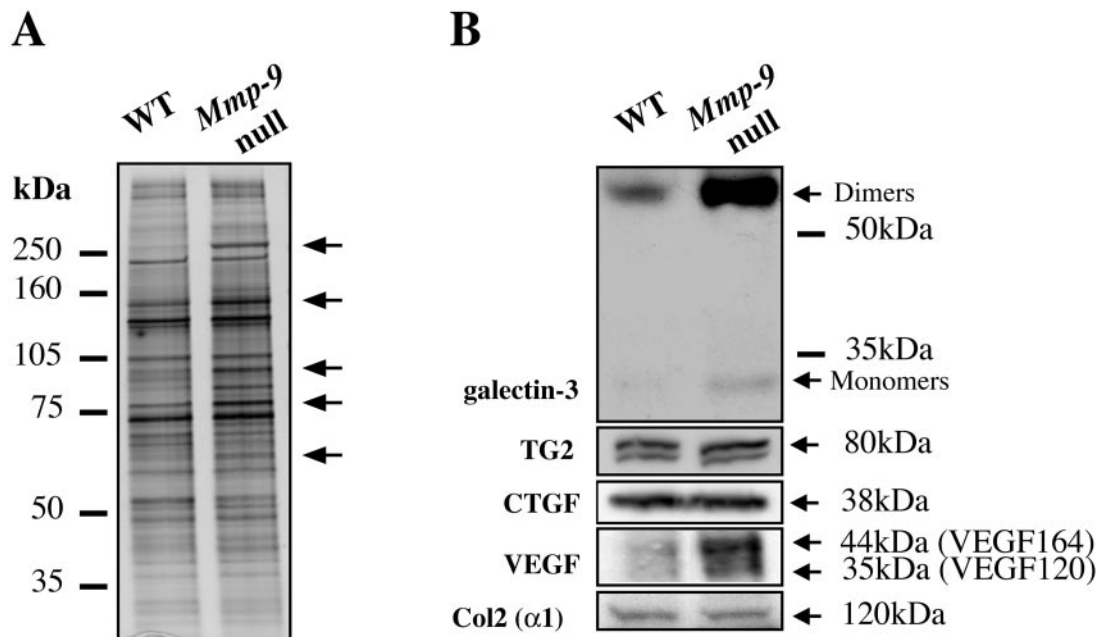
1997). *Mmp-13* and *Op* mRNAs appeared in the center of WT humeri in the most terminal rows of HC and in the primary ossification center where osteoclasts and osteoblasts are recruited (Figure 4A, i and k). In *Mmp-9* null mice, although the *Mmp-13* and *Op* expression domains appeared to be diminished overall because of a delay in the establishment of the ossification center, their expression domains within the hypertrophic cartilage (delimited by a dotted line) was expanded and revealed that the accumulation of late HC begins at an early embryonic stage of endochondral ossification (Figure 4A, j and l).

*Galectin-3* mRNA expression was observed in two distinct zones of the developing bone. It was localized primarily in proliferating and prehypertrophic chondrocytes, and, to a lesser extent, in late HC (Figure 4Ac). A second domain of strong *galectin-3* mRNA expression in the ossification center overlapped with the expression domain of *Mmp-13*, *OP*, and *Mt1-Mmp* (Figure 4A, i, k, and m), most likely in osteoclasts and osteoblasts (Niida *et al.*, 1994; Aubin *et al.*, 1996; Colnot *et al.*, 1999). The expression domain of *galectin-3* was slightly expanded in early HC in *Mmp-9* null embryos, where it overlapped with the *Col10* expression domain (Figure 4Ad). As in WT mice, the signal decreased markedly in late HC. Because formation of the primary ossification center was delayed, preosteoclast recruitment and vascular invasion had not yet occurred in *Mmp-9* null humeri. Accordingly, no cells expressing *galectin-3* had migrated into the ossification center, but instead a few *galectin-3*-positive cells were present in the perichondrium (Figure 4Ad). Osteoblasts expressing *Mmp-13* and *Op* were detected only in the perichondrium, as were osteoclasts expressing *Mt1-Mmp* and TRAP (Figure 4A, j, l, n, and p). These results show a delay in the recruitment of osteoclasts and osteoblasts from the perichondrium to the primary ossification center. This delay in recruitment paralleled the delay in the initial removal of late hypertrophic chondrocytes in *Mmp-9* null mice at E15.0.

At E16.0, when vascular invasion was well underway in WT embryonic bones, *Mmp-9* mRNA was highly expressed in cells located at the primary ossification front, as is typical of *Mmp-9* expression during endochondral bone formation from embryonic stages through adulthood. The *Mmp-9* expression pattern partially overlapped with the localization of *galectin-3* at the front of ossification and coexpression of these genes was detected in a limited cell population, most likely comprised of (pre-) osteoclasts (Figure 4B). The remaining *galectin-3*-positive cells, which did not express *Mmp-9*, may represent another population of ECM resorbing cells expressing *Mt1-Mmp* and/or osteoblasts expressing *Mmp-13* and *Op* as suggested by the overlapping expression profiles observed at E15.0 (Figure 4A).

Taken together, these results indicate that the expansion of late HC and the delay in their disappearance observed in *Mmp-9* null mice begins early in bone development. Concomitant with these defects, we observed an expansion of *galectin-3* expression in the upper HC zone and a decrease in the initial recruitment of *galectin-3*-positive cells in the primary ossification center. These observations suggests that *galectin-3* as a downstream regulator of MMP-9 at two steps in endochondral bone formation: HC differentiation, and osteoclast recruitment and differentiation.

**Accumulation of Galectin-3 Protein at the Chondro-osseous Junction of *Mmp-9* Null Mice Overlaps with MMP-9 Localization** At postnatal 2 wk, *galectin-3* mRNA was highly expressed in proliferating and prehypertrophic chondrocytes with an expansion of this expression domain in *Mmp-9* null mice. *Galectin-3* mRNA was strongly decreased in late



**Figure 3.** Accumulation of uncleaved galectin-3 in the growth plate of *Mmp-9* null mice. (A) Protein extracts from WT and *Mmp-9* null hypertrophic chondrocyte zones were analyzed by SDS-PAGE and silver staining. Differences in protein profiles were observed as increases in the intensity of several bands in *Mmp-9* null samples, as indicated by arrows. (B) Protein extracts from WT and *Mmp-9* null hypertrophic chondrocyte zones were analyzed for expression of galectin-3, tissue transglutaminase (TG2), CTGF, VEGF, and  $\alpha 1$  chain of collagen type II (Col2;  $\alpha 1$ ) by Western blotting. Note the increase in intact galectin-3 dimers and VEGF in *Mmp-9* null mice compared with WT samples.

HC, although a few sparsely distributed cells exhibited a higher expression (Figure 5, A and B). An antibody to galectin-3-stained proliferating chondrocytes strongly and HC less intensely in WT growth plates (Figure 5C), as previously described (Colnot *et al.*, 1999). At the chondro-osseous junction, galectin-3 was also detected in mononuclear cells, preosteoclasts, some vascular cells and multinucleated osteoclasts (unpublished data). However, in *Mmp-9* null samples, the majority of HC stained very strongly positive for galectin-3, and the number of positive cells at the chondro-osseous junction also increased (Figure 5D). In *Mmp-9* null mice, galectin-3 protein accumulated in the enlarged late HC in nuclei, along the plasma membrane, extracellularly in the perilacunar space and to a lesser extent weakly in the proximal ECM (Figure 5F). In contrast, in WT mice few late HC exhibited intense nuclear galectin-3 staining, and membrane, perilacunar, and ECM staining were very weak (Figure 5E). Strongly galectin-3-positive HC were only  $20.2 \pm 4.2\%$  of the total HC population in WT mice, versus  $43.5 \pm 5.1\%$  ( $p \leq 0.005$ ;  $n = 6$ ) in *Mmp-9* null mice (Figure 5G).

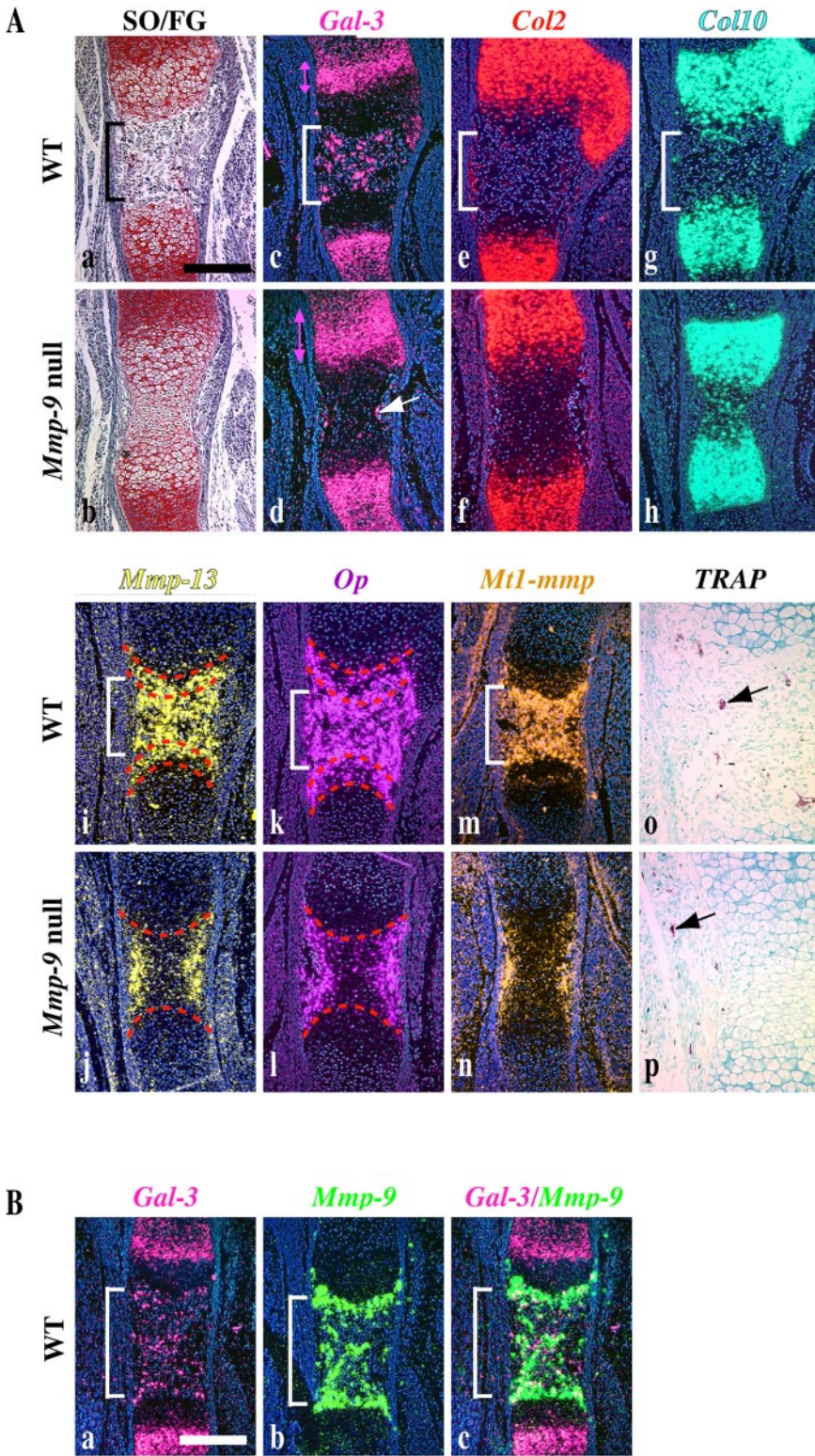
The galectin-3 staining in the lacunae and ECM in *Mmp-9* null mice increased and persisted in the last rows of HC (Figure 5I). In contrast, the numerous empty lacunae observed at the chondro-osseous junction in WT mice had little galectin-3 staining in the surrounding matrix (Figure 5H). At the chondro-osseous junction, the number of recruited galectin-3-positive cells per section increased to  $20.3 \pm 1.0$  in *Mmp-9* null mice compared with  $9.2 \pm 1.3$  ( $p \leq 0.005$ ,  $n = 6$ ) in WT (Figure 5J). Some of these cells were vascular cells, whereas others were preosteoclasts; osteoblasts did not show high expression of galectin-3 (unpublished data).

Thus, the slightly expanded region of *galectin-3* mRNA expression in *Mmp-9* null mice owed to a modest expansion of the prehypertrophic zone. In the absence of MMP-9, galectin-3 protein accumulated in regions where MMP-9 was

located in WT animals, along the last row of HC, their ECM, and the chondro-osseous junction. We did not observe an increase in galectin-3 protein in bone marrow of *Mmp-9* null mice, indicating that this accumulation does not result from a global effect of MMP-9 deficiency on galectin-3 translation. The fact that galectin-3 accumulates in a context of decreased ECM remodeling strongly suggests a decrease in galectin-3 degradation. Therefore, galectin-3 appears to be downstream of MMP-9, most likely as a direct substrate, in regulating the final stages of HC differentiation and also in regulating osteoclastic recruitment and/or survival during endochondral bone formation.

#### Excess Extracellular Galectin-3 Phenocopies Early MMP-9 Deficiency

A feature of the *Mmp-9* null growth plates was the presence of extracellular galectin-3. This raises the question of whether the growth plate phenotype in *Mmp-9* null mice is caused by an overabundance of uncleaved extracellular galectin-3. If this is the case, then addition of excess galectin-3 should delay the initial formation of the growth plate and concomitantly increase the size of the hypertrophic cartilage zone in skeletal elements undergoing endochondral ossification. We tested this hypothesis in organotypic cultures of embryonic metatarsals. We treated metatarsals dissected from E16.5 WT or *Mmp-9* null mice for 4 d with recombinant uncleaved (multimeric) or MMP-9-cleaved galectin-3. In three independent experiments, WT embryonic metatarsals treated with intact galectin-3 mimicked the embryonic *Mmp-9* null phenotype, exhibiting delayed and decreased recruitment of preosteoclasts to the primary ossification center coupled with an increase in the HC zone (Figure 6). This resembles the early growth plate phenotype of metatarsals

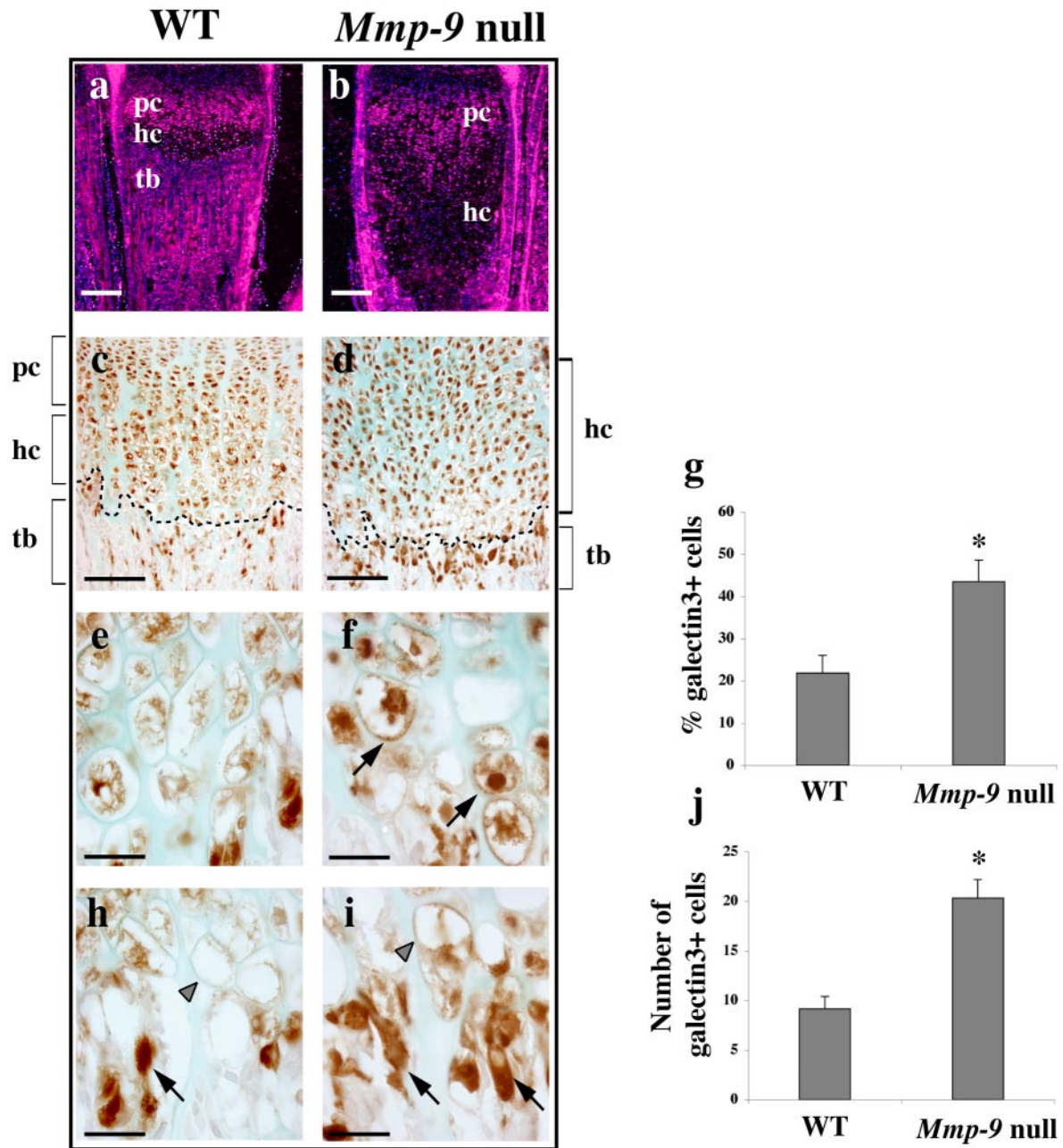


**Figure 4.** Galectin-3 expression is disturbed during the initial stage of endochondral bone formation in *Mmp-9* null mice. (A) Serial longitudinal sections of humeri from E15.0 WT and *Mmp-9* null mice were stained with Safranin O/fast green (SO/FG; a and b) and for TRAP activity (o and p, TRAP-positive cells are indicated by black arrows). Adjacent sections were hybridized with antisense RNA probes for *Galectin-3* (*Gal-3*, fuchsia), *Collagen type 2* (*Col2*, red), *Collagen type 10* (*Col10*, green), *Mmp-13* (yellow), *Osteopontin* (*Op*, purple), or *Mt1-mmp* (orange). Note the increase in *Galectin-3* expression in *Mmp-9* null mice as indicated by double pink arrows. Rare *Galectin-3*-expressing cells are found in the perichondrium of *Mmp-9* null samples (white arrow). Red dotted lines indicate the limits of *Mmp-13* and *Op* expression domains in late HC. (B) Serial longitudinal sections of femurs from E16.0 WT mice were hybridized with antisense RNA probes for *Galectin-3* and *Mmp-9* as indicated. Merging of both signals shows partial overlap between *Galectin-3* and *Mmp-9* expression. Brackets indicate the primary ossification center in wild-type sections. Scale bars, (A) 250  $\mu\text{m}$ ; (B) 500  $\mu\text{m}$ .

from developing embryonic *Mmp-9* null mice in vivo and in culture with decreased vascular invasion and recruitment of TRAP-positive cells and an increased HC zone (Engsig *et al.*, 2000). In contrast, we could not detect any significant phenotypic effects in embryonic metatarsals treated with MMP-9-cleaved galectin-3. *Mmp-9* null embryonic metatarsals,

which already have an endogenous excess of galectin-3, were unaffected by these treatments (unpublished data). Thus, either excess uncleaved multimeric galectin-3, or ablation of *Mmp-9* interferes with two pathways, one that directs late differentiation of HC and a second that controls osteoclast differentiation and migration.



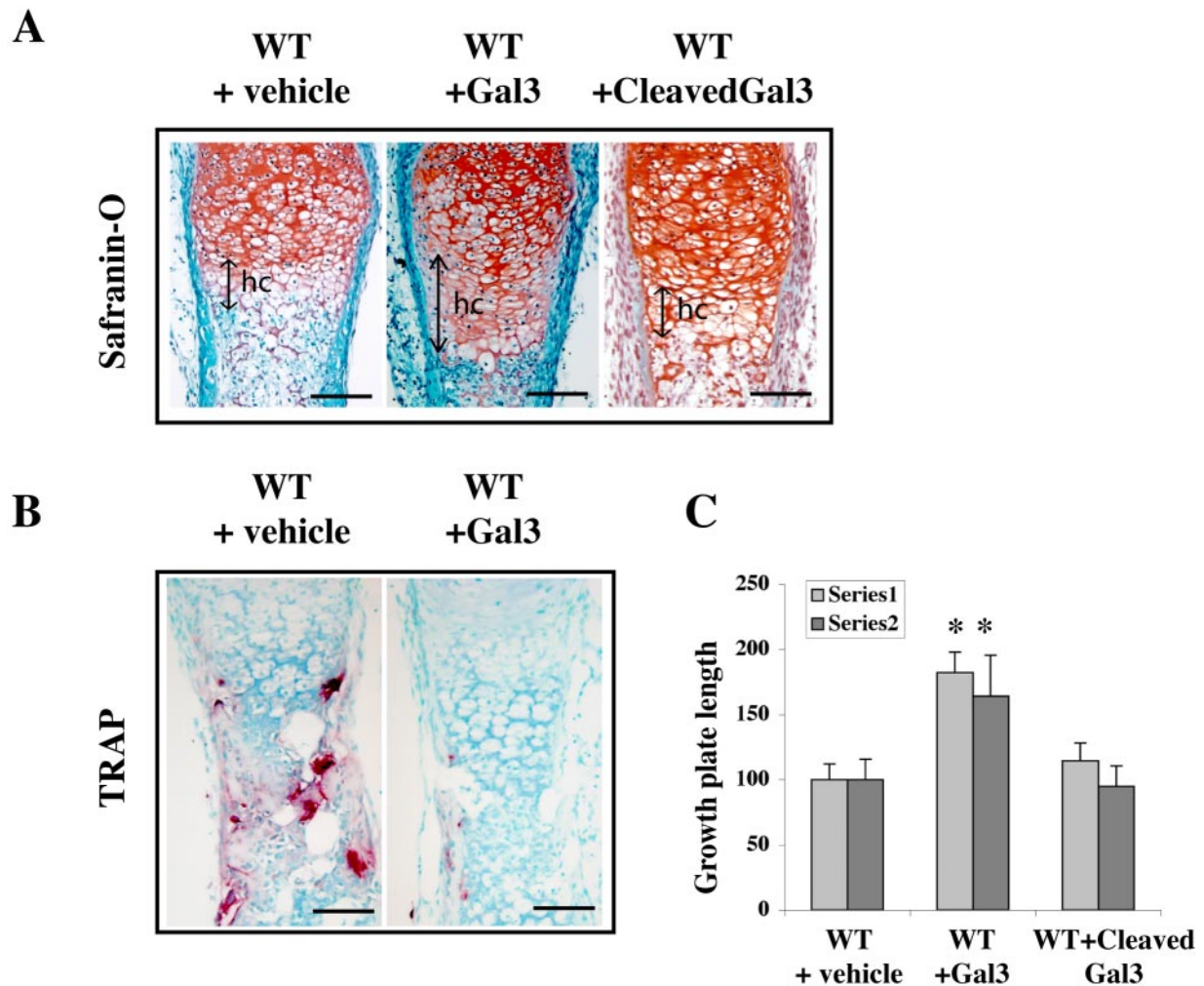


**Figure 5.** Galectin-3 accumulates at the chondro-osseous junction of *Mmp-9* null mice. Paraffin sections of the growth plates of 2-wk-old *Mmp-9* null and WT metatarsals. (a and b) In situ hybridization with antisense RNA probe for *Galectin-3*. (c and d) Immunostaining with monoclonal rat anti-galectin-3. (e and f) Higher magnification of (c and d) showing the increase in galectin-3 protein in hypertrophic chondrocytes (hc) of *Mmp-9* null mice. Black arrows indicate galectin-3 staining in perilacunar space. (h and i) Higher magnification of (c and d) showing the increase in recruitment of galectin-3-positive cells at the chondro-osseous junction (indicated by dotted line) in *Mmp-9* null mice (black arrows) and the increase in galectin-3 staining in the pericellular matrix of the last row of hypertrophic chondrocytes (gray arrowheads). (g) Quantification of hypertrophic chondrocytes exhibiting high levels of galectin-3 immunostaining. Results are expressed as percentage of total hypertrophic chondrocytes counted. (n = 6 different metatarsals; \*p ≤ 0.005). (j) Quantification of galectin-3-positive cells recruited at the chondro-osseous junction expressed as total number of galectin-3-positive cells per section along the ossification front. (n = 6 different metatarsals; \*p ≤ 0.005). pc, proliferating chondrocytes; hc, hypertrophic chondrocytes; tb, trabecular bone. Scale bars, (a and b, 250 μm); (c and d, 150 μm); (e, f, h, and i, 30 μm).

## DISCUSSION

*Mmp-9* deficiency leads to abnormal endochondral bone formation, with decreased rates of HC apoptosis and trabecular bone formation (Vu *et al.*, 1998). These defects are ini-

tially derived from a delay in vascular invasion and osteoclast recruitment at an early stage in development (Engsig *et al.*, 2000). The growth plate of *Mmp-9* null mice is characterized by an extension of the lifespan of late HC, as well as the accumulation of full-length galectin-3, in and around late



**Figure 6.** Ectopic addition of multimeric uncleaved galectin-3 partially mimics MMP-9 deficiency. Sections from E16.5 WT metatarsals cultured for 4 d in presence of vehicle alone, full-length mouse recombinant galectin-3 or MMP-9-cleaved galectin-3 stained with (A) Safranin O or (B) for TRAP activity. (C) Quantification of the increase in growth plate length following galectin-3 treatment. Results are expressed as the percentage of increase compared with untreated WT and the results from two representative independent experiments are shown (series 1,  $n = 5$  for vehicle alone,  $n = 4$  for full length galectin-3 and  $n = 4$  for MMP-9 cleaved galectin-3,  $*p \leq 0.005$ ); (series 2,  $n = 5$  for vehicle alone,  $n = 5$  for full length galectin-3 and  $n = 3$  for MMP-9 cleaved galectin-3,  $*p \leq 0.005$ ).

HC. Ectopic extracellular galectin-3 produces delayed osteoclast recruitment as well as expansion of the hypertrophic cartilage zone due to the delayed removal of HC at the chondro-osseous junction. Our results point to galectin-3 as a downstream regulator of MMP-9 function at two distinct points during endochondral bone formation, differentiation of late HC and control of the recruitment and/or differentiation of TRAP-positive osteoclasts or osteoclast progenitors to the front of ossification.

#### **Mmp-9 Null Mice Show Altered Expression and Extracellular Activities of Galectin-3 during Endochondral Bone Formation**

Galectin-3 is synthesized in proliferating and prehypertrophic chondrocytes, with a clear nuclear and cytoplasmic localization in WT mice. During cartilage differentiation, galectin-3 synthesis decreases gradually until the late hypertrophic stage (Colnot *et al.*, 1999). We report here that the *Mmp-9* null phenotype is characterized by an abnormal accumulation of galectin-3 in and around late HC, even

though there is no increase in mRNA in this particular zone of the growth plate. Galectin-3 also accumulated in and around (pre)osteoclasts

In culture, exogenous intact galectin-3 disrupted normal embryonic bone development, increasing the HC zone and decreasing recruitment and/or differentiation of preosteoclasts. These effects mimic the phenotype previously observed in embryonic bones of *Mmp-9* null mice (Engsig *et al.*, 2000). Galectin-3 cleaved by MMP-9 (monomeric) had no effect in our *in vitro* metatarsal culture experiments. Thus, we hypothesize that the functions of intact galectin-3 may be regulated through inactivation of the protein by proteolytic cleavage during endochondral bone formation on both sides of the chondro-osseous junction, in late HC and preosteoclasts or osteoclasts.

The slight expansion in the region of *galectin-3* expression in proliferating and prehypertrophic zones of the growth plate suggests that MMP-9 may also regulate factors involved in the transition from chondrocyte prehypertrophy to hypertrophy. The expanded *galectin-3* mRNA expression

early in development was conserved in 2-wk-old mice and could have contributed to the increased HC zone in *Mmp-9* null mice during the phase of rapid bone growth through its antiapoptotic effects. However, the expansion of the HC zone in *Mmp-9* null mice does not result from an increase in chondrocyte proliferation or in expression of major differentiation markers such as Col10 (Vu *et al.*, 1998).

### ***Galectin-3 Is a Downstream Regulator of MMP-9 in Hypertrophic Chondrocyte Terminal Differentiation***

Numerous studies have established intracellular galectin-3 as an antiapoptotic factor. Indeed, galectin-3 contains the anti-death domain characteristic of the Bcl-2 protein family (NWGR; Akahani *et al.*, 1997), and cells that over express galectin-3 display increased resistance to apoptotic stimuli (for review see Krzeslak and Lipinski, 2004; Wang *et al.*, 2004). Galectin-3 is the only member of the galectin family that promotes cell survival (Akahani *et al.*, 1997; Matarrese *et al.*, 2000). It is now becoming clear that galectin-3 is an extracellular modulator of cell/ECM interactions that exhibits pro- and antiadhesive properties in different processes such as kidney development, angiogenesis, and metastasis (Nangia-Makker *et al.*, 2000; Bullock *et al.*, 2001; Fukushi *et al.*, 2004; Takenaka *et al.*, 2004). However, the previous studies do not differentiate between intracellular and extracellular functions of galectin-3.

We propose that extracellular galectin-3 negatively regulates terminal differentiation of HC, possibly by acting as an antiapoptotic matricellular protein that maintains ECM anchorage. In support of this hypothesis, exogenous galectin-3 expands the population of HC in cultured embryonic metatarsals. This is further supported by the galectin-3 null-mutant phenotype, where increased programmed cell death is observed at the chondro-vascular junction in the absence of galectin-3 (Colnot *et al.*, 2001). It is possible that ECM remodeling by MMPs, particularly MMP-9 present along the last transverse septa, degrades galectin-3, thereby derepressing terminal differentiation or programmed cell death of HC and allowing endochondral ossification to proceed.

The HC of *Mmp-9* null mice reached late differentiation as evidenced by their expression of *Mmp-13* and *Op*. However, very few HC expressing *Mmp-13* and *Op* were detectable in WT mice because of the rapid exit of these chondrocytes from the growth plate during the first 3 wk of postnatal life, when growth of long bones is maximal. We conclude that the *Mmp-9* null phenotype results from a delay in the disappearance of late HC, characterized by galectin-3 accumulation and is probably due to a defect in the switch to terminal differentiation or programmed cell death.

Because there was no difference in *galectin-3* expression in late HC between WT and *Mmp-9* null mice, we argue that persistence of galectin-3 in late HC results from a defect in its degradation. We cannot exclude that MMP-9 deficiency induces an increase in galectin-3 because of an indirect role in controlling other proteases involved in galectin-3 degradation, but intriguingly, at this locale, galectin-3 and MMP-9 colocalize and the galectin-3 detected is present in *Mmp-9* null mice as intact dimers. Moreover, our immunostaining data support that hypothesis, as strong staining for galectin-3 was seen all around the lacuna in hypertrophic cartilage of *Mmp-9* null mice, where late HC survived and accumulated abnormally. The presence of galectin-3 dimers may promote HC survival through maintenance of a strong adhesion to the ECM. Indeed, *galectin-3* overexpression can protect cells from apoptosis by improving cell adhesion (Matarrese *et al.*, 2000), which is dependent on oligomerization of galectin-3, particularly through TG2 activity (Mehul

*et al.*, 1995; Mahoney *et al.*, 2000). Because TG2 is expressed at identical levels in WT and *Mmp-9* null chondrocytes, we hypothesize that the observed accumulation of galectin-3 dimers does not result from an increase in TG2 activity but more likely from an increase in the half-life of the dimers.

In vitro, galectin-3 is a direct substrate for MMP-9, MMP-2, and MMP-13, which cleave the amino terminal domain of the molecule at a common site (Ochieng *et al.*, 1994; Guevremont *et al.*, 2004). Because MMP-13 is expressed in *Mmp-9* null HC, and *Mmp-13* null mice exhibit only a modest expansion of the HC zone (Stickens *et al.*, 2004), we suggest that MMP-9 is the dominant MMP for cleaving the N-terminal domain of galectin-3 in and around late HC. As this domain allows oligomerization of galectin-3, its integrity may be required for adhesion of HC to the ECM and consequent antiapoptotic effects. Cleavage of this domain by MMP-9 would therefore modify the affinity of galectin-3 for its ligands and promote HC terminal differentiation or programmed cell death. As such, we propose that galectin-3 is a downstream regulator of MMP-9 controlling terminal differentiation of HC at the chondro-osseous junction.

### ***Aberrant Accumulation of Extracellular Galectin-3 Interferes with Recruitment of Osteoclast Progenitors***

We observed two distinct effects of galectin-3 during endochondral ossification on cells of the osteoclast lineage. In our embryonic metatarsal culture experiments, an excess of exogenous intact galectin-3 decreased the initial TRAP-positive cells (preosteoclasts and osteoclasts) invading the chondro-osseous junction. Addition of galectin-3 cleaved by MMP-9 had no such effect on recruitment of TRAP-positive cells. In contrast, we observed *increased* TRAP staining at the front of ossification in 2-wk-old *Mmp-9* null mice, in parallel with an increase in the number of galectin-3-positive cells. Osteoclasts and osteoclast precursors, which are monocyte-derived, express *galectin-3* (Niida *et al.*, 1994). Interestingly, galectin-3 has been described as a chemoattractant for monocytes and macrophages (Sano *et al.*, 2000). It is likely that full-length galectin mediates this effect since both N- and C-terminal domains are required. *Galectin-3* null mice have defective macrophage survival and adhesion (Hsu *et al.*, 2000). However, no defect in osteoclast differentiation was reported in *galectin-3* null mice and MMP-9-positive cells seemed to be recruited normally at the chondro-osseous junction (Colnot *et al.*, 2001). It is possible that aberrant persistence of intact galectin-3 induces abnormal osteoclast survival and excess bone turnover.

### ***Mmp-9 Null Mice Have Altered ECM and Disruption of Growth Plate Homeostasis***

Homeostasis of the growth plate during long bone growth depends on a delicate balance between the rate of chondrocyte proliferation and the rate of HC disappearance or death. The critical role of metaphyseal vascular supply in this homeostasis has been demonstrated (Trueta and Amato, 1960), and VEGF has been identified as one of the major angiogenic factor involved in this process. Inhibition of VEGF by circulating soluble inhibitors (Gerber *et al.*, 1999) or deficiency in VEGF long isoform expression (VEGF<sub>121/121</sub>; Maes *et al.*, 2002; Zelzer *et al.*, 2002) results in an enlarged HC zone, disturbed vascularization and altered bone mineralization. Moreover, trabecular bone volume, HC apoptosis, and osteoclast recruitment decrease.

The striking phenotypic similarities between these VEGF mutant mice and the *Mmp-9* null mice indicate an important link between these factors. We found that many proteins accumulate aberrantly in extracts from *Mmp-9* growth

plates. Two mechanisms may contribute to these increases: prolonged synthesis of these proteins by late HC that normally would have exited the growth plate and/or reduced degradation of these proteins in the absence of MMP-9 activity. We identified two of these proteins as VEGF and galectin-3. In contrast to galectin-3, which accumulates most probably because of a decrease in its degradation, VEGF mRNA persists in the expanded hypertrophic cartilage zone of *Mmp-9* null mice (Engsig *et al.*, 2000), indicating that late HC synthesize VEGF as long as they survive. Because VEGF is essential for osteoclast recruitment into developing long bones (Engsig *et al.*, 2000) and is a chemoattractant for the monocyte/macrophage lineage (Barleon *et al.*, 1996; Clauss *et al.*, 1996), this increase in VEGF protein may contribute to the increase in TRAP- and galectin-3-positive cells at the chondro-osseous junction of *Mmp-9* null mice. It follows then that a decrease in VEGF should result in decreased numbers of TRAP-positive cells, which is indeed the case (Gerber *et al.*, 1999; unpublished observations).

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