Osteopontin Expression Is Required for Myofibroblast Differentiation

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Abstract—Osteopontin (OPN) is a multifunctional cytokine that is strongly expressed in healing wounds and fibrotic lesions, both of which are characterized by the formation of myofibroblasts. We examined the role of OPN in myofibroblast differentiation induced by the profibrotic cytokine transforming growth factor-β1. In cultured cardiac or dermal fibroblasts treated with transforming growth factor-β1, there was a 2- to 5-fold increase in the expression of the myofibroblast markers α-smooth muscle actin and extradomain A fibronectin but no significant increase of these proteins in OPN-null fibroblasts. Phalloidin staining for actin filaments and immunostaining for α-smooth muscle actin and focal adhesion proteins showed reduced stress fibers, focal adhesions, and lamellipodia in OPN-null fibroblasts compared with wild-type cells. OPN-null fibroblasts exhibited 40% to 60% less spreading, 50% less resistance to detachment by shear force, and a ≈3-fold reduction in collagen gel contraction. These defects were partially rescued by ectopic expression of OPN. Mass spectrometric analysis of proteins in focal adhesions formed on collagen type I beads revealed an enrichment of HMGB1 protein in wild-type cells, whereas HMGB1 was not detected in OPN-null cells. Treatment of wild-type cells with small interfering RNA to knock down OPN reduced transforming growth factor-β1-induced α-smooth muscle actin and HMGB1 to levels observed in OPN-null cells. These studies demonstrate that OPN is required for the differentiation and activity of myofibroblasts formed in response to the profibrotic cytokine transforming growth factor-β1. (Circ Res. 2008;102:319-327.)

Key Words: osteopontin • myofibroblasts • HMGB1 • differentiation

Fibrotic repair is associated with tissue injury, chronic inflammation, and abnormal granulation tissue in several organs including lung, liver, kidney, and heart and may result in loss of function.1-4 Fibrosis can be lifesaving when it maintains the integrity of a functional organ such as the injured myocardium. However, excessive fibrosis results in reduced cardiac muscle compliance, impaired diastolic function, and progressive heart failure.5,6

During fibrotic repair of the myocardium, there is increased expression of cytokines such as interleukin (IL)-10, transforming growth factor (TGF)-β1, angiotensin II, and osteopontin (OPN): mediators that stimulate fibroblast proliferation and new matrix formation.6,7 In cardiac tissues, increased levels of OPN are associated with heart failure, and OPN may be a potential prognostic marker for clinical outcome after myocardial infarction.8 Furthermore, OPN may control remodeling of the cardiac extracellular matrix9,10 and inflammatory mediators expression,11 as well as contribute to the development of cardiomyopathy10 and myocardial hypotrophy. Functional analyses of tissue repair after experimental myocardial infarction show >30% reduction of ejection fraction and impaired ventricular contractile ability in OPN-null mice compared with controls,2 suggesting that OPN regulates the postinfarction fibrotic response.

OPN can modulate a variety of cellular activities associated with fibrotic responses, including proliferation, adhesion, survival, motility, and phagocytosis.1-4 The temporospatial expression of OPN in the injured myocardium is associated with upregulation of fibronectin (FN) and TGF-β1 expression.12,13 TGF-β1 and OPN expression are also temporally associated with fibroblast proliferation, matrix formation, adhesion to matrix, and cell survival11,14; moreover, OPN expression is upregulated in fibroblastic cells after TGF-β1 treatment.15 Whereas the profibrotic activity of TGF-β1 is well defined, the role of OPN and its relationship to TGF-β1-mediated functions are not understood. Notably, myofibroblast differentiation is regulated by TGF-β1, which induces the expression of α-SMA and extradomain (ED)-A16,17 and morphological features of well-developed stress fibers and mature integrin-associated adhesion complexes.18,19

The enhanced expression of OPN in cardiac disease7,9,10,13 and its association with fibroblastic adhesion complexes and enhanced migration,4,14 together with its role in fibroblast survival,2 motivated studies to determine the importance of

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OPN in myofibroblast differentiation and activity. Our main findings are that OPN is required for TGF-β1–induced expression of α-SMA and ED-A in differentiating myofibroblasts, as well as the formation of mature focal adhesions containing OPN and the high-mobility group box 1 protein (HMGB1).

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture and Myofibroblast Differentiation
Primary cardiac fibroblasts were isolated from neonate and 8-week-old wild-type (WT) mice and from OPN-null C57Bl/6 mice as described. To improve survival and proliferative capacity, neonatal cardiac fibroblasts were plated at low density in low (3%) oxygen conditions (online data supplement, cell culture section). WT and OPN-null dermal fibroblast cell lines (clone 135 to 3T3 from strain 129 C57Bl/6 F2 mice) and an OPN-null cell line (ROPN) expressing full-length human OPN under the control of a metallothionein promoter were induced to express OPN by adding zinc sulfate (2 mM) for 8 hours in serum-free medium, as described, 12 hours before TGF-β1 or vehicle control treatment. Myofibroblasts formation studied in cultures serum-starved for 48 hours to deplete OPN, a component of serum, and to optimize conditions for myofibroblast formation. Myofibroblast differentiation was induced by administration of 10 ng/mL TGF-β1 for 48 hours or vehicle control in all assays unless stated otherwise.

Immunoblotting
Cells were lysed and proteins were separated by electrophoresis in 10% SDS-PAGE gels and then transferred to poly(vinylidene difluoride) membranes, which were blocked and then incubated with antibodies to α-SMA, ED-A, or OPN (online data supplement, reagents section). Membranes were subsequently stripped and re-probed for GAPDH antibody as a loading control.

Transcription Analyses
RNA was isolated from lysed cell suspensions with QIA homogenizers (Qiagen) and DNase-I–treated. For quantitative RT-PCR, RNA was reverse-transcribed using TaqMan primers (Applied Biosystems) for mouse transcripts of: GAPDH, OPN, connective tissue growth factor (CTGF), and HMGB1. Real-time fluorescence detection of PCR products was performed (7900HT; Applied Biosystems), and quantification of gene expression was estimated in relation to GAPDH and expressed as the ΔΔCt and the fold increase (2−ΔΔCt).

RNA Interference
An OPN-specific small interfering (si)RNA oligonucleotide (GCUUUCAGGCACCAGGACGCT) and a control oligonucleotide sequence CAGUACAGCACCACU6GCAIdTdT were synthesized. Cells were grown to 50% confluence, transfected with 0.9 µg/mL siRNA in a 1:2 dilution of X-tremeGENE transfection reagent for 4 hours and then washed, and stimulated with TGF-β1.

Adhesion, Spreading, and Gel Contraction of Fibroblasts
For studies of adhesion, cells were incubated for 1 hour with fluorescent beads (2 µm; Molecular Probes, Eugene, Ore) previously coated in solutions of 5 mg/mL BSA, 3 mg/mL collagen, or 5 mg/mL hyaluronan (HA). Bead adherence to cells was analyzed by flow cytometry as described previously. For estimation of whole cell adhesion strength to substrates, plated cells were subjected to shear forces, and the numbers of attached cells after washes were counted.

ed. For examination of cell spreading, cells were allowed to attach to fibronectin-, BSA-, or HA-coated slides for 2 hours and were washed 3× to remove nonadherent cells. Attached cells were stained with tetramethylrhodamine B isothiocyanate (TRITC)-phalloidin, and the projected area of single cells was measured with ImageJ software. Analysis of floating collagen gel contraction was performed as described in floating collagen gels containing 3.6×106 cells per gel treated with TGF-β1 or carrier (control). Changes in surface area measured every 24 hours for 3 days. Cell migration was evaluated as described previously in Transwell chambers using serum or TGF-β1 as chemoattractants.

Isolation of Bead-Associated Adhesion Complexes
Cells attached to coated ferric oxide beads (Sigma) were sonicated, and proteins enriched in bead adhesion complexes were isolated as described and analyzed by immunoblotting or by tandem mass spectrometry (online data supplement, adhesion complexes section). For these experiments, attachment complex extracts were digested overnight with trypsin and were analyzed by matrix-assisted laser desorption ionization (MALDI) in linear mode on a QSTAR XL MALDI QTOF mass spectrometer (337-nm laser). The mass spectra were externally calibrated from molecular weights of a mixture of standard peptides.

Statistical Analysis
Data are expressed as means±SEM. Means between 2 groups were compared using the 2-tailed Student’s t test. Differences between multiple groups were analyzed by 1-way ANOVA. Resistance to shear force was analyzed by linear regression and parametric tests. Differences of P<0.05 were considered statistically significant. For all experiments, at least 3 replicates were included, and experiments were repeated at least 3 times.

Results
Myofibroblast Differentiation
To examine whether myofibroblast differentiation is affected by the absence of OPN, the expression of α-SMA and ED-A in response to TGF-β1 treatment (48 hours) was measured by immunoblotting (Figure 1). In primary WT cardiac fibroblasts, there was a 2-fold increase of α-SMA expression (Figure 1A). In contrast, OPN-null fibroblasts showed no response to TGF-β1 stimulation and expressed basal levels of α-SMA that were comparable to untreated WT cells (Figure 1A). Similarly, embryonic dermal cell lines treated with TGF-β1 showed a ≈5-fold increase of α-SMA expression (P<0.05; Figure 1B) and a ≈3-fold increase of ED-A expression (P<0.05) compared with controls (Figure 1C), whereas no significant changes of α-SMA or ED-A were detected in the TGF-β1–treated OPN-null cell line (Figure 1B and 1C; P>0.1). Following induction of OPN expression in the ROPN cells, TGF-β1 stimulated a 4-fold increase of α-SMA expression (Figure 1B; P<0.05) and >3-fold increase of ED-A expression (Figure 1C; P<0.05). Notably, there were no significant differences of β-actin expression in either WT or OPN-null fibroblasts after TGF-β1 treatment (data not shown), indicating that TGF-β1 did not affect the expression of all actin isoforms.

Requirement of OPN in α-SMA Expression
We next determined whether OPN expression was required for TGF-β1–stimulated myofibroblast differentiation and α-SMA expression. RNA interference was used to selectively knock down OPN expression in a WT cardiac cell line stimulated for myofibroblast differentiation with TGF-β1
The efficacy of the OPN knockdown in WT cardiac fibroblasts was examined by immunoblotting, which showed large reductions of OPN (to 30% and 50% of the levels in mock and nonspecific siRNA-transfected controls, respectively, $P<0.05$; Figure 2A). Mock and scrambled siRNA controls both exhibited increased -SMA expression after TGF-$\beta_1$ treatment, whereas WT fibroblasts treated with OPN siRNA did not respond to TGF-$\beta_1$ stimulation and showed similar levels of -SMA expression as did OPN-null fibroblasts (Figure 2B; $P<0.05$). CTGF expression is an early mediator of TGF-$\beta_1$ fibrotic signaling. The expression of -SMA and OPN by a murine WT cardiac cell line transfected with siRNA was analyzed by Western blotting after treatment with or without TGF-$\beta_1$. A. Both the mock- and the nonspecific siRNA control-transfected cells expressed elevated levels of -SMA in response to TGF-$\beta_1$ treatment. Transfection of an OPN-specific siRNA significantly impaired expression of -SMA in WT fibroblasts relative to the mock and nonspecific siRNA controls, the levels being reduced to those observed in the OPN$^{-/-}$ cells ($P<0.05$). B. The OPN-specific siRNA significantly reduced expression of OPN relative to the mock and nonspecific siRNA controls ($P<0.05$), whereas no OPN was detected for the OPN$^{-/-}$ cells. Densitometry results were normalized to GAPDH content, and the results are expressed as means±SD.
cription was >3-fold greater in WT fibroblasts (75.06±3.8) in comparison to the OPN-null fibroblasts (22.78±2.1).

**Actin filaments and α-SMA Distribution**

We assessed the effect of OPN on actin stress fibers and on the spatial distribution of α-SMA in response to TGF-β1 (Figure IA in the online data supplement) and focal adhesion–related proteins: α-actinin, paxillin, and vinculin in cardiac fibroblasts plated on fibronectin (supplemental Figure IB). Unstimulated WT and OPN-null fibroblasts displayed sparse, thin stress fibers that were not well-developed. TGF-β1 treatment increased the number and thickness of stress fibers in WT cells, a characteristic of differentiated myofibroblasts. TGF-β1 treatment of OPN-null fibroblasts minimally increased the number of stress fibers, but their arrangement was disorganized. α-SMA, α-actinin, paxillin, and vinculin staining of WT and OPN-null fibroblasts plated on fibronectin showed high-intensity fluorescence of WT fibroblasts whereas OPN-null fibroblasts showed diffuse low intensity staining.

**Contractile Ability of OPN-Null Fibroblasts**

Generation of tensile forces by myofibroblasts can be modeled by floating collagen gels in which contraction is enhanced by TGF-β1 and α-SMA expression. Because primary cardiac OPN-null fibroblasts exhibit poor survival properties and limited replication in vitro, embryonic dermal fibroblast cell lines were used for collagen gel contraction assays. WT fibroblasts treated with TGF-β1 contracted the gels to <20% of their original area within the first day (Figure 3) and to <10% of the original area by day 3 (Figure 3B). In contrast, untreated control WT cells demonstrated a slower rate of contraction, which resulted in a final collagen gel surface area ~40% of its original size (Figure 3B). TGF-β1–treated OPN-null cells exhibited slow gel contraction, similar to the untreated WT group, whereas control OPN-null cells showed no significant change of gel area (Figure 3B).

**Cell Adhesion, Spreading, and Migration**

OPN promotes cell adhesion through integrins and CD44 receptors. To determine whether OPN expression is required for cell adhesion, beads coated with BSA, collagen, or HA were incubated with WT and OPN-null primary cardiac fibroblasts for 1 hour, and, after removing unbound beads, cells were analyzed by flow cytometry (Materials and Methods and the online data supplement) as described. The WT fibroblasts exhibited >3-fold greater collagen bead binding compared with OPN-null cardiac fibroblasts (Figure 4A). Significantly more collagen beads attached to WT cells, and the differences between WT and OPN-null cells were more marked in cells with high numbers of attached beads (P<0.001; Figure 4B). Similar patterns of differences were observed for HA-coated beads. WT fibroblasts exhibited >6-fold greater HA bead binding than OPN-null fibroblasts (Figure 4C). ROPN fibroblasts treated with zinc sulfate to restore OPN expression showed 2-fold more binding of HA-coated beads compared with untreated ROPN cells, which demonstrated similar binding as OPN-null cells.

The strength of cellular adhesion was estimated by examining the ability of cells to resist detachment by jet washing as described. In untreated cells, attaching to plastic or collagen-coated substrates, the resistance to shear forces was 2-fold greater after the first 2 washes in WT cells compared with OPN-null cells (Figure 5A and 5B). Whereas the adhesion of noninduced ROPN cells was no different from parental OPN-null fibroblasts, adhesion was increased 25% to 70% when cells were treated with zinc sulfate. Adhesion of WT cells to HA-coated surfaces was only 25% higher than OPN-null cells and only after the forth wash (Figure 5C).

Consistent with the adhesion results, embryonic dermal WT fibroblasts demonstrated extensive spreading and extension of lamellipodia by most of the cells, whereas OPN-null fibroblasts did not spread as well on the various substrates and retained a more rounded morphology (supplemental Figure I). To quantitatively evaluate differences in spreading between OPN-null and WT control fibroblasts, cells were allowed to attach for 2 hours on fibronectin, BSA, or HA substrata and then stained with TRITC–phalloidin, and images of single cells were measured for surface area. The surface area of OPN-null fibroblasts was ~3 times greater in the OPN-/- groups compared with the WT groups, respectively. The migration of cells is dependent on the remodeling of the actin cytoskeleton, which appears to be
impaired in the absence of OPN, we studied the migration of cardiac and embryonic dermal fibroblasts in Transwell chambers. Greater numbers of WT cardiac fibroblasts (>70%) migrated in response to TGF-β1 using serum. OPN-/- dermal fibroblasts exhibited reduced migration in response to serum and restoration of OPN expression in ROPN cells increased the migration of the OPN-null fibroblasts to levels of WT cells (supplemental Figure II).

**Characterization of Adhesion Complexes**

MALDI mass spectrometric analysis of WT cardiac fibroblast focal adhesion extracts compared with the OPN null extracts exhibited wider spectrum of tryptic peptides recovered from both the collagen-associated (Figure 6A) and HA-associated (data not shown) adhesion complexes. Notably, a major difference was found for peptides representing HMGB1/amphoterin, which were highly enriched in the WT cell-derived extracts (Figure 6A). To confirm this finding, collagen and HA bead-associated focal adhesion proteins were

**Figure 4.** Fibroblast binding to collagen- and HA-coated beads. Coated microsphere beads were incubated with WT and OPN-/- primary cardiac fibroblasts for 1 hour and analyzed by single-cell flow cytometry. A, WT cells bind 3 times more collagen-coated microspheres than OPN-/- cells. Thirty-seven percent (±1.05%) of WT fibroblasts attached to 1 or more beads, whereas only 11% (±1.16%) of the OPN-/- fibroblasts attached to 1 or more beads (*P<0.05). B, Fractionated flow showed more WT fibroblasts attached to 1, 2, and 4 or more beads (*P<0.05), whereas greater significance was evident for attachment to 3 beads (**P<0.01). C, Similar results were demonstrated for HA-coated beads (**P<0.01). In response to induction of OPN expression, ROPN fibroblasts also increased their attachment to HA-coated beads relative to controls (*P<0.05).

**Figure 5.** Strength of focal adhesions. A, WT, OPN-null embryonic, and ROPN cell lines were plated on plastic and collagen- and HA-coated culture wells. Attached cells subjected to shear forces generated by jet washing. The mean number of cells per culture well that resisted shear forces during 1, 2, 4, 8, 16, and 32 washes were counted. The WT fibroblasts on untreated plastic culture wells resisted shear forces by 70% to 100% more than the OPN-/- cell line. The WT phenotype was partially restored in the zinc-sulfate–stimulated ROPN cell line. Control ROPN cells retained the null phenotype. B, Similar results were obtained when cell lines were incubated on collagen-coated wells. C, On HA-coated culture wells, OPN-/- resistance was decreased by only 25%, compared with WT cells.
eluted and analyzed by immunoblotting (Figure 7B). Immunoblotting of focal adhesion-associated proteins prepared from OPN-null dermal fibroblasts and neonatal cardiac fibroblasts showed significantly less HMG1 in cells derived from WT mice (Figure 7B).

**Temporal and Spatial Expression of HMG1 and OPN in TGF-β1-Treated Fibroblasts**

Western blot analysis revealed that HMG1 in WT embryonic dermal and cardiac fibroblast focal adhesions and cell extracts increased >10-fold \((P<0.01)\) in response to TGF-β1 stimulation, whereas HMG1 was not increased significantly in the corresponding OPN-/- cell line (Figure 7C). Knockdown of OPN by siRNA reduced HMG1 in WT cardiac fibroblasts to levels observed in OPN-/- cardiac fibroblasts (data not shown).

Immunostaining of WT and OPN-null embryonic dermal cell lines was performed in cells stimulated with or without TGF-β1 and incubated with collagen-coated beads. Dual staining of HMG1 and OPN revealed diffuse OPN staining in WT control cells, whereas HMG1 localized to the nucleus (Figure 8A). In OPN-/- cells, the staining for HMG1 in the nucleus was much weaker than in the WT cells. After stimulation with TGF-β1, HMG1 staining was intensified in the nucleus and also detectable in the cytoplasm of WT cells but not in the OPN-/- cells. Notably, OPN and HMG1 colocalized to some of the collagen-coated beads, sites of focal adhesion formation (Figure 8B). In OPN-null cells, only background staining was evident in the nucleus for HMG1. After TGF-β1 treatment, HMG1 became much more difficult to detect in OPN-/- fibroblasts anywhere in the cells and its intensity decreased.

**Discussion**

Our studies have shown that OPN expression, which is correlated with the development and maintenance of cardiac...
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OPN absence of OPN, the ability of TGF-β to induce CTGF in the absence of OPN expression indicates that OPN exerts its effects on fibrosis early during induction of myofibroblast differentiation, is consistent with compromised stress fiber formation and reduced contractile capacity. Similarly, the anticipated TGF-β upregulation of mRNA for CTGF, which mediates TGF-β fibrotic effects, was abrogated in the OPN−/− fibroblasts. That the responses of the OPN-null cells were attributable to the lack of OPN expression, rather than an impairment of collagen genes affected by the targeted disruption of the OPN gene, was confirmed using siRNA to downregulate OPN mRNA in TGFβ1-stimulated WT cells. Suppression of the OPN mRNA in TGF-β-stimulated cells was accompanied by a reduction in α-SMA expression comparable to that observed in the OPN−/− fibroblasts.

**Development of Focal Adhesion Complexes Is Impaired in OPN-Null Fibroblasts**

OPN−/− fibroblasts displayed significant reductions in attachment to collagen and HA, resistance to shear forces, cell surface area, lamellipodia formation, and cell migration. The impairments in attachment and cytoskeletal defects in the OPN−/− fibroblasts are consistent with the reduced ability of these cells to contract collagen gels and formation of supermature focal adhesions containing α-actinin, vinculin, and paxillin that can be used to confirm the presence of mature functional myofibroblasts. The reduced levels of these proteins in the OPN−/− fibroblasts correlated with the poor spreading and resistance to shear force, indicating a reduction of supermature focal adhesions and resulting in weak adhesions to the extracellular matrix (ECM). This, in turn, is associated with a lack of α-SMA expression and colocalization with the stress fibers. Although OPN is a ligand for integrins, as well as CD44, and could assist in the formation of focal adhesions, many of the assays described in this study were performed over short time periods (40 to 60 minutes), during which time, little OPN would have been secreted. Thus, some of the OPN effects may involve an intracellular form of OPN that we have shown previously to colocalize with the CD44:ERM (ezrin/radixin/moesin) adhesion complexes in migrating fibroblasts. The involvement of the intracellular form of OPN is also indicated from the short-term rescue experiments performed with the ROPN cells. Notably, induction of OPN expression in the ROPN cells was able to largely restore cytoskeletal deficits of adherence and response to TGF-β1. In contrast, HMGB1 was not observed in the OPN−/− cells, indicating that OPN is required for the recruitment of this DNA-binding protein in focal adhesion assembly.

**OPN Is Required for TGF-β-Induced Myofibroblast Differentiation**

Because α-SMA and ED-A are expressed in unstimulated OPN-null fibroblasts under in vitro conditions that induce spontaneous myofibroblast differentiation, it is apparent that, although OPN may not be essential for myofibroblast differentiation per se, it is required for TGF-β1-stimulated myofibroblast differentiation. In the OPN−/− cells, the inability of TGF-β to stimulate the expression of α-SMA, ED-A, and CTGF, which have been shown to be expressed de novo during induction of myofibroblast differentiation, is consistent with compromised stress fiber formation and reduced contractile capacity. Similarly, the anticipated TGF-β upregulation of mRNA for CTGF, which mediates TGF-β fibrotic effects, was abrogated in the OPN−/− fibroblasts. That the responses of the OPN-null cells were attributable to the lack of OPN expression, rather than an impairment of collagen genes affected by the targeted disruption of the OPN gene, was confirmed using siRNA to downregulate OPN mRNA in TGFβ1-stimulated WT cells. Suppression of the OPN mRNA in TGF-β-stimulated cells was accompanied by a reduction in α-SMA expression comparable to that observed in the OPN−/− fibroblasts.
spreading, together with the upregulation of α-SMA and ED-A by TGF-β.

**Absence of HMGB1 in Focal Adhesions of OPN−/− Fibroblasts**

Proteomic analysis of bead-associated adhesion complexes using MALDI identified HMGB1 in WT but not OPN−/− fibroblasts. The mass spectrometric finding, which was confirmed in cell and bead extracts by Western blotting was surprising because neither the presence of HMGB1 in focal adhesion complexes nor the association with OPN has been described previously. HMGB1 is primarily considered to be a nonhistone DNA-binding protein that is a critical cofactor for normal transcription control in somatic cells, without which lethal hypoglycemia typically occurs in HMGB1-null newborn mice. Moreover, similarly to OPN, HMGB1 has a role during stress and pathologic conditions and is considered a strong proinflammatory cytokine that protects against endotoxemia, sepsis, cardiac damage, and arthritis. HMGB1 is released into the ECM following cellular necrosis and acts as a potent “necrosis marker.” In the ECM, HMGB1 triggers a robust inflammatory reaction initiating reparative processes following wounding and is also found in tissues with pathological ECM deposition in synovial tissues of rats with rheumatoid arthritis and systemic lupus erythematosus. Both OPN and HMGB1 expression also have been associated with neoplastic progression and metastases.

**Temporal Expression and Spatial Distribution of HMGB1 Is Influenced by OPN**

The increase in HMGB1 observed in Western blots following TGF-β1 stimulation appears to reflect translocation of the HMGB1 from the nucleus into the cytoplasm and focal adhesions containing the intracellular form of OPN, as indicated from the immunostaining results (Figure 8A). Notably, translocation of HMGB1 from the nucleus to the cytoplasm was recently described in immune cells, which is confirmed in cell and bead extracts by Western blotting was surprising because neither the presence of HMGB1 in focal adhesion complexes nor the association with OPN has been described previously. HMGB1 is primarily considered to be a nonhistone DNA-binding protein that is a critical cofactor for normal transcription control in somatic cells, without which lethal hypoglycemia typically occurs in HMGB1-null newborn mice. Moreover, similarly to OPN, HMGB1 has a role during stress and pathologic conditions and is considered a strong proinflammatory cytokine that protects against endotoxemia, sepsis, cardiac damage, and arthritis. HMGB1 is released into the ECM following cellular necrosis and acts as a potent “necrosis marker.” In the ECM, HMGB1 triggers a robust inflammatory reaction initiating reparative processes following wounding and is also found in tissues with pathological ECM deposition in synovial tissues of rats with rheumatoid arthritis and systemic lupus erythematosus. Both OPN and HMGB1 expression also have been associated with neoplastic progression and metastases.

The increase in HMGB1 observed in Western blots following TGF-β1 stimulation appears to reflect translocation of the HMGB1 from the nucleus into the cytoplasm and focal adhesions containing the intracellular form of OPN, as indicated from the immunostaining results (Figure 8A). Notably, translocation of HMGB1 from the nucleus to the cytoplasm was recently described in immune cells, which release HMGB1 on activation. The paucity of HMGB1 in focal adhesions of OPN−/− cells suggests that OPN is required for the recruitment of HMGB1 following its translocation into the cytoplasm, stimulated by TGF-β1. Notably, HMGB1 shares several pathophysiological properties with OPN, such as control of the transition between apoptosis and cell necrosis, as well as their high concentration in peripheral blood during organ destruction and intense inflammatory reaction, possibly through their impact on the secretion of cytokines such as macrophage inflammatory protein-1α and tumor necrosis factor-α and other potent proinflammatory mediators such as IL-1α, -6, -8, and -12. Thus, the similar distribution and upregulation of HMGB1 and OPN in various pathologies, as well as in focal adhesions, suggests a functional association between these proteins.

Collectively, these results demonstrate the importance of OPN expression in TGF-β1–mediated fibrotic signaling and myofibroblast differentiation. OPN expression is required for α-SMA expression, reorganization of the actin cytoskeleton, and stable cell–matrix adhesions containing HMGB1 and OPN. This study provides a cellular basis for the impaired fibrotic response in the OPN-null mouse and provides novel insights for the development of antifibrotic therapies.

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**Disclosures**

None.

**References**


OSTEOPONTIN EXPRESSION IS REQUIRED FOR
MYOFIBROBLAST DIFFERENTIATION

Online Data Supplements

Materials and Methods

Reagents

TGF-β1 obtained from R&D Systems (Minneapolis, MN). The RNeasy Plus Mini Kit was from Qiagen, DNase-I was from Invitrogen (Santa Clara, CA), the cDNA Synthesis Kit was from Fermentas, siRNA oligonucleotides were from Ambion (Austin, TX) and the X-tremeGENE transfection reagent was from Hoffmann-La Roche (Basel, Switzerland). TRITC-phalloidin, mouse α-SMA monoclonal antibody (clone 1A4), mouse Paxillin monoclonal antibody (clone PXC-10), Mouse Vinculin monoclonal antibody (clone VIN-11-5) and β-actin monoclonal antibody (clone AC-15) were from Sigma (Mississauga, ON). Mouse EDA-FN antibody (Clone IST-9) was from Oxford Biotechnology (Oxfordshire, UK). Mouse human α-actinin monoclonal antibody (Clone BM-75.2) and rabbit HMGB1 antibody (ab11972) were from AbCam (Cambridge, MA). Goat anti-mouse OPN antibody (BDO02) was from Cedarlane Laboratories (Hornby, ON). Rabbit OPN antibody (affinity-purified) has been described 1, 2, 4. Mouse glyceraldehyde-3-phosphate dehydrogenase antibody (clone MAB374) was from Chemicon International (Temecula, CA). FITC-conjugated affiniPure F(ab’)_2 fragment goat anti-mouse IgG antibody and donkey anti-goat IgG antibody were from Jackson ImmunoResearch (West Grove, PA). Bead coatings were prepared as described 3 using 3 mg/ml collagen (Vitrogen-100, Collagen Corp., Santa Clara, CA), 5 mg/ml hyaluronic
acid (HA, Sigma), 5 mg/ml fibronectin (FN, Sigma) or 5 mg/ml bovine serum albumin (BSA, Bioshop, Burlington, ON).

Cell Culture
Primary cardiac fibroblasts were isolated from neonate and 8 week-old wild-type (WT) mice and from OPN-null C57Bl/6 mice by sequential collagenase digestion as described 4. To improve survival and proliferative capacity, neonatal cardiac fibroblasts were plated at low density in low (3%) oxygen conditions 5. Cultures exhibiting fibroblastic cell growth were sub-cultured and from passage 7 exhibited increased proliferation and shortening of doubling time. Cells were subsequently passaged every 3-4 days when cultures reached 80% confluence. At passages 11-13, cells were transferred back to 20% oxygen conditions. Cells at passages 17-22 were frozen and thawed without loss of proliferative capacity. Embryonic WT and OPN-null dermal fibroblast cell lines (clone 135-3T3 from strain 129 C57Bl/6 F2 mice), and an OPN-null cell line (ROPN) expressing full-length human OPN under the control of a metallothionein promoter, were used as described 4. Cells were grown to 80% confluence in HG-DMEM containing 10% FBS and antibiotics. For all experiments cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. Cells were grown in HG-DMEM containing 10% FBS and antibiotics.

Myofibroblast differentiation
Cells were serum-starved for 48 hours to deplete OPN, a component of serum, to optimize conditions for myofibroblast formation 6, induced by administration 10 ng/ml TGF-β1 for 48 hours. OPN transcription in ROPN cells was induced by adding zinc sulfate (2 μM) for 8 hours, after which the medium was changed to serum-free medium.
for 12 hours prior to TGF-β1 or vehicle control treatment. Induced ROPN produce and secret OPN and in 24 hours conditioned medium ~0.4 mg/litre can be purified. Myofibroblast differentiation was assessed by examination of stress fibers and immunoblotting for α-SMA and EDA-FN.

**Immunoblotting**

Cells were lysed and proteins were separated by electrophoresis in 10% SDS-PAGE gels and then transferred to PVDF membranes, which were blocked and then incubated with antibodies to α-SMA (1:1000), β-actin (1:1000), EDA-FN (1:50) or OPN (0.1 μg/ml). Membranes were subsequently stripped and re-probed for GAPDH antibody as a loading control. Immunoreactive bands were visualized by ECL chemiluminescence. Blot densities were estimated with Image J software (NIH) and normalized to GAPDH blot densities.

**Transcription Analyses**

RNA was isolated from lysed cell suspensions with QIA homogenizers (Qiagen CA). RNeasy Plus (Qiagen) was used to extract RNA, which was quantified by spectrophotometry. For quantitative RT-PCR, RNA was reverse-transcribed using TaqMan® primers (Applied Biosystems) for mouse transcripts of: GAPDH, Osteopontin, TGF-β1, CTGF and HMGB1. Real-time fluorescence detection of PCR products was performed (7900HT; Applied Biosystems) and the cycle number at which a fluorescent signal rose statistically above background levels was computed. Quantification of gene expression was estimated in relation to GAPDH and expressed as the ΔΔCt and the fold-increase \(2^{-\Delta\Delta Ct} \).
RNA Interference

An osteopontin-specific siRNA oligonucleotide \(^8\) (GUUUCACAGCCACAAGGAC\text{tt}) and a control oligonucleotide sequence CAGUACAACGCAUCUGGC\text{AdTdT} were synthesized (Ambion Inc., Austin, TX). Cells were plated \((2 \times 10^5\) cells per 35 mm well), grown to 50\% confluence, transfected with 0.9 \(\mu\)g/ml siRNA in a 1:2 dilution of X-tremeGENE transfection reagent for 4 hours and then washed, serum-starved for 48 hours and treated with 10 ng/ml TGF-\(\beta\)1 for 48 hours.

Actin localization and immunostaining

Actin filaments were stained for 15 minutes with TRITC-phalloidin in PBS-0.3\% Nonidet P-40. Cells were immunostained with \(\alpha\)-SMA antibody \((1:50)\) or \(\alpha\)-actinin antibody \((1:100)\) or Paxillin antibody \((1:300)\) or Vinculin antibody \((1:50)\) at 37\(^o\)C for 1hr. For HMGB1, cells were stained overnight \((1:100\) HMGB1 antibody). For OPN, cells were stained with a \((1:500\) OPN antibody) for 3hr. Immunostained cells were imaged with a confocal microscope (Leica, Heidelberg, Germany) and optical sections were obtained at a nominal thickness of 1 \(\mu\)m.

Collagen Gel Contraction

Analysis of floating collagen gel contraction was performed as described \(^9\). Briefly, collagen solutions were prepared and, after incorporating \(3.6 \times 10^6\) WT or OPN-null cells, allowed to gel in 35 mm bacteriological dishes. Floating collagen gels were treated with 10 ng/ml TGF-\(\beta\)1 or carrier (control) and incubated for 3 days, during which time the surface area of the collagen gel surface was measured every 24 hours using Image J software (NIH). Three replicates were used for each group, and experiments were repeated three times.
**Adhesion and Spreading of Fibroblasts**

For studies of adhesion, cells were incubated for one hour with beads (2 μm, Molecular Probes, Eugene, OR) previously coated with BSA, collagen or hyaluronan (HA). Bead adherence to cells was analyzed by flow cytometry (Altra, Beckman-Coulter, Mississauga, ON). Fibroblasts incubated with beads at a ratio of 4 beads per cell for 1 hour only to assess binding and not internalization of beads. Cells prepared as single-cell suspensions for single cell analyses by Flow cytometry. Background fluorescence was determined by analyzing fluorescence beads alone. At least 10^5 cells were analyzed for each culture dish of triplicate samples per group. For estimation of whole cell adhesion strength to substrates, plated cells were subjected to shear and the numbers of attached cells after washes were counted. For examination of cell spreading, cells were allowed to attach to FN, BSA or HA-coated slides for 2 hours and were washed 3X to remove non-adherent cells. Attached cells were stained with TRITC-phalloidin and the projected area of single cells was measured with Image J software. 10 cells or more were measured each in each of the triplicate dishes and experiments were repeated three times.

**Cell Migration**

Cells were allowed to attach to FN-coated polycarbonate membranes (8 μm pore size) in Transwell chambers® for 2 hours. Unattached cells were washed away and new medium was added containing 2% fetal calf serum in the upper chamber and 10 ng/ml TGF-β1, vehicle control or 20% fetal calf serum medium in the lower chamber. Cells were incubated for an additional 4 hours to allow migration. Membranes were removed and cells were fixed. Cells were scraped from the lower side of the membrane and washed.
Cells were stained with DAPI and the numbers of migrating cells were counted in five fields of triplicate chambers, by fluorescence microscopy.

**Isolation of Bead-associated Adhesion Complexes**

Proteins enriched in bead adhesion complexes were isolated as described 3. Ferric oxide beads (Sigma) were coated in solutions of 5 mg/ml BSA, 3 mg/ml collagen, or 5 mg/ml HA. Cells with attached beads were collected into 50 mM Tris-HCl (with 10 mM CaCl₂, 0.01% Triton-X, pH 8.0) and sonicated. Attachment complexes were isolated at 4°C using a side-pull magnetic isolation apparatus (Dynal, Lake Placid, NY) and analyzed by immunoblotting or by tandem mass spectrometry. For these experiments, attachment complex extracts were digested overnight with trypsin and were analyzed by Matrix Assisted Laser Desorption Ionisation (MALDI) in linear mode on a QSTAR XL MALDI QTOF mass spectrometer (337 nm laser) as described 12. The mass spectra were externally calibrated from molecular weights of a mixture of standard peptides. Three replicates were included, and experiments were repeated three times.

**Statistical Analysis**

Data are expressed as means ± SEM. Means between two groups were compared using the two-tailed Student’s t-test. Differences between multiple groups were analyzed by one-way ANOVA. Resistance to shear force was analyzed by linear regression and parametric tests. Differences of p<0.05 were considered statistically significant.
References


**Online Figure I. Immunostaining of WT and OPN-/- fibroblasts.** Cell lines and established cardiac fibroblasts were stimulated with TGF-β1 or grown on a fibronectin substratum and examined for stress fiber formation and α-SMA, Paxillin, Vinculin and α-actinin distribution. (A) WT Control fibroblasts displayed some stress fibers that were homogenously distributed and aligned along the long axis of the cells spanning the cytoplasm and ending in the cortex. Upon stimulation with 10 ng/ml TGF-β1 for 48 hours, the intensity of stress fiber staining was increased markedly, characteristic of differentiated myofibroblasts. Unstimulated OPN-/- fibroblasts contained very few short, blind-ending stress fibers that did not appear aligned within the cytoplasm and did not extend into the cortex. Upon stimulation with TGF-β1, the amount of stress fiber staining did not increase appreciably, and the disorganized arrangement did not change. (B) WT and OPN-/- cells were plated and allowed to spread on a fibronectin substratum and then stained for α-SMA, Paxillin, Vinculin and α-actinin. The WT fibroblasts stained strongly for α-SMA and focal adhesions formation markers at the cell periphery whereas the staining intensity in the OPN-/- fibroblasts was markedly reduced.

**Online Figure II. OPN affects fibroblast migration.** Results from Transwell chamber analyses demonstrate that WT fibroblasts migrated ~3-fold faster than OPN-/- fibroblasts (p <0.05*). When induced to express OPN by zinc treatment ROPN fibroblasts demonstrated a significant improvement in migratory ability relative to control (p <0.01**).
Online Figure I

A  Phalloidin Staining

Control

WT  WT  OPN/-  OPN/-

10 ng/ml TGF-β1

WT  WT  OPN/-  OPN/-

B

α–SMA  Paxillin  Vinculin  α–Actinin

OPN/-  OPN/-  OPN/-  OPN/-

WT  WT  WT  WT
Online Figure II

![Bar graph showing Migrating Cells (%)](image-url)

- WT
- OPN-/-
- ROPN

**Zn -**

**Zn +**

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*Online Figure II*