Molecular mechanisms linking wound inflammation and fibrosis: knockdown of osteopontin leads to rapid repair and reduced scarring

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Previous studies of tissue repair have revealed osteopontin (OPN) to be up-regulated in association with the wound inflammatory response. We hypothesize that OPN may contribute to inflammation-associated fibrosis. In a series of in vitro and in vivo studies, we analyze the effects of blocking OPN expression at the wound, and determine which inflammatory cells, and which paracrine factors from these cells, may be responsible for triggering OPN expression in wound fibroblasts. Delivery of OPN antisense oligodeoxynucleotides into mouse skin wounds by release from Pluronic gel decreases OPN protein levels at the wound and results in accelerated healing and reduced granulation tissue formation and scarring. To identify which leukocytic lineages may be responsible for OPN expression, we cultured fibroblasts in macrophage-, neutrophil-, or mast cell–conditioned media (CM), and found that macrophage- and mast cell–secreted factors, specifically platelet-derived growth factor (PDGF), induced fibroblast OPN expression. Correspondingly, Gleevec, which blocks PDGF receptor signaling, and PDGF-Rβ–neutralizing antibodies, inhibited OPN induction by macrophage-CM. These studies indicate that inflammation-triggered expression of OPN both hinders the rate of repair and contributes to wound fibrosis. Thus, OPN and PDGF are potential targets for therapeutic modulation of skin repair to improve healing rate and quality.

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From late stages in fetal development onward, tissue repair is always accompanied by a robust recruitment of inflammatory cells to the wound site. Although this inflammatory response is clearly necessary for defense against invading microbes, recent studies in several organisms have shown that it is not essential for the tissue repair process, per se, and may even be causal of a fibrotic response (1). This may contribute not only to scarring of skin wounds, but may also underlie fibrotic pathologies in other tissues (2–4). Our recent analysis of tissue repair in PU.1-null mice, which lack neutrophils, macrophages, and mast cells, and therefore are genetically incapable of raising a standard inflammatory response, revealed a similar time course of wound healing to that of wild-type siblings, but PU.1-null wounds appear largely free of fibrosis as a consequence of this lack of inflammation (5). Our microarray studies of wound tissues from wild-type mice versus PU.1-null siblings revealed a portfolio of genes that are expressed only in the presence of a robust inflammatory response (6). One of these inflammation-dependent genes was osteopontin (OPN), which is expressed by wound granulation tissue fibroblasts coincident with the wound inflammatory response. These data, along with microarray studies in other models that have shown elevated OPN expression in fibrosis of other organ systems (e.g., lung [7] and liver [8]), support the idea that OPN may functionally contribute to inflammation-associated fibrosis. OPN is known to act both as a secreted chemokine-like protein (9), as well as part of an intracellular signaling complex (10), depending on the cellular context. It has many and diverse cellular effects, including the capacity to regulate cell adhesion, migration, and survival, as well as several functions in immune regulation, and a key role in the interplay between osteoblasts and osteoclasts as bone is remodeled (11); yet OPN-null mice develop normally and

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heal skin wounds with a similar time course to their wild-type siblings, with only a slight alteration in matrix architecture and collagen fibril formation in the deep layers of the wound (12).

We report the consequences of acute local knockdown of OPN on skin wound healing using antisense oligodeoxynucleotides (AS ODNs). We show that OPN AS ODN treatment leads to accelerated repair and decreased granulation tissue and scar formation. We go on to show that it is macrophages, specifically macrophage-derived platelet-derived growth factor (PDGF)-BB, which appear to be responsible for the inflammation-induced OPN expression in wound fibroblasts.

RESULTS AND DISCUSSION

To address our hypothesis that inflammation-dependent OPN expression in healing wounds may contribute to fibrosis and scarring, we sought to down-regulate its expression at the wound site. AS ODNs were designed and optimized (Supplemental materials and methods, Table S1, and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071412/DC1), and these, or a corresponding scrambled-sequence control ODN, were applied to in vivo 4-mm punch biopsy wounds on the backs of adult mice, at a concentration of 1 μM in 30% Pluronic gel. Standardly, OPN is expressed in the wound granulation tissue by 6 h after wounding, with levels peaking at 3 d, and then diminishing to prewound levels by

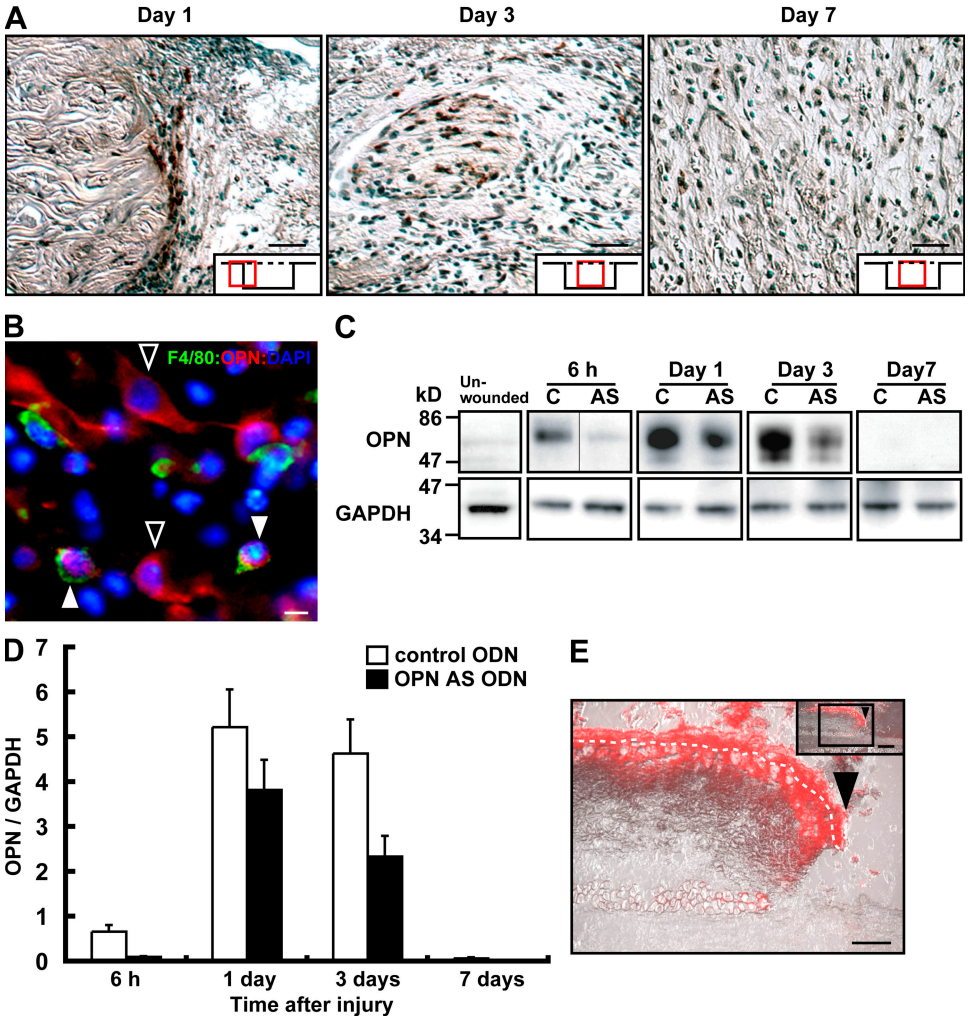

Figure 1. AS ODNs against OPN efficiently decrease OPN protein levels in in vivo mouse wounds. (A) Immunostaining for OPN (brown) reveals expression by wound connective tissue cells at day 1 and 3, which is resolved by 7 d after wounding. (B) Double immunofluorescence of wound tissue at 3 d reveals OPN expression (red) in both F4/80-positive (green; closed arrowheads) and -negative cells (open arrowheads). (C) Western blot analysis of OPN in control- and OPN AS ODN–treated wound tissue confirms the temporal profile of the immunostaining in A, and indicates that AS ODN treatment leads to depletion of OPN. (D) Band intensities were quantified by densitometric analysis on blots from six independent experiments, and values for the OPN/GAPDH ratio are shown (mean ± SEM). (E) Cy3-tagged OPN AS ODNs (red) were applied to wounds to assess to what depths and in which tissues OPN was being depleted (evaluated at 6 h). Dotted line indicates the epidermal/connective tissue interface, and the arrowhead indicates the epidermal wound edge. Inset shows a low-magnification view of the same section. Bars: (A) 50 μm; (B) 10 μm; (E) 100 μm; (E, inset) 200 μm.

7 d (Fig. 1 A). Our immunofluorescence studies with antibodies against OPN and the macrophage-specific marker F4/80 indicate that although some wound macrophages express OPN at a low level, the majority of OPN-positive cells are F4/80 negative, and thus are mesenchymally derived and most likely fibroblasts, although we cannot exclude that some may be pericytes (Fig. 1 B). Western blot analyses of wound tissues indicate that our AS knockdown reproducibly reduced OPN levels to ∼25% of control levels at 6 h, and 50% of control levels by 3 d after wounding (Fig. 1, C and D). This reduction was consistent with reduced immunohistochemical (IHC) staining for OPN in sections from AS ODN–treated wounds at these time points (unpublished data). To visualize the uptake and localization of delivered ODNs, we generated a Cy3-tagged OPN AS ODN that shows uptake in a non–cell lineage–specific manner, extending up to 1 mm back from the wound margin and in cell layers up to 300 μm deep within the wound (Fig. 1 E).

OPN AS ODN improves the rate and quality of wound healing

Macroscopic analyses of time-matched OPN knockdown versus control wounds showed that closure is markedly accelerated at early time points during repair in OPN AS

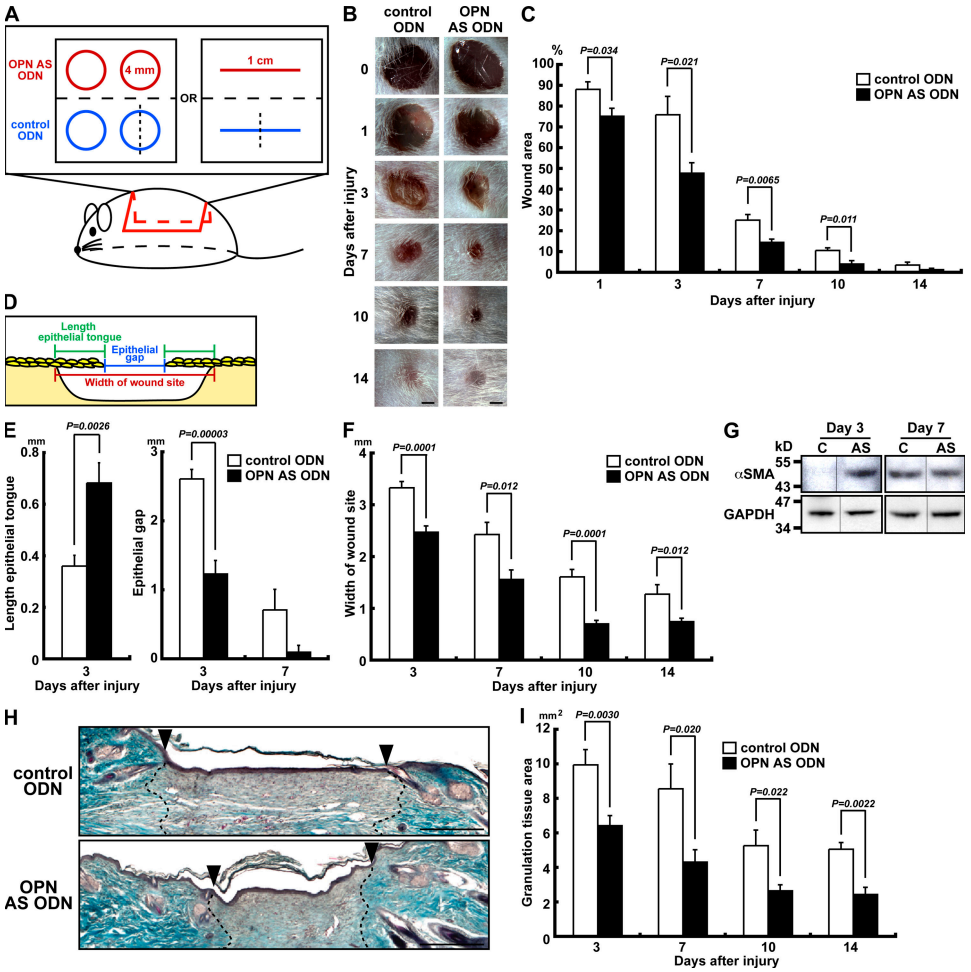

Figure 2. Skin wound healing is accelerated in OPN AS ODN–treated wounds. (A) A schematic diagram illustrating the location and dimensions of the full-thickness excisional and incisional wounds made to shaved dorsal skin of adult male ICR mice. Dotted lines indicate the axes of sections. (B) Macroscopic observation of excisional control- and OPN AS ODN–treated wounds at various time points after wounding. (C) The proportion of the wound remaining open relative to the initial wound area at each time point after the injury in control- (open bars) versus OPN AS ODN–treated (filled bars) wounds (mean ± SEM; $n = 6$). (D) Schematic to indicate measurements derived from histological sections. (E) Wound reepithialization at 3 and 7 d after wounding ($n = 9$ for both). (F) Connective tissue wound width remaining after 3, 7, 10, and 14 d of repair in control- versus OPN AS ODN–treated wounds (day 3 and 7, $n = 9$; day 10 and 14, $n = 6$). (G) Expression of α-smooth muscle actin at 3 and 7 d using Western blotting. Images shown are representative of four independent experiments. (H) Tissue from excisional wounds was harvested at 14 d after wounding and stained with Masson's Trichrome, and the extent (cross-sectional area) of granulation tissue was visualized and quantified at the mid-point of the wound (indicated by dotted line). (I) The area of granulation tissue was significantly decreased by OPN AS ODN treatment (filled bars), relative to control (open bars; day 3 and 7, $n = 9$; day 10 and 14, $n = 6$). Bars: (B and H) 1 mm.

ODN–treated wounds (Fig. 2, B and C), with a mean of 56% of complete closure achieved by 3 d, compared with 24% closure in controls. Histological analysis enables us to dissect the contributions made by reepithelialization and connective tissue contraction (Fig. 2 D) and indicates that reepithelialization, at least up to 3 d, is significantly faster in OPN AS ODN–treated wounds (Fig. 2 E), and that connective tissue wound width is less than controls at all stages during the healing process, suggesting enhanced connective tissue contraction from the earliest time points studied (Fig. 2 F). In concord with this, we see α-smooth muscle actin, which is a marker of contractile myofibroblasts, expressed on day 3 in OPN AS ODN–treated wound sites, which is earlier than in control wounds (Fig. 2 G). That AS ODN-treated wounds are capable of complete and efficient closure suggests that OPN does not play a pivotal role in skin repair, which is consistent with observations of wound repair in OPN-null mice (12), but opposes the interpretation that the lower OPN expression observed in a diabetic mouse model is functionally responsible for slower wound healing (13). Rather, it seems that OPN expressed by wound fibroblasts as a consequence of signals from inflammatory cells may hinder the rate of repair.

Sections of excisional wounds were stained with Masson's Trichrome to reveal the cross-sectional area of wound granulation tissue in the mid-wound region (Fig. 2 H). At all time points analyzed, OPN knockdown wounds had a considerably reduced cross-sectional area of granulation tissue compared with controls (at 10 and 14 d, 50% less; Fig. 2, H and I). These data implicate OPN expression at the wound site as being at least partially causal of the extensive granulation tissue formation and subsequent fibrosis that we see during repair of standard skin lesions. Our data do not distinguish whether OPN might be directly responsible for these changes or whether they are an indirect consequence of the reduced inflammatory response. Although expression of OPN has previously been correlated with several fibrotic conditions in other anatomical sites (e.g., lung [7], liver [8], and heart [14]),

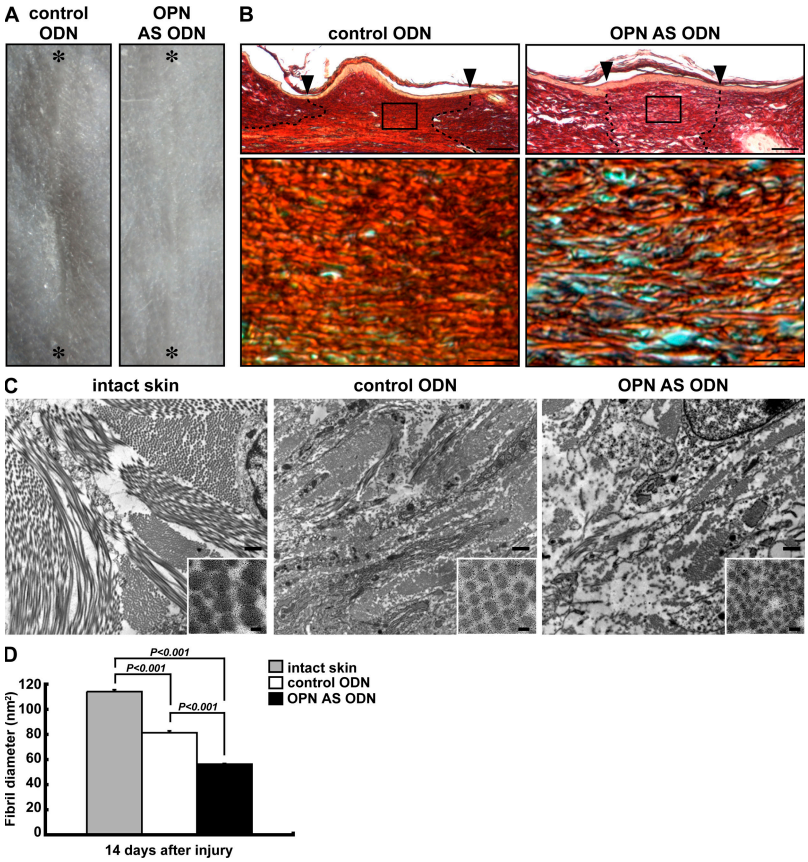

Figure 3. Altered collagen organization after OPN knockdown in wound sites. (A) Macroscopic views of scars of control- and OPN AS ODN–treated incisional wounds at 21 d after injury. *, wound edge. Images shown are representative of eight independent experiments. (B) Picrosirius red–stained sections of control- and OPN AS ODN–treated incisional wounds at 21 d. Representative of five independent experiments. High-magnification details from boxed areas indicated are differential interference contrast images. Arrowheads show wound edge. (C) TEM images of connective tissue from unwounded skin, control-, and AS ODN–treated 14 d wounds. Higher magnification insets illustrate differing collagen fibril diameters in these tissues. (D) Graphic representation of the mean diameter of collagen fibrils (± the SEM) from at least 5 fields of view of intact, unwounded skin, control ODN-, and OPN AS ODN–treated wounds; unwounded skin (striped bar, $n = 200$), control ODN (open bar, $n = 300$), OPN AS ODN (filled bar, $n = 150$). Bars: (B; low magnification) 100 μm; (B; high magnification) 25 μm; (C) 1 μm; (C, inset) 100 nm.

this study of skin repair is the first functional demonstration that OPN expression may contribute to fibrosis.

Altered collagen organization at OPN AS ODN–treated wound sites

To address whether knockdown of OPN influences the level of scarring resulting from the repair process, we turned to 1-cm incisional wounds, which were monitored daily for up to 3 wk. Macroscopic analyses 21 d after injury showed that the control wounds developed a thin linear white scar, which was noticeably reduced in OPN knockdown wounds (Fig. 3 A). To further compare developing scar tissue in control- and OPN AS ODN–treated wounds, Picrosirius red staining and transmission electron microscopy (TEM) analysis of 3 wk wound sections were undertaken to reveal gross collagen bundling patterns and ultrastructural analysis of individual collagen fibril diameter and density of fibrils in wound connective tissues, respectively (Fig. 3, B–D). The Picrosirius red histology revealed less dense "scar collagen" in the OPN AS ODN–treated wounds (Fig. 3 B), and our ultrastructural studies showed more "vacant" extracellular space (i.e., space between the collagen fibrils and between bundles), such that 153 $nm^2/\mu m^2$ of the cross-sectional area through the mid-region of AS ODN–treated wounds was "empty" of collagen fibers compared with 114 $nm^2/\mu m^2$ in control ODN-treated wounds, and 132 $nm^2/\mu m^2$ in unwounded skin (Fig. 3 C). Moreover, a significant reduction in collagen fibril diameter was observed in OPN AS ODN–treated wounds in comparison to controls, both of which displayed smaller diameters than unwounded skin (56 nm vs. 81 nm vs. 114 nm, respectively; Fig. 3, C and D). We speculate that these differences in collagen fibril diameter and bundling density at the wound site may reflect and give rise to the later differences in extent of fibrosis seen in OPN AS ODN–treated wounds.

Collagen fibrillogenesis is known to be regulated by several enzymes, including members of the ADAMTS family, BMP1, and the mammalian tolloids, as well as by interaction with extracellular matrix proteins (15). Our observations, together with data from OPN-null mice where the diameter of collagen fibrils at deep dermal wound sites are also smaller than in wild-type mice (12), suggest that this process may also be affected by OPN. Indeed, it has been reported that OPN interacts directly with collagen type I and III, which are the chief isoforms of intact and repairing skin connective tissue, respectively (16). Alternatively, OPN could affect collagen fibril formation indirectly, e.g., by altering fibronectin levels, which we demonstrate are reduced in AS OPN-treated wounds (Fig. 4 C). Studies of fibronectin-null fibroblasts have shown that the interaction of these two molecules is a key step in collagen assembly (17).

Leukocyte recruitment is significantly reduced in OPN AS ODN–treated wounds

Several lineages of inflammatory cells are recruited to sites of tissue damage with overlapping time courses during the repair process. Both neutrophils and macrophages are known to influence various aspects of repair, and we observe that the numbers of both of these cell types are significantly reduced as a consequence of OPN AS ODN treatment at times when their numbers are peaking in control wounds (i.e., 1 d for neutrophils and 7 d for macrophages; Fig. 4, A and B). Our data are consistent with other in vivo evidence that OPN may function as a chemoattractant for neutrophil and macrophage lineages (18). Mast cells, which are also implicated in tissue remodeling at wound sites (19), exhibit reduced numbers by comparison to control wounds at 10 d (Fig. 4, A and B). Associated with these reduced numbers of inflammatory cells at the wound site are reduced levels of mRNA encoding the potent inflammatory cell chemoattractant, Ccl2, as well as TGFβ1 (Fig. 4 C), suggesting that a positive feedback loop leading to amplification of the inflammatory episode may be disrupted by OPN knockdown.

Altered extracellular matrix deposition and angiogenesis in OPN AS ODN–treated wounds

The reduced extent of granulation tissue in OPN knockdown wounds and the associated down-regulation of TGFβ1 (a major fibrogenic factor during skin wound healing [20]) led us to investigate alterations in matrix deposition at the wound site. We show that fibronectin and type I collagen α1 mRNA are reduced at OPN AS ODN–treated wounds, and that the wound-induced metalloproteinase, MMP9, is also expressed at significantly lower levels from 7 d on (Fig. 4 C).

Angiogenesis is intimately associated with granulation tissue formation during wound repair. We have evaluated angiogenesis by IHC for platelet/endothelial cell adhesion molecule 1 (CD31) in control and OPN AS ODN wounds. OPN AS ODN led to significantly increased numbers of vessel lumens in the mid-granulation tissue zone at 7 d after wounding compared with control wounds (Fig. 4 D and E). This difference in the extent of wound vascularity was resolved by 14 d, and suggests an increased rate of vessel invasion at early time points in the repair process. This appears not to be caused by altered levels of the wound angiogenic factor vascular endothelial growth factor, as we see no significant difference in OPN AS ODN–treated wounds in this regard (unpublished data). Recent evidence suggests that OPN has the capacity to directly inhibit angiogenesis (21), but we also note that during repair of skin wounds in PU.1-null mice, where there is no inflammatory response and reduced scarring, there is also significantly increased vascularity within wound granulation tissue (5). Similarly, TNF-Rp55 knockout mice that lack TNF-α–mediated signaling display increased angiogenesis at skin wound sites (22). These results indicate that the inflammatory response and inflammatory cytokines may negatively regulate angiogenesis, and this may encourage fibrosis during normal wound repair.

Inflammation-dependent regulation of OPN expression is mediated largely by macrophages

Our recent comparison of the gene expression profiles of tissue repair in wild-type versus PU.1-null mice revealed that

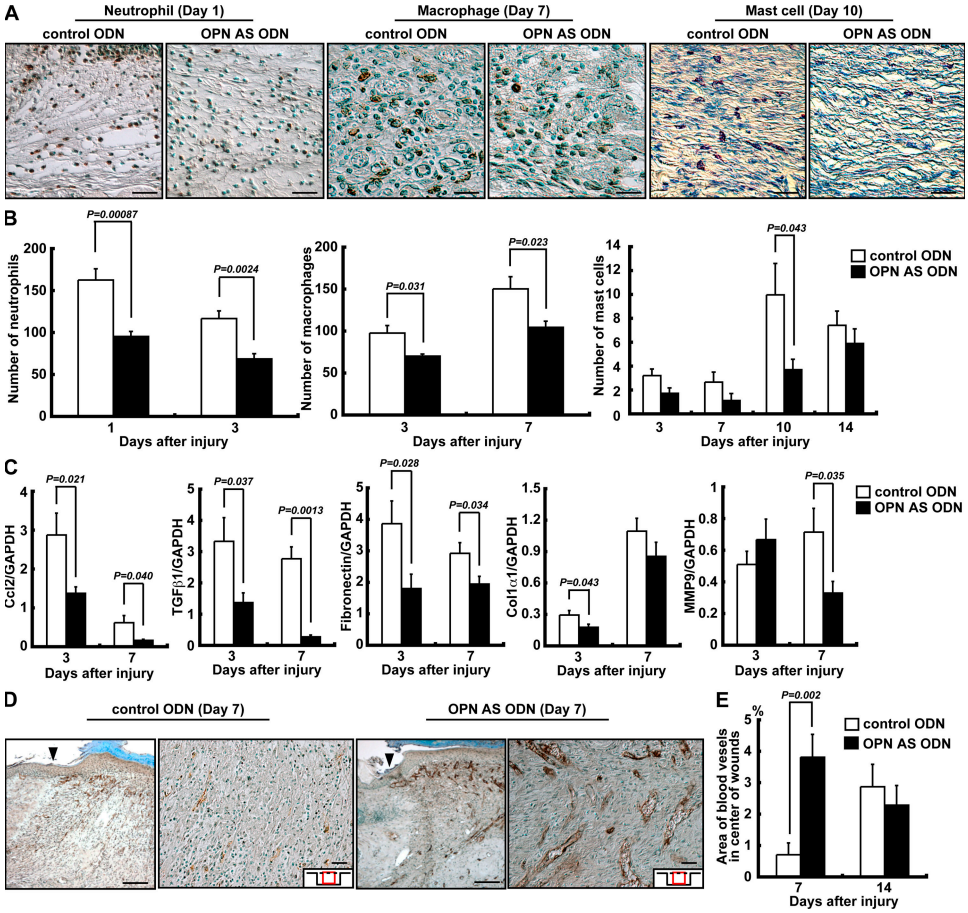

Figure 4. Knockdown of OPN dampens the recruitment of inflammatory cells and increases neovascularization. (A) Immunohistochemistry (IHC) for myeloperoxidase (neutrophils) and F4/80 (macrophages) or Toluidine blue staining (mast cells) reveals significantly fewer leukocytes in the wound granulation tissue of AS ODN–treated versus control wounds. (B) The number of neutrophils, macrophages, and mast cells at wound sites were quantified for control- (open bars) and OPN AS ODN–treated wounds (filled bars; mean ± SEM; $n \geq 5$). (C) Real-time RT-PCR analysis determined the expression of Ccl2, TGFβ1, fibronectin, collagen type1α1, and MMP9 at wound sites, relative to GAPDH, in control ODN (open bars) and OPN AS ODN wounds (filled bars; mean ± SEM; $n = 9$). (D) CD31/platelet/endothelial cell adhesion molecule 1 IHC reveals blood vessels in control- (left) and OPN AS ODN–treated wounds (right) at 7 d after injury (original wound margins indicated by arrowheads). (E) Graphical representation of the number of vessel lumens in the central wound granulation zone at 7 and 14 d. Bars: (low magnification) 200 μm; (high magnification) 50 μm.

OPN is an inflammation-dependent repair gene, but because the PU.1-null mice lack neutrophils, macrophages, and mast cells, it was unclear which of these leukocytic lineages might be responsible for inducing OPN expression in the normal repair situation. To address this, Swiss 3T3 fibroblasts were cultured in the presence of conditioned media (CM) from equal numbers of J774.1 macrophages, primary mouse neutrophils, RBL mast cells, or primary bone marrow–derived macrophages (Fig. 5 A). Western blot analysis showed that factors secreted by all macrophages tested, and mast cells, but not neutrophils, can trigger 3T3 fibroblast expression of OPN (Fig. 5 B). Normal human dermal fibroblasts similarly respond to macrophage-CM with a robust up-regulation of OPN (unpublished data). Induction of OPN expression in in vivo wounds coincides temporally with the first influx of macrophages to the wound, whereas mast cell numbers peak much later after injury, and are relatively few in number throughout repair (Fig. 4) (19, 23). We reasoned that macrophages are likely to be providing the major inductive signal for OPN up-regulation during in vivo healing, although our data do not rule out a contribution from mast cells. Six growth-factors/cytokines known to be secreted by macrophages (24) were then tested for their ability to induce OPN expression in Swiss 3T3s. PDGF-BB, but not EGF, TGFβ1, TGFβ3, IL-6, or TNF-α, was able to significantly up-regulate OPN expression (Fig. 5 C). To confirm that PDGF was, indeed, mediating the macrophage-CM induction of OPN, we suppressed PDGF-R signaling with Gleevec or PDGF-Rβ neutralizing antibodies during fibroblast exposure to macrophage CM (Fig. 5, D and E), and showed that OPN expression was significantly reduced. Induction of OPN could similarly be blocked with wortmannin (Fig. 5 F), which implicates the PI3K signaling cascade down-stream of PDGF in transducing this signal. In an in vivo setting, release of PDGF by degranulating platelets

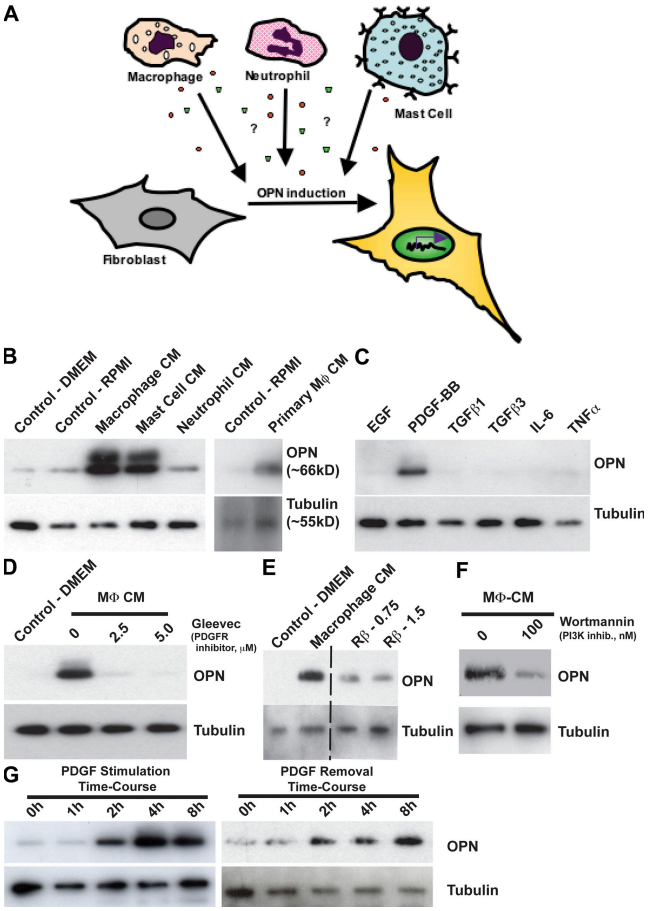

Figure 5. Regulation of OPN expression by inflammatory cells. (A) Schematic diagram of our in vitro assay to determine which leukocytes and which secreted factors are responsible for induction of OPN expression in fibroblasts. Western blot analyses of Swiss 3T3 fibroblast cells treated for 8 h with CM from various leukocytic cell lineages (B) or EGF, PDGF-BB, TGFβ1, TGFβ3, IL-6, or TNF-α (C) to determine which conditions lead to increased OPN expression. (D) Fibroblasts were cotreated with macrophage-CM and Gleevec, PDGF-R neutralizing antibodies (E), or wortmannin (F). (G) Swiss 3T3 fibroblasts were stimulated with 20 ng/ml PDGF-BB for the time indicated, and either immediately analyzed (left), or rinsed in fresh media and cultured for the remainder of 8 h before being analyzed for OPN expression (right).

occurs immediately, but transiently, after injury, and is subsequently supplemented by local up-regulation by resident wound cells and invading macrophages. We performed a time course experiment to address the minimum window of exposure to PDGF required for OPN expression, and our data suggest that OPN levels peak ∼8 h after initial exposure and require at least a 2-h window of exposure (Fig. 5 G). These findings, considered together with our observation that PU.1-null mice, which have normal platelet biology, but do not express OPN in wounds (6), suggest that platelet-derived PDGF exposure is not sufficient on its own to induce OPN expression; rather, sustained exposure of fibroblasts to macrophage-derived PDGF is responsible for the OPN induction that we observe in vitro, and by implication, in vivo as well.

Although OPN expression at sites of tissue repair appears to have largely detrimental consequences on healing, the same is not true for PDGF, as highlighted by the observation that Gleevec treatment delivered from the outset of the repair process significantly slows wound closure (25). Indeed, topical application of PDGF-BB to wounds has been successful in accelerating the healing of chronic ulcers (26), but there is evidence that this improvement in healing speed is accompanied by increased granulation tissue and scarring (27). We propose that these negative side-effects may be attributable to PDGF-induced OPN expression, and we further speculate that down-regulation of OPN may be a molecular mechanism by which Gleevec reduces pulmonary and dermal fibrosis (28, 29). Clearly, any clinical efforts to therapeutically dampen PDGF signaling to improve the quality of tissue repair would require careful consideration of ways that would enable positive contributions while blocking the later "fibrotic" influence. Fibrosis is likely to be a multifactorial driven process, and, accordingly, we are not suggesting that macrophage-secreted PDGF and consequent OPN induction alone are responsible for wound fibrosis. There is considerable evidence that the balance of TGFβ isoforms, for example, is also critical in directing the extent of scarring at a healing skin wound (30). However, our studies indicate that neither TGFβ1 nor β3 is the inductive signal responsible for triggering OPN expression by wound fibroblasts; yet acute knockdown of OPN improves both the rate of healing and the eventual quality of the repair.

Together, our in vivo wound studies and tissue culture experiments indicate that inflammatory cell-mediated signals (we have identified macrophage-derived PDGF as a likely player) trigger expression of OPN in wound fibroblasts, and this may both retard repair and be partially responsible for the fibrosis resulting from wound healing. How OPN expression might lead to increased fibrosis is not yet clear, but our data hint that it may have pleiotropic effects, influencing wound angiogenesis, amplifying the inflammatory response, and possibly even directly modulating the expression of matrix genes in fibroblasts (although preliminary in vitro studies with 3T3 fibroblasts indicate that neither collagen nor fibronectin expression levels are significantly altered directly upon OPN exposure). Further studies will indicate which of these predominates and how this may influence the "window of opportunity" for therapeutic intervention.

MATERIALS AND METHODS

Wound model and ODN treatment. All experiments were conducted according to UK Home Office regulations. Mice (ICR age-matched males; 7–11 wk old) were halothane-anaesthetized, and four full-thickness excisional wounds (4-mm biopsy punch; Kai Industries) or two full-thickness incisional wounds of 1 cm were aseptically made to the shaved dorsal skin (Fig. 2 A). ODNs (control or OPN AS; described in Table S2, available at http://www.jem.org/cgi/content/full/jem.20071412/DC1) were topically applied by pipette into the wound cavity immediately after wounding (50 μl; 1, 2, or 5 μM in 30% Pluronic F-127 gel [see Supplemental materials and methods]; Sigma-Aldrich). Wounds were recorded using a microscope (SteREO Lumar.V12; Carl Zeiss, Inc.), and areas were calculated using Openlab

4.0.2 (Improvision) software. Wound tissue was harvested with a 6-mm biopsy punch.

Histology. Tissue was fixed in 10% formalin or 4% paraformaldehyde for embedding in paraffin or OCT compound (Tissue-Tec), respectively. 6-μm sections were subjected to hematoxylin and eosin, Masson's Trichrome, Toluidine blue or Picrosirius red staining, F4/80, or CD31 IHC (StreptAB-Complex/HRP; DAKO), MPO IHC (Envision; DAKO), or F4/80-OPN double immunofluorescence. Antibody information can be found in Table S3 (available at http://www.jem.org/cgi/content/full/jem.20071412/DC1).

TEM. Specimens were fixed at 4°C overnight in 2% gluteraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, and subsequently for 1 h in 1% osmium tetroxide, and then processed for TEM (model CM100; Philips). Details of collagen bundle density and fiber diameter measurements are outlined in the Supplemental materials and methods.

RNA isolation and RT-PCR analysis. Total RNA was extracted using TRIzol (Invitrogen) and further purified using RNeasy MinElute Cleanup kit (QIAGEN). RNA (5 μg) was reverse transcribed (SuperScriptIII; Invitrogen), and the expression of OPN and GAPDH was analyzed by standard PCR (HotStarTaq; QIAGEN; Supplemental materials and methods). TaqMan Gene Expression Assays (Applied Biosystems) were used for amplification and real-time detection of transcript abundance.

Western blotting. Protein samples (20 μg; collected in T-PER Reagent; Thermo Fisher Scientific) were separated on Tris-Glycine Gels (Invitrogen), transferred to PVDF, and blotted according to standard protocols (antibody details are shown in Table S3). Protein bands were visualized by chemiluminescence (Roche), and band intensity was calculated using Image J 1.34s software (National Institutes of Health).

Cell culture. Swiss 3T3, J774.1 macrophages, and RBL mast cells were cultured in DMEM plus 10% fetal calf serum. Primary neutrophils were harvested by peritoneal lavage 4 h after thioglycollate-induced peritonitis, and primary macrophages were derived from mouse bone marrow and activated with LPS (500 ng/ml). Both primary cell types were maintained in RPMI 1640 with 10% serum. CM was prepared by culturing cells (2.5×10^5 cells/ml) under standard conditions for 48 h, and media was filtered (0.2 μm). Swiss 3T3 cells were treated with CM, or the following growth factors/cytokines for 8 h or periods thereof: OPN, PDGF-BB, EGF, TGFβ1 or 3, IL-6, TNF-α, Gleevec (STI571, imatinib mesylate; Novartis), wortmannin, or PDGF-Rβ neutralizing antibodies (concentrations and suppliers are shown in Table S4, available at http://www.jem.org/cgi/content/full/jem.20071412/DC1).

Online supplemental material. Supplemental materials and methods are provided. Also, sequences for deoxyribozymes (Dzs) and ODNs, and antibody and growth factor information are described in Table S1–S4. Fig. S1 shows the results of experiments to test the abilities of various Dzs and ODNs to cleave OPN mRNA in vitro and to decrease OPN expression in vitro and in vivo. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20071412/DC1.

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