Altered fracture repair in the absence of MMP9

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SUMMARY

The regeneration of adult skeletal tissues requires the timely recruitment of skeletal progenitor cells to an injury site, the differentiation of these cells into bone or cartilage, and the re-establishment of a vascular network to maintain cell viability. Disturbances in any of these cellular events can have a detrimental effect on the process of skeletal repair. Although fracture repair has been compared with fetal skeletal development, the extent to which the reparative process actually recapitulates the fetal program remains uncertain. Here, we provide the first genetic evidence that matrix metalloproteinase 9 (MMP9) regulates crucial events during adult fracture repair. We demonstrate that MMP9 mediates vascular invasion of the hypertrophic cartilage callus, and that Mmp9−/− mice have non-unions and delayed unions of their fractures caused by persistent cartilage at the injury site. This MMP9-dependent delay in skeletal healing is not due to a lack of vascular endothelial growth factor (VEGF) or VEGF receptor expression, but may instead be due to the lack of VEGF bioavailability in the mutant because recombinant VEGF can rescue Mmp9−/− non-unions. We also found that Mmp9−/− mice generate a large cartilage callus even when fractured bones are stabilized, which implicates MMP9 in the regulation of chondrogenic and osteogenic cell differentiation during early stages of repair. In conclusion, the resemblance between Mmp9−/− fetal skeletal defects and those that emerge during Mmp9−/− adult repair offer the strongest evidence to date that similar mechanisms are employed to achieve bone formation, regardless of age.

Key words: Matrix metalloproteinase 9, Vascular endothelial growth factor, Endochondral ossification, Cartilage, Bone healing, Mechanical environment, Mouse

INTRODUCTION

The bony skeleton possesses an astounding regenerative potential. Unlike other adult tissues, which generate scar tissue at the site of an injury, the skeleton heals by forming new bone that is indistinguishable from adjacent, uninjured tissue. There are aspects of this adult regenerative process that bear a resemblance to fetal skeletal tissue development. For example, both fetal and adult skeletal progenitor cells aggregate to form cell condensations that eventually differentiate into skeletal tissue (Hall, 1988; Thompson et al., 2002). The same molecular markers of chondrogenesis and osteogenesis are expressed during development and repair, which suggests that the regulation of cell differentiation is also conserved (Ferguson et al., 1999; Karsenty and Wagner, 2002; Vortkamp et al., 1998). In both processes, vascularization is a prerequisite for ossification (Thompson et al., 2002; Vu et al., 1998), and disruptions in extracellular matrix remodeling can delay subsequent bone formation.

There are also notable differences between the adult reparative process and fetal skeletal development. For example, mechanical forces have not been implicated in the initiation of chondrogenesis or osteogenesis during fetal development, but the role of the mechanical environment during adult repair (Carter et al., 1998; Probst and Spiegel, 1997), and its effect on cartilage and bone formation during fracture healing (Le et al., 2001; Thompson et al., 2002) has been well documented. An inflammatory reaction exerts a substantial effect on adult skeletal repair (Simon et al., 2002), whereas the immune system has no known role in fetal skeletogenesis. Likewise, skeletal progenitor cells abound in the fetus, whereas they may be limited in number in the adult (Bruder et al., 1994; Ekholm et al., 2002).

We found the parallels between fetal skeletal development and adult repair particularly intriguing, and pursued this issue in the context of fracture healing. Our investigation focused on the extent to which the functions of one molecule, matrix metalloproteinase 9 (MMP9), were equivalent during skeletal development and fracture healing. We developed several novel models of skeletal repair, and exploited the Mmp9−/− mouse in order to gain the first molecular insights into the regulation of angiogenesis during skeletal tissue regeneration.

MATERIALS AND METHODS

Non-stabilized and stabilized fractures

Mmp9−/− mice [3- to 5-month old; 30-35 grams (g)] and their wild-type littermates were anesthetized with an intraperitoneal injection of 2% Avertin (0.015 ml/g body weight). Closed, standardized non-
stable fractures were produced following protocols approved by the UCSF Committee on Animal Research. The tibia was placed on the fracture jig and 460 g weight was dropped from 14 cm to create a closed, transverse fracture by three-point bending, which was confirmed by radiography. These non-stabilized fractures heal through the formation of a cartilage intermediate (Thompson et al., 2002). To produce stabilized fractures, which heal without a cartilage intermediate, an external fixation device was placed at the time of fracture (Tay et al., 1998; Thompson et al., 2002). Mice with non-stabilized fractures were sacrificed by cervical dislocation following deep inhalation anesthesia (Metofane) at 3 (n=6), 6 (n=6), 10 (n=10), 14 (n=19), 21 (n=6) and 28 (n=6) days post-fracture. Mice with stabilized fractures were sacrificed at 3 (n=6), 7 (n=8), 10 (n=11), 14 (n=16), 21 (n=10) and 28 (n=10) days post-fracture.

**Pin implantation**

Mice were anesthetized as described above. Using a percutaneous approach, stainless steel pins (0.25mm diameter) were inserted through the marrow and both tibial cortices; pin ends were cut flush with the skin. Mice were sacrificed 7 and 10 days after surgery, and pins were removed from the tibiae following decalcification.

**Treatment with recombinant vascular endothelial growth factor (VEGF) protein**

Mmp9+/– tibiae were fractured as described above and recombinant vascular endothelial growth factor (rVEGF) protein (5.0 µg/injection; Genentech) was delivered at four separate time-points (6, 7, 8 and 9 days post-fracture). Following inhalation anesthesia, percutaneous injections were made directly into the fracture site using a 30 gauge needle. Extreme care was taken to avoid displacing the bone ends during these injections. Control Mmp9+/+ mice received injections of an equivalent volume of vehicle (PBS) at the same time-points. PBS and rVEGF-injected mice were sacrificed at 10 (n=5 PBS-injected, n=5 rVEGF-injected) and 14 (n=5 PBS-injected, n=5 rVEGF-injected) days post-fracture.

**Biomechanical analyses (distraction to failure testing)**

Closed tibial fracture tissues were collected at 14 and 19 days post-fracture. The tibiae were carefully dissected free of surrounding soft tissue, placed in normal saline and stored overnight at 4°C. The tibiae were prepared for mechanical testing by placing 0.25 mm transfixion pins (Fine Science Tools, Foster City, CA) into the bone at locations that were proximal and distal to the fracture site. The proximal and distal ends of the bone, including the pins, were prepared for mechanical testing by placing 0.25 mm transfixion pins (Fine Science Tools, Foster City, CA) into the bone at locations that were proximal and distal to the fracture site. The proximal and distal ends of the bone, including the pins, were embedded in polymethylmethacrylate (PMMA), leaving the fracture callus exposed. Tissues were kept moist until they were loaded onto the materials testing system (Bionix 858; MTS, Eden Prairie, MN) with a precision force transducer (100# [454 N] Load Cell Model 31, Senstec, Columbus, OH). The ends of the bone were secured to the machine using a custom-made clamp. The tibiae were then loaded in distraction at a rate of 0.25 mm/minute with simultaneous force and displacement data recorded. Maximum force at failure (in Newtons, N) was calculated for each specimen.

**Histology and immunohistochemistry**

Under RNase-free conditions, callus tissues were fixed overnight at 4°C in 4% paraformaldehyde. Callus tissues were decalcified at 4°C in 1% EDTA (pH 7.4) for 10-14 days, then dehydrated in a graded ethanol series and embedded in paraffin. The entire callus was sectioned (10 µm thick), and adjacent sections were analyzed using a variety of histological and cellular analyses. Safranin-O/Fast Green (SO) staining was performed as described (Thompson et al., 2002). Trichrome staining was performed to analyze bone formation in the fracture callus, the Aniline Blue (AB) component of the trichrome stain was selected for image analyses. Tartrate resistant acid phosphatase (TRAP) staining was performed using a leukocyte acid phosphatase kit (Sigma, St. Louis, MO). For platelet endothelial cell adhesion molecule (PECAM)- and MPP9-antibody staining, sections were de-paraffinized, immersed in 5% H2O2/PBS for 5 minutes, washed in PBS, treated with 0.1 M glycine for 30 seconds, washed in PBS, and then incubated in the following blocking solutions followed by intermediate PBS washes: 5% powdered milk for 10 minutes; 1.0 mg/ml ovalbumin for 10 minutes; and 5% sheep serum for 30 minutes. Sections were incubated overnight at 4°C in monoclonal rat anti-mouse PECAM1 (BD Pharmingen, San Diego, CA) or a polyclonal rabbit anti-mouse MPP9, washed in PBS, blocked in 5% sheep serum for 30 minutes, and incubated for 1 hour at room temperature in biotinylated anti-rat IgG (BD Pharmingen, San Diego, CA) or biotinylated anti-rabbit IgG (Jackson Immunoresearch). Slides were washed in PBS and incubated in horseradish peroxidase-conjugated streptavidin (Amersham, Cleveland, OH). Signal was revealed by incubation in a diaminobenzidine solution containing 1% CoCl2 and 1% NiSO4 for PECAM immunostaining (Vu et al., 1998). For MMP9-TRAP double staining, TRAP staining was performed following MMP9 immunostaining.

**In situ hybridization**

In situ hybridization was performed using mouse cDNAs for Mmp9, ColIIa (Col2a1 – Mouse Genome Informatics), ColIX (Col10a1 – Mouse Genome Informatics), Oc (Tcf1l1 – Mouse Genome Informatics), Vegf (Vegfa – Mouse Genome Informatics), and the Vegf receptors Flk-1, Flt1 (Kdr – Mouse Genome Informatics) and neuropilin 2. Sections were de-waxed, fixed in 4% PFA, treated with 20.0 µg/ml Proteinase K, fixed with 0.1% sodium borohydride/PBS and acetylated in a solution of 0.1 M triethanolamine-HCl. Sections were then hybridized with 35S-labeled denatured probes overnight at 45°C. Sections were then washed in 5xSSC containing 20 mM β-mercaptoethanol at 45°C, then in 50% formamide containing 2xSSC at 45°C, followed by a wash in 2xSSC, and lastly in 0.1xSSC at room temperature. Sections were then dehydrated in a graded ethanol series. Emulsion coating was performed as described (Albrecht et al., 1997). Image analyses were performed as described previously (Ferguson et al., 1999).

**Histomorphometric measurements**

At 10 and 14 days, the fracture callus was composed of cartilage, islands of bone, and fibrous tissue. To determine the volume of the callus and the cartilage within each callus, and to circumvent difficulties in assessing a heterogenous tissue such as the fracture callus, we first sectioned the entire callus. From the resulting 300 tissue sections (each 10 m thick), histomorphometric analyses were performed. In our initial studies we analyzed tissue sections every 30 µm; we later determined that tissue sections taken at a 300 µm interval produced the same results. Thus, for each callus, an average of 10-15 tissue sections were used to determine callus and cartilage volumes. Sections were stained with SO and images of each section were photographed using a digital camera. Images were imported into Adobe Photoshop, and the software was used to quantify the area of the callus and the area of cartilage (which stained red after SO histological analysis). The areas of the callus and the cartilage were both determined empirically in a double-blinded manner, and checked by an independent investigator. These data were used to calculate the total volume of each callus and the total volume of cartilage in each callus.

**RESULTS**

MMP9 is expressed throughout the entire process of adult skeletal regeneration

The initial stage of fracture healing is characterized by an acute inflammatory reaction, which has been proposed to stimulate mesenchymal cell proliferation at the site of injury (Einhorn et
Fracture repair in the absence of MMP9 (Le et al., 1995). In mice, this inflammatory period encompasses the first 3-4 days post-fracture (Le et al., 2001). During this window of time, we noted abundant Mmp9 mRNA and MMP9 protein expression in mesenchymal and inflammatory cells surrounding the fracture site (Fig. 1, 3 days). The inflammatory stage is closely followed by the soft callus phase of repair, during which time mesenchymal cells differentiate into chondrocytes and begin their maturation process to a state of hypertrophy. In mice, this stage lasts up to 10 days post-fracture and is characterized by the formation of a cartilage callus that acts to stabilize the fractured bone ends (Le et al., 2001). During the early part of the soft callus phase, a large number of MMP9-positive and TRAP-positive preosteoclasts were detected at the fracture site (Fig. 1, 6 days). During the latter part of the soft callus phase we noted that Mmp9 expression in osteoclasts/chondroclasts interposed between the hypertrophic cartilage and the newly forming bone of the fracture callus (Fig. 1, 10 days; and Fig. 5A).

The hard callus phase of repair is characterized by the replacement of a cartilage scaffold with bone, and this occurs between 10 and 21 days post-fracture in mice (Le et al., 2001). At 14 days, MMP9-positive cells were found within the hypertrophic cartilage callus localized to sites of vascular invasion (Fig. 1, 14 days; and Fig. 5). During the remodeling phase of healing, which in mice extends from 14 days post-fracture until a minimum of two weeks afterwards, MMP9 was strongly expressed in osteoclasts that were in the process of degrading new bone matrix (Fig. 1, 21 days).

Mmp9−/− mice display abnormal fracture healing
Given the expression of MMP9 throughout the course of bone repair, we speculated that some aspect of adult skeletal repair would be compromised in Mmp9−/− mice. The most obvious difference we noted was during the hard callus phase of repair. Whereas the wild-type cartilage callus was undergoing rapid degradation and remodeling by 14 days, the Mmp9−/−
The *Mmp9*^−/−^ mutation affects cartilage remodeling but not chondrocyte maturation

One possible explanation for the *Mmp9*^−/−^ repair defect we had observed was that *Mmp9*^−/−^ chondrocytes were slower to mature, and that this ultimately affected their rate of fracture repair. We tested this hypothesis by examining chondrocyte maturation in wild-type and mutant mice at numerous time-points during the healing process. At 7 and 10 days post-fracture, most wild-type callus chondrocytes expressed mRNA for collagen type Ila (*CollIa*), a marker of resting, proliferating and mature chondrocytes (Sandell et al., 1997). The same was true of chondrocytes in the *Mmp9*^−/−^ callus (data not shown). Likewise, the area of collagen type X (*ColIX*) expression, a marker of hypertrophic chondrocytes (Hiltunen et al., 1993), was equivalent in wild-type and *Mmp9*^−/−^ calluses (Fig. 3), indicating that the *Mmp9*^−/−^ mutation did not affect the transition of chondrocytes from a mature to a hypertrophic state.

By sharp contrast, the removal of hypertrophic *ColIX*-expressing cartilage was profoundly affected in *Mmp9*^−/−^ mice (Fig. 3). We noted that in wild-type mice, the *ColIX* domain was much smaller at 14 days than at 10 days post-fracture, indicating that the onset of hypertrophic cartilage degradation began between these two time points (Fig. 3). However, in *Mmp9*^−/−^ mice the *ColIX* domain remained large, even after 14 days (Fig. 3). Thus, MMP9 was not required for chondrocyte maturation but it did play a role in the degradation and removal of hypertrophic cartilage. Once again, this phenotype bore a striking resemblance to the defect we had observed during fetal development (Vu et al., 1998).

The *Mmp9*^−/−^ mutation affects the biomechanical properties of the fracture callus

A crucial aspect of bone healing is that the regenerated tissue must provide sufficient strength to the injured limb in order for the animal to regain function. We sought to determine if the *Mmp9*^−/−^ mutation compromised the strength and stability of an injured bone. We first used a biomechanical test to measure callus strength. We chose the distraction-to-failure model as a mode of testing because the geometry of a non-stabilized fracture is highly variable, which renders more standard biomechanical tests less accurate. Wild-type and *Mmp9*^−/−^ calluses were subjected to a gradual distractive force and the maximum force required to cause rupture of the callus was determined (White et al., 1977). At 14 days, we found the maximum force at failure was greater in wild-type [5.19±0.63 Newtons (N), ±s.e.m.; *n*=8] than in *Mmp9*^−/−^ calluses (4.29±1.06 N; *n*=13; *P*=0.045, Student’s *t*-test), indicating that *Mmp9*^−/−^ calluses were structurally weaker than wild-type calluses. By 19 days, the maximum force at failure was equivalent in wild-type (6.99±1.22 N, *n*=11) and *Mmp9*^−/−^ calluses (6.47±0.97 N; *n*=10; *P*=0.41), which supported our previous observations that the *Mmp9*^−/−^ defect resolved around the onset of bony remodeling. Collectively, these data indicate that MMP9 serves at least two functions during skeletal tissue regeneration. First, MMP9 is necessary for the efficient degradation and remodeling of the hypertrophic cartilage callus. Second, MMP9 participates in the regeneration of osseous tissue in the callus. Ultimately, these data indicate that MMP9 activity is crucial for the regeneration of functional skeletal tissue.
et al., 2002). We stabilized wild-type and (Carter and Giori, 1991; Probst and Spiegel, 1997; Thompson differentiation of skeletal progenitor cells into osteoblasts and instead form bone through the direct observation that immobilized fractures heal with little or no ossification. This repair model was based on the clinical evidence that pin implant sites healed through intramembranous ossification. By contrast, abundant cartilage formed at the Mmp9+/− implant site (54%, n=13; Fig. 4B), similar to the stabilized fracture phenotype we had observed previously. However, the cartilage was located at the osteoimplant surface rather than in the endosteum. These data suggested that skeletal progenitor cells in the periosteum are particularly susceptible to the Mmp9-null mutation, and either are delayed in their differentiation into osteoblasts, or are misdirected in their cell fate specification.

The Mmp9+/− mutation impairs ossification during fracture healing

Our previous analyses of the cranial and appendicular skeletons of prenatal and early postnatal Mmp9+/− mice had failed to reveal defects in intramembranous ossification (Vu et al., 1998). However, in our fracture healing model we had observed that intramembranous ossification was disrupted. Using osteocalcin (Oc) expression as an indicator of osteogenesis (Lian et al., 1978), we ascertained that Oc was expressed at very low levels, and only at the periphery of the callus, in Mmp9+/− mice, whereas Oc was expressed throughout wild-type calluses by day 14 (Fig. 3). This retardation in intramembranous ossification persisted in Mmp9+/− mice into the remodeling phase of fracture repair (Fig. 3).

We initially postulated that a delay in intramembranous ossification during Mmp9+/− fracture healing was due to a primary defect in cartilage removal, which indirectly delayed subsequent bone formation. An alternative possibility was that the delay in intramembranous ossification represented a separate, and primary, defect in bone formation. To resolve this issue, we developed another model of skeletal repair that allowed us to evaluate whether Mmp9+/− bones would heal properly if the primary mode of repair was intramembranous ossification. This repair model was based on the clinical observation that immobilized fractures heal with little or no cartilage, and instead form bone through the direct differentiation of skeletal progenitor cells into osteoblasts (Carter and Giori, 1991; Probst and Spiegel, 1997; Thompson et al., 2002). We stabilized wild-type and Mmp9+/− tibiae with an external device that immobilized the fractured bone segments (Thompson et al., 2002), then assessed the calluses for the onset of osteoblast differentiation after 7, 10, 14 and 21 days. As expected, the majority of wild-type calluses formed bone without evidence of chondrogenesis (81%, n=31; Fig. 4A). By contrast, the majority of Mmp9+/− mice exhibited late onset, and low expression, of Oc, indicating that intramembranous ossification was greatly delayed in the mutant mice (88%, n=16; Fig. 4A). However, more surprising was the fact that skeletal progenitor cells in the Mmp9+/− calluses were not simply delayed in their differentiation to osteoblasts. Instead, the cells differentiated into chondrocytes despite the fact that the bone ends were immobilized (Fig. 4A).

The Mmp9+/− stabilized fracture phenotype may arise because skeletal progenitor cells are mis-specified to a chondrogenic cell fate, or are delayed in their differentiation to an osteogenic lineage. However, the source(s) of these skeletal progenitor cells was unclear. Because periosteum and endosteum are both probable repositories of skeletal progenitor cells (Einhorn, 1998; Pechak et al., 1986), we sought to localize the cell lineage/differentiation defect to Mmp9+/− periosteum or endosteum. We devised another skeletal repair model to test whether periosteal or endosteal regeneration was compromised in Mmp9+/− mice by inserting an implant that penetrated both tibial cortices and the bone marrow cavity (Fig. 4B). This repair model injured both tissues, but essentially compartmentalized periosteal regeneration from endosteal repair by blocking the space between the two tissues with the implanted pin. In wild-type animals, new bone formed in the periosteum and endosteum at the implant site, with no evidence of ColIIa expression or proteoglycan staining in either location (91%, n=11; Fig. 4B). These findings confirmed that pin implant sites healed through intramembranous ossification. By contrast, abundant cartilage formed at the Mmp9+/− implant site (54%, n=13; Fig. 4B), similar to the stabilized fracture phenotype we had observed previously. However, the cartilage was located at the periosteal surface rather than in the endosteum. These data suggested that skeletal progenitor cells in the periosteum are particularly susceptible to the Mmp9-null mutation, and either are delayed in their differentiation into osteoblasts, or are misdirected in their cell fate specification.

The pin implant model thus uncovered a potential new role for Mmp9 in the commitment of skeletal progenitor cells to an osteogenic lineage. We reasoned that if MMP9 was involved in such a cell fate specification, the protein should be expressed at the time when such decisions are made during fracture repair. We had already observed that cells within the fracture site express chondrogenic and osteogenic markers within 3 days of fracture (Le et al., 2001); using a functional approach we now demonstrated that cells became specified in their fate
within this same time frame. By permitting motion at the site of a fracture for 0, 24 or 48 hours, or for 5 days, and subsequently stabilizing the bone segments until day 10, we were able to show that healing occurred by endochondral ossification if they were mobile for longer than 48 hours (Fig. 4C). Fractures unstable for 24 hours or less healed by intramembranous ossification (Fig. 4C).

These functional data strongly suggested that skeletal progenitor cells could commit to a chondrogenic or an osteogenic fate within 48 to 72 hours following injury. We used immunohistochemistry to show that MMP9 protein was expressed within that same time frame. MMP9 protein was detected in TRAP-negative mesenchymal cells located on the endosteal and periosteal surfaces of the fractured tibia, in the extracellular matrix surrounding the fracture site (Fig. 4D), and in TRAP-negative inflammatory cells such as neutrophils (Fig. 4D). After 5 days, we also detected MMP9 in TRAP-positive pre-osteoclasts (Figs 1, 5) (Engsig et al., 2000). Taken together these data indicate that MMP9 protein is expressed at sites where skeletal progenitor cells are proposed to exist, and that MMP9 is expressed during the period when these cells adopt and commit to chondrogenic or osteogenic fates following injury.

**Fig. 4.** *Mmp9*+/– mice exhibit an ossification defect during stabilized fracture repair. In A and B, wild-type is left, *Mmp9*+/– is right. (A) SO-FG stained sections through the stabilized fracture site at 10 days confirm the presence of abundant new bone (arrows indicate *Oc* expression) and the absence of cartilage in the wild-type callus (no *ColIIa* signal is evident despite hybridization with this RNA probe). By sharp contrast, abundant cartilage forms in the *Mmp9*–/– callus regardless of stabilization of the bone segments, as shown by the expression of *ColIIa* (pink), *Oc* expression (black) is also detected in the peristeum, adjacent to the fracture site (arrows) and in the endosteum. (B) SO-FG staining of sections at a pin implant (*) at 10 days shows that although no cartilage is detected in wild-type animals, abundant cartilage (red) is present in *Mmp9*+/– animals. This cartilage is restricted to the peristeal surface (po), *Oc* expression (black) is localized both at the peristeal and endosteal (en) surfaces (arrows) in wild-type and *Mmp9*+/– animals. (C) SO-FG stained sections through the wild-type cartilage illustrate that if fractures are left unstable for 24 hours (left, 24h) and subsequently stabilized, they heal without evidence of cartilage (10 days; arrow indicates the healing fracture). However, fractures that are unstable for 48 hours (right, 48h) and subsequently stabilized tend to heal with abundant cartilage (red). (D) MMP9 immunostaining and double-staining with TRAP illustrate that MMP9 protein (brown) is detected by day 3 in the endosteal matrix (box 1), in inflammatory cells (box 2), in mesenchymal cells within the fracture gap and surrounding soft tissues (box 3), and in the peristeum (box 4). These MMP9-positive cells are TRAP-negative. TRAP-positive osteoclasts/chondroclasts are present at the epiphysial growth plates of the fractured bone (not shown), and in the cortical bone. bm, bone marrow; c, cortex. Scale bars: in A, C, 1 mm; in B,D (low magnification), 500 μm; in D (high magnification), 10 μm.

The *Mmp9*–/– fracture repair defect is caused by delayed vascularization of the cartilage callus

The delayed repair defect in *Mmp9*–/– mice resembles a hypertrophic non-union, a human condition characterized by persistent hypertrophic cartilage that is attributed to disruptions in the vascular network of the fracture callus (Einhorn, 1999). Based on the similarities between the mouse phenotype and the human condition, we investigated the extent to which vascularization was compromised in the *Mmp9*–/– callus. Because extracellular matrix remodeling and angiogenesis are closely linked during skeletal tissue development, we included molecular markers of both in this analysis. By 10 days post-fracture, the periphery of the wild-type callus was surrounded by MMP9-expressing, TRAP-positive osteoclasts, but these cells were absent from the *Mmp9*–/– callus (Fig. 5A). By day 14, osteoclasts had removed most of the wild-type cartilage callus and new bone occupied the space (Fig. 5A). By contrast, very few osteoclasts were detectable in the *Mmp9*–/– unremodeled cartilage callus (Fig. 5A). This failure to recruit TRAP-positive cells to the *Mmp9*–/– callus was paralleled by a delay in vascular invasion. Whereas PECAM-positive endothelial cells had accumulated at the periphery of the wild-type hypertrophic cartilage callus, very few endothelial cells were found adjacent to the *Mmp9*–/– hypertrophic cartilage (Fig. 5A). By day 14, endothelial cells invaded the wild-type callus cartilage, whereas in the *Mmp9*–/– cartilage the endothelial cells remained restricted to the periphery (Fig. 5A).

A delay in endothelial cell recruitment could indicate a delay in cartilage calcification. We performed a series of molecular and histological analyses to test this possibility, but failed to...
Fracture repair in the absence of MMP9

Fracture repair in the absence of MMP9
detect any defect in cartilage matrix mineralization (Fig. 3, and
data not shown). Another explanation for the
Mmp9–/– dependent delay in vascular invasion might be the lack, or
decreased expression, of an angiogenic signal or some part of
the angiogenic signaling machinery. We examined Mmp9–/–
calluses at multiple time points during fracture repair for
changes in the expression of Vegf and the VEGF receptors Flt1,
Flk1 and neuropilin 1 and 2. All of these molecules were
expressed within the Mmp9–/– callus (Fig. 5B, and data not
shown). The only difference we detected was in levels of
expression: the number of cells expressing Vegf was actually
higher in the Mmp9–/– callus, which was due to the persistence
of Vegf-positive hypertrophic cartilage (Fig. 5B) (see Colnot
and Helms, 2001). We also noted reduced Flk1 expression,
reflecting the paucity of endothelial cells in the Mmp9–/– callus
(Fig. 5B). Collectively, these data indicated that the Mmp9–/–
angiogenic defect was not caused by the failure of expression
of a potent angiogenic stimulator or its receptors during
skeletal repair. Another possible explanation was that MMP9
might be regulating the bioavailability of VEGF, as it does
during some disease processes (Bergers et al., 2000).

Exogenous VEGF rescues the Mmp9-dependent
skeletal repair defect

We reasoned that if functional VEGF is limiting during
Mmp9–/– skeletal repair, then exogenously applied VEGF
should rescue the Mmp9–/– defect. We injected recombinant
human VEGF protein (rVEGF), or PBS as a control, into the
fracture site of Mmp9–/– mice, beginning on day 6 (the time at
which Vegf is normally expressed in the callus), then daily for
three more days. We then compared the callus tissues of
Mmp9–/– mice that received rVEGF with those that received
PBS. By 14 days, we noted a clear difference between the two
groups: Mmp9–/– calluses treated with rVEGF had significantly
less cartilage than the controls (Fig. 6A,B). Not only did
rVEGF injection reduce the amount of hypertrophic cartilage

Fig. 5. Mmp9–/– fracture healing is hindered
by the reduction in chondroclasts/osteoclasts
and a delay in vascular invasion. 10 days, left
half; 14 days right half. (A) Top row, left
panels show that in wild-type mice at 10 days
post-fracture, Mmp9–expressing
chondroclasts/osteoclasts (inset shows cells
with characteristic ruffled borders) begin to
accumulate at the wild-type cartilage/bone
boundary (dotted red line). MMP9 is not
expressed in null-mutant mice. In an adjacent
section (middle row, left panels), TRAP
activity highlights the location of osteoclasts
in the wild-type callus (arrowheads), which
are largely absent from the Mmp9+/– callus. b,
bone; c, cartilage. In near-adjacent sections
(bottom row, left panels), PECAM-expressing
endothelial cells (arrow) have accumulated at
the border between wild-type hypertrophic
cartilage (c) and newly forming bone
(b; arrow). Few PECAM-positive cells are
detected in the Mmp9+/– callus (arrow). By 14
days post-fracture, abundant MMP9 protein
(upper right panels, brown) and TRAP activity
(middle right panels, arrowheads) indicate that
osteoclasts-mediated degradation of the
cartilage callus is well underway (dotted red
line indicates the cartilage-bone junction).
This degradation activity is associated with
increased vascular invasion as illustrated by
PECAM-expressing endothelial cells
(arrows). Although more PECAM-positive
cells are present at 14 days in the Mmp9+/–
callus (arrow), they are restricted to the edge
of the cartilage-bone junction (dotted red
line). (B) Tissue sections adjacent to those
analyzed in A were examined for the
expression of Vegf (top, fuchsia) and one of its
receptors, Flk1 (bottom, green). Vegf
transcripts were detected in late hypertrophic
chondrocytes in both wild-type and Mmp9+/–
calluses at 10 and 14 days post-fracture.
Transcripts for Flk1 were detected in
endothelial cells surrounding the cartilage callus of both wild-type and Mmp9+/– calluses. b, bone; c, cartilage. The dotted lines in B delimit the
boundary of the cartilage in the callus. Scale bars: in A (low magnification) and in B, 200 μm; in A (high magnification), 20 μm.
Fig. 6. rVEGF rescues the Mmp9−/− fracture repair defect. (A) Tissue sections from PBS- and rVEGF-injected Mmp9−/− calluses were stained with SO-FG at 10 and 14 days post-fracture to illustrate that, during the maturation phase of fracture healing (10 days), there is no difference in either the amount of cartilage (red) or the size of the callus (indicated by dotted black line). However, by 14 days post-fracture, the amount of cartilage was substantially reduced in Mmp9−/− mice that received rVEGF compared with those that received PBS. These histological observations were confirmed by histomorphometric measurements of the total callus volume and the cartilage volume (B). By 14 days post-fracture, there was a statistically significant decrease in both the total callus volume (left graft, asterisks) and in the cartilage volume (right graft, asterisks) of Mmp9−/− mice that received rVEGF (n=10) versus PBS (n=10), as assessed by ANOVA (P=0.03 and P=0.02, respectively; bars represent means±s.e.m.). (C) By 14 days post-fracture, substantially less hypertrophic cartilage was detected in the Mmp9−/− calluses that had been treated with rVEGF compared with their PBS counterparts. Left panels show the yellow ColX hybridization signal superimposed upon a tissue section stained with SO-FG. Higher magnification of area boxed in red illustrates that ossification was also accelerated by rVEGF when compared with PBS controls [Analine Blue, (AB) stain superimposed upon an adjacent tissue section stained with SO-FG]. Higher magnification of the area boxed in black illustrates that rVEGF induced substantially greater osteoclast-mediated degradation of the callus compared with PBS controls (arrowheads in upper and lower panels indicate TRAP immunostaining, red dotted line demarcates the boundary between the remaining hypertrophic cartilage and the newly forming bone). Higher magnification of this same region demonstrates that rVEGF resulted in an increased vascular invasion of the callus, as shown by the presence of PECAM-positive endothelial cells penetrating the Mmp9−/− hypertrophic cartilage callus (arrows, bottom right panel). Conversely, endothelial cells remain at the periphery of the PBS-injected Mmp9−/− callus. Scale bars: in A,C (SO/ColX panel), 1 mm; in C (TRAP and PECAM panels), 200 μm.

(ColX; Fig. 6C), it also resulted in an increased synthesis of bone matrix (Fig. 6C, boxed area in red, AB). rVEGF treatment also resulted in an increase in TRAP activity (Fig. 6C) and in the numbers of endothelial cells that invaded the Mmp9−/− callus (Fig. 6C, arrows). These observations demonstrated that rVEGF compensated for the lack of Mmp9 by stimulating the recruitment and/or differentiation of the three cell types that express the VEGF receptor: chondroclasts/osteoclasts that remodel hypertrophic cartilage, endothelial cells that form vascular channels, and osteoblasts that generate new bone at the injury site.

**DISCUSSION**

**Fetal skeletogenesis and adult skeletal repair: two sides of the same coin?**

The similarities between Mmp9−/− skeletal defects that emerge during fetal development and those that occur during adult repair offer the strongest evidence to date that the same molecular mechanisms are employed to achieve bone formation, regardless of age. At a histological level, fracture healing closely resembles fetal skeletal development. Mesenchymal cells alter their extracellular matrix and cell-cell contacts, and aggregate, thus forming condensations that will either form cartilage or bone (Le et al., 2001; Probst and Spiegel, 1997). The molecular programs regulating chondrogenesis and osteogenesis are also conserved (Ferguson et al., 1999; Vortkamp et al., 1998). These observations have led us and others to hypothesize that fracture healing recapitulates fetal skeletogenesis (Ferguson et al., 1998; Vortkamp et al., 1998). However, the differences between a fetal developmental program and an adult reparative process cannot be underestimated. Inflammation plays a crucial role in healing, as do mechanical forces that act upon cells in the fracture callus (Carter et al., 1998; Einhorn et al., 1995). The presence, or paucity, of stem cells in an adult, and their developmental potency, is certain to influence adult healing as well. The contributions of these factors to skeletal tissue regeneration remain largely unknown and most probably under appreciated. We undertook this study to gain an appreciation for the roles of one MMP in the
process of skeletal tissue formation. Our previous work had uncovered a role for MMP9 in fetal skeletogenesis (Vu et al., 1998), and in this study we elucidated additional functions for MMP9 in adult skeletal repair.

**MMP9 regulates hypertrophic cartilage angiogenesis**

Our data indicate that MMP9 stimulates angiogenesis of the hypertrophic cartilage callus (Fig. 5). One model that may account for the Mmp9−/− repair defect is that the loss of MMP9 affects the bioavailability of a potent angiogenic molecule, VEGF. The fact that VEGF cannot accelerate healing in wild-type animals (data not shown) but can rescue the phenotype of Mmp9−/− mutants indicates that VEGF is a limiting factor in the absence of MMP9, which suggests an interaction between the two molecules. MMP9 may regulate vascular invasion by releasing VEGF that is bound to the hypertrophic cartilage matrix. Once released, VEGF could bind to its receptors on endothelial cells, osteoclasts and osteoblasts (Nakagawa et al., 2000; Risau, 1997), stimulating their migration and activity at the fracture site. Precisely how MMP9 and VEGF coordinate the activity of three cell types (osteoblasts, osteoclasts/chondroclasts and endothelial cells) during the process of skeletal repair is still a puzzle. Other angiogenic factors are clearly involved in skeletal repair, as the Mmp9−/− defect is transient (Fig. 2). Likewise, other proteases may mediate angiogenic activity (Yamagiwa et al., 1999), as is suggested by skeletal defects in M1/Mmp−/− mice (Holmbeck et al., 1999).

Understanding the identities and contributions of other angiogenic regulators and proteases will undoubtedly provide a more complete view of how extracellular matrix remodeling and angiogenesis are synchronized during skeletal tissue regeneration.

**MMP9 mediates an angiogenic switch during bone regeneration**

The transition of an anti-angiogenic tissue, cartilage, to an angiogenic one, bone, is a crucial feature of adult bone regeneration (Fig. 5). Tumors undergo a similar angiogenic switch, heralding their progression to a more aggressive form of the disease (Bergers et al., 2000; Huss et al., 2001; Semenza, 2000). Angiogenic switches also occur during the development of other tissues, such as the lung and the mammary gland (Muratore et al., 2000; Pepper et al., 2000).

During fracture healing, a failure of cartilage to undergo an angiogenic switch is associated with a pathological skeletal condition known as hypertrophic non-union. The Mmp9−/− fracture healing phenotype is a prime example of how a genetic mutation can impair adult healing in a phenotypically normal individual. As illustrated by the Mmp9−/− mouse, a complete loss of MMP9 can be compatible with life and normal reproduction; humans with mutations in MPP2 are also viable (Martignetti et al., 2001). It will be interesting to ascertain if a genetic predisposition underlies human skeletal healing defects that are associated with perturbations in vascular remodeling, and if biologically based therapies can be developed for the treatment of such recalcitrant skeletal injuries.

**MMP9 and cell fate specification**

MMP9 plays another role during the early phase of stabilized skeletal repair. Although stabilized skeletal injuries heal through intramembranous ossification in wild-type mice, the same injuries heal through endochondral ossification in Mmp9−/− mice (Fig. 4). There are a number of possible explanations for this curious phenotype. For example, MMP9 may participate in the mobilization of osteoprogenitor cells from the periosteum and/or from the bone marrow. In the bone marrow, MMP9 is involved in the mobilization and activation of hematopoietic and endothelial stem cells, and MMP9-expressing cells may in fact share a common lineage with these cells (Heissig et al., 2002). The Mmp9−/− intramembranous ossification defect may therefore be caused by a failure to release and/or activate osteoprogenitor cells from either the periosteum or the bone marrow cavity.

Alternatively, the differentiation of skeletal progenitor cells may be delayed in Mmp9−/− stabilized injuries. MMP9 appears to release VEGF from its extracellular matrix stores (Bergers et al., 2000), and a failure to activate one of the VEGF receptors on osteoprogenitor cells (Deckers et al., 2000; Midy and Plouët, 1994) may ultimately affect the rate at which these cells differentiate. As the Mmp9−/− ossification defect is transient, it is highly likely that other signals also participate in osteoblast differentiation during repair. The nature of these compensatory signals is, at present, unknown.

Another possibility is that the MMP9 mutation may disrupt the formation of an intact vascular network during the initial stages of stabilized repair. A stable environment has long been thought to favor the formation of an intact vascular network (Claes et al., 2002; Glowacki, 1998), and this oxygen-rich environment appears to support the differentiation of skeletal progenitor cells into osteoblasts. One possibility is that a vascular network fails to form around the Mmp9−/− stabilized fracture or the Mmp9−/− implant site (Fig. 3). However, we think this is the least likely explanation, as rVEGF failed to rescue the Mmp9−/− stabilized healing defect. Other angiogenic molecules, such as CTGF, the angiopoietins (Street et al., 2002; Thurston et al., 1999) and the fibroblast growth factors (Kawaguchi et al., 1994), may regulate the establishment of a vascular network during early stages of fracture healing.

**The mechanical environment influences progenitor cell fate decisions**

Until recently (Henderson and Carter, 2002), it was thought that the most prominent difference between fetal and adult bone formation was the fact that fetal endochondral ossification takes place independently of mechanical stimuli, whereas the fate of progenitor cells in the fracture callus depends upon the mechanical environment. Clinical examination, biomechanical data (Carter and Giori, 1991; Carter et al., 1998) and biological observations (Claes et al., 1998; Park et al., 2003; Thompson et al., 2002) clearly indicate that stabilization favors the differentiation of cells into osteoblasts, whereas the lack of stabilization leads initially to the production of chondrocytes. In Mmp9−/− mice, the majority of progenitor cells in the fracture callus adopt a chondrogenic fate regardless of whether the fracture is stabilized or not. Precisely how the mechanical environment and MMP9 function are coordinated is still open to speculation. The differences in stabilized and non-stabilized fracture healing may be attributable to the extent of tissue disruption, and therefore may not be directly comparable. For example, stabilized fractures and implant models may be characterized by minimal tissue disruption following acute injury,
whereas non-stabilized fractures may be subjected to continued tissue disruption. These differences might trigger signaling pathways that are not induced in a stable mechanical environment and therefore healing between the two models may be difficult to compare. However, there is no current data to support or refute the hypothesis that the extent of mechanical disruption leads to the activation of different molecular pathways.

In vitro studies show that stretch and compression forces applied to tissues from a fracture site upregulate the expression of some MMPs (Haas et al., 1999; Rubin et al., 1999), and this upregulation occurs through cell adhesion molecules, such as integrins (Spessotto et al., 2002; Sugiuira and Berditchevski, 1999). Thus it is possible that different MMP-induced responses to varying mechanical stimuli may modulate the activity of different sets of growth factors, analogous to the case of hematopoietic reconstitution (Heissig et al., 2002). Probable candidate growth and differentiation factors include VEGF (Villars et al., 2000), and members of the transforming growth factor β (Alliston et al., 2001; Bonevand and Dallas, 1994; Centrella et al., 1994), bone morphogenetic protein (Fromigue et al., 1998), hedgehog protein (Pola et al., 2001; Spinella-Jaegle et al., 2001) and fibroblast growth factor (Liu et al., 2002; Scutt and Bertram, 1999) families. It is equally probable that subtle shifts in the balance of these and other growth factors subsequently affect the differentiation, or survival and proliferation, of osteo- or chondroprogenitor cells that populate the fracture site.

Inflammation and skeletal repair

Inflammation plays an important role in bone repair (Altmann et al., 1995; Banovac et al., 1995; Zhang et al., 2002). The mechanical environment influences the inflammatory response (Hankemeier et al., 2001), although the mechanisms by which this is achieved are unclear. MMP9 participates in the inflammatory response associated with skeletal injury, as demonstrated by the fact that neutrophils and macrophages strongly express the protein in the early fracture callus and in the implant site. The function(s) of MMP9 in the inflammatory response associated with the early stages of skeletal repair is still unclear. MMP9 released from neutrophils, mast cells and macrophages may be involved in remodeling the early fracture callus. Inflammatory cells expressing MMP9 may preferentially accumulate in a stable mechanical environment, which would result in the delivery of more cytokines to these sites of injury. In turn, these cytokines may stimulate osteogenesis to a greater degree than is observed in a non-stabilized fracture.

In conclusion, these data reveal new roles for MMP9 in fracture healing. The continued close scrutiny of the parallels, and differences, between fetal and adult programs of cell differentiation, extracellular matrix remodeling and angiogenesis will surely yield new insights into these crucial events in tissue regeneration.

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Fracture repair in the absence of MMP9


