TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Abundant Nucleostemin Expression Supports the Undifferentiated Properties of Germ Cell Tumors

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Nucleostemin (NS) is a nucleolar GTP-binding protein that is involved in ribosomal biogenesis and protection of telomeres. We investigated the expression of NS in human germ cell tumors and its function in a mouse germ cell tumor model. NS was abundantly expressed in undifferentiated, but not differentiated, types of human testicular germ cell tumors. NS was expressed concomitantly with OCT3/4, a critical regulator of the undifferentiated status of pluripotent stem cells in primordial germ cells and embryonal carcinomas. To investigate the roles of NS in tumor growth *in vivo*, we used a mouse teratoma model. Analysis of teratomas derived from embryonic stem cells in which the NS promoter drives GFP expression showed that cells highly expressing NS were actively proliferating and exhibited the characteristics of tumor-initiating cells, including the ability to initiate and propagate tumor cells *in vivo*. NS-expressing cells exhibited higher levels of GTP than non-NS-expressing cells. Because NS protein is stabilized by intracellular GTP, metabolic changes may contribute to abundant NS expression in the undifferentiated cells. OCT3/4 deficiency in teratomas led to loss of NS expression, resulting in growth retardation. Finally, we found that teratomas deficient in NS lost their undifferentiated characteristics, resulting in defective tumor proliferation. These data indicate that abundant expression of NS supports the undifferentiated properties of germ cell tumors. (*Am J Pathol* 2013; 183: 592–603; <http://dx.doi.org/10.1016/j.ajpath.2013.04.018>)

Testicular germ cell tumors (TGCTs) are the most common solid tumor in young adult males.^{1,2} Histologically, TGCTs consist of two groups: seminomas and nonseminomas. The nonseminomas include embryonal carcinoma (undifferentiated), teratoma (differentiated), and yolk sac tumor and choriocarcinoma (extra-embryonic). TGCTs are thought to be initiated *in utero* as intratubular germ cell neoplasias unclassified, which originate from developing germ cells, primordial germ cells (PGCs), or gonocytes.

PGCs are derived from epiblasts during early embryogenesis.³ Epiblasts are pluripotent cells that differentiate to form all three layers of the trilaminar germ disk in a process called gastrulation. Consistently, pluripotent stem cell lines, termed epiblast stem cells, can be established from epiblasts *in vitro*.^{4,5} In mice, PGCs become identifiable as a cluster of cells at the base of the allantois at 7.25 days after coitus

(dpc).³ The PGCs express OCT3/4, which support pluripotency. By 11.5 dpc, PGCs migrate from the allantois through the hindgut and settle in the gonadal ridge. By 13.5 dpc, PGCs start to display sexually dimorphic patterns of development. In males, PGCs become surrounded by differentiating Sertoli cells and grow continuously until 16.5 dpc, when they undergo mitotic arrest and become gonocytes.⁶ The differentiation of a PGC into a gonocyte restricts its subsequent developmental potential to the generation of male germ cells and is associated with down-regulation of

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markers of pluripotency.^{7,8} TGCTs and PGCs or gonocytes have similar gene expression profiles.⁹ Embryonal carcinomas highly express genes that support pluripotency of embryonic stem (ES) cells, including genes for OCT3/4, SOX-2, and NANOG.^{10,11} OCT3/4 and NANOG are sensitive and specific markers for primary seminoma and embryonal carcinoma, whereas SOX-2 is not expressed in seminomas.¹⁰ These data suggest that the differences among the subtypes of TGCTs may depend on the differentiation status of the tumor.

Nucleostemin (NS), alias guanine nucleotide-binding protein-like 3 (GNL3), is a nucleolar GTP-binding protein that is expressed preferentially in the nucleolus¹² but it can also shuttle to and from the nucleoplasm.¹³ NS belongs to the class of nucleolar GTPases that includes yeast Nug1, which exports pre-60S ribosomal subunits out of the nucleolus.¹⁴ In *Caenorhabditis elegans*, *nst-1* mutants exhibit reduced rRNA levels, suggesting a critical role for NS in ribosome biogenesis.¹⁵ NS knockdown apparently delays processing of 32S pre-rRNA into 28S rRNA and is accompanied by a substantial decrease both in protein synthesis and in the levels of rRNAs and some mRNAs.¹⁶ NS is also involved in regulating telomere integrity. NS and another protein in the same family, GNL3-like (GNL3L), interact with one of the telomeric proteins, telomeric repeat-binding factor 1 (TRF1), preventing telomere damage.^{17–19} NS and GNL3L form a complex with the telomerase catalytic subunit, human telomerase reverse transcriptase (hTERT).²⁰ NS deficiency in mice is early embryonic lethal,^{17,21} and NS thus plays a critical role in fetal development *in vivo*. NS was originally reported to be highly expressed in stem cells from several different tissues, including ES cells, immature hematopoietic cells, and neural stem/progenitor cells.¹² A study using tetracycline (Tet)–inducible NS-deficient ES cells showed that NS is essential for the survival of ES cells.²²

Our research group has previously characterized NS expression in murine male germ cells.²³ In that study, NS protein was highly expressed in the nucleoli of PGCs. In addition, we successfully identified NS-expressing neonatal germ cells as spermatogonial stem cells with long-term repopulating capacity. In the present study, we investigated the expression pattern of NS in human germ cell tumors and its function in a mouse teratoma model and found that NS is essential for maintaining the undifferentiated status of germ cell tumors.

Materials and Methods

Animals

The NS-GFP transgenic mice were generated as described previously.²³ BALB/c nu/nu mice (6 to 8 week old males) used as xenograft recipients were purchased from Sankyo Labo Service (Tokyo, Japan). All procedures were performed in accordance with the animal care guidelines of Kanazawa University, Kanazawa, Japan. For analysis of

timed pregnant mice, the date the vaginal plug was observed was defined as 0.5 dpc of gestation.

Human Samples

Tissue microarray sections consisting of human samples from patients with germ cell tumors (catalog no. TE2081) and of human fetal tissue (catalog no. BE01015) were purchased from US Biomax (Rockville, MD). For detailed immunohistochemical analysis, formalin-fixed, paraffin-embedded sections of surgically resected specimens from 13 germ cell tumors (4 seminomas, 4 mature teratomas, and 5 mixed germ cell tumors composed of teratoma and embryonal carcinoma) were obtained from surgical specimens in the Department of Human Pathology, Kanazawa University School of Medicine. All human materials and protocols were approved by the ethics committees of Kanazawa University. Informed consent was obtained from all patients at the time of their surgery. Surgical human specimens were immediately fixed in 10% neutral-buffered formalin and were embedded in paraffin. Serial sections (4 μ m thick) were prepared from each formalin-fixed, paraffin-embedded block. Several of the sections were routinely stained for histological evaluation, and the remainder were processed for immunohistochemistry.

Cell Culture

Mouse ES cells (E14K) were maintained on a layer of mitomycin C–treated mouse embryonic fibroblasts in KnockOut Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 1% nonessential amino acids, 2 mmol/L L-glutamine, 50 units/mL penicillin plus 50 μ g/mL streptomycin, 55 μ mol/L 2-mercaptoethanol (all from Life Technologies—Invitrogen, Carlsbad, CA), and 1000 units/mL leukemia inhibitory factor (ESGRO; Millipore—Chemicon International, Temecula, CA). The NS-GFP mouse ES cells were established by transfection of an NS-GFP fragment²³ into parental ES cells by electroporation followed by subcloning. For labeling of ES cells with orange fluorescence, we transfected the NS-GFP ES cells with a Kusabira Orange expression vector (CAG-KO), in which Kusabira Orange cDNA (Medical & Biological Laboratories, Nagoya, Japan) was cloned into pCAGGS vector plasmid. ES cells with Tet-inducible OCT3/4 deficiency (ZHBTcH4),²⁴ ES cells with Tet-inducible NS deficiency,²² and control ES cells (ERBTcH3)²² were maintained without feeder cells, as described previously.^{22,24} These ES cells were cultured with Glasgow minimum essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mmol/L L-glutamine, 50 U/mL penicillin plus 50 mg/mL streptomycin, 1 mmol/L sodium pyruvate (Gibco), 55 mmol/L 2-mercaptoethanol, and 1000 U/mL leukemia inhibitory factor. The differentiation of ES cells was prevented by passaging the cells every 2 or 3 days. For Western blotting analysis, ES cells were incubated in Glasgow minimum

essential medium containing 10 µg/mL Doxycycline (DOX) (Sigma-Aldrich).

Mouse Teratoma Model

All ES cells were trypsinized and resuspended in 1:1 PBS/Matrigel (BD Biosciences, San Jose, CA). Viable ES cells (1×10^6 to 5×10^6 cells in 100 µL) were injected subcutaneously into the left and right dorsal flanks of BALB/c nu/nu mice. The mice were monitored for 5 weeks for teratoma formation. Any teratoma observed was measured with calipers, and size was calculated as length (mm) $\times$ width (mm). When appropriate, mice were given 0.2 mg/mL Dox (Sigma-Aldrich) in drinking water.

Histology and Immunohistochemistry

For immunohistochemistry using human specimens, including those on tissue array, the deparaffinized and rehydrated sections were microwaved in citrate buffer (pH 6.0) for OCT3/4 or EDTA buffer (pH 9.0) for NS in a microwave oven for 20 minutes. After endogenous peroxidase was blocked, these sections were incubated at 4°C overnight with antibodies against OCT3/4 or NS (both from Abcam, Cambridge, UK) and then at room temperature for 1 hour with anti-rabbit IgG conjugated to a peroxidase-labeled dextran polymer (N-Histofine Simple Stain MAX PO kit; Nichirei Bioscience, Tokyo, Japan). After a benzidine reaction, the sections were lightly counterstained with hematoxylin. Mouse teratomas were fixed with 4% paraformaldehyde at 4°C overnight and embedded in paraffin. For some experiments, testes and teratomas were fixed with periodate-lysine paraformaldehyde for 1 hour and dehydrated with 15% sucrose for 3 hours. Sections fixed with periodate-lysine paraformaldehyde were embedded in optimal cutting temperature frozen-embedding medium (OCT Tissue-Tek; Sakura Finetek, Tokyo, Japan). Sections were immunostained with the following primary antibodies: anti-NS (Abcam and Novus Biologicals, Littleton, CO), anti-OCT3/4 (Santa Cruz Biotechnology, Santa Cruz, CA; BD Pharmingen, San Diego, CA; and Abcam), anti-Ki-67 (BD Biosciences), anti-bromodeoxyuridine (anti-BrdU) (Calbiochem; Merck Millipore, Darmstadt, Germany), and anti-GFP (Medical & Biological Laboratories and Aves Labs, Tigard, OR). The staining signals in paraffin-embedded sections were visualized with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Chalfont St Giles, UK, and Dako, Carpinteria, CA) and a coloring reaction using a 3,3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories, Burlingame, CA), counterstained with hematoxylin, and viewed using a microscope (Axio Imager A1; Carl Zeiss, Jena, Germany). The staining signals in frozen sections were visualized with Alexa Fluor dye-conjugated secondary antibodies: anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG (Life Technologies—Invitrogen). For visualization of nuclei, specimens were stained with TOTO-3 dye (Life Technologies—Invitrogen). Immunostaining

was visualized under a confocal microscope (FV1000; Olympus, Tokyo, Japan).

Flow Cytometry

To investigate the expression of GFP, dissociated cells were analyzed by flow cytometry. In brief, teratomas were dissected and incubated with 1% collagenase type B (Roche Diagnostics, Meylan, France), 2.5 mmol/L CaCl₂, and 1 unit/mL Dispase II (Roche Diagnostics) in PBS and were dissociated with a pipetting procedure. Dissociated teratoma cells were stained with phycoerythrin-conjugated anti-CD45 and anti-Ter119 antibodies (BD Pharmingen). CD45⁺ and Ter119⁺ cells were negatively selected on an LD MACS column using anti-phycoerythrin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Finally, the flow-through fraction was incubated with 1 µg/mL propidium iodide to allow the removal of dead cells. Teratoma cell sorting and analysis were performed using a FACSaria fluorescence-activated cell sorting system (BD Biosciences) and a JSAN cell sorter (Bay Bioscience, Kobe, Japan). Sorted cells were resuspended in PBS containing 10% fetal bovine serum or KnockOut Dulbecco's modified Eagle's medium, and were prepared for further analysis.

Colony Assay and Alkaline Phosphatase Assay

Cells fractionated by flow cytometry were plated at 3000 cells per well in 24-well dishes. Colonies were visualized and counted after 1 week. The colonies were rinsed three times with PBS and fixed with 4% paraformaldehyde for 10 minutes at 4°C. The fixed colonies were washed with Tris-buffered saline and were measured with alkaline phosphatase (ALP) staining. ALP activity was detected with a fuchsin⁺ substrate-chromogen system (Dako, Glostrup, Denmark).

Real-Time PCR

RNA samples were purified from fractionated teratoma cells using an RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed using an Advantage RT-for-PCR kit (Clontech Laboratories, Mountain View, CA). Real-time RT-PCR was performed with an Mx3000P quantitative PCR system (Stratagene, La Jolla, CA) and SYBR premix Ex Taq (Takara, Shiga, Japan), according to the manufacturer's recommended protocol. The following primers were used: GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3', NS forward 5'-GAGTGGACAGGTGCCTCATTAGGTTACT-3' and reverse 5'-GATGGCTTACCTGCTGTTGATTGCTC-3', and OCT3/4 forward 5'-GGCGTTCTCTTTGGAAGGTG-TTC-3' and reverse 5'-CTCGAACCACATCCTTCTCT-3'.

Quantification of Metabolites

For capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) analysis, 8×10^5 NS-GFP^{neg}, NS-GFP^{low},

or NS-GFP^{high} cells from teratomas were lysed to extract metabolites. Metabolomic profiling and data analysis were performed twice, as described previously.^{25,26}

BrdU Incorporation *in Vivo*

For BrdU labeling experiments, mice were injected intraperitoneally with 100 mg/kg body weight BrdU (Sigma-Aldrich), and the tissues were removed 2 hours later. Detection of BrdU-labeled cells was achieved with a BrdU labeling kit (Calbiochem; Merck Millipore), according to the manufacturer's instructions.

Western Blotting Analysis

ES cells were lysed with cell lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 8.0, 1% NP-40 detergent, and Roche Diagnostics protease inhibitor cocktail); these were then used as total cell lysates. Protein concentrations were measured with a Pierce BCA protein assay (Thermo Fisher Scientific, Rockford, IL), and equal amounts of protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in PBS containing Tween 20 for 1 hour at room temperature. Membranes were then incubated with a goat anti-NS antibody (1:1000; Neuromics, Edina, MN) or an anti-OCT3/4 antibody (1:2000; BD Pharmingen) for 16 hours at 4°C, or a mouse anti- β -actin antibody (1:1000; Sigma-Aldrich) for 1 hour at room temperature. Immune complexes were detected using peroxidase-conjugated secondary antibodies (1:1000; 30 minutes incubation at room temperature; GE Healthcare and Dako) and an ECL Prime Western blotting detection system (GE Healthcare).

Detection of Apoptosis

To detect apoptosis *in vivo*, freshly isolated teratomas were immediately fixed with 4% paraformaldehyde, and tissue sections were prepared. The sections were stained by using a DeadEnd colorimetric TUNEL system (Promega, Madison, WI), according to the manufacturer's instructions.

Statistical Analysis

Statistical significance was determined using the unpaired Student's *t*-test.

Results

Abundant Expression of NS in Undifferentiated, but Not Differentiated, Germ Cell Tumors in Human TGCTs

To analyze expression of NS in germ cell tumors, we stained tissue sections of human TGCTs (including seminomas, embryonal carcinomas, and mixed germ cell tumors) with an anti-NS antibody. NS expression was detected in 18/28 (64%)

embryonal carcinomas and in 52/86 (60%) seminomas (Figure 1, Supplemental Figure S1, and Supplemental Table S1). NS protein was localized mainly in nuclei, and was not present in the cytoplasm. In most NS⁺ cells, NS protein was detected in both the nucleoplasm and nucleoli (Figure 1 and Supplemental Figure S1). There were no remarkable differences in expression pattern or abundance of NS between embryonal carcinomas and seminomas. NS expression was detected in undifferentiated components, including the embryonal carcinoma portion of all mixed germ cell tumors, and most tumor cells in the embryonal carcinoma component highly expressed NS (Figure 2, A and B). NS was not expressed in most interstitial cells, but was expressed in a few undifferentiated mesenchymal cells within the embryonal carcinoma (Figure 2, A and B). NS expression was not detected in the well-differentiated intestine-like component of a mature teratoma (Figure 2C), but weak nucleolar expression was seen in epidermal squamous cells (Figure 2D). Thus, NS was highly expressed in the embryonal carcinoma component, but not in well-differentiated mature teratoma tissues. Because it has been reported that OCT3/4, which is a critical regulator of the undifferentiated status of ES cells, controls the malignant fate of germ cell tumors,²⁷ we next compared NS expression with OCT3/4 expression. OCT3/4 protein was detected in all mixed germ cell tumors. It was highly expressed in the embryonal carcinoma component (Figure 2E), but not in the well-differentiated mature teratoma components, including the intestine-like cells and epidermal cells (Supplemental Figure S2). Double staining with anti-OCT3/4

Figure 1 NS and OCT3/4 expression in human seminomas. Representative sections of NS⁺ seminomas. Sections were stained with an anti-NS or anti-OCT3/4 antibody, followed by a DAB peroxidase reaction; sections were counterstained with hematoxylin. **A:** Seminoma. H&E stain. **B:** Immunohistochemistry for NS. Most tumor cells in the seminoma are positive for NS. **C:** Higher magnification of a section stained with anti-NS antibody. NS is strongly expressed within the nuclei and nucleoplasm of seminoma cells (arrow). Infiltrating lymphocytes within tumor nests are negative for NS (arrowhead). **D:** Immunohistochemistry for OCT3/4. Nuclei of tumor cells within a seminoma are positive for OCT3/4 (arrow). Infiltrating lymphocytes within tumor nests are negative for OCT3/4 (arrowhead). The insets show digitally enlarged images of regions with cells indicated by arrows in the insets. Original magnification: $\times 100$ (A and B); $\times 400$ (C and D).

Figure 2 Abundant expression of NS in undifferentiated cells in human mixed-type germ cell tumors. Expression of NS and OCT3/4 in human germ cell tumors of mixed type (both embryonal carcinoma and mature teratoma cells). Sections were stained with an anti-NS or anti-OCT3/4 antibody, followed by a DAB peroxidase reaction; sections were counterstained with hematoxylin. **A:** Immunohistochemistry for NS in the embryonal carcinoma component. Most tumor cells of the embryonal carcinoma are positive for NS (asterisk), as are a few undifferentiated mesenchymal cells (double asterisk). **B:** Higher magnification of a portion of **A**. NS is expressed in the nucleoli of embryonal carcinoma cells (asterisk) and undifferentiated mesenchymal cells (double asterisk). **C** and **D:** Immunohistochemistry for NS in the intestine-like component (**C**) and in squamous cells of the epidermis (**D**). No positive cells were found in the intestine-like component. Weak nucleolar expression was found in the epidermal squamous cells. The insets show digitally enlarged images of the boxed regions. **E:** Immunohistochemistry for OCT3/4. Many OCT3/4⁺ cells were seen in the embryonal carcinoma (asterisk) but none in the undifferentiated mesenchymal components (double asterisk). **F:** Double immunohistochemical staining for NS (brown) and OCT3/4 (green). Most embryonal carcinoma cells express both NS and OCT3/4. Original magnification: $\times 200$ (**A** and **C**); $\times 400$ (**B** and **D–F**).

and anti-NS antibodies clearly showed that both proteins were expressed in the same cells (Figure 2F). Thus, coexpression of NS and OCT3/4 was observed mainly in undifferentiated germ cell tumors (embryonal carcinomas).

PGC-Specific Expression of NS

Because TGCTs possibly originate from PGCs, we evaluated NS expression in developing gonadal tissues in mice. Previously, we had generated transgenic reporter mice expressing GFP under the control of the NS promoter (NS-GFP Tg mice) and reported that the GFP successfully reflects NS expression in the developing testes.²³ Consistent with that earlier study, we found particular cell populations expressing high levels of GFP (corresponding to NS-expressing cells) in the gonadal ridge at 10.5 dpc and in

embryonic testes at 14.5 dpc (Figure 3A). Approximately 60% of NS-GFP–positive cells were OCT3/4⁺ at 10.5 dpc (Oct4⁺/GFP⁺, $62.73 \pm 7.14\%$; Oct4[−]/GFP⁺, $35.12 \pm 10.87\%$; Oct4⁺/GFP[−], $2.15 \pm 3.72\%$; $n = 3$). At 14.5 dpc, most NS-GFP–positive cells were OCT3/4⁺ (Oct4⁺/GFP⁺, $97.50 \pm 2.23\%$; Oct4[−]/GFP⁺, $2.49 \pm 2.23\%$; Oct4⁺/GFP[−], 0% ; $n = 3$). Thus, GFP expression was almost coincident with that of OCT3/4 at 14.5 dpc; because OCT3/4 is a PGC marker, this indicates that NS is expressed in PGCs. In neonates, OCT3/4 expression dramatically diminished, indicating that there is discrepancy between NS and OCT3/4 in gonocytes. We also analyzed the expression of NS in human embryonic testes. As previously reported,²⁸ OCT3/4-immunopositive staining was restricted to a particular subset of germ cells (Figure 3B). The staining pattern of NS was similar to that of OCT3/4 (Figure 3B). Double-staining analysis showed that, although some cells expressed OCT3/4 but not NS, NS was expressed only in cells that also expressed OCT3/4 (Figure 3B). Thus, common expression of NS and OCT3/4 in developing gonadal tissues was observed in both mouse and human tissues, indicating that NS is expressed in PGCs.

Characterization of Tumor Cells That Highly Express NS in a Teratoma Model

ES cells are commonly used to generate teratomas (ie, tumor-like formations containing tissues belonging to all three germ layers), which are used as a model of germ cell tumors. Because mouse teratomas derived from ES cells contain an OCT3/4⁺ undifferentiated cell component,²⁷ this model may be similar to human mixed-type germ cell tumors. We therefore chose this model for evaluating NS expression. We established single-clone-derived ES clones containing the NS-GFP fragment and used them to inoculate mice subcutaneously (Figure 4A). Tumors became detectable approximately 2 weeks later and grew continuously until the mice were sacrificed at 5 weeks. The NS-GFP ES cell–derived tumors were identified as teratomas, because several different cell components were included (eg, epithelial cells, muscles, intestine, and neurons; data not shown). In a histological analysis, NS-GFP was highly expressed only in particular areas (Figure 4B). Most cells expressing NS-GFP exhibited high levels of endogenous NS protein (Figure 4C). The NS-GFP–expressing cells also expressed OCT3/4 (Figure 4C), but they did not express the differentiation markers TuJ1 and SMA (Figure 4C). We did not detect metastatic NS-GFP–expressing cells in the recipient mice. The NS-GFP–positive cells appeared to be actively proliferating, based on their Ki-67 positivity (Figure 4C). These data clearly indicate that NS is abundantly expressed in proliferating, undifferentiated cells in the ES-derived mouse teratomas, which is similar to NS expression in human mixed-type teratomas.

To further investigate the characteristics of NS-expressing cells, we dissociated teratoma tissues with collagenase/trypsin, then analyzed them with flow cytometry. Although

Figure 3 Coexpression of NS and OCT3/4 in mouse and human embryonic testes. **A:** Immunohistochemical analyses of developing gonad sections from NS-GFP Tg mouse embryos (10.5 and 14.5 dpc) and neonates [0.5 days after birth (dpp)]. Sections were stained with anti-GFP (green) and anti-OCT3/4 (red) antibodies, and with TOTO3 (blue) to visualize nuclei. **B:** Immunohistochemical analyses of testicular sections from human embryos (5 or 6 months of gestation). Sections were stained with an anti-OCT3/4 or anti-NS antibody, followed by a DAB peroxidase reaction (**upper panels**). Double staining was performed with anti-NS (brown) and anti-OCT3/4 (green) antibodies (**lower panels**). The sections were counterstained with hematoxylin. The **right lower panel** is a magnified view of the **boxed region** in the **left lower panel**; the **boxed region** in the **right lower panel** is shown at higher magnification in the **inset**. NS⁺ (brown)/OCT3/4⁺ (green) cells are indicated by **arrows**; NS-negative (no brown staining)/OCT3/4⁺ (green) are indicated by **arrowheads**. Scale bars: 25 μ m (**A**, **middle** and **lower panels**), 50 μ m (**A**, **upper panels**, and **B**, **right lower panel**); 100 μ m (**B**, **upper panels**); 200 μ m (**B**, **left lower panel**).

the NS-GFP ES cells in our experiments expressed high levels of GFP, the majority of teratoma cells exhibited a lower level of GFP expression (**Figure 5A**), indicating that GFP expression was down-regulated during teratoma formation. The NS-GFP–positive cells could be fractionated into two populations, based on GFP intensity (**Figure 5B**). We compared the properties of the NS-GFP^{neg}, NS-GFP^{low}, and NS-GFP^{high} cell populations. NS-GFP intensity increased as the amount of endogenous NS mRNA increased, confirming that NS-GFP properly reflected the level of NS mRNA expression (**Figure 5C**). In addition, NS-GFP intensity and OCT3/4 mRNA were correlated, indicating that NS-GFP^{high} cells are OCT3/4⁺ undifferentiated cells.

To investigate the functions of the NS-expressing cells, we cultured isolated cells in ES medium *in vitro*. By day 7 of culture, ES-like cell colonies derived from teratomas had emerged. The NS-GFP^{high} cells produced higher numbers of

ES-like colonies (identified by expression of ALP, a marker of undifferentiated cells) than did NS-GFP^{neg} or NS-GFP^{low} cells (**Figure 5D**). To evaluate the functions of ES-derived teratoma cells alone, without including cells in the tumor tissues derived from the recipient (host) mice, we established a teratoma model using NS-GFP ES cells labeled with Kusabira Orange fluorescence (**Supplemental Figure S3**, A and B). Consistent with data in **Figure 5D**, colony-forming assays with teratoma cells positive for Kusabira Orange showed that NS-GFP^{high} cells produced high numbers of ES-like colonies (**Supplemental Figure S3C**). Moreover, NS-GFP^{high} cells formed significantly larger tumors *in vivo* than did NS-GFP^{low} cells (**Figure 5E**). No tumor formation from NS-GFP^{neg} cells was observed. These data indicate that high NS expression represents undifferentiated status and that at least some NS-expressing cells can function as tumor-initiating cells.

Figure 4 NS is expressed in proliferating cells that exhibit undifferentiated characteristics in a mouse teratoma model. **A:** Experimental design of a mouse teratoma model using NS-GFP ES cells. GFP expression is driven by the NS gene promoter in the NS-GFP ES cells. The teratomas are generated by injection of NS-GFP ES cells into the dorsal flanks of BALB/c nu/nu mice. In teratomas derived from NS-GFP ES cells, endogenous NS gene expression can be monitored by GFP. **B:** A representative section of anti-GFP immunohistochemistry in a teratoma derived from NS-GFP ES cells. Sections were stained with an anti-GFP antibody, followed by a DAB peroxidase reaction. The **inset** shows digitally enlarged image of the **boxed region**. **C:** Immunohistochemical analyses of teratomas derived from NS-GFP ES cells. Sections were stained with an anti-GFP antibody (green) and anti-NS, anti-OCT3/4, anti-TuJ1, or anti-Ki67 antibodies (red), followed by TOTO3 (blue) to visualize nuclei. Scale bars: 50 μ m (**C**, **first row**); 200 μ m (**B** and **C**, **remaining rows**).

Expression of NS is regulated both transcriptionally and post-translationally. Stabilization of the NS protein is supported by intracellular GTP.^{29,30} These data prompted us to examine whether the undifferentiated cell fractions in the teratomas have a high level of GTP *in vivo*. We used CE-TOFMS to evaluate metabolite concentrations^{25,26} in NS-GFP^{neg}, NS-GFP^{low}, and NS-GFP^{high} cells. Although there was no significant difference in GTP concentration between NS-GFP^{high} and NS-GFP^{low} cells, both NS-GFP^{high} and NS-GFP^{low} cells had significantly more GTP than did NS-GFP^{neg} cells (Figure 5F). These findings suggest that NS protein stability may be supported by GTP in NS-GFP^{high} and NS-GFP^{low} cells, but not in NS-GFP^{neg} cells. Because the amount of NS mRNA in NS-GFP^{high} cells was greater than in NS-GFP^{low} cells (Figure 5C), we assumed that the high level of NS protein in NS-GFP^{high} is supported by

transcriptional activity and protein stability. Similarly, NS-GFP^{neg} cells showed a low level of ATP (Figure 5F); the low level of this energy source in NS-GFP^{neg} cells may be related to the fact that majority of NS-GFP^{neg} cells are not proliferating (Figure 4C). Thus, the regulation of metabolites may support the characteristics of undifferentiated cells in the germ cell tumors.

Loss of NS Expression in OCT3/4-Deficient Teratomas *in Vivo*

It has been reported that OCT3/4 deficiency in a teratoma model results in the loss of undifferentiated status and malignant properties.²⁷ We therefore investigated whether NS expression is affected by OCT3/4 deficiency. For this purpose, we used Tet-inducible OCT3/4-deficient ES cells

Figure 5 Characterization of NS-expressing cells in a mouse teratoma model. **A:** Flow cytometry analysis of GFP expression in NS-GFP teratomas and ES cells. The teratomas were generated by injection of NS-GFP ES cells into the dorsal flanks of BALB/c nu/nu mice (Figure 4A). Nonhematopoietic cells from teratoma tissues were isolated by depletion of CD45⁺Ter119⁺ cells. Data shown are representative flow cytometry analyses of GFP in CD45⁺Ter119⁺PI⁺ cells from NS-GFP teratoma cells (black line) and in NS-GFP ES cells (gray line). GFP expression was remarkably down-regulated during tumor formation. **B:** Flow cytometry analysis of GFP expression in wild-type and NS-GFP teratoma cells. GFP was not detected in wild-type teratomas, but was detected in almost half of the cells in the NS-GFP teratomas. The teratoma cells (nonhematopoietic cells) from the NS-GFP teratomas were fractionated into GFP^{neg}, GFP^{low}, and GFP^{high} subpopulations. Data are expressed as the percentage of the specified subpopulation among CD45⁺Ter119⁺PI⁺ cells and are representative of 20 independent experiments. **C:** Expression of endogenous NS and OCT3/4 in the NS-GFP subpopulations. Total RNA was purified from the subpopulations (**B**), and mRNA levels of NS and OCT3/4 were evaluated by quantitative real-time RT-PCR. The RNA data were normalized to the expression of GAPDH. Data are expressed as means $\pm$ SD of GFP^{low} or GFP^{high} values relative to those of GFP^{neg} cells. **D:** ALP⁺ colony formation of the NS-GFP subpopulations. Fractionated cells (**B**) were cultured for 7 days, followed by staining with ALP. **E:** Teratoma formation of the NS-GFP subpopulations *in vivo*. Fractionated cells (Supplemental Figure S3B) were inoculated subcutaneously in BALB/c nu/nu mice. The tumor volume was measured 5 weeks later. Teratoma size was calculated as length (mm) $\times$ width (mm). **F:** Quantification of metabolites in the NS-GFP subpopulations. GTP and ATP levels were evaluated in the subpopulations (**B**) by CE-TOFMS analysis. Data are expressed as means $\pm$ SD (**C–F**) or as both means $\pm$ SD and individual values (**E**). $n = 3$ (**F**); $n = 5$ (**C**); $n = 6$ (**D**); $n = 8$ per group (**E**). * $P < 0.05$; ** $P < 0.01$.

(ZHBTcH4).²⁴ Both endogenous alleles of OCT3/4 are disrupted in these ES cells, but OCT3/4 cDNA under the control of a Tet-responsive transcriptional activator is introduced. The exogenous OCT3/4 cDNA is induced in OCT3/4-deficient ES cells in the absence of Tet (Dox), whereas its expression is down-regulated in the presence of Dox. The ES cells thus lose OCT3/4 expression when treated with Tet (forming Tet-induced OCT3/4-deficient ES cells). After teratomas were formed in nude mice by injection of Tet-inducible OCT3/4-deficient ES cells, the mice

were administered Dox to eliminate OCT3/4 expression. At day 14, the OCT3/4 protein was completely lost in teratoma tissues treated with Dox, but not in control tissues (Figure 6, A and B). In addition, the OCT3/4-deficient teratoma cells lost Ki-67 expression, indicating that OCT3/4 deficiency resulted in inhibition of proliferation, which is consistent with previous report.²⁷ NS protein was reduced also in the OCT3/4-deficient teratomas (Figure 6B), as were the amounts of OCT3/4 and NS mRNA (Figure 6C). These data indicate that the teratomas that lost the undifferentiated cell

Figure 6 Loss of NS in OCT3/4-deficient teratomas. **A** and **B**: Immunohistochemical analyses of teratoma sections derived from Tet-inducible OCT3/4-deficient ES cells in immunocompromised (BALB/c nu/nu) mice. After teratoma formation, the mice were administered Dox orally for 14 days. The sections were stained with an anti-OCT3/4, anti-NS, or anti-Ki-67 antibody, followed by a DAB peroxidase reaction. Sections were counterstained with hematoxylin. **C**: NS and OCT3/4 mRNA in the teratomas with and without Dox. Total RNA was purified from teratomas, and mRNA levels of NS and OCT3/4 were evaluated with quantitative real-time RT-PCR. RNA data were normalized to expression of GAPDH. Data are expressed as means $\pm$ SD of values of samples with Dox relative to those of samples without Dox. $n = 5$. ** $P < 0.01$. Scale bars: 200 μ m.

component did not express NS and that NS is therefore specifically expressed in undifferentiated cells in teratomas *in vivo*.

Essential Role of NS in the Maintenance of Undifferentiated Properties of Germ Cell Tumors

To investigate the roles of NS in undifferentiated germ cell tumors, we generated teratomas by using Tet-inducible NS-deficient ES cells. The gene for NS can be deleted from ES cells by tetracycline treatment, as in the Tet-induced OCT3/4-deficient ES cells described above.²² We confirmed that Dox treatment *in vitro* efficiently reduced NS protein in the ES cells but did not affect OCT3/4, consistent with previous report²² (Supplemental Figure S4). When the teratomas formed (3 weeks after inoculation), the mice were administered Dox to induce NS deficiency. The histological analysis showed that the Dox administration resulted in loss of the undifferentiated cell components in teratomas derived from the Tet-inducible NS-deficient ES cells (Figure 7A). NS protein clearly disappeared in the teratomas after Dox administration; also, the NS-deficient tumors lost OCT3/4 expression (Figure 7B), and this loss was associated with reduced Ki-67 expression and BrdU incorporation (Supplemental Figure S5, A and B). A TUNEL assay did not show any remarkable induction of apoptosis after NS deletion *in vivo* (Supplemental Figure S5C). The neuronal marker TuJ1, however, was

remarkably induced (Figure 7B and Supplemental Figure S5D). These data indicate that loss of NS induces differentiation of teratoma cells. To investigate whether loss of NS affects tumor growth, we started Dox administration at the same time as the ES cells were inoculated; teratoma growth was strongly reduced, compared with the control (Figure 7C). These data indicate that NS is essential for maintaining undifferentiated properties, thus leading to the promotion of germ cell tumors.

Discussion

In the present study, we found similar expression patterns for NS and OCT3/4 in undifferentiated germ cell tumors in humans and in PGCs in both humans and mice (Figures 1, 2, and 3). Because previous gene expression profiling showed that PGCs and germ cell tumors have similar gene expression signatures,⁹ it has been proposed that PGCs are one source of germ cell tumors. Embryonic stages and malignancy are thought to be closely related, because embryonic antigens, such as α -fetoprotein, commonly emerge during carcinogenesis. Thus, NS may be a critical link between embryonic development and malignancy.

In the mouse teratoma model, NS was abundantly expressed in proliferating, undifferentiated cells (Figure 4). Furthermore, we clearly demonstrated that NS can be an indicator of tumor-initiating cells (alias cancer stem cells)

Figure 7 Inhibition of teratoma growth by NS deficiency. **A** and **B**: Immunohistochemical analyses of teratoma sections derived from Tet-inducible NS-deficient ES cells in immunocompromised (BALB/c nu/nu) mice. After teratoma formation (3 weeks after inoculation), the mice were given Dox orally. **A**: Sections of teratomas obtained at days 0, 4, 8, and 12 after the initiation of Dox administration, stained with H&E. The undifferentiated cell component disappeared with Dox treatment. **Boxed regions** in the **upper row** are shown at higher magnification in the **lower row**. Undifferentiated cell components (**asterisk**) disappear with loss of NS. **B**: Sections of teratomas treated without or with Dox for 14 days were stained with an anti-NS, anti-OCT3/4, or anti-TuJ1 antibody, followed by a DAB peroxidase reaction. Sections were counterstained with hematoxylin. **C**: Effect of NS deficiency on teratoma formation. The immunocompromised mice were inoculated with control or ES cells with Tet-inducible NS-deficiency; Dox was administered orally at the same time. Teratoma size was calculated as length (mm) $\times$ width (mm). Data are expressed as means $\pm$ SD. $n = 8$ per group. $**P < 0.01$. Scale bars: 1 mm (**A**, **upper panels**); 100 μ m (**A**, **lower panels**); 200 μ m (**B**).

(Figure 5). Although NS was originally reported to be highly expressed in stem cells from several tissues, including ES cells, immature hematopoietic cells, and neural stem/progenitor cells,¹² it has also been reported to be expressed in postmitotic, terminally differentiated cells (myotubes and myofibers).³¹ Our research group previously reported abundant NS protein in mature hepatocytes.³² Thus, the expression pattern of NS appears to be complex. Furthermore, the NS-GFP reporter system allowed us to identify a specific fraction of neonatal germ stem cells as undifferentiated cells,²³ developing liver stem/progenitor cells,³² and neural stem cells.³³ We also combined the NS reporter system with a mouse brain tumor model and demonstrated the existence of an undifferentiated tumor-initiating cell population in a highly aggressive brain tumor by analyzing GFP fluorescence intensity.³³ Consistent with the present data, a report using a bacterial artificial chromosome transgenic mouse line expressing GFP from the NS promoter showed that

NS-enriched mammary tumor cells are highly tumorigenic *in vitro* and *in vivo*.³⁴ Although the mechanisms are still unclear, we assume that there are particular programs that control the promoter activity and that these are commonly up-regulated in undifferentiated cells in both normal and malignant tissues.

Loss of NS resulted in decreased OCT3/4 expression in mouse teratomas (Figure 7). This finding may conflict with our other findings in the present study (Supplemental Figure S4) and with those of others,^{21,22} that NS deletion did not affect OCT3/4 expression in embryos and ES cells. NS also appears to be controlled independently of mechanisms involving the OCT3/4–SOX-2 core complex, which is specific for the maintenance of undifferentiated status of ES cells. OCT3/4 has a bipartite POU domain, which mediates DNA binding for the regulation of gene expression networks.^{35–37} A number of studies have shown that OCT3/4 cooperates with SOX-2 to regulate gene expression

in ES cells.^{38–41} Although a possible HMG/POU motif is present in the NS promoter,⁴² there is no evidence that the OCT3/4–SOX-2 complex binds to the NS locus.^{43,44} There are several possible reasons why NS deletion could lead to loss of OCT3/4 in teratomas. For example, loss of NS may primarily inhibit cell proliferation, thereby inducing differentiation of the immature cell component of teratomas, which is associated with loss of OCT3/4 expression. Another possibility is that cells expressing OCT3/4 disappear because of apoptosis after NS deletion. Although in one study NS loss induced apoptosis and reduced cell proliferation in ES cells *in vitro*,²² we did not find that NS deficiency led to remarkable induction of apoptosis in teratomas; however, it may be that apoptotic cells were immediately removed by phagocytosis *in vivo*. Given that we did find remarkable up-regulation of the differentiation marker TuJ1, cell differentiation induced by NS loss may cause down-regulation of OCT3/4 in mouse teratoma in an indirect manner. Recently, Qu and Bishop⁴⁵ reported that NS is elevated during reprogramming of somatic cells to pluripotent status and that ectopic NS cooperates with OCT3/4 and SOX-2 for this reprogramming. Although it is unknown how NS is involved in the reprogramming, such mechanisms may underlie the maintenance of the undifferentiated status of germ cell tumors.

Currently, treatment for TGCTs is a cisplatin-based combination chemotherapy.⁴⁶ Although patients with TGCTs benefit from this treatment, novel therapeutic approaches are needed to achieve a cure. To eradicate all tumor cells, it would be important to particularly target the cells that express NS and OCT3/4, because, as we have shown with the present study, NS depletion contributes to the suppression of tumor progression. The investigation of other potent chemical compounds may lead to novel therapeutic approaches for the successful eradication of TGCTs.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.04.018>.

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