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
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% CO₂.

Construction and of NOB1 shRNA lentivirus

The corresponding sequences of the NOB1 short hairpin RNA (shRNA) and non-silencing control shRNA were 5’- CTAGCCCGGTTCTCCGAACTGTGTCACGTACTCCGAGATACGGTGATCCGGAGAAATT TTTTTATAAT-3’ and 5’- CTAGCCCGGTTCTCCGAACTGTGTCACGTACTCCGAGATACGGTGATCCGGAGAAATT TTTTTATAAT-3’, respectively. shRNA expressing vector pH1UGW and lentiviral packaging vectors pSVG and pCMVADR 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 × 10⁵ 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 containing the lentivirus. After 24 h, 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 fluorescence protein (GFP) containing lentivirus particles under fluorescent microscope 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 were: for NOB1 forward primer 5’-AACGAGAGAG AGGAGGAGG-3’ and reverse primer 5’-ACTTTCTCTC AGGTCTTGTTCT-3’, Actin forward primer 5’-GGGATACCCGCAAAAGG-3’ and reverse primer 5’-AAAGGGTGAACGCAACTA-3’. Relative gene expression levels compared to Actin was calculated using 2⁻ΔΔCT 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 cytomter following propidium iodide (PI) staining. The infected cells were seeded on a 6 well plate at a cell density of 1 × 10⁵. 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
an effective method to reduce the higher expression level that the lentiviral mediated infection of NOB1 siRNA is MB-231 cells (Figure 4B). Therefore, it was confirmed siRNA infection in both MCF-7 (Figure 1C) and MDA-control siRNA infected group. Similarly the NOB1 protein in NOB1 siRNA infected cells were significantly (p<0.01) reduced with NOB1 following NOB1 siRNA infection was observed NOB1 siRNA infection compared to non-silencing in MCF-7 cells were significantly (p<0.01) reduced with in Fig. 1B the relative mRNA expression level of NOB1 by real time PCR and Western blot analysis. As shown NOB1 RNAi is observed by florescence imaging. The NOB1 expression down regulation by NOB1 siRNA shown in Figure 1A and 4A both the breast cancer cells transfected to the cells together with NOB1 siRNA. As positive cells under the green florescence as GFP is also NOB1 siRNA infected cells were detected as GFP 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
Collectively, this study showed potential future prospects for the inhibition of breast cancer cell proliferation. NOB1 gene has inhibitory effects on the breast cancer growth. Therefore, siRNA mediated silencing of the NOB1 gene has played an important role in cell survival. The proliferation rate, 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 higher 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 for the inhibition of breast cancer cell proliferation.
of using NOB1 gene therapy as an effective breast cancer treatment method.

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