CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Oncostatin M, an Inflammatory Cytokine Produced by Macrophages, Supports Intramembranous Bone Healing in a Mouse Model of Tibia Injury

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Accepted for publication
November 12, 2014.

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Different macrophage depletion strategies have demonstrated a vital role of macrophages in bone healing, but the underlying molecular mechanisms are poorly understood. Here, with the use of a mouse model of tibia injury, we found that the cytokine oncostatin M [OSM or murine (m)OSM] was overexpressed during the initial inflammatory phase and that depletion of macrophages repressed mOSM expression. In *Osm*^{-/-} mice, by micro-computed tomography and histology we observed a significant reduction in the amount of new intramedullar woven bone formed at the injured site, reduced number of Osterix⁺ osteoblastic cells, and reduced expression of the osteoblast markers runt-related transcription factor 2 and alkaline phosphatase. In contrast, osteoclasts were normal throughout the healing period. One day after bone injury, Stat3, the main transcription factor activated by mOSM, was found phosphorylated/activated in endosteal osteoblastic cells located at the hedge of the hematoma. Interestingly, we observed reduced activation of Stat3 in *Osm*^{-/-} mice. In addition, mice deficient in the mOSM receptor (*Osmr*^{-/-}) also had reduced bone formation and osteoblast number within the injury site. These results suggest that mOSM, a product of macrophages, sustains intramembranous bone formation by signaling through *Osmr* and Stat3, acting on the recruitment, proliferation, and/or osteoblast differentiation of endosteal mesenchymal progenitor cells. Because bone resorption is largely unaltered, OSM could represent a new anabolic treatment for unconsolidated bone fractures. (*Am J Pathol* 2015, 185: 765–775; <http://dx.doi.org/10.1016/j.ajpath.2014.11.008>)

With an aging population, fractures are becoming a common clinical problem, especially fractures associated with nonunion or delayed healing.¹ Each year, 2% of the population would be affected after a trauma, infection, cancer, or orthopedic implant, for example. Antiresorptive agents such as bisphosphonates efficiently reduce the risk of fracture through inhibition of bone resorption by osteoclasts, but subsequently bone formation by osteoblasts is suppressed.² Indeed, only a few truly anabolic bone therapies are available to induce bone reconstruction. Parathyroid hormone (PTH) or bone morphogenetic proteins are known to improve fracture healing in animal models, but their complexity of action on both bone formation and resorption and their relative inefficacy in clinical trials highlights our need for new anabolic agents.^{1,3,4}

Fracture healing is a multistep process that involves recruitment of mesenchymal progenitor cells and differentiation into osteoblasts to form a hard callus that is secondarily remodeled by osteoclasts.^{4,5} Different types of bone reconstruction can be observed, through either endochondral ossification, whereby a cartilaginous model is first produced and then mineralized, or intramembranous

Supported by INSERM, the region Pays de la Loire (CIMATH2 project), the French Agency for Biomedicine, and fellowship from le French Ministry of Research (P.G.).

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Disclosures: None declared.

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Figure 1 mOSM is expressed in the bone injury site. mRNA expression of indicated genes was analyzed by quantitative real-time RT-PCR in the mouse tibial injury model at different times after surgery. Gapdh was used as an invariant control. Similar results were obtained in another independent experiment. The **dotted lines** represent the expression amount without bone injury. Data are expressed as means $\pm$ SD. $n = 3$ mice for each time point. Alp, alkaline phosphatase; Bsp, bone sialoprotein; Ctsk, cathepsin K; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Opg, osteoprotegerin; mOSM, murine oncostatin M; Osx, osterix; Rankl, receptor activator of NF- κ B ligand; Runx2, runt related transcription factor 2; Tnf- α , tumor necrosis factor α ; Trap, tartrate-resistant acid phosphatase.

ossification, whereby the bone matrix is directly formed.^{4,5} Noncritical bone fractures heal mainly through intramembranous ossification, but, in the case of nonunion and unconsolidated fractures, endochondral ossification is largely involved. More recently, it has become clear that inflammation plays a key role in fracture repair. Indeed, during the first few days, a hematoma is formed with infiltration of inflammatory cells and secretion of cytokines and growth factors.⁶ In murine models, tumor necrosis factor α and IL-6 are produced at the fracture site within 24 hours of injury and are implicated in the recruitment and differentiation of mesenchymal progenitor cells to promote bone healing.^{1,7} However, inflammation must be tightly controlled and resolved, with a key role here for anti-inflammatory cytokines such as IL-10.⁸

In the field of osteoimmunology, the innate and adaptive immune systems were shown to regulate the bone tissue, and there is an intense crosstalk between bone and immune cells.⁹ In the absence of lymphocytes, fracture healing is accelerated, whereas depletion of macrophages prevents intramembranous bone healing.^{4,8} Macrophages thus appear to have a vital role in the regulation of bone formation during inflammation and bone injury. With the use of a high-throughput approach in culture models, we previously demonstrated that monocytes/macrophages secrete a major cytokine of the IL-6 family,

oncostatin M [OSM or murine (m)OSM], to promote osteogenesis in mesenchymal stem cells (MSCs).¹⁰ These results were confirmed in two other studies.^{11,12} In mice models, injection of recombinant mOSM enhances calvarial thickness, and overexpression of mOSM in long bone induces woven bone formation that resembles intramembranous bone healing.^{10,13} The bone phenotype of *Osm*^{-/-} mice was not yet published, but mOSM receptor (*Osmr*)-deficient mice were previously analyzed.¹³ *Osmr*^{-/-} mice have reduced number of osteoblasts and osteoclasts, resulting in osteopetrotic bones. Unfortunately, these mice were not used to assess the role of the Osm-Osmr signaling in fracture healing.

Here, with the use of a noncritical injury model in tibia of mice, we show that mOSM is expressed early during the inflammatory phase mainly by macrophages and that *Osm* or *Osmr* deficiency leads to delayed bone healing.

Materials and Methods

Murine Model of Bone Injury and Knockout Mice

All research involving animals was conducted according to the institutional guidelines and was approved by the French ethical committee CEEA.PdL.06 and by local veterinary

Figure 2 Macrophage depletion represses mOSM expression. **A–F:** Mice were treated with the macrophage-depleting agent clodro or control PBS liposome. Eight hours after tibial injury (dotted areas) animals were sacrificed, and tibial sections were stained for IBA1 (pan-macrophages in brown) (**A**, **C**, and **E**) or mOSM (**B**, **D**, and **F**). Representative images are shown in the bone injury site (defect) and the contralateral noninjured leg (control). **C** and **D** are enlarged images of the boxed areas in **A** and **B**. Asterisks indicate hematoma with eventual bone debris; black arrows, endosteal osteoblasts; blue arrows, osteocytes; red arrows, macrophages. **G:** mOSM, Il6, and Il1β mRNA expression was analyzed by quantitative real-time RT-PCR 8 hours after surgery. Hprt was used as an invariant control. Data are expressed as means ± SD. $n = 3$ mice per group (**A–F**); $n = 4$ mice in PBS + control, clodro + control, and PBS + defect groups (**G**); $n = 3$ mice in the clodro + defect group (**G**). * $P < 0.05$ versus PBS. Original magnification: $\times 20$ (**A** and **B**); $\times 40$ (**C–F**). Clodro, clodronate liposome; Hprt, hypoxanthine-guanine phosphoribosyltransferase; IBA1, ionized calcium-binding adapter molecule 1; mOSM, murine oncostatin M; PBS, phosphate-buffered saline.

services (license no. D-44015) and was performed under the supervision of authorized investigators. The generation and genotyping of *Osm* and *Osmr*^{-/-} mice, backcrossed onto C57BL/6, was described previously^{14,15} and were kindly provided by Dr Atsushi Miyajima (University of Tokyo, Japan). All experiments were conducted with wild-type (WT) and *Osm*^{-/-} (or *Osmr*^{-/-}) littermates obtained from our breeding colony by using heterozygous breeding pairs. Mice were housed in pathogen-free facilities and under conditions controlled for light (12 hours light/dark cycle), temperature (22°C to 25°C), and humidity (50% to 60%).

For noncritical bone injury, 7- to 8-week-old mice were anesthetized by inhalation of an isoflurane–air mixture (2%, 0.2 L/minute) and injected with 20 μg/kg Buprecaire (Animalcare, Dunnington, UK). Hair was removed from the left hindlimb, and an incision was made in the skin over the medial part of the proximal tibia. Soft tissue was removed, and a hole (1.0 mm in diameter) was created through cortical and medullar bone (the opposite side of cortical bone was preserved) by using a 19-gauge needle attached to an electric drill (Surgic XT; NSK, Paris, France). Because no differences were observed in bone

healing between male and female mice, both sexes were used indifferently.

Macrophage Depletion Using Clodronate Liposome

Mice were treated daily by intraperitoneal injection of 10 μ L/g clodronate or control phosphate-buffered saline (PBS) liposomes (ClodronateLiposomes.com; Haarlem, The Netherlands) as described previously.^{4,16} After 3 days, the bone injury was performed, and mice received an additional 50 μ L of intradefect injection of clodronate or PBS liposomes. Animals were sacrificed 8 hours later for immunohistochemistry and real-time PCR analysis.

microCT

Bone architecture was analyzed at the indicated time on anesthetized animals [8% xylazine (Rompun; Bayer, Deerfield, IL) and 13% ketamine (Imalgène 500; Merial, London, UK) in PBS; 100 μ L/10 g] by using the high-resolution SkyScan-1076 X-ray micro-computed tomography (microCT) system for small animal imaging (SkyScan, Kartuizersweg, Belgium). All tibias were scanned with the same variables (pixel size, 18 μ m; 50 kV; 0.5-mm Al filter). The relative trabecular bone volume and other trabecular variables (number, thickness, and separation) were quantified with the SkyScan CtAn software version 1.13. For the bone phenotype of *Osm* or *Osmr*^{-/-} mice, the metaphyseal secondary spongiosa of the proximal tibia of 7- to 8-week-old littermates was analyzed. For bone defect experiments, the area of interest corresponded to the injured area.

Real-Time PCR

After sacrifice by CO₂ inhalation, soft tissue of left hindlimbs was removed (soft or hard callus around bone defect was preserved). Total RNA was extracted from bone defect-containing tissue samples of 0.5 cm long by using TRIzol reagent and DI25 Ultra-Turrax (IKA, Staufen, Germany) on ice and at the indicated time after surgery. First-strand cDNA was synthesized from 1 μ g of total RNA by using ThermoScript RT-PCR System (Invitrogen Life Technologies, Cergy-Pontoise, France). The real-time PCR contained 10 ng of reverse-transcribed total RNA, SYBR green buffer (Bio-Rad, Marnes-la-Coquette, France), and 300 nmol/L previously described primers.^{10,17,18} Quantitative PCRs were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). Analysis was performed with glyceraldehyde-3-phosphate dehydrogenase or hypoxanthine-guanine phosphoribosyltransferase used as invariant controls and results were expressed as 2^{- Δ Ct}.

Histology and Immunohistochemistry

Hindlimbs were fixed in 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO) at room temperature and then

Figure 3 Delayed bone healing in *Osm*^{-/-} mice. **A:** At indicated days after injury (D3, D7, and D14), tibias from *Osm*^{-/-} and WT littermates were analyzed by X-ray microCT scan. Representative sagittal sections are shown. **B:** Quantification of newly formed bone within the injury site by microCT (BV/TV). **C** and **D:** Relative Tb.Th (**C**) and Tb.N (**D**) of the intramedullary woven bone. Data are expressed as means $\pm$ SEM. $n = 4$ to 9 mice in each group as indicated in **B**. * $P < 0.05$ versus WT. BV/TV, trabecular bone volume; microCT, micro-computed tomography; Tb.N, trabecular number; Tb.Th, trabecular thickness; WT, wild-type.

decalcified for 96 hours in 4.13% EDTA (Sigma-Aldrich) and 0.2% paraformaldehyde in PBS at pH 7.4 to 7.6 by using the KOS Microwave Histostation (Milestone, Kalamazoo, MI). Once decalcified, all samples were embedded in paraffin.

Sections (4 μ m thick) were stained by Masson trichrome staining as described previously.¹⁹ Tartrate-resistant acid phosphatase (Trap) staining was performed by 1-hour incubation in 1 mg/mL naphthyl phosphate and 1 mg/mL Fast Red Salt (Sigma-Aldrich) solution (pH 5.2). The counterstain was done with Mayer hematoxylin. Immunostaining was performed as described previously^{10,18} with the following primary antibodies at the indicated concentration: rabbit anti-osterix (OSX; dilution 1:800; Abcam, Cambridge, MA), goat anti-ionized calcium-binding adapter molecule 1 (IBA1; dilution 1:1000;

Figure 4 *Osm*^{-/-} mice have reduced number of osteoblasts within the injury site. **A** and **B**: Seven days after injury animals were sacrificed, and tibial sections were stained with Masson trichrome (collagen fibers stained in green) or for Osterix (osteoblastic cells stained in brown) and Trap (osteoclasts stained in red). Representative images are shown. Injury sites are indicated by dotted areas. The upper panels in **B** are enlarged images of the boxed areas in **A**. **C**: Histomorphometric values within the injury site were calculated at indicated time points after bone injury by using the NDPView2 software for bone formation parameters (N.Ob/B.Pm and Ob.S/BS) and for bone resorption parameters (N.Oc/B.Pm and Oc.S/BS). The N.Osx⁺/T.Ar and the Oc.Ar/T.Ar were quantified with ImageJ software. **D**: mRNA expression of indicated genes was analyzed 7 days after injury by real-time PCR. Hprt was used as an invariant control. The dotted line represents threshold under which *Osm* is considered not expressed. Data are expressed as means ± SEM. *n* = 3 mice in each group at each time point, except *n* = 4 mice in the WT group at day 7 (**C**); *n* = 4 mice in each group (**D**). **P* < 0.05 versus WT. Original magnification: ×5 (**A**); ×40 (**B**). Alp, alkaline phosphatase; Ctsk, cathepsin K; N.Ob/B.Pm, number of osteoblasts/bone perimeter; N.Oc/B.Pm, number of osteoclasts/bone perimeter; N.Osx⁺/T.Ar, number of Osterix⁺ osteoblastic cells/tissue area; Ob.S/BS, osteoblast surface/bone surface; Oc.Ar/T.Ar, osteoclast area/tissue area; Oc.S/BS, osteoclast surface/bone surface; Opg, osteoprotegerin; mOSM, murine oncostatin M; Rankl, receptor activator of NF-κB ligand; Runx2, runt related transcription factor 2; Trap, tartrate-resistant acid phosphatase; WT, wild-type.

Abcam), goat anti-mOSM (dilution 1:50; R&D Systems, Minneapolis, MN), goat anti-Osmr (dilution 1:100; R&D Systems), and rabbit anti-phospho-Stat3 (Tyr705; dilution 1:400; Cell Signaling Technologies, Beverly, MA). Histomorphometric values were calculated after Trap staining, within the secondary spongiosa or in selected area in bone defect, using the NDPView software version 2.3.1 (Hamamatsu, Massy, France) for bone formation parameters [number of osteoblasts/bone perimeter (N.Ob/B.Pm), osteoblast surface/bone surface (Ob.S/BS)] and for bone resorption parameters (N.Oc/B.Pm and Oc.S/BS) following the recommendations of the American Society for Bone and Mineral Research.²⁰

The number of osterix+ osteoblastic cells/tissue area (N.Osx+/T.Ar) and the osteoclast area/tissue area (Oc.Ar/T.Ar) were quantified with the ImageJ software version 1.43u (NIH, Bethesda, MD) as described.¹⁰ All analyses were assessed after scanning the slides with the Nano-Zoomer 2.0-RS system (Hamamatsu).

Statistical Analysis

Results were analyzed with Mann-Whitney or unpaired *t*-test by using GraphPad InStat software version 3.02 (GraphPad Inc., San Diego, CA). Results are given as means $\pm$ SD or SEM as indicated, and *P* < 0.05 was considered significant.

Results

OSM Is Expressed in the Bone Injury Site

We used a tibia injury model in C57BL/6 mice in which bone heals mainly through intramembranous ossification.⁴ In this model, the standard phases of stabilized fracture healing were observed, that is, an initial inflammatory phase within the first 3 days, a bone modeling phase with intramedullar woven bone deposition that culminates around day 7, and later a bone remodeling phase in which osteoclasts largely resorbed the woven bone (Supplemental Figure S1). Rarely, cartilaginous tissues were observed in periosteal area but never in the medullar cavity (not shown). Interestingly, mOSM mRNA amount was induced during the initial inflammatory phase (8 hours after injury), together with Il-6 and Il-1 β but not tumor necrosis factor α (Figure 1). Expression of the anti-inflammatory cytokine IL-10 peaked later at day 3, whereas typical osteoblast and bone formation markers (runt-related transcription factor 2, *Osx*, alkaline phosphatase, and bone sialoprotein) arose at approximately days 5 to 7. Osteoclast-related markers [receptor activator of NF- κ B ligand (*Rankl*), cathepsin K, and *Trap*] were highly induced at day 10 after injury (Figure 1). Expression of osteoprotegerin (*Opg*), a *Rankl* decoy receptor, followed the expression profile of bone formation markers; the *Rankl*/*Opg* ratio being thus strongly induced after day 10 (Figure 1).

Figure 5 Activation of Stat3 is reduced in *Osm*^{-/-} mice. Activation of Stat3 was analyzed 1 day after injury by immunohistochemistry by using an anti-P-Stat3 antibody. Representative images from two different animals are shown. A serial section is shown for Osterix staining. The insets (boxed areas) are enlarged images of dotted boxes. Asterisks indicate hematoma with eventual bone debris. *n* = 3 mice in each group. Original magnification: $\times 10$ (main images); $\times 40$ (insets). P-Stat3, phospho-Stat3; WT, wild-type.

OSM Expression Is Repressed by Macrophage Depletion

Efficient macrophage depletion during 7 to 8 days or more is known to repress physiologic bone formation, PTH bone anabolic actions,^{21,22} or bone reconstruction by osteoblasts in this tibia injury model,⁴ and we next wondered whether it would also repress mOSM expression. As revealed by immunohistochemistry with the pan-macrophage marker IBA1, macrophages were located throughout the bone marrow and eventually in contact with the endoste (Figure 2, A, C, and E). Three days after treatment with the macrophage-depleting agent clodronate liposome largely reduced the number of IBA1⁺ macrophages, whereas osteoblasts or osteocytes were not altered (Figure 2, A, C, and E and Supplemental Figure S2). Eight hours after bone injury, IBA1⁺ macrophages were numerous at the periphery of the hematoma and again were efficiently depleted with clodronate liposomes (Figure 2, A, C, and E). At this location of the tibia (diaphysis) and early time point after bone injury (8 hours), osteoclasts were rare in PBS or clodronate liposome-treated mice (not shown) (Supplemental Figure S1). Therefore, this short-term treatment with clodronate liposome resulted in a specific depletion of macrophages in the bone marrow and in the bone injury site, osteoblasts and osteocytes being unaltered, and osteoclasts being almost absent.

By immunohistochemistry, mOSM protein was found in the cytoplasm of cells throughout the bone marrow and in the periphery of the hematoma (Figure 2, B, D, and F). mOSM⁺ cells had a structure compatible with IBA1⁺ macrophages and were also drastically depleted with clodronate liposomes (Figure 2, B, D, and F). As described previously,¹³ osteoblasts and osteocytes were also found to express mOSM, but they were not modified after this short treatment with clodronate liposome (Figure 2F). Depletion of macrophages repressed the early induction of mOSM mRNA expression 8 hours after bone injury, and similar results were also observed for Il-6 or Il-1b expression (Figure 2G). Together, these results strongly suggested that soon after bone injury macrophages were major cell sources of these inflammatory cytokines. Note that clodronate liposomes did not ablate mOSM expression in the absence of bone injury (Figure 2G), suggesting that other cells such as osteoblasts or

osteocytes could be implicated in the basal expression of this cytokine.

Osm Deficiency Leads to Delayed Bone Reconstruction

To study the potent role of mOSM in bone healing, we next used *Osm*-deficient mice. On the basis of microCT, quantitative bone histomorphometry, and immunohistochemistry, *Osm*^{-/-} mice appeared to have a grossly normal bone phenotype by 7 to 8 weeks of age (Supplemental Figure S3). Compared with WT littermates, trabecular bone volume, thickness, and number and osteoblast or osteoclast number and surface were identical, and only a slight decrease in the trabecular separation was noticed. However, 7 to 9 days after tibial injury, we observed a significant reduction in the amount of new intramedullar bone formed at the injured site for *Osm*^{-/-} mice by microCT (Figure 3). By day 12 to 14 after injury, corresponding to the remodeling phase, the intramedullar bone was reduced and the cortical bone was formed in both WT and *Osm*^{-/-} mice, and, overall, no difference was found anymore in bone volumes between the two genotypes (Figure 3, A and B). Reduced new intramedullar bone in *Osm*^{-/-} mice at day 7 to 9 was because of reduced thickness of the newly formed bony trabeculae, trabecular number being not statistically different (Figure 3, C and D).

Masson trichrome staining revealed a reduced amount of collagen deposition in the injured tibia of *Osm*^{-/-} mice at day 7 (Figure 4A). The intramedullar new woven bone appeared as an irregular network of bony trabeculae, and it was reduced and less dense in *Osm*^{-/-} mice. As early as day 3 until day 14, there was also a significant reduction in osteoblast number (N.Ob/B.Pm) and surface (Ob.S/BS), and in the number of *Osx*⁺ osteoblastic cells (N.*Osx*⁺/T.Ar) in *Osm*^{-/-} mice (Figure 4, A–C). *Osx*⁺ cells were mainly osteoblasts lining the bony trabeculae, but pre-osteoblasts (not in contact with bone) or osteocytes (embedded into bone matrix) were eventually also positive for *Osx* staining (Figure 4B and Supplemental Figure S1). In contrast, *Trap*⁺ osteoclast number (N.Oc/B.Pm), surface (Oc.S/BS), or area (Oc.Ar/T.Ar) was normal throughout the healing period in *Osm*^{-/-} mice (Figure 4, B and C). At day 7, mRNA amounts of runt-related transcription factor 2, alkaline phosphatase, and the *Rankl*/*Opg* ratio were all reduced, and cathepsin K and *Il-6* amounts were normal, whereas *Il-1β* amount was elevated in *Osm*^{-/-} mice (Figure 4D). Together, these results indicated that *Osm* deficiency led to a transient delay only in the initial modeling phase of the bone healing process, with reduced woven bone apposition by osteoblasts. In contrast, bone remodeling and osteoclasts were not affected despite altered expression of inflammatory/osteoclastogenic cytokines (*Rankl*/*Opg* and *Il-1β*).

Stat3 Is Activated during the Initial Inflammatory Phase

STAT3 is the main transcription factor activated by OSM and is necessary for this cytokine to induce osteogenesis in

MSCs.¹⁰ One day after bone injury, Stat3 was found phosphorylated and thus activated in a discrete population of endosteal cells located at the edge of the hematoma (Figure 5). On serial sections, these cells stained positive for *Osx* and were therefore of the osteoblastic lineage (Figure 5 and Supplemental Figure S1). Interestingly, we observed reduced activation of Stat3 in *Osm*^{-/-} mice (Figure 5), indicating that mOSM was implicated early in activation of Stat3 in endosteal osteoblastic cells. At later time points, Stat3 activation was low in WT or *Osm*^{-/-} mice, especially in mature osteoblasts in contact with the newly formed woven bone (data not shown).

Osmr Deficiency Leads to Reduced Bone Healing

In human MSCs, OSM recruits a receptor composed of Gp130 and OSMR to activate STAT3 and osteogenesis.¹⁰ Murine osteoblasts also express the *Osmr*,¹³ and we determined that macrophage depletion with clodronate liposome did not alter *Osmr* expression in osteoblasts (Supplemental Figure S2). As observed with *Osm*^{-/-} mice, 7- to 8-week-old *Osmr*^{-/-} mice had normal bone architecture, osteoblasts, and osteoclasts (Supplemental Figure S3). However, 7 days after tibia injury, they had reduced woven bone formation (Figure 6A), osteoblast number and surface, and reduced number of *Osx*⁺ osteoblastic cells within the injury site (Figure 6, B–D), a result similar to the one observed with *Osm*^{-/-} mice (Figures 3 and 4). Unexpectedly, *Osmr*^{-/-} mice also had reduced osteoclast number, surface, and area compared with WT littermates in this bone injury model (Figure 6, C and D), in contrast to *Osm*^{-/-} mice which had normal osteoclasts (Figure 4).

Discussion

The innate immune system is activated within a few hours after bone injury, with platelets, neutrophils, and macrophages being the first cells present in the inflamed fractured site. At that time, a wide variety of inflammatory cytokines, growth factors, and chemokines are released to further fuel leukocyte recruitment and to start bone healing through activation of mesenchymal progenitor cells. With the use of the mouse model, we show that mOSM is part of this early anabolic cytokine cocktail with an important role in the initial phase of bone apposition, that is, bone modeling, whereas the subsequent remodeling phase does not implicate this cytokine. Preliminary results also indicated that OSM is highly expressed in human fractured bones (data not shown). Although additional experiments are compulsory, it is tempting to speculate that OSM also has a role in human bone healing.

Macrophages and Bone Healing

Macrophages are detected early during human or mouse fracture healing, and their depletion by using clodronate

Figure 6 *Osmr*^{-/-} mice have reduced bone healing. **A:** Seven days after injury, quantification of newly formed bone within the injury site was realized by micro-computed tomography (relative bone volume). **B** and **C:** Seven days after injury, animals were sacrificed and analyzed for osterix (osteoblastic cells stained in brown) and Trap (osteoclasts stained in red) staining. Representative images are presented. Injury sites are indicated by dotted areas. **D:** Histomorphometric values within the injury sites were calculated with the NDPView2 software for bone formation parameters (N.Ob/B.Pm and Ob.S/BS) and for bone resorption parameters (N.Oc/B.Pm and Oc.S/BS). The N.Osx⁺/T.Ar and the Oc.Ar/T.Ar were quantified with ImageJ software. **E:** Proposed model for the role of mOSM during bone healing. Eight hours after bone injury, mOSM is secreted by macrophages (1). At 24 hours, the *Osmr*-Stat3 or other pathways are activated in endosteal osteoblastic cells (2). At 3 to 7 days, this leads to sustained proliferation, migration, and/or differentiation of osteoblasts, and thus enhanced intramembranous bone formation (3). Data are expressed as means ± SEM. *n* = 4 WT mice (**A**); *n* = 3 *Osmr*^{-/-} mice (**A**); *n* = 4 mice in each group (**D**). **P* < 0.05 versus WT. Original magnification: ×5 (**B**); ×40 (**C**). BV/TV, trabecular bone volume; N.Ob/B.Pm, number of osteoblasts/bone perimeter; N.Oc/B.Pm, number of osteoclasts/bone perimeter; N.Osx⁺/T.Ar, number of osterix⁺ osteoblastic cells/tissue area; Ob.S/BS, osteoblast surface/bone surface; Oc.Ar/T.Ar, osteoclast area/tissue area; Oc.S/BS, osteoclast surface/bone surface; mOSM, murine oncostatin M; Trap, tartrate-resistant acid phosphatase.

liposome was shown to suppress bone deposition during bone healing.^{4,23} Until recently, however, the identity of the macrophage-secreted factor(s) that drive(s) bone reconstruction remained elusive. With the use of *in vitro* human primary cultures, we previously showed that the main bone anabolic factor produced by monocytes/macrophages is OSM.¹⁰ Here, with the use of a mouse tibia injury model, a macrophage-depletion strategy with clodronate liposome and *Osm* knockout mice, we propose that early after bone injury IBA1⁺ macrophages efficiently produce mOSM to promote bone repair (Figure 6E). However, this assumption needs to be confirmed, for example, by using mice with specific *Osm* deficiency in macrophages. Indeed, other cell types such as osteoblasts, osteocytes, T lymphocytes, or neutrophils are known to produce this cytokine,^{13,24,25} but after bone injury the main cell sources of mOSM appeared to be macrophages that are highly sensitive to clodronate liposome.

Anabolic Pathways for Bone Formation

OSM is not the sole anabolic agent implicated in bone healing and should act together with other important soluble mediators. Previous studies demonstrated that OSM acts in synergy with bone morphogenetic protein 2 to induce osteogenesis in human MSCs.¹² Through inhibition of sclerostin expression in osteocytes, mOSM could also release the Wnt signaling pathways to induce bone formation *in vivo*.¹³ Moreover, *Osmr* expression is induced by PTH and the anabolic effect of PTH is reduced in *Omsr*^{-/-} mice, suggesting important crosstalk between the OSM and PTH signaling pathways.²⁶ It should be noted that tumor necrosis factor α and OSM, only when applied together, increased alkaline phosphatase activities and *in vitro* calcification by vascular smooth muscle cells.²⁷ Future studies should certainly unravel how these bone anabolic agents act in network to sustain bone healing.

We and others previously described that two other IL-6-type cytokines have a role in induction of osteogenesis: IL-6 itself and leukemia inhibitory factor (LIF).^{10,28} All these cytokines activate the common Gp130 receptor chain and STAT3, and they most presumably induce additive or redundant signals during bone healing. Indeed, Gp130-deficient mice or mice with osteoblast-specific disruption of the Gp130 (*Il6st*) or *Stat3* gene have much more severe bone phenotype than *Osm* or *Osmr*^{-/-} mice, with reduced bone formation and osteopenia.^{29–32} Moreover, STAT3 mutations cause a rare human immunodeficiency disease called hyper-IgE syndrome that presents reduced bone mineral density and recurrent pathologic fractures.³³ We show here that young adult *Osm* or *Osmr*^{-/-} mice do not have an abnormal bone phenotype, but after bone injury these mice have reduced bone healing, with reduced number of osteoblastic cells and reduced early activation of Stat3 in osteoblastic endosteal cells. These results suggest that mOSM does not have a significant role during bone development or physiologic bone remodeling in young adult mice, but after bone injury this cytokine is highly expressed and has an important role in the activation of mesenchymal progenitor cells.

Other studies described that *Osmr*^{-/-} mice are osteoprotic with reduction in osteoblasts and osteoclasts.¹³ This discrepancy with our results could be explained by differences in animal housing and breeding, but these protocols are rarely published and thus difficult to compare. In addition, *Osmr*^{-/-} neonates were already shown to have normal osteoblasts or osteoclasts (as in the present study that used 7- to 8-week-old mice), and only 12-week-old *Osmr*^{-/-} mice had altered bone cells,¹³ suggesting that reduced bone remodeling is associated with aging in *Osmr*^{-/-} mice. Importantly, *Osmr*^{-/-} mice also develop adipose tissue inflammation, insulin resistance, and obesity by 16 to 35 weeks of age,³⁴ conditions that can have important confounding effects on bone cells. Similarly *Il6*-deficient mice, despite having a normal bone phenotype, develop osteopenia with aging and have delayed callus mineralization in the early stages of fracture healing.^{7,35} More recently, it was shown that mice with osteoblast-specific disruption of *Gp130* do not have osteopenia until 12 weeks of age.³²

Intramembranous versus Endochondral Ossification during Bone Healing

Bone fractures can heal through different modes of ossification. In the bone injury model used in the present study, intramembranous bone apposition is mainly involved with the bone matrix being directly produced by osteoblasts. *Osm* deficiency impairs the early steps of intramembranous bone formation in this model, presumably by acting directly on the recruitment, proliferation, and/or osteoblast differentiation of mesenchymal progenitor cells initially located at the endosteal surface near the fractured inflamed site (Figure 6E). Indeed, early activation of Stat3, expression of several osteoblast markers, and collagen deposition are reduced in the absence of mOSM. However, mOSM does not have a role

later for cortical bone reconstruction, at a time when its expression is largely normalized. In culture, OSM can induce proliferation, osteogenesis, and bone matrix formation by purified MSCs.^{10,36} Injection of mOSM also induces an increase of mouse calvarial thickness, a model of intramembranous ossification,¹³ and OSM can enhance bony filling in a rabbit critical calvarial defect model.³⁷ In contrast to calvarial bones, long bones develop and can heal through both intramembranous and/or endochondral ossification, the later being predominant for large critical bone defects. We previously described that OSM could induce the terminal stage of chondrogenesis to the hypertrophic chondrocyte, which is a necessary step for endochondral bone formation especially at the growth plate.³⁸ In addition, mOSM overexpression induces abnormal cartilaginous growth (chondrophyte) formation and growth plate damage.³⁹ In *Osm* or *Osmr*^{-/-} mice, we did not observe alteration at the growth plate, long bone sizes were normal, and the amount of cartilaginous matrix formed after bone injury was comparable with WT littermates, although periosteal endochondral ossification was only rarely observed in this bone injury model (data not shown). Whether mOSM has an important role in consolidation of larger critical long bone defects through sustained intramembranous and/or endochondral ossification thus deserves further investigations.

mOSM and Bone Resorption

The last step of bone healing relies on bone remodeling. At this stage, osteoclasts resorb the woven bone, which is later replaced by more mature lamellar bone especially cortical bone. mOSM is able to induce osteoclast formation through increased Rankl expression in osteoblastic or chondroblastic cells.^{13,38} With the use of *Osmr*^{-/-} mice and a Lifr receptor (Lifr) antagonist, it was suggested that mOSM, when acting through the *Osmr*, induces Rankl, bone resorption, and bone formation, whereas mOSM, when acting through the Lifr, reduces sclerostin expression in osteocytes and thus induces bone formation independently of bone resorption.¹³ In addition, Lifr- or Lifr-deficient mice have a striking increased number of giant osteoclasts,^{40,41} suggesting that mOSM sustains bone resorption through the *Osmr* but restrains it through the Lifr. Another cytokine, Il-31, can also recruit and activate the *Osmr*, but it did not appear to have a role on bone cells.^{10,13} After bone injury, we observed that *Osmr*^{-/-} mice had reduced osteoclasts, but *Osm*^{-/-} mice, despite having a reduced Rankl/Opg ratio, had no alteration of osteoclasts or cathepsin K expression. These results would suggest that an additional receptor for mOSM, maybe the Lifr, effectively exists in mice and counteracts the *Osmr* effect on osteoclast formation. Another possibility, not excluding the first one, is that compensatory mechanisms maintain osteoclast formation in *Osm*^{-/-} mice, such as induced expression of Il-1 β (Figure 4D) which is a known inflammatory cytokine that directly stimulates osteoclastogenesis and survival of osteoclast precursors.⁴²

Together these data suggest that mOSM, when signaling through the *Osmr*-Stat3 or additional pathways, is an important factor in the first stages of bone healing (Figure 6E). This inflammatory cytokine appears unique in that it induces intramembranous bone formation but leaves bone resorption largely unaltered. mOSM could therefore represent an interesting new anabolic treatment for unconsolidated bone fractures.

Acknowledgments

We thank Prof. Jean-Claude Lecron (University of Poitiers, France) and Dr. Hans Yssel (INSERM U945, Paris, France) for providing *Osm*- and *Osmr*-deficient mice, Prof. François Gouin (INSERM U957, Nantes, France) for human bone samples, and Dr. Frédéric Lezot (INSERM U957, Nantes, France) for helpful advice on mouse breeding.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.11.008>.

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