RESEARCH COMMUNICATION

siRNA Mediated Silencing of NIN1/RPN12 Binding Protein 1 Homolog Inhibits Proliferation and Growth of Breast Cancer Cells

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Abstract

The gene encoding the Nin one binding (NOB1) protein which plays an essential role in protein degradation has been investigated for possible tumor promoting functions. The present study was focused on NOB1 as a possible therapeutic target for breast cancer treatment. Lentivirus mediated NOB1 siRNA transfection was used to silence the NOB1 gene in two established breast cancer cell lines, MCF-7 and MDA-MB-231, successful transfection being confirmed by fluorescence imaging. NOB1 deletion caused significant decline in cell proliferation was observed in both cell lines as investigated by MTT assay. Furthermore the number and size of the colonies formed were also significantly reduced in the absence of NOB1. Moreover NOB1 gene knockdown arrested the cell cycle and inhibited cell cycle related protein expression. Collectively these results indicate that NOB1 plays an essential role in breast cancer cell proliferation and its gene expression could be a therapeutic target.

Keywords: Breast cancer - NOB1 - proliferation - colony formation - siRNA

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Introduction

Breast cancer is one of the leading causes of death among women worldwide. Therefore, identification of effective treatments to prevent breast cancer is highly researched. As breast cancers are tend to arise due to genetic alterations in cells which will lead to uncontrolled growth and survival advantage (Hanahan, 2000). Therefore, it could be suggested that the regulation of genes involved in breast cancer progression would be a promising target for breast cancer therapy. In that regard RNA interference (RNAi) mediated cancer therapy has been identified as a novel and effective method of treatment compared to conventional methods (Mello, 2004). In the last few years, small interference RNA (siRNA) has gained more attention as a more specific and efficient approach of cancer therapy. The function of siRNA mediated cancer therapy is to down regulate the mutant cancer relevant transcripts and restoring wild-type function in heterozygous cancer cell models (Størvold, 2006). For this therapy identification of genes which have a significant role in cancer development and progression is of utmost importance.

In malignant the ubiquitin proteasome pathway of protein degradation which plays a critical role in gene transcription, signal transduction and modulation of protein turnover in cell cycle is mostly mutated (Hershko, 1998; Ferrell, 2000). This mutation directly leads to cancer development and malignancy. Nin one binding protein (NOB1p), encoded by the NOB1 gene plays a crucial role in protein degradation pathway as it binds together the 20S proteasome with the 19S regulatory particle in the nucleus to facilitate the maturation of the 20S proteasome (Tone, 2002). Recently it has been found that the down regulation of over expressed NOB1 in ovarian cancer tissues lead to the suppressed cancer cell survival. Therefore, it was suggested that NOB1 may act as an oncogenic factor and it could be used as a therapeutic marker (Lin, 2012). In the light of these previous experiments we hypothesized that highly malignant breast cancer cells may have higher expression levels of this oncogenic gene NOB1 and silencing this gene would be effective for the regulation of breast cancer cell progression. To test this hypothesis highly malignant breast cancer cell models were used and tested for the expression levels of NOB1 and lentivirus mediated transfection of NOB1 siRNA was used as an approach to silence the NOB1 gene. Then the effect of silencing NOB1 on the breast cancer cell proliferation and survival was analyzed.

Materials and Methods

Reagents and plasmids

AgeI, EcoRI, and SYBR Green Master Mix Kits were purchased from TaKaRa (Dalian, China). pVSVG, pFH1UGW, pCMV◢R 8.92 plasmids were purchased from Hollybio (Shanghai, China). RNeasy Midi Kit was from Qiagen (Valencia, CA, USA). Dulbecco's

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modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Cambrex, MD, USA). Lipofectamine 2000, TRIzol and Super ScriptII reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA). The antibodies used were as follows: anti-NOB1 (1:1,000 dilution; Abcam, ab87151); anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, 1:2,000 dilution; Santa Cruz Biotechnology, Inc., sc-32233) anti-p21 (1:1,000 dilution; Cell signaling, #2947); anti-CDK4 (1:2,000 dilution; Cell signaling, #2906); Cyclin D1 (1:500 dilution; Marine Biological Laboratory, MD-17-3).

Cell culture

Breast cancer cells (MCF-7 and MDA-MB-231) and human embryonic kidney cells (293T) were obtained from American Type Culture Collection (ATCC). The cells were maintained in Penicillin/streptomycin treated DMEM supplemented with 10% FBS at 37 °C in humidified atmosphere of 5% CO_2.

Construction and of NOB1 shRNA lentivirus

The corresponding sequences of the NOB1 short hairpin RNA (shRNA) and non-silencing control shRNA were 5'- CTAGCCCGGTTCTCCGAACGTGTCA CGTATCTCGAGATACGTGACACGTTCGGAGAATT TTTTTAAT-3' and 5'-CTAGCCCGGCC AAGGAAGTGCAATTGGTTTTTTGTTAAT-3, respectively. shRNA expressing vector pFH1UGW and lentiviral packing vectors pVSVG and pCMV◢R 8.92 (Shanghai, Hollybio, China) was used to insert these shRNA into the plasmid. DNA sequencing was performed to confirm the lentiviral based shRNA expressing vectors. Then the lentiviral basedsh-RNA were transfected into HEK293T cells cultured in 10 cm dishes at a cell density of 1×10^7 using standard protocols (Soneoka, 1995). Two hours before transfection, the medium was replaced with serum-free DMEM and the three plasmids including 20 μg of silencing sequence or non-silencing sequence, 15 μg packaging vecto and 10 μg VSVG expression plasmid were added to 200 μl Opti-MEM and 15 μl of Lipofectamine 2000. The supernatant was collected 48 h after transfection and lentiviral particles were harvested by ultra-centrifugation (4,000 g) at 4 °C for 10 min.

NOB1 shRNA infection into the breast cancer cells

NOB1 and non-silencing control shRNA were infected into breast cancer cells seeded in 96 well plates (50,000 cells per well) by replacing the medium with Opti-MEM medium containing the lentivirus. After 24h, the virus containing medium was replaced with fresh medium and further incubated for another 72 h. The successful infection was examined by counting the green fluorescence emitted by the green florescence protein (GFP) containing lentivirus particles under fluorescence microscopy following 96 h of infection.

RNA extraction and real-time PCR analysis

RNA for RT-PCR was extracted from breast cancer cells after 5 days of infection. Cells were lysed with Trizol reagent (Invitrogen) and total RNA was extracted from the lysate using standard procedures. The cDNA synthesis was carried out using Promega M-MLV cDNA synthesis kit according to the manufactures instructions. For the RT-PCR analysis Actin was used as the reference housekeeping gene. The forward and reverse primers used are; for NOB1 forward primer 5'-AAGTGAGGAGG AGGAGGAG-3' and reverse primer 5'-ACTTTCTTC AGGGTCTTGTTC -3', Actin forward primer 5'-GTGGACATCCGCAAAGAC -3' and reverse primer 5'-AAAGGGTGTAACGCAACTA-3'. Relative gene expression levels compared to Actin was calculated using $2^{-\Delta\Delta CT}$ analysis method.

Western Blot analysis

Total protein was isolated from cells infected for 5 days and the isolated protein was quantified by BSA protein analysis method. Protein (20 μg) was loaded onto a 10% SDS-PAGE and electrophoresed at 60 V for 4 h. Then the protein in the gel was transferred to polyvinylidene difluoride membrane following electrophoresis (Millipore). The proteins levels were detected by respective antibodies following detection with ECL kit (Amersham) and exposed to X-ray film. The GAPDH was used as control and detected by an anti-GAPDH antibody (Santa Cruz Biotechnology). Bands on X-ray films were quantified with an ImageQuant densitometric scanner (Molecular Dynamics).

MTT analysis

Breast cancer cells were seeded into a 96 well plate 5 days after infection at a concentration of 2,000 cells/well. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analysis was performed in different time intervals (1, 2, 3, 4 and 5 days after incubation) to find the viability of cells at tested time periods. After the specified incubation time, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. then the medium and MTT from the wells were removed and 100 µl of DMSO was added to each well. The optical density was, measured using a micro-plate reader at 490 nm. Experiments were performed in triplicates.

Colony forming assay

The cells were transfected for 5 days and seeded into 6 well plates at a density of 500 cells per well. The medium was changed every 3 days until 2 weeks of culture. Then the cells were fixed with 4% paraformaldehyde and the fixed cells were stained by adding freshly prepared diluted Giemsa stain for 20 min. The cells were rinsed with distilled water and colonies with more than 50 cells were counted under a fluorescence microscope.

Cell cycle analysis

The cell cycle distributions in the infected cells were analyzed using flow cytometer following propidium iodide (PI) staining. The infected cells were seeded on a 6 well plate at a cell density of 1×10^6. After 24 h the cells were collected, washed with ice cold PBS and were fixed with 70% ethanol and incubated for another 30 min at 4 °C. The

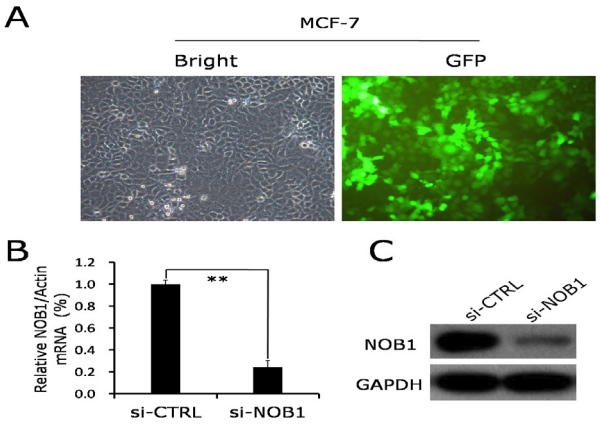

Figure 1. Silencing of NOB1 Suppressed the NOB1 mRNA and Protein Expression Levels in MCF-7 Cells. A. Light microscopic and fluorescent microscopic pictures of MCF-7 cells 96 h after infection with lentivirus containing NOB1 siRNA and non-silencing siRNA at the magnification of × 200. B. Quantitative real time PCR data of siRNA mRNA levels following the silencing compared to non-silenced control group. C. NOB1 protein expression levels of NOB1 in NOB1 siRNA infected and control siRNA infected MCF-7 cells. The values represents the mean from three independent experiments; bars represent SD. **P<0.01 in comparison with control

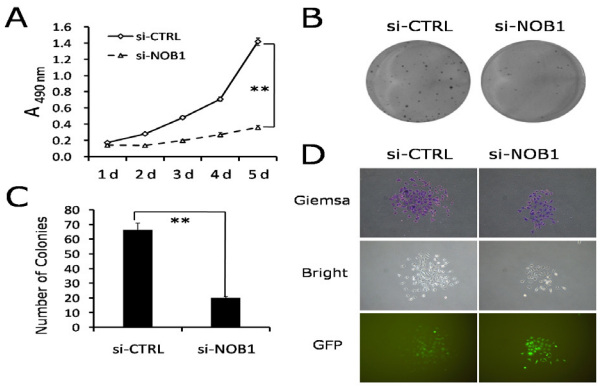

Figure 2. Effect of NOB1 Silencing on the Proliferation and Colony Forming Ability of MCF-7 Cells. A. The cell proliferation levels in NOB1 silenced and non-silenced MCF-7 cells. The values represent the mean from three independent experiments; bars represent SD. B. Giemsa staining of the cancer cell colonies under light microscope. C. Numerical representation of the number of colonies in the NOB1 silenced group and non-silenced group. D. Images of a single colony after Giemsa staining, in bright field and under fluorescence microscope. The values represents the mean from three independent experiments; bars represent SD. **P<0.01 in comparison with control

ethanol was removed by centrifugation and the remaining cell pellets were suspended in 100 μg/ml of DNase-free RNase for 30 min at 37 °C. Finally, the PI solution (100 µg/ml) was added to the cell suspension and analyzed by flow cytometer (FACS Cali-bur, BD Biosciences) after filtering through a 50-μm nylon mesh.

Statistical analysis

All data were expressed as mean ± SD of three independent experiments conducted in triplicates. The Students t test was used to evaluate the differences between the control cells and NOB1 silenced cells in SPSS 13.0 software. P < 0.01 was considered as statistically significant.

Results

NOB1 expression down regulation by NOB1 siRNA

The successful infection with lentivirus mediated NOB1 RNAi is observed by florescence imaging. The NOB1 siRNA infected cells were detected as GFP positive cells under the green florescence as GFP is also transfected to the cells together with NOB1 siRNA. As shown in Figure 1A and 4A both the breast cancer cells MCF-7 and MDA-MB-231 are successfully infected with the NOB1 siRNA. Further the gene expression levels of NOB1 following NOB1 siRNA infection was observed by real time PCR and Western blot analysis. As shown in Fig. 1B the relative mRNA expression level of NOB1 in MCF-7 cells were significantly (p<0.01) reduced with the NOB1 siRNA infection compared to non-silencing control siRNA infected group. Similarly the NOB1 protein expression was also markedly inhibited due to NOB1 siRNA infection in both MCF-7 (Figure 1C) and MDA-MB-231 cells (Figure 4B). Therefore, it was confirmed that the lentiviral mediated infection of NOB1 siRNA is an effective method to reduce the higher expression level of NOB1 in breast cancer cells.

NOB1 siRNA infection suppressed the proliferation of breast cancer cells

The effect of silencing NOB1 on breast cancer cell proliferation was analyzed by MTT assay. It could be clearly observed that the proliferation of both NOB1 siRNA infected MCF-7 (Figure 2A) and MDA-MB-231 (Figure 4C) cell proliferation was time dependently decreased. At the 5th day of observation, the cell viability was significantly (p<0.01) reduced in both cell lines compared to the non-silencing control siRNA infected cell groups. These results indicate that the higher expression levels of NOB1 are closely related to the proliferation of breast cancer cells.

Colony formation of breast cancer cells were suppressed by the silencing of NOB1

Both MCF-7 and MDA-MB-231 cells are growing in groups and therefore the effect of NOB1 silencing on this colony forming ability of breast cancer cells was investigated by performing colony formation assay following Giemsa staining. The number and the size of the colonies were observed in both NOB1 siRNA infected cell group and the non-silencing control siRNA infected group. The reduction in the number and the size of the colonies were clearly observed under the light microscope in NOB1 siRNA infected MCF-7 cells (Figure 2B). Further the numbers of colonies were counted in both cell lines infected with NOB1 and control siRNA. As shown in Figure 2C and Figure 4D NOB1 siRNA infection significantly (p<0.01) reduced the number of colonies by 69.8% in MCF-7 cells and 59.5% in MDA-MB-231 cells. The Figure 2D evidently show that the GFP expressing NOB1 siRNA infected cell colonies have reduced size compared to the control group. Collectively, these results strongly support that in the absence of NOB1 both the

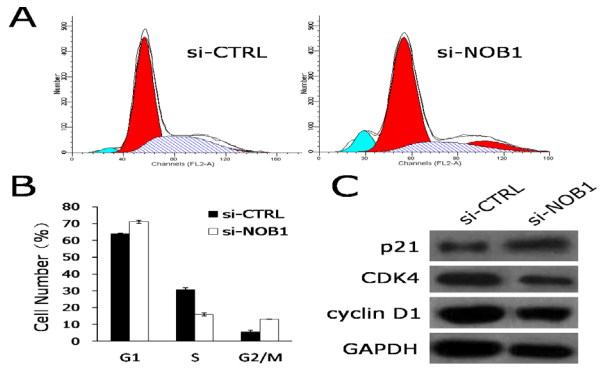

Figure 3. Effect of NOB1 Silencing on the Cell Cycle Distribution of MCF-7 Cells. A. The histograms obtained from flow cytometric analysis of NOB1 silenced and non-silenced cells. B. The percentages of MCF-7 cells at different phases of the cell cycle following NOB1 siRNA or non-silencing siRNA infection. C. The protein expression levels of cell cycle and apoptosis related proteins. The values represents the mean from three independent experiments; bars represent SD. **P<0.01 in comparison to control

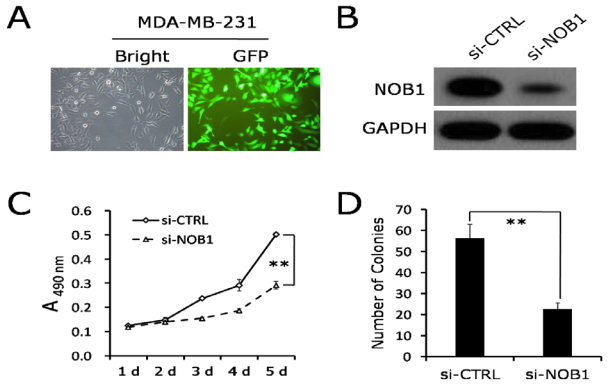

Figure 4. The Effect of NOB1 Deletion on the MDA-MB-231 Breast Cancer Cells. A. Light microscopic and fluorescent microscopic pictures of MDA-MB-231 cells 96 h after infection with lentivirus containing NOB1 siRNA and non-silencing siRNA at the magnification of × 200. B. NOB1 protein expression level in the absence and presence of NOB1 gene. C. The effect of NOB1 silencing on the proliferation of MDA-MB-231 cells. D. The effect of NOB1 silencing on the colony forming ability of the MDA-MB-231 cells. The values represents the mean from three independent experiments; bars represent SD. **P<0.01 in comparison with control

number and the size of the colonies are suppressed.

Infection with NOB1 siRNA arrested the cell cycle of MCF-7 cells

The effect of NOB1 on the cell cycle progression was analyzed by flow cytometry by using both NOB1 silenced and non-silenced MCF-7 cells. As depicted in the Figure 3A and 3B thenumber of cells in the G0/G1 phase has increased by 9.9% due to NOB1 siRNA infection. And also the number of events in the S phase of the cell cycle is decreased by 47.8% and the events in G2/M phase have increased by 57.8% as a result of NOB1 gene knockdown. This significant (p<0.01) arrest of cell cycle in NOB1 silenced MCF-7 cells were further confirmed by investigating cell cycle related protein expression levels. As shown in Figure 3C the cell cycle related proteins CDK4 and cyclin D1 are inhibited due to the absence of NOB1. Furthermore, the anti-proliferative protein p21 expression was markedly increased in the NOB1 absent cell group. Therefore, it is clear that the presence of NOB1 is a determining factor of breast cancer cell proliferation and cell cycle progression.

Discussion

Breast cancer is the most common mortality cause of women worldwide despite of higher research on controlling the cancer progression. Therefore, novel methods of cancer treatments are approached recently. Among them cancer therapy at the gene level through understanding of the molecular functions of cancer cell survival has gained more attention. This study was focused to identification of an oncogenic target in breast cancer cells and investigation of the effects of silencing the respective gene on breast cancer cell proliferation. NOB1 gene was found as an oncogene responsible for the higher rates of proliferation in cancer cells. In humans NOB1 gene is located on chromosome 16q22.1, composed of nine exons and 1,749 bp long (Zhang, 2005). The translation product of NOB1, NOB1 protein is mainly localized in the nucleus of the mammalian cells. It has been found that NOB1 plays essential roles in proteasome biogenesis (Pertschy, 2009). And several recent studies have reported that repression of NOB1 gene inhibit the growth of ovarian cancer and hepatocellular carcinoma (Lin, 2012; Lu, 2012). Therefore, this study the level of NOB1 proliferation in highly malignant breast cancer cell models MCF-7 and MDA-MB-231 were investigated and the results showed that both cell lines expressed significantly high levels of NOB1. These significant higher levels of NOB1 expression was down regulated by transfecting with NOB1 siRNA in order to find whether NOB1 down regulation has any effect on the breast cancer cell survival.

The results demonstrated that siRNA mediated silencing of NOB1 gene has a significant influence on the survival of breast cancer cells. The proliferation rate, colony forming ability and the cell cycle progression was strongly inhibited by the absence of NOB1 gene. Hence it is clear that NOB1 gene has played an important role in the proliferation and progression of breast cancer cells. The eukaryotic proteasome which degrade or process intracellular proteins control the cell cycle proteins such as cyclins, CDK4 and apoptosis proteins (Adams, 2004). NOB1 protein plays a major role in the proteasome by forming a complex between 19S regulatory particle of the 26S proteasome where the latter catalyze the protein degradation through ubiquitin–proteasome pathway for the cell cycle progression (Shirane, 1999; Xu, 2008; Fasanaro, 2010). Therefore, it could be suggested that by silencing the NOB1 gene the functions of the NOB1 protein in cell cycle progression was inhibited and thereby the breast cancer cells proliferation is suppressed.

In conclusion through this study identifies NOB1 as a critical gene in the breast cancer cell survival and growth. Therefore, siRNA mediated silencing of the NOB1 gene has inhibitory effects on the breast cancer cell proliferation by inhibiting the cell cycle progression. Collectively, this study showed potential future prospects

of using NOB1 gene therapy as an effective breast cancer treatment method.

References

Adams J (2004). The proteasome: a suitable antineoplastic target. *Nat Rev Cancer,* **4**, 349-60.

Fasanaro P, Capogrossi MC, Martelli F (2010). Regulation of the endothelial cell cycle by the ubiquitin-proteasome system. *Cardiovasc Res*, **85**, 272-80.

Ferrell K, Wilkinson CR, Dubiel W, Gordon C (2000). Regulatory subunit interactions of the 26S proteasome, a complex problem. *Trends Biochem Sci*, **25**, 83-8.

Hanahan Da WRA (2000). The hallmarks of cancer. *Cell*, **100**, 57-70.

Hershko Aa CA (1998). The ubiquitin system. *Annual Review Biochem*, **67**, 425-79.

Lin Y, Peng S, Yu H, Teng H, Cui M (2012). RNAi-mediated downregulation of NOB1 suppresses the growth and colony-formation ability of human ovarian cancer cells. *Med Oncol*, **29**, 311-7.

Lu Z, Guo Q, Shi A, Xie F, Lu Q (2012). Down regulation of NIN/RPN12 binding protein inhibit the growth of human hepatocellular carcinoma cells. *Mol Biol Rep*, **39**, 501-7.

Mello CC, Conte D Jr. (2004). Revealing the world of RNA interference. *Nature*, **431**, 338-42.

Pertschy B, Schneider C, Gnadig M, et al (2009). RNA helicase prp43 and its co-factor Pfa1 promote 20 to 18S rRNA processing catalyzed by the endonuclease Nob1. *J Biol Chem*, **284**, 35079–91.

Shirane M, Harumiya Y, Ishida N, et al (1999). Down-regulation of p27(Kip1) by two mechanisms, ubiquitin-mediated degradation and proteolytic processing. *J Biol Chem*, **274**, 13886-93.

Soneoka Y, Cannon PM, Ramsdale E, et al (1995). A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Res*, **23**, 628-33.

Størvold GL, Andersen TI, Perou CM, Frengen E (2006). siRNA: a potential tool for future breast cancer therapy? *Crit Rev Oncog*, **12**, 127-50.

Tone Y, Toh-E A (2002). Nob1p is required for biogenesis of the 26S proteasome and degraded upon its maturation in Saccharomyces cerevisiae. *Genes Dev*, **16**, 142-57.

Xu G, Bernaudo S, Fu G, et al (2008). Cyclin G2 is degraded through the ubiquitin-proteasome pathway and mediates the antiproliferative effect of activin receptor-like kinase 7. *Mol Biol Cell*, **19**, 4968-79.

Zhang Y, Ni J, Zhou J, et al (2005). Cloning, expression and characterization of the human NOB1 gene. *Mol Biol Rep*, **32**, 185-9.