Gene expression profiling of HL-60 cells following knockdown of nucleostemin using DNA microarrays

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Abstract. Nucleostemin (NS) plays an important role in tumorigenesis and progression. Most studies consider that NS plays its role through combining with p53 and inhibiting it, however our previous studies revealed that NS could also function without the existence of p53. To date, few studies have focused on the p53-independent pathway of NS, and its molecular mechanism remains unknown. The aim of the present study was to investigate the p53-independent pathway of NS in the human acute myeloid leukemia cell line HL-60 which was p53-null by using the DNA microarray technique. Lentivirus-mediated RNA interference technique was used to knock down NS expression in HL-60 cells, and then DNA microarray and bioinformatics were used to analyze the gene expression profiling changes. The microarray data showed that after knocking down NS in HL-60 cells, 2,628 differentially expressed genes were identified through ≥2 or ≤0.5-fold-change, in which 818 genes were upregulated and 1,810 genes were downregulated. Real-time quantitative polymerase chain reaction (qPCR) validated the reliability of DNA microarray data. Pathway analysis showed extensive signal pathways in HL-60 cells were influenced by inhibiting NS expression. In particular, the inhibition of PI3K-AKT pathway, JAK-STAT pathway, RAS-RAF-MEK-ERK1/2 pathway and activation of JNK pathway, p38 MAPK pathway may associate with the apoptosis of HL-60 cells after knocking down NS. The findings of this study provide insight to further explore the specific molecular mechanism of NS function in p53-null leukemia and they also lay the foundations for exploring new therapeutic targets for p53-null leukemia and even p53-null tumors.

Introduction

Nucleostemin (NS) is a protein related to cell proliferation which was originally identified in 2002, and located in the nucleolus; it was first found in some early pluripotent cells such as embryonic, neural and bone marrow-derived stem cells in rat, but was not expressed in terminally differentiated cells (1,2). NS is highly expressed in several types of tumor tissues and cells, such as prostate and esophageal cancer (3,4). After knocking down NS expression in tumor cells, its proliferation became weak, and most studies suggest that NS regulates cell proliferation through blocking the cells at the G0/G1 or G2/M point and then leads the cells out of the cell cycle (5-7). These studies indicated that NS is essential to maintain proliferation and undifferentiated state of stem and tumor cells.

Most traditional studies on NS action mechanism suggest that NS could combine with p53 to inhibit its tumor suppressor function, and then lead to tumorigenesis (1). In addition, NS could protect telomere, its overexpression could regulate the telomere length negatively and then delay cell senescence; however, the absence of NS could increase the probability of telomere damage and mutation and then result in cell senescence (8). This may also be one type of mechanism of NS in tumorigenesis and tumor development.

However, in our previous studies, we used human acute myeloid leukemia cell line HL-60 which was p53-null as the research material. After inhibiting NS expression, HL-60 cells presented more apoptosis (9). Thus, we speculated that NS may also play its role independent of p53. Similar views have been reported by others, including Beekman *et al* (10), Jafamejad *et al* (11) and Nikpour *et al* (12). However, to date, only few studies have focused on the detailed mechanism of this pathway.

Clinically, the therapeutic effect of tumors is closely associated with p53. P53-null or mutate is one of the main reasons for drug-resistant, poor prognostic and therapeutic effects. Thus, exploring effective treatment targets and measures for p53-null and p53-mutate tumors is a critical issue that requires immediate attention. The present study analyzed the gene expression profiling changes of HL-60 cells after knocking down NS, aiming to explore the detailed mechanism of NS p53-independent pathway. These findings may lay the foundations for drug development for p53-null leukemia and even p53-null tumors.

Materials and methods

Cell culture. HL-60 cells were cultured in RPMI-1640 medium (Gibco-BRL) supplemented with 10% fetal bovine

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Table I. Sequences of two single-stranded DNA oligonucleotides.

No.	5'	STEM	Loop	STEM	3'
NS-RNAi-1	ccgg	agCAAGTATTGAAGTAGTAAA	CTCGAG	TTTACTACTTCAATACTTGCT	TTTTTg
NS-RNAi-2	aattcaaaaa	agCAAGTATTGAAGTAGTAAA	CTCGAG	TTTACTACTTCAATACTTGCT	

serum (FBS; Gibco-BRL), 100 U/ml of penicillin, 100 μg/ml streptomycin and were incubated at 37˚C with 5% humidified CO_2. The cells were changed medium every 2 days.

Construction of NS-siRNA lentiviral vector. RNA interference (RNAi) sequence was designed corresponding to NS gene (Genbank NM_014366). Two single-stranded DNA oligonucleotides containing NS-RNAi sequence, loop circle, *Age*I/*Eco*RI enzyme cutting site and termination signal sequence were synthesized (Table I) and annealed to a double-stranded DNA template. Subsequently, the template was inserted into the *Age*I and *Eco*RI enzyme sites of GV248 lentiviral vector (Genechem, Shanghai, China) with catalysis by T4 DNA ligase to construct recombinant vectors expressing NS-shRNA. The recombinant vectors were transformed into competent *E.coli* and then confirmed by DNA sequencing. The recombinant vector and packaging vectors were co-transfected into 293T cells, and the supernatants containing packaged vectors were harvested 48 h later. Subsequent purification using ultracentrifugation was performed and the titer of lentiviruses was determined.

Validation of the RNA-interference effect. Fresh medium for HL-60 cells was changed 24 h before transfection to ensure the cells in logarithmic phase at transfection. At the time of transfection, the cells were harvested and centrifuged, and then suspended in fresh medium with 10% FBS and seeded into a 6-well plate. A certain amount of NS-shRNA lentivirus was added to HL-60 cells according to lentiviral titer and MOI of HL-60 cells. At the same time, to exclude the influence of blank lentiviral vector and off-target silencing effect mediated by specific-shRNA, the blank control and the negative control group were also set. The same amount of blank lentiviral vectors and negative control lentivirus was added to HL-60 cells as the blank control group and negative control group, respectively. The final volume in each well was 2 ml. Sixteen hours after transfection, the medium was changed and continued to culture. Seventy-two hours after transfection, the plate was placed under inverted fluorescence microscope to observe the transfection efficiency.

Cells (5-10x10^6) in each group were harvested 96 h after transfection. Total-RNA was extracted from cells using TRIzol reagent (Invitrogen). The reverse transcription reaction was performed with PrimeScript® RT reagent Kit with gDNA Eraser (Takara). Expression of NS mRNA was detected by ABI 7500 real-time PCR instrument using SYBR® Premix Ex TapTMII (Tli RNaseH Plus) Kit (Takara). The sequence of primers for NS and GAPDH are shown in Table II. Relative gene expression was quantified to calculate the inhibition efficiency.

Table II. Primer pairs used for quantitative RT-PCR.

Gene	Primer pairs
PIK3CD	F: 5'-TTT CTC ATG GCT GTC CTT CAG-3' R: 5'-CAG GAG AAT CTA ACG GAT GC-3'
AKT2	F: 5'-CAT CAC ATC TGG TTT CCT TGG-3' R: 5'-AAC TGG AAA TGT AAT TTT GGG-3'
STAT3	F: 5'-ACC AGC AAT ATA GCC GAT TCC-3' R: 5'-CCA TTG GCT TCT CAA GAT ACC-3'
STAT5A	F: 5'-ATT ATC TCA GCC CTG GTG ACC-3' R: 5'-CTG CTG CTC ACT GAT GAT GGT-3'
GRB2	F: 5'-GGA CAT CCT CAA GGT TTT GAA C-3' R: 5'-CGC TCT CAC TCT CTC GGA TAA G-3'
HRAS	F: 5'-AGC TGA TCC AGA ACC ATT TTG T-3' R: 5'-GTT GAT GGC AAA CAC ACA CAG-3'
MAPK9	F: 5'-CTG CGT CAC CCA TAC ATC AC-3' R: 5'-CTT TCT TCC AAC TGG GCA TC-3'
MAPK13	F: 5'-AGG TCT CTG GGG GTT GAG TTG GG-3' R: 5'-AGG GGC AGC AAC GTC TCA TTG C-3'
GAPDH	F: 5'-TGA CTT CAA CAG CGA CAC CCA-3' R: 5'-CAC CCT GTT GCT GTA GCC AAA-3'
NS	F: 5'-TAGAGGTGTTGGATGCCAGAG-3' R: 5'-CACGCTTGGTTATCTTCCCTTTA-3'

Microarray hybridization. Total RNA was purified using the RNase Mini Kit (Qiagen p/n 74104), according to the protocol for Quick Amp Labeling Kit, One-Color (Agilent p/n 5190-0442), reverse transcript total-RNA to cDNA, and then further transcripted cDNA to cRNA with Cy3 labeling and purifying it. Next, the purified Cy3-labeled cRNA was used for hybridization on Agilent 4x44K Human Whole-Genome 60-mer oligonucleotide microarrays following the protocol for Agilent Gene Expression Hybridization Kit (p/n 5188-5242).

Microarray data acquisition, processing and analysis. Microarrays were scanned with Agilent DNA Microarray Scanner (Agilent p/n G2565BA), using the setting parameters recommended by Agilent Technologies (Green PMT were set at XDR Hi 100% and XDR Lo 10%; scan resolution was set to 5 μm). Then, the Agilent Feature Extraction software v11.0.1.1 was used to analyze acquired microarray images, and the resulting text files extracted from it were imported into the GeneSpring GX v12.0 software package (Agilent

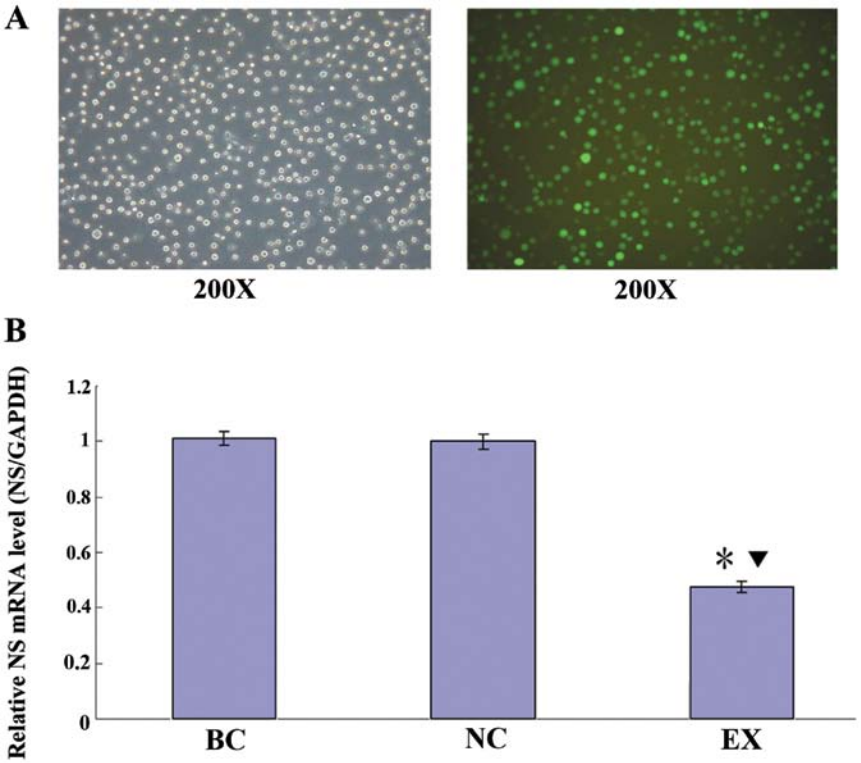

Figure 1. The interference effect of NS-shRNA lentivirus in HL-60 cells. (A) HL-60 cells transfected with NS-shRNA lentivirus were observed under an inverted microscope using general light (left) and fluorescence (right), respectively. (B) Detection of relative NS mRNA level in each group by real-time PCR. *P<0.05 compared with the blank control (BC) group, ▼P<0.05 compared with the negative control (NC) group; EX, experimental group.

Technologies) for further analysis. After quantile normalization of the raw data, genes that at least 2/2 samples have flags in Detected ('All Targets Value') were chosen for further data analysis. Differentially expressed genes with statistical significance were identified through fold-change filtering for 2 compared samples. Pathway analysis of the differentially expressed genes was performed using the KEGG Pathway Database (http://www.genome.jp/kegg).

Real-time quantitative PCR. Reverse transcription was performed using PrimeScript® RT reagent Kit with gDNA Eraser (Takara). Quantification of gene expression was performed by ABI 7500 real-time PCR instrument using SYBR® Premix Ex TapTMII (Tli RnaseH Plus) Kit (Takara). The expression level of GAPDH was used as an internal control. The expression of the following genes was analyzed: PIK3CD, AKT2, STAT3, STAT5A, GRB2, HRAS, MAPK9, MAPK13. The primers are listed in Table II.

Statistical analysis. The experiments were repeated at least three times and the data are presented as means ± SD (standard deviation). Statistical software SPSS 17.0 was used for the assessment. The Student's t-test was used to compare means of two groups. Fisher's exact test was used to analyze the significance of the pathway. P<0.05 was considered to indicate statistically significant differences.

Results

Downregulation of NS in HL-60 cells after NS-shRNA lentivirus transfection. The lentiviral vectors transfected HL-60 cells successfully and the transfection efficiency was >80% which could be observed under an inverted fluorescence microscope (Fig. 1A). The NS mRNA expression was determined by real-time qPCR. As shown in Fig. 1B, there was no significant difference between the blank control group (BC) and the negative control group (NC; P>0.05); compared with the BC and the NC group, the levels of NS mRNA in the experimental group (EX) were significantly reduced by 52.9 and 52.3%, respectively (P<0.05). To maximize reducing the off-target effect, we chose the NC and the EX group for further DNA microarray analysis.

Data analysis: general features. Total-RNA isolated from the EX and NC group cells was used to synthesize cDNA. Probe labeling, hybridization and scanning were performed by Shanghai Kangcheng Co., Ltd (China). To identify differentially expressed genes with statistical significance, a fold change filtering between two samples was performed and the default threshold was ≥2.0 fold-change. Results showed that 2,628 genes, 818 upregulated and 1,810 downregulated, were differentially expressed in HL-60 cells following downregulation of NS.

Real-time qPCR validation. To evaluate the reliability of the array results, eight differentially expressed genes (PIK3CD, AKT2, STAT3, STAT5A, GRB2, HRAS, MAPK9, MAPK13), which had >2-fold-changes according to the microarray assay results, were selected for further validation by real-time qPCR. The real-time qPCR results of these genes were generally in agreement with microarray data (Fig. 2). Although the fold-changes of these genes determined respec-

Table III. Pathway analysis of upregulated genes.

PathwayID	Definition	Fisher's P-value	Genes
hsa05322	Systemic lupus erythematosus - *Homo sapiens* (human)	7.77667E-06	C7//CTSG//ELANE//HIST1H2AC//HIST1H2AI//HIST1H2BB//HIST1H2BF//HIST1H2BH//HIST1H2BK//HIST1H2BL//HIST1H2BO//HIST1H3B//HIST1H3H//HIST1H4H//HIST1H4L//HIST2H2BE//HIST2H2BF
hsa05144	Malaria - *Homo sapiens* (human)	0.000416632	CCL2//DARC//HBB//HBD//HGF//IL1B//IL8//THBS1
hsa04621	NOD-like receptor signaling pathway - *Homo sapiens* (human)	0.001132948	CASP5//CCL2//CXCL1//IL1B//IL8//MAPK13//MAPK9//NLRP1
hsa04340	Hedgehog signaling pathway - *Homo sapiens* (human)	0.003689235	BMP7//CSNK1E//CSNK1G1//PRKACB//PTCH1//SUFU//WNT4
hsa05146	Amoebiasis - *Homo sapiens* (human)	0.004635966	COL11A2//CTSG//CXCL1//HSPB1//IL1B//IL8//LAMC1//PRKACB//RAB5A//SERPINB13
hsa04010	MAPK signaling pathway - *Homo sapiens* (human)	0.007661671	CACNA1B//CACNA1E//FGF4//FGF8//FGFR3//FLNA//HSPB1//IL1B//JUN//MAPK13//MAPK8IP2//MAPK9//NTRK2//PLA2G2A//PRKACB//RAC1//RAPGEF2//RASGRP3
hsa05020	Prion diseases - *Homo sapiens* (human)	0.007782237	C7//EGR1//IL1B//LAMC1//PRKACB
hsa05120	Epithelial cell signaling in *Helicobacter pylori* infection - *Homo sapiens* (human)	0.01072619	CXCL1//IL8//JAM2//JUN//MAPK13//MAPK9//RAC1
hsa04620	Toll-like receptor signaling pathway - *Homo sapiens* (human)	0.01085929	IL1B//IL8//JUN//LY96//MAPK13//MAPK9//RAC1//SPP1//TLR6
hsa04512	ECM-receptor interaction - *Homo sapiens* (human)	0.01106789	CD44//COL11A2//ITGAV//LAMC1//SPP1//SV2B//SV2C//THBS1
hsa05132	Salmonella infection - *Homo sapiens* (human)	0.01264349	CXCL1//FLNA//IL1B//IL8//JUN//MAPK13//MAPK9//RAC1
hsa05217	Basal cell carcinoma - *Homo sapiens* (human)	0.01355671	FZD4//FZD5//FZD7//PTCH1//SUFU//WNT4
hsa05200	Pathways in cancer - *Homo sapiens* (human)	0.01360474	CCND1//CYCS//FGF4//FGF8//FGFR3//FZD4//FZD5//FZD7//HGF//IL8//ITGAV//JUN//LAMC1//MAPK9//PTCH1//RAC1//RARA//RUNX1//SUFU//WNT4
hsa04310	Wnt signaling pathway - *Homo sapiens* (human)	0.01989438	CCND1//CSNK1E//FZD4//FZD5//FZD7//JUN//MAPK9//PPP2R5B//PRKACB//RAC1//WNT4
hsa04962	Vasopressin-regulated water reabsorption - *Homo sapiens* (human)	0.02006348	ARHGDIB//DYNLL2//PRKACB//RAB11A//RAB5A
hsa04510	Focal adhesion - *Homo sapiens* (human)	0.02786726	CAPN2//CCND1//COL11A2//FLNA//HGF//ITGAV//JUN//LAMC1//MAPK9//PXN//RAC1//SPP1//THBS1
hsa04210	Apoptosis - *Homo sapiens* (human)	0.03465665	ATM//CAPN2//CYCS//IL1B//IL1RAP//PRKACB//TNFRSF10C
hsa04722	Neurotrophin signaling pathway - *Homo sapiens* (human)	0.03905394	ARHGDIB//JUN//MAPK13//MAPK9//NTRK2//PDK1//RAC1//SH2B2//YWHAE

Table III. Continued.

PathwayID	Definition	Fisher's P-value	Genes
hsa05014	Amyotrophic lateral sclerosis (ALS) - *Homo sapiens* (human)	0.04095384	ALS2//CYCS//MAPK13//RAB5A//RAC1
hsa04964	Proximal tubule bicarbonate reclamation - *Homo sapiens* (human)	0.0480162	ATP1B1//GLS//SLC38A3
hsa05133	Pertussis - *Homo sapiens* (human)	0.0495752	IL1B//IL8//JUN//LY96//MAPK13//MAPK9

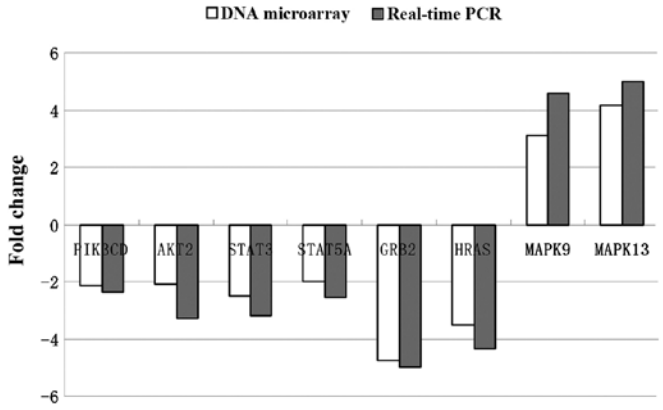

Figure 2. Quantitative real-time PCR confirmation of the microarray data. The real-time PCR results of these genes were in agreement with the microarray data. GAPDH was used as the internal control, and each sample was detected in triplicate.

tively by microarray and real-time qPCR were not completely the same, the general trends were. This was due to some noise that was inevitable. Nevertheless, these results still confirm our findings of differential gene expression by microarray analysis.

Pathway analysis. We proceeded with pathway analysis based on the KEGG database to illustrate all the available pathways containing differentially expressed genes. The significant pathways affected due to upregulated and downregulated genes are shown in Tables III and IV, respectively.

Discussion

Nucleostemin (NS) is a protein mainly expressed on stem and tumor cells, and it is important to maintain early embryonic development and tumor cell proliferation (1,3,4). Most studies consider that NS could inhibit the antineoplastic function of p53 through combining with it (1), but some researchers still consider that NS could also play its function independent of p53 (10-15). However, the specific mechanism of this p53-independent pathway has yet to be elucidated.

In this study, we used lentivirus-mediated RNA interference and DNA microarray technology to investigate the differences of gene expressing profiling after knocking down NS in HL-60 cells, which was p53-null, in order to further explore the specific mechanism of NS p53-independent pathway. Our study showed that knockdown of NS in HL-60 cells could modulate the expression of extensive genes. Next, we proceeded to pathway analysis to explore how NS plays its function independent of p53. In all the pathways which changed significantly, the acute myeloid leukemia pathway (Pathway ID: has05221) warranted further attention, since HL-60 was an acute myeloid leukemia cell line. The PI3K-AKT, JAK-STAT and MAPK pathways are three main pathways included in acute myeloid leukemia (Fig. 3).

The PI3K-AKT pathway is one of the most important cellular signal transduction pathways involved in the regulation of proliferation. It plays its role of inhibiting apoptosis and promoting proliferation in cells through impacting the activated state of a variety of downstream effectors, and is closely related to the occurrence of many human tumors. After PI3K is activated by upstream molecules, it phosphorylates PI (4,5) P2 on plasma membrane to PI (3,4,5) P3, the latter could translocate AKT to plasma membrane to activate it. Next, the activated AKT could activate or inhibit its downstream target proteins such as Bad, Caspase 9, NF-κB, Forkhead, mTOR, Par-4 and P21 through phosphorylation, which mediate cell growth and promote cell survival induced by insulin and types of growth factors; it is an important antiapoptotic factor (16,17). In addition, AKT could regulate the activity of IKK, cause the nuclear translocation of NF-κB, and then promote the transcription of NF-κB-dependent survival genes to facilitate cell survival (18); also, the inhibition of NF-κB could promote cell apoptosis (19). In this research, after knocking down NS expression in HL-60 cells, the key point in the PI3K-AKT pathway including PI3K (PIK3CD), AKT2, IKK (IκBKG) and NF-κB were all downregulated (Fig. 3). This suggested that the inhibition of the PI3K/AKT/NF-κB pathway may participate in the apoptosis caused by knocking down NS in HL-60 cells.

The continuous activation of the JAK-STAT pathway is widespread in leukemia cells; the activated key point STAT could induce abnormal expression of genes closely related to cell proliferation, differentiation and apoptosis, and then promote cell proliferation and inhibit apoptosis through various pathways (20,21). STAT family members are closely related to tumors, especially STAT3 and STAT5A. Some studies reported that inhibition of STAT5A could block the

Table IV. Pathway analysis of downregulated genes.

PathwayID	Definition	Fisher's P-value	Genes
hsa03040	Spliceosome - *Homo sapiens* (human)	3.71589E-08	ACIN1//BUD31//CHERP//CTNNBL1//DDX39B//DHX38//HNRNPA1L2//HNRNPA3//HNRNPC//HNRNPK//HSPA8//ISY1//LSM4//NHP2L1//PPIE//PQBP1//PRPF31//PRPF6//PRPF8//RBMX//SF3B2//SF3B4//SNRNP40//SNRNP70//SNRPA//SNRPB//SNRPC//SRSF3//SRSF9//TCERG1//THOC1//THOC3//THOC4//TRA2B
hsa03013	RNA transport - *Homo sapiens* (human)	2.51665E-05	AAAS//ACIN1//DDX39B//EEF1A1//EIF2B4//EIF2B5//EIF3C//EIF3F//EIF3G//EIF4A1//EIF4B//EIF4G1//GEMIN8//NUP188//NUP210//NUP214//NUP62//NUP93//NXF1//POM121//PRMT5//RNPS1//SEC13//SNUPN//TACC3//THOC1//THOC3//THOC4//THOC5//THOC6//WIBG//XPO5
hsa03410	Base excision repair - *Homo sapiens* (human)	0.000263071	APEX1//FEN1//HMGB1//LIG1//MPG//NTHL1//OGG1//PARP1//POLD2//POLE//XRCC1
hsa00520	Amino sugar and nucleotide sugar metabolism - *Homo sapiens* (human)	0.00053131	GALE//GALK1//GALT//GMPPA//GMPPB//GNPDA1//GNPNAT1//HEXA//NAGK//NPL//PMM1//PMM2//TSTA3
hsa05221	Acute myeloid leukemia - *Homo sapiens* (human)	0.001151056	AKT2//ARAF//CEBPA//GRB2//HRAS//IKBKG//MAP2K2//PIK3CD//RAF1//RELA//RPS6KB1//STAT3//STAT5A//ZBTB16
hsa04142	Lysosome - *Homo sapiens* (human)	0.001392349	ABCA2//AP1M1//AP4B1//ATP6AP1//ATP6V0D1//CLTCL1//CTSA//CTSC//CTSD//CTSL1//DNASE2//GALNS//GBA//GGA1//GNPTG//HEXA//IGF2R//LAMP2//MAN2B1//MCOLN1//NAGLU//PPT1//PSAP
hsa04141	Protein processing in endoplasmic reticulum - *Homo sapiens* (human)	0.001583112	AMFR//BAK1//CANX//CAPN1//DDIT3//DNAJC5G//EDEM2//ERP29//HSP90AA1//HSP90AB1//HSPA8//LMAN1L//LMAN2//MAN1B1//NSFL1C//P4HB//PDIA4//PPP1R15A//PRKCSH//RAD23B//RPN1//RRBP1//SEC13//SEC61A1//SSR2//STUB1//UBE2J2//UBQLN4//UBXN6
hsa03050	Proteasome - *Homo sapiens* (human)	0.003429877	PSMA7//PSMB9//PSMC3//PSMC4//PSMC5//PSMD13//PSMD2//PSMD3//PSMD8//PSME1//PSMF1
hsa05213	Endometrial cancer - *Homo sapiens* (human)	0.003806897	AKT2//ARAF//AXIN1//ELK1//FOXO3//GRB2//HRAS//ILK//MAP2K2//PDPK1//PIK3CD//RAF1
hsa04666	Fc γ R-mediated phagocytosis - *Homo sapiens* (human)	0.004610906	AKT2//ARPC1B//CFL1//DNM2//GSN//HCK//LAT//NCF1//PIK3CD//PIP5K1C//PRKCD//PTPRC//RAC2//RAF1//RPS6KB1//VASP//WAS//WASF2
hsa04662	B cell receptor signaling pathway - *Homo sapiens* (human)	0.005562133	AKT2//CD79B//CD81//GRB2//HRAS//IFITM1//IKBKG//LILRB3//MAP2K2//NFKBIB//PIK3CD//PTPN6//RAC2//RAF1//RELA
hsa00310	Lysine degradation - *Homo sapiens* (human)	0.006858597	DOT1L//EHMT2//GCDH//GLT25D1//HADHA//MLL4//PLOD1//SETD1B//SETD2//SUV39H1//WHSC1
hsa04910	Insulin signaling pathway - *Homo sapiens* (human)	0.007757841	AKT2//ARAF//CALML3//ELK1//EXOC7//FLOT1//FLOT2//GRB2//HRAS//MAP2K2//MKNK2//PCK2//PDPK1//PHKG2//PIK3CD//PPP1CA//PRKAG1//PRKAR1B//PYGB//RAF1//RPS6//RPS6KB1//TSC2
hsa05130	Pathogenic *Escherichia coli* infection - *Homo sapiens* (human)	0.008284432	ABL1//ARHGEF2//ARPC1B//TUBA1A//TUBA1C//TUBA3C//TUBA4A//TUBB//TUBB2C//TUBB8//WAS//YWHAZ
hsa04150	mTOR signaling pathway - *Homo sapiens* (human)	0.0108534	AKT2//DDIT4//EIF4B//MLST8//PDPK1//PIK3CD//RPS6//RPS6KA1//RPS6KB1//TSC2//ULK3

Table IV. Continued.

PathwayID	Definition	Fisher's P-value	Genes
hsa04722	Neurotrophin signaling pathway - *Homo sapiens* (human)	0.01165631	ABL1//AKT2//ARHGDIA//CALML3//CAMK2G//FOXO3//GRB2//HRAS//IRAK1//MAP2K2//MAPKAPK2//NFKBIB//PIK3CD//PRKCD//RAF1//RELA//RPS6KA1//RPS6KA4//YWHAE//YWHAZ//ZNF274
hsa00511	Other glycan degradation - *Homo sapiens* (human)	0.02057153	GBA//HEXA//MAN2B1//MAN2B2//MAN2C1
hsa03030	DNA replication - *Homo sapiens* (human)	0.0211445	FEN1//LIG1//MCM5//MCM7//POLA2//POLD2//POLE//RFC2
hsa00480	Glutathione metabolism - *Homo sapiens* (human)	0.02164977	G6PD//GGT1//GPX2//GPX4//GSS//GSTK1//GSTZ1//IDH2//PGD//SRM
hsa05140	Leishmaniasis - *Homo sapiens* (human)	0.02422244	C3//CYBA//ELK1//IRAK1//ITGA4//ITGB2//NCF1//NCF4//NFKBIB//PTPN6//RELA//TAB1//TGFB1
hsa05220	Chronic myeloid leukemia - *Homo sapiens* (human)	0.02422244	ABL1//AKT2//ARAF//CTBP1//GRB2//HRAS//IKBKG//MAP2K2//PIK3CD//RAF1//RELA//STAT5A//TGFB1
hsa03015	mRNA surveillance pathway - *Homo sapiens* (human)	0.03338131	ACIN1//CPSF1//DDX39B//NXF1//PABPN1//PPP2R1A//PPP2R3B//PPP2R5D//RNGTT//RNPS1//SMG5//SMG6//THOC4//WIBG
hsa03420	Nucleotide excision repair - *Homo sapiens* (human)	0.04168682	DDB2//ERCC1//ERCC2//GTF2H4//LIG1//POLD2//POLE//RAD23B//RFC2

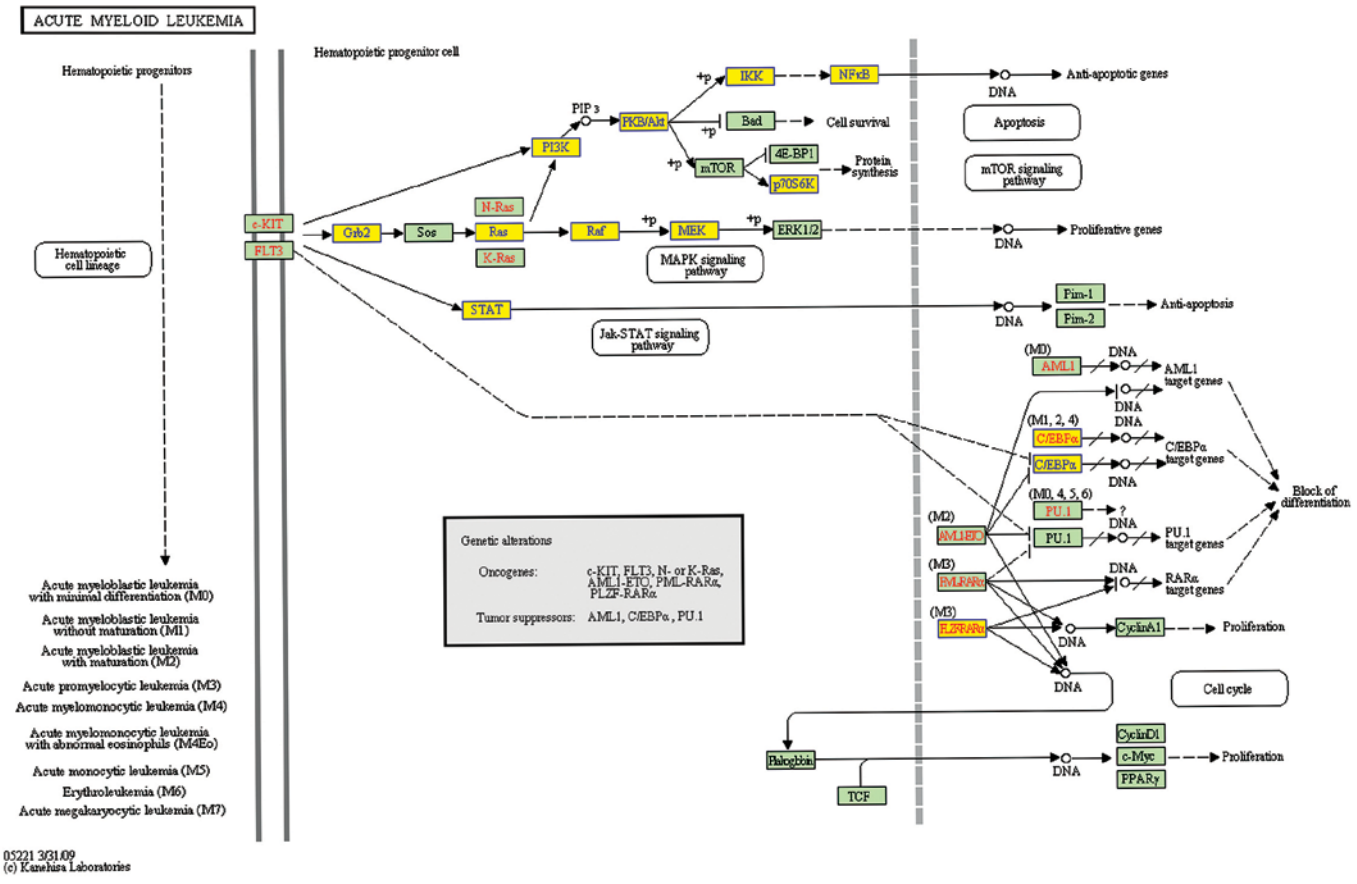

Figure 3. Acute myeloid leukemia pathway. Yellow marked nodes are associated with downregulated genes, green nodes have no significance.

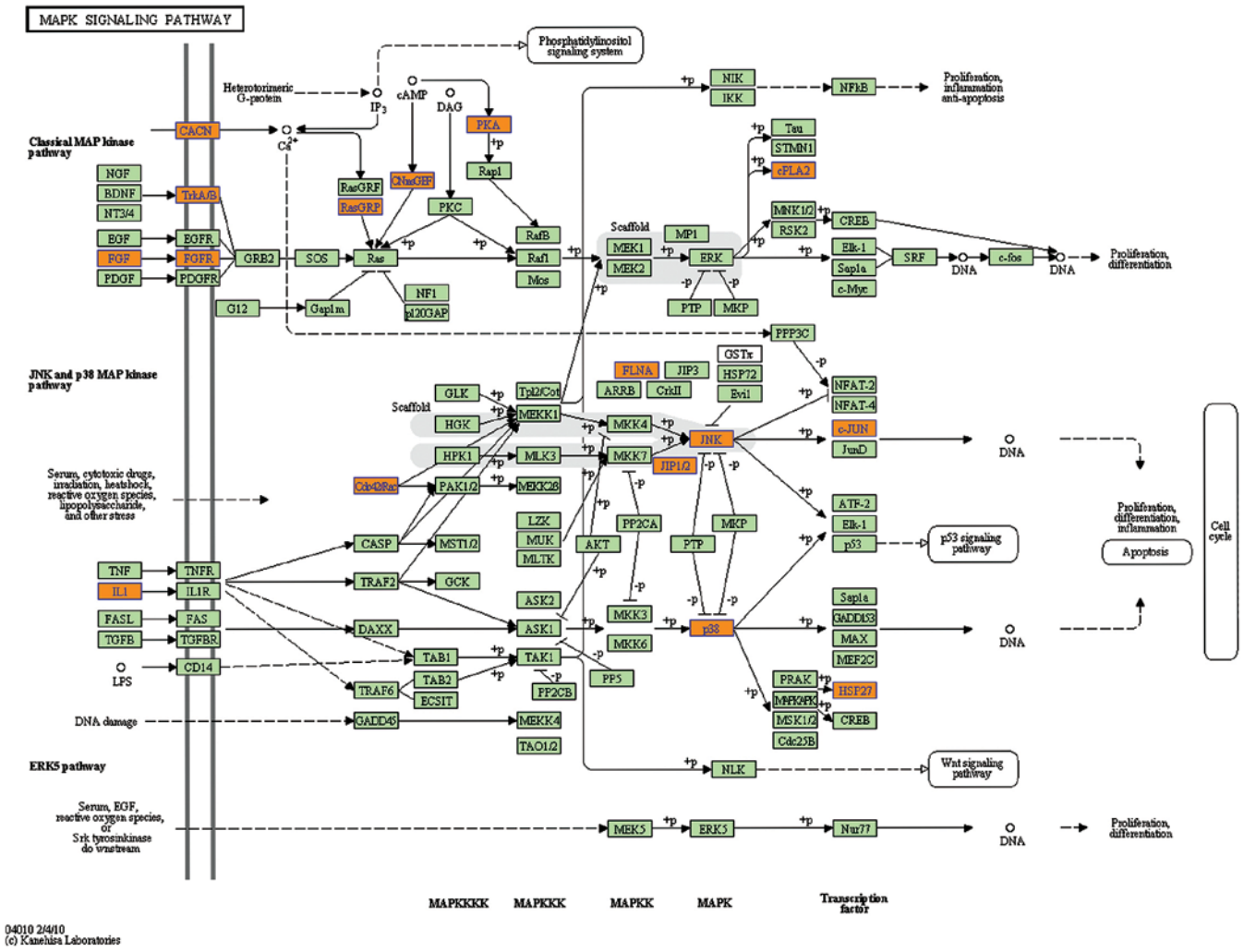

Figure 4. MAPK signaling pathway. Orange marked nodes are associated with upregulated or only whole dataset genes, green nodes have no significance.

growth of prostate cancer cells (22); inhibition of STAT3 could promote the apoptosis of bladder cancer cells (23); high-dose methylprednisone induced HL-60 cell apoptosis, and this process was accompanied by downregulation of STAT5A protein (24). In the present study, STAT3 and STAT5A were both downregulated after the inhibition of NS, indicating that downregulation of STAT may be another reason for cell apoptosis after knocking down NS in HL-60 cells.

Mitogen-activated protein kinase (MAPK) is a type of serine-threonine protein kinase existing in cells and plays an important role in cell signal transduction. MAPK pathways mainly include the RAS-RAF-MEK-ERK1/2, the JNK and the P38MAPK pathway (25). A previous study reported that the RAS-RAF-MEK-ERK1/2 pathway may have an important impact on the proliferation and differentiation of melanoma cells (26); inhibiting this pathway could be an effective approach for antitumor therapy (27-29). Some antineoplastic drugs could induce cell apoptosis mainly through the activation of JNK and P38MAPK pathway (30-32). Herein, we found genes involved in the RAS-RAF-MEK-ERK1/2 pathway, including GRB2, RAS (HRAS), RAF (RAF1), MEK2 (MAP2K2), were all downregulated (Fig. 3), while JNK (MAPK9) and P38 (MAPK13) were upregulated (Fig. 4). This suggested that the inhibition of RAS-RAF-MEK-ERK1/2 pathway and activation of JNK as well as P38MAPK pathway may also involved in HL-60 cell apoptosis caused by knocking down the expression of NS.

The pathway analysis of downregulated genes showed that spliceosome (Pathway ID: hsa03040) and RNA transport (Pathway ID: hsa03013) were much more significant. Spliceosome plays a critical role in processing pre-mRNA and folding mRNA. The data of our research showed that many genes participated in the formation of spliceosome, therefore it was speculated that knocking down NS may influence the cellular gene expression through preventing RNA processing and transport. Romanova *et al* presented a similar viewpoint, that knocking down NS had an impact on the processing of rRNA, folding and stabilization of other RNAs (33,34).

In conclusion, we detected the changes of gene expression profiles in HL-60 cells which were p53-null after knocking down NS expression, in order to explore how NS plays its function without the existence of p53. The DNA microarray data showed a large number of genes were differentially expressed. Pathway analysis indicated that after NS was inhibited in HL-60 cells, especially inhibition of the PI3K-AKT pathway, the JAK-STAT pathway and RAS-RAF-MEK-ERK1/2, and the activation of JNK pathway as well as P38MAPK pathway may be involved in the cell apoptosis caused by knocking down

NS. This study provides insight into exploring the functional mechanism of NS independent of p53, and lays the foundations for the search of new effective therapeutic targets of p53-null leukemia and even p53-null tumors.

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