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Role of matrix metalloproteinase 13 in both endochondral and intramembranous ossification during skeletal regeneration.

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INTRODUCTION

Bone is noteworthy in that it does not heal through the formation of scar tissue, but rather through a regenerative process that resembles fetal skeletal development. During bone healing much of the initial developmental program is conserved, from the various cell types involved to the genetic mechanisms regulating cell differentiation [1–3]. As in development, repair of long bones by endochondral ossification begins with the production of cartilage at the injury site. The chondrocytes of the fracture callus deposit an extracellular matrix (ECM) comprised of type II collagen (Col2) and aggrecan, then differentiate into hypertrophic chondrocytes that deposit an ECM comprised of type X collagen (Col10). This ECM is then partially mineralized, resorbed and replaced by a matrix comprised predominantly of type I collagen (Col1). This process is regulated by the action of invading osteoblasts and osteoclasts. These cells, whose development and function are intimately linked, continue to remodel this regenerated tissue into mature bone until the fracture is consolidated. Other aspects of skeletal repair differ from skeletal development. For example, skeletal repair may be influenced by the mechanical environment. While non-stabilized fractures heal via endochondral ossification, stabilized fractures heal via intramembranous ossification. In this process, mesenchymal precursors recruited to the site of injury differentiate along the osteoblastic lineage and produce both compact (cortical) and spongy ( cancellous) bone in the absence of cartilage production [4,5].

Skeletal elements are rich in ECM and the remodeling of this ECM is central to both development and repair [3,5]. Matrix remodeling in both of these processes is regulated by many of the same proteases, and these enzymes may determine the rate and effectiveness of the development and repair programs [6]. The role of matrix metalloproteinasces (MMPs) during bone development has been studied extensively [7–12]. MMP13 promotes both the resorption of hypertrophic cartilage from the growth plate and the remodeling of newly deposited trabecular bone during long bone development [9,10]. Moreover, other work has pointed to the requirement for MMPs used in bone development for bone repair [3,5,13,14]. These reports have led us to ask whether MMP13 also participates in skeletal repair. In the present study, we employed several models of repair by both endochondral and intramembranous ossification, including non-stabilized and stabilized fracture models as well as a cortical defect model, to elucidate the role of MMP13 during the various stages of healing.

RESULTS

Mmp13 is expressed in the non-stabilized fracture callus

We first established which cells express Mmp13 during endochondral ossification in non-stabilized fractures. We performed in situ
hybridization analyses on histological sections through fracture calluses from wild-type (WT) mice using probes for *Mmp13* and a number of cell type-specific marker genes (Fig. 1). At 3 days post-fracture, we observed *Mmp13* expression in regions of activated peristomeum that also expressed *Col1* (data not shown). Likewise, at 6 days post-fracture, we observed *Mmp13*-expressing cells within the *Col1* expression domain in osteoclasts (OC)-negative portions of the peristomeum, indicating that *Mmp13* is expressed during the early stages of healing by immature osteoblasts (Fig. 1A–D and data not shown). The *Mmp13* expression pattern differed from that of *Mmp9*, indicating that *Mmp13* was not upregulated in newly recruited inflammatory cells and osteoclasts (Fig. 1B–D) as observed previously (Colnot et al., 2003). At 10 days post-fracture, *Mmp13* expression in the cartilage overlapped with *Col10* and vascular endothelial growth factor (Vegf) expression, indicating that *Mmp13* is expressed by hypertrophic and late hypertrophic chondrocytes (Fig. 1E–H). At 14 days post-fracture, *Mmp13* expression colocalized with *Col1* and *Oc* expression, suggesting that by this time point *Mmp13* is expressed in both immature and mature osteoblasts within the callus (Fig. 1I–L). *Mmp13* expression was detected in osteoblasts throughout the remodeling phase of healing and was not associated with osteoclasts (Fig. 1A–D and data not shown). These results demonstrate that the expression pattern of *Mmp13* in both cartilage and bone tissues during fracture healing parallels that seen during development via endochondral ossification [10,15,16] and suggest that MMP13 may play a role in both cartilaginous and bony tissues during fracture healing.

**Mmp13−/−** mice accumulate cartilage during non-stabilized fracture healing but cartilage differentiation to hypertrophy is normal

Cartilage begins to develop in the non-stabilized fracture callus by days 3 to 7 post-fracture (soft callus phase), peaks at day 10, is being remodeled by day 14 (hard callus phase) and is fully resorbed by day 28 (remodeling phase; [5]). To study the effect of MMP13 deficiency on this process, we created closed, non-stabilized fractures in WT and *Mmp13−/−* adult mice and examined the timing and extent of cartilage and bone formation throughout the healing process. Histological analyses indicated that cartilage is formed in *Mmp13−/−* calluses, but its remodeling and removal are delayed (Fig 2A). Quantitative analyses confirmed these observations. Histomorphometric analyses revealed that there was no significant difference in total callus volume between WT and *Mmp13−/−* samples at any time point examined (Fig. 2B), but there was a significantly greater volume of cartilage and of cartilage as a proportion of total callus volume in *Mmp13−/−* calluses at 7, 14 and 21 days post-fracture (Fig. 2B). Examination of the percentage of samples that exhibited cartilage confirmed the delay in cartilage removal in *Mmp13−/−* calluses. At day 21, all *Mmp13−/−* calluses contained cartilage while only one third of WT calluses did. At day 28, cartilage was still present in one third of *Mmp13−/−* calluses but was never observed in WT samples.

To characterize the differences observed during the early stages of healing, we analyzed *Col2* expression in fracture calluses at day 5 by in situ hybridization. We did not detect an alteration in the initial differentiation of chondrocytes in *Mmp13−/−* calluses as

**Figure 1. Mmp13 expression during non-stabilized fracture healing.** (Left column) Safranin-O/Fast Green (SO) and Trichrome (TC) stained sagittal sections through the WT callus at 6 (A), 10 (E) and 14 (I) days post-fracture. Cartilage (red) develops during the soft callus phase of healing (A), is resorbed during the hard callus phase (E) and is replaced by bone (blue, I). (Middle/Right column) In situ hybridization analyses of *Mmp13* expression and osteoblast/chondrocyte differentiation markers. (B–D) At 6 days post-fracture, *Mmp13* is expressed in the callus and overlaps with *Col1*-expressing cells (early osteoblasts) but not *Mmp9*-expressing cells (osteoclasts and inflammatory cells). (F–H) At day 10, *Mmp13* mRNA is detected in hypertrophic chondrocytes also expressing *Col10* and Vegf. (J–L) At day 14, *Mmp13* is expressed in mature osteoblasts co-expressing *Col1* and/or *Oc*. Scale bars: A, E, I = 1mm; B–D, F–H, J–L = 200 μm. doi:10.1371/journal.pone.0001150.g001
compared to WT (Fig. 3A), indicating that the difference in cartilage volume observed by day 7 was transient. We next examined chondrocyte hypertrophy in the Mmp13$^{-/-}$ calluses by investigating Col10 expression (Fig. 3A). The Col10-expression domain was comparable between WT and Mmp13$^{-/-}$ calluses at days 10 and 14 post-fracture, suggesting that chondrocyte hypertrophy was not altered in the absence of MMP13. These studies demonstrate that the absence of MMP13 did not affect the overall amount of cartilage produced in the callus or its differentiation to hypertrophy, but did affect the removal of hypertrophic cartilage.

To understand the basis for the delayed hypertrophic cartilage removal, we examined angiogenesis and ECM remodeling, two key components of endochondral ossification (Fig. 3B; [5,10,12,17]). Platelet endothelial cell adhesion marker (PECAM) immunostaining (left column) demonstrated that the observed delay in cartilage remodeling/removal was not due to delayed vascular invasion into the Mmp13$^{-/-}$ callus. Staining for Tartrate-resistant acid phosphate (TRAP)-positive cells (Fig. 3B, middle column) demonstrated the delay was also not due to delayed osteoclast recruitment into the Mmp13$^{-/-}$ callus. However, these recruited blood vessels and osteoclasts did not invade the hypertrophic cartilage matrix, suggesting the inability of the cartilage matrix itself to become invaded. We next stained for an epitope of aggrecan, the major proteoglycan of the cartilage ECM [18] which is degraded during the very last stages of chondrocyte differentiation/removal [19]. Cleavage of aggrecan by MMPs, as assessed by immunostaining for the cryptic DIPEN epitope (Fig. 3B, right column; [20]), decreased in the Mmp13$^{-/-}$ callus indicating that processing of the cartilage ECM in the Mmp13$^{-/-}$ callus was delayed.

Mmp13$^{-/-}$ mice have increased bone density during non-stabilized fracture healing

Since hypertrophic cartilage remodeling is necessary for the replacement of cartilage by bone during healing [5], we asked whether delayed removal of hypertrophic cartilage affects ossification of the fracture callus in Mmp13$^{-/-}$ mice. Histomorphometric analyses revealed a significantly smaller bone volume in Mmp13$^{-/-}$ samples at day 7 post-fracture (Fig. 4B), suggesting that the increase in cartilage formation at this early time point may have compromised the initial stages of bone formation. By day 14, however, we did not detect a significantly decreased bone volume...
nor a significantly decreased bone volume as a proportion of total callus volume in Mmp13<sup>−/−</sup> samples compared to WT (Fig. 4B). This suggests that the increased cartilage deposition/decreased cartilage removal observed at this time point does not impair new bone deposition. At later stages of repair, during active remodeling of the new bone in WT calluses, Mmp13<sup>−/−</sup> calluses had significantly greater callus bone volume and a significantly greater bone volume as a proportion of total callus volume, both at days 28 and 56 post-fracture (Fig. 4B).

By histological examination, we observed that spongy bone accumulated in Mmp13<sup>−/−</sup> calluses while the reconstitution of the bone marrow cavity was more advanced in WT calluses (Fig. 4A, day 28, middle column; day 56, right column). Histomorphometric analyses performed on the volumes of new bone in WT and Mmp13<sup>−/−</sup> calluses at 21 and 28 days post-fracture confirmed differences in the spongy and compact bone in Mmp13<sup>−/−</sup> calluses. At 28 days post-fracture, there was a significantly greater volume of spongy bone in Mmp13<sup>−/−</sup> calluses as compared to WT (Fig. 4C). By contrast, there was no significant difference observed in compact bone volume, regardless of timepoint or genotype. Taken together, these results suggest that the increased bone volume observed in the Mmp13<sup>−/−</sup> non-stabilized fracture callus resulted from an increase in the spongy bone of the callus.

Furthermore, we performed micro-CT analyses on non-stabilized fracture calluses from WT and Mmp13<sup>−/−</sup> mice at 21 and 28 days post-fracture. While no difference in overall callus volume was detected by micro-CT regardless of healing time point or genotype (data not shown), a significant increase in bone mineral density was observed in Mmp13<sup>−/−</sup> calluses as compared to WT at 21 and 28 days post-fracture (Fig. 4D). Given that histomorphometric analyses indicated no significant increase in bone volume at 21 days, these results suggest that the bone matrix...
was not fully mineralized in Mmp13\(^{-/-}\) calluses at this time point. By 28 days, the micro-CT results correlated with that of histomorphometry confirming that Mmp13 is required for proper bone remodeling in the non-stabilized fracture callus.

Since the increase in Mmp13\(^{-/-}\) callus bone volume observed by histomorphometry persisted through 8 weeks post-fracture, these results reflected a prolonged change in the bone volume of the mutant callus. We considered the possibility that the impact of Mmp13 deficiency on bone volume and bone mineral density in the non-stabilized fracture callus could affect the mechanical properties of these calluses. To test this, we performed mechanical analyses on non-stabilized fracture calluses from WT and Mmp13\(^{-/-}\) mice at 14, 21 and 28 days post-fracture (Table 1). We observed no significant impact on the maximum force at failure of fracture calluses regardless of healing timepoint or genotype (Table 1). Therefore, increased bone volume within the spongous bone compartment of the callus did not change the overall mechanical properties of the tissue.

**WT bone marrow-derived cells do not rescue the Mmp13\(^{-/-}\) non-stabilized fracture healing phenotype**

Osteoclasts are the major bone resorbing cells. Although we did not detect Mmp13 in osteoclasts, other cells of hematopoietic origin, specifically in the monocyte/macrophage lineage, can express Mmp13 [21]. To distinguish the effects of the Mmp13 mutation on osteoblasts and chondrocytes from its effects on cells derived from the hematopoietic lineage, we asked whether transplantation of WT bone marrow could rescue the Mmp13\(^{-/-}\) phenotype. As previously observed in WT hosts [22], we confirmed that bone marrow was derived from donor mice (Fig. 5A left panel), while chondrocytes (Fig. 5A, middle panel) and osteoblasts/osteocytes in the callus (Fig. 5A, right panel) were host derived. Histology (Fig. 5B) and histomorphometric analyses (Fig. 5C) showed that there was no difference in the proportion of cartilage in the callus at 14 days post-fracture, or in the proportion of bone in the callus at 28 days post-fracture between Mmp13\(^{-/-}\) mice that received WT (WT\(\rightarrow\)Mmp13\(^{+/+}\)) and Mmp13\(^{-/-}\) (Mmp13\(^{-/-}\)\(\rightarrow\)Mmp13\(^{+/+}\)) bone marrow. These results demonstrate that Mmp13-competent hematopoietically derived cells, including osteoclasts are insufficient to restore the timely resorption of cartilage and bone in Mmp13\(^{-/-}\) fracture callus. This suggests that there is an intrinsic defect in chondrocytes, osteoblasts and/or their matrices as the source of the Mmp13\(^{-/-}\) healing phenotype.

**Mmp13 is required for healing by intramembranous ossification**

To differentiate the consequences of the Mmp13\(^{-/-}\) mutation on cartilage and bone during repair, we examined healing via intramembranous ossification in fractures that were stabilized with a rigid external fixator [4, 5] and in a cortical defect model [23, 24]. WT and Mmp13\(^{-/-}\) mice that received stabilized fractures were examined at 10 and 28 days post-fracture (Fig. 6A). Unlike the Mmp9\(^{-/-}\) mice, which display aberrant cartilage formation at 10 days post-fracture (Fig. 6A bottom left panel; [5]), Mmp13\(^{-/-}\) stabilized fracture calluses (Fig. 6A, middle left panel) did not display cartilage and were comparable to WT (Fig. 6A, top left panel). At 28 days post-fracture, histological analyses suggested that Mmp13\(^{-/-}\) stabilized fracture calluses (Fig. 6A, middle right panel), were larger and contained more bone as compared to WT. However, since differences in callus sizes and bone content may result from variability in the alignment of bone ends at the fracture site, we turned to another model of intramembranous bone healing for quantification.

**DISCUSSION**

**Mmp13 is required for cartilage resorption**

We demonstrate here that the absence of Mmp13 does not affect the overall amount of cartilage produced in the callus during non-stabilized fracture healing, but rather affects the removal of hypertrophic cartilage from the callus. This is reminiscent of skeletal development in the absence of Mmp13, where an accumulation of hypertrophic cartilage results from its delayed

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**Table 1. Mechanical properties of intact tibiae and unstabilized fracture calluses.**

| Healing time (days) | Intact | 14 | 21 | 28 |
|---------------------|--------|----|----|----|
| Moment (N*mm)       | WT 612.418±236.998 | WT 681.582±342.389 | WT 470.850±277.999 | WT 639.629±344.144 |
| Mmp13\(^{-/-}\) | 539.063±215.845 | 610.200±458.343 | 735.207±454.051 | 512.438±268.329 |

Results are given as mean ± SD of sample group (intact WT \(n=10\), Mmp13\(^{-/-}\) \(n=10\); 14 days post fracture WT \(n=7\), Mmp13\(^{-/-}\) \(n=5\); 21 days WT \(n=2\), Mmp13\(^{-/-}\) \(n=2\).

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removal from the endochondral growth plate [9,10]. Our study has provided evidence for the importance of MMP13 activity during skeletal repair, which is both complementary to and distinct from its role during development.

Previous studies have also revealed a mechanistic link between fetal skeletal development and adult skeletal repair [3,5]. The delayed cartilage removal in Mmp13−/− mice resembles the phenotype that was observed in Mmp9−/− mice during develop-

Figure 5. Transplant of WT bone marrow does not rescue the Mmp13−/− non-stabilized fracture healing phenotype. (A) Immunostaining for GFP on callus tissues from Mmp13−/− mice transplanted with bone marrow from β-actin GFP mice (GFP→Mmp13−/− mice). (Left panel) Bone marrow cells (bm) are positive for GFP (black staining) showing they are donor-derived but the adjacent cortex (c) is negative. (Middle panel) Chondrocytes at day 14 and (Right panel) osteocytes embedded in the new bone (arrows) at day 28 do not stain for GFP, showing they are host-derived. (B, Left column) SO and (Right column) Masson’s Trichrome staining of non-stabilized fracture calluses from Mmp13−/− mice transplanted with WT bone marrow (WT→Mmp13−/−) and Mmp13−/− mice transplanted with Mmp13−/− bone marrow (Mmp13−/−→Mmp13−/−) show no difference in the amount of cartilage volume at 14 days post-fracture (WT→Mmp13−/− n=6, Mmp13−/−→Mmp13−/− n=5) and no difference in the amount bone at day 28 (WT→Mmp13−/− n=7, Mmp13−/−→Mmp13−/− n=4). (C) Histomorphometric analyses of total cartilage volume as a proportion of total callus volume (CV/TV; day 14) and total bone volume as a proportion of total callus volume (BV/TV; day 28) demonstrate no significant difference between WT→Mmp13−/− and Mmp13−/−→Mmp13−/− animals, suggesting that bone marrow transplant does not rescue the Mmp13−/− non-stabilized fracture healing phenotype. Bonferroni corrected t-test, bars represent means ± SD. Scale bars: (A, left and middle) = 50 μm, (A, right) = 25 μm, B = 1 mm. doi:10.1371/journal.pone.0001150.g005

Figure 6. MMP13 is required for normal healing by intramembranous ossification. (A, Left column) SO stain of stabilized fracture calluses at day 10 post-fracture show that unlike Mmp9−/− mice, no cartilage is formed during stabilized fracture healing in Mmp13−/− mice (n=14) compared to WT mice (n=3). (A, Right column) At day 28, stabilized fracture calluses in Mmp13−/− mice (n=12) appear to have increased bone volume as compared to WT (n=14) by histology. (B) Masson’s Trichrome staining of cortical defect samples at 21 and 28 days post-surgery suggests an increased amount of bone in Mmp13−/− mice. Labels designate compact (Co) and spongy (S) regions of defect. Histomorphometric analyses (within the boxed area) of WT (d21 n=5, d28 n=6) and Mmp13−/− (d21 n=6, d28 n=6) cortical defect samples confirm that there is an increase in spongy bone volume (SV/DV) but not compact volume (CoV/DV) in the defect area measured at day 21 and 28 (*p<0.05) in Mmp13−/− as compared to WT. Bonferroni corrected t-test, bars represent means ± SD. Scale bars: A = 1 mm, B = 500 μM. doi:10.1371/journal.pone.0001150.g006
MMP13 is required in bone remodeling

We found other differences between Mmp13<sup>−/−</sup> and Mmp9<sup>−/−</sup> phenotypes when we analyzed the production and remodeling of new bone in the fracture callus. In Mmp9<sup>−/−</sup> mice, the delayed removal of hypertrophic cartilage correlated with a delay in ossification. On the contrary, Mmp13<sup>−/−</sup> mice did not exhibit a delay in the deposition of new bone as a consequence of the cartilage phenotype. Instead, we observed an increase in bone volume at later stages of healing. This increase was restricted to the areas of spongy bone, much like those observed in Mmp13<sup>−/−</sup> bones during development [10]. Since spongy bone is lower in density and strength than compact bone, this result may explain why no significant difference was detected when we compared the strength of WT and Mmp13<sup>−/−</sup> calluses at late stages of healing.

Examination of stabilized fractures and cortical defects also revealed a role for MMP13 in the timely resorption of the transient compartments of spongy bone during healing via intramembranous ossification. Unlike development, where MMP13 activity is required for the formation of endochondral but not intramembranous bone, these observations suggest that its proteolytic activity is required during repair regardless of the type of ossification involved. These results support the idea that the bone defect observed during adult skeletal repair in the absence of MMP13 is caused by a defect intrinsic to osteoblasts or the matrix they produce. Furthermore, WT osteoclasts did not accelerate the resorption of spongy bone in the Mmp13<sup>−/−</sup>-callus. Similar to its role in hypertrophic cartilage, MMP13 present in new bone matrix may be required for the initial degradation of ECM components allowing the timely remodeling of the bone callus (Fig. 7).

Cartilage-bone interaction in the Mmp13<sup>−/−</sup>-fracture callus

That bone remodeling was delayed in both intramembranous and endochondral ossification during bone repair demonstrates that in the absence of MMP13 the cartilage phenotype manifests independently of the bone phenotype. These results are in accordance with the observations reported for skeletal development in the absence of MMP13 by Stickens et al. (2004). In addition, the observed delay in cartilage resorption did not affect the deposition of bone in the Mmp13<sup>−/−</sup>-callus at day 14 post-fracture. While bone and cartilage have been shown to provide regulatory feedback to each other [28], these results indicate that proper remodeling of the ECM depends on unique signals or properties intrinsic to the bone and cartilage.

Regulatory interactions between bone and cartilage also take place during the early stages of fracture healing [5]. During the early stages of non-stabilized fracture healing in the absence of MMP13, we observed a delay in initial bone deposition correlated with an increase in cartilage formation. This might indicate a transient inhibition of osteogenesis due to increased chondrogenesis or a decreased ability of MMP13-deficient mesenchymal precursor cells to differentiate into fully mature osteoblasts [29]. This early imbalance was not observed during stabilized fracture healing in either WT or Mmp13<sup>−/−</sup> mice, which healed via intramembranous ossification. This stands in contrast with Mmp9<sup>−/−</sup> mice that heal stabilized fractures via an aberrant endochondral process [5].

A role for MMP13 in osteoclasts?

The independent manifestation of the cartilage and bone phenotypes may be due to the fact that osteoclasts are not affected in Mmp13<sup>−/−</sup> mice. Several studies point to MMPs, possibly even MMP13, as potentially important mediators of osteoclast recruitment during development and repair [30–33] and a number of factors that promote bone resorption/remodeling are able to affect Mmp13 gene expression [34–38]. Our results indicate that Mmp13 is not co-expressed with Mmp9 in osteoclasts. Furthermore, our bone marrow transplantation studies indicate that WT osteoclasts were not able to support normal ECM remodeling in the Mmp13<sup>−/−</sup> environment, suggesting that they did not provide...
a source of MMP13. This result points again to a defect that is
intrinsic to the ECM. MMP13 activity may be required prior to
the onset of osteoclastic and MMP9 activity to prepare the bone
matrix for resorption [Fig. 7]. Indeed, prior work has suggested
that MMP13 does participate in the resorption of the organic bone
matrix, but that this is through the action of mesenchymal stem
cell-derived cells, not osteoclasts [39]. Consequently, WT (and
therefore MMP13-competent) osteoclasts are unable to perform
their normal function due to a deficiency in this preparatory step
in the Mmp13+/− ECM.

Implications for normal skeletal repair
In humans, although the mode of healing may vary depending on
the site of injury, extent of trauma, and fixation, most fractures heal
through enchondral ossification. Therefore in the majority of
clinical cases, remodeling of both cartilage and bone is critical
for bone bridging and full recovery of mechanical integrity. While
most fractures heal spontaneously, 5 to 10% fail to heal in a timely
manner [40]. Risk factors such as age, injury type and socio-
economic conditions have been shown to impact the outcome of
healing, yet little is known about the genetic predispositions to
impaired healing. Although mutations in MMP genes have been
linked to skeletal defects in humans [12,41], none have been
associated with delayed healing. Our results show that MMP13
and other MMPs such as MMP9 play a role in all stages of fracture
healing from the early deposition of cartilage and bone to the late
stages of callus remodeling. Perturbations in any of these processes
may compromise healing. Although Mmp13+/− mice have
phenotypic manifestations similar to Mmp9+/−/− mutants, such as
late onset of hypertrophic cartilage removal, we show that there
are intrinsic differences in the way these two proteases act in
fracture repair and that treatment strategies to overcome these
defects differ. These observations point to the need for a better
understanding of the underlying causes of delayed healing in
humans in order to plan efficient therapies. From this perspective,
the detailed analyses of Mmp9+/−/− and Mmp13+/−/− mutant mice
shed light into the normal process of bone healing and potential
approaches to treat impaired healing.

MATERIALS AND METHODS
Non-stabilized and stabilized fractures
Mmp13+/−/− mice (3- to 6-month-old males; 30–35 grams (g)) and
their WT littermates were anesthetized with an intraperitoneal
injection of 50 mg/ml Ketamine/0.5 mg/ml Medetomidine
(0.03 ml/mouse). Closed, standardized non-intraperitoneal fractures
were produced as previously described [5]. For stabilized fractures,
an external fixator was placed as previously described [4]. Mice
were sacrificed by cervical dislocation following an intraperitoneal
injection of 2% Avertin (0.5 ml/mouse). Non-stabilized fractures
were collected at 5, 10, 14, 21, 28, and 56 days post-fracture.
Stabilized fractures were collected at 10 and 28 days post-fracture.
All protocols were approved by the Institutional Animal Care and
Use Committee at UCSF.

Bone marrow transplantation
10-week-old male Mmp13+/−/− mice were lethally irradiated with
two 5 Gy doses of γ-irradiation 3–4 hours apart. Bone marrow
cells from WT, Mmp13+/−/− and β-actin Green Fluorescent Protein (GFP)
mice were transplanted into irradiated Mmp13+/−/− mice from the
same FVB/N background as previously described (Colnot et al.,
2006). Following a recovery period (Colnot et al. 2006), non-
stabilized fractures were produced in chimeric mice as described
above. Fracture tissues were collected at 14 and 28 days post-
fracture and processed for histological analyses.

Cortical defects
Monocortical defects (1mm in diameter) were produced on the
anterior-proximal tibia as previously described [23,24]. Samples
were collected at 21 and 28 days post-surgery and processed for
paraffin embedding. Longitudinal sections parallel to the plane of
the defect were collected and processed for histological analyses.

Histology and immunohistochemistry
Callus tissues were fixed overnight at 4°C in 4% paraformalde-
yde, decalcified at 4°C in 19% EDTA (pH 7.4) for 10–14 days,
then dehydrated in a graded ethanol series and embedded in
paraffin. Sections (10 μm thick) were stained with Safranin-O/
Fast Green (SO) to detect cartilage formation as described [4]. A
modified Milligan’s Trichrome (TC) staining using Analine Blue
was performed to analyze bone formation in the fracture callus.
Tartrate-resistant acid phosphatase (TRAP) staining was per-
formed using a leukocyte acid phosphatase kit (Sigma, St. Louis,
MO) as previously described [16]. Immunohistochemistry for
platelet endothelial cell adhesion molecule-1 (PECAM) was done
as previously described [5,8]. Immunohistochemistry for the
DIPEN epitope was performed as described previously [10].

Calluses collected from Mmp13+/−/− mice transplanted with bone
marrow from β-actin GFP mice were fixed and decalcified as
mentioned above, then cryo-embedded in OCT. Sections (8 μm)
were cut on a cryostat (Leica). For GFP immunostaining,
cyosections were treated with 0.3%H2O2 in methanol, digested
with ficin (Zymed), and blocked with 5% milk in PBS and 5% normal
goose serum in PBS. Antibody staining was done using rabbit
anti-GFP antibody (Abcam) followed by goat anti-rabbit
secondary antibody conjugated to horseradish peroxidase. Slides
were developed with diaminobenzidine and counterstained with
0.1% fast green.

In situ hybridization
In situ hybridization was performed using 35S-labeled probes from
mouse cDNAs for Mmp13, Col1 α1 chain (Col1a1 – Mouse Genome
Informatics), Mmp9, Col10 (Col10a1 – Mouse Genome Informatics),
Vegf (Vega – Mouse Genome Informatics), Ocs (Tregl – Mouse
Genome Informatics) and Col2 (Col2a1 – Mouse Genome Informatics)
as previously described [16]. Emulsion coating and image
analyses were performed as described previously [2,42].

Histomorphometric measurements
Histomorphometry on fracture samples was performed as pre-
viously described [5,43]. To determine the amount of cartilage
within each callus, every thirtieth section (300 μm) was stained
with SO. Adobe Photoshop was used to capture images from
a Leica DM 5000 B light microscope (Leica Microsystems GmbH,
Wetzler, Germany) that was equipped with a camera (Diagnostic
Instruments, Inc., Sterling Heights, MI). To determine the amount
of bone within each callus, adjacent sections were stained with TC
and photographed. The area of the callus, cartilage and bone
(including compact and spongy bone [44] was determined using
Adobe Photoshop.

To determine the amount of bone within cortical defects, 2
central sections within the defect were selected and stained with
TC. In Adobe Photoshop, a standard box (as shown in Figure 6)
was used to select an area in the center of the defect, including the
damaged cortical region and the underlying bone marrow space.
The total area of bone within the defect, as well as the areas of
Mechanical testing

Mechanical testing was performed on intact tibiae from 12-week-old mice and tibiae from mice that had received non-stabilized fractures collected at 14, 21 and 28 days post-fracture. The proximal and distal end of each tibia was potted in a cryovial cap [46]. The distal end of the specimen was rigidly held to the test frame while the proximal end was attached to a single-cable pulley system. Movement of the test specimen was rigidly held to the test frame to prevent gravitational loading of the specimen by the PMMA endcaps [46]. The distal end of the specimen was attached to a uniaxial load cell (Sensotec Model 129, 25 lb capacity, Honeywell International, Inc., Columbus, OH) was used to monitor cable tension, and the moment applied to the specimen during a uniaxial load test frame to prevent gravitational loading of the specimen by the PMMA endcaps [46]. The distal end of the specimen was rigidly held to the test frame while the proximal end was attached to a single-cable pulley system. Movement of the test frame actuator caused tension in the cable, which was positioned around a loading ring so as to generate a pure moment, or couple, loading condition. A uniaxial test frame (Sensotec Model 129, 25 lb capacity, Honeywell International, Inc., Columbus, OH) was used to monitor cable tension, and the moment applied to the specimen was calculated as the product of the cable tension and the diameter of the loading ring \((N\cdot\text{mm})\). Moment and crosshead displacement were monitored, and the ultimate moment over the entire destructive test cycle was recorded as the failure strength of the specimen.

Statistical analyses

For non-stabilized fracture studies, Wilcoxon rank sum tests were used at each time point to examine whether differences between WT and Mmp13−/− samples in cartilage, callus, and bone volumes were statistically significant \((p<0.05)\). Bonferroni adjustments were performed. For mechanical testing of non-stabilized fracture calluses, force data were analyzed using GraphPad Prism 4 by one-way analysis of variance (ANOVA). For bone marrow transplant and cortical defect studies, histomorphometry data were analyzed using ANOVA followed by Bonferroni corrected t-tests for data sets where ANOVA, \(p<0.05\).

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Author Contributions

Conceived and designed the experiments: CC. Performed the experiments: CC ZK SL DB. Analyzed the data: CC DB. Contributed reagents/materials/analysis tools: JB JL ZW. Wrote the paper: CC DB. Other: Critically revised the manuscript: ZK JB RM ZW TM.

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