Neuronal Nitric Oxide Synthase Contributes to the Regulation of Hematopoiesis

Peter Krasnov,[1,2] Tatyana Michurina,[1] Michael A Packer,[1,2] Yuri Stasiv,[1,2] Naoki Nakaya,[1,2] Kateri A Moore,[3] Kenneth E Drazan,[2] and Grigori Enikolopov[1]

[1]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA; [2]Arginox Pharmaceuticals, Inc., Redwood Shores, California, USA; [3]Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA

Nitric oxide (NO) signaling is important for the regulation of hematopoiesis. However, the role of individual NO synthase (NOS) isoforms is unclear. Our results indicate that the neuronal NOS isoform (nNOS) regulates hematopoiesis in vitro and in vivo. nNOS is expressed in adult bone marrow and fetal liver and is enriched in stromal cells. There is a strong correlation between expression of nNOS in a panel of stromal cell lines established from bone marrow and fetal liver and the ability of these cell lines to support hematopoietic stem cells; furthermore, NO donor can further increase this ability. The number of colonies generated in vitro from the bone marrow and spleen of nNOS-null mutants is increased relative to wild-type or inducible- or endothelial NOS knockout mice. These results describe a new role for nNOS beyond its action in the brain and muscle and suggest a model where nNOS, expressed in stromal cells, produces NO which acts as a paracrine regulator of hematopoietic stem cells.

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INTRODUCTION

Nitric oxide (NO), a crucial regulator of vasodilation, immunity, and neurotransmission, is also involved in regulating the balance between proliferation and differentiation in several developmental and differentiation settings (1-7). In several instances, this action of NO is based on its antiproliferative properties, such that suppression of NO synthase (NOS) activity by pharmacologic or genetic means increases the number of dividing cells in a tissue and delays or hinders differentiation (2-4,6,8-14). NO can act in both autocrine and paracrine signaling modes, activating soluble guanylate cyclase, combining with reactive oxygen species, modifying proteins, and inducing both short- and long-term signaling cascades (1,5,7,15-17).

In the hematopoietic system, NO contributes to the regulation of hematopoietic stem and progenitor cells in the bone marrow; exposure of mice to NOS inhibitors, either directly or after irradiation and bone marrow transplantation, increases the number of stem and progenitor cells in the bone marrow (18). Moreover, in the transplantation model, this increase is followed by a transient increase in the number of neutrophils in the peripheral blood (18), indicating that modulation of NOS activity may be used for therapeutic intervention.

There are three NOS genes in the mammalian genome, coding for the neuronal, endothelial, and inducible isoforms of NOS (nNOS, eNOS, and iNOS, respectively), and mRNA of each NOS isoform can be reliably detected in mouse bone marrow (18). Furthermore, nNOS mRNA has been detected in neutrophils (19-22), eNOS mRNA has been detected in lymphocytes, megakaryocytes, and platelets (19,23), and iNOS mRNA has been found in megakaryocytes, eosinophils, and unstimulated monocytes (21,24).

Although the action of NO in the hematopoietic system can be readily demonstrated (18,25-29), neither the contribution of individual NOS isoforms nor their mode of action (autocrine vs. paracrine) is understood. We show here that NOS proteins are expressed in bone marrow stroma, that expression of the nNOS isoform strongly correlates with the ability of a panel of stromal cell lines to support hematopoietic stem and early progenitor cells in vitro, and that genetic inactivation of the nNOS gene increases the number of colonies that can be generated from bone marrow and spleen. Our results suggest that nNOS acts as a paracrine effector to regulate hematopoiesis.

MATERIALS AND METHODS

Animals

We used 6- to 12-week-old C57Bl/6 female and male mice (Jackson Laboratories, Bar Harbor, ME, USA, or Taconic Farms, Germantown, NY, USA). Mice with genetically inactivated eNOS gene (B6.129 P2-NOS3^{tm1Unc}) and iNOS gene

Address correspondence and reprint requests to *Grigori Enikolopov, Cold Spring Harbor Laboratory, Bungtown Road, Cold Spring Harbor, NY 11724.. Phone: (516) 367-8316; Fax: (516) 367-6805; E-mail: enik@cshl.edu.*
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(B6.129P2-NOS2^{tm1Lau}) were purchased from Jackson Laboratories. Generation of nNOS-null mutant mice, lacking exon 6 of the nNOS gene (nNOSKOex6 mice), has been described (10). All mice were bred and maintained at the Cold Spring Harbor Laboratory Animal Care Facility in microisolator cages. Wild-type, iNOS$^{-/-}$, and eNOS$^{-/-}$ mice were provided with autoclaved standard diet. nNOS$^{-/-}$ mice were provided with autoclaved liquid diet. All mice received acidified water ad libitum.

Isolation of Bone Marrow, Spleen, and Fetal Liver Cells

Animals were killed by cervical dislocation. Bone marrow cells were isolated from femurs and tibias by repeatedly flushing the bones with Iscove's modified DMEM medium (Iscove's MDM) (Invitrogen) supplemented with 2% heat-inactivated fetal bovine serum (FBS) using a 1-mL syringe and a 21G needle. The cells were then passed three times through a 26G needle. Spleens were minced with fine scissors in Iscove's MDM containing 2% FBS. Cell aggregates were disrupted by drawing the cell suspension up and down nine times through a blunt 18G needle (StemCell Technologies) using a 3-mL syringe, followed by three to four times with a 21G needle. The resulting suspension was filtered using a 40-μm cell strainer (Falcon). For isolation of e14 fetal liver cells, pregnant female mice after anesthesia were perfused with Hanks solution, and embryos were isolated. Livers were dissected from e14 embryos or newborn p1 mice and minced with fine scissors in Hanks solution. After centrifugation (160 g, 10 min), cells were resuspended in 1 mL collagenase-containing Liver Digest solution (Gibco) and incubated for 10 min at 37°C and then EDTA was added to final concentration 5 mM for collagenase inactivation. The cell suspension was filtered using a 40-μm cell strainer (Falcon). The aliquots of cell suspensions were diluted with 3% acetic acid with Methylene Blue (StemCell Technologies), and nuclear cells were counted using a hemocytometer.

Primary Cell Cultures

For primary cell cultures, bone marrow or spleen cells were seeded on 35-mm plates (5×10^6 per plate) in Myelocult medium supplemented with 1 μM hydrocortisone (both StemCell Technologies) and cultured at 33°C in a humidified 5% CO_2 atmosphere for 14 days. One-half of the culture medium was replaced weekly with fresh MyeloCult-hydrocortisone medium. For the analysis of NOS expression in bone marrow stroma, the cells were grown for 3 weeks until they reached 85% to 95% confluence and then exposed directly on the plate to 15 Gy γ irradiation (Marc I irradiator with cesium-137 source). After irradiation, cell cultures were further maintained for at least 4 days to remove destroyed hematopoietic cells. For the measurement of the endogenous NO production, irradiated stromal cells were kept in culture, and half of the media was replaced weekly. Isolated liver cells were seeded on 100-mm plates (10×10^6 per plate) in the media containing 50% Myelocult, 35% α-MEM, 15% FBS, and 1 μM hydrocortisone and cultured at 37°C in a humidified 5% CO_2 atmosphere. Half of the culture medium was replaced weekly.

Stromal Cell Lines

Stromal cell lines from were grown in DMEM supplemented with 10% FBS and 50 μm β-mercaptoethanol at 33°C in a humidified 5% CO_2 atmosphere.

Quantification of Committed Progenitors (CFCs)

All reagents for the analyses were purchased from StemCell Technologies, and the assay was performed according to the manufacturer's instructions. For colony-forming cell (CFC) quantification, semisolid Methocult GF M3434 medium was used. Mononuclear bone marrow cells from femurs or spleen cells of at least three animals of each genotype were seeded in triplicate on 35-mm nonadhesive plates in 1 mL of the medium at a density of 2×10^4 and 1×10^5 cells per plate, respectively. The cultures were maintained for 12 d at 37°C in a humidified 5% CO_2 atmosphere, and the total number of colonies containing >50 cells was counted using an inverted microscope. To analyze the CFC content in bone marrow or spleen primary cell cultures, after 14 days of cultivation floating hematopoietic cells were collected and attached hematopoietic and stromal cells were collected after treatment of cultures with trypsin in citrate saline (StemCell Technologies) and combined with the floating hematopoietic cells. After washing with DMEM supplemented with 2% FBS, the cells were differentially counted using size discrimination criteria in a hemocytometer chamber, seeded in Methocult GF M3434 medium, cultured, and analyzed as described above. In a separate series of experiments, we examined the effect of the NO donor SNAP, added during cultivation, and found that both the number of colonies and the number of cells in each colony was decreased.

Quantification of Long-Term Culture-Initiating Cells

The LTC-IC assay was performed as described (18).

Quantification of Mesenchymal Stem Cells (CFU-F)

For colony-forming unit, fibroblast (CFU-F) quantification in the bone marrow, MesenCult MSC basal medium supplemented with mouse Mesenchymal Stem Cell Stimulatory Supplements (both from StemCell Technologies) was used according to the manufacturer's instructions. Briefly, 0.5×10^6 bone marrow mononuclear cells from femurs of at least three animals of each genotype were seeded in triplicates on 35-mm adhesive plates in 2 mL of the media and cultured without media change for 14 days at 37°C in a humidified 5% CO_2 atmosphere. Cells were then washed with PBS, fixed with methanol for 5 min at room temperature, and stained with diluted (1:20) Giemsa staining solution (StemCell Technologies) for 5 min. After washing, mesenchymal cell colonies were counted using an inverted microscope.

Quantitation of the NOS Isoform-Specific Transcripts

Total RNA was extracted from tissues and cultured cells using TRIzol reagent (Gibco) according to the manufacturer's instructions. The amount of NOS isoform-specific transcripts was quantified using real-time RT-PCR. cDNA was synthesized from 2 μg total RNA using random hexamers and Taqman Multiscribe reverse transcriptase (125 units) (Applied Biosystems). For negative controls, reverse transcriptase was omitted. Diluted cDNA was then mixed with 2× SYBR Green PCR Master Mix (Applied Biosystems) including 0.3 μM both forward and reverse primers and amplified in an optical plate set in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers for selective cDNA amplification of nNOS, eNOS, iNOS, or β-actin were designed using Primer Express software (Applied Biosystems) to achieve the melting temperature value of 58°C to 60°C and the amplicon size of 70 to 150 bp. Primers are as follows:

nNOS, 5′-AAAACCTGCAAAGTCCTAAATCCA-3′ (forward) and 5′-CTCCTGATTCCCGTTGGTGT-3′ (reverse);

eNOS, 5′-GATGGGCCCTGTACCTCAA-3′ (forward) and 5′-GTGGGCCGGCTCTGTAACT-3′ (reverse);

iNOS, 5′-TCCACAGTATGTGAGGATCAAAAAC-3′ (forward) and 5′-ATGTGGCCTTGTGGTGAAGAGT-3′ (reverse);

β-actin, 5′-CGTGAAAAGATGACCCAGATCA-3′ (forward) and 5′-CACAGCCTGGATGGCTACGTA-3′ (reverse);

The amplification conditions were as follows: 1 cycle at 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 1 min. Each sample was analyzed in duplicate. During the amplification, the fluorescence of each sample was detected in real time, and a respective amplification curve was generated using the Sequence Detection System software (Applied Biosystems). Data from NOS amplifications were normalized for each sample using the Ct value (threshold cycle number, a cycle number at which the curve starts to rise over the background noise) for β-actin. Relative differences in the amounts of individual NOS mRNAs between analyzed samples were calculated.

Immunoblotting

For immunoblotting experiments, primary monoclonal antibodies (mAbs) against nNOS (cat. no. 31020) and eNOS (30020) and polyclonal antibodies (pAbs) against iNOS (32030), all from BD Transduction Laboratories, were used at 1:2000, 1:1000, and 1:2000 dilutions, respectively. mAbs against β-actin (Sigma) were used at 1:10,000 dilution. Secondary antibodies (sheep anti-mouse and goat anti-rabbit Ig-horseradish peroxidase conjugates, Pierce), were used at 1:25,000 dilution. Cell suspensions or tissue homogenates in PBS were mixed with an equal volume of 2× SDS-buffer (4% SDS, 120 mM Tris-HCl, pH 6.8) and supplemented with protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mM PMSF final concentration). Lysates were passed three times through a 26G needle to disrupt DNA, boiled for 4 min, and centrifuged at 16,000g for 5 min. After protein determination using BCA reagent system (Pierce), lysates were mixed with one-third volume of 50% glycerol containing 450 mM dithiothreitol and Bromophenol Blue and boiled for 4 min. Gel electrophoresis, membrane transfer, and protein detection were performed as described (30).

Determination of Nitrates/Nitrites

Nitrite and nitrate concentrations were determined in cell culture medium as described (31-33), using the fluorescent 2,3-diaminonapthalene (DAN, Fluka 88461), against nitrite and nitrate standard curves (0 to 1000 nM). For each sample, measured in quadruplet, nitrates were reduced to nitrites using nitrate reductase (NAD(P)H: nitrate oxidoreductase, EC 1.7.1.2 Boehringer Mannheim, cat. no. 981249) following standard methods (34,35) and compared with nitrite measurements alone. Briefly, conditioned cell culture media and nitrite and nitrate standard solutions (40 μL) were treated in a microtiter tray well with 10 μL nitrate reductase cocktail containing (final concentrations in the well) 0.1 U/mL nitrate reductase, 5 μM FAD, 1 μM NAD(P)H), 0.5 mM glucose-6-phosphate (G-6-P), and 0.4 U/mL and G-6-P dehydrogenase (Sigma, G4134) in 100 mM Tris-HCl (pH 7.4) for 60 min at room temperature in the dark. DAN reagent (100 μL) at 0.02 mg/mL concentration in 248 mM HCl diluted from a 1.58 mg/mL stock in dimethyl formamide was added, and the samples were incubated for 30 min in the dark. NaOH (40 μL of 2.8 M) was then added, and the plate was analyzed on a fluorescent plate reader exciting at 360 ± 20 nm and reading at 440 ± 20 nm.

Immunocytochemistry

Sections (40 μm thick) were cut from fetal liver (e14 or p1) on a cryostat and collected on gelatin-subbed slides. The sections were washed twice in PBS (all PBS solutions contained 0.01% thiomersal) and treated with 50% ethanol at room temperature for 30 min followed by blocking with 10% normal goat serum (NGS), 2% Triton X-100 in PBS for 3 h, then incubated at 4°C for 94 h with rabbit anti-nNOS antibody (Zymed, cat. no. Z-RNN3) at 0.25 μg/mL concentration. Sections were washed four times with PBS-T (PBS plus 1% Triton X-100) over a period of 3 h at 4°C and then with PBS with 4% NGS and 1% Triton X-100. Secondary antibody (Alexa fluor 594; Molecular Probes) was applied at 5 μg/mL for 3 h in PBS-T at 4°C, then sections were washed with PBS-T for 3 h at room temperature and overnight at 4°C. Finally, the sections were rinsed with water, air dried, and mounted using Prolong media (Molecular Probes). Confocal microscopy was performed on a Zeiss LSM 510 machine using multitracking, where excitation and emission could not bleed through between channels. Optical slices (0.3 to 0.4 μm thick) were collected that overlapped through the entire section in the z-plane, so projections were continuous.

RESULTS

Expression of NOS Isoforms in Stromal Cells

We have previously shown that mRNA of each NOS isoform can be detected in bone marrow cells by RT-PCR (18). However, it was not known whether these isoforms are expressed in the hematopoietic or the stromal cells of the bone marrow. We have now compared NOS mRNA expression in preparations of cultured stromal and hematopoietic cells from mouse bone marrow. nNOS RNA expression levels were seven times higher in stromal cells than in hematopoietic cells, and for eNOS the difference was 85 times; furthermore, expression of NOS isoforms was higher in stromal cells than in the preparation of freshly isolated bone marrow, 1.4, 315, and 39 times for nNOS, eNOS, and iNOS, respectively (Figure 1A).

We also found that whereas the iNOS protein, but not the other isoforms, can be detected in the lysates of freshly isolated bone marrow, high levels of eNOS protein are detected in the bone marrow and in stromal cells after cultivation (Figure 1B). Low levels of eNOS and iNOS can also be detected in hematopoietic cells after cultivation (Figure 1B).

In agreement with the RNA and protein analyses, we found that cultured stromal cells but not bone marrow cells (isolated and cultivated for 1 day) produced significant amounts of NO (20 nmol total nitrates/nitrites per 10^6 cells per day). Together, these results indicate that stromal cells of the bone marrow are the main source of NOS mRNA, NOS protein, and NO. They also suggest that stromal cells may affect hematopoietic cells through paracrine signaling by NO.

nNOS Is Expressed in the Fetal Liver

Fetal liver becomes the major site of hematopoiesis in the mouse after embryonic day 11 (e11) and stays so until the end of gestation, when hematopoiesis switches over to bone marrow (36,37). We compared the expression of NOS isoforms in the fetal liver at e14, immediately after birth (postnatal day 1, p1), and in the adult liver. Expression of nNOS mRNA was significantly higher in e14 fetal liver than in p1 or adult liver (3.7 and 5.5 times, respectively; Figure 2A). Also, levels of eNOS mRNA were 2.0 and 1.7 times higher in e14 and p1 liver compared with adult liver, whereas for iNOS the difference was 0.5 and 0.1 times (Figure 2A).

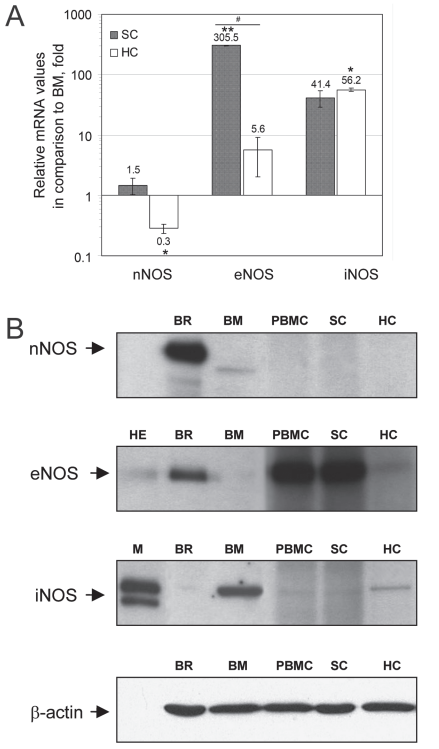

Figure 1. NOS expression in bone marrow cells. (A) Q-PCR analysis of NOS isoform expression. mRNA expression of each NOS isoform was determined in cultured stromal cells (SC) and hematopoietic cells (HC) using Q-PCR and isoform-specific primers. Data are presented as a ratio of expression level of NOS isoform in stromal cells to that in bone marrow. Data were analyzed using *t* test; $*P < 0.005$, $**P < 0.0001$, $\#P < 0.005$. (B) Western blotting analysis of NOS isoform expression. Lysates of bone marrow (BM), primary bone marrow culture (PBMC), SC, HC, human endothelial cells (HE), and brain (BR) (latter two as control and reference) were analyzed by electrophoresis and blotting using antibodies against individual NOS isoforms and β-actin (loading control).

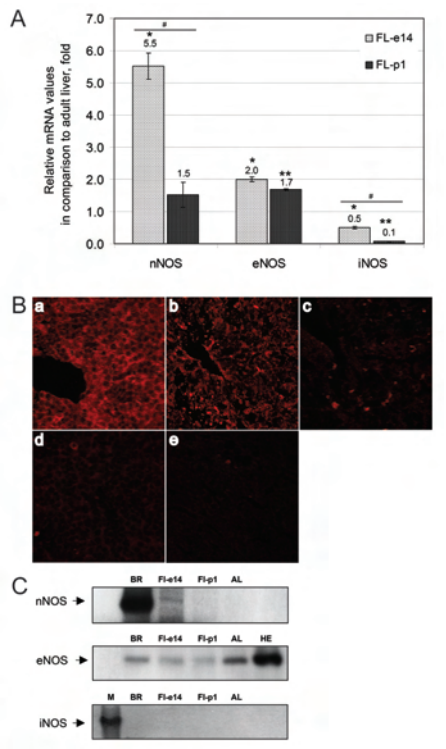

Figure 2. NOS expression in the fetal liver. (A) Fetal liver is enriched in nNOS. RNA expression of NOS isoforms was examined by Q-PCR in liver at 14 days of embryonic development (e14) and postnatal day 1 (p1) and in adult liver. Data for each NOS isoform are presented as ratio of expression level of NOS isoform in e14 and p1 liver to that in adult liver. Data were analyzed using *t* test; $*P < 0.05$, $**P < 0.005$, $\#P < 0.005$. (B) nNOS expression in the fetal liver. Expression of nNOS was determined in sections of e14 (a), p1 (b), and adult (c) liver using monoclonal antibodies against nNOS. (d, e) Negative controls (omitting primary antibody) for e14 (d) and adult (e) liver. (C) Western blotting analysis of NOS isoform expression in the liver. Lysates of fetal liver at embryonic day 14 (Fl-e14), fetal liver at perinatal day 1 (Fl-p1), adult liver (AL), human endothelial cells (HE), macrophage (M), and brain (BR) (latter three as control and reference) were analyzed by electrophoresis and blotting using antibodies against individual NOS isoforms.

We confirmed the expression of nNOS in the fetal liver by immunocytochemistry: it was significantly higher at e14 than at p1 or in the adult liver (Figure 2B).

These results were also confirmed using the Western blot analysis of cell extracts (Figure 2C). Furthermore, we found accumulation of nitrates/nitrites in cultured e14 fetal liver cells (7 nmol total nitrates/nitrites per 10^6 cells per day) but not in cultured cells of the p1 liver (not detectable). Together, these results demonstrate that nNOS is expressed in the fetal liver when this organ serves as the major site of embryonic hematopoiesis, but by birth, when bone marrow becomes the major site of hematopoiesis, expression of nNOS in the liver is greatly reduced.

Expression of nNOS Correlates with the Supportive Abilities of Stromal Cell Lines

Hematopoietic stem cells are rapidly depleted upon cultivation in vitro. Their survival can be supported by cultivating them with appropriate stromal cell lines. Such supporting cell lines have been derived from all of the major embryonic and adult regions of definitive hematopoiesis-embryonic aorta-gonads-mesonephros (AGM) region, fetal liver, and adult bone marrow (38-41). Among the derived cell lines some, but not all, can support the maintenance of repopulating hematopoietic stem cells.

Because bone marrow stromal cells are enriched in NOS RNA and protein and nNOS is expressed in the e14 fetal liver, we examined the relationship between NOS expression and the ability of stromal cells to support hematopoietic stem cells. We examined the expression of NOS isoforms in a panel of stromal cell lines widely used to support cultures of hematopoietic cells. Among them is a group of four immortalized stromal cell lines isolated from murine fetal liver (38,39); these lines were generated as a set, making comparisons between them more straightforward. These cell lines had been tested for their ability to support hematopoietic stem cells during prolonged cultivation. Among this set is a line (AFT024) that provides excellent support for hematopoietic stem cells, a line (2012) with limited supporting abilities, and two lines (2018 and BFC) that

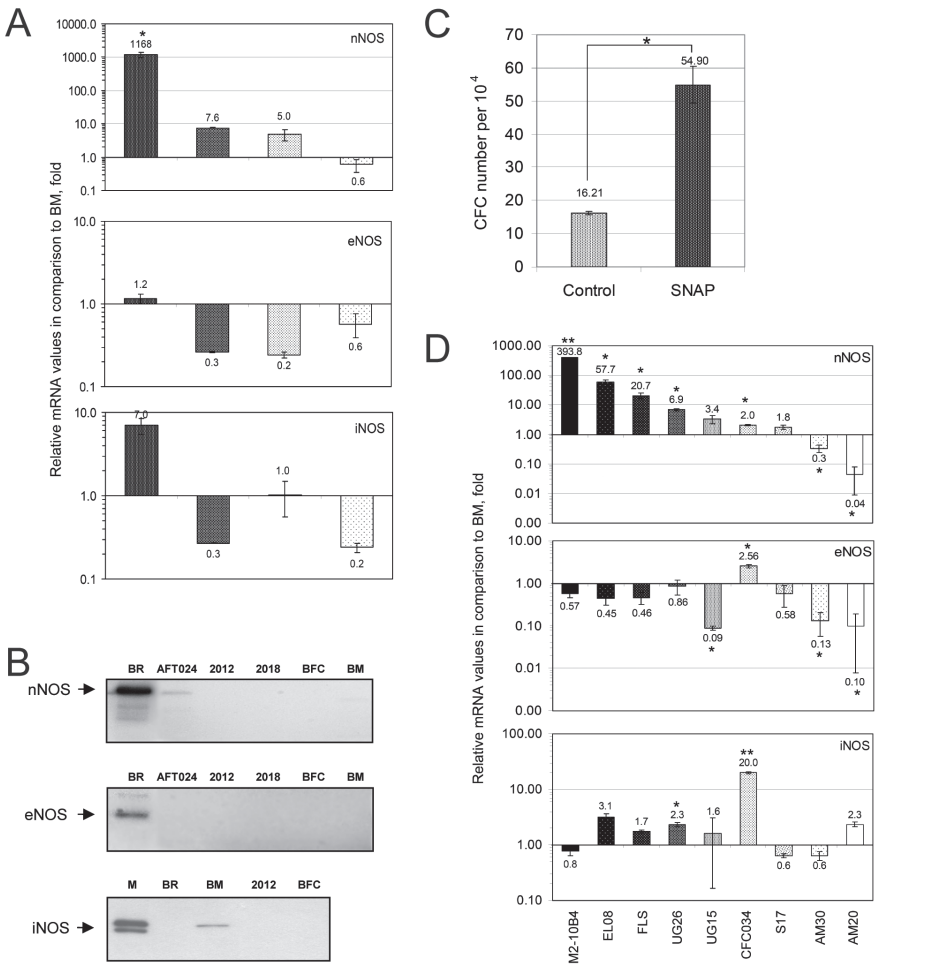

Figure 3. Expression of nNOS correlates with the supporting ability of stromal cell lines. (A) nNOS expression in a set of fetal liver-derived stromal cell lines with different supporting capability for hematopoietic stem cells. mRNA expression for each NOS isoform was determined by Q-PCR. Data are presented as a ratio of expression level of NOS isoform in a stromal line to that in murine bone marrow. Data were analyzed using *t* test; $*P < 0.02$. (B) Western blotting analysis of NOS expression in fetal liver-derived stromal cell lines. Lysates of stromal cells, bone marrow, and brain (as control and reference) were analyzed after electrophoresis using antibodies against NOS isoforms. (C) LTC-IC assay. The number of early hematopoietic progenitors was determined by cultivating hematopoietic cells on the AFT024 stromal cell layer for 4 weeks and replating in methylcellulose for colony formation. Data are presented as the number of LTC-IC per 1×10^4 bone marrow cells taken for analysis. Data were analyzed using *t* test; $*P < 0.02$ (D) nNOS expression in selected stromal cell lines. mRNA expression of NOS isoforms in several stromal lines differing in their ability to support hematopoietic stem cells and in cultured stromal cells from fetal liver (FLS) determined by Q-PCR. Data are presented as a ratio of expression level of NOS isoform in a line to that in murine bone marrow. Data were analyzed using *t* test; $*P < 0.05$, $**P < 0.001$.

can support short- but not long-term hematopoietic cells (38,39).

We were able to detect expression of NOS isoforms in each of these fetal liver-derived cell lines. Interestingly, expression of the nNOS mRNA correlated with the ability of these cell lines to support long-term hematopoietic stem cells: when compared with bone marrow (taken as a common standard in these experiments),

nNOS levels in AFT024, 2012, 2018, and BFC cells were, respectively, 1176, 7.5, 4.6, and 0.5 times the levels in the bone marrow (Figure 3A). Expression of eNOS and iNOS mRNA was also higher in the AFT024 cells than in 2012, 2018, and BFC cells, although the differences were much smaller and the overall levels (compared with bone marrow) were lower. Furthermore, nNOS protein was detected in AFT024 cells but not in 2012, 2018, and BFC cells (Figure 3B). To further examine the role of NO in the ability of stromal cells to support long-term hematopoietic progenitors, we performed a LTC-IC assay with cells cultivated on the AFT024 stromal cell layer with and without an NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and determined the number of early hematopoietic progenitor using the CFC assay. Addition of NO increased the number of CFC colonies 3.5 times (Figure 3C), demonstrating that NO helps to support early hematopoietic progenitors during cultivation.

To further examine the potential relationship between NOS expression and the supporting ability of stromal cell lines, we analyzed a panel of stromal lines used as a feeder layer for hematopoietic cell cultivation and established from adult bone marrow [M2-10B4 (42) and S17 (43)], early embryonic tissue (EL08, UG26, UG15, AM20, and AM30 (40,41), and fetal (e14) liver stroma [CFC034 (38)], as well as a primary culture of cells isolated from the fetal liver which also are capable of supporting hematopoietic stem cells. We found that those cell lines that have been reported to effectively support hematopoietic stem cells (M2-10B4, EL08, and primary fetal liver cells) were strongly enriched in nNOS mRNA (up to 400 times for the M2-10B4 line, compared with bone marrow), whereas those that did not support or had only poor to moderate supporting abilities [UG15, AM20, AM30, and S17 (39,41)] were not enriched (Figure 3D). Interestingly, of the UG26-UG15 pair, UG26 line (a good supporter) expressed all three NOS isoforms at higher levels than its UG15 counterpart (a weaker supporter). Of further note, CFC034 line was only two-fold enriched in nNOS but had 20 times higher levels of iNOS than bone marrow.

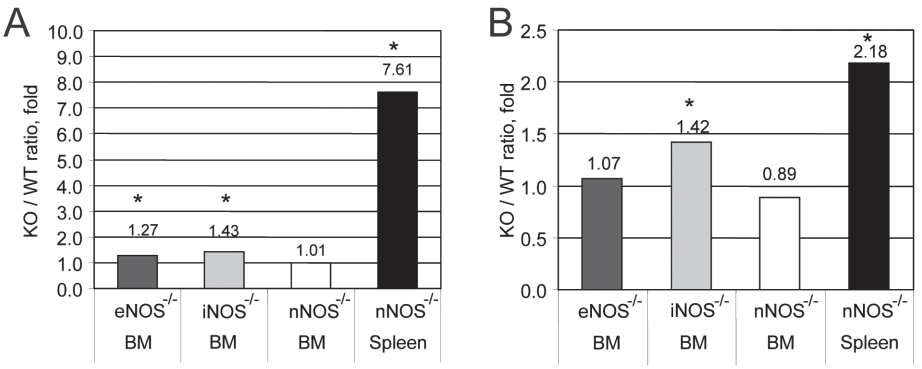

Figure 4. Hematopoietic progenitors in bone marrow and spleen of NOS-knockout mice. Number of CFCs was determined for the bone marrow (BM) of wild-type (WT) and nNOS-, eNOS-, and iNOS-knockout mice and in the spleen of wild-type and nNOS-knockout mice. For each NOS knockout, data are presented as a ratio of frequency (number of CFCs per 10^4 cells) (A) or total number of CFCs per femur in knockouts to those in wild-type mice (B). Data were analyzed using *t* test; $^*P < 0.05$.

Together, these results demonstrate a strong correlation between the hematopoietic supporting ability of stromal cell lines and the levels of expression of nNOS and show that this ability can be increased by the addition of NO. They further support the idea that nNOS, produced by stromal cells, may regulate hematopoietic cells in a paracrine manner.

Hematopoiesis in nNOS-Knockout Animals

We next examined the potential role of NOS isoforms in hematopoiesis in more detail, analyzing committed hematopoietic progenitors (CFCs) in mice with inactivated eNOS (44) and iNOS (45) genes, as well as the nNOSKOex6-null mutant mouse line (10); in these nNOS-knockout mice, disruption of the heme-binding domain of nNOS results in a complete loss of nNOS protein activity. We analyzed the population of committed hematopoietic progenitors by comparing the numbers of CFCs in the bone marrow and spleen of wild-type and mutant animals. We found a modest increase (1.4 times, $P < 0.05$) in the number of CFCs in the bone marrow of the iNOS-knockout mice (both in frequency and in the total number of CFCs per femur), but did not see significant differences in the bone marrow of the eNOS and nNOS knockouts (Figure 4A). However, we found a strong increase in the frequency (7.6 times, $P < 0.05$) and the total number (2.2 times, $P < 0.05$) of CFCs in the spleen of the nNOS-knockout animals (Figure 4B).

We further examined the status of hematopoietic progenitors by cultivating cells from the bone marrow or spleen for 14 days in vitro and then testing them in the CFC assay. Cultures from the bone marrow of the nNOS-knockout mice showed a 2.4-fold increase in the number of CFCs compared with cultures from the wild-type mice, whereas cultures from the eNOS and iNOS knockouts produced the same number of CFCs as the wild-type controls (Figure 5A). Notably, cultures from the spleens of the nNOS-knockout mice produced 27.5 times more CFCs than the wild-type controls (Figure 5A). Furthermore, the increased number of the CFCs in the cultures from the nNOS knockouts was paralleled by a 2-fold (for bone marrow) and an 18.2-fold (for spleen) increase in the number of hematopoietic cells in cultures from the nNOS-deficient animals (Figure 5B); in addition, the number of stromal cells was increased 3.3-fold in cultures from spleens of the nNOS knockouts (Figure 5C). Note that

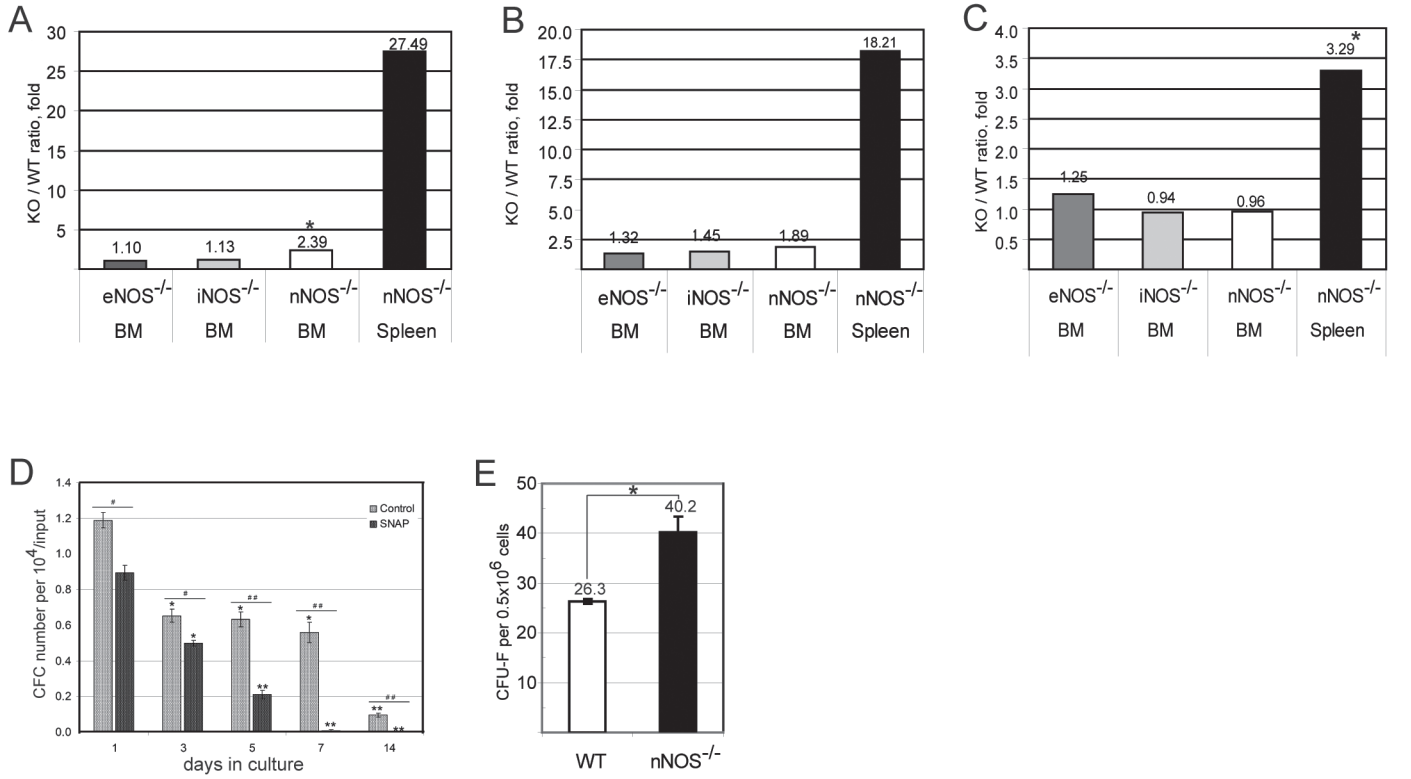

Figure 5. Hematopoietic progenitors and mesenchymal stem cells in the NOS knockouts. Number of CFCs (A), hematopoietic cells (B), and stromal cells (C) per culture after cultivation of bone marrow cells from wild-type (WT) and NOS-knockout mice and spleen cells from wild-type and nNOS knockouts. Data are presented as a ratio of colonies or cells per culture in knockouts to those in wild-type mice. Data were analyzed using *t* test; $*P < 0.05$. (D) Addition of SNAP during cultivation decreases the number of hematopoietic progenitors. Bone marrow cells were cultivated in the Myelocult media with or without 250 μM SNAP for the indicated number of days, and the number of committed progenitors was determined using the CFC assay. The data are presented as a ratio of colonies per 10^4 cells after cultivation to the number of colonies in the starting culture (input), i.e., determined at day 0. Similar results were obtained when the CFC number per culture was calculated. Data were analyzed using *t* test; $*P < 0.01$, $**P < 0.001$, $\#P < 0.01$, $\#\#P < 5 \times 10^{-7}$. (E) Number of CFC-Fs per 0.5×10^6 cells in the bone marrow of wild-type and nNOS-knockout mice. Data were analyzed using *t* test; $*P < 0.05$.

whereas the number of CFC cells in the wild-type or nNOS-deficient bone marrow decreased 11.6 and 5.1 times, respectively, during cultivation (from 17,700 to 1500 and from 19,500 to 3800 per culture), or stayed the same for the wild-type spleen (600 vs. 700 per culture), the number of CFCs in cultured nNOS-deficient spleen cells increased 4.4-fold (from 4400 to 19,300 per culture).

Together, the results with hematopoietic progenitors from NOS mutant mice indicate that reduction of NO production results in an increased number of early hematopoietic progenitors. In a converse series of experiments, we found that addition of the NO donor SNAP results in a dramatic decrease in the number of progenitors to <3% of the control, when subsequently probed using the CFC assay (Figure 5D) (this was observed whether calculated per culture or per 10,000 cells).

The results above suggest that the stroma from nNOS-knockout animals may be enriched in mesenchymal stem cells. We measured these cells in the CFC-F assay, comparing the bone marrow from the nNOS-deficient and wild-type animals, and found 1.53 times more CFC-Fs in the bone marrow of nNOS-knockout than that of their wild-type littermates (Figure 5E).

Together, these results demonstrate that spleens from nNOS-deficient animals are enriched in committed colony-producing progenitors (CFCs). Furthermore, they suggest that bone marrow and spleen of nNOS knockouts are enriched in less mature CFC precursor cells or may provide a better support for the cultivation and maintenance of hematopoietic cells in vitro.

DISCUSSION

We present evidence that NO, produced in stromal cells by nNOS, may act as a paracrine effector to regulate hematopoiesis in the bone marrow and spleen. We show that mRNA of all NOS isoforms is produced in bone marrow stromal cells and that nNOS, in particular, is expressed in bone marrow stroma, fetal liver, and fetal liver-derived cell lines. We further show that expression of

nNOS correlates with the ability of a set of stromal cell lines to support long-term hematopoietic stem cells and that addition of an NO donor increases this ability; furthermore, several other stromal cell lines, widely used to maintain hematopoietic stem cells in culture, express high levels of nNOS. This potential role of nNOS in regulating hematopoiesis is confirmed in our experiments with mutant animals, where inactivation of the nNOS gene results in an increased number of committed progenitors (CFCs) in the spleen and, upon further cultivation of cells, in a greatly increased number of CFCs in the spleen and the bone marrow.

Together, our data suggest a model in which NO, produced by nNOS in the stromal cells of the bone marrow and spleen, acts in a paracrine manner to negatively control hematopoiesis. This model is compatible with the observations that NO can act as a negative regulator of hematopoietic stem and progenitor cells in vitro and in vivo. Donors of NO can change the extent of hematopoietic maturation in vitro (26,29), and inhibitors of NOS activity affect the action of TNFα, IFNγ, and GM-CSF on hematopoiesis in vitro (26-29). Importantly, we have shown that NO regulates hematopoietic stem and early progenitor cell activity in vivo such that treatment with NOS inhibitors increase the number of stem cells in the bone marrow (18). We posit that the experiments with the NO donors and the NOS inhibitors reflect the action of the stroma-derived NO on hematopoietic cells in the bone marrow. In each case, NO arises as a negative regulator of hematopoietic stem and progenitor cells, such that suppression of NOS activity increases the number of hematopoietic cells in vitro and in vivo.

A putative regulatory role for NO may be related to the microenvironment of hematopoietic stem cells (46). The stem cell niche in bone marrow is hypoxic, and analysis of the proteome of hematopoietic stem cells shows adaptation for an anaerobic environment, including increased expression of antioxidant enzymes (47). Hematopoietic stem cells are highly sensitive to reactive oxygen species (ROS) (46,48,49), and nNOS-produced NO, a potent antioxidant, may contribute to the defense against ROS by scavenging superoxides, preventing heme oxidation, suppressing Fenton chemistry, and attenuating lipid peroxidation (7,15,17); furthermore, NO controls expression of a wide range of cell-protective and antiproliferative genes (50-52). Notably, post-translational control plays a prominent role in regulating hematopoietic stem cells in their hypoxic niche (47), and nNOS may be particularly well suited for controlling the niche because it is upregulated by hypoxia; moreover, chronic hypoxia induces a translationally efficient isoform of nNOS mRNA that is resistant to the global translational repression induced by hypoxia (53). Furthermore, NO increases the expression of angiopoietin 1 and its receptor Tie2 (54), whose interactions are crucial for maintaining the quiescence of hematopoietic stem cells in the bone marrow niche (55). Overall, the functional role of NO in hematopoietic tissues may be to act as an antioxidant and to establish a dominant antiproliferative tone to help protect the stem cell pool from premature depletion when challenged with stressful stimuli (18,56). Such a role for NO is compatible with the expression of nNOS in the supporting stromal lines derived from fetal liver and adult bone marrow (sites of definitive hematopoiesis); in these tissues, their ability to maintain hematopoietic stem cells rather than simply to induce them (as in the yolk sac or AGM, the sources of primitive hematopoietic stem cells), becomes of paramount importance, and nNOS may play a significant role in this regulatory step.

There are several other possible links between NO and hematopoiesis, including its potential to regulate proteolysis and to affect mobilization of hematopoietic stem cells (25,57). Whereas the role of eNOS (most probably, produced by endothelial cells) in mobilization of hematopoietic stem cells has been convincingly demonstrated (25), the somewhat unexpected role of nNOS may have been overlooked so far. Selective inactivation of the nNOS gene in stromal cells may provide further evidence for the importance of the paracrine signaling by NO for hematopoiesis.

The regulatory function of NO in the bone marrow and fetal liver that we found may parallel the role of NO in the developing and adult brain (10,13,14,58), where suppression or upregulation of nNOS activity results in an increase or a decrease, respectively, in neurogenesis. It will be important to determine whether NO uses the same molecular mechanisms to control neurogenesis and hematopoiesis.

In addition to revealing a link between NO and hematopoiesis, our results point to nNOS as a possible pharmacological target and suggest that nNOS-selective inhibitors may have a therapeutic potential for hematopoiesis-related disorders.

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