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Tumor-Initiating Function of Nucleostemin-Enriched Mammary Tumor Cells

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Abstract

Nucleostemin (NS) is highly expressed in normal stem cells and tumors and is upregulated by estradiol in MCF7 breast cancer cells. To investigate the role of NS in mammary tumorigenesis, we established first that NS is expressed at higher levels in the basal cell type than in the luminal cell type in mouse mammary tumors and human breast cancer cells. NS expression was also increased during progression of mammary tumors in MMTV-Wnt1 and MMTV-PyMT transgenic mice and by the tumor sphere culture. To determine the function of NS-enriched tumor cells, we generated a BAC transgenic mouse line expressing GFP from the NS promoter and bred it to MMTV-Wnt1 mice, so that NS-expressing cells can be prospectively isolated based on their GFP levels. Notably, NS-enriched mammary tumor cells exhibited stronger in vitro and in vivo tumorigenic activities and expressed higher levels of K5, CD133, Oct4, TERT and CXCL12 compared to NS-deficient mammary tumor cells. Furthermore, knockdown of NS dramatically reduced the sphere-forming activity of MDA-MB-231 and MCF7 human breast cancer cells. Our findings establish the tumor-initiating and molecular features of NS-enriched mammary tumor cells, suggesting that NS may offer a valuable therapeutic target.

Keywords

nucleostemin; MMTV-*wnt-1*; mammary tumor; breast cancer; cancer stem cells

Introduction

The existence of stem-like cells in cancer, better known as cancer stem cells (CSC) or tumor-initiating cells (TIC), was initially observed in acute myeloid leukemia (1,2) and later found in solid tumors of the breast (3), brain (4), and other tissues (5–9). They are thought to be responsible for the recurrence, metastasis, and drug resistance of high-grade tumors (10). The most commonly used markers for FACS-based enrichment of human mammary CSC (mCSC) are CD44⁺CD24^{low}Lin[−] (3,11,12). For mouse normal or cancerous mammary stem cells, various sets of markers have been reported, including CD29^{high}CD24⁺Lin[−] (13), CD49^{high}CD24^{med}Lin[−] cells (14), Thy1⁺CD24⁺ (15), CD24⁺CD29⁺Lin[−] (16,17), CD61 (18), Oct4 (19), CD133 (20), Sca-1 (21,22), and ESA (23). Most of the sorting schemes require multi-parameter FACS, yield rather heterogeneous cell populations, and are unclear in their biological meaning. Some of the markers, such as Sca-1 and CD24, have also been used as negative selectors for normal mouse mammary stem cells or human mCSC

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(3,13,14). Enrichment of mCSC can also be based on their aldehyde dehydrogenase (ALDH) activity (24).

Nucleostemin (NS) was first isolated as a gene upregulated in MCF7 cells upon 17 β -estradiol treatment (25), and later shown to function in maintaining the continuous proliferation of neural stem cells (NSCs) and cancer cells (26). Since then, high levels of NS expression have been reported in ES cells, mesenchymal stem cells (MSCs), germline stem cells, and human cancers (26–29). Together, these findings suggest a potential link between NS and mCSC. To investigate this possibility, we chose the MMTV-*wnt-1* mice as our mammary tumor model (30). The relevance of MMTV-*wnt-1* mammary tumors to human breast cancers is supported by frequent deregulations or mutations of the genes involved in the canonical Wnt signaling pathway (31), particularly in the basal-like breast cancers (BLBCs) (31–35). Several Wnts are found overexpressed in human breast cancer tissues and cell lines (32) and necessary for their survival (31,36–38). Finally, the MMTV-*wnt-1* mammary tumor exhibits mixed cell lineages of basal and luminal cells (39–41), which resembles the broad spectrum of heterogeneity in human breast cancer samples and suggests perturbation at the mCSC/progenitor cell level (39,40). Here, we report that NS expression correlates with the basal subtype of mammary tumor cells, and that mammary tumor cells expressing higher levels of NS harbor stronger *in vitro* and *in vivo* tumorigenic activities. We also establish that NS is functionally required for the sphere-forming activity of human breast cancer cells.

Materials and Methods

Animal Care

All animals were housed in the Program for Animal Resources of the TAMHSC-Houston, and handled in accordance with the principles and procedure of the *Guide for the Care and Use of Laboratory Animals*.

Immunofluorescence and western blot antibodies

Ab2438 was raised in chickens (Aves Labs) against mouse NS. Ab3404 was also raised in chickens against mouse GNL3L. Other primary antibodies include anti-K5 (Covance), K8 (TROMA-I, DSHB), K19 (TROMA-III, DSHB), PCNA (PC10, DSHB), K6 (Covance), and SMA (Sigma). The antibody specificities were confirmed by staining parallel sections or blots of antigen-negative tissues.

Mammary tumor sphere and semisolid cultures

Mammary tumors were harvested from MMTV-*wnt-1*/FVB/NJ female transgenic mice (Jackson Laboratory). Tumor tissues were minced and digested with collagenase and hyaluronidase. After filtering through 40 μ m Nylon mesh, dissociated cells were cultured in DMEM/F12 medium, supplemented with apo-transferrin, selenium, insulin, FGF2 (10ng/ml), and EGF (20ng/ml) in the attached, suspension, or semisolid culture. Fresh FGF2 and EGF were added daily, and medium was changed on a 3-day schedule. MDA-MB231 and MCF7 cells were maintained in DMEM-10%FBS. For sphere culture, dissociated cells were plated in Corning ultra-low attachment plates and incubated at 37C under constant shaking. For semisolid cultures, 200 μ l of 0.6% low melting-point agar in experimental media was plated at the bottom of a 24-well plate. Single cells were resuspended in 0.3% agar in experimental media. Cell/agar mixtures of 5000, 2500, and 1250 cells were plated in each well. Fresh media were changed every three days on top; colony formation was monitored daily and counted on day 12.

Flow cytometry

Dissociated cells were removed of lineage marker (CD5⁺, CD45R⁺, CD11b⁺, Ly-6G/C⁺, 7-4, and Ter-119⁺) positive cells by magnetic bead sorting (Miltenyl Biotec). Lin⁻ cells were resuspended at a concentration of 1×10^6 cells in 100 μ l and labeled with biotinylated anti-CD24 antibody (clone M1/69), PE-conjugated anti CD29 antibody (clone HMB1-1), and APC-conjugated streptavidin (eBioscience). Cell sortings were performed by MoFlo (Beckman Coulter Corporation) equipped with 3 lasers (water cooled UV, air cooled argon, and an air cooled HeNe). Dead cells, debris, and doublets were removed by forward and side scatter gating. The remaining live cell populations were then gated based on the signal intensities of positive and negative control samples. For GFP sorting, the GFP⁺ cells were further divided into three equal parts (GFP^{low}, GFP^{med}, and GFP^{high}).

In vivo tumorigenic assay

GFP-sorted tumor cells were resuspended in 100 μ l of DMEM/F-12 medium at different cell densities and injected into the 4th inguinal mammary glands of 6–8 week-old athymic nude female mice. Tumor formation and growth were monitored twice a week, and the final count was tallied at 7 weeks after the injection. The TIC percentages in the injected cells were calculated by the L-Calc software. Tumor volume was calculated by the formula: $0.52 \times (\text{width})^2 \times \text{length}$.

siRNA knockdown of NS

One day after plating, cells were incubated with siRNA duplexes (100nM, Dharmacon Research, CO) complexed with oligofectamine (Invitrogen) for 24 hours, and then cultured in fresh medium for 48 hours. The sense siRNA sequences are: 5'-GAA CUA AAA CAG CAG CAG AdTdT-3' (siNS) and 5'-UGA CGA UCA GAA UGC GAC UdTdT-3' (siScr).

Results

NS is expressed higher in the basal cell type than in the luminal cell type of mammary tumors

First, the expression of NS in normal mammary glands was analyzed by immunofluorescence. The NS signals were detected in the nucleolus of both keratin 5-positive (K5⁺) basal and K8⁺ luminal cells, but the strongest signals were found in scattered basal cells (arrows) (Fig. 1A). To determine the expression cell type(s) of NS in mammary tumors, MMTV-*wnt-1* tumors were dissociated and triple-stained for NS, K8, and K5 (Fig. 1B2) or NS, K5, and α -smooth muscle actin (SMA) (Fig. 1B3). Tumor cells can be divided into the luminal (K8⁺), basal (K5⁺), or myoepithelial (SMA⁺/K5⁻) types (Fig. 1B1). The NS-high basal, luminal, and myoepithelial cells make up 6.2%, 0.4%, and 1.5% of the total tumor epithelial cells, and the NS-low basal, luminal, and myoepithelial cells make up 15.8%, 56.7%, and 19.2% of the total tumor epithelial cells, respectively (Fig. 1B4). The NS^{high}-to-NS^{low} ratios in the K5⁺, K8⁺, and SMA⁺ groups are 0.404, 0.007, and 0.073, respectively ($p < 0.01$ between any two groups, t-test, $n = 4$). We also sorted MMTV-*wnt-1* tumor cells into 4 subgroups based on their CD24 and CD29 expression. qRT-PCR assays showed that the CD24^{high}CD29⁻, CD24^{med}CD29⁺, and CD24^{high}CD29⁺ populations contain predominantly the K8⁺, SMA⁺, and K5⁺ cells, respectively, and that the NS expression level is about two-fold higher in the SMA and K5-enriched subgroups than in the K8-enriched subgroup (Fig. 1C). Here, we did not observe a difference in the NS transcript levels between the K5⁺ and SMA⁺ groups as we did by immunofluorescence, which compares the signal intensity of NS proteins in the nucleolus. In support of the higher level of NS in basal than in luminal tumor cells, western blots showed that the MMTV-*wnt-1* tumors express higher levels of NS, K5, and SMA and a lower level of K19 than MMTV-

PyMT (polyomavirus middle T antigen) tumors, which contain predominantly luminal cells (Fig. 1D). In human cancer cell lines, the level of NS protein is also significantly higher in the MDA-MB231 basal breast cancer cells than in the MCF7 luminal breast cancer cells (Fig. 1E). Thus, we conclude that the basal subtype of mammary tumor cells expresses more NS than the luminal subtype.

NS expression correlates with MMTV-wnt-1 and MMTV-PyMT tumor progression

To determine the relationship between NS expression and mammary tumor progression, western blotting was performed on samples collected from normal mammary glands (MG) and the small (≤ 0.5 cm in diameter) and large (≥ 1.0 cm in diameter) MMTV-*wnt-1* tumors (Fig. 2A). The luminal (K19⁺) and basal (K5⁺) cell contents increase in the cancerous tissues compared to the normal glands, but differ very little between the small and large tumors. NS is undetectable in normal glands with the amount of protein loaded, but increases dramatically in the large tumors compared to the small tumors, suggesting that its expression is up-regulated as tumors advance. This progression-associated increase of NS expression is relatively specific and not seen in GNL3L. It is also not a by-product of increased cell proliferation, as determined by the amount of PCNA (proliferative cell nuclear antigen). To identify the tumor cell type that expresses NS in the large tumors, double-labeled IF was performed for NS and either K8 or K5. The results showed that the NS signals are more intense in the K5⁺ cells than in the K8⁺ cells (Fig. 2B). Compared to the normal mammary epithelium, a slight increase of NS expression was noted in the hyperplastic mammary epithelium of MMTV-*wnt-1* mice prior to the emergence of tumor foci (Fig. 1S). To strengthen the correlation between NS expression and tumor progression, the NS levels were quantified during tumor progression in the MMTV-*PyMT* transgenic model. MMTV-*PyMT* mice form mammary hyperplasia, adenoma/MIN, and carcinoma at 4, 8, and 12 weeks of age, respectively, which mimics human breast cancer progression (42). Western blotting showed that the epithelial components are similar in the adenoma/MIN isolated from the 8-week-old mice and the carcinoma isolated from the 12-week-old mice (0.7 vs.1.0 for K5 and 0.8 vs.1.0 for K19) (Fig. 2C). More importantly, the NS protein level is 10-fold higher in the carcinoma than in the adenoma/MIN. Immunofluorescence showed that this stage-dependent increase of NS expression in MMTV-*PyMT* mice correlates with an increased number of cells with strong NS signals and weak K8 signals (Fig. 2D). These data demonstrate a positive correlation between NS expression and tumor progression in two different mouse mammary tumor models.

NS-expressing cells are enriched by tumor sphere culture

Increased NS expression during tumor progression suggests that NS-enriched tumor cells may be more tumorigenic and possess more mCSC properties than NS-deficient cells. To investigate this possibility, tumor spheres were grown from dissociated MMTV-*wnt-1* mammary tumor cells, passaged every 8–13 days, and collected from passage 1 (P1) to P3 (Fig. 3A). Western blots showed that P1 tumor spheres expressed significantly higher levels of NS, K5, and K6 (a putative marker of mammary progenitor cells (14,39)) than the P0 primary tumor cells (Fig. 3B). The levels of NS and K5 also increase slightly from the P1 spheres to the P2 spheres. This pattern of increase in NS expression matches the increase of sphere-forming efficiency from the P0 to P1 culture ($p < 0.01$, t-test, $n=3$) (Fig. 3C). By contrast, K19, SMA, and PCNA display the opposite expression pattern. These findings suggest that NS-enriched cells may be better at forming spheres *in vitro* and initiating tumors *in vivo*.

Tumor cells expressing more NS display higher *in vitro* sphere-forming activities and *in vivo* tumorigenicity

Next, we tested whether tumor cells with higher NS expression levels may show stronger tumorigenic activities. To purify live NS-expressing cells, we created a bacterial artificial chromosome (BAC)-based transgenic model, in which the expression of GFP is instructed by the NS promoter. The transgenic construct was built from a BAC clone (RP23-102M6), where GFP was fused in-frame to the start codon of NS, thus placing the expression of GFP under the control of the 224-kb genomic sequence surrounding the NS locus (Fig. 4A). Two transgenic lines were created and verified for their transgene integrity and expression (43); one of which, GFP^{NS} (#14), was bred into the MMTV-*wnt-1* mice. Mammary tumors were harvested from the GFP^{NS} × MMTV-*wnt-1* mice, dissociated, and FACS-sorted based on their GFP expression levels into the NS-negative (NS^{neg}), low (NS^{lo}), medium (NS^{med}), and high (NS^{hi}) groups (Fig. 4B). qRT-PCR assays verified the relative NS expression levels in these four groups of cells, and showed that the NS^{hi} group expresses the highest level of CD61, Thy1, and CD24 (Fig. 4C). This expression profile fits the published results, which used the CD61⁺ or Thy1⁺CD24⁺ sorting paradigm to isolate TIC from MMTV-*wnt-1* tumors (15,18).

To compare their tumorigenic activities *in vitro*, the GFP-sorted cells were plated in suspension culture (1×10^6 cells per well). Only the NS^{med} and NS^{hi} cells exhibit strong activities to form spheres with size larger than 50 μ m in diameter ($p < 0.05$, t-test, $n=9$) (Fig. 4D). The numbers of small-sized spheres ($\leq 50 \mu$ m), which represent the non-self-renewing progenitor cells, are comparable between the NS^{lo}, NS^{med}, and NS^{hi} groups. The NS^{neg} cells did not form even small-sized spheres, suggesting that they lack any progenitor functions. The GFP-sorted cells were also measured for their abilities to form colonies in semisolid medium. In support of the tumor sphere findings, NS^{med} and NS^{hi} cells formed three times more colonies ($> 50 \mu$ m in diameter) in the semisolid culture than NS^{lo} cells did ($p < 0.001$, t-test, $n=9$), and no colony was formed in the NS^{neg} group (Fig. 4E).

To determine the *in vivo* tumorigenic potential of NS-expressing cells, these GFP-sorted mammary tumor cells were transplanted into the 4th inguinal mammary fat pads of nude mice in limiting dilutions (1×10^2 , 1×10^3 , 1×10^4 , and 1×10^5 ; 10 transplanted sites for each cell concentration). The appearance of tumors at the transplanted site was monitored twice a week for up to 7 weeks. Our results showed that the NS^{neg} cells have no tumorigenic activity, and that the TIC frequency of NS^{hi} cells is 4- and 84-fold higher than that of NS^{med} and NS^{lo} cells, respectively, demonstrating that the NS^{hi} cells are highly enriched for TIC (Fig. 5A and S2). Once tumors formed at the transplanted sites, those derived from NS^{hi} cells grew at the same rate (volume double time [T_2]=5.2 days) as the ones derived from NS^{med} cells (T_2 =4.9 days) and NS^{lo} cells (T_2 =6.6 days) (Fig. 5B).

The NS expression shows high degrees of correlation with that of K5, CD133, Oct4, TERT, and CXCL12

To distinguish the molecular identities of NS^{hi} versus NS^{lo} cells, qRT-PCR assays were performed to determine their expressions of genes related to stem cells (*e.g.* CD133, Oct4, TERT [telomerase reverse transcriptase], Sca-1, and Bmi-1) or the malignant progression of breast cancers (*e.g.* CXCL12 [C-X-C chemokine ligand 12], CXCR4 [C-X-C chemokine receptor 4], and MMPs [matrix metalloproteinase]). Since none of these genes is specifically expressed in only one cell group, we measured their expression profiles across all four groups of GFP-sorted mammary tumor cells and calculated their correlation co-efficiencies (r) with NS. Based on the calculation that the significance of correlation coefficient ≥ 0.95 equals p value ≤ 0.05 (two-tailed, $n=4$), we conclude that the expression of NS shares significant correlation with that of K5, CXCL12, CD133, Oct4, and TERT (Fig. 5C).

NS depletion blocks the sphere-forming activity of MDA-MB231 and MCF7 human breast cancer cells

The high tumorigenic activity of NS^{hi} mammary tumor cells suggests that NS may be functionally important for mammary tumor progression. To examine this possibility, we measured the effect of NS knockdown on the tumor sphere-forming activity of MDA-MB231 and MCF7 cells. NS knockdown was introduced by oligofectamine-mediated transfection of a NS-specific siRNA duplex (siNS). Control samples were treated with a siRNA duplex targeting a scrambled sequence (siScr). Two days after the knockdown, these cells were plated in suspension culture and allowed to grow for 12 days. The siNS treatment led to more than 90% knockdown of the NS protein in MDA-MB231 cells and 75% knockdown of NS proteins in MCF7 cells (Fig. 6A). It also caused a significant decrease of K5 in MDA-MB231 cells. Importantly, NS knockdown dramatically reduced the ability of MDA-MB231 to form tumor spheres – only 2.8 spheres were detected from 10⁴ siNS-treated cells plated, while 180 spheres were found in the siScr-treated group ($p < 0.001$, t-test, $n=18$) (Fig. 6B, C). Even though the luminal MCF7 cells expressed only low levels of NS and formed much fewer spheres compared to MDA-MB231 cells, NS depletion still significantly decreased their sphere-forming activity ($p < 0.001$, t-test, $n=12$), suggesting that NS may be important for the tumor-initiating potential of a wide range of breast cancers.

Discussion

Mammary TIC express high levels of NS

Several pieces of evidence indicate that TIC express more NS than non-tumor-initiating cells in mammary tumors. First, the expression level of NS is higher in the basal subtype than in the luminal subtype of human and mouse mammary tumor cells, and correlates with the progression of mammary tumors in MMTV-*wnt-1* and MMTV-*PyMT* mice. Although consistent with its basal cell expression, the differential expression level of NS in MDA-MB231 versus MCF7 cells may also result from other known differences between these two cell lines, including their hormone status and genetic makeup. Second, we show that the NS level is significantly increased from the P0 to P1 sphere culture, which parallels the increase of sphere-forming activity from P0 to P1. Most importantly, NS-enriched tumor cells display stronger tumorigenic capacities *in vitro* and contain more TIC *in vivo* compared to NS-deficient mammary tumor cells. TIC is 80-fold enriched in the NS^{hi} population compared to the NS^{lo} population. This enrichment scale is better than the combinatorial use of several markers to enrich for TIC in mouse models of breast cancer. For example, in tumors arising in MMTV-*wnt1* mice, selection for Lin⁻/CD24⁺/Thy1⁺ enriches for TIC by 49-fold (15), and Lin⁻/CD29^{lo}/CD24⁺/CD61⁺ enriches for TIC by 20-fold (18). In the *p53*-null model of breast cancer, Lin⁻/CD24⁺/CD29⁺ markers enrich for TIC by 71-fold (16). In human breast cancer samples, selection for Lin⁻/CD44⁺/CD24^{-low} enriches for TIC by 10- to 50-fold (3). Therefore, NS represents a new protein whose expression level can effectively differentiate between TIC and their differentiated progeny.

The tumor-initiating function of NS-enriched cells

Our results demonstrate that NS-enriched tumor cells display stronger tumorigenic activities *in vitro*, and are able to generate significantly more secondary tumors after limiting dilution transplantation than NS-deficient tumor cells. The relative TIC frequency of NS^{hi} cells is 4- and 84-fold higher than that of NS^{med} and NS^{lo} cells, respectively. The absolute TIC frequency of NS^{hi} cells is only 1 in 6,006, which may be due to the physical damage to the cells during the FACS procedure and the cellular heterogeneity of the NS^{hi} group, as seen with other sorting paradigms as well. GFP^{NS} mammary tumors clearly contain two distinct populations, *i.e.* the GFP⁺ and GFP⁻ cells. According to their marker expression profiles

(Fig. 5C), these two groups may represent the epithelial and non-epithelial components of the tumors, respectively. Within the GFP⁺ group, signals are distributed in continuum, indicating that the differences in NS expression between different subsets of epithelial tumor cells are relative rather than absolute and that the TIC frequency of NS^{hi} cells may be further improved by a higher gating threshold. However, once formed, the transplanted tumors derived from the NS^{hi} cells display the same growth rate as those derived from the NS^{med} and NS^{lo} cells. A direct involvement of NS in mammary tumor progression is shown by the knockdown experiments, which demonstrate that NS depletion significantly reduces the sphere-forming activity of human breast cancer cells. Based on these findings, we conclude that NS and NS-enriched mammary tumor cells are functionally involved in mammary tumor progression and possibly recurrence.

Molecular identity of NS-enriched mammary tumor cells

To elucidate the molecular property of NS-enriched mammary tumor cells and to gain insight on the pathways co-regulated with NS, we analyzed the expression profiles of the four GFP-sorted tumor cells, and showed that the NS expression correlates significantly with the expressions of K5, CXCL12, CD133, Oct4, and TERT ($p < 0.05$). Since the probability of two different cell types cosegregating in all four GFP-sorted groups is low, we conclude that tumor cells expressing more NS also express more K5, CXCL12, CD133, Oct4, and TERT. The CXCL12 cytokine is the ligand for CXCR4 that plays a role in mediating the *in vitro* invasive property and *in vivo* metastatic property of mammary tumors. TERT mediates telomere lengthening and is highly expressed by the majority of human cancer cells to maintain their telomere integrity. CD133 and Oct4 are neural stem cell and ES markers that have been used to enrich for mCSC as well (19,20). It should be noted that in normal mammary glands, the expressions of CD133, Sca-1, and Bmi-1 are not restricted to the stem cell compartment. CD133 and Sca-1 are also expressed by the ER⁺ luminal cells in normal mammary glands (44,45). On the other hand, even though Bmi-1 promotes stem and progenitor cell expansion, it is commonly expressed in all mammary cells (46,47). These differences may highlight the molecular distinction between mCSCs and normal mammary stem cells.

In conclusion, NS is preferentially expressed by the basal subtype of mammary tumor cells and mCSC. NS-enriched mammary tumor cells express more CSC-related genes and show higher tumorigenic activities compared to NS-deficient tumor cells. In addition, NS is required for the sphere-forming activity of human breast cancer cells. Therefore, NS may be a potential target for CSC-based breast cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. NS is expressed at higher levels by the basal subtype than by the luminal subtype of mammary tumor cells

(A) Normal mouse mammary glands were co-stained for NS and K8. Dashed lines demarcate the boundary between the basal and luminal compartments. Arrows point to the strong NS-positive basal cells. The rectangle indicates an enlarged region shown on the right. Bars, 40 μ m (left) and 8 μ m (right). (B1) Dissociated MMTV-*wnt-1* mammary tumors consist of K8⁺ luminal (L), K5⁺ basal (B), and SMA⁺ (smooth muscle actin)/K5⁻ myoepithelial (S) cell types. (B2-B3) The most intense NS signals (NS^{hi}) are found mainly in the K5⁺ basal cells, whereas most of the NS^{low} (NS^{lo}) cells are luminal cells. Bars, 15 μ m. (B4) The NS^{hi}-to-NS^{lo} cell ratio (NS^{hi/lo}) is highest in the K5⁺ cells, followed by the SMA⁺ and K8⁺ cells ($p < 0.01$ between any two groups, $n = 4$, t-test). (C) CD29/CD24 sorting separated MMTV-*wnt-1* tumor cells into 4 distinct subpopulations. qRT-PCR assays demonstrated that the CD24^{high}CD29⁻ (R2), CD24^{med}CD29⁺ (R3), and CD24^{high}CD29⁺ (R4) subgroups contain predominantly the K8⁺, SMA⁺, and K5⁺ cells, respectively, and that the NS expression level is about two-fold higher in the SMA⁺ and K5⁺ subgroups than in the K8⁺ subgroup. Bars, mean $\pm$ s.e.m. ($n = 6$); **, $p < 0.001$; ***, $p < 0.0001$. (D) Western

comparison between MMTV-*wnt-1* and MMTV-*PyMT* mammary tumors (2 samples each) showed that NS expression correlates with the levels of K5 and SMA and is opposite to that of K8. (E) The basal type human cancer cells (MDA-MB231) express more NS than the luminal type human cancer cells (MCF7). Tub, α -tubulin.

Figure 2. The NS expression is increased during the progression of mammary tumors in MMTV-*wnt-1* and MMTV-*PyMT* mice

(A) Western analyses of NS expression and cell type composition in virgin mammary glands (MG), and small (≤ 0.5 cm) and large (≥ 1.0 cm) MMTV-*wnt-1* mammary tumors. PCNA, proliferating cell nuclear antigen. (B) Large MMTV-*wnt-1* mammary tumors were co-stained for NS and K8 (B1) or NS and K5 (B2). Strong NS signals were found more in the K5⁺ cells than in the K8⁺ cells. Right panels represent enlarged areas indicated by rectangles in the left panels. Dashed lines show the boundary between the basal and luminal cells. Scale bars: 40 μ m (left) and 10 μ m (right). (C) Western analyses of NS expression levels in MMTV-*PyMT* mammary lesions collected at 4, 8, and 12 weeks of age. Compared to the 8-week tumors, the 12-week tumors express significantly more NS but similar amounts of epithelial components (K5 and K19). (D) NS/K8 co-staining of MMTV-*PyMT* mammary tumors showed a time-dependent increase of cells with strong NS and weak K8 signals. Insets, enlarged views of regions indicated by rectangles. Bars, 40 μ m.

Figure 3. NS-expressing cells are enriched by tumor sphere culture

(A) Tumor spheres were cultured from MMTV-*wnt-1* mammary tumors. Phase-contrast images were taken on the 10th day of each passage. Bar, 50μm. (B) Primary tumor spheres (P1) express significantly more NS, K5, and K6 and less K19, SMA, and PCNA compared to the primary tumor (P0). The increase of NS and K5 levels moderates after P1. (C) The expression patterns of NS and K5 match the significant increase of sphere-forming efficiency from the P0 to P1 culture ($p < 0.01$, t-test, n=3).

Figure 4. NS-enriched mammary tumor cells show higher tumorigenic activity *in vitro*
(A) A schematic diagram of the original RP23-102M6 BAC containing the NS locus (top), the modified BAC transgene containing a GFP-Kan cassette fused in-frame to the start codon of NS, and the final BAC transgene without the Kan cassette. **(B)** (Left panel) Dissociated cells were collected from mammary tumors of the $GFP^{NS} \times MMTV-wnt-1$ mice, removed of dead cells, debris, and doublets (31.7%), and sorted based on their GFP levels into negative (NS^{neg}), low (NS^{lo}), medium (NS^{med}), and high (NS^{hi}) groups. (Right panel) The GFP-negative control plot. **(C)** qRT-PCR assays confirmed the relative expression levels of NS in these four sorted groups and showed that NS^{hi} cells express significantly higher levels of CD61 and Thy1 and a moderate increased CD24 compared to the other groups. **(D)** The NS^{med} and NS^{hi} groups display significantly higher sphere-forming activities than the NS^{neg} and NS^{lo} groups. Y-axis marks the number of tumor spheres generated from 1×10^6 plated cells. Black bars represent spheres $>50\mu m$ in diameter. Grey bars show spheres of $20-50\mu m$ in diameter. **(E)** The four GFP-sorted cell groups were cultured in semisolid medium. The number of isolated colonies ($>50\mu m$) in the NS^{hi} and NS^{med} groups are three times higher than that in the NS^{lo} group ($n=9$). Bars, $100\mu m$; bar graphs, means $\pm$ s.e.m.

Figure 5. NS-enriched mammary tumor cells display higher tumor-initiating activities *in vivo* and express several stem cell-related genes at higher levels than do NS-deficient mammary tumor cells

(A) The *in vivo* tumor-initiating activities of GFP-sorted MMTV-*wnt-1* mammary tumors were examined by the limiting dilution transplantation experiment. Data represent the tumor incidence (numerator) recorded at 7 weeks after the transplantation from ten transplanted events (denominator) performed for each category. Freq. TICs indicates estimated frequencies of tumor-initiating cells. (B) Tumors derived from the NS^{hi}, NS^{med}, and NS^{lo} cells show similar growth rates. X-axis, time after the initial tumor detection; Y-axis, the average tumor volume. The tumor volume first detected in each animal is set as 1 at time point 0. The NS^{lo} group developed only one tumor, which appeared two weeks later than those in the NS^{med} and NS^{hi} groups. Error bars represent s.e.m. (C) qRT-PCR measurements of stem cell and breast cancer-related genes in GFP-sorted MMTV-*wnt-1* mammary tumor cells. The NS expression shows a significant correlation with that of K5, CXCL12, CD133, Oct4, and TERT ($p < 0.05$, $n = 6$). Bars, mean $\pm$ s.e.m. The correlation coefficients (r) of each gene compared with NS are shown in parentheses.

Figure 6. NS depletion significantly reduces the sphere-forming activities of MDA-MB231 and MCF7 cells

(A) MDA-MB231 and MCF7 cells were treated with control (siScr) or NS-specific (siNS) siRNA duplexes. Western blots show a > 90% and a 75% knockdown efficiency of NS protein in the siNS-treated MDA-MB231 and MCF7 cells, respectively. (B) The sphere-forming activities of siScr and siNS-treated cells were determined after 12 days of culture. MDA-MB231 and MCF7 cells were plated at 2500 and 2×10^4 cells per well (24-well plate), respectively. Bars, 300 μ m. (C) The sphere-forming frequency (>50 μ m spheres per 10^4 plated cells) of both MDA-MB231 and MCF7 cells are significantly reduced by NS knockdown. Bars, mean $\pm$ s.e.m.