Osteopontin Regulates Actin Cytoskeleton and Contributes to Cell Proliferation in Primary Erythroblasts*

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Erythropoietin and stem cell factor are the key cytokines that regulate early stages of erythroid differentiation. However, it remains undetermined whether additional cytokines also play a role in the differentiation program. Here, we report that osteopontin (OPN) is highly expressed and secreted by erythroblasts during differentiation. We also demonstrate that OPN-deficient human and mouse erythroblasts exhibit defects in F-actin filaments, and addition of exogenous OPN to OPN-deficient erythroblasts restored the F-actin filaments in these cells. Furthermore, our studies demonstrate that OPN contributes to erythroblast proliferation. OPN knock-out male mice exhibit lower hematocrit and hemoglobin levels compared with their wild-type counterparts. We also show that OPN mediates phosphorylation or activation of multiple proteins including Rac-1 GTPase and the actin-binding protein, adducin, in human erythroblasts. In addition, we show that the OPN effects include regulation of intracellular calcium in human erythroblasts. Finally, we demonstrate that human erythroblasts express CD44 and integrins β1 and α4, three known receptors for OPN, and that the integrin β1 receptor is involved in transmitting the proliferative signal. Together these results provide evidence for signaling transduction by OPN and contribution to multiple functions during the erythroid differentiation program in human and mouse.

Early stages of erythroid cell differentiation are regulated by multiple growth factors including interleukin-3, erythropoietin (EPO), and stem cell factor (SCF) (1, 2). EPO and SCF have distinct functions. The predominant role of EPO is to deliver survival signals and maintain cell viability (3, 4), whereas SCF provides signals for cell proliferation (4–6). Together these two growth factors guide the erythroid differentiation program from the early basophilic stage through the late polychromatic stage of maturation. However, the effects of these cytokines explain only the early stages of erythropoiesis. We were interested in identifying additional cytokines and/or factors involved in the erythroid differentiation program, especially factors that regulate the remodeling of the cytoskeleton. To achieve these objectives we developed methods to obtain extremely pure primary erythroblasts that synchronously differentiate into reticulocytes. Utilizing these cells, we screened a cDNA microarray and identified OPN as one of the cytokines that is highly expressed by erythroblasts during differentiation.

OPN is a multifunctional cytokine that is highly expressed during bone remodeling and has pro-inflammatory effects (7–12). OPN has anti-apoptotic, chemotactic, and proliferative properties, depending on the cell type and context. It also plays a vital role in the delayed-type immune response and is known to be secreted by activated T cells and macrophages (13). OPN knock-out mice are viable and live a normal life span but suffer from bone defects and problems with wound and fracture healing (14). To date, OPN has not been shown to be expressed by erythroblasts, nor has it been implicated in functions associated with erythroid cell maturation. Here, we demonstrate that OPN is expressed by erythroblasts and contributes to the regulation of actin cytoskeleton and proliferation. We also demonstrate that stimulation of erythroblast cells by OPN induces activation and/or phosphorylation of Rac-1 GTPase and other intracellular proteins, including efflux of intracellular calcium. Finally, our studies show that OPN receptors CD44 and several integrins are expressed in these cells and suggest that integrin β1 is responsible for transmitting the proliferative signal. Collectively, our data define a cytokine important in the regulation of multiple functions during the erythropoiesis.

**EXPERIMENTAL PROCEDURES**

Antibodies and Reagents—Initial microarray studies that identified OPN expression by erythroblasts were carried out by Memorec Biotech Inc. in Cologne, Germany (a Miltenyi Biotec company). Both fluorochrome-conjugated and non-conjugated glycophorin A (GlyA) antibodies were purchased from BD Biosciences, Inc. The transferrin receptor (CD71) antibody was purchased from Beckman Coulter, Inc. Recombinant OPN and recombinant SCF were from R & D, Inc. The anti-phospho adducin Ser-724 antibody (cat. no. 05-587), which also recog-
nizes phospho adducin Ser-726 in human cells, was purchased from Upstate Biotechnology, Inc. The anti-phosphotheonine (cat. no. 71-8200)-specific antibody was purchased from Zymed Laboratories Inc. The anti-OPN antibody used for immunoblot analysis and immunofluorescence was from R & D, Inc. Fluorescently labeled phalloidin was purchased from Molecular Probes. Fluo-3/AM was purchased from VWR International. The A23187 calcium ionophore was purchased from Calbiochem, Inc. The Rac inhibitor, NSC23766 (cat. no. 553502) was purchased from Calbiochem Inc.

Primary Human Erythroid Cultures and Flow Cytometry—Human primary erythroblasts were generated by culturing CD34+ early hematopoietic progenitors initially isolated from growth factor-mobilized peripheral blood (purchased from ALL Cells, Inc.) using an Isoplex 300i cell selection device. The culture contained 15% fetal bovine serum, 15% human serum, Iscove’s modified Dulbecco’s medium (IMDM), 10 ng/ml interleukin-3, 2 units/ml EPO, and 50 ng/ml SCF. During the initial 8 days of culture, cells were fed on days 3 and 6 by adding equal volumes of fresh culture media supplemented with growth factors. However, no new interleukin-3 was added after the initial addition on day 0, and the amount of SCF added to the fresh media was gradually decreased at each feeding (day 3, 25 ng/ml; day 6, 10 ng/ml; day 8, 2 ng/ml). The amount of EPO added was 2 units/ml during each feeding. On day 8 of culture, cells were further purified by flow cytometry sorting for Gly A/CD71 or CD71 cells using a MoFlo high speed flow cytometer. The purity of the population isolated by this method was 98–99%. Sorted cells were cultured in the same media as before with EPO and SCF, except the concentration of SCF was reduced to 2 ng/ml. Cells were fed one more time on day 10 of culture by adding equal volumes of fresh media with only EPO (2 units/ml) during this final feeding. Cells were collected at various time points during the culture and stained with benzidine and hematoxylin, as described previously, to monitor the differentiation program (15). Bone marrow-derived erythroblasts were isolated by selection of CD71-positive cells from mononuclear cells obtained from bone marrow aspirates of volunteer donors. CD71-positive cells were cultured until used for qPCR analysis.

PCR Amplification—Real-time PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen, Inc.) on the MyiQ instrument (Bio-Rad) using OPN and 18 S ribosomal RNA gene primers (OPN: forward, 5'-TTGCAGTGGATTTCTTTGC-3'; reverse, 5'-GTCATGGCTTTCTGTGGACT-3'; 18s: forward, 5'-ATGCCGGTCTTACGTTGTG-3'; reverse, 5'-CGC-TGAGCCAGTCAGTGTAG-3'). PCR conditions were as follows: 95 °C for 15 min followed by 40 cycles of 94 °C, 15 s, 55 °C, 30 s, and 72 °C, 30 s. The melting curve analysis was performed by increasing the temperature from 55 to 95 °C at 0.5 °C/10 s and measuring the fluorescence intensity at the interval. Relative RNA levels of the target gene in each sample were determined by the ΔΔCt method and normalized against the RNA level of 18 S in each sample.

OPN Stimulation, Immunoblot Analysis, and ELISA—Primary erythroblast cells on day 9 or 10 of culture were collected and washed twice with IMDM media to eliminate any secreted OPN from the culture media. Cells were then incubated in serum-free media (IMDM/1% bovine serum albumin) for 4.5 h prior to stimulation with OPN. Immunoblot analysis was performed as described previously (16). An ELISA for OPN was performed using medium collected from cultures where cells had been seeded at a concentration of 1 × 10⁶/ml. After 3–4 days of culture, the medium was collected and subsequently used in the ELISA assay. Also, an OPN ELISA was performed using a commercially available kit according to the manufacturer’s instructions (Assay Design, Inc.).

Isolation and Culture of Mouse Erythroblasts from OPN−/− Mice—Mice (B6.Cg-Spp1tm1Blh/J, cat. no. 004936) were purchased from The Jackson Laboratory. The genotype of mice was confirmed by PCR of tail DNA using primers, as suggested by the Jackson Laboratory. Pure C57BL/6 mice (WT) were purchased from The Jackson Laboratory as experimental controls. All animal research was approved by the University of Chicago Institutional Animal Care and Use Committees. To obtain bone marrow cells, mouse femurs and tibiae were flushed with phosphate-buffered saline (PBS) containing citrate and passed through a 20-gauge syringe to obtain a single cell suspension. To isolate erythroblasts, the cells were stained with PE-conjugated Ter119 antibodies and sorted using a MoFlo-HTS cell sorter (Dako Cytomation) or selected for CD71 using the EasySep magnetic selection method. Selected and/or sorted cells were cytospun onto slides and stained with benzidine and hematoxylin for morphological evaluation and immobilized on Alcian Blue (Sigma)-treated coverslips for immunofluorescence analysis to evaluate F-actin distribution. In the experiment where the effect of OPN on F-actin cytoskeleton was determined, CD71-positive erythroblasts were cultured in 30% bovine serum, 0.1 mmol/liter α-thioglycerol, IMDM, and 1 unit/ml EPO for 16 h in the presence or absence of 1 μg/ml OPN prior to immunofluorescence analysis for F-actin. As a control, WT mouse erythroblasts were also cultured under similar conditions but without OPN. In experiments where Complete Blood Count (CBC) for mouse blood was obtained, about 20 μl of mouse blood were collected from the mouse tail vein. HEMVET 850 was used to determine CBC for blood samples within 3 h.

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed on human and mouse cells after immobilizing and fixing cells on Alcian Blue-treated coverslips. Cells were permeabilized in 0.1% Triton X-100 in PBS for 5 min and washed 5 min prior to blocking with 10% fetal bovine serum in PBS for 45 min at room temperature. In experiments where OPN was localized in human erythroblasts, cells were incubated with anti-OPN antibody diluted in PBS containing 1% fetal bovine serum and 0.01% Triton X-100 at 37 °C for 1 h, and washed in PBS containing 0.01% Triton X-100. Cells were then incubated with Alexa Fluor-conjugated anti-goat IgG antibody at room temperature for 45 min before washing in the same buffer as before. Incubation with anti-GlyA antibody was performed at room temperature for 1 h. Cells were washed in the same buffer before the incubation of Alexa Fluor-conjugated anti-mouse IgG antibodies at room temperature for 30 min. Cells were washed in PBS, the coverslips were allowed to air-dry, and were mounted with Prolong Gold mounting medium (Invitrogen). In experiments where the localization of
F-actin was performed, both human and mouse erythroblasts were stained using Texas Red-conjugated phalloidin according to the manufacturer’s suggested procedure (Molecular Probes). All photographs were taken using a Leica SP2 AOBS spectral confocal microscope under a ×63 oil immersion lens.

siRNA Transfection—Day 6 erythroblasts (basophilic) or day 9 GlyA/CD71 cells (polychromatic) were plated at a density of 1 × 10⁶ cells in 0.5 ml of growth medium per well in a 12-well plate. In a 1.5-ml tube, 100 µl of serum-free medium and 3 µl of the TransIT-siQUEST transfection reagent (Mirus, Bio Corp.) were mixed and incubated at room temperature for 20 min. 50–100 nM of either control siRNA or OPN siRNA (Dharmacon, Inc.) were added and incubated at room temperature for 20 min. The TransIT-siQUEST reagent/siRNA complex mixture was added dropwise to the cells. The plate was gently rocked and incubated for 24–72 h. The knock-down of target gene expression was tested by real-time PCR or Western blot analysis. The suppression of OPN expression was effective for up to 72 h.

Rac-1 Assay—Erythroblasts (day 9) in culture were placed in fresh serum-free medium for 2 h with or without the Rac inhibitor prior to stimulation with OPN. Equal amounts of lysates (500 µg) were incubated with GST-Pak1-PBD (to pull-down active GTP-bound Rac-1) in the presence of SwellGel™-immobilized glutathione at 4 °C for 1 h in a spin column (Pierce EZ-Detect Rac1 Activation kit). The samples were subsequently analyzed for bound Rac-1 by immunoblot analysis using an anti-Rac-1 antibody.

Calcium Efflux Assay—Day 10 human erythroblasts were centrifuged in medium at 400 x g, and the medium was aspirated. Cells were washed three times in HEPES-G (123 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 25 mM HEPES, 10 mM glucose, pH 7.4) and consecutively centrifuged at 800, 600, and 500 x g. 40 µl of cells were added to 20 ml of HEPES-G in a 50-ml sterile conical tube (0.2% Hct). The tube was covered with aluminum foil to exclude light. 20 µl of 2 mM fluo-3/AM stock were added to the tube. The sample was incubated at 37 °C for 15 min with shaking. An additional 20 µl of 2 mM fluo-3/AM stock were added to each tube. The sample was incubated at 37 °C for an additional 45 min with shaking. The sample was centrifuged at 400 x g for 5 min at 4 °C. The sample was washed twice in PBS-G (PBS, 10 mM glucose, 0.5% bovine serum albumin, pH 7.4) and centrifuged at 400 x g for 5 min at 4 °C. The sample was transferred to 15-ml conical tubes, washed once in HEPES-G, and centrifuged at 400 x g for 5 min at room temperature. The supernatant was aspirated. The cells were resuspended in 2 ml of HEPES-G (1% Hct) and incubated at room temperature for 15 min. Prior to flux studies, 100 µl of loaded cells were added to 1400 µl of HEPES-G in a glass cuvette. Cells were stimulated at 60 s with no stimulant, 1 µg/ml OPN, or 1 µM A23187. A fourth sample was preincubated with 1 µg/ml OPN for 3 min and stimulated at 60 s with 1 µM A23187. Loaded cells were excited at 506 nm, and the fluorescence emission was recorded at 530 nm using a fluorimeter (Aminco Bowman Series 2 Luminescence Spectrometer). Triplicate trials were run for each sample.

Detection of Receptors for OPN Engagement—Day 10 erythroblasts were used to detect the cell surface expression of CD44, CD44v6, integrins β₁, α₁β₁, and α₁β₃ along with glycophorin A by flow cytometry. Antibodies against CD44, integrin β₁ (CD29), integrin α₁ (CD49d), and integrin β₃ were from eBioscience Inc. The antibody against CD44v6 was purchased from R & D Inc. The antibody against integrin α₁β₃ was from Santa Cruz Biotechnology Inc. Peripheral blood mononuclear cells (PBMC) and Chinese hamster ovary (CHO) cells were used as positive controls for receptors that did not show expression in erythroblasts.

Cell Proliferation Assays—In the experiments where proliferation effects of OPN were determined, mouse CD71-positive erythroblasts from WT and OPN⁺⁻/⁻ mice were cultured for 48 h prior to determining the level of proliferation. In the experiment where the effect of neutralizing antibodies on cell proliferation was determined, day 9 human erythroblasts were cultured for 24 h in the presence or absence of 20 µg/ml for each antibody. None of the antibodies utilized in cell culture contained sodium azide, which is used as an antibacterial agent in most commercial antibodies. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay according to the manufacturer’s suggested protocol (Sigma).

RESULTS

OPN Expression by Highly Purified Human Erythroblasts—To obtain an extremely pure erythroid cell population that differentiates in a synchronous manner, we used a primary human erythroid cell culture system, which was modified from our previous work (17). We initially cultured CD34-positive early hematopoietic cells under conditions that promote commitment and differentiation to the erythroid lineage. During the mid-phase of in vitro culture, we selected a highly synchronous erythroblast population by flow cytometry sorting for GlyA- and CD71-positive cells (Fig. 1A). Sorted cells were 98–99% positive for the GlyA/CD71 population. We also confirmed the absence of other myeloid and lymphoid cell types after purification by flow cytometry analysis (data not shown). These cells were then recultured for another 6–8 days under conditions optimal for terminal differentiation to reticulocytes (Fig. 1B). Cells were collected at several time points during culture and stained for hemoglobin (benzidine) to monitor terminal differentiation (Fig. 1B). The OPN expression was evaluated using qPCR for OPN mRNA at the following stages of the differentiation program: CD34-positive cells (day 0), polychromatic erythroblasts (day 9), orthochromatic erythroblasts (day 12), and enucleating late stage erythroblasts (day 13) (Fig. 1C). Transcripts for OPN were present in CD34-positive early hematopoietic cells and at all stages of differentiation, but the highest levels were observed at later time points peaking on day 12 of culture (Fig. 1C). To exclude the possibility that OPN is expressed by erythroblasts as a result of growth factor mobilization (source of our CD34 cells), we also cultured erythroblasts (CD71-selected) isolated directly from bone marrow aspirates of volunteer donors. These studies confirmed that OPN is expressed by bone marrow-isolated erythro-
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FIGURE 1. Expression of OPN in highly purified human erythroblasts. A, flow cytometry analysis of human erythroblasts on day 8 of culture prior to sorting for GlyA and CD71 (transferrin receptor) (left panel) and after sorting (right panel). The purity of the sorted population was 98–99%. B, photomicrographs of differentiating human erythroblasts stained with hematoxylin and benzidine on day 6 (basophilic erythroblasts), day 9 (polychromat), and days 12 and 16 (orthochromatic) of culture. C, expression of OPN in glycophorin A/CD71-sorted cells determined by real-time qPCR and normalized against 18 S RNA. Levels are compared with the level of OPN expression in day 0 cells. The results are the mean of triplicate determinations. Bars indicate medians with S.D. *p < 0.01 compared with day 9; **, p < 0.01 compared with day 12. D, qPCR analysis of OPN expression in bone marrow-derived and growth factor-mobilized peripheral blood (mPB)-derived erythroid progenitors cultured until day 9. Levels are compared with the level of OPN expression in mPB. Bars indicate medians with S.D. The results are the mean of triplicate determinations.

The two erythroleukemia cell lines, K562 and HEL, which were used as negative controls, do not express OPN. We then reproped the same immunoblot against anti-tubulin antibody to verify equal protein loading and with antibodies against protein band 3 (anion exchanger) to verify terminal differentiation. Band 3 is an erythroid transmembrane protein, which appears relatively late during the differentiation program, and is therefore an excellent marker of erythroid differentiation.

Cellular Secretion and Localization of OPN—Because OPN is a secreted cytokine, we examined whether purified erythroblasts secrete OPN during their differentiation process. OPN was readily detectable by ELISA in culture medium of GlyA/CD71-positive erythroblasts (Fig. 2B). The level of OPN was especially high between days 8 and 11 of culture, during which time these cells rapidly synthesize hemoglobin as well as several other cytoskeleton proteins (18). Interestingly, OPN secretion persisted even during the very late stages of erythroid differentiation (days 14–19), suggesting continued autocrine and/or paracrine functions (Fig. 2B).

We then examined the intracellular localization of OPN in differentiating erythroblasts on day 10 of culture. Using fluorescently labeled antibodies against OPN and GlyA (both indirect fluorescent conjugates), we observed the localization of OPN in GlyA-positive cells (day 10), further confirming that OPN is expressed by erythroid progenitors (Fig. 2C). OPN had a punctate appearance in cells and was distributed throughout the cytoplasm, whereas GlyA was localized to the cell membrane. Fluorochrome-labeled secondary antibody alone was used as a control, which did not react with erythroblast cells (Fig. 2C).

OPN Regulates F-actin Filament Formation and Distribution—To investigate the functional relevance of OPN expression, we depleted OPN expression in human erythroblasts by siRNA. By transfecting cells with a pool (Smart Pool™) of siRNA directed against OPN, we were able to effectively inhibit the expression of OPN at the RNA and protein levels (Fig. 3, A and B). Because OPN regulates F-actin polymerization in the bone, we examined the impact of OPN deple- tion on F-actin reorganization during erythroid differentiation. In erythroblasts, F-actin is normally present in the peri-membrane region of the cells. The depletion of OPN resulted in a dramatic rearrangement of the distribution and appearance of F-actin in differentiating erythroblasts as determined by fluorescence microscopy using Texas-Red-conjugated phalloidin. The knock-down of OPN in human erythroblasts resulted in the reduction of actin filaments in the peri-membrane region of the cells and the appearance of aggregated bundles throughout the cytoplasmic region, which was evident under the oil immersion lens (Fig. 3C). We then examined cultured erythroblasts from the bone marrow of WT and OPN−/− mice, which showed that in OPN−/− mice, there were no definitive F-actin filaments along the peri-membrane region compared with WT mice. In these OPN−/− cells, F-actin had diffusely spread throughout the cytoplasm of cells (Fig. 3D). Furthermore, when we cultured the OPN−/− erythroblasts with recombinant mouse OPN, we were able to restore the F-actin filaments in the peri-membrane region of the cells (Fig. 3D). The changes in actin filaments were visible only at high magnification, although photomicrographs of larger fields allowed us to observe the overall expression in the total cell population.

Signal Transduction by OPN—To identify the upstream signaling elements responsible for the regulation of actin polymerization by OPN in erythroblasts, we investigated whether Rac-1 GTPase, a known signaling intermediate of actin remodeling, is activated by OPN. Rac-1 belongs to the...
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FIGURE 2. Expression, secretion, and localization of OPN in human erythroblasts. A, primary erythroblasts were collected and analyzed for the levels of OPN protein at various times during the differentiation program. The same blot was reprobed with an anti-tubulin antibody (middle panel) and an anti-band 3 antibody (bottom panel) to monitor the extent of differentiation and to establish protein loading controls, respectively. On a separate immunoblot, total lysates collected from erythroblastoid cell lines, K562 and HEL were probed with the same anti-OPN antibody as a negative control, followed by immunoblotting against anti-tubulin antibodies as the protein loading control. B, quantitation of OPN in the culture media during erythroid differentiation. ELISA was performed using an OPN ELISA kit to determine the amount of secreted OPN present in the culture media at various time intervals during erythroid differentiation. All data are presented as mean ± S.D. The results are the mean of triplicate determinations. *, p < 0.01 compared with media; **, p < 0.01 compared with day 8–11; and ***, p = 0.055 compared with day 11–14. C, OPN (green) was localized to cells expressing Gly A (red) by confocal immunofluorescence microscopy on day 10 of culture using specific antibodies to OPN and Gly A. The first panel shows DIC micrographs of the cells. Images of larger fields are shown for the OPN, Gly A, and secondary antibody controls. Images of two single cells expressing OPN and Gly A are shown under high power.

Rho family of GTPases and binds its downstream effector PAK-1 to initiate the signaling cascade upon Rac-1 activation by extracellular ligands (19). We tested Rac-1 activation in response to OPN by performing a Rac-1 binding assay on total cell lysates from human erythroblasts. Stimulation by exogenous OPN resulted in a 50% increase in Rac-1 binding to PKA-1, compared with the unstimulated sample (Fig. 4A). Moreover, pretreatment of cells with a Rac GTPase inhibitor (NSC23766) prior to OPN stimulation suppressed the extent of binding, demonstrating that Rac-1 GTPase is a target of OPN (Fig. 4A).

We then focused on adducin, an actin-binding protein that plays an important role in actin filament regulation in the erythroid cytoskeleton. Three isoforms (α, β, γ) of adducin are present in the erythroblast cytoskeleton and have phosphorylation sites that are substrates for multiple kinases. Although protein kinases A and C are known to phosphorylate adducin (20), cytokine-induced phosphorylation of adducin is not known. To determine if OPN stimulation leads to adducin phosphorylation, total cell lysates were collected after OPN stimulation (for various times or for 30 min with increasing concentrations of OPN) and immunoblotted with an anti-phosphoserine 726 antibody. These experiments showed that OPN stimulation leads to phosphorylation of α and/or β isoforms (120/110 kDa) in a dose- and time-dependent manner that exceeds any basal phosphorylation levels, as indicated by the relative ratios of the band intensities (Fig. 4, B and C).

To further explore whether other proteins are also phosphorylated in response to OPN, we used pan anti-phosphothreonine antibody to investigate general phosphorylation patterns in erythroblasts. This experiment revealed that at least three other proteins were phosphorylated at threonine sites, suggesting the involvement of OPN as a signal transducer in erythroid cells (Fig. 4D). The molecular sizes of these proteins were ~120, 30, and 27 kDa. The kinetics of phosphorylation of all proteins showed a maximum level of phosphorylation between 5 and 15 min after exposure, indicating that the OPN effect on erythroblasts in terms of signal transduction is quite rapid (Fig. 4D).

Intracellular Calcium Is Regulated by OPN—Because OPN has been linked to regulation of Ca2+ absorption in bone, we used a fluorescence assay to investigate the role of OPN in the regulation of intracellular Ca2+. Exogenous OPN stimulation of erythroblasts induced efflux of Ca2+ from the cells, as demonstrated by decreased fluorescence intensity compared with control samples that were not stimulated by OPN (Fig. 5). As a positive control, cells were stimulated with A23187, a Ca2+ ionophore, which induced a dramatic increase in fluorescence intensity corresponding to rapid Ca2+ influx. The incubation of OPN in A23187-treated cells resulted in a relative reduction of Ca2+ influx compared with the positive control. Because calcium often acts as a signaling mediator, OPN stimulation of erythroblasts likely induces specific signaling cascades, resulting in or involving calcium efflux.

Hematologic Analysis in OPN-deficient Mice—We then investigated whether hematologic parameters are affected in OPN knock-out mice (Table 1). By comparing the total blood counts of 19 OPN−/− mice and 20 wild-type mice we found that both Hct and Hb levels were 10–12% lower in OPN−/− male mice compared with the wild-type mice. No differences were observed in female knock-out mice. The mean corpuscular volume of OPN−/− and wild-type mouse erythrocytes were not statistically different, nor were there striking abnormalities in erythrocyte morphology. Also, the white blood cell and platelet counts were within normal limits, and blood smears appeared
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FIGURE 3. OPN regulates localization and distribution of F-actin in human and mouse erythroblasts. A and B, OPN expression was knocked down in human erythroblasts by transfection of OPN-specific siRNA into day 6 cells. Real time RT-PCR and immunoblot analysis were performed to confirm the efficient knock-down of the gene 24-h, post-transfection. A set of nonspecific siRNA was also transfected as a control. C, distribution of F-actin in human erythroblasts transfected with control siRNA (Ctrl) and cells transfected with OPN-specific siRNA 48-h post-transfection by immunofluorescence analysis using Texas Red-conjugated phalloidin. The top panel shows DIC micrographs of cells. D, localization of F-actin in erythroblasts of wild-type and OPN knock-out male mice. CD71-selected erythroblasts were cultured in vitro for 16 h with or without OPN as indicated in the case of OPN knock-out mice (OPN−/−) and without added OPN in the case of WT mice. Cells were harvested and analyzed for F-actin filaments by Texas Red-conjugated phalloidin. DIC and fluorescence micrographs of two individual cells (under high power) and larger fields are depicted.

normal (data not shown). We also examined the serum iron levels and interleukin-6 in OPN−/− male mice, two factors that are associated with anemia, and found that serum iron and interleukin-6 levels were comparable to the WT mice (data not shown).

Expression of OPN Receptors in Human Erythroblasts—Because of the effects of OPN on erythroblasts, we examined the expression of known OPN receptors in human erythroblasts. The receptors we examined were integrins β1, β3, α4, α5, β5, CD44, and CD44v6. Day 10 human erythroblasts were incubated with antibodies against each of these receptor proteins along with GlyA, and expression levels were determined by flow cytometry. Integrins β1, α4, and CD44 but not CD44v6, integrins β5 and α5β3 were expressed on day 10 erythroblasts (polychromatophilic stage) (Fig. 6, A and B). PBMNC and CHO cells were used as positive controls in these experiments (Fig. 6C).

OPN Contributes to Erythroblast Proliferation But Not to Differentiation and Apoptosis—We then investigated the role of OPN in cell proliferation, differentiation, or prevention of apoptosis. To determine whether OPN promotes erythroblast proliferation we cultured CD71-selected bone marrow-derived erythroblasts obtained from WT and OPN−/− mice for 48 h in vitro and performed an MTT cell proliferation assay. We also cultured a sample from OPN−/− mice in the presence of added OPN. The OPN WT cells proliferated at a much higher rate than the early erythroblast cells obtained from OPN−/− mice during the 48-h culture period (Fig. 7A). The addition of exogenous OPN to OPN−/− cells restored the proliferation rate to the level of WT cells suggesting that OPN-deficient cells are able to respond to exogenous OPN to the same extent as cells producing OPN (Fig. 7A).

We also investigated whether OPN contributes to erythroblast differentiation and/or prevention of apoptosis. To ascertain whether OPN contributes to erythroblast differentiation, we cultured the murine CD71-selected erythroblasts from WT and OPN−/− mice for 72 h and examined the levels of Ter119 by flow cytometry. Normally, Ter119 expression is highly up-regulated during erythroid differentiation. We did not observe any difference in the level of this differentiation marker between WT and OPN−/− cells indicating that OPN does not play a role in erythroblast differentiation (data not shown). Finally, we examined whether OPN contributes to erythroblast survival using human erythroblast cells. We cultured day 8 erythroblasts for 24 h with or without EPO but added OPN into these cultures to determine whether OPN could provide protection against apoptosis induced by the absence of EPO. Analysis of these cells by annexin and propidium iodide for apoptosis revealed that OPN did not provide any protection against apoptosis (data not shown).
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Integrin β1 Mediates the Cell Proliferative Effect in Human Erythroblasts—To determine the receptors responsible for the OPN-mediated increase in cell proliferation, we carried out MTT cell proliferation assay on human erythroblast cells after culturing them for 24 h with neutralizing antibodies against integrin β1, α4, and CD44, the three receptors that were expressed on these cells. We included anti-CD44v6-neutralizing antibody as a control, although this molecule is not expressed in erythroblasts. Our data showed that only exposure to the integrin β1 neutralizing antibody slowed the proliferation rates of erythroblasts (Fig. 7B). Based on these data, engagements of the integrin β1 receptor, most likely by OPN, seem to contribute to the proliferative signal in erythroblasts, although further studies will precisely identify whether OPN binds this receptor in erythroblasts.

DISCUSSION

We studied the regulation of erythropoiesis using a culture system of pure erythroblasts with synchronous maturation. This system was critical in assuring that erythroblasts did not contain non-erythroid cells such as monocytes and macrophages, which are known to secrete chemokines and cytokines including osteopontin (13). Using these cells, we demonstrated that OPN mRNA is expressed throughout erythroid differentiation and were able to study its role in erythroid cell physiology. OPN mRNA was expressed in CD34-positive uncultured (day 0) cells, although at lower levels compared with cells that were at late stages of the differentiation program. Furthermore, we confirmed that OPN expression was not due to growth factor mobilization as OPN transcripts are also present in non-mobilized bone marrow-derived erythroid progenitors.

OPN protein expression peaked between days 6 and 9 of culture, corresponding to the time of maximum proliferation, but cells continued to secrete OPN even at late stages of differentiation. We confirmed that OPN is produced in polychromatic cells by localizing OPN in GlyA-positive cells. Previous studies have shown that OPN is secreted by several hematopoietic cell types such as monocytes, macrophages, and natural killer cells in the context of inflammation and immune function (13, 21). To our knowledge, this is the first report demonstrating expression and secretion of OPN by a hematopoietic cell type not associated with an immune-related function.
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Because of the essential role of F-actin profilaments in the erythrocyte cytoskeleton and its central role in OPN-mediated bone remodeling, we investigated whether OPN plays a role in the regulation of F-actin in erythroblast cells. We observed abnormal structure and distribution of actin profilaments in human OPN knock-down and mouse OPN knock-out erythroblasts compared with untreated and wild-type cells. In OPN\(^{-/-}\) erythroblasts, F-actin profilaments diffuse away from the peri-membrane region and aggregate within the cytoplasm. Interestingly, addition of OPN to OPN\(^{-/-}\) mouse erythroblasts allowed proper formation and localization of F-actin confirming the OPN role in the regulation of the actin cytoskeleton and the ability of OPN\(^{-/-}\) cells to respond to OPN. Therefore, we conclude that OPN plays an important role in the regulation of actin filament formation in erythroid progenitors.

### TABLE 1

| Genotype       | No. | Hb level (g/dL) | Hct (%) |
|----------------|-----|----------------|---------|
| WT (male)      | 10  | 14.1 ± 0.89    | 44.0 ± 5.02 |
| OPN\(^{-/-}\) (male) | 10  | 12.9 ± 0.85\(^a\) | 38.4 ± 4.48\(^a\) |
| WT (female)    | 10  | 14.7 ± 1.62    | 45.1 ± 6.28 |
| OPN\(^{-/-}\) (female) | 9    | 14.3 ± 1.10    | 45.2 ± 2.77 |

\(^a\)\(p = 0.006\) versus WT at base line. 
\(^b\)\(p = 0.012\) versus WT at base line.

FIGURE 6. Expression of receptors for OPN in human erythroblasts. Flow cytometry analysis was performed on day 10 human erythroblasts to detect the expression of various OPN receptors. A, integrin \(\beta_1\), CD44, and integrin \(\alpha_v\) expression in GlyA-positive erythroblasts. B, CD44v6, integrin \(\beta_3\), and \(\alpha_v\beta_3\) in GlyA-positive erythroblasts. C, PBMNC was used as a positive control for CD44v6 and integrin \(\alpha_v\beta_3\) and CHO cells as a positive control for \(\beta_3\) to confirm the presence of receptors that were not observed in erythroblast cells.
studies revealed that integrins through non-RGD-dependent interactions (31, 32). Our CD44 and CD44v6 also have been shown to engage OPN sequence present in OPN. Additional receptors such as integrins in particular were identified as OPN receptors for proliferation, differentiation, survival, and migration (13). The importance of OPN for erythropoiesis in vivo and in vitro is indicated by the decreased iron uptake, lower Hct and Hb in male mice in addition to decreased cell proliferation in mouse erythroblasts. Finally, we showed that the action of OPN on cell proliferation is mediated through the integrin β1. Because actin plays a central role in cell adhesion and motility in association with integrin receptors it is conceivable that OPN acts as a bridge to facilitate interaction between erythroblasts and the mouse bone stroma, perhaps by regulating the release of nascent reticulocytes into the peripheral circulation. Additional studies are needed to further characterize the functions of OPN in the context of erythroid maturation and blood island formation within the bone marrow stromal microenvironment.

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FIGURE 7. OPN promotes cell proliferation of erythroblasts. A, erythroblasts from bone marrows (CD71-selected) of WT and OPN−/− mice were cultured with or without OPN as indicated. The cell proliferation was assessed by MTT assay after 48 h in culture. All data are presented as mean ± S.D. The results are from the mean of triplicate determinations. *, p = 0.02 compared with WT cells; ‡, integrin β1 receptor contributes to cell proliferation in human erythroblasts. Day 9 human erythroblasts were cultured for 24 h in the presence or absence of various neutralizing antibodies (20 μg/ml) as indicated prior to determining the level of proliferation by an MTT assay. All data are presented as mean ± S.D. The results are from the mean of determinations. ‡, p = 0.001 compared with control.

the wild-type mice. There was no difference in the mean corpuscular volume between OPN knock-out and the wild-type male mice. Additionally, no major defects such as fragmented erythrocytes or other morphologically abnormal erythrocytes were observed in OPN knock-out mice in contrast to observations in Rac-1/Rac-2 double knock-out mice (23). However, our data demonstrating that OPN contributes to erythroblast proliferation may in part explain the reason for lower Hct and Hb in OPN−/− mice compared with the WT mice. Although SCF is critical for erythroblast proliferation, the data in our current study suggest that additional cytokines such as OPN also contribute to cell expansion in the erythroid lineage.

In additional studies, we examined the expression of several surface receptors that have been shown to interact with OPN and deliver diverse biological responses including cell proliferation, differentiation, survival, and migration (13). Integrins in particular were identified as OPN receptors because of their interaction through the Arg-Gly-Asp (RGD) sequence present in OPN. Additional receptors such as CD44 and CD44v6 also have been shown to engage OPN through non-RGD-dependent interactions (31, 32). Our studies revealed that integrins β1, α4, and CD44 receptors are highly expressed in erythroblasts, and integrin β1 is important for cell proliferation. Previous studies have demonstrated that OPN promotes cell proliferation in addition to cell survival in several hematopoietic cell types such as pro-B cell line, Ba/F3, and mouse bone marrow cells although through the engagement of CD44 (33). Based on our current studies, OPN does not seem to have a role in cell survival, unlike EPO, which is required for maintenance of cell viability in erythroblasts. Future studies will determine whether CD44 and integrin α4 receptors engage OPN in erythroblasts and their role in OPN-mediated signal transduction and gene expression in this lineage.

In summary, our studies identify erythroblasts as a source of OPN secretion. We also showed that OPN modulates multiple signaling events in erythroblasts, influences Ca2+ efflux, and plays an important role in F-actin filament formation. A functional consequence of OPN deficiency is lower Hct and Hb in male mice in addition to decreased cell proliferation in mouse erythroblasts. Finally, we showed that the action of OPN on cell proliferation is mediated through the integrin β1. Because actin plays a central role in cell adhesion and motility in association with integrin receptors it is conceivable that OPN acts as a bridge to facilitate interaction between erythroblasts and the mouse bone stroma, perhaps by regulating the release of nascent reticulocytes into the peripheral circulation. Additional studies are needed to further characterize the functions of OPN in the context of erythroid maturation and blood island formation within the bone marrow stromal microenvironment.
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