Identification of NOL8, a nucleolar protein containing an RNA recognition motif (RRM), which was overexpressed in diffuse-type gastric cancer

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(Received January 13, 2004/Revised March 6, 2004/Accepted March 9, 2004)

In an attempt to identify novel therapeutic targets for diffuse-type gastric cancer, we had previously compared expression profiles of 20 diffuse-type gastric-cancer tissues with corresponding non-cancerous mucosae by means of a cDNA microarray consisting of 23,040 genes. Among 153 genes whose expression levels were elevated in cancers compared to non-cancerous mucosae, we focused on a gene termed *NOL8* that encodes a putative 150-kDa protein with an RNA-recognition motif (RRM) domain in its amino-acid terminal region. Comparison of expression profiles between diffuse-type and intestinal-type gastric cancers showed that NOL8 was specifically up-regulated in diffuse-type cancers. Northern blot analysis revealed that *NOL8* was expressed in skeletal muscle, but not expressed or hardly detectable in 22 other tissues examined. Immunocytochemical staining of NOL8 showed specific localization in the nucleolus. Subsequent protein phosphatase analysis coupled with western analysis revealed the presence of the phosphorylated form. Furthermore, transfection of short-interfering RNA (siRNA) specific to *NOL8* into three diffuse-type gastric cancer cells, St-4, MKN45 and TMK-1, effectively reduced expression of this gene and induced apoptosis in these cells. These findings provide a new insight into diffuse-type gastric carcinogenesis and may contribute to the development of new therapeutic strategies for diffuse-type gastric cancer. (Cancer Sci 2004; 95: 430–435)

Gastric cancer is the fourth leading cause of cancer-related death in the world.[1)] Development of a diagnostic strategy using endoscopy has facilitated the early detection of gastric cancers that are curable by surgical resection. However, prognosis of gastric cancer at an advanced stage remains very poor due to the limited effectiveness of chemotherapy for gastric cancer; the response rates of presently available anticancer drugs such as 5-FU, doxorubicin, epirubicin, mitomicin C, etoposide and cisplatin range between 19 to 36%.[2)] Furthermore, since these drugs have cytotoxic and/or cytostatic effects on both normal and cancer cells, severe or even lethal adverse reactions are observed in a subset of patients. In addition, radiotherapy for cancers in the upper abdomen is technically difficult and often poorly tolerated. Therefore development of novel therapeutic approaches is an issue of great and urgent clinical importance.

Recently, intensive studies on the molecular mechanisms of carcinogenesis have led to the identification of molecular targets for development of new anti-tumor agents. For example, HER2/neu, a receptor that is overexpressed in approximately 30% of breast cancers, mediates growth signals in response to binding of its specific ligand. A combination therapy of anticancer drugs and an anti-HER2 monoclonal antibody, trastuzumab, that antagonizes growth signaling by this ligand-receptor, improved clinical response as well as overall survival in a subset of breast-cancer patients who had HER2/neu overexpression.[3)] A tyrosine kinase inhibitor, STI-571, which selectively inactivates the bcr-abl fusion protein, is very effective for the treatment of chronic myelogenous leukemias where constitutive activation of bcr-abl tyrosine kinase plays a crucial role in the transformation of leukocytes.[4)] Recently, a novel drug designed to inhibit EGFR tyrosine kinase, EGFR-TKI (Gefitinib) or ZD1839 (Iressa), has been reported to be highly effective against non-small-cell lung cancer, because the EGFR-signaling pathway plays an important role in tumor progression, including cell proliferation, angiogenesis and inhibition of apoptosis.[5)] These results suggest that inhibition of gene products with oncogenic activity that are up-regulated specifically in cancer cells is a promising strategy for development of novel anti-cancer agents. This notion prompted us to survey genes up-regulated in cancers in our microarray data.

The nucleolus is the site of ribosome biogenesis, where ribosomal RNA is transcribed, modified, assembled with ribosomal proteins and exported to the cytoplasm to form mature ribosomes. The nucleolus is also involved in mitosis and meiosis, regulation of cell cycle progression, and gene expression.[6)] An active nucleolus is crucial for nuclear architecture, functional compartmentalization of the nucleus and control of cell proliferation. Proteomic analyses suggested that more than 200 proteins function in the nucleolus. These proteins include ribosomal proteins, proteins associated with ribosome biogenesis or mRNA metabolism, translation factors, chaperones, fibrous proteins and others. To clarify the molecular mechanisms underlying the biological processes in the nucleolus, it is necessary to uncover the functions of nucleolar proteins.

Gastric cancers are histologically classified into diffuse type (infiltrating, poorly differentiated, non-cohesive cancer cells with vast fibrous stroma) and intestinal type (cohesive, glandular-like cell groups).[7)] These two types have significant differences in their epidemiology, etiology, pathogenesis and biological behavior. The diffuse type is observed in relatively younger individuals regardless of gender, and often metastasizes to peritoneum or lymph nodes, having a poorer prognosis as a result. To identify novel therapeutic targets for diffuse-type gastric cancer, we compared in our previous study the expression profiles of 20 diffuse-type gastric cancer tissues with those of the corresponding non-cancerous mucosae by means of a cDNA microarray consisting of 23,040 genes. In the present study, we demonstrate that a novel nucleolar protein, NOL8, is frequently up-regulated in diffuse-type gastric cancer, and that its decreased expression results in induction of apoptosis in cancer cells. These data should lead to a better understanding of gastric tumorigenesis, and thereby facilitate the development of novel strategies for diagnosis and/or treatment of diffuse-type gastric cancer.

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Materials and Methods

Cell lines and clinical materials. Human diffuse-type gastric cell lines (MKN45 and MKN1), mouse fibroblast cell line (NIH3T3), transformed human embryonic kidney cell line (293T) and monkey kidney cell line (COS-7) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Other human diffuse-type gastric cell lines, St-4 and TMK-1, were kindly provided by Dr. Tsuruo (Cancer Institute, Tokyo) and Dr. Yasui (Hiroshima University School of Medicine), respectively. All cells were cultured as monolayers in appropriate media; RPMI1640 (Sigma-Aldrich Corp., St. Louis, MO) for St-4, MKN45 and MKN1; DMEM (Sigma-Aldrich) for TMK-1, 293T, COS7 and NIH3T3; each was supplemented with 10% fetal bovine serum (Cansera International, Inc., Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO_2. Cancerous tissues and corresponding non-cancerous mucosae were excised from 20 patients during surgery, after informed consent had been obtained.

Multiple-tissue northern blot analysis. Human multiple-tissue blots (BD Bioscience, Palo Alto, CA) were hybridized with a ^{32}P-labeled PCR product of *NOL8*, which had been labeled by random-oligonucleotide priming with a Mega Label kit (Amersham Biosciences, Buckinghamshire, UK). The PCR product was prepared by RT-PCR using the primer set 5′-TTAAGCAATTCTACAAACAGAGG-3′ and 5′-AGCCTTGTTCACATTCAGTATC-3′. Prehybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at −80°C for 7 days.

Semi-quantitative RT-PCR. Total RNA was extracted from cultured cells using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Extracted RNA was treated with Dnase I (Roche Diagnostics, Mannheim, Germany) and reverse-transcribed to single-stranded cDNAs using oligo$(dT)_{12-18}$ primer with Superscript II reverse transcriptase (Invitrogen). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring the farnesyl-diphosphate farnesyltransferase 1 gene (*FDFT1*) and glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) gene as a quantitative control for RT-PCR of aRNAs and siRNA experiments, respectively. Primer sequences were 5′-TGTGTGGCTGGGACCTTTAGGAA-3′ and 5′-TCATTCTAGCCAGGATCATACTAAG-3′ for *FDFT1*; and 5′-ACAACAGCCTCAAGATCATCAG-3′ and 5′-GGTCCACCACTGACACGTTG-3′ for *GAPDH*; and 5′-ATATGAGCAGGAACTCTGGGAG-3′ and 5′-CTCAGGATAGAGGGCAAAGAGA-3′ for *NOL8*. All of the reactions involved initial denaturation at 94°C for 2 min followed by 25 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s (for *FDFT1*); or 18 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s (for *GAPDH*); or 30 cycles at 94°C for 30 s, 61°C for 30 s and 72°C for 30 s (for *NOL8*), on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA).

Construction of psiH1BX-NOL8. Plasmids expressing siRNAs specific to NOL8 were prepared by cloning the following double-stranded oligonucleotides into psiH1BX3.0 vector that was constructed by our group as described previously[8]: 5′-TCCCGTGCAGAAGGATGAGAGTTTTCAAGAGAAACTCTCATCCTTCTGCAC-3′ and 5′-AAAAGTGCAGAAGGATGAGAGTTTCTCTTGAAAACTCTCATCCTTCTGCAC-3′ for siA; 5′-TCCCTGGCTCATTAGGTGAAGTGTTCAAGAGACACTTCACCTAATGAGCCA-3′ and 5′-AAAATGGCTCATTAGGTGAAGTGTCTCTTGAACACTTCACCTAATGAGCCA-3′ for siB. The forward oligonucleotides and their complements were each phosphorylated by incubation with T4-polynucleotide kinase at 37°C for 30 min, followed by boiling and slow cooling to anneal the two oligonucleotides. Each product was ligated into psiH1BX3.0 to create a siRNA expression vector against *NOL8*. Total RNA was extracted from the cells 48 h after transfection and the gene-silencing effect of NOL8-siRNA was subsequently examined by semi-quantitative RT-PCR.

Growth-suppressive effect of NOL8-siRNAs. St-4, MKN45 and TMK-1 cells plated onto 10-cm dishes (1×10^6 cells/dish) were transfected with psiH1BX-NOL8 or psiH1BX-EGFP plasmids using a Nucleofector kit V according to the supplier's recommendations (Amaxa Biosystems, Cologne, Germany), and maintained in media containing 10% fetal bovine serum with appropriate concentrations of Geneticin for 10 days. The cells were then fixed with 100% methanol and stained with Giemsa solution. Cell viability was additionally measured by MTT assay using a cell-counting kit 8 (Dojindo, Kumamoto) as described elsewhere.[8]

Western blotting and λ-protein phosphatase assay. The entire coding region of human *NOL8* was amplified by RT-PCR using primers 5′-CCAGGTACCTGCCCAGCCTTCATGAAAGTGAAC-3′ (forward) and 5′-CATGAGCTCTTTTGGTTTCATTTTCCTTTTTGCGTC-3′ (reverse), and the PCR product was cloned into an appropriate enzyme site of the pcDNA3.1-myc/His expression vector (Invitrogen). Plasmids expressing NOL8 (pcDNA3.1-myc/His-NOL8), or the control vector (pcDNA3.1-myc/His) were transfected into 293T cells. Preparation of cell extract and PAGE were performed as described previously.[9] Extracts from 293T cells transfected with pcDNA3.1-myc/His-NOL8 were incubated with or without Lambda protein phosphatase (λ-PPase) (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Immunoblotting was carried out with mouse anti-c-Myc monoclonal antibody (9E10) (Santa Cruz Biotechnologies, Santa Cruz, CA) as the first antibody, and HRP-conjugated sheep anti-mouse IgG (Amersham Pharmacia, Little Chalfont, UK) served as the secondary antibody for the ECL detection system (Amersham Pharmacia).

Immunocytochemical staining. St-4, MKN1 and COS-7 cells transfected with pcDNA3.1/Myc-His-NOL8 (Invitrogen) were fixed and stained as described previously.[9] Mouse anti-c-Myc 9E10 monoclonal antibody (Santa Cruz Biotechnologies) diluted 1:1000 was used for the first antibody, and the reaction was visualized after incubation with fluorescein-conjugated anti-mouse IgG secondary antibodies (ICN Biomedicals, Inc., Aurora, OH). Nuclei were counter-stained with 4′,6′-diamidine-2′-phenylindole dihydrochloride (DAPI). Fluorescence images were obtained with a TCS SP2 AOBS Spectral Confocal Scanning System (Leica).

FACS (fluorescence-activated cell sorter) analysis. Cells were fixed with 75% ethanol at 4°C, treated with PBS containing RNase at 37°C for 30 min, and then stained with propidium iodide (40 μg/ml). The percentages of sub-G1 nuclei in the population were determined from at least 2×10^4 ungated cells in a flow cytometer (FACScalibur; Becton Dickinson, San Diego, CA).

Results

Up-regulation of B3873N in diffuse-type gastric cancer. Our genome-wide expression analysis of 20 diffuse-type gastric cancers using a cDNA microarray consisting of 23,040 genes identified 153 genes that exhibited more-than-two fold elevated expression in cancer tissues compared to non-cancerous mucosae in more than 50% of the cases examined. Among the 153 genes, we focused on a gene with an in-house identification number of B3873N, because it was overexpressed in 7 of 12 diffuse-type cancers (data not shown), but in none of the 20 intestinal-type cancers previously analyzed.[10] Subsequent semi-quantitative RT-PCR using RNA samples that had served for

the microarray analysis corroborated its enhanced expression in 6 of the 8 cancers (Fig. 1A). A homology search with the sequence of B3873N in public databases revealed that this gene corresponded to a human gene that had been annotated as *Homo sapiens* hypothetical protein FLJ20736 (GenBank accession number; NM_017948).

Expression and genomic structure of *NOL8*. Northern blot analysis using B3873N cDNA as a probe identified a 4.4-kb transcript that was expressed in human skeletal muscle but was not or was hardly detectable in the remaining 22 tissues examined (Fig. 1B). Since assembled cDNAs sequences of B3873N reported in the public databases did not cover the entire transcript, we carried out an exon-connection experiment on the basis of computer-predicted exon-like sequences in the human genome sequence harboring the gene (GenBank accession number; NT_008476) using polyA RNA from St-4 cells as a template. As a result, we obtained a cDNA sequence of 4302 nucleotides (GenBank accession number; AB105104) encoding a putative 1167-amino acid protein with a predicted molecular mass of 131.6 kDa. Comparison of the cDNA sequence with the genomic sequence allowed us to determine that the *NOL8* gene consists of 16 exons and spans a genomic region of approximately 30 kb on chromosomal band 9q22.32. A search for protein motifs in the predicted amino-acid sequence using SMART (http://smart/embl-heidelberg/de/), and CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) identified an RRM domain in the amino-acid terminal between codons 9 and 85 and a KOG4365 domain between codons 582 and 1154 (Fig. 1C). Furthermore, the PSORT II program (http://psort.nibb.ac.jp/form2.html) predicted the subcellular localization of B3873N in the nucleus. According to its subcellular localization as described later in the results, we termed this gene *NOL8* (Nucleolar Protein 8).

We next examined expression of *NOL8* in gastric cancer cell lines MKN1, MKN7, MKN28, MKN74, St-4, MKN45 and Kato III, as well as skeletal muscle, by semi-quantitative RT-PCR (Fig. 1D); the expression level of NOL8 in the seven gastric cancer cells was relatively higher than in skeletal muscle. Among the seven cell lines examined, Kato III most abundantly expressed *NOL8*, and MKN1 showed the lowest expression level.

Phosphorylation and nucleolar localization of NOL8. To investigate the function of NOL8 protein, we amplified and cloned its entire coding region into pcDNA3.1/Myc-His vector (pcDNA3.1/Myc-His-NOL8). Immunoblot analysis using cellular extracts of 293T transfected with pcDNA3.1/Myc-His-NOL8 revealed a band of approximately 150 kDa that was larger than the predicted molecular weight of myc-/His-tagged NOL8 and non-specific bands between 75 and 90 kDa. Therefore, we hypothesized that NOL8 was modified in mammalian cells. Since the amino acid sequence contained several putative serine/threonine/tyrosine phosphorylation sites predicted by the NetPhos 2.0 Server computer program (http://www.cbs.dtu.dk/services/NetPhos/), we examined the protein phosphorylation status. We incubated extracts from the transfected cells in the presence or absence of λ-protein phosphatase, and analyzed the molecular weight of tagged NOL8 protein by western blot analysis. As expected, the measured weight of the tagged NOL8 protein in the extracts treated with phosphatase was smaller than that in the untreated cells, and corresponded to the expected molecular weight of 140 kDa. These data indicated that NOL8 protein was possibly phosphorylated in mammalian cells (Fig. 2A). To investigate its subcel-

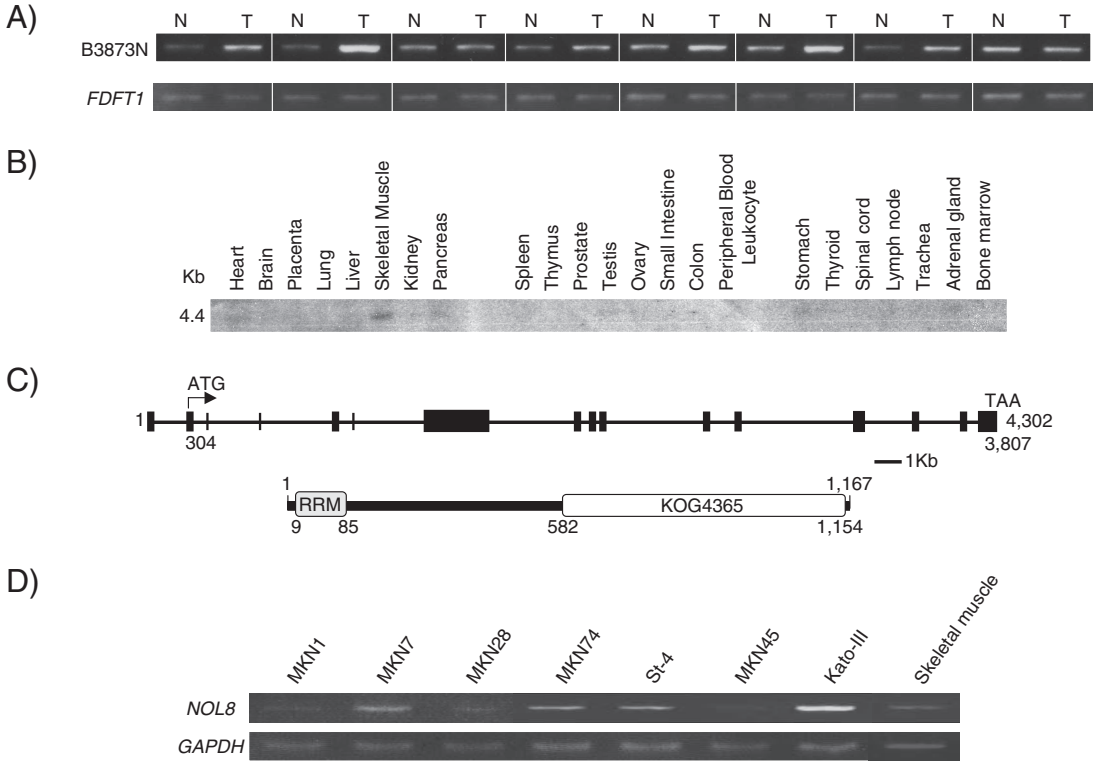

Fig. 1. Expression and genomic structure of *NOL8*. A) Expression of B3873N in diffuse-type gastric cancers (T) and corresponding non-cancerous epithelia (N) by semi-quantitative RT-PCR. Expression of *FDFT1* served as an internal control (FDFT1 showed the smallest T/N fluctuation in our microarray data). B) Northern blot analysis of B3873N in various human tissues. The transcript of B3873N is approximately 4.4 kb in size. C) Genomic structure of the *NOL8* gene and motifs of the predicted NOL8 protein. RRM, RNA recognition motif; KOG4365, a conserved domain in eukaryotes with unknown function. D) Comparison of *NOL8* expression level in seven gastric cancer cell lines and skeletal muscle by semi-quantitative RT-PCR. Expression of *GAPDH* served as an internal control.

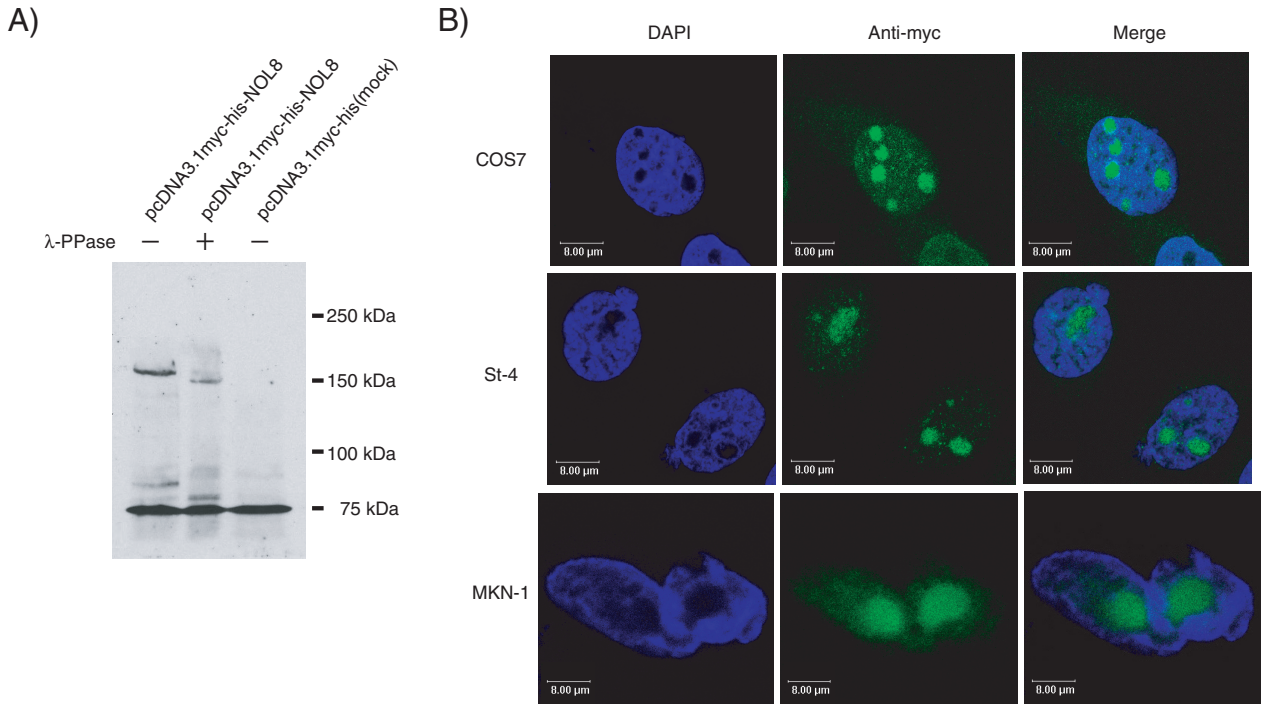

Fig. 2. Phosphorylation and subcellular localization of myc-tagged NOL8 protein. A) Phosphatase (λ-PPase) assay of myc-tagged NOL8 protein in 293T cells transfected with pcDNA3.1/Myc-His-NOL8 (lane 1, whole extract; lane 2, whole extract treated with λ-PPase or mock vector (lane 3)). B) The cells were stained with mouse anti-myc monoclonal antibody and visualized by FITC-conjugated anti-mouse IgG secondary antibody. Nuclei were counter-stained with DAPI.

lular localization, we transfected COS-7, St-4 and MKN1 cells with pcDNA3.1/Myc-His-NOL8 and performed immunocytochemical staining with anti-c-Myc antibody. The tagged NOL8 protein was selectively stained in nucleoli of all cells examined, but was not observed in mock-transfected cells (Fig. 2B).

Effect of NOL8-siRNAs on growth of diffuse-type gastric cancer cells. We then constructed plasmids expressing siRNAs specific to *NOL8* under the control of the H1RNA promoter (psiH1BX-NOL8). We transfected psiH1BX-NOL8-siA, psiH1BX-NOL8-siB or control vector (psiH1BX-siEGFP) into three gastric cancer cells, St-4, MKN45 and TMK-1 and examined the expression of *NOL8* at 48 h after transfection. Semi-quantitative RT-PCR revealed that psiH1BX-NOL8-siA significantly suppressed endogenous expression of *NOL8* in the cells, while no effect was observed in the cells transfected with psiH1BX-NOL8-siB, or the control (Fig. 3A, data not shown for MKN45). As a consequence of this reduced expression, the growth of St-4, MKN45 and TMK-1 cells transfected with psiH1BX-NOL8-siA was suppressed; the number of viable cells was significantly fewer than those transfected with psiH1BX-NOL8-siB or the control plasmid (Fig. 3, B and C, and data not shown for MKN45).

Effect of NOL8-siRNAs on apoptosis. We further investigated whether the growth-suppressive effect of NOL8-siRNAs was associated with apoptosis induction by FACS analysis. We transfected St-4, MKN45 and TMK-1 cells with psiH1BX-NOL8-siA, psiH1BX-NOL8-siB or psiH1BX-siEGFP and measured the proportions of the sub-G1 fraction; a significant increase of sub-G1 population was observed in response to NOL8-siA compared to NOL8-siB or siEGFP, indicating that suppression of *NOL8* expression induced apoptosis (Fig. 4, A and B, data not shown for St-4 and MKN45). This result suggests that expression of *NOL8* is essential for the survival of these gastric cancer cells.

Discussion

In the study presented here, we revealed that a novel gene, *NOL8* encoding a nucleolar protein was up-regulated in the majority of diffuse-type gastric cancers, but not in intestinal-type gastric cancers. We revealed that reduced expression of *NOL8* suppressed the growth of diffuse-type gastric cancer cells. We also transferred *NOL8* into NIH3T3 cells, but failed to observe an oncogenic effect on the cells (data not shown). We think that the elevated expression of this gene alone does not have growth-enhancing activity, but the lack of this gene product has a critical effect on the survival of diffuse-type gastric cancer cells.

The nucleolus has many important functions, including ribosome biogenesis and modifying small RNAs, assembling ribonucleoprotein, sequestering regulatory molecules, regulating cell-cycle progression and controlling aging.[11] Since the putative amino acid sequence of NOL8 contains an RNA recognition motif (RRM), one of the most common and evolutionarily-conserved motifs in the RNA-binding protein family,[12] NOL8 is likely to function as a novel RNA-binding protein. In eukaryotic cells, a large number of different RNA-binding proteins form functional ribonucleoprotein (RNP) complexes associated with various RNA species. RRMs are found in a variety of RNA-binding proteins, including heterogeneous nuclear ribonucleoproteins (hnRNPs), proteins implicated in regulation of alternative splicing, and protein components of small nuclear ribonucleoproteins (snRNPs). In view of its nucleolar localization, NOL8 may also be involved in regulation of gene expression at the post-transcriptional level or in ribosome biogenesis in cancer cells.

Since suppression of *NOL8* by siRNAs resulted in significant growth suppression in diffuse-type gastric cancer cells through induction of apoptosis, expression of *NOL8* could be essential

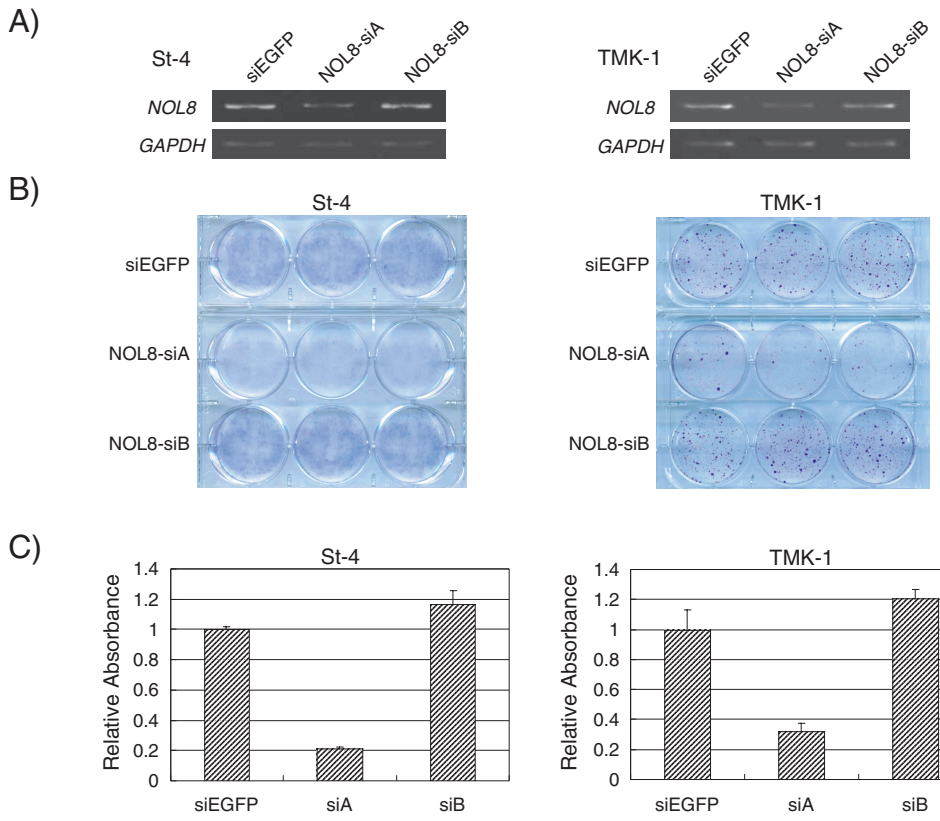

Fig. 3. Growth-suppressive effect of NOL8-siRNAs. A) Knockdown effect of siRNAs on expression of NOL8. Expression of NOL8 at 48 h after transfection was analyzed by semi-quantitative RT-PCR in St-4 and TMK-1 cells. B) Colony formation assays of St-4 and TMK-1 cells transfected with siEGFP, NOL8-siA and NOL8-siB. Transfected cells were selected by treatment with Geneticin for 10 days. C) Quantitative analysis of viable cells in response to the control and NOL8-siRNAs was carried out in triplicate using a Cell Counting Kit 8.

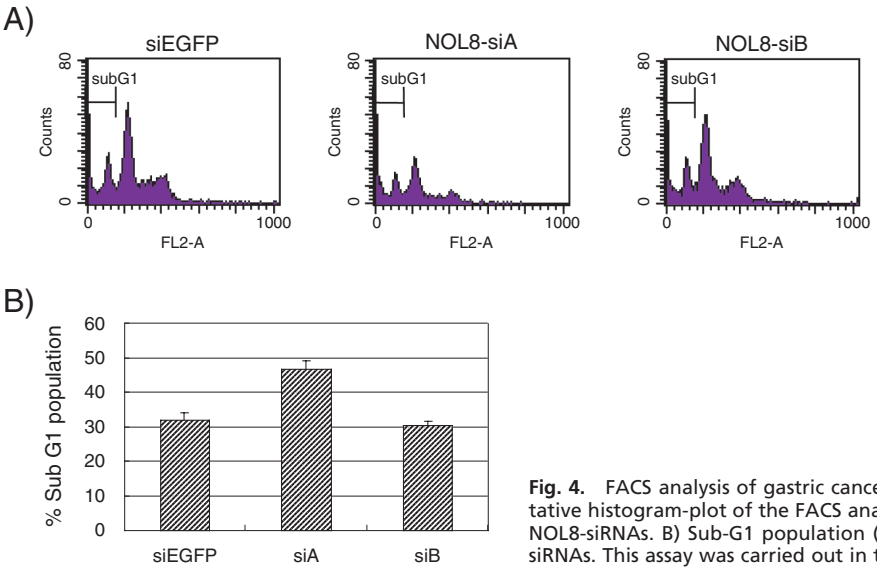

Fig. 4. FACS analysis of gastric cancer cells transfected with NOL8-siRNAs. A) Representative histogram-plot of the FACS analysis of TMK-1 cells treated with control (siEGFP) or NOL8-siRNAs. B) Sub-G1 population (%) of TMK-1 cells in response to control or NOL8-siRNAs. This assay was carried out in triplicate.

for the survival of diffuse-type cancer cells. Recently, it was reported that some nucleolar proteins play an important role in apoptosis. For example, overexpression of nucleolar PHGPx significantly decreased cell death that was induced by actinomycin D and doxorubicin in rat RBL2H3 cells, suggesting that this nucleolar PHGPx may function as an inhibitor of apoptosis and/or a suppressor of nucleolar damage.[13] One of the major nucleolar proteins, nucleolin, is directly involved in the regulation of ribosome synthesis, the regulation of cell proliferation and growth, cytokinesis, replication, embryogenesis and nucleogenesis, indicating that it is fundamental to the survival and proliferation of cells.[14] In addition, nucleolin has been reported to play an important role in apoptosis of leukemia cells,[15] and to act as an activator of HPV18 oncogene transcription in cervical cancer.[16] Therefore, NOL8 may independently play a crucial role for the survival of cancer cells as well. Alternatively, NOL8 may interact with PHGPx or nucleolin, and modulate their function. During the preparation of this manuscript, another group reported that NOL8 binds to G proteins, RRAG A/C/D in the nucleolus.[17] Although they termed the gene as Nop132, we used *NOL8* because it is an approved symbol of HUGO.

Since the expression of *NOL8* was enhanced in the majority of diffuse-type gastric cancers, it may be a good molecular target for treatment of diffuse-type gastric cancer. In this study, we also demonstrated that NOL8 is a phosphorylated protein. A computer program, NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/), predicted a total of 121 putative Ser-Thr-Tyr phosphorylation sites in NOL8 protein. Since phosphorylation is one of the major control mechanisms of protein function, it is of great importance to examine whether phosphorylation is associated with the function of NOL8. Further investigations are of necessity to disclose which amino acid residue(s) is phosphorylated, which molecule phosphorylates NOL8, and how it is regulated. Functional analysis of NOL8 should provide new insight concerning gastric tumorigenesis and may contribute to the development of novel therapeutic strategies for diffuse-type gastric cancer.

We thank Ms. Yumi Nakajima and Ms. Tae Makino for technical assistance, and Dr. Takashi Shimokawa for helpful discussions. This work was supported in part by a Research for the Future Program Grant (00L01402) from the Japan Society for the Promotion of Science.

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