Effects of Nucleostemin gene silencing on morphology and cytochemistry of HL-60 cells

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Abstract

Objective. To explore the effect of Nucleostemin (NS) gene silencing on morphology and cytochemistry of HL-60 cells, and to study the relationship between the changes of morphology and cytochemistry and biological behaviors. *Methods.* Nucleostemin specific short hairpin RNA (NS-shRNA) was transfected into HL-60 cells directly. The effects of NS gene silencing and inhibition of NS protein were detected by RT-PCR and Western blot. The morphology of HL-60 cells were observed under inverted microscope and using Wright-Giemsa staining analysis. The volume, granularity and size variation of HL-60 cells were assayed by blood cell analyzer. HL-60 cells were analyzed by myeloperoxidase (MPO), α-naphthol acetate esterase (α-NAE) and periodic acid-schiff (PAS) staining. *Results.* The characters of differentiation and maturation in karyon and cytoplasm were observed by silencing NS gene *in vitro*. The nuclear fragmentations (karyorrhexises) and apoptosis bodies were observed in cells by Wright-Giemsa staining. The size of cells increased. Small cells, karyorrhexises and cell-debrises without nucleus were detected by blood cell analyzer. MPO and α-NAE activities and PAS positive rate were increased after NS-shRNA transfection. *Conclusion.* The redifferentiation and apoptosis of HL-60 cells can be induced by silencing NS gene *in vitro.* Meantime, the morphology and cytochemistry characters of HL-60 cells were altered in accordance with differentiation and apoptosis. These changes may be one of the evidences to study the biological behaviors in cells. [Life Science Journal. 2008; 5(2): 9 – 14] (ISSN: 1097 – 8135).

Keywords: Nucleostemin; leukemia; morphology; cytochemistry; HL-60 cell

1 Introduction

Nucleostemin (NS), a kind of protein related with cell proliferation, was identified in 2002. NS is expressed in multipotential stem cells which have the ability of infinite proliferation, but isn't expressed in committed and terminally differentiated cells[1–5]. NS is probably the essential protein which is involved in proliferation of stem cells and cancer cells, and keeps cells in non-differentiated state. It is significant to study the function and regulation of NS protein which exists in stem cells and tumor cells[6,7]. The study of the relationships between NS and the stem cells, and the role of NS on the occurrence and development of tumor cells, are currently starting in the world.

Leukemia belongs to malignant disease of haematopoietic stem cells (HSCs). And the cells are arrested at certain differentiation stages, producing increasing numbers of poorly differentiated and immature leukemia cells in the marrow and these leukemia cells are in the state of infinite proliferation. Prior researches of our research group had certified the presence of NS gene in leukemia cells, and the protein encoded by NS gene is abundantly expressed[8]. According to further investigations, by silencing NS gene *in vitro,* the proliferation of leukemia cells is inhibited, and the redifferentiation and apoptosis should be induced. Meantime, the morphology and cytochemistry of leukemia cells may be altered.

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2 Materials and Methods

2.1 Cell culturing

The leukemia cell of HL-60 was kindly provided by Chinese Academy of Medical Science. The cell line was cultured in RMPI1640 medium, supplemented with 10% fetal bovine serum (containing 100 U/ml penicillin and 100 μg/ml streptomycin) at 37 ºC with 5% CO_2.

2.2 Designing and synthesizing of NS-shRNA

2.2.1 Designing of NS-shRNA. 1833 bp consensus sequences were selected from three variants (variant, NM014366, NM206825, NM206826) of complete cDNA in human. Other homologous coding sequences and expressed sequence tag (EST) homologous sequences were discarded according to the principals of siRNA designing. Finally, two of 21 bp sequences were determined as targeting cDNA sequences, connected by a 9 bp loop [aaguucucu] and ended in a 2-uridine 3'-overhang. The total length of the designed NS-shRNA was 49 bp.

2.2.2 Synthesizing of NS-shRNA. First of all, the complementary single DNA templates for NS-shRNA, which contained T7 promoter sequence, were constructed *in vitro*, and then they were annealed to generate double DNA template. Two NS-shRNA were synthesized *in vitro* by T7 RNA polymerase promoter, and a more effective NS-shRNA was screened for post research.

2.3 Transfection of NS-shRNA into leukemia cells

Logarithmic growth phase cells were adjusted to the density of 4×10^5 cells/μl with whole medium, then aliquoted into 24-well plates for 500 μl per well. In order to evaluate the suppression efficacy of NS-shRNA, cells were grouped into the transfected group, the negative control group and the blank control group. For transfecting shRNA into HL-60 cells, 2 μl Code Breaker siRNA transfection agent (Promega Company) was added into 125 μl non-serum medium, the transfected group was added NS-shRNA to the final concentration of 10 nmol/L according to preliminary experiment, while the negative control group and the blank group were added non-related shRNA and transfection agent respectively.

2.4 Detection of silencing NS gene and inhibition of NS protein synthesizing

72 hours after NS-shRNA transfected into HL-60 cells, the total RNA and protein were extracted from above three groups for RT-PCR and Western blot. The forward primer of NS gene was 5'-AAAGCCATTCGGGTTGGAGT-3', and the reverse primer was 5'-ACCACAGCAGTTTGGCAGCAC-3', with the expected size of 418 bp. β-actin gene was taken as internal control with the expected size of 315 bp. Its forward primer was 5'-TCCTGTGGCATCCACGAAACT-3', and the reverse primer was 5'-GAAGCATTTGCGGTGGACGAT-3'.

2.5 Morphology changes in HL-60 cells

The HL-60 cells both in transfected group and control group were observed under inverted microscope 48 hours after transfection. The volume, granularity and size of HL-60 cells were assayed by K4500 (Sysmex Company) and SF3000 (Sysmex Company) blood cell analyzer. Cells were harvested 72 hours after transfection, then centrifuged to condense and smeared. The morphology and structure of HL-60 cells were observed by microscope and Wright-Giemsa staining[9].

2.6 Observation of apoptosis induced by NS-shRNA

After 48-hours' culture, the HL-60 cells apoptosis was examined by dUTP nick end labelling (TUNEL) technology. The cells were harvested and smeared 72 hours after transfection, and observed by Wright-Giemsa staining and under the microscope.

2.7 Myeloperoxidase (MPO), α-naphthol acetate esterase (α-NAE) and periodic acid-schiff (PAS) in HL-60 cells with NS silencing

Cells were collected after 24 hours, 48 hours, and 72 hours respectively, and centrifuged to condense and smear. MPO, α-NAE, NaF inhibitory assays and PAS staining[9] of HL-60 cells were determined, and the positive cells were counted under the microscope.

2.8 Statistical analysis

All experimental data were processed by 13.0 software. *t* test was used to evaluate the significance, and data was reported as $\bar{X}$ ± SD. Statistical significance was set *P* less than 0.05.

3 Results

3.1 Detection of silencing gene and the NS-mRNA expression

3.1.1 NS mRNA. The expression of NS-mRNA in HL-60 cells was down-regulated after 24-hour's culture. The expression of NS mRNA was inhibited by NS-shRNA, and the effect was especially significant after 48 hours and after 72 hours, with the peak inhibiting rate of (68.3 ± 2.15)%. But the NS mRNA expression after 48-hours' treatment wasn't different from the expression after 72-

hours' treatment ($t = 0.98$, $P > 0.05$, Figure 1 & Figure 2).

3.1.2 Inhibition of NS protein expression. As shown by Western blot, the electrophoresis band was narrow and thin in the transfected group, in contrast with those in control groups. The intensity ratio of Western blot product showed that the inhibiting rate of NS protein was 78.2%. It suggested that synthesizing NS protein was significantly down-regulated after blocked by NS-shRNA (Figure 3).

3.2 Morphology changes in HL-60 cells

3.2.1 Morphology changes *in vitro*. Cells in control groups were suspended and aggregated, the sizes were even, and the shapes were smooth and glossy. But compared with those in control group, cell densities and aggregative degrees were decreased and the sizes of cells were obviously different in the NS-shRNA group 48 hours after transfection. Part of cells in transfected group changed to fusiform even pseudopod (Figure 4).

3.2.2 Volume and size of HL-60 cells. Sizes of HL-60 cells were even in control group according to blood cell analyzer. As shown by volume histogram, the curve was approximate to normal distribution, and the peak amplitude was around 200 fl, with few less than 150 fl. 48 hours after transfection, the peak amplitude and the normal curve was disappeared, bulks of HL-60 cells were more than 150 fl, and the sizes were unequal (Figure 5). In the scattergraph, two high-density areas were in transfected group, and only one in control group. The "low angle scattered light (LASL)" on Y-axis had positive correla-

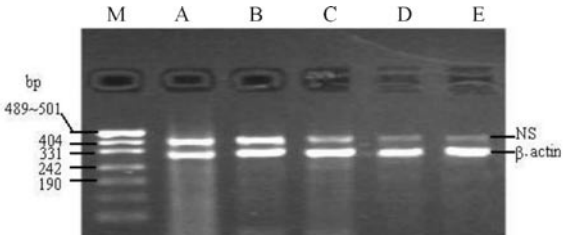

Figure 1. Effects of NS-shRNA on NS-mRNA expression in HL-60 cells. M: Molecular weight standard; A: The blank control group; B: The negative control group; C: 24 hours; D: 48 hours; E: 72 hours.

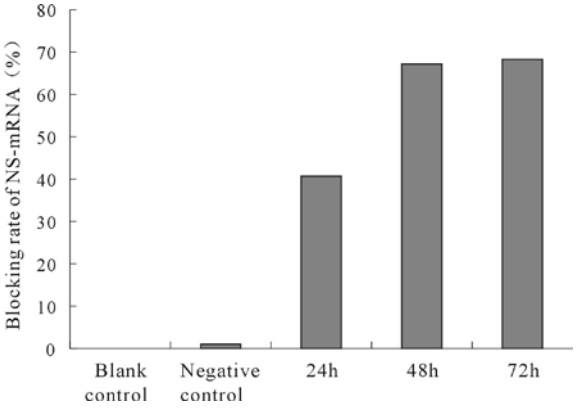

Figure 2. Effects of NS-shRNA on inhibiting NS-mRNA expression in HL-60 cells within 24 – 72 hours.

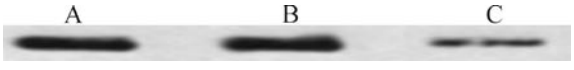

Figure 3. Result of Western blot 72 hours after transfected with NS-shRNA. A: Blank control group; B: Negative control group; C: Transfected group.

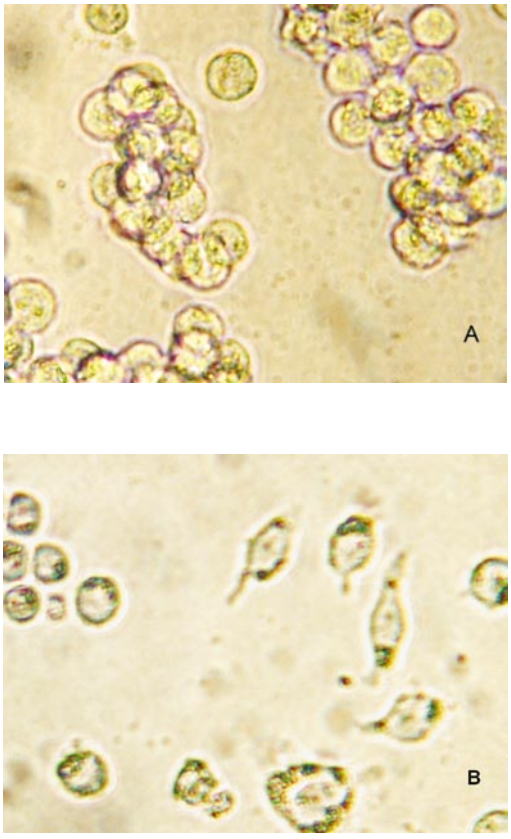

Figure 4. Morphology of HL-60 cells transfected with NS-shRNA 48 hours later (× 400). A: The control group; B: The NS-shRNA group.

tion with the size of cells. In transfected group, the "center" moved down, which suggested that a great amount of small-volume cells exist. The "high angle scattered light (HASL)" on X-axis had positive correlation with the size of karyons and the quantity of granulations in cells. And the "center" shifted left in transfected group, which indicated the increase of nuclear fragmentations and cell-debrises (Figure 6).

3.2.3 Morphology observation by Wright-Giemsa staining. The sizes of cells in control group were equal, and larger than those in transfected group. Shapes of cells were round or elliptical. Nucleus was round, taking about 3/4 area of the cell, with 3 – 4 nucleoli in it, and the chromatin was exquisite. The cytoplasm was just a small amount, and was dark blue by Wright-Giemsa staining. Aggregated prunosus granulations and several foamy vacuoles could be seen (Figure 7A). Compared with the control group, variation of cell sizes increased in transfected group. Cell shapes change to irregular even pseudopod appeared. Nucleus was compacted in small cells, some were quite cavate and the cytoplasm turned eosinophilic at the same time. The chromatin was aggregated, and nucleolus was obsolete or disappeared (Figure 7B).

3.2.4 Induced HL-60 cells apoptosis. Nuclear fragmentations and "apoptotic bodies" were seen in treated HL-60 cells. The apoptosis characteristics were observed: the shrunk cells, karyorrhexises and pycnosises, various cell debris, and "apoptosis bodies" (Figure 7C). The positive rate detected by TUNEL was (27.3 ± 3.21)% in transfected group, more than (3.3 ± 1.52)% in control group (t = 11.704, P < 0.01). It was indicated that cell apoptosis could be induced by NS-shRNA in HL-60 cells.

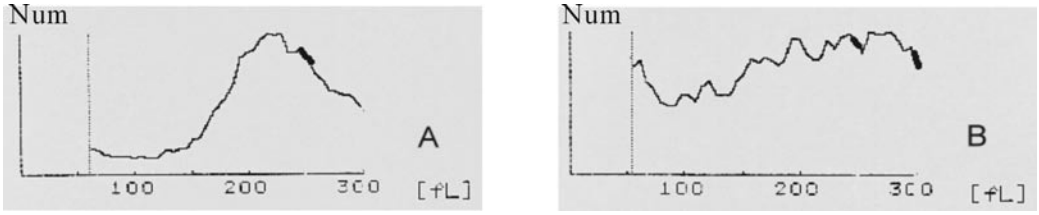

Figure 5. Volume histogram of HL-60 cells 48 hours after transfection. A: Control group; B: Transfected group.

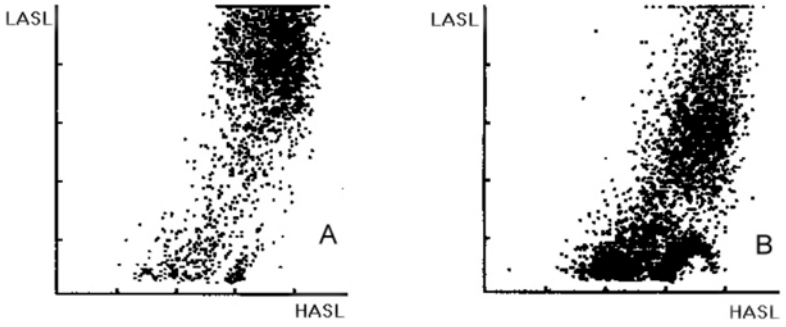

Figure 6. Granularity analysis of HL-60 cells in the scattergraph after transfection. A: Control group; B: Transfected group.

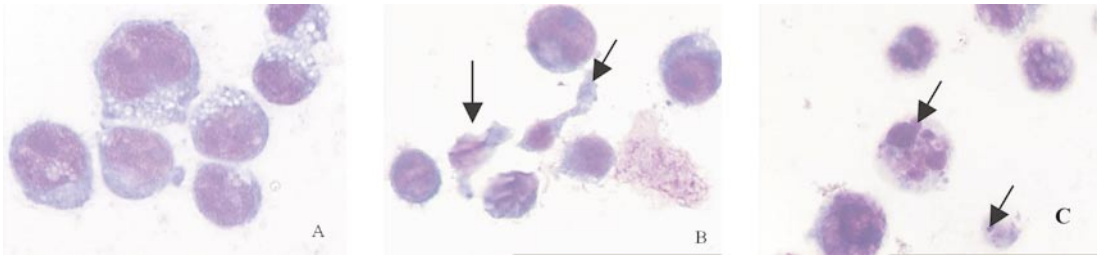

Figure 7. Morphology changes in HL-60 cells after transfected by NS-shRNA (× 1000). A: Control group; B: Irregular shape with pseudopod and pycnosises, the chromatin was aggregate, and nucleolus disappeared; C: Cell apoptosis.

3.3 Detection of cytochemistry changes

3.3.1 MPO staining. MPO positive rate was higher than that in control group ($t = 4.553$, $P < 0.01$) 48 hours after transfection, and was continuously increasing to the 72 hours after transfection ($t = 16.997$, $P < 0.01$). But in the initial 24 hours, no visible changes were observed compared with the control group (Figure 8, Table 1).

3.3.2 α-NAE staining. 72 hours after transfection, α-NAE positive cells reached to 13.0%, and brownish-black product appeared in cytoplasm (Figure 9B). This positive reaction could be inhibited by NaF, which suggests that some monocytic α-NAE be created by NS-shRNA (Figure 9C). The α-NAE was negative and no change all the time in control group (Figure 9A).

Table 1. Variation of MPO positive rate in HL-60 cells after transfection (%)

Group	0 hour	24 hours	48 hours	72 hours
Control	11.0 ± 3.53	11.7 ± 2.77	11.4 ± 2.49	11.7 ± 3.61
Transfected	11.0 ± 3.53	12.5 ± 3.28	21.4 ± 3.23[#]	27.2 ± 2.82[#]

[#]: Comparison between transfected group and control group at the same time, except for the effect of 24 hours ($P > 0.05$).

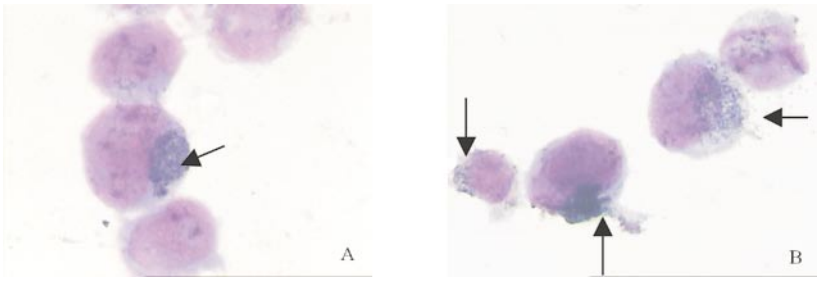

Figure 8. MPO staining in HL-60 cells (the dark-blue pointed by the arrow was MPO positive) (× 1000). A: Control group; B: Transfected group.

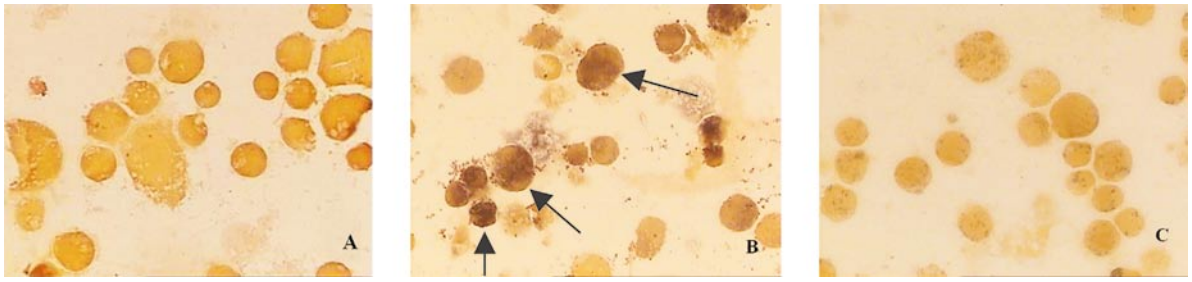

Figure 9. α-NAE and α-NAE + NaF reaction in HL-60 cells after treated with NS-shRNA (pointed by the arrow was α-NAE positive). A: Control group; B: Transfected group; C: NaF test in transfected group.

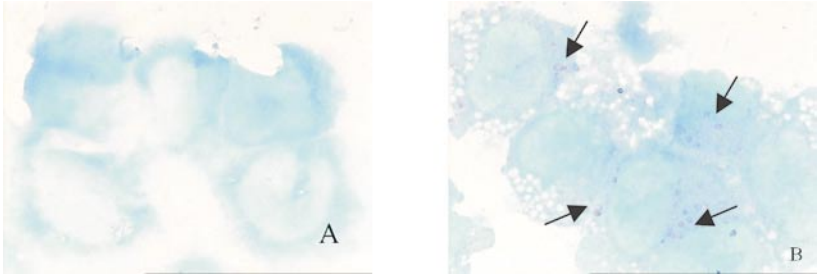

Figure 10. PAS reaction in HL-60 cells after treated with NS-shRNA (pointed by the arrow was PAS positive). A: Control group; B: Transfected group.

3.3.3 PAS staining. 72 hours after transfection, 72.0% PAS positive cells appeared in the transfected group, and the positive product was prunosus mass or granulation (Figure 10B). The PAS reaction was negative and no change all the time in control group (Figure 10A).

4 Discussion

Down-regulation of NS gene could induce cell out of normal cell cycle and result in differention[10–13]. We observed that the redifferentiation of HL-60 cells could be induced by silencing NS gene *in vitro*. Meantime, the characters of morphology and cytochemistry of HL-60 cells would be altered accordingly. Some of HL-60 cells treated with NS-shRNA were changed from round to fusiform or pseudopod, observed in culture or by Wright-Giemsa staining. Monocytic α-NAE presented in cytoplasm. The amount of this cytotype was in accordance with that of CD14 positive cells detected by flow cytometry (FCM). It proves the differentiation toward monocytes definitely. As discovered by continuous observation, MPO was increasing in transfected HL-60 cells, which suggested further mature to granulocytes. The increase of glycogen material in HL-60 cells observed by PAS staining also indicated the maturity and redifferentiation after the inhibition of NS gene. Nucleus shrank and turned cavate, the chromatin was compact, and eosinophilic cytoplasm appeared in HL-60 cells, observed by Wright-Giemsa staining. On the whole analysis, silencing NS-gene has contribution to the maturing and redifferentiation of HL-60 cells. Morever, as shown by differentiation antigen, it is multi-directed differentiation that aims at granulocyte series and monocyte series. Our research group assayed the volume and granularity, and size of HL-60 cells by blood cell analyzer which evaluated both the external and internal characteristics in single cells, and reflected the differences among cells. According to analysis of the size and granularity, small cells, karyorrhexises and cell-debrises without nucleus appeared in the cultivate system after transfected by NS-shRNA. Meanwhile, the evidence of apoptosis is that nuclear fragmentations and small nucleuses are observed under the microscope with Wright-Giemsa staining. And the small-nucleus cell with just a little cytoplasm is the "apoptosis body". Moreover, the silencing NS gene induced apoptosis in HL-60 cells was supported and certified by FCM and TUNEL experiment. NS protein is probably a specific regulatory factor responsible for stem cells and cancer cells acrossing G_2/M checkpoint. It is also p53 binding protein and functions with the dynamic separating or binding with wild-type p53 protein. Cells used for our study are p53-absent type, but still a series of changes on the biological behaviors occurred after NS gene silencing. As is newly reported according to Jafarnejad's research, the proliferation and differentiation of cells isn't necessarily depended on the interaction with p53[14]. Thus, it can be concluded that the relationship between NS protein and p53 is not that simple, may be other links or even another pathway exists to affect the series of biological behaviors in cells.

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