**Forced Expression of Nanog or Esrrb Preserves the ESC Status in the Absence of Nucleostemin Expression**

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**Key Words.** Embryonic stem cells • Epiblast stem cells • Leukemia inhibitory factor • Pluripotency

**ABSTRACT**

Nucleostemin (NS) is a nucleolar GTP-binding protein that is involved in a plethora of functions including ribosomal biogenesis and maintenance of telomere integrity. In addition to its expression in cancerous cells, the NS gene is expressed in stem cells including embryonic stem cells (ESCs). Previous knockdown and knockout studies have demonstrated that NS is important to preserve the self-renewality and high expression levels of pluripotency marker genes in ESCs. Here, we found that forced expression of Nanog or Esrrb, but not other pluripotency factors, resulted in the dispensability of NS expression in ESCs. However, the detrimental phenotypes of ESCs associated with ablation of NS expression were not mitigated by forced expression of Rad51 or a nucleolar localization-defective NS mutant that counteracts the damage associated with loss of NS expression in other NS-expressing cells such as neural stem/progenitor cells. Thus, our results indicate that NS participates in preservation of the viability and integrity of ESCs, which is distinct from that in other NS-expressing cells. **Stem Cells** 2015;33:1089–1101

**INTRODUCTION**

Nucleostemin (NS) (also known as GNL3) is a nucleolar GTP-binding protein that is involved in numerous biological functions including ribosomal biogenesis, protection against telomere damage, and destabilization of p53 protein [1–5]. Recently, a genome-protecting role of NS has been uncovered in neural stem cells (NSCs) [6] and regenerating hepatocytes [7], in which NS protein recruits Rad51 to foci of DNA damage to facilitate DNA repair. NS was first discovered in NSCs [1] and later in numerous types of stem/progenitor cells and cancer cells. Stem cells are defined by their abilities to produce multiple distinct cell types (multipotency) and self-renew while maintaining their multipotency. However, each stem cell type is usually only able to generate cell types that coexist in certain tissues [8]. For example, NSCs produce only neurons, astrocytes, and oligodendrocytes in the brain, at least in a physiological context [9]. In terms of self-renewality, the durations are different depending on the stem cell type, but it is never permanent. Embryonic stem cells (ESCs) are also categorized as stem cells, although their differentiation potential and self-renewal properties are much greater compared with those of other stem cell types, because they can convert to any cell type in the body, including germ cells, and maintain self-renewality indefinitely [10–12]. NS is also expressed in ESCs, and we have previously demonstrated such expression is essential to preserve cell viability [13]. Another study [4] has shown that NS expression is important for rapid transit through G1 phase to sustain the robust proliferation of ESCs and is also crucial to maintain high expression levels of pluripotency marker genes. However, the molecular mechanisms of NS in ESCs remain largely unexplored. Moreover, it is unknown whether NS expression is crucial for other pluripotent cells, that is, epiblast stem cells (EpiSCs) [14, 15].

Here, we demonstrate that ablation of NS expression leads to a substantial decline in the expression levels of pluripotency marker genes and extensive cell death of both EpiSCs and ESCs. However, unlike in NSCs, the effects of NS expression ablation are not mitigated by forced expression of Rad51, implying a pluripotent cell-specific function of NS. Our data also demonstrate that the changes associated with NS expression ablation in ESCs, but not in EpiSCs, are almost completely rescued by forced expression of either Nanog or Essrb pluripotency factors.
MATERIALS AND METHODS

Cell Culture

Wild-type (CMTI-1) and NS tet-off ESCs, in which NS gene expression from the ROSA26 locus can be controlled by the tetracycline-off system [13], were cultured under a feeder-free condition with standard ESC medium containing leukemia inhibitory factor (LIF) and serum [16]. To generate NS tet-off EpiSCs, NS tet-off ESCs were marked with fluorescent Kusabira Orange (KBO) by stable integration of a KBO expression vector with a puromycin resistance gene [17] and then injected into blastocysts. After transfer to surrogate ICR mice, embryos at ~6.5 days postcoitum (dpc) were recovered to establish EpiSCs according to Brons et al. [15]. The EpiSCs were cultured on fibronectin-coated dishes with medium containing 20 ng/ml human activin A (338-AC-050) (R&D Systems, Minneapolis, MN, http://www.rndsystems.com) and 12 ng/ml murine basic fibroblast growth factor (450-33) (PeproTech, Rocky Hill, NJ, http://www.peprotech.com) as described by Gillich et al. [18]. To suppress NS expression from the ROSA26 locus in the inducible NS tet-off ESCs and EpiSCs, 1 μM doxycycline (Dox) was added to the culture medium.

TUNEL Assay

For TUNEL assays, cells were plated on gelatin-coated Cell Disks. TUNEL-positive apoptotic cells were detected using an In situ Cell Death Detection kit, Fluorescein (11684795910) (Roche Applied Science, Mannheim, Germany, http://www.roche-applied-science.com).

Hydroxyurea Treatment

CMTI-1 ESCs were transiently transfected with expression vectors for either wild-type or the ΔB mutant of NS [19] and then treated with 2 mM hydroxyurea (HU) for 20 hours. Subsequently, HU-containing medium was replaced by standard ESC medium and then cultured for 4 hours before subjecting to immunocytochemistry using an antibody against γ-H2A-X. CMTI-1 ESCs transfected with an empty vector were used as a control.

Microarray Analysis

Biotin-labeled cRNA was synthesized as described by the Affymetrix guidelines. Labeled samples were hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays according to the manufacturer’s instructions. Microarray expression data were background subtracted and normalized with the robust multivariate analysis method using statistical software R. Gene ontology (GO) analyses were conducted using DAVID web tools (http://david.abcc.ncifcrf.gov). The selected GO terms were further subjected to analyses using AmiGO1 (http://amigo1.geneontology.org/cgi-bin/amigo/go.cgi) and REVIGO (http://revigo.irb.hr) web sites to eliminate redundancy. For gene set enrichment analysis (GSEA), we used GSEA software v2.0.14.

Quantitative RT-PCR

Quantitative RT-PCR was conducted with the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) using cDNAs generated by reverse transcription. The TaqMan-based reactions quantified the expression levels of Gapdh, Nanog, Oct3/4, Sox2, Salt4, Utf1, Tbx3, Tcl1, Esrrb, Rex1, Klf2, Klf4, Gata4, Gata6, T, Cdx2, and Pax6. SYBR Green-based reactions were used to quantify the expression levels of Prdm14 with the following primer set: forward, 5′-GGC CAT ACC AGT GCG TGT A-3′; reverse, 5′-TGC TGT CTG ATG TGT GTT CG-3′. The results were normalized to Gapdh expression levels.

Alkaline Phosphatase Staining

ESCs (2,500 cells) were plated in each well of a gelatin-coated six-well plate and cultured with or without Dox. Then, the cells were stained with an AP staining kit (AP100R-1) (System Biosciences, Inc., Mountain View, CA, http://www.systembio.com).

Knockdown of NS Expression in Human-Induced Pluripotent Stem Cells

Human-induced pluripotent stem cells (hiPSCs) (SC102A-1) were cultured according to the supplier’s instructions (System Biosciences, Inc.). Inhibition of NS expression by shRNA-mediated knockdown in hiPSCs was conducted as described previously [20] with the sequence (shNS1) used by Okamoto et al. [21].

Accession Number

DNA microarray data have been deposited in the NCBI Gene Expression Omnibus under accession number GSE56797.

Materials used in this study, vector constructions, Western blot, and immunostaining methods were provided in Supporting Information Materials and Methods.

RESULTS

Decline in the Expression Levels of Pluripotency Marker Genes After Ablation of NS Expression in ESCs

We have previously demonstrated that ablation of NS expression leads to extensive cell death of ESCs using inducible NS tet-off ESCs in which the NS gene was homozygously disrupted, but NS cDNA was introduced into the ROSA26 locus together with the tetracycline-off system [13]. Furthermore, we demonstrated with that the expression levels of pluripotency markers such as Oct3/4 and UTF1 are not noticeably influenced by the loss of NS expression in this ESC line. However, a recent report with knockdown experiments of NS expression in ESCs demonstrated that a decline in NS expression levels does not provoke a strong apoptotic phenotype, but lowers the expression levels of numerous pluripotency marker genes [4]. We considered that these apparent discrepancies may be attributed to a difference in the levels of residual NS expression in NS-knockout and -knockdown ESCs, resulting in a milder phenotype in NS-knockdown ESCs with respect to cell viability. In terms of pluripotency marker gene expression, we assumed that the extensive cell death phenotype of NS-knockout ESCs had prevented us from correctly quantifying the expression levels of pluripotency marker genes. To address the latter possibility, we first examined Oct3/4 expression in NS-knockout ESCs by immunocytochemical analyses. Although our previous Western blot and mRNA quantification analyses did not indicate a decline in Oct3/4 expression after Dox administration [13], immunocytochemistry showed some differences in Dox-untreated (NS expressing)
and -treated (NS nonexpressing) NS tet-off ESCs. Indeed, cells with a low intensity of Oct3/4 immunostaining were apparent among Dox-treated NS tet-off ESCs. However, most of them were not completely negative, but retained at least certain signal for Oct3/4. Therefore, the proportion of completely negative cells for Oct3/4 was similar between Dox-treated and -untreated NS tet-off ESCs. These results were in marked contrast to the results obtained with Dox-untreated NS tet-off ESCs that showed homogeneous Oct3/4 signals (Fig. 1A). We also performed immunocytochemical analyses of Esrrb. Consistent with previous reports [22–24], Esrrb expression was heterogeneous among ESCs with only approximately 60% of ESCs positive for Esrrb even among Dox-untreated NS tet-off ESCs (Fig. 1A). However, the proportion of cells negative for Esrrb became much more prominent following Dox treatment, implying that the overall expression level of Esrrb was also

Figure 1. Most pluripotency marker genes undergo a decline in expression after ablation of NS expression in ESCs. (A): Immunocytochemical analyses of Oct3/4 and Esrrb in Dox-untreated and -treated NS tet-off ESCs. NS tet-off ESCs were cultured on cell disks in the absence or presence of Dox for 5 days before immunocytochemistry. The ratio of Oct3/4- or Esrrb-positive cells among DAPI-positive cells is shown as the mean ± SD (n = 3). Scale bar = 50 μm. (B): Quantitative RT-PCR analyses of pluripotency and differentiation marker expression. Data are the mean ± SD (n = 3). Each value from Dox-untreated NS tet-off ESCs is set to one. *, p < .05; **, p < .01; and ***, p < .001. (C): Western blot analyses of pluripotency marker and p53 proteins. NS tet-off ESCs were treated with Dox and recovered at the indicated days. Abbreviations: ESC, embryonic stem cell; NS, nucleostemin.
decreased upon NS expression ablation. Furthermore, analyses of the expression of Nanog, which is also a pluripotency marker with rather heterogeneous expression in ESCs [23], revealed that the proportion of Nanog-negative cells was increased significantly after ablation of NS expression in ESCs (Supporting Information Fig. S1).

Because our immunocytochemical analyses indicated downregulation of the expression of Oct3/4, Esrrb, and Nanog, we determined whether other pluripotency marker genes underwent downregulation upon Dox-mediated ablation of NS expression in ESCs. Quantitative RT-PCR analyses demonstrated that most of the examined pluripotency marker genes underwent significant downregulation of expression. However, some pluripotency marker genes such as Oct3/4, Sox2, and Salt4 were not significantly influenced by the loss of NS expression. The UTF1 expression level was even elevated in NS-null ESCs (Fig. 1B). Thus, these findings partly account for the previous discrepancy in the decline of pluripotency marker gene expression in Dox-treated NS tet-off ESCs [13]. Differential effects of NS expression ablation on gene expression among pluripotency markers were also consistent with a previous report [4]. Western blot analyses also showed decreases in the expression of all the pluripotency marker proteins, particularly Nanog, Esrrb, and Klf4, upon Dox-mediated loss of NS expression (Fig. 1C). Again, the decline in the expression level of Oct3/4 was not very obvious compared with that of other pluripotency markers such as Nanog and Esrrb. Thus, our data indicate that pluripotency marker genes can be categorized into two groups according to rapid or gradual declines in their expression levels (first and second groups, respectively). Therefore, a reduction in the expression levels of second group genes such as Oct3/4 may be due to a secondary effect of the reduction in the expression levels of first group genes such as Klf4 and Esrrb. Consistent with this notion, we observed a strong rescue effect by forced expression of certain first group genes (see below). With respect to the expression of differentiation marker genes, we noted induction of T (Brachyury) expression, implying initiation of mesodermal induction. However, there was no elevation in the expression levels of ectodermal (Pax6) or endodermal (Gata4) marker genes, while Gata6 and Sox17, other endodermal markers, showed increases in their expression levels upon loss of NS expression in ESCs. Unexpectedly, we found the trophectodermal marker gene Cdx2 underwent a profound increase in expression (Fig. 1B), although ESCs do not intrinsically have the ability to differentiate into the trophectodermal cell lineage. However, we do not know the reason for the abnormal expression pattern of the differentiation marker genes, especially the strong induction of Cdx2 in Dox-treated NS tet-off ESCs.

Decline in the Phosphorylation Level of Stat3 in NS tet-off ESCs

To understand the molecular basis of the extensive cell death and decline in the expression levels of pluripotency genes, we explored methods to sustain the ESC status in the absence of NS gene expression. Pluripotency marker genes including Klf4 and Esrrb show higher expression in ESCs than that in EpiSCs [25]. Furthermore, these pluripotency genes were more predominantly downregulated by NS expression ablation than other genes (Oct3/4 and UTF1) showing apparently equivalent expression levels (Fig. 1B). Therefore, we examined the possibility that the LIF-Stat3 signaling cascade was impaired in NS tet-off ESCs, because ESCs, but not EpiSCs, are crucially dependent on this signaling cascade. Figure 2A shows that, although the total amount of Stat3 did not change, the amount of phosphorylated (active) Stat3 was decreased significantly upon loss of NS expression in ESCs. We also noted that the amount of phosphorylated Akt, which cooperatively participates with Stat3 to maintain ESC pluripotency [26, 27], was also decreased in NS tet-off ESCs, although the decrease was less significant compared with that of phosphorylated Stat3. However, phosphorylation levels of Erk1/2 and GSK3β were unchanged by NS expression ablation. These results prompted us to examine the effect of forced expression of the constitutively active form of Stat3 [28] and Akt on NS tet-off ESCs. These analyses revealed that both constitutively active Stat3 and Akt yielded some alkaline phosphatase-positive viable colonies (Fig. 2B), although most of the cells underwent cell death in both cases. However, both Stat3- and Akt-dependent colonies showed extremely slow cell proliferation (refer Fig. 3C for Stat3; data not shown for Akt) and substantial cell death occurred during each passage. Therefore, we could not maintain either Stat3- or Akt-dependent colonies over several passages. The dependency of ESCs on LIF-Stat3 signaling is much weaker in ESCs under the 2i plus LIF condition compared with that in the conventional LIF and serum condition [29, 30]. Moreover, previous knockdown studies have demonstrated that the decline in the expression levels of pluripotency marker genes is much less significant when NS expression in ESCs is knocked down under the 2i plus LIF condition compared with that in the conventional LIF and serum condition [4]. Therefore, we examined the effect of NS knockout in ESCs under the 2i plus LIF condition. Similar to forced expression of constitutively active Stat3, we observed some alkaline phosphatase-positive viable cells (Fig. 2B), although the vast majority of NS tet-off ESCs underwent cell death. We assumed that the less potent effect of the 2i plus LIF condition on NS-knockout ESCs compared with that in NS-knockdown ESCs [4] was again attributed to the difference in residual NS expression levels in NS-knockout and -knockdown ESCs. Because we obtained a partial rescue effect by Stat3, Akt, or the 2i condition, we next examined the effect of the combination of all three components. The results revealed that the rescue effect was more significant when these components were applied in combination compared with the individual components in terms of both the number and size of the rescued alkaline phosphatase (AP)-positive colonies (Fig. 2B).

**NS Expression Becomes Dispensable by Forced Expression of Nanog or Esrrb in ESCs**

Next, we examined whether forced expression of pluripotency marker genes shows rescue effects in NS tet-off ESCs, because a decline in the expression of pluripotency marker genes is one of the prominent features of these cells. Although most of the pluripotency factors did not exert any noticeable effects on the viability of NS tet-off ESCs (Supporting Information Fig. S2), forced expression of Nanog or Esrrb produced a number of viable and alkaline phosphatase-positive colonies (Fig. 3A). These effects were much more prominent even when compared with those obtained by simultaneous expression of Stat3 and Akt in the 2i condition.
Indeed, the numbers of viable colonies were similar for Dox-treated and -untreated NS tet-off ESCs that had been rescued by Nanog or Esrrb expression. These rescued NS tet-off ESCs could be passaged and expanded without affecting their viability or the undifferentiated morphology of the colonies. Western blot analyses revealed that, unlike NS tet-off ESCs transfected with Dox and then protein extracts were prepared at the indicated days. **(A):** Western blot analyses to examine the phosphorylation levels of signal transduction kinases that are related to ESC pluripotency. NS tet-off ESCs were treated with Dox and then protein extracts were prepared at the indicated days. **(B):** Partial rescue of NS tet-off ESCs by either constitutively active Stat3, Akt, or exposure to the 2i condition. Upper right panel shows a Western blot for confirmation of the exogenously overexpressed Stat3 mutant and Akt. The combinatorial effect was also examined for all three components. Lower left and right bar graphs show the relative number of AP-positive colonies and the total occupied area of all rescued colonies, respectively, in which data from Dox-treated NS tet-off ESCs are set to 1 in both cases. NS tet-off ESCs and those overexpressing constitutively active Stat3 and/or Akt were treated as indicated for 6 days, and then subjected to AP staining. Scale bar = 100 μm. Abbreviations: ESC, embryonic stem cell; NS, nucleostemin.

Figure 2. Incomplete counteraction of NS expression ablation-mediated detrimental phenotypes of ESCs by constitutive activation of Stat3 Akt or exposure to the 2i condition. **(A):** Western blot analyses to examine the phosphorylation levels of signal transduction kinases that are related to ESC pluripotency. NS tet-off ESCs were treated with Dox and then protein extracts were prepared at the indicated days. **(B):** Partial rescue of NS tet-off ESCs by either constitutively active Stat3, Akt, or exposure to the 2i condition. Upper right panel shows a Western blot for confirmation of the exogenously overexpressed Stat3 mutant and Akt. The combinatorial effect was also examined for all three components. Lower left and right bar graphs show the relative number of AP-positive colonies and the total occupied area of all rescued colonies, respectively, in which data from Dox-treated NS tet-off ESCs are set to 1 in both cases. NS tet-off ESCs and those overexpressing constitutively active Stat3 and/or Akt were treated as indicated for 6 days, and then subjected to AP staining. Scale bar = 100 μm. Abbreviations: ESC, embryonic stem cell; NS, nucleostemin.
Effects of Forced Expression of Nanog and Esrrb on the Global Expression Profile of NS Expression-Ablated ESCs

To systematically quantify the similarity in the overall gene expression profiles of Nanog- or Esrrb-rescued NS tet-off ESCs and Dox-untreated NS tet-off ESCs, we performed DNA microarray analyses and used the obtained data for cluster analyses. Data from 10 samples were clustered into two main groups in which one group mainly consisted of Dox-untreated NS tet-off ESCs, while three types of Dox-treated NS tet-off ESCs were assigned to the other group.
ESCs (Stat3mut, 2i, and empty) belonged to the other group. However, we noted that Nanog- and Esrrb-rescued Dox-treated NS tet-off ESCs showed extremely similar expression patterns. They were clearly segregated from the group of Dox-treated NS tet off ESCs, but belonged to the first group mainly including Dox-untreated NS tet-off ESCs. This result provided additional evidence that the forced expression of Nanog or Esrrb had counteracted NS expression ablation-mediated disruption of ESC pluripotency (Fig. 4A). MA (log ratios/mean average) plots comparing gene expression profiles (Fig. 4B) provided data consistent with this notion. The expression profile of NS tet-off ESCs was greatly altered upon ablation of NS expression with Dox and neither constitutively active Stat3 nor exposure to the 2i condition were able to alleviate this change significantly. However, forced expression of Nanog or Esrrb made NS tet-off ESCs rather refractory against this NS expression ablation-mediated change of their global expression profile. The ESC status is preserved by exquisite interplay of three transcription subnetworks termed Core, Myc, and PRC modules [31]. Our analyses of the DNA microarray data revealed that these three subnetworks were significantly unbalanced in Dox-treated NS tet-off ESCs, and neither expression of constitutively active Stat3 mutant nor exposure to the 2i plus LIF condition appreciably attenuated this unbalance. However, we found that these subnetworks were fairly normalized in both Nanog- and Esrrb-rescued NS tet-off ESCs (Fig. 4C). In addition, we used the microarray data to examine the expression levels of individual genes related to pluripotency and cellular differentiation. We found that trophoblast marker genes and pluripotency genes were significantly upregulated and downregulated, respectively (Fig. 4D). Some, but not all, mesendodermal marker genes showed elevated expression levels in Dox-treated NS tet-off ESCs. However, we assume that this observation did not represent the normal differentiation process of ESCs, because no clear upregulation of primitive ectodermal marker genes such as Fgf5 and Otx2 [32] was evident in Dox-treated NS tet-off ESCs at any time point. More importantly, such expression changes observed in parental NS tet-off ESCs were not influenced by constitutively active Stat3 or exposure to the 2i plus LIF condition, but fairly minimized by forced expression of Nanog or Esrrb. We also investigated the expression dynamics of 49 genes that have been previously denoted as Nanog-sensitive genes [33]. These genes were identified by two strict criteria: they have been demonstrated as Nanog target genes by genome-wide chromatin immunoprecipitation analyses and show immediate alterations in gene expression levels after transcriptional activation of Nanog. GSEA revealed that most genes with positively regulated expression levels by Nanog showed a decline in their expression levels after ablation of NS expression in ESCs, while genes subjected to negative regulation by Nanog tended to show upregulated expression levels upon NS expression ablation in ESCs. However, such a tendency was not observed in Nanog- or Esrrb-rescued NS tet-off ESCs (Supporting Information Fig. S3). It may be noteworthy that Nanog-rescued NS tet-off ESCs tended to show relatively higher expression of genes subjected to positive regulation by Nanog when the cells were treated with Dox compared with that in Dox-untreated cells. These data imply that abnormally high levels of Nanog in Nanog-overexpressing Dox-untreated cells may be detrimental for the expression of Nanog target genes.

**GO Analyses of Genes Showing Differential Expression Levels by Ablation of NS Expression in ESCs**

Next, to correlate upregulation and downregulation of these genes by NS expression ablation with overall molecular functions, we conducted GO analyses. To this end, we first constructed Venn diagrams with genes that showed altered expression levels by more than twofold in Dox-treated NS tet-off ESCs that were transfected with either Nanog, Esrrb, or an empty vector (Fig. 5A). We found that the expression levels of most genes (933 out of 1,181) that were upregulated by more than twofold in Dox-treated NS tet-off ESCs were normalized by forced expression of Nanog or Esrrb. Similarly, 731 out of 938 genes that were downregulated in Dox-treated NS tet-off ESCs were normalized by forced expression of Nanog or Esrrb. We also conducted GO classification analyses. A number of GO terms, especially those related to morphogenesis and development, were significantly enriched for genes upregulated but normalized by Nanog or Esrrb (Fig. 5Ba), while only two GO terms ("lipid catabolic process" and "cell cycle") were represented as prominent terms among genes downregulated but normalized by Nanog or Esrrb. These results indicate that the major role of NS in ESCs is suppression of cellular differentiation and morphogenesis, which can be substituted by forced expression of either Nanog or Esrrb. These data also indicated that, unlike the role of upregulated genes, the downregulated genes are a gene set without an apparent overall biological significance. We also subjected other groups of genes in the Venn diagrams shown in Figure 5A to GO analyses. However, none of the GO terms were selected by the analyses except for genes with downregulated expression in NS tet-off ESCs irrespective of the forced expression of either Nanog or Esrrb. In this case, genes categorized into a GO term (antigen processing and presentation of peptide antigen via MHC class I) were found to be enriched, but the physiological significance of identification of this term is unknown at present (Fig. 5Bc).

**Figure 3.** Strong rescue effects by forced expression of Nanog or Esrrb in NS tet-off embryonic stem cells (ESCs). (A): Forced expression of Nanog or Esrrb allowed self-renew for a prolonged period even after ablation of NS expression in ESCs. After stable integration of expression vectors for Nanog or Esrrb with puromycin selection, the cells were cultured in the presence or absence of Dox with standard ESC medium. After 6 days, the cells were examined under a conventional bright field microscope and then subjected to alkaline phosphatase staining. Scale bar = 500 μm (upper panel); 100 μm (lower panel). (B): Western blot analyses of cells that were partially rescued by exposure to the 2i condition or with constitutively active Stat3 and those rescued by forced expression of Nanog or Esrrb. The cells were cultured in the presence or absence of Dox for 6 days. (C): Growth curves of NS tet-off ESCs overexpressing either constitutively active Stat3, Nanog, or Esrrb, or exposed to the 2i condition. NS tet-off ESCs transfected with an empty vector were used as controls. Cell numbers were counted at the indicated days, divided by \(1 \times 10^3\), and then plotted on the semilogarithmic graph. The number of cells at day 0 was \(4 \times 10^5\). Data from Dox-untreated and -treated cells are plotted in the left and right panels, respectively. (D): TUNEL assay of Nanog- or Esrrb-overexpressing NS tet-off ESCs. The cells were cultured in the presence of Dox for 6 days. The ratio of TUNEL-positive cells to DAPI-positive cells is shown with the SD. ***, \(p < .001\). Scale bar = 10 μm. Abbreviation: NS, nucleostemin.
Evidence of a NS-Mediated Rad51-Independent Mechanism to Preserve Cell Viability and Expression of Pluripotency Marker Genes in ESCs

Recently, it has been demonstrated that recruitment of Rad51 to foci of stalled replication-induced DNA damage to maintain genomic stability is the major role of NS in NSCs [6]. To investigate whether the same mechanism underlies the apoptotic phenotypes of NS tet-off ESCs, we first examined whether there was a striking increase of γ-H2AX-positive cells among NS tet-off ESCs, which is observed with NS-null NSCs. We found no evident increase of γ-H2AX-positive cells for the first 2 days after Dox addition. We did observe γ-H2AX-positive NS tet-off ESCs at 4 days post-Dox administration. However, these cells never showed dot-like signal pattern, but homogeneously stained pattern in the nucleus, implying that double-stranded DNA breaks marked by γ-H2AX positivity were not the primary cause, but rather a secondary consequence of apoptotic cell death of NS tet-off ESCs (Fig. 6A) [34, 35]. Differences in the molecular mechanisms of NS tet-off ESC and NSC apoptosis were also suggested by the fact that, unlike NS knockout NSCs, overexpression of neither Rad51 (Fig. 6B) nor the nucleolar localization-defective NS mutant (NSΔB) (Fig. 6C)
mitigated the detrimental phenotypes of NS tet-off ESCs. Thus, these results indicate that loss of the stabilizing effect of NS on the genome does not account for the major cause of the extensive cell death phenotypes of NS tet-off ESCs. However, these results do not necessarily eliminate the possibility that NS also participates in genomic stabilization of ESCs. NS protein in ESCs may exert dual functions to preserve pluripotency and cell viability by preservation of genome integrity, as demonstrated in other NS-expressing cells such as NSCs, and some ESC-specific function. To test this hypothesis, we overexpressed NS in wild-type ESCs and compared the levels of γ-H2AX positivity after induction of DNA damage by HU treatment with those in ESCs transfected with an empty vector. These analyses revealed that the numbers of γ-H2AX-positive cells were noticeable declined by NS overexpression and this effect was evident even with NSΔB (Fig. 6D). Taken together, these results indicate that the genome-protective role of NS is operative at least among ESCs, NSCs, and regenerating hepatocytes, and probably more widely among NS-expressing cells. Our data also indicate that NS in ESCs is involved in an additional mechanism in parallel to preserve pluripotency and cell viability.

**Crucial Role of NS in Preservation of Pluripotency Marker Gene Expression and Cell Viability of EpiSCs**

Both ESCs and EpiSCs exhibit pluripotency [14, 15]. Therefore, we determined whether NS also participates in preserving the pluripotency of EpiSCs. To test this hypothesis directly, we

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**Figure 5.** Characterization of the effects of forced expression of Nanog or Esrrb on the global expression profile of nucleostemin expression-ablated embryonic stem cells (ESCs). (A): Venn diagram illustrating relative relationships of upregulated (left) and downregulated (right) genes in Dox-treated ESCs with stable integration of either Nanog (blue), Esrrb (green) or empty (red) expression vectors. Small letters (a, b, and c) in circles represent groups of gene sets that were suggested to be enriched with certain biologically related genes by the gene ontology (GO) analyses shown in (B). (B): GO analyses of the gene sets depicted in (A) using DAVID. The p-value cutoff was set to \(<1 \times 10^{-3}\). All gene sets (seven groups in each panel) were subjected to the analyses. However, prominent GO terms that satisfied the criteria of selection (\(p < 1 \times 10^{-3}\)) were only identified for the three gene sets denoted with a, b, and c in (A).
generated NS tet-off EpiSCs (Fig. 7A) and then cultured these cells with or without Dox. Microscopic observation revealed that Dox-treated NS tet-off EpiSCs showed severely impaired cell growth (Fig. 7B). Similar to ESCs, this severe phenotype was not alleviated by forced expression of Rad51 (Supporting Information Fig. S4). A substantial number of cells also

![Figure 6](image_url)

**Figure 6.** Evidence of an additional role of NS other than a genome-protective role in ESCs. (A): Examination of γ-H2A.X signals in NS tet-off ESCs. NS tet-off ESCs were cultured with Dox for 0, 2, 4, and 6 days, and then immunostained with an anti-γ-H2A.X antibody. Scale bar = 10 μm. (B): Alkaline phosphatase staining of control and Flag-Rad51-overexpressing NS tet-off ESCs cultured in the absence or presence of Dox for 6 days. Scale bar = 100 μm (left panel). Western blot analyses were also performed to confirm the exogenously overexpressed Flag-tagged Rad51 with an antibody against Rad51 (right panel). (C): Alkaline phosphatase staining of control, Flag-NS-, and Flag-NSΔB-overexpressing NS tet-off ESCs that were cultured either with or without Dox for 5 days. Scale bar = 100 μm (left panel). Immunostaining of these cells with an anti-Flag antibody was performed to confirm nucleolar and nuclear localization of wild-type NS and the NSΔB mutant, respectively. Scale bar = 10 μm (right upper panel). Right lower panel shows a Western blot of exogenously overexpressed wild-type NS and the NSΔB mutant on HU-mediated genomic damage in ESCs. Levels of genomic DNA damage were evaluated by positivity for γ-H2A.X in immunocytochemical analyses. *, p < .05. Abbreviations: ESC, embryonic stem cell; NS, nucleostemin.
features of NS tet-off ESCs, we examined whether p53 protein was similarly accumulated in NS tet-off EpiSCs. Our analyses revealed that a substantial number of cells expressed p53 protein in their nucleus after Dox-mediated ablation of NS expression (Fig. 7E). Next, we examined the consequence of Nanog or Esrrb overexpression in NS tet-off EpiSCs. These analyses revealed that, unlike NS tet-off ESCs, forced expression of neither Nanog nor Esrrb overrode the requirement of NS for the preservation of robust cell proliferation, cell viability, and homogeneous expression of Oct3/4 in EpiSCs (Supporting Information Fig. S5). Similar to ESCs, these results indicate that NS crucially participates in preservation of the cell viability of EpiSCs. However, there is a definite difference in the responses to forced expression of either Nanog or Esrrb in NS tet-off ESCs and EpiSCs. We will discuss about these data more in details later (see Discussion).

Role of NS Expression in Human iPSCs

To extend our findings concerning the role of NS expression in mouse ESCs and EpiSCs, we examined the role of NS expression in the preservation of human iPSC viability by knockdown experiments. We found that a proportion of human iPSCs bearing activated caspase-3/7 became significantly larger as a result of shRNA-mediated knockdown of NS expression compared with control cells in which shRNA-mediated knockdown targeted unexpressed luciferase (Luc) (Supporting Information Fig. S6). This result implies that NS also plays crucial roles in preserving the viability of human iPSCs.

**DISCUSSION**

NS is involved in numerous biological functions, and expressed in various stem/progenitor cell types, including ESCs. Knockout mouse analyses have demonstrated overt morphological abnormalities in NS knockout embryos during blastocyst formation, in which a discernible inner cell mass is absent at 3.5 dpc and the overall structure of the embryo resembles morulae without a blastocoeI cavity [3]. In accordance with these findings, we have previously demonstrated that NS expression is essential to preserve the cell viability of ESCs that are derivatives of the inner cell mass of blastocysts [13]. Subsequently, Qu and Bishop [4] have demonstrated that NS expression is important to preserve the unique cell cycle kinetics observed in mouse ESCs that show an unusually low proportion of cells in G1 phase. One of the major findings in this study is that forced expression of Nanog or Esrrb rendered NS expression in ESCs redundant. Indeed, Nanog- or Esrrb-overexpressing NS tet-off ESCs were able to maintain normal expression levels of pluripotency marker genes and propagate robustly for prolonged periods without showing any apparent apoptotic phenotype. Strong counteracting effects of Nanog and Esrrb against the changes associated with NS expression ablation in ESCs were also suggested by analyses of the global gene expression profile. Approximately 80% of genes with altered expression levels by more than twofold were not strongly affected by the loss of NS expression in Nanog- or Esrrb-overexpressing ESCs. GO analyses revealed that a number of terms related to morphogenesis and development were significantly represented among genes.
upregulated by more than twofold in NS expression-ablated ESCs, but became insensitive to the loss of NS expression by forced expression of Nanog or Esrrb. The same analyses of downregulated genes but normalized by Nanog or Esrrb revealed only two GO terms (lipid catabolic and cell cycle). Therefore, these results indicate that the major functions of NS in ESCs involve suppression of cellular differentiation and promotion of robust cell proliferation, which can be replaced by Nanog or Esrrb. Recently, Esrrb has been shown to be one of the major targets of Nanog [33]. Indeed, Esrrb can functionally substitute Nanog in ESCs. Therefore, it is possible that the rescue effect of Nanog overexpression may be largely explained by its function in supporting Esrrb gene expression.

It has been recently demonstrated that NS protein is involved in maintaining genomic stability of NSCs and regenerating liver cells by facilitating recruitment of Rad51 to foci of stalled DNA replication-induced DNA damage [6, 7]. However, our data obtained by forced expression of Rad51 and other experimental data demonstrate that impairment of this mechanism at least does not account for the major causes of the detrimental phenotypes observed in NS tet-off ESCs and EpiSCs. These data imply the presence of a pluripotent cell-specific function of NS protein. Similar to ESCs, our data also showed that NS expression is crucial in EpiSCs to preserve their pluripotency, self-renewal, and cell viability. However, unlike in ESCs, no apparent rescue effect by forced expression of Nanog or Esrrb was evident in NS-null EpiSCs. The endogenous expression levels of Nanog and particularly Esrrb were significantly low in EpiSCs compared with those in ESCs [36]. Therefore, we assume that these differential counteracting effects by forced expression of Nanog or Esrrb against NS expression ablation in ESCs and EpiSCs are due to the lack of additional factors and/or cofactors in EpiSCs, which are required for the effectiveness of the forced expression of these factors.

Our data demonstrated that overexpression of NS mitigated the DNA damage caused by addition of HU, suggesting that the genome-protecting role of NS in NSCs and regenerating hepatocytes is also operative in ESCs. However, our data demonstrated that an additional mechanism is operating in parallel to preserve pluripotency and cell viability, although the molecular basis of such a role is unknown at present except that it can be suppressed by forced expression of Nanog or Esrrb. Our finding of the partial rescue of NS tet-off ESCs by Stat3, Akt, and the 2i condition may help to elucidate the pluripotent cell-specific role of NS. Prominent stabilization of p53 in NS tet-off ESCs may be an alternative, but not mutually exclusive, clue to understand the molecular basis of NS function in pluripotent cells, because p53 is known to repress the expression of numerous pluripotency maker genes including Nanog [37]. It is also important to determine whether the pluripotent cell-specific role of NS we advocated in this study is truly specific in pluripotent cells or operates in other cell types. Significant similarity has been demonstrated in the gene expression profiles of pluripotent cells and cancer cells [31, 38]. Therefore, it is tempting to speculate that NS participates in preserving the undifferentiated state and cell viability of cancer cells, particularly cancer stem cells, in a similar manner that operates in ESCs and EpiSCs. This hypothesis can be tested experimentally, because cancer cells such as glioma cells can be theoretically generated from ESC-derived NSCs/progenitor cells [39, 40].

**CONCLUSIONS**

Previous knockdown and knockout studies have demonstrated that NS is important to preserve the self-renewal and high expression levels of pluripotency marker genes in ESCs. In this study, we demonstrate that NS expression is also essential to preserve the self-renewal and pluripotent properties of EpiSCs. Furthermore, our data demonstrate that forced expression of Nanog or Esrrb renders NS expression dispensable in ESCs, but not EpiSCs.

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**AUTHOR CONTRIBUTIONS**

M.K.: conception and design, collection and assembly of data, data analysis, interpretation, and manuscript writing; M.E., Y.N., Y.M., M.H., A.S., A.U., M.N., and S.T.: collection and analysis of data and data analysis and interpretation; Y.O.: data interpretation, financial, and administrative support; A.O.: conception and design, interpretation, manuscript writing, financial and administrative support, and final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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