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Nucleostemin is indispensable for the maintenance and genetic stability of hematopoietic stem cells

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ABSTRACT

Nucleostemin is a nucleolar protein known to play a variety of roles in cell-cycle progression, apoptosis inhibition, and DNA damage protection in embryonic stem cells and tissue stem cells. However, the role of nucleostemin in hematopoietic stem cells (HSCs) is yet to be determined. Here, we identified an indispensable role of nucleostemin in mouse HSCs. Depletion of nucleostemin using short hairpin RNA strikingly impaired the self-renewal activity of HSCs both *in vitro* and *in vivo*. Consistently, nucleostemin depletion triggered apoptosis rather than cell-cycle arrest in HSCs. Furthermore, DNA damage accumulated during cultivation upon depletion of nucleostemin. The impaired self-renewal activity of HSCs induced by nucleostemin depletion was partially rescued by p53 deficiency but not by $p16^{Ink4a}$ or $p19^{Arf}$ deficiency. Taken together, our study demonstrates that nucleostemin protects HSCs from DNA damage accumulation and is required for the maintenance of HSCs.

1. Introduction

Hematopoietic stem cells (HSCs) reside at the apex of the blood system hierarchy. HSCs can give rise to daughter stem cells by self-renewal and also to multipotent progenitors (MPPs) by differentiation, which in turn give rise to a variety of committed progenitors and mature blood cells. HSCs preserve homeostasis by serving as a lifelong source of blood cells. As such, any mis-repair of DNA damage can be transmitted to their daughter cells [1], and accumulation of DNA damage in the HSC pool can result in decreased regenerative capacity—one of the physiological hallmarks of aging—or the development of hematological malignancies upon acquisition of oncogenic mutations [2]. Therefore, the HSC genome must be strictly protected from various genotoxic insults such as ionizing radiation [3], reactive oxygen species [4], endogenous metabolites [5], and replicative stress [6]. HSCs use complex mechanisms to maintain their genome integrity, including DNA repair, transient cell-cycle arrest, senescence, and apoptosis [1], and there is growing evidence to support apoptosis as an important mechanism for controlling the quality of the HSC pool and maintaining homeostasis in the hematopoietic system [7,8]. However, much remains to be clarified regarding how stem cells manage DNA damage to maintain the homeostatic balance while avoiding aging or cancer development.

Nucleostemin is a GTP-binding protein present in the nucleoli of neural stem cells, embryonic stem cells (ESCs), other stem cell-enriched populations such as hematopoietic stem and progenitor cells, and also several cancer cell lines [9,10]. The importance of nucleostemin in stem cells is emphasized by the fact that deletion of nucleostemin in mouse ESCs causes failure to enter S-phase, increased senescence and apoptosis, and eventually embryonic death at the blastocyst stage [11–13]. The evidence that nucleostemin can directly associate with p53 raises the possibility that nucleostemin regulates the activity of p53 [14]. However, because loss of p53 does not rescue nucleostemin-null mice from embryonic lethality, nucleostemin appears to regulate stem cell fate via other mechanisms. Recent studies have revealed that nucleostemin prevents DNA damage accumulation and subsequent proliferation defects caused by telomere dysfunction [15] and replicative stress [16,17]. In addition to its role in normal stem cells, abundant expression of nucleostemin is also critical for the maintenance of cancer stem cells [18]. In this respect, nucleostemin is a key regulator in both normal and cancer stem cells. Therefore, a better understanding of the role of nucleostemin in stem cells will provide an important insight into the molecular mechanism underlying 'stemness'. However, the role of nucleostemin has yet to be explored in HSCs, for which stem cell activity can be precisely evaluated using established experimental systems.

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Here, we investigated the role of nucleostemin in HSCs. We evaluated the effect of nucleostemin depletion in HSCs/MPPs by phenotypic and functional analyses following nucleostemin knockdown with a short hairpin RNA (shNS). We show that nucleostemin is indispensable for HSC self-renewal. Nucleostemin plays an essential role in maintaining the genetic stability and cell viability of HSCs/MPPs in the proliferative state, which is to some extent dependent on p53 but not on $p16^{Ink4a}$ or $p19^{Arf}$. These results reveal how nucleostemin contributes to the maintenance of HSC activity.

2. Materials and methods

2.1. Mice

C57BL/6 mice (Ly5.1 and Ly5.2) were purchased from Sankyo Labo Service (Tokyo, Japan). To obtain p53-null hematopoietic cells, *Mx1-Cre*; $p53^{flox/flox}$ mice, generated by crossing $p53^{flox/flox}$ mice [19] and *Mx1-Cre* mice [20], were intraperitoneally administered with 400 μg pIpC (Sigma–Aldrich, St. Louis, MO, USA) five times every other day at least 4 weeks before collecting bone marrow. The $p16^{Ink4a-/-}p19^{Arf-/-}$ mice were described previously [21]. All procedures in this study were performed in accordance with the guidelines for animal and recombinant DNA experiments of Keio University School of Medicine, with the approval of the institutional review board of Keio University.

2.2. Antibodies for flow cytometry

The antibodies used for flow cytometry and cell isolation are listed in Supplementary Table S1. A cocktail of monoclonal antibodies against CD4, CD8, B220, Ter119, Gr-1, and Mac-1 was used to identify lineage$^+$ cells.

2.3. Flow cytometric analysis and isolation of hematopoietic cells

Bone marrow cells were collected from mouse femurs and tibias for analysis and isolation of HSCs. The methods used to isolate HSCs and other hematopoietic cells were described previously [22]. Cell-cycle analysis using a monoclonal anti-Ki67 antibody was performed as described previously [23]. For apoptosis detection, cells stained for cell surface markers were further incubated for 15 min with APC-conjugated Annexin V (BD Biosciences, San Jose, CA, USA) and DAPI (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. All data collection was performed on FACS Calibur or FACS Aria II instruments (BD Biosciences) and data were analyzed with FlowJo software (Treestar, Ashland, OR, USA).

2.4. Retroviral transduction

shRNA sequences targeting nucleostemin (#1: GTAAGAAGC TGATACTTGTAT, #2: GGAGTAAGAAGCTGATACTTG) were cloned

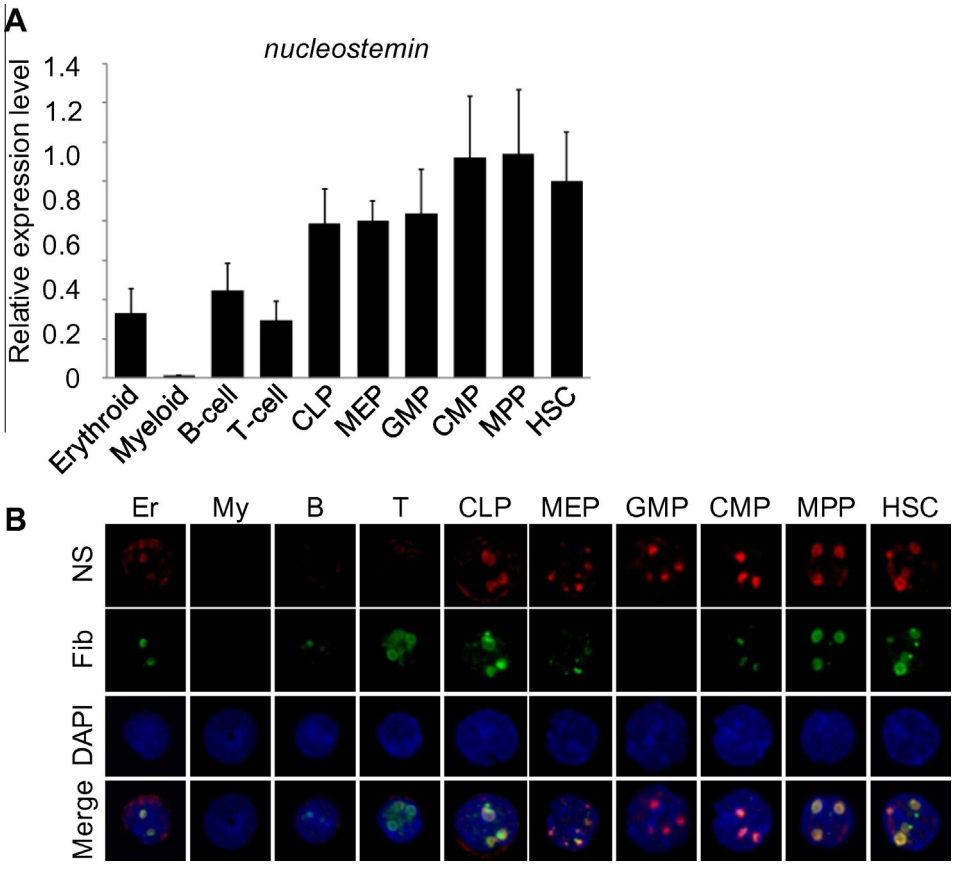

Fig. 1. Expression of nucleostemin in hematopoietic cells. (A) qRT-PCR analysis of nucleostemin in various hematopoietic cells. Relative expression levels of nucleostemin in erythroid cells (Ter119$^+$), myeloid cells (Gr1$^+$Mac1$^+$), B cells (B220$^+$CD19$^+$), T cells (CD3$^+$), CLPs (Lin$^-$Sca1^{low}cKit^{low}Flt3$^+$IL-7Rα$^+$), MEPs (Lin$^-$Sca1$^-$cKit$^+$CD34$^-$FcγRII/III^{low}), GMPs (Lin$^-$Sca1$^-$cKit$^+$CD34$^+$FcγRII/III^{high}), CMPs (Lin$^-$Sca1$^-$cKit$^+$CD34$^+$FcγRII/III^{low}), MPPs (CD150$^-$Lin$^-$Sca1$^+$cKit$^+$), and HSCs (CD150$^+$CD41$^-$CD48$^-$Lin$^-$Sca1$^+$cKit$^+$) sorted from adult murine bone marrow are shown ($n = 3$). (B) Immunocytochemistry of nucleostemin in hematopoietic cells. Nucleostemin (*red*) and a nucleolar marker, fibrillarin (*green*), co-immunostained in the hematopoietic cells described above. Nuclei were counterstained with DAPI (*blue*). Er, My, B, and T represent erythroid cells, myeloid cells, B cells, and T cells, respectively. NS: nucleostemin, Fib: fibrillarin.

into the pReGS retroviral vector [24]. The constructed vectors were transfected into Plat-E cells with FuGENE HD (Roche Diagnostics GmbH, Mannheim, Germany), and the supernatant was used as the retrovirus solution. Retroviral transduction of lineage$^-$ Sca1$^+$ cKit$^+$ (LSK) cells was performed on RetroNectin (Takara Bio, Otsu, Japan) using Magnetofection (OZ Biosciences, Marseille, France), as described previously [25].

2.5. Bone marrow transplantation

LSK cells from C57BL/6-Ly5.1 congenic mice were isolated and subjected to retroviral transduction as described above. Two days later, 5000 GFP$^+$ LSK cells were isolated by FACS and transplanted into 9.5 Gy-irradiated C57BL/6 mice (Ly5.2) together with 4×10^5 BM MNCs from C57BL/6 mice (Ly5.2). The reconstitution of donor-derived cells was monitored by staining peripheral blood samples with monoclonal antibodies against Ly5.1 and Ly5.2 every 4 weeks. The chimerism of BM was analyzed 4 months after transplantation.

2.6. Colony assay

Two hundred transduced GFP$^+$ LSK cells were plated in 1 ml of methylcellulose medium (Methocult GF M3434; StemCell Technologies, Vancouver, Canada) on 35-mm petri dishes in triplicate. Colony-forming units in culture (CFU-C) and high proliferative potential-colony forming cells (HPP-CFC) were scored on days 10 and 14 of culture, respectively.

2.7. Immunocytochemistry

Sorted LSK cells were placed onto a glass slide, fixed with ice-cold methanol followed by ice-cold acetone, and stained with anti-nucleostemin (R&D Systems, Minneapolis, MN, USA; 2.5 μg/mL), anti-fibrillarin (Abcam, Cambridge, UK; 5 μg/mL), and anti-53BP1 (Novus Biologicals, Littleton, CO, USA; 5 μg/mL) antibodies. Nuclei were identified by staining with DAPI (Molecular Probes). Images were collected using a FV1000 confocal laser-scanning microscope and FV10-ASW software Version 2.0 (Olympus, Tokyo, Japan).

2.8. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was obtained using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using Superscript III reverse transcriptase and random hexamer primers (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using 2× Fast Universal master mix and TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). Sample RNA content was normalized based on β-actin amplification.

2.9. Statistical analysis

An unpaired two-tailed Student's *t*-test was used for comparisons between the two groups. Data are shown as the means ± S.D. $^*P < 0.05$ was considered as significant, and $^{**}P < 0.01$ as highly significant.

3. Results

3.1. Nucleostemin is abundantly expressed in hematopoietic stem and progenitor cells

The expression of nucleostemin was reported to be enriched in Sca1$^+$, AA4.1$^+$, c-kit$^+$ fetal liver hematopoietic stem cells [26], and adult Lin$^-$cKit$^+$ cells [9]. To further examine the expression of

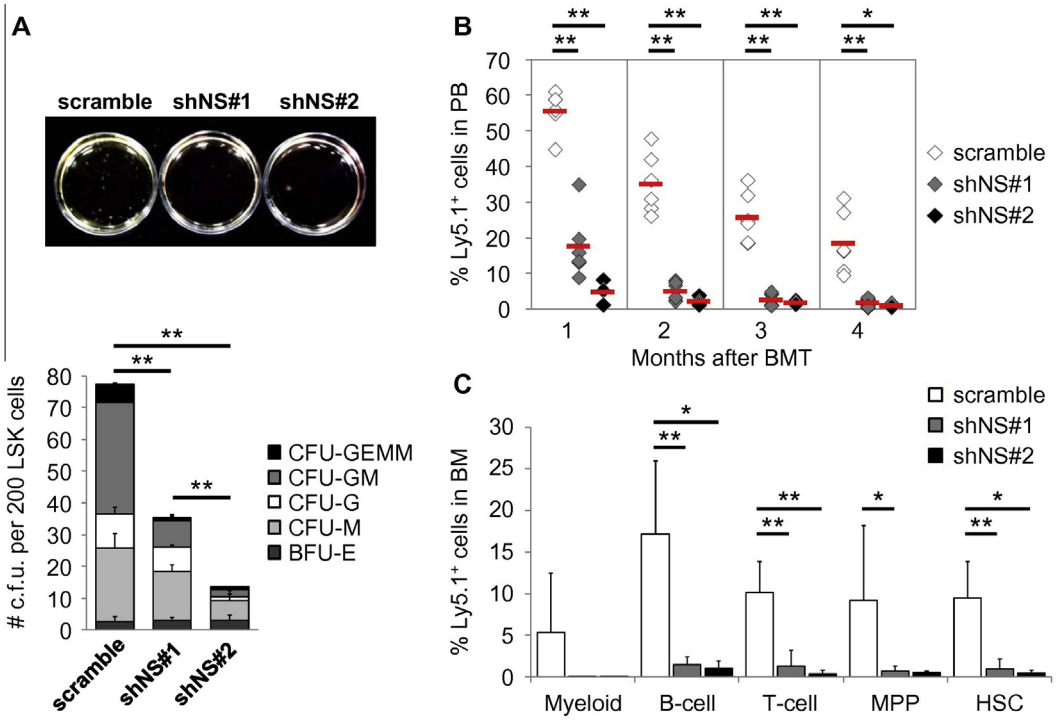

Fig. 2. Colony formation and bone marrow reconstitution capacity of HSCs after nucleostemin depletion. (A) CFU-C assay using 200 LSK cells transduced with scrambled shRNA or shNS. Representative images of colonies formed in methylcellulose (*upper*) and a stacked bar graph indicating the number of colonies in each category (*lower*) are shown ($n = 3$). (B and C) Competitive bone marrow transplantation using LSK cells transduced with scrambled shRNA or shNS ($n = 6$ for scrambled shRNA and shNS#1; $n = 3$ for shNS#2). 5000 Ly5.1$^+$ transduced LSK cells were transplanted into lethally irradiated recipient mice (Ly5.2) together with 4×10^5 competitor BM MNCs (Ly5.2). The percentages of donor-derived (Ly5.1$^+$) cells were examined every 4 weeks in peripheral blood (B) and at 16 weeks post-transplantation in bone marrow (C).

nucleostemin in hematopoietic cells, we sorted various hematopoietic cells from adult mouse bone marrow and evaluated the expression of nucleostemin by qRT-PCR (Fig. 1A). Consistent with previous reports, nucleostemin was highly expressed in $Lin^{-}cKit^{+}$ immature hematopoietic cells such as MEPs, GMPs, CMPs, MPPs, and HSCs. Immunocytochemical analysis revealed that nucleostemin was predominantly expressed in the nucleoli by co-immunostaining with the nucleolar marker, fibrillarin (Fig. 1B). These results suggest that nucleostemin is abundantly expressed in immature hematopoietic cells, including HSCs.

3.2. Nucleostemin is indispensable for the maintenance of HSCs

To assess the function of nucleostemin in HSCs, we evaluated the effect of nucleostemin depletion in HSCs. Retroviruses carrying shRNA against nucleostemin (shNS) were prepared, and an approximate 50% reduction in endogenous nucleostemin mRNA expression was observed following transduction with shNS#1 and shNS#2 (Supplementary Fig. S1A). The level of nucleostemin protein was also efficiently reduced (Supplementary Fig. S1B). Using these retroviruses, LSK cells, which consist of HSCs and MPPs, were transduced with scrambled shRNA or shNS, and colony formation assays were performed. Nucleostemin was successfully down-regulated after the transduction of shNS (Supplementary Fig. S1C), and the colony-forming capacity of nucleostemin-depleted LSK cells was significantly decreased as measured by CFU-C and HPP-CFC assays (Fig. 2A and Supplementary Fig. S1D). Consistent with these results, the long-term reconstitution capacity was strikingly impaired in nucleostemin-depleted LSK cells (Fig. 2B and C). These results demonstrate an indispensable role of nucleostemin for the maintenance of HSCs.

3.3. Depletion of nucleostemin triggers apoptosis in HSCs

Depletion of nucleostemin can induce cell-cycle arrest and/or apoptosis in ESCs and tissue stem cells [13,27], although this effect of nucleostemin has not yet been documented in HSCs. We therefore analyzed cell-cycle status and apoptosis in LSK cells 2 days after transduction with scrambled shRNA or shNS. Despite the significant reduction in proliferation and self-renewal capacity, LSK cells treated with shNS did not show major changes in cell cycle status as shown by Ki67 staining (Fig. 3A). A BrdU short labeling assay revealed that the population of cells in S-phase was comparable or slightly increased, suggesting that entry into S-phase was not disturbed by the depletion of nucleostemin (data not shown) and that cell-cycle arrest is not a main cause for the reduction of proliferation and self-renewal capacity. Instead, the percentage of apoptotic cells was significantly elevated in nucleostemin-depleted LSK cells (Fig. 3B), and the rate of apoptosis was inversely related to the colony-forming capacity and bone marrow reconstitution capacity. These results indicate that nucleostemin depletion induces apoptosis rather than cell-cycle arrest, thereby reducing the self-renewal capacity of HSCs.

3.4. Nucleostemin depletion induces DNA damage accumulation

Apoptosis can be induced by various cellular stresses, including DNA damage, and proliferation can be accompanied by replicative

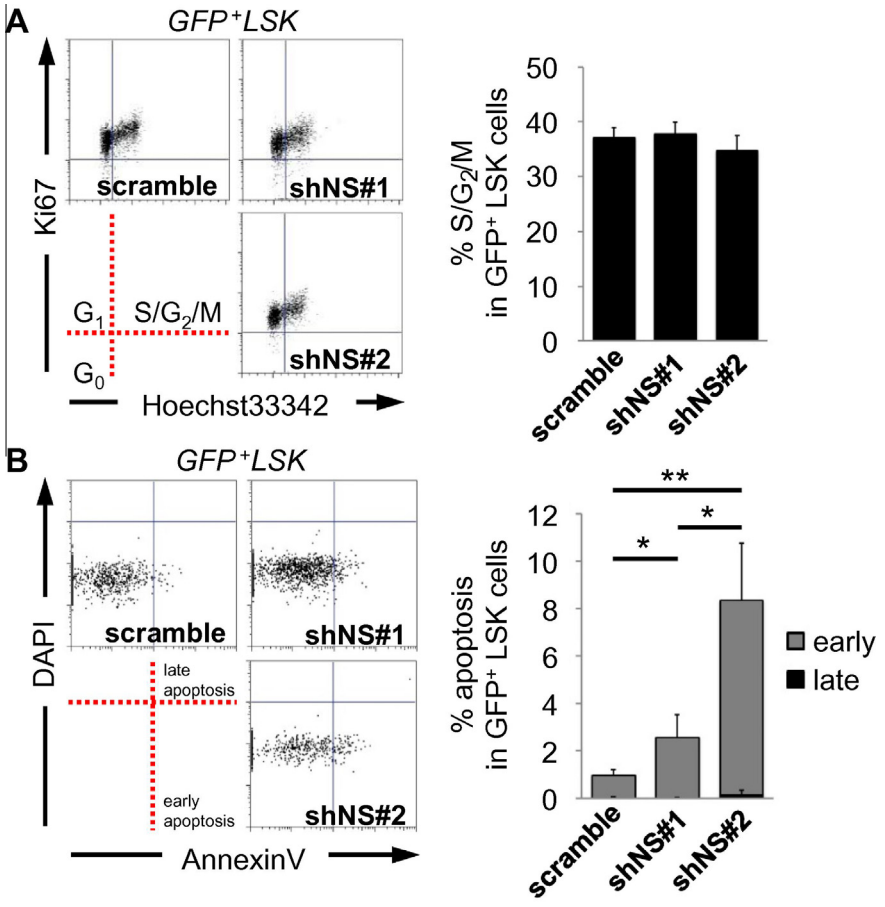

Fig. 3. Cell cycle and apoptosis in HSCs after nucleostemin depletion. Flow cytometric analysis of LSK cells transduced with scrambled shRNA or shNS using Ki67 and Hoechst 33347 (A), and Annexin V and DAPI (B) staining. Representative plots (*left*) and the percentages of cells in each state (*right*) are shown ($n = 3$).

stress, which is one of the physiological sources of DNA damage accumulation [28]. Recently, it was reported that deletion of nucleostemin induces γH2AX foci in neural stem cells [16] and hepatocyte progenitor cells [17] during proliferation. To assess the possibility that nucleostemin also prevents DNA damage in HSCs, we examined the accumulation of 53BP1, another marker of the DNA damage response, in LSK cells 2 days after transduction with scrambled shRNA or shNS. Consistently, nucleostemin-depleted LSK cells showed a remarkable increase in the number of 53BP1 foci (Fig. 4A). Meanwhile, we observed significant up-regulation of *p21* and *Bax* mRNAs in nucleostemin-depleted LSK cells (data not shown), indicating that nucleostemin reduction causes DNA damage in HSCs, which induces apoptosis and reduces the self-renewal capacity as a result.

3.5. HSC impairment induced by nucleostemin depletion is partly mediated by p53 but not by $p16^{Ink4a}/p19^{Arf}$

Following induction of DNA damage, distinct factors detect, transmit, and amplify the DNA damage signal. This DNA damage response (DDR) converges on p53, which regulates different cellular outcomes depending on the target genes activated, such as *p21* for transient cell-cycle arrest, *Bax*, *Puma*, and *Noxa* for apoptosis, and $p16^{Ink4a}$ and $p19^{Arf}$ for senescence [1]. To assess whether the reduction of self-renewal capacity by nucleostemin depletion is mediated through the activation of p53 or $p16^{Ink4a}/p19^{Arf}$, we introduced shNS into $p53^{\Delta/\Delta}$ or $p16^{Ink4-/-}p19^{Arf-/-}$ LSK cells. Interestingly, depletion of nucleostemin still reduced colony forming activity in the absence of p53 or $p16^{Ink4a}/p19^{Arf}$ (Fig. 4B and Supplementary Fig. S2A and B), although loss of p53 partially rescued the reduction of colony formation capacity (Fig. 4C). These results demonstrate that reduction of the self-renewal capacity of HSCs by nucleostemin depletion is partly mediated through the activation of p53, but not through $p16^{Ink4a}/p19^{Arf}$.

4. Discussion

We first established direct evidence that nucleostemin is essential for HSC self-renewal. Our findings indicate that nucleostemin protects HSCs from genotoxic insults and subsequent apoptosis, thereby maintaining cell viability. Because the expression of nucleostemin is comparably high in committed progenitor cells with limited differentiation but high proliferation potential (Fig. 1A and B), and because differentiation capacity is not altered after nucleostemin reduction (Fig. 2C), it seems that the function of nucleostemin might be associated with proliferation. Proliferation essentially accompanies replicative stress, one of the intrinsic sources of DNA damage, and indeed nucleostemin plays an important role in preventing DNA damage accumulation during short-term culture when cells are enforced to proliferate with cytokines (Fig. 4A).

The observation that the depletion of nucleostemin causes DNA damage in proliferating HSCs/MPPs is quite consistent with recent studies suggesting that DNA damage accumulates in neural stem cells and hepatocyte progenitor cells after conditional deletion of nucleostemin [17,19]. Regarding the underlying mechanisms, there is emerging evidence that nucleostemin recruits RAD51, a key molecule for homologous recombination in mammals, to DNA lesions [15,17,19]. TERT can associate with nucleostemin [15,18], and we have previously shown that TERT prevents HSCs from DNA damage and subsequent apoptosis following reactive oxygen species stress [23]. Considering both findings, nucleostemin may play a significant role in the genetic stability of the telomeric region in collaboration with TERT.

Damaged cells may undergo transient cell-cycle arrest, senescence, or apoptosis, depending on the extent, type, and duration of DNA damage. Following the massive accumulation of 53BP1 foci in the nuclei after nucleostemin depletion, apoptosis rather than cell-cycle arrest appeared to function as a compensatory

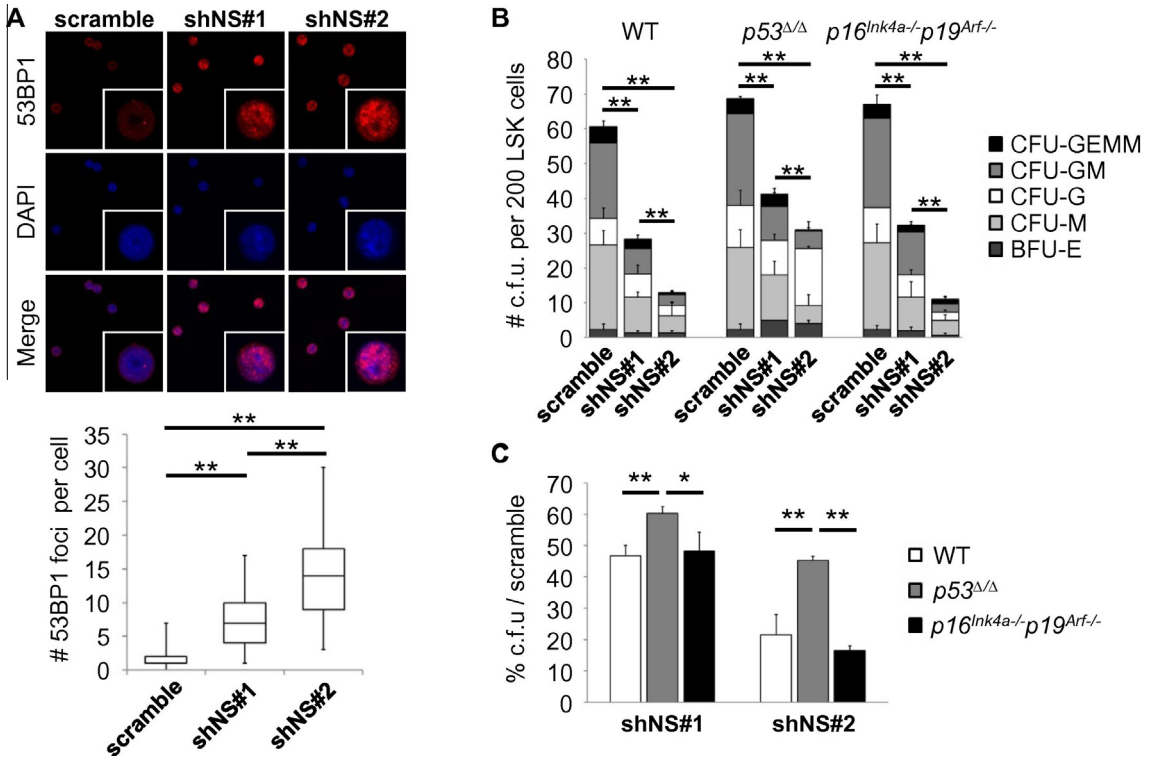

Fig. 4. DNA damage response in nucleostemin-depleted HSCs. (A) Immunocytochemical staining of 53BP1 in LSK cells transduced with scrambled shRNA or shNS. Representative images (*upper*) and a boxplot of 53BP1 foci per nucleus (*lower*) are shown ($n = 100$). (B and C) CFU-C assay using $p53^{\Delta/\Delta}$ and $p16^{Ink4a-/-}p19^{Arf-/-}$ LSK cells transduced with scrambled shRNA or shNS. The number of colonies classified into each category (B) and the ratio of the total number of colonies formed by LSK cells transduced with shNS to the total number of colonies formed by LSK cells transduced with scrambled shRNA (C) are shown ($n = 3$).

mechanism for removal of the cells with unrepairable DNA damage (Fig. 3B). In the present study, the decreased number of CFU-C caused by nucleostemin depletion was partially rescued by p53 deficiency but not by $p16^{Ink4a}$ or $p19^{Arf}$ deficiency (Fig. 4B), indicating that nucleostemin depletion triggers apoptosis and diminishes HSC self-renewal partly through the activation of p53.

In conclusion, our present study established a vital role for nucleostemin in the maintenance of HSCs. Nucleostemin makes a significant contribution to the conservation of genetic stability in HSCs. Because nucleostemin is preferentially expressed in stem cells, is localized in nucleoli, and regulates genetic stability, further investigation into its molecular function will enable a better understanding of how stem cells use their nucleoli to manage DNA damage for the maintenance of self-renewal and to avoid premature aging and cancer development.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.032.

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