Nucleophosmin Regulates Cell Cycle Progression and Stress Response in Hematopoietic Stem/Progenitor Cells*

Received for publication, February 13, 2006, and in revised form, April 10, 2006. Published, JBC Papers in Press, April 11, 2006, DOI 10.1074/jbc.M601386200

June Li†, Daniel P. Sejas‡, Reena Rani§, Tara Koretsky¶, Grover C. Bagby#, and Qishen Pang‡‡

From the †Division of Experimental Hematology, Cincinnati Children’s Hospital Medical Center and the ‡Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45229 and the §Oregon Health Sciences University Cancer Institute, School of Medicine, Oregon Health Sciences University, Portland, Oregon 97201

Nucleophosmin (NPM) is a multifunctional protein frequently overexpressed in actively proliferating cells. Strong evidence indicates that NPM is required for embryonic development and genomic stability. Here we report that NPM enhances the proliferative potential of hematopoietic stem cells (HSCs) and increases their survival upon stress challenge. Both short term liquid culture and clonogenic progenitor cell assays show a selective expansion of NPM-overexpressing HSCs. Interestingly, HSCs infected with NPM retrovirus show significantly reduced commitment to myeloid differentiation compared with vector-transduced cells, and majority of the NPM-overexpressing cells remains primitive during a 5-day culture. Bone marrow transplantation experiments demonstrate that NPM promotes the self-renewal of long term repopulating HSCs while attenuated their commitment to myeloid differentiation. NPM overexpression induces rapid entry of HSCs into the cell cycle and suppresses the expression of several negative cell cycle regulators that are associated with G1-to-S transition. NPM knockdown elevates expression of these negative regulators and exacerbates stress-induced cell cycle arrest. Finally, overexpression of NPM promotes the survival and recovery of HSCs and progenitors after exposure to DNA damage, oxidative stress, and hematopoietic injury both in vivo and in vitro. DNA repair kinetics study suggests that NPM has a role in reducing the susceptibility of chromosomal DNA to damage rather than promoting DNA damage repair. Together, these results indicate that NPM plays an important role in hematopoiesis via mechanisms involving modulation of HSC/progenitor cell cycle progression and stress response.

Nucleophosmin (NPM) plays important roles in the regulation of cell proliferation and apoptosis. NPM is found to be more abundant in tumor and growing cells than in normal resting cells (1–5). The synthesis of NPM is rapidly increased at early G1 phase and is closely correlated with proliferative induction by various mitogens (1, 2). NPM is also identified as a major gene product required for stem cell development. Conversely, NPM expression is down-regulated in cells undergoing differentiation or apoptosis (6, 7). NPM appears to be the target for certain transforming oncogenes such as c-Myc (8, 9). In the context of hematopoiesis, NPM is frequently found in the chromosomal translocation associated with several hematopoietic malignancies, such as acute promyelocytic leukemia, anaplastic large cell lymphomas, and myelodysplastic syndrome/acute myeloid leukemia (10–12). Interestingly, certain mutations in the NPM gene cause aberrant cytoplasmic localization of the mutated NPM, which has been proposed as a biomarker for certain acute myelogenous leukemia (AML) (13–15). Most recently, studies with Npm-deficient mice have shown that loss of NPM impairs embryonic development and leads to premature cellular senescence and genomic instability (16, 17).

We previously demonstrated that elevated NPM expression in the lymphoblasts derived from a patient with Fanconi anemia (FA), which express extremely low level of NPM, significantly reduced stress-mediated apoptosis (18). More recently, we have shown that NPM acts as a cellular p53 negative regulator to protect cells from stress-induced apoptosis (19, 20). Although these findings are consistent with the notion that NPM modulates stress-induced apoptosis, the influence of NPM on cellular proliferation and apoptosis in the context of hematopoiesis remains unknown. Here we show that NPM regulates differentiation, cell cycle progression, and stress response in hematopoietic stem/progenitor cells.

MATERIALS AND METHODS

Mice—WT and Fancc−/− mice were generated by interbreeding the heterozygous Fancc+/− mice (a generous gift from Dr. Manuel Buchwald, University of Toronto; Ref. 21). The genetic background of the mice is C57BL/6. All of the mice were used at ~8–10 weeks of age. All experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center.

Isolation of BM LSK Cells—BM mononuclear cells were depleted of lineage-committed cells using the magnetic-activated cell separation (MACS) cell separation columns (Miltenyi Biotec Inc.). Lin-ScAl+c-kit+ (LSK) cells were then purified by staining the Lin− cells with Sca-1-PE and c-kit-APC antibodies (BD Pharmingen) followed by cell sorting using a FACS Calibur (Becton Dickinson).

Construction of Retroviral Expression Vectors—The full-length human NPM cDNA (GenBank™ sequence accession number BC009623) was amplified by polymerase chain reaction, using Pfu DNA polymerase (Stratagene) and the following primers: 5′-ATAAGAATGGCGGCCGCCCAC-CATGGGAAATTGATGGACATG and 5′-GTCCTAGAAAGAGACTTCCCTCCACTG. The resulting PCR fragments were subcloned into the NotI and XbaI sites of p3×FLAG-CMV-14 (Sigma) to create p3×FLAG-CMV-14-NPM. The FLAG-tagged NPM fragments was then removed and subcloned into the retroviral vector MIEG3 (a gift from Dr. Yi Zheng, Cincinnati Children’s Hospital Medical Center) to create MIEG3-NPM.
The MIEG3 and MIEG3-NPM plasmids (10 μg each) were used to produce retroviral supernatant.

Retroviral Production and Infection—The MIEG3 and MIEG3-NPM retroviruses were prepared by Dr. van der Loo at the Viral Vector Core, Cincinnati Children’s Research Foundation, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH. Retroviral supernatant was collected at 36, 48, and 60 h, respectively, after transfection. Lin− or LSK cells were plated onto Retronectin-coated (Takara-Shuzo) non-tissue culture 24-well plates and prestimulated for 2 days in IMDM medium containing 20% fetal calf serum, 100 ng/ml stem cell factor (SCF), 20 ng/ml interleukin-6 (IL-6), and 50 ng/ml Flt-3 ligand (Flt-3L) (Peprotech). Cells were then exposed to the retroviral supernatant at multiplicity of infection (MOI) of 2–5 for 3 h at 37 °C in the presence of 4 μg/ml polybrene (Sigma). Cells were centrifuged at 600 × g for 45 min. Infection was repeated two times and infection efficiency was assessed by the detection of green fluorescent protein (GFP)-positive cells by fluorescence-activated cell sorting (FACS).

Clonogenic Progenitor Assays—The transduced cells were cultured at 1000 cells/ml in a 35-mm tissue culture dish in 4 ml of semisolid medium containing 3 ml of MethoCult M 3134 (Stem Cell Technologies) and the following growth factors: 100 ng/ml SCF, 10 ng/ml rhIL-3, 100 ng/ml granulocyte colony-stimulating factor (G-CSF), and 4 units/ml erythropoietin (Peprotech). On day 10 after plating, the colony number was counted and photographed. Colony growth results were expressed as mean (of triplicate plates) ± S.D. per 1000 cells plated. Levels of significance were determined using Student’s t distribution.

Single Cell Analysis—Single LSK GFP+ cells were sorted and deposited into each well of 96-well plates and cultured in 15 μl of IMDM medium containing cytokines. The presence of a single cell in individual wells was verified, and wells with no cell or more than one cell were excluded from analysis. Cell division was monitored at 24-h intervals for at least 4 days.

Apoptosis Assay and Cell Cycle Analysis—Cells were stained with annexin V and propidium iodide using BD ApoAlert Annexin V kit (BD PharMingen) in accordance with the manufacturer’s instructions. Apoptosis was analyzed by quantification of annexin V positive cell population by flow cytometry. For cell cycle analysis, cells were fixed by 1 ml of 70% ethanol at −20 °C. After air drying, cells were resuspended in 1 ml of 0.25% formaldehyde in phosphate-buffered saline, permeabilized with 0.3% Nonidet P-40, and then stained with propidium iodide containing 1 mg/ml RNase A, followed by FACS analysis of the G0/G1, S, and G2/M populations.

Immunoblotting—Whole cell extracts (100 μg of total proteins) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots were incubated with the antibodies specific for cyclins A, B, or E, cdc2 (Oncogene Research Products), Cdk2, 4, or 6, NPM (Santa Cruz Biotechnologies), p53, p21 (Cell Signaling Technology), or β-actin (Sigma) for 12–16 h at 4 °C.

Immunocytochemistry—Cells were cytospun onto slides and fixed in ice-cold methanol for 5 min in −20 °C. After air drying, cells were blocked for 1 h with 5% normal serum. Then cells were incubated with primary antibodies (p53 or p21, both from Cell Signaling Technology) in phosphate-buffered saline with 2% normal serum at room temperature for 1 h. After extensive washes, cells were incubated with PE-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, ME). DNA was then labeled with DAPI (4,6-diamidino-2-phenylindole, Sigma). Slides were finally mounted in mounting medium (Vector). Cells were viewed and photographed using a Leica DM IRB microscope at ×10 magnification with an ORCA-ER C4742–95 camera (Hamamatsu). The captured images were processed using OpenLab 3.1 software (Improvision) and displayed with Adobe Photoshop V6.0.

RNA Isolation and Reverse Transcription (RT)-PCR—Total RNA was prepared with RNaseasy kit (Qiagen) following the manufacturer’s procedure. Reverse transcription was performed with random hexamers and Superscript II RT (Invitrogen) and was carried out at 42 °C for 60 min and stopped at 95 °C for 5 min. These reactions were followed by PCR using two primers specific for the respective NPM and 3′xFLAG sequences: 5′-TCCGCTGCAGACAGTGGCAG-3′ (sense; within NPM sequence) and 5′-CATCGCGCAATCCAGCGACG-3′ (antisense; within 3′xFLAG sequence), with thermal cycling parameters: 95 °C for 5 min; 15, 20, or 30 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min.

Short and Long Term Competitive Repopulating Analysis—Age-matched female congenic B6.SJL-PtprcPep3b/BoyJ (B6.BoyJ; CD45.1+) mice (Jackson Laboratories) were used as transplant recipients. These mice were lethally irradiated (9.5 Gy, 110 cGy/min, 137Cs γ-rays) and injected intravenously with 1 × 106 Lin− BM cells transduced with MIEG3 or MIEG3-NPM viruses, mixed with 5 × 105 competitor cells. Donor-derived repopulation in recipients was assessed by the proportion of leukocytes in peripheral blood that expressed the GFP protein by flow cytometry. Short and long term engraftment and multilineage repopulation analysis of donor cells were performed at 1 month and 4 month post-transplantation. For secondary BM transplantation, BM cells from all individual recipients were pooled and then 5 × 104, 2 × 105, and 2 × 106 bone marrow cells were injected into cohorts of eight female lethally irradiated recipients per cell dose. Recipients were analyzed at 4 and 16 weeks post-transplant. For transplants using LK5+ cells, recipient mice received 1,000 freshly isolated cells and were analyzed at 1, 4, and 6 month intervals.

Comet Assay—The generation of DNA strand breaks was assessed by the single cell gel electrophoresis (comet) assay (22), using a Fpg-FLARE fragment length analysis using repair enzymes comet assay kit in accordance with the manufacturer’s instructions (Trevigen). For each experimental point at least three different cultures were analyzed, and 50 cells were evaluated from each culture. Comet tail length and tail moment were measured under a fluorescence microscope (Nikon model 027012) using an automated image analysis system based on a public domain NIH Image program (23).

Hematopoietic Stress Treatment of Transplanted Mice—Two weeks after transplantation, recipients were injected intraperitoneal with a single dose of 5-fluorouracil (5-FU; Sigma; 150 mg/kg). GFP-positive donor-derived cells were counted weekly post-injection. Peripheral blood leukocytes were analyzed for myeloid (Gr-1/CD11b) and lymphoid (B220/CD3) reconstitution.

Statistics—Data were analyzed statistically using a two-tail Student’s t test. The level of statistical significance stated in the text was based on the p values. p < 0.05 was considered statistically significant.

RESULTS

NPM Promotes HSC and Progenitor Cell Expansion—To gain insight into the role of NPM in hematopoiesis, we overexpressed FLAG-tagged NPM in murine BM LSK cells by retroviral gene transfer (Fig. 1A). We purified GFP-positive cells by FACS and confirmed the expression of the NPM transgene by RT-PCR using primers complementary to the NPM and FLAG-coding sequences (Fig. 1B). We generally achieved transduction efficiency of greater than 50% (Fig. 1C). We cultured vector- and NPM-transduced LSK cells for 10 days and analyzed cell proliferative potential. As shown in Fig. 2A, NPM-transduced cells expanded significantly faster than vector-
NPM Promotes HSC and Progenitor Cell Proliferation

FIGURE 1. A, retroviral constructs. MIEG3 retrovirus with an internal ribosomal entry site (IRES) and GFP-encoding sequences. NPM-FLAG encodes full-length human NPM with a FLAG tag on its C terminus. B, RT-PCR analysis, showing NPM-FLAG expression in NPM-transduced cells using primers complementary to NPM and FLAG coding sequence, respectively. Equal RNA used for PCR amplification from vector- and NPM-transduced cells was ensured by amplification of the housekeeper gene GAPDH. C, transduction efficiency of vector (MIEG3) and NPM-3FLAG (NPM) retrovirus (MOI, 2–5) determined by quantification of the fraction of GFP⁺ cells (gated in R2).

FIGURE 2. NPM promotes HSC/progenitor cell growth. A, expansion of vector- and NPM-transduced LSK cells. B, cell death at different time points of 6-day culture. C, growth advantage of NPM-transduced (GFP-positive) LSK cells. Cells were cultures in cytokine-supplemented medium, and percentage of GFP⁺ cells was analyzed by flow cytometry. D, growth of clonogenic progenitors (CFC) of vector- and NPM-transduced LSK cells. E, NPM overexpression increased series replating efficiency of hematopoietic progenitors. Data represent the mean ± S.D. of triplicate experiments. *, statistical significance between paired samples at p < 0.05.
duced cells during 4 days of culture (4.2- and 6.4-fold at days 3 and 4, respectively, compared with 1.7- and 2.3-fold with vector-transduced cells). Notably, we observed that the growth rate of NPM-transduced cells peaked at days 5 and 6. Further analysis indicated a modest increase in cell death (Fig. 2B) and erythroid lineage (Ter119+/H11001) differentiation in these cells (Table 1). Thus, a modest decline in proliferation of the NPM-transduced cells might be caused by an increase in terminal differentiation, which could lead to an increase in cell death. Indeed, we found that NPM expression increased erythroid reconstitution in our bone marrow transplantation experiments (see Fig. 4, below). We next determined whether NPM confers growth advantage to HSC and progenitor cells. During the 10-day period, the content of GFP+ cells in vector group (cells transduced with vector alone) remained steady at ~50% (Fig. 2C), which was close to initial transduction efficiency of 52%. Significantly, NPM-transduced cells (54% transduction efficiency) exhibited a significant growth advantage to total cells, with more than 80% of the cells expressed eGFP/NPM at day 10 (Fig. 2C).

We next asked if NPM expression increases clonogenic progenitor activity. We found that total number of colonies formed by NPM-transduced cells was more than 2-fold higher than that of vector-transduced cells (Fig. 2D). Significantly increased series-plating efficiency was also observed with NPM-transduced LSK cells compared with vector-transduced cells (Fig. 2E). In addition, NPM-expressing LSK cells generally formed larger colonies than vector-transduced cells did (data not shown). Taken together, these results indicate a selective expansion of NPM-overexpressing HSC and progenitor cells.

NPM Overexpression Decreases Differentiation of Myeloid-committed Progenitors—Given that NPM overexpression promotes HSC expansion, we wondered if NPM affected differentiation of these primitive cells. Interestingly, NPM expression decreased the differentiation of LSK cells toward myeloid lineage, with a 3-fold decrease in cell population expressing myeloid marker Gr-1 and CD11b (Fig. 3).
FIGURE 4. Overexpression of NPM increases repopulating capacity of HSC/progenitor cells. A, representative results and statistic analysis of short term engraftment. B, representative results and statistic analysis of long term engraftment. Data represented as the mean ± S.D. (n = 6). *, p < 0.05 between vector and NPM-transplanted mice. C, NPM promotes the self-renewal of long term repopulating HSCs while attenuated their commitment to myeloid differentiation. PB cells from long term (16-week) transplanted mice were stained with antibodies that recognize Gr-1 and CD11b or B220 and CD3 or Ter119, and lineage distribution was analyzed by flow cytometry in GFP + and GFP - cell populations. Shown is representative flow cytometric presentation of two independent experiments (n = 6). Numbers in the corners indicate percent of events in that quadrant. D, summary of the flow data shown in C. All data are expressed as mean ± S.D. *, p < 0.05 between vector and NPM-transplanted mice.

NPM Promotes HSC and Progenitor Cell Proliferation
Moreover, more than 50% of NPM-expressing cells retained expression of primitive marker Sca1 and c-Kit compared with 20% of vector-transduced cells (Fig. 3C). Consistent with this, NPM-expressing cells showed loss of granulocyte maturation and accumulation of myeloblasts and early progenitors (mostly promyelocytes) at day 10 of culture (Fig. 3D). These results indicate a selective expansion of NPM-expressing HSC and progenitor cells.

**NPM Expression Increases HSC Repopulating Capacity**—Next, we performed bone marrow transplantation to evaluate the *in vivo* function of NPM-expressing hematopoietic progenitor cells. Significantly, NPM-transduced cells constituted more than 40% of the peripheral blood cells at 16 weeks after transplantation, compared with less than 10% reconstitution by vector-transduced cells (Fig. 4B). Examination of lineage distribution of GFP+/GFP- cells in the peripheral blood from the 16-week post-transplanted mice shows that 10% of the cells were Gr-1/CD11b+, 30% were either B220+ or CD3ε+, and 16% were Ter119+, indicating that HSCs expressing NPM has the ability of multilineage reconstitution. However, the percentage of myeloid (Gr-1/CD11b+) cells was significantly reduced in NPM/GFP+ fraction compared with that reconstituted by non-transduced cells (Fig. 4, C and D). This is in agreement with the *in vitro* data (Fig. 3) showing that NPM inhibits myeloid differentiation. Interestingly, NPM expression increased proportion of erythroid (Ter119+) cells (16% compared with 6% in vector/GFP+ fraction; p < 0.05; Fig. 4, C and D), suggesting that NPM may be a positive regulator of erythropoiesis. Similar results were obtained with secondary transplantation, which shows that NPM promoted the self-renewal of long term repopulating HSCs while attenuated their commitment to myeloid differentiation (Table 2).

**NPM Is a Positive Regulator of HSC Cell Cycle Progression**—To address how NPM expression enhances HSC and progenitor cell expansion, we analyzed cell cycle profile of NPM-transduced LSK cells. As shown in Fig. 5A, NPM overexpression prominently reduced cells in the G0/G1 phase (55.6% compared with 65.4% with vector-transduced cells). This coincided with a significant increase in cells in the S phase (34% compared with 26.1% with vector-transduced cells). BrdU labeling experiments show that the percentage of cells entering the cell cycle was significantly higher in NPM-transduced cells than in vector-transduced cells.

**TABLE 2**

Secondary transplantation

| Virus | All WBCs | Myeloid | Lymphoid | Erythroid |
|-------|----------|---------|----------|-----------|
| Vector | 3 ± 0.2  | 16 ± 3.2| 12 ± 2.6 | 15 ± 5.7  |
| NPM   | 10 ± 1.6*| 6 ± 0.8*| 12 ± 3.5 | 25 ± 4.3* |

* Statistical significance between vector and NPM samples at p < 0.05.

**FIGURE 5.** NPM promotes G1 to S transition. A, cell cycle analysis of vector and NPM transduced LSK cells. B, transduced LSK cells were pulsed with BrdU, and percentage of cells incorporated with BrdU was determined by staining the cells with FITC-conjugated anti-BrdU antibody. C and D, NPM overexpression promotes HSC cell division. Single LSK cell was sorted into 96-well plates and the cumulative percentage of cells that have undergone at least one cell division after 24 h (C) and 48 h (D) were calculated. All data are expressed as mean ± S.D. *, p < 0.05 between vector and NPM-transduced cells.
NPM Promotes HSC and Progenitor Cell Proliferation

FIGURE 6. NPM overexpression is associated with reduced expression of negative cell cycle regulators. Vector- or NPM-transduced LSK (A) or Lin– (B–D) BM cells were analyzed for the expression of G1/S cell cycle regulators by immuno-fluorescence (A), Western blotting (B and D), or RT-PCR (C).

cells (45% compared with 30%; Fig. 5B). To further determine the cellular mechanism by which NPM promotes HSC and progenitor cell expansion, we employed a single-cell assay to see if overexpression of NPM regulates the recruitment of HSCs into the cell cycle in response to cytokine stimulation. At 24 h, about 20% of NPM-transduced LSK cells underwent at least one division compared with ~12% of vector-transduced cells (p < 0.05; Fig. 5C). Less dramatic but still significant (p < 0.05) result was obtained with the pair of cell lines at 48 h when nearly 70% of NPM-transduced cells have undergone division compared with 56% of vector-transduced cells (Fig. 5D).

NPM Suppresses the Expression of Negative Cell Cycle Regulators—To investigate the molecular mechanism whereby NPM promotes HSC and progenitor cell cycle progression, we examined the expression of cell cycle regulatory molecules in vector- and NPM-transduced cells. We focused on p53, p21WAF1, p27KIP1, p16INK4A, and p19Arf, which have been known to negatively regulate cell cycle progression in HSC and progenitor cells (24, 25). We found that more than 70% of the control vector-transduced LSK cells were stained positive for p53, significantly higher than NPM-transduced cells (20%; p < 0.05; Fig. 6, A and B). Higher accumulation of p21WAF1 was also observed in vector-transduced cells than in NPM-transduced cells. Gene expression profiling by RT-PCR shows that the expression of p16INK4A was significantly decreased by NPM expression (Fig. 6C). We also examined the expression of the positive regulators of G1-to-S transition including cyclins A, B, D, and E, and cyclin-dependent kinases cdc2 (Cdk1), Cdk2, -4, and -6. Among them, we found only cyclin A that is up-regulated by NPM overexpression (Fig. 6D). It should be noted that the levels of phosphorylated Cdk1 and Cdk2 and the kinase activities of Cdk1, -2, and -4 were significantly higher in NPM-transduced cells than in vector cells (data not shown).

NPM Enhances HSC and Progenitor Survival in Response to DNA Damage and Hematopoietic Stress—Because we previously showed that forced expression of NPM reduces cell death induced by tumor necrosis factor α in lymphocytes derived from a Fanconi anemia group C patient (18), and because cells from FA patients exhibit hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC) and oxidative stress (26–30), we investigated the effect of NPM expression in HSC and progenitor cells isolated from mice deficient for the Fanconi group C gene (Fancc−/−). Transduced WT and Fancc−/− LSK cells were challenged with DNA damage (MMC) or oxidative stress (H2O2). Fancc−/− HSC and progenitor cells were extremely sensitive to MMC with virtually no viable cells left after 4 days (Fig. 7A). NPM expression dramatically increased survival of Fancc−/− cells exposed to MMC or H2O2 (Fig. 7, A and B). A milder but still significant increase in survival was also observed in WT NPM-transduced cells. This was apparently because of a significant reduction in stress-induced apoptosis in the NPM-transduced cells, as determined by annexin V binding (Fig. 7C).

To further investigate the in vivo role of NPM in the response of HSC and progenitor cells to stress, we determined the effect of NPM overexpression on hematopoietic recovery in mice treated with 5-FU. Analysis of peripheral blood from 5-FU-treated recipients showed that NPM-transduced cells were able to recover from hemoablation rapidly and by only 7 days post 5-FU treatment, the number of GFP-positive cells has reached at least the pretreatment level (data not shown). Furthermore, mice transplanted with NPM-transduced HSC and progenitors showed accelerated multilineage recovery within 7 days after 5-FU treatment (Table 3). In contrast, vector-transduced cells recovered slowly and reached ~70% of the level before 5-FU treatment (data not shown).

To further investigate the mechanism by which NPM protects HSC and progenitor cells from DNA-damaging stresses, we determine oxidative stress-induced DNA damage in HSC/progenitor cells by the comet assay (31, 32), which measures specifically oxidative DNA damage including single- and double-strand DNA breaks (32). To augment the stress effect, we also used Fancc−/− HSC and progenitor cells which are hypersensitive to H2O2 treatment (Fig. 7). NPM expression significantly reduced oxidative DNA damage in both untreated and H2O2-treated Fancc−/− HSC and progenitor cells, as well as in H2O2-treated WT cells (data not shown). The reduced levels of oxidative DNA dam-
The age observed in NPM-overexpressing HSC/progenitor cells suggest that NPM could protect DNA from oxidative attack, thereby decreasing DNA strand breaks, or alternatively, NPM could enhance the damage response/repair process. To distinguish between these possibilities, we treated BM HSC and progenitor cells transduced with vector alone or NPM with H$_2$O$_2$ and conducted a time course study to assess DNA repair kinetics by examining the levels of DNA strand breaks. NPM-overexpressing HSC/progenitor cells consistently showed lower levels of DNA strand breaks than vector-transduced cells (data not shown). However, there was no significant difference between the two cell lines.

**TABLE 3**

Peripheral blood parameters of mice following hemoablation with 5-FU

| Peripheral Blood Counts | Vector Day post-5-FU | NPM Day post-5-FU | Relative ratio (vector/NPM) Day 3 | Relative ratio (vector/NPM) Day 7 |
|-------------------------|---------------------|-------------------|-----------------------------------|----------------------------------|
| WBC                     | Day 0 | Day 3 | Day 7 | Day 0 | Day 3 | Day 7 | Day 0 | Day 3 | Day 7 | Day 0 | Day 3 | Day 7 | Day 0 | Day 3 | Day 7 |
| 7.9 ± 0.3               | 3.5 ± 0.4          | 2.0 ± 0.2         | 8.1 ± 0.6          | 4.5 ± 0.4          | 3.0 ± 0.4 | 1.3 | 1.5 |
| 6.9 ± 0.3               | 6.6 ± 0.3          | 4.3 ± 0.5         | 7.5 ± 0.6          | 7.2 ± 0.3          | 7.2 ± 0.7* | 1.1 | 1.67 |
| 9.7 ± 0.2               | 8.0 ± 0.1          | 4.8 ± 0.2         | 9.5 ± 0.7          | 7.3 ± 0.2          | 7.0 ± 0.5* | 0.9 | 1.46 |

| PB WBC composition     | Day 0 | Day 3 | Day 7 | Day 0 | Day 3 | Day 7 | Day 0 | Day 3 | Day 7 | Day 0 | Day 3 | Day 7 | Day 0 | Day 3 | Day 7 |
|------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Gr-1/CD11b             | 7.2 ± 0.5       | 5.8 ± 0.2       | 0.1 ± 0.0       | 7.3 ± 0.4       | 5.5 ± 0.3       | 0.1 ± 0.0       | 0.9 | 1.0 |
| B220                   | 4.6 ± 0.2       | 2.4 ± 0.3       | 1.3 ± 0.1       | 4.4 ± 0.3       | 2.5 ± 0.2       | 1.7 ± 0.8       | 1.0 | 1.3 |
| CD4/CD8                | 2.2 ± 0.1       | 0.6 ± 0.2       | 0.6 ± 0.3       | 2.3 ± 0.4       | 0.9 ± 0.5       | 1.4 ± 0.3       | 1.5 | 1.8 |

*Statistical significance between vector and NPM samples at $p < 0.05$. 

**FIGURE 7.** NPM overexpression enhances survival of HSC/progenitor cells in response to DNA damage and oxidative stress. A, survival rates of LSK cells from WT and Fancc$^{-/-}$ mice treated with MMC (40 nM). B, survival rates of LSK cells in A treated with H$_2$O$_2$ (50 μM). Data shown in A and B represent mean ± S.D. of triplicate determinations. C, apoptosis of LSK cells treated with MMC (40 μM).
NPM Promotes HSC and Progenitor Cell Proliferation

in terms of the kinetics of DNA damage repair, as measured by the remaining amounts of DNA strand breaks over a period of 60-min post-treatment. This suggests that cells expressing NPM accumulated less oxidative DNA damage probably because of its role in reducing the susceptibility of chromosomal DNA to damage rather than promoting DNA damage repair.

DISCUSSION

Hematopoiesis is an ordered process of proliferation and differentiation, which leads to the generation of mature blood cells from a rare population of pluripotent HSCs (33). The hematopoietic system utilizes a variety of homeostatic mechanisms for regulation of HSCs in order to sustain blood cell production throughout life. A fine balance between self-renewal and differentiation allows for the size of the HSC pool to be maintained and for the body to continually supply with the lymphoid, myeloid, and erythroid lineages. The fate of HSCs for self-renewal or for differentiation is decided by both extrinsic and intrinsic factors. Studies with mutant gene knock-in in mice have provided insights into some of positive and negative regulators for hematopoiesis (34). For example, certain cytokines such as stem cell factor, thrombopoietin, flt-3 ligand, and their receptors have been shown to play important roles in the regulation of HSC self-renewal and differentiation (35–37). Recently, other receptor/ligand signaling pathways such as the Notch and Wnt signaling pathways have been found to regulate HSC self-renewal (38, 39). And a variety of transcription factors such as Ikaros and Bmi-1 and cell cycle regulators like the G1-specific inhibitor p21WAF1 have emerged as key regulatory components involved in proliferation and differentiation of HSCs (24, 25, 40). Here we report that NPM is another regulatory molecule that plays a role in HSC proliferation, differentiation, and stress response.

Our results show for the first time that NPM enhances the proliferative potential of HSC and progenitor cells, as demonstrated by using both ex vivo and BM transplantation models. This finding is consistent with a recent report by Grisendi et al. (16) showing that NPM is essential for primitive hematopoiesis. Mice deficient for NPM die at early embryonic stage because of severe anemia (16). We attribute the enhanced proliferative effect of NPM to its ability to induce rapid entry of hematopoietic progenitors into the cell cycle, probably via promoting G1-to-S transition. Our study of the mechanism underlying this phenomenon reveals that the positive role of NPM in HSC and progenitor cell cycle progression is associated with suppression of the negative cell cycle regulators p53, p21WAF1 and p16INK4a. Recent findings in mice deficient for Bmi-1, ATM, and JunB have highlighted the impact of G1-S regulation by p53, p21WAF1, and p16INK4a in primitive hematopoiesis (24, 25, 33). In contrast to previous reports that NPM binds the tumor suppressors p53 and p19ARF and may play a role in the stability of these proteins (17, 41), overexpression of NPM in HSC and progenitor cells reduced p53 protein levels, which was correlated with decreased expression of p53 target protein p21WAF1, whereas down-regulation of NPM by siRNA elevated p53 levels accompanied by increased p21WAF1 in response to stress. We failed to detect the effect of NPM on p19ARF in either NPM-overexpressing or NPM-knockdown HSC and progenitor cells. Among the positive regulators of G1-to-S transition, we found only cyclin A that is up-regulated by NPM overexpression in BM progenitors. Notably, overexpression of cyclin A in cultured cells affects cell cycle progression and leads to accelerated entry into S phase (42). However, down-regulation of endogenous NPM in HSC and progenitor cells did not affect cyclin A expression in the steady state or in response to stress. This suggests that cyclin A may not be the target of NPM overexpression. It has been reported that NPM is a substrate of cyclin-dependent kinases Cdk1 (cdc2) and Cdk2 (43, 44), which could complex with cyclin A in the process of cell cycle progression. We are in the process of investigating whether NPM plays a role in regulation of the formation or/and activity of cyclin A/Cdk complexes.

Intriguingly, we observed the attenuation of myeloid differentiation in HSC/progenitor cells overexpressing NPM. Significantly lower levels of mature myeloid markers (Gr-1/CD11b) were expressed in NPM-overexpressing LSK progeny or mice reconstituted with NPM-transduced progenitors. In addition, majority of the NPM-overexpressing cells in day-5 liquid culture and in the increased series-plating colonies showed loss of granulocyte maturation and accumulation of myeloblasts and early progenitors (mostly promyelocytes) and expressed primitive progenitor markers Sca1+c-kit+, thus arguing for a direct effect of NPM overexpression on differentiation of myeloid progenitor cells. The detection of significantly lower myeloid (Gr-1/CD11b-1) cells in recipients reconstituted with NPM-transduced BM progenitors compared with that reconstituted by non-transduced cells further supports the notion that the observed block in myeloid differentiation is intrinsic to the NPM-transduced BM progenitor cells. The cause for this phenomenon may involve preferential proliferation over differentiation of NPM-overexpressing myeloid precursors. It has been long known that NPM expression is elevated in actively proliferating cells but decreased when cells are induced to differentiate (1, 2, 6). However, the mechanism by which NPM selectively inhibits myeloid differentiation remains to be determined.

Overexpression of NPM protects HSCs and progenitors exposed to MMC or hydrogen peroxide, suggesting that NPM can protect cells from DNA damage and oxidative stress. We recently demonstrated that NPM protects cells from apoptotic cell death induced by diverse stresses through a mechanism involving inhibition of the p53 tumor suppressor protein (19, 20). Others have reported that UV radiation induces expression and nuclear translocation of NPM, which in turn enhances DNA damage repair and prevents apoptosis (17, 45). More recently, work with NPM-deficient mice strongly indicates that NPM is essential for the maintenance of genomic stability and cell survival (16, 17). Still, it seems to be contradictory that NPM protects cells from DNA damage and at the same time suppresses p53 in response to stress, the latter of which may allows cells to proceed with cycling without repairing the DNA damage. However, p53 is also a major factor that influences life-or-death decision of the cell. By regulating p53 in response to DNA-damaging stress, NPM may provide a survival mechanism which allows the cell to ultimately repair the damage. This may be particularly important for such types of cells as HSC and progenitor cells, which are needed for the production of billions of blood cells each day.

How does NPM protect cells from DNA damage induced by genotoxic stress? NPM-overexpressing HSC and progenitor cells accumulated less stress-induced DNA damage. By examining the kinetics of DNA repair in these cells, we show that NPM could protect DNA from oxidative damage, thereby reducing the levels of DNA strand breaks. NPM binds both DNA and RNA (46), and functions as a histone chaperone during the assembly of new nucleosomes and after DNA lesions are repaired (47). NPM also plays roles in chromatin remodeling and assembly (48). NPM has been shown to enhance UV-induced DNA repair (49), is a component B cell-specific multiprotein complex that possesses DNA recombination activity (50). In vitro experiments with recombinant NPM showed that the protein promoted DNA single-strand reannealing and mediated D-loop formation (50). However, our results with repair kinetics do not support the idea that NPM enhances or facilitates the repair of DNA strand breaks induced by oxidative...
stress. Instead, we argue a role for NPM in reducing the susceptibility of chromosomal DNA to damage. Taken together, our study indicates that NPM plays an important role in hematopoiesis via mechanisms involving modulation of HSC and progenitor cell differentiation, cell cycle progression, and stress response.

Acknowledgments—We thank Dr. Manuel Buchwald (Hospital for Sick Children, University of Toronto) for the Fanc+/- mice, Dr. Xiaoling Zhang for technical assistance and discussion, Dr. van der Loo (Cincinnati Children’s Hospital Medical Center) for the preparation of retroviruses.

REFERENCES

1. Chan, W. Y., Liu, Q. R., Borjigin, J., Busch, H., Rennert, O. M., Tease, L. A., and Chan, P. K. (1998) Biochim. Biophys. Acta 1353, 1033–1039
2. Feuerstein, N., Chan, P. K., and Mond, J. J. (1988) J. Biol. Chem. 263, 10608–10612
3. Nozawa, Y., Van Belzen, N., Van der Made, A. C., Djinjens, W. N., and Bosman, F. T. (1996) J. Pathol. 184, 48–52
4. Subong, E. N., Shue, M. J., Epstein, J. I., Briggman, J. V., Chan, P. K., and Partin, A. W. (1999) Proc. Natl. Acad. Sci. USA 96, 298–304
5. Tanaka, M., Sasaki, H., Kino, I., Sugimura, T., and Terrada, M. (1992) Cancer Res. 52, 3372–3377
6. Patterson, S. D., Grossman, J. S., D’Andrea, P., and Latter, G. I. (1995) N. Engl. J. Med. 337, 254–266
7. Feuerstein, N., Chan, P. K., and Mond, J. J. (1988) J. Immunol. 140, 1818–1822
8. Zeller, K. I., Hagerty, T. J., Barrett, J. F., Guo, Q., Wosney, D. R., and Dang, C. V. (2001) J. Biol. Chem. 276, 48285–48291
9. Neiman, P. E., Ruddell, A., Jasoni, C., Loring, G., Thomas, S. J., and Brandvold, K. A. (2001) Proc. Natl. Acad. Sci. USA 98, 6378–6383
10. Redner, R. L. (2002) Leukemia 16, 1927–1932
11. Yoneda-Kato, N., Look, A. T., Kirstein, M. N., Valentine, M. B., Raimondi, S. C., Tigaud, I., Castaigne, S., Raffoux, E., De Botton, S., Fenaux, P., Dombret, H., and C., Helin, K., Falini, B., and Pelicci, P. G. (2005) Proc. Natl. Acad. Sci. USA 101, 1501–1509
12. Rosenberg, A. R., Zindy, F., Le Deist, F., Moudy, H., Metezeau, P., Brechot, C., and Lamas, E. (1995) Oncogene 10, 1501–1509
13. Feuerstein, N., and Randazzo, P. A. (1991) Exp. Cell Res. 206, 899–902
14. Boisvert, N., Rennert, A., Biggio, V., Heng, K., and Bagby, G. C. (2003) J. Biol. Chem. 278, 41709–41717
15. Li, J., Zhang, X., Sejas, D. P., Bagby, G. C., and Pang, Q. (2004) J. Biol. Chem. 279, 41275–41279
16. Li, J., Zhang, X., Sejas, D. P., and Pang, Q. (2005) Leukemia 19, 1415–1423