RESEARCH ARTICLE

In vitro Study of Nucleostemin as a Potential Therapeutic Target in Human Breast Carcinoma SKBR-3 Cells

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Abstract

Although nucleolar protein nucleostemin (NS) is essential for cell proliferation and early embryogenesis and expression has been observed in some types of human cancer and stem cells, the molecular mechanisms involved in mediation of cell proliferation and cell cycling remains largely elusive. The aim of the present study was to evaluate NS as a potential target for gene therapy of human breast carcinoma by investigating NS gene expression and its effects on SKBR-3 cell proliferation and apoptosis. NS mRNA and protein were both found to be highly expressed in all detected cancer cell lines. The apoptotic rate of the pcDNA3.1-NS-Silencer group (12.1-15.4±3.8%) was significantly higher than those of pcDNA3.1-NS (7.2-12.0±1.7%) and non-transfection groups (4.1-6.5±1.8%, $P<0.01$). MTT assays showed the knockdown of NS expression reduced the proliferation rate of SKBR-3 cells significantly. Matrigel invasion and wound healing assays indicated that the number of invading cells was significantly decreased in the pcDNA3.1-NS-siRNA group ($P<0.01$), but there were no significant difference between non-transfected and over-expression groups ($P>0.05$). Moreover, RNAi-mediated NS down-regulation induced SKBR-3 cell G1 phase arrest, inhibited cell proliferation, and promoted p53 pathway-mediated cell apoptosis in SKBR-3 cells. NS might thus be an important regulator in the G2/M check point of cell cycle, blocking SKBR-3 cell progression through the G1/S phase. On the whole, these results suggest NS might be a tumor suppressor and important therapeutic target in human cancers.

Keywords: Nucleostemin - RNA interference - SKBR-3 cells - proliferation - apoptosis

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Introduction

Nucleostemin (NS) was initially described by Tsai & Makay in 2002, located at 3p21.1, which is highly expressed in embryonic stem cells, neural stem cells, hematopoietic primitive cells and tumor cell lines, but not in differentiated progeny, and down-regulated during differentiation (Tsai et al., 2002; Fan et al., 2006). NS possesses two putative GTP-binding motifs, and has been proposed as a marker for an undifferentiated or dedifferentiating state and for TICs in highly aggressive brain tumors (Kafienah et al., 2006; Tamase et al., 2009). The level of NS decreases drastically prior to cell cycle exit upon differentiation of the stem cells, suggesting that this protein may be an imperative protein for regulating the proliferation of stem cells and cancer cells, and in maintenance of their undifferentiated features (Jafarnejad et al., 2008; Nomura et al., 2009; Gao et al., 2012).

Several nucleolar proteins, such as ARF, ribosomal protein (RP), have been shown to induce p53 activation by inhibiting MDM2 activity and consequently to trigger cell cycle arrest and/or apoptosis (Zhu et al., 2006; Meng et al., 2008; Romanova et al., 2009). Specifically, recent studies have shown that expression of NS delays the onset of cellular senescence by regulating telomere length by promoting the degradation of telomeric repeat-binding factor 1 (TRF1) and that depletion of NS causes G1 arrest in a p53-dependent manner (Tsai 2009; Zhu et al., 2009; Gil-Ranedo et al., 2011). Hence, maintaining a fine balance of NS levels is essential for cellular homeostasis.

However, it is unclear how the reduction of NS levels brings about cell cycle arrest. Our study investigated the expression of NS in 5 cancer cell lines and normal liver cells. Furthermore, we utilized RNA interference (RNAi) technique to knockdown the expression of NS in SKRB-3 cells, and in this way we could learn the changes of proliferation and tumorigenesis. On this basis, the feasibility of NS served as a potential molecular target for anti-cancer drug development was evaluated.

Materials and Methods

Cell Culture

Human cervical cancer (Hela) cells, human breast carcinoma (SKBR-3) cells, human breast cancer (T47-D) cells, human hepatocarcinoma (HepG-2) cells, human

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melanoma (A375) cells were bought from American Type Culture Collection (Rockville, MD). Normal human liver cells were obtained from Chinese Academy of Agricultural Science and cultured in the current laboratory. Cells were cultured in DMEM or RPMI 1640 containing 10% FBS at 37°C, in 5% CO_2 (Sakamoto et al., 2013).

RNA Extraction, RT-PCR and Western blot analysis

Total RNAs from all five cancer cells and normal human liver cells were extracted and reversibly transcribed into cDNA using oligo dT18 primer and M-MLV Reverse Transcriptase (Promega, USA). RT products were then used as templates to examine the differential expression of NS using PCR. Cells were lysed using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, China). Proteins were separated by 12% SDS-PAGE gel electrophoresis and electro-blotted to PVDF membranes (Millipore, USA) using a semi-dry blotting apparatus. Membranes were incubated with anti-NS (1:500) or anti-β-actin (1:500) primary antibody (Santa Cruz, USA), and incubated with rabbit or mouse HRP-conjugated secondary antibodies (Zhong et al., 2013). Blots ware visualized with the enhanced chemiluminescent method (ECL, Beyotime, China), and analyzed using a gel image analysis system (Bio-rad Geldoc XR, USA).

Construction of siRNA and over-expression vector

U6 snRNA promoter of mouse was amplified by PCR, U6F: 5'-GATTTCGACGCCGCCATCTCTAGG (EcoR I site underlined) and U6R:5'-AAACAAGGCTTTTCTCCA AGGG, and ligased with pGEM-T vector (Pormega, USA). Furthermore, pGEM-T-U6-promoter was used as template to construct the frame of three NS-siRNA coding sequences by PCR, U6F and NS-siRNA1 primer (5'-AGATCTAAAAAAGAAGCCTAGGAAAGACCCA GGATCTCTTGAATCCTGGGTCTTTCCTAGGCGTCA AACAAGGCGTTTCTCCAAGGG) (Figure 2). And PCR product was digested by EcoR I and Bgl II, and then the cutout fragment was inserted into the pcDNA3.1 (+) vector, named pcDNA3.1-NS-siRNA1.

The specific PCR primers sequences amplifying full-length NS cDNA (AY825265) were NS-F3: 5'-CCAAGCTTCGATGAAAAAGCCTAAGTTA-3' (Hind III site underlined), NS-R3 5'-CGGGATCCTTACAC ATAATCTGTACTGA-3' (BamH I site underlined) . PCR product was inserted into the pcDNA3.1 (+)vector, named pcDNA3.1-NS.

Cell Transfection and Screening of Cell Clones

Large quantities of pcDNA3.1-NS-siRNA1, NS-siRNA2, NS-siRNA3 and pcDNA3.1-NS vectors were produced using a plasmid isolation kit (Omega Corporation). After linearization, the expression vectors were respectively transfected into SKBR-3 cells using Lipofectamine™ 2000 (Invitrogen Corp., Carlsbad, CA, USA). SKBR-3 cells were harvested at 3 days after transfection, respectively. Stable transfectants were obtained by screening G418 resistance and monoclonic culture for 1 month. After further expanding culture, we isolated total RNA from these cells, and evaluated NS expression and related genes differentially expression by RT-PCR.

Growth curve and Cell Proliferation Assays

Cells (1.5×10^4/well) of the pcDNA3.1-NS-siRNA1, pcDNA3.1-NS and non-transfection were respectively inoculated into 24- well cluster plates in triplicate. After culturing for 7 days, three wells were monitored and recorded on a daily basis for density until they reached the plateau phase. Finally, we drew growth curves for each group (Gu et al., 2006). Cell proliferation was evaluated by 3- (4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Each experiment was performed in six replicate wells and independently repeated three times, and absorbance values were normalized to media control.

Cells apoptosis and Cell cycle Assays

Cells apoptosis of NS-siRNA1, pcDNA3.1-NS and non-transfection was evaluated using AO/EB method, Annexin V/FITC kit (Beyotime, Jiangsu, China), respectively. Morphological alterations of apoptosis from different groups were observed by confocal microscopy (Nikon TE-2000-E, Tokyo, Japan) with the excitation wavelengths of 405 nm, 488 and 543 nm. The determination of cells in G0/G1, S and G2 phase was carried out by Flow Cytometry (Cytomics FC 500, Beckman Coulter, USA).

Transwell Migration, Invasion and Wound Healing Assays

The migration of breast cancer cells and their subclones was performed using a 24-well transwell chamber (Corning) containing gelatin-coated polycarbonate membrane filter (6.5 mm diameter, 8 μm pore size), as described previously (Kano et al., 2010; Ma et al., 2013). The invasion of the cancer cells were performed by the same procedure as in the migration assay except that the chamber filter were coated with matrigel (BD Biosciences) and 5×10^5 cells were seeded into the upper chamber. 10^5 cells were added to each well of 12-well plate and cultured at 37 °C in 5% CO_2 until more than 80% confluent. They were then scratched with a standard 200 μl pipette tip, wounded monolayers were washed twice to remove non-adherent cells and images were captured at 0 h, 24 h and 48 h after wounding using a Fluorescent microscope.

Results

NS mRNA and Protein differentially expression in Tumor cells

Under the culture condition *in vitro*, cell morphology showed that five cancer cell lines and normal human liver cell grow well, and more in logarithmic phase (Figure 1B). In terms of the results of RT-PCR and Western blot, highly levels of NS mRNA and protein in all five tumor cell lines could be detected. SKRB-3 cell, T47-D cells and HepG-2 had the highest levels of NS expression (Figure 1C). Relative expression rates of NS/GAPDH mRNA in five tumor cell lines were 19.8±4.6%, 38.2±3.0%, 44.4±6.2%, 64.0±12.1%, 36.3±3.6, respectively. Relative expression rates of NS/β-actin protein were 18.2±6.2%, 28.8±4.4%, 27.3±4.8%, 19.6±7.2%, 16.1±7.0%, respectively (Figure 1A). However, no NS expression could be found in normal human liver cells.

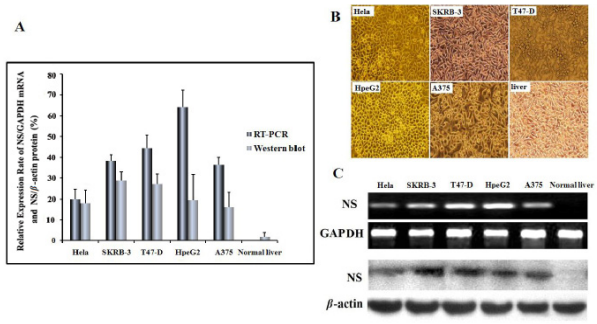

Figure 1. Relative Expression Levels of NS mRNA and Protein in Five Tumor Cell Lines and Normal Human Liver Cell. (A) NS relative expression rates in six cell lines using RT-PCR and Western blot; (B) Culture and Morphology of six cell lines; (C) Relative expression rates of NS/GAPDH mRNA and NS/β-actin protein

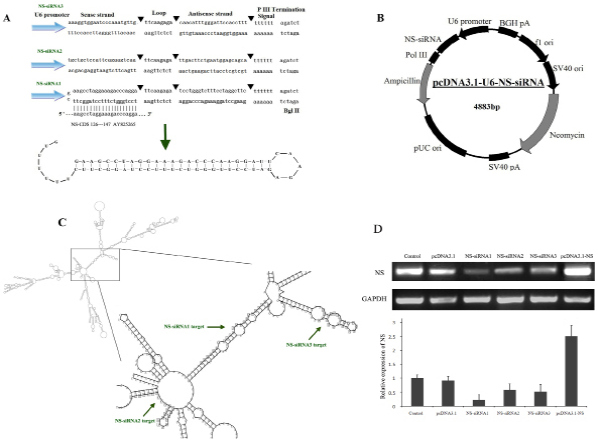

Figure 2. Construction of pcDNA3.1-NS-Silencer Vector and Relative Expression Levels after Transfection of pcDNA3.1-siRNA and pcDNA3.1-NS. (A)The coding sequence of NS-siRNA; (B) pcDNA3.1-NS-Silencer vector cycle map; (C) NS mRNA secondary structure at 37°C was calculated to analyze mRNA target structural effects on RNAi efficiency; (D) Relative expression levels after transfection of pcDNA3.1-siRNA and pcDNA3.1-NS

Up-regulation and down-regulation of NS in SKBR-3 cells

The NS-siRNA expression frame included three parts, the U6 promoter, coding sequences of NS-siRNA and RNA Pol III termination signal. The coding sequence of NS-siRNA also had three parts, the sense strand (22 bp), the loop (9 bp) and antisense strand (22 bp) (Figure 2A, B). The pcDNA3.1-NS-siRNA target regions in NS mRNA secondary structure are shown in Figure 2C. Individual siRNA efficiency in inducing NS silencing in SKBR-3 cells was tested in transiently transfected cells. Out of the three siRNA constructs, siRNA1 presented the highest efficiency in silencing NS expression in SKBR-3 cells, with a reduction of approximately 77% at Day 3 after transfection compared with the control, whereas in the other two, although significantly high, these were less than 60% (Figure 2D). Moreover, the level of NS mRNA increased up to 2.4–2.8 folds in pcDNA3.1-NS groups. These results demonstrated that transient transfection of pcDNA3.1-NS effectively up-regulated NS expression, while pcDNA3.1-NS-siRNA1 obviously down-regulated NS expression in SKBR-3 cells. Thus, siRNA1 was chosen for flowing establishment of NS-knockdown SKBR-3 cell lines.

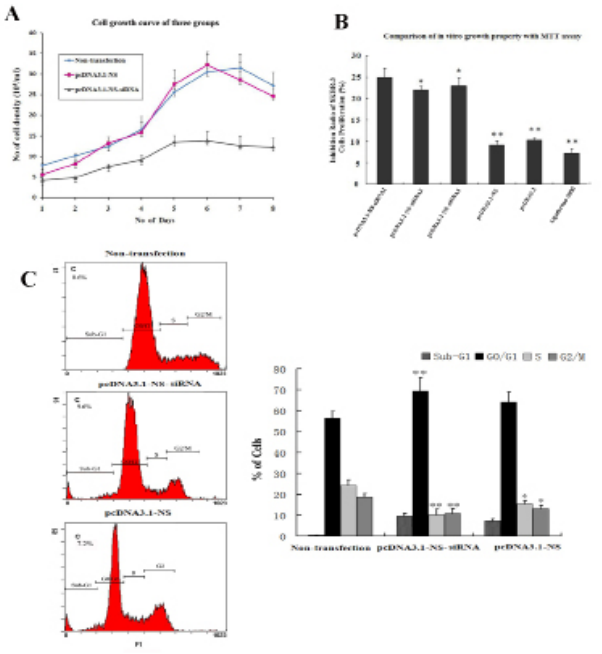

Figure 3. Growth Curve and Changes of Cell Cycle of SKBR-3 Cells Transfected with pcDNA3.1-NS-siRNA1. (A) Cell growth curve of three groups; (B)Comparison of *in vitro* growth property with MTT assay at 3 day;(C) The change of Cell Cycle of SKBR-3 cells transfected with Non-transfection, pcDNA3.1-NS-siRNA1and pcDNA3.1-NS; Relative rate of three groups. *P<0.05, ** P<0.01

NS silencing inhibited Cell Cycle and Cell proliferation

The MTT assay and cell growth curves revealed significant cell growth inhibition in pcDNA3.1-NS-siRNA1 transfectants, showing a 44.8%-60.5% decrease of non-transfected group in the number of total cells at 2 day to 8 day (Figure 3A, B). Cell proliferation between non-transfected and over-expression group were similarly unaffected. Transfection of with pcDNA3.1-NS for 3 days decreased proportion of cells in S phase from 24.3 to 15.4%, and in G2/M phase from 18.6% to 13.1 (P<0.05), while increased cells in G1 phase from 56.5 to 64.3% (P<0.05), when compared to that of controls (Figure 3C). RNAi directed against NS significantly inhibited cell growth, showing a 20–60% decrease in the number of total cells recovered from cell culture (P<0.01). Transfection of SKBR-3 cells with pcDNA3.1-NS-siRNA1 for 3 days had decreased proportions of cells in S phase from 24.3 to 10.1% (P<0.01) and in G2/M phase from 18.6 to 10.9% (P<0.05), and increased proportion of cells in G1 phase from 56.5 to 69.4% (P<0.01) when compared to controls (Figure 3C).

Effect of NS knockdown and over-expression on Cells apoptosis of SKBR-3 cells

Morphological alterations and apoptosis rates of different groups were observed by confocal microscopy and flow cytometry. The results showed in Figure 4, the apoptotic rate of the pcDNA3.1-NS-Silencer group (12.1-15.4±3.8%) was significantly higher than those

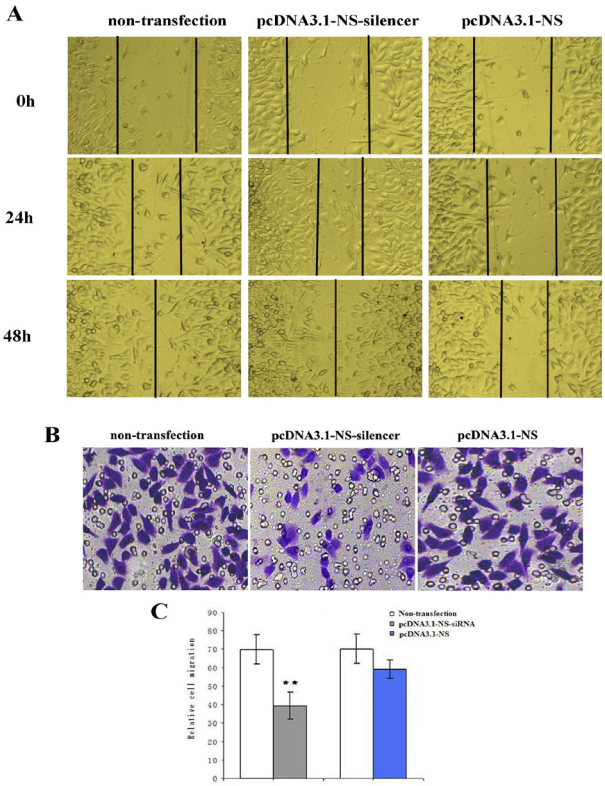

Figure 4. The Migration and Invasion Capacity of Three Groups were Measured by Wound Healing Assay and Matrigel Invasion Assay. (A) The migration capacity of three groups was measured by wound healing assay. (B) Cell invasion was evaluated using the Matrigel invasion assay 48h after treatment; (C) Quantification of cell invasion. The data are presented as relative cell invasion numbers and are representative of three independent experiments (*P<0.05, **P<0.01)

of pcDNA3.1-NS and non-transfection group (P < 0.01), and non-transfection group was minimum (4.1-6.5±1.8%). Moreover, apoptotic rate of pcDNA3.1-NS over-expressed group (7.2-12.0±1.7%) obviously higher than non-transfection group (P < 0.05).

Inhibition of NS Generation Decreased Cell Motility and Invasion

The transwell migration and invasion assay showed that the number of invading cells was significantly decreased in pcDNA3.1-NS-siRNA group (P<0.01, Control, 69.2±7.9; pcDNA3.1-NS-siRNA, 39.5±7.6), but there were no significant difference between non-transfected and over-expression group (P>0.01, pcDNA3.1-NS, 61.5±4.7, Figure 5B). In agreement with the Transwell assay, the wound healing assay showed significantly delayed the closure of the knockdown group compared to non-transfected ells after scratch assay (Figure 5A).

Discussion

Tsai and McKay (2002) had identified NS, a novel p53-binding protein that was abundantly present in stem cells and stem cell-enriched tissues (Kafienah et al., 2006; Tamase et al., 2009). Interestingly, both NS mRNA and protein are absent in adult differentiated cells/tissues, although highly expressed in different human cancer cell

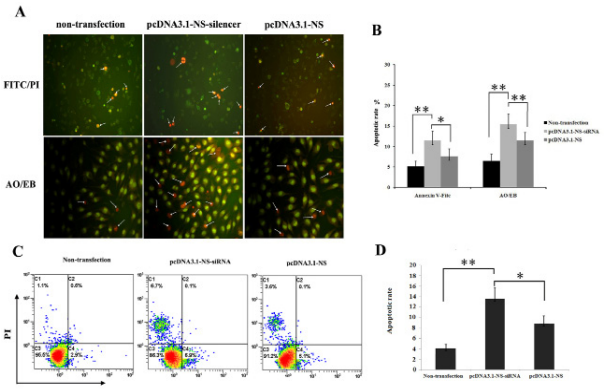

Figure 5. Effect of NS Knockdown or Over-expression on Cell Apoptosis in SKBR-3 Cell. (A) Comparison of cell apoptosis rates of pcDNA3.1-NS-silencer, non-transfection and pcDNA3.1-NS group using Annexin V-FITC/PI and AO/EB combined stain, respectively; (B) Apoptosis rates of three experimental groups were detected by confocal microscopy; (C) Cell apoptosis of three experimental groups were determined using AnnexinV-FITC/PI by flow cytometry; (D) Apoptosis rates of three experimental groups were detected by flow cytometry. *P<0.05, ** P<0.01

lines. Many studies suggested that NS may have similar functions in stem cells and tumor cells, such as self-renewal, proliferation and differentiation regulation, and therefore may be important in tumor genesis, development and metastasis. Thus, it is thought that NS protein may be marker of stem cells and cancer cells. When stem cells were in multi-potential stage, NS had high expression levels, but its expression vanished completely and abruptly when stem cells began to differentiate. From the results of RT-PCR and western blot analysis, we found that all these detected cancer cell lines in our program had high levels of NS expression, which indicated NS played a really important role in the self-renewal of these cancer cells. We demonstrate and identified NS can have an oncogenic function and can act as a tumor suppressor through the direct control of NS expression in breast cancer cell lines.

Our study demonstrated that RNAi directed against NS significantly inhibited cell growth. Transfection of SKBR-3 cells with pcDNA3.1-NS-siRNA1 for 3 days had decreased proportions of cells in S phase from 24.3 to 10.1% and in G2/M phase from 18.6 to 10.9%, and increased proportion of cells in G1 phase from 56.5 to 69.4%. The apoptotic rate of the pcDNA3.1-NS-siRNA group was significantly higher than those of pcDNA3.1-NS and non-transfection group. The Matrigel invasion assay showed that the number of invading cells was significantly decreased in pcDNA3.1-NS-siRNA group. These results indicated that the RNAi-mediated NS down-regulation induced SKBR-3 cells G1 phase arrest, inhibited cell growth, and promoted the p53 pathway cell apoptosis. NS might be an important regulator in the G2/M check point of cell cycle, which blocked SKBR-3 cells to pass through G1/S phase.

However, unlike other known nucleolar regulators, NS surprisingly plays a dual role, as both aberrant over-expression and depletion of NS inhibit cell proliferation (Schon et al., 2002). In this study, we showed that aberrantly low levels of NS induce G1 arrest and reduce

cell proliferation rates in agreement with previous report. However, there was no significant difference about the cell proliferation between non-transfected and over-expression groups. Therefore, NS just as c-Myc is more like a proliferation marker rather than a molecule only involved in the cell cycle control of stem and cancer cells. From expression and functional points of view, c-Myc and NS share certain similarities: (1) both are abundantly present in stem cells and down-regulated in differentiated cells and (2) their expression levels should be within a limited window, and both depletion and over-expression lead to cell growth arrest and/or apoptosis.

In conclusion, NS mRNA and protein were both high level expressed in all detected cancer cell lines. RNAi-mediated NS silencing induced SKBR-3 cells G1 phase arrest, inhibited cell growth and cell invasion, and promoted the p53 pathway cell apoptosis in SKBR-3 cells. NS might be an important regulator in the G2/M check point of cell cycle, which blocked SKBR-3 cells to pass through G1/S phase. We suggested NS can be used as tumor suppressor and important therapeutic target in human cancers.

Acknowledgements

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