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Tumorigenesis and Neoplastic Progression

NOLC1, an Enhancer of Nasopharyngeal Carcinoma Progression, Is Essential for TP53 to Regulate *MDM2* Expression

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Nasopharyngeal carcinoma (NPC) is one of the most common cancers among Chinese living in South China, Singapore, and Taiwan. At present, its etiological factors are not well defined. To identify which genetic alterations might be involved in NPC pathogenesis, we identified genes that were differentially expressed in NPC cell lines and normal nasomucosal cells using subtractive hybridization and microarray analysis. Most NPC cell lines and biopsy specimens were found to have higher expression levels of the gene encoding nucleolar and coiled-body phosphoprotein 1 (NOLC1) as compared with normal cells. Severe combined immunodeficiency mice bearing NPC xenografts derived from NOLC1-short hairpin-RNA-transfected animals were found to have 82% lower levels of tumor growth than control mice as well as marked tumor cell apoptosis. Measuring the expression levels of genes related to cell growth, apoptosis, and angiogenesis, we found that the *MDM2* gene was down-regulated in the transfectants. Both co-transfection and chromatin immunoprecipitation experiments showed that tumor protein 53-regulated expression of the *MDM2* gene requires co-activation of NOLC1. These findings suggest that NOLC1 plays a role in the regulation of tumorigenesis of NPC and demonstrate that both NOLC1 and tumor protein 53 work together synergistically to activate the *MDM2* promoter in NPC cells. ***(Am J Pathol 2009, 175:342–354; DOI: 10.2353/ajpath.2009.080931)***

Nasopharyngeal carcinoma (NPC) is a malignant tumor with specific racial and geographic distribution patterns. Although it is common in southern China, Taiwan, Singapore, and southeastern Asia, it is rare in Western countries and in neighboring Asian countries, such as Japan.[1] The incidence of NPC in southern China, especially in Guangdong, has been reported to be 25 to 50 per 100,000 individuals.[2] Emigrants from endemic countries to nonendemic areas, such as the United States, maintain this high risk, whereas second- and third-generation offspring have slightly lower risk.[2] The etiology of NPC is multifactorial, but to date, not well defined. However, it has been suggested that environmental factors such as the long-term consumption of salted fish in Hong Kong[3,4] and Malaysian Chinese[5] and the long-term exposure to sulfuric acid vapor in Taiwan,[6,7] can induce the formation of NPC. Genetic factors may also play some role in its development,[7] though until now no gene has been associated with the carcinogenesis of NPC.[8] The Epstein-Barr virus (EBV) has, however, been closely associated with its progression.[2,9–18]

Tumor markers for NPC are urgently needed, but the molecular mechanisms of NPC tumorigenesis remain obscure.[2,9,18] Suppression subtractive hybridization (SSH) has been proven powerful in isolation of differential expressed genes, especially in isolation of rare transcripts.[19–21] Combination of SSH and microarray provides an advantage in the global investigation of changes in gene expression in the biological system.[22,23] In this

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study, we performed these two methods to investigate the differentially expressed genes between NPC and normal nasomucosal (NNM) cells and found high expressions of the gene encoding nucleolar and coiled-body phosphoprotein 1, *NOLC1*, previously also called *hNOPP140* gene, in most NPC cell lines, but low expressions in NNM cells.

Human NOLC1 has a high degree (72% to 73%) of sequence homology with the well-characterized rat homologue, the nucleolar phosphoprotein NOPP140.[24] This protein contains a nuclear localization signal binding sequence and is thought to shuttle between the nucleolus and the cytoplasm.[24] A previous study found NOLC1 to have transcription factor-like activity.[25] By binding to the transcription factor C/EBPβ (also known as AGP/EBP or NF-IL6), NOPP140 can indirectly activate the transcription of the α-1 acid glycoprotein gene.[25] Overexpression of the partial or whole NOLC1 cDNA resulted in mislocalization of nucleolar proteins, improper formation of the nucleolus, and inhibition of rRNA gene transcription. These observations suggest that hNopp140 is crucial for normal cell growth. We were not compelled to study NOLC1 because of these reasons, but because it was overexpressed in NPC cells and may be associated the tumorigenesis of NPC.

The *MDM2* gene is a cellular proto-oncogene, that is often amplified in ~7% of all human cancers.[26] Two promoters have been identified in *MDM2* gene structure: a constitutive promoter and a TP53-response intronic promoter (P2).[27,28] From our previous study, the expression of *MDM2* gene can be indirectly enhanced in the EBV-infected NPC cells through enhancement of TP53 activation.[29]

Using RNA interference *in vivo* to examine the role of NOLC1 in the pathogenesis of NPC, we found that NOLC1 was crucial for NPC cell growth and that reduction of its expression in transfected xenografts resulted in retardation of tumor growth and apparent apoptosis and necrosis. We subsequently examined several genes related to this function and found that the depletion of NOLC1 resulted in a reduction of the *MDM2* expression. Moreover, we found that NOLC1 and TP53 synergistically co-regulated *MDM2* expression in NPC cells.

Materials and Methods

Cell Lines, Tissues, and Surgical Specimens

Fourteen NPC cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Gibco BRL, Gaithersburg, MD). They included NPC-TW01, 02, 03, 04, 05, 06, 07, 08, 09, and 10, all established in our laboratory[30,31]; NPC-CGBM-1, a gift from Dr. S. K. Liao (Chang-Gung University, Taoyuan, Taiwan)[32]; and three other lines CNE1, CNE2, and HONE-1 cells, all originating from China.[33,34] The NPC-TW06 cell line contains a heterozygous point mutation in the tumor protein 53 (*TP53*) gene; the TP53 protein is retained in the cytoplasm and lost the transcriptional activity.[35] TW01, 02, 05, and 08 are keratinizing squamous cell carcinoma lines (World Health Organization Type I), and TW03 is an undifferentiated carcinoma (World Health Organization Type III), also called lymphoepitheliometous carcinoma. The other cell lines were all undifferentiated carcinoma types (World Health Organization Type III). The four NNM cell cultures, NNM-9, NNM-11, NNM-12, and NNM-13, were primary cultured cells from nasal polyps as described previously,[17] and were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum. NPC paraffin blocks were obtained from the archives of the Department of Pathology, National Taiwan University Hospital, Taipei, Taiwan. The use of human specimens in this research was approved by the Institutional Review Board (IRB-926170459) of National Taiwan University Hospital.

RNA Isolation and SSH

SSH was used to isolate genes present in the NPC-TW04 or NNM cells. Total RNAs from these cells were isolated using the acid guanidinium thiocyanate-phenol-chloroform method (TRIzol; Invitrogen Life Technologies, Carlsbad, CA). The mRNA from NPC-TW04 cells was used as the "tester" and the mRNA from NNM cells was used as the "driver" for cDNA subtraction. The construction was performed following the SSH procedure using a PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA). Briefly, equal amounts of mRNA from the tester and driver populations were converted to double-stranded cDNA by reverse transcription followed by digestion with RsaI separately. The digested tester cDNA was subdivided into two populations, each ligated with a different adaptor. Ligation efficiency was evaluated using PCR using primers specific to chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and the adaptor sequences. Following ligation, two hybridization steps was performed. For the first hybridization, an excess of driver was added to each tester, which was denatured, and allowed to anneal. The target sequences in the testers became enriched for differentially expressed genes in NPC-TW04 cells (NPC-TW04$_{\text{-NNM}}$ cDNA). The same procedure was repeated using mRNA from NNM cells as tester and mRNA from NPC-TW04 cells as a reference for cDNA subtraction. This produced other target sequences that also became enriched for differentially expressed in NNM cells (NNM$_{\text{-NPC-TW04}}$ cDNA). In the second hybridization step, the subtracted target cDNAs were specifically amplified by nested PCR using adaptor-specific primer pairs and labeled with the addition of digoxigenin- or biotin-labeled nucleotides (Roche Molecular Biochemicals, Indianapolis, Ind) to obtain the digoxigenin-NPC-TW04$_{\text{-NNM}}$ cDNA and biotin-NNM$_{\text{-NPC-TW04}}$ cDNA, respectively.

cDNA Microarray Analysis and Screening of the cDNA Library with Subtracted Probes

A nylon microarray membrane containing 9600 cloned expressed sequence tag DNAs was constructed using cDNA clones from the National Taiwan University Hospi-

tal Microarray Core Facility for Genomic Medicine, Taiwan. The DNA microarray analysis was based on the colorimetry detection method on human eye recognition. The probes derived from the subtraction experiments were added to the membrane, the substrate was added, and the two colors developed. The image of each DNA dot was digitized by scanning on a high-resolution flat bed scanner (Umax Magic Scan at 3000 dpi). The digitized images were separated into cyan and magenta colors. The most significantly different hybridizing cDNAs were selected for further analysis. Clones containing the chosen expressed sequence tag cDNA were used to screen an NPC cDNA library prepared from the NPC-TW01 cell line in 1998 by Dr. P. Ouyang of the Department of Anatomy, Chang-Gung University, Taiwan, following a standard procedure.[36] The full-length nucleotide sequences were compared with sequences in the database using nucleotide BLAST from the National Center for Biotechnology Information BLAST website (*http://www.ncbi.nlm.nih.gov/BLAST/*).

Touchdown and Quantitative Reverse-Transcription PCR analysis

RNA was obtained from 14 NPC, three NNM, *NS* (nonspecific)-short hairpin (sh)RNA-NPC-TW03, shNOLC1-1-NPC-TW03, and shNOLC1-2-NPC-TW03 cell lines, and four xenograft tumors, as described above in the RNA isolation method. Reverse transcription was performed using the SuperScript First-Strand Synthesis Kit (Invitrogen Life Technologies, Carlsbad, CA). Touchdown PCR was performed for each of 29 genes (Table 1) following procedures described in Don et al,[37] with denaturation performed at 95°C for 30 seconds, anneling of 10 cycles at 65–56° C and 20 cycles at 55°C for 30 sec per cycle, and extension at 72°C for 1 minute. The PCR products were analyzed on a 1.2% agarose gel. The endogenous reference gene was *ACTB* (encoding β-actin). We performed quantitative reverse transcription (QRT-PCR) analysis using the comparative threshold cycle method using an ABI PRISM 7700 Sequence Detector System and SYBR Green PCR Master Mix Kit (Perkin Elmer, Applied Biosystems, Wellesley, MA) , according to the manufacturers' instructions and as described in our recent paper.[17] The endogenous reference gene used was *GAPDH*.

Immunohistochemical Staining

Both NPC cell lines and NPC biopsy specimens were subjected to routine immunohistochemical staining using a monoclonal antibody directed against NOLC1,[38] according to a previously described method.[13] Immunoreactivity, defined as the number of positive tumor cells over total tumor cells, was scored independently by two researchers. The number of NOLC1-positive and negative NPC cells was counted under light microscope at a magnification of ×400, with only the cells displaying brown nucleoli on the section considered NOLC1-positive. For each slide, 7 to 10 microscopic fields were randomly chosen. Positive scores were the categorized into weak staining (only one nucleolus was stained), moderate staining (more than one nucleolus was stained), and strong staining (both nucleus and nucleolus of the tumor cell staining). The average percentage of NOLC1-positive NPC cells was then calculated for each group.

Western Blot Analysis

Lysates from the cultured cells were subjected to routine Western blotting as described previously.[39] The antibodies used were monoclonal anti-mouse antibodies against NOLC1,[38] TP53, MDM2, and α-tubulin, and polyclonal rabbit antibodies against MMP9 and CASP1. Antibodies of TP53, MDM2, MMP9, α-tubulin, and CASP1 were purchased from Lab Vision Co. (Fremont, CA). The results shown are representative of two independent experiments.

Establishment of Stable shRNA Transfectants

The shRNA constructs described in Table 2 were purchased from Open Biosystems (Huntsville, AL). When the NPC cultured cells had reached 70% to 80% confluence, the shRNA constructs were transfected into the NPC cells using the Arrest-In Transfection Reagent for RNAi (Open Biosystems). After incubation for 48 hours, the cells were selected with puromycin to establish of two stable lines: shNOLC1-1-NPC-TW03 and shNOLC1-2-NPC-TW03 line. To avoid the individual clonal variation of gene expression, we used mixed clones of all of the candidate cells that were successfully selected after adding the antibiotics. Then we checked the RNA and protein levels to confirm that these selected clones contained shRNA.

Tumor Growth in Severe Combined Immunodeficient Mice

To establish an animal model for the functional analysis of NOLC1, 24 six-week-old NOD/severe combined immunodeficient female mice were obtained from the National Taiwan University Hospital Experimental Animal Center. The animals were divided into four groups. We injected 1×10^7 NPC-TW03 cells, transfected with NOLC1-1 shRNA, NOLC1-2 shRNA, or the control vector, or the same number of untransfected cells, subcutaneously into the right flanks of six mice in each group separately. One week after cell transplantation, two dimensions of the tumor size were measured with the calipers. The tumor volume was estimated using the equation: length $\times$ width^2 $\times$ 0.52.[29] The tumor sizes were measured once per week. Values are presented as the mean values $\pm$ SEM. After 11 weeks, all animals were examined by routine autopsy and all xenografts were excised, fixed in 4% paraformaldehyde, and embedded in paraffin blocks, or stored at −80°C for QRT-PCR analysis and other experimental use. The use of animals was approved by the Institutional Animal Care Use Committee.

Table 1. The Primers Used for RT-PCR and QRT-PCR

A. For RT-PCR

UniGene number	*Gene symbol*	Official full name	Primers
Hs.467020	*BCL-2*	B-cell CLL/lymphoma 2	5′-ACTTGTGGCCCAGATAGGCACCCAG-3′ 5′-CGACTTCGCCGAGATGTCCAGCCAG-3′
Hs.631546	*BAX*	BCL2-associated X protein	5′-GCTCTGAGCAGATCATGAAGACAG-3′ 5′-CACAAAGATGGTCACGGTCTGC-3′
Hs.2490	*CASP1*	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	5′-TTTGATTGACTCCGTTATTC-3′ 5′-TCTCTGCCGACTTTTGTTTC-3′
Hs.23960	*CCNB1*	cyclin B1	5′-GAAGCTACTGGAAACATG-3′ 5′-CTACAGCTCGTTGTATGA-3′
Hs.153752	*CDC25B*	cell division cycle 25B	5′-CACGCCCGTGCAGAATAAGC-3′ 5′-ATGACTCTCTTGTCCAGGCTACAGG-3′
Hs.370771	*CDKN1A*	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	5′-ATGTCAGAACCGGCTGGGGATG-3′ 5′-GCAGGCCAAGGCCCCGCAC-3′
Hs.644056	*CSNK2A1*	casein kinase 2, alpha 1	5′-ATGACCACCAGTCACGGCTTAC-3′ 5′-GGTTCAGACACGGTGCTTCTG-3′
Hs.488293	*EGFR*	epidermal growth factor receptor	5′-CATAGACGACACCTTCCTCC-3′ 5′-GGGTCTAAGAGCTAATGCGG-3′
Hs.244139	*FAS*	Fas (TNF receptor superfamily, member 6)	5′-TAGCTCCTATATTTTCGGCTT-3′ 5′-CTCACCAGCAACACCAAGTGC-3′
Hs.396530	*HGF*	hepatocyte growth factor	5′-ACTGGCTCTTTTAGGCACTGACTC-3′ 5′-TGTTCCCTTGTAGCTGCGTCCTTT-3′
Hs.132966	*MET*	Met proto-oncogene (hepatocyte growth factor receptor)	5′-ACTCCCCCTGAAAACCAAAGCC-3′ 5′-GGCTTACACTTCGGGCACTTAC-3′
Hs.567303	*MDM2*	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	5′-GCAGGGGAGAGTGATACAGAT-3′ 5′-GATGGCTGAGAATAGTCTTCA-3′
Hs.513617	*MMP2*	Matrix metallopeptidase 2	5′-GGGGCCTCTCCTGACATT-3′ 5′-CATTCCCTGCAAAGAACACA-3′
Hs.297413	*MMP9*	Matrix metallopeptidase 9	5′-TGGGCTACGTGACCTATGAC-3′ 5′-CAAAGGTGAGAAGAGAGGGC-3′
Hs.202453	*MYC*	v-myc myelocytomatosis viral oncogene homolog	5′-GTGGCACCTCTTGAGGACCA-3′ 5′-TGGTGCTCCATGAGGAGACA-3′
Hs.463456	*NME1*	Non-metastatic cells 1, protein (NM23A)	5′-TGCTGCGAACCACGTGGGT-3′ 5′-ATGTGGTCTGCCCTCCTGT-3′
Hs.645227	*TGFB1*	Transforming growth factor, beta 1	5′-CTCCGAGAAGCGGTACCTGAAC-3′ 5′-CACTTGCAGTGTGTTATCCCT-3′
Hs.522632	*TIMP-1*	TIMP metallopeptidase inhibitor 1	5′-CTGGAAAACTGCAGGATGGA-3′ 5′-CGCTGAGCTAAGGTCAGGCT-3′
Hs.633514	*TIMP-2*	TIMP metallopeptidase inhibitor 2	5′-CTCATTGCAGGAAAGGCCGA-3′ 5′-TGGGTGGTGCTCAGGGTGTC-3′
Hs.591665	*TIMP-4*	TIMP metallopeptidase inhibitor 4	5′-CCAGAGGTCAGGTGGTAA-3′ 5′-ACAGCCAGAAGCAGTATC-3′
Hs.241570	*TNF*	Tumor necrosis factor (TNF superfamily, member 2)	5′-CTTCTGCCTGCTGCACTTTGGA-3′ 5′-TCCCAAAGTAGACCTGCCCAGA-3′
Hs.81791	*TNFRSF11B*	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	5′-AGGAAATGCAACACACGACAAC-3′ 5′-AGGAACAGCAAACCTGAAGAATG-3′
Hs.156346	*TOP2A*	topoisomerase (DNA) II alpha 170kDa	5′-TGGTCAGAAGAGCATATGAT-3′ 5′-CTCACAATCTGATCAGCTAC-3′
Hs.408312	*TP53*	Tumor protein p53	5′-CTATGTCGAAAAGTGTTTCTGTCATC-3′ 5′-CAGCCAAGTCTGTGACTTGCACGTAC-3′
Hs.111779	*SPARC*	Secreted protein, acidic, cysteine-rich (osteonectin)	5′-ACTGAAGCTTCCCAGCACCATG-3′ 5′-GAGAGGATCCGGTACTGTGG-3′
Hs.585572	*SOX5*	SRY (sex determining region Y)-box 5	5′-CAACCTTGGTGCTGCTGTATCT-3′ 5′-GTCTTGGGTTTAGCTGATAGGTTCA-3′
Hs.73793	*VEGF*	Vascular endothelial growth factor A	5′-CGATCGTTCTGTATCAGTCTTTCC-3′ 5′-GAAGTGGTGAAGTTCATGGATGTC-3′
Hs.523238	*NOLC1*	Nucleolar and coiled-body phosphoprotein 1	5′-AGAAAAGAAAAAGGCGGCAG-3′ 5′-TCCTCATCAAGACCCTCACC-3′
Hs.520640	*ACTB*	Actin, beta	5′-CACTCTTCCAGCCTTCCTTC-3′ 5′-GCCATGCCAATCTCATCTTG-3′
Hs.544577	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	5′-CGGGAAGCTTGTGATCCATGG-3′ 5′-GGCAGTGATGGCATGGACTG-3′

(*table continues*)

Table 1. *Continued*

B. For QRT-PCR	
Gene	Primers
ARF	5′-GAAGCCAAGGAAGAGGAATGAG-3′
	5′-CAAATATGTTCCCCCCTTCAGA-3′
BAX	5′-ATGTTTTCTGACGGCAACTTCA-3′
	5′-CAGTTCCGGCACCTTGGT-3′
CASP1	5′-GCAGGACAACCCAGCTATGC-3′
	5′-TCTGCCGACTTTTGTTTCCAT-3′
MMP9	5′-AGTTTGCCGGATACAAACTGGTA-3′
	5′-GAAACACTCCAACAAAAAACAAAGGT-3′
NOLC1	5′-GACTGCATCTTCTCGTTTTTTACAGTATA-3′
	5′-GATCAGTGATTCTCAACCATGTAGGA-3′
MDM2	5′-CCTTAGCTGACTATTGGAAATGCA-3′
	5′-CAGGAAGCCAATTCTCACGAA-3′
TNF	5′-CCTGCCCCAATCCCTTTATT-3′
	5′-CCCTAAGCCCCCAATTCTCTT-3′
TP53	5′-GGGTTAGTTTACAATCAGCCACATT-3′
	5′-GGGCCTTGAAGTTAGAGAAAATTCA-3′
VEGF	5′-TCTACCTCCACCATGCCAAGT-3′
	5′-CTGCGCTGATAGACATCCATGA-3′
β-actin	5′-ACGTGGACATCCGCAAAGAC-3′
	5′-CTCAGGAGGAGCAATGATCTTGAT-3′
GAPDH	5′-TGGTATCGTGGAAGGACTCA-3′
	5′-AGTGGGTGTCGCTGTTGAAG-3′
TP53 binding site of MDM2 promoter	5′-TAGTCTGGGCGGGATTG-3′
	5′-TGCAGTTTCGGAACGTG-3′
Exson 2 of MDM2 promoter	5′-TGGCGATTGGAGGGTAGA-3′
	5′-ACCTGGATCAGCAGAGAA-3′
GAPDH promoter	5′-TCCAAGCGTGTAAGGGT-3′
	5′-GAAGGGACTGAGATTGGC-3′
Beta-Hemoglobulin promoter	5′-ATCTGAGCCAAGTAGAAGACCTTTTC-3′
	5′-TCTGCCTGGACTAATCTGCAAG-3′

Transient Cotransfection for Reporter Induction

NPC-TW03, shNOLC1-1-NPC-TW03, NS-shRNA-NPC-TW03, and NPC-TW06 cells were seeded into six-well plates, grown to a density of 80% confluence, and transfected separately with a transfection mixture that included fluorescent Arrest-In Transfection Reagent. The transfection mixture, containing the appropriate reporter and effector plasmids (including pGL2-MDM2-Luc, pGL3-SV40-TP53, pGL3-SV40-TP53 null, and pGL3-CMV-βgal) and a lipophilic reagent were mixed in serum-free medium and applied to the cells according to the manufacturer's instructions. After three hours, complete medium was added. After 48 hours, the cells were harvested for analysis. Luciferase enzyme assays and colorimetric β-galactosidase assays were performed according to the manufacturer's instructions (Promega, Madison, WI). Luciferase activity was normalized to β-galactosidase activity to assess the transfection efficiency. Each transfection experiment was repeated three times.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's instructions (Upstate, Charlottesville, VA). Briefly, 1×10^5 NPC-TW04 cells were fixed with 1% formaldehyde (Sigma, St. Louis, MO) in culture medium, and then total DNA was extracted and sonicated to an average size of 250 bp. ChIP assay was performed by adding NOLC1 or TP53 monoclonal antibodies, agarose-conjugated protein A beads, and then incubated for 2 hours. Immunoprecipitates were then washed, processed, and eluted for QRT-PCR analysis. The primers are shown in Table 1.

Table 2. The Construct Sequences of shRNA

shRNA symbol	Sequence
NOLC1-1 shRNA	5′-TGCTGTTGACAGTGAGCGCGACATCTAAGTCTGCAGTTAATAGTGAAGCCACAGAT GTATTAACTGCAGACTTAGATGTCTTGCCTACTGCCTCGGA-3′
NOLC1-2 shRNA	5′-TGCTGTTGACAGTGAGCGAACAGTTAAAGCTCAGACTAAATAGTGAAGCCACAGAT GTATTTAGTCTGAGCTTTAACTGTCTGCCTACTGCCTCGGA-3′
NS-shRNA	5′-TGCTGTTGACAGTGAGCGAACCACTAAGCTTCTGTCTTAATAGTGAAGCCACAGAT GTATTAAGACAGAAGCTTAGTGGTCTGCCT5′-ACTGCCTCGGA-3′

Effect of EBV Infection on NOLC1 Expression in NPC Cell Lines

The procedures used to isolate EBV particles and to identify viral DNA in the isolated viral solution were as described previously.[13] EBV infection using IgA receptor (secretary component protein)-mediated endocytosis in the NPC-TW01, NPC-TW03, NPC-TW04, NPC-TW06, and CG-BM-1 cell lines was performed as previously reported.[13,29,40] After infection, the cells were cultured for 10 days, collected, and used in the detection of the EBV by EBNA-1 immunostaining,[29] and subjected to the QRT-PCR analysis of *NOLC1* gene expression.

Statistical Analysis

Data were described as the means ± SE of mean of the indicated number of separate experiments. Statistical significance was determined by the paired two-tailed Student's *t*-test. *P* values less than 0.05 were considered significant.

Results

Genes Selected by SSH and Microarray

After the mRNAs of NPC and NNM cells were subjected to each other using SSH, two populations of differentially expressed cDNA in both cell types were generated, respectively. To identify these two differentially expressed transcripts, the subtractive cDNA of digoxigenin- or biotin-labeled NPC-TW04$_{-NNM}$ and NNM$_{-NPC\text{-}TW04}$ were monitored by screening with a cDNA microarray of expressed sequence tag clones (see supplemental Figure S1 at *http://ajp.amjpathol.org*). After hybridization, only red and blue spots represented that these two transcripts are only up-regulated or down-regulated genes in NPC-TW04 compared with NNM cells. To verify the expression of candidate genes in other NPC cells, candidate expressed sequence tag genes (see supplemental Table S1 at *http://ajp.amjpathol.org*), which were highly expressed in NPC or NNM cells, were screened by a phage λgt11 expression library of NPC genes. After sequence analysis and database searches with BLAST, three genes, *NOLC1*, *MRPL19*, and *AL359844* (see supplemental Table S2 at *http://ajp.amjpathol.org*), were identified. Because *NOLC1* was the most highly expressed gene among these three genes in NPC cells, it was chosen for further study.

NOLC1 *Gene Expression is Up-Regulated in Most NPC Tumor Cells*

To verify the differences in expression of the selected gene, we performed QRT-PCR analysis (Figure 1A) for *NOLC1* gene expression in 14 NPC and four NNM cell lines. The expression of the *NOLC1* gene was elevated in most of the NPC cell lines (12/14 lines), with the highest expression of both the *NOLC1* mRNA (Figure 1A) and protein (Figure 1B) in NPC-TW03 cells. NNM cells exhibited minimal or no expression (Figure 1, A and B). Immunohistochemical localization of NOLC1 protein clearly showed the reaction product in the nucleoli of all NPC cells (Figure 1C, left) but no reaction product in the nucleoli of control IgG (Figure 1C, right). These results demonstrate that NOLC1 is highly expressed in NPC cells, but in normal cells it is very weak if not nil.

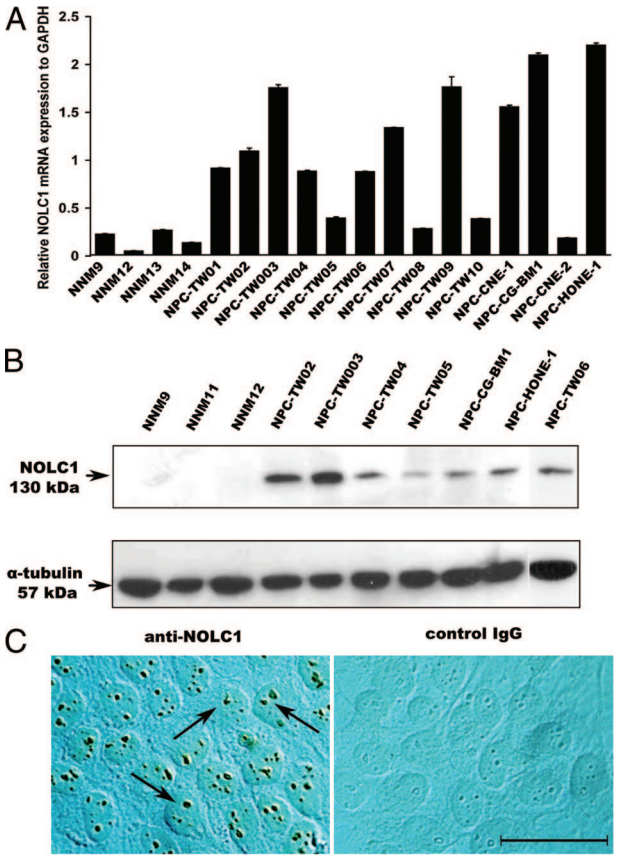

Figure 1. *NOLC1* mRNA and protein expression in NPC cell lines. **A:** *NOLC1* mRNA levels detected by QRT-PCR in 14 different NPC lines and four NNM cells. The *NOLC1* gene was highly expressed in most of the NPC lines compared with the NNM cells. Experiments were repeated three times and normalized to the *GAPDH* control. **B:** Western blot analysis of NOLC1 protein expression. A 130-kDa band of NOLC1 protein is shown in the NPC cell lines. In contrast, NNM cells showed no detectable signals. α-tubulin was used as an internal control. **C:** Immunohistochemical localization of NOLC1 protein in NPC cells. The **left panel** was stained with anti-NOLC1 antibody and **right panel** was stained with the nonspecific negative control IgG. NOLC1 immunoreactivity was seen clearly in the nucleoli of the NPC-TW03 cells (**arrows**). The control IgG staining shows no reaction product. Scale bar = 10 μm.

NOLC1 Is Highly Expressed in the NPC Biopsy Specimens

To find out if NOLC1 protein was highly expressed in NPC patients, we performed immunohistochemical localization of this protein in 30 NPC biopsies. As shown in Figure 2, the nucleoli of the tumor cells in the NPC biopsies were found to have NOLC1 immunoreactivity (Figure 2, A, C–E), while those of the control IgG showed no reaction product (Figure 2, B and F). All 30 specimens, including

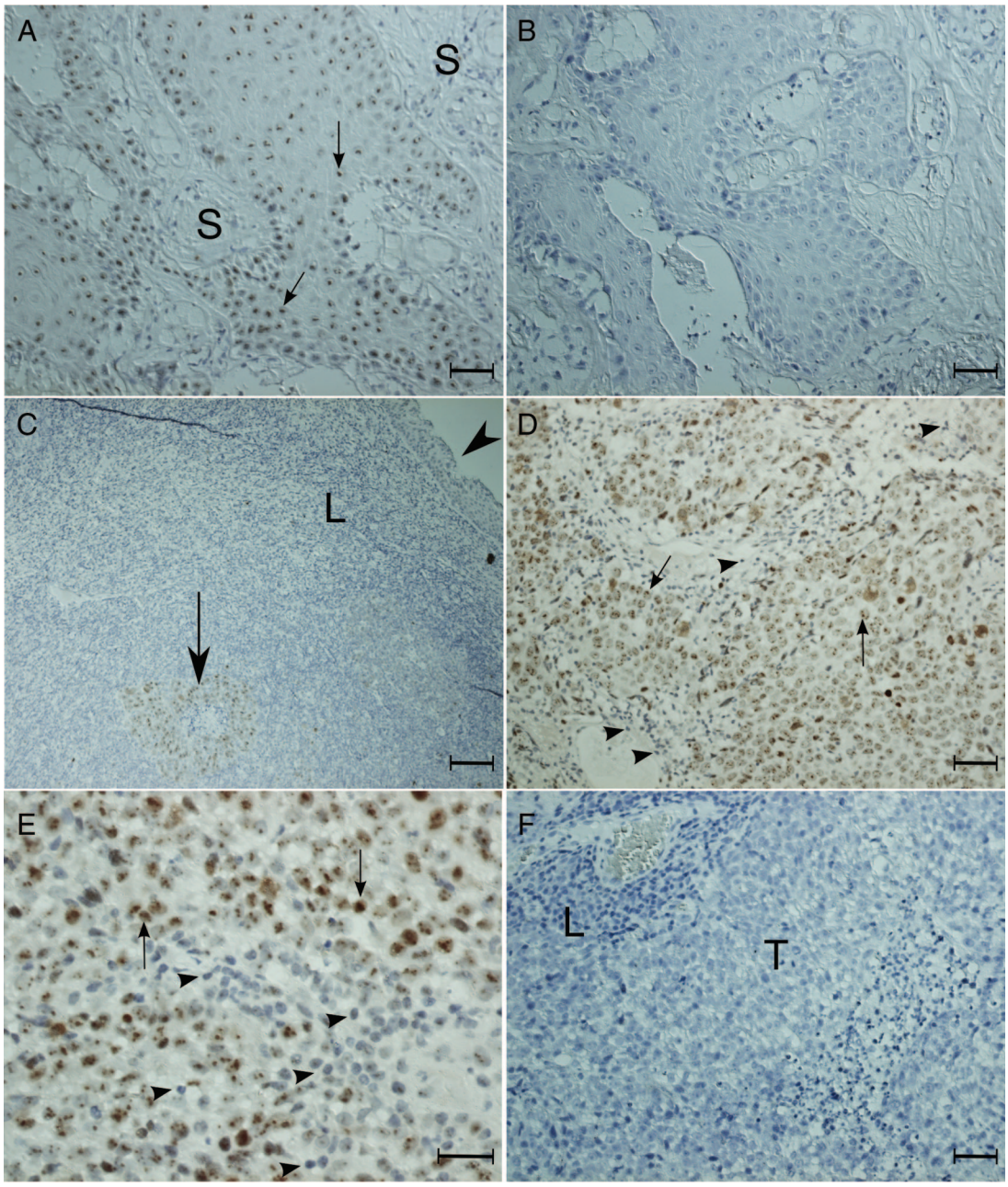

Figure 2. Representative pictures of NOLC1 protein staining in different NPC biopsy specimens. **A–B:** Type I NPC biopsy specimens. **C–F:** Type III NPC biopsy specimens. The paraffin sections were stained with either monoclonal anti-NOLC1 (**A, C–E**) or the control IgG (**B, F**). The NOLC1 immunoreactivity was seen clearly in the nucleoli of the tumor cell (**arrows**) in the tumor nests, but not in the stromal cells or in the normal squamous metaplastic epithelial cells (**C**, **arrowhead**). No reaction product was seen in the control sections. S: stromal cells; T: tumor nest; L: lymphocyte. Scale bars = 25 μm (**A, B, D–F**); 5 μm (**C**).

World Health Organization type I (keratinizing squamous cell carcinoma) and World Health Organization type III (undifferentiated carcinoma), expressed the NOLC1 proteins. Looking at all of the staining data, we found that regardless of pathological type, all expressed NOLC1 proteins, average staining of the positive-NOLC1 NPC cells being 93%. There was weak staining in 14%, moderate staining in 33%, and strong staining in 46% of the positive-NOLC1 NPC specimens. However, no reaction product was found in the stromal cells (Figure 2A, *S*), lymphocytes (Figure 2C, *L*), or normal mucosal epithelia (Figure 2C, arrowhead). These results clearly demonstrate that NOLC1 is highly expressed in NPC cells both *in vitro* and *in vivo*.

Inhibition of NOLC1 Expression Decreases Proliferation and Increases Apoptosis in NPC Cells

To investigate the function of NOLC1 protein in NPC cells, shRNA vectors directed against NOLC1 (shNOLC1-1 and shNOLC1-2) were transfected into NPC-TW03 cells separately to establish two stable cell lines. NPC-TW03

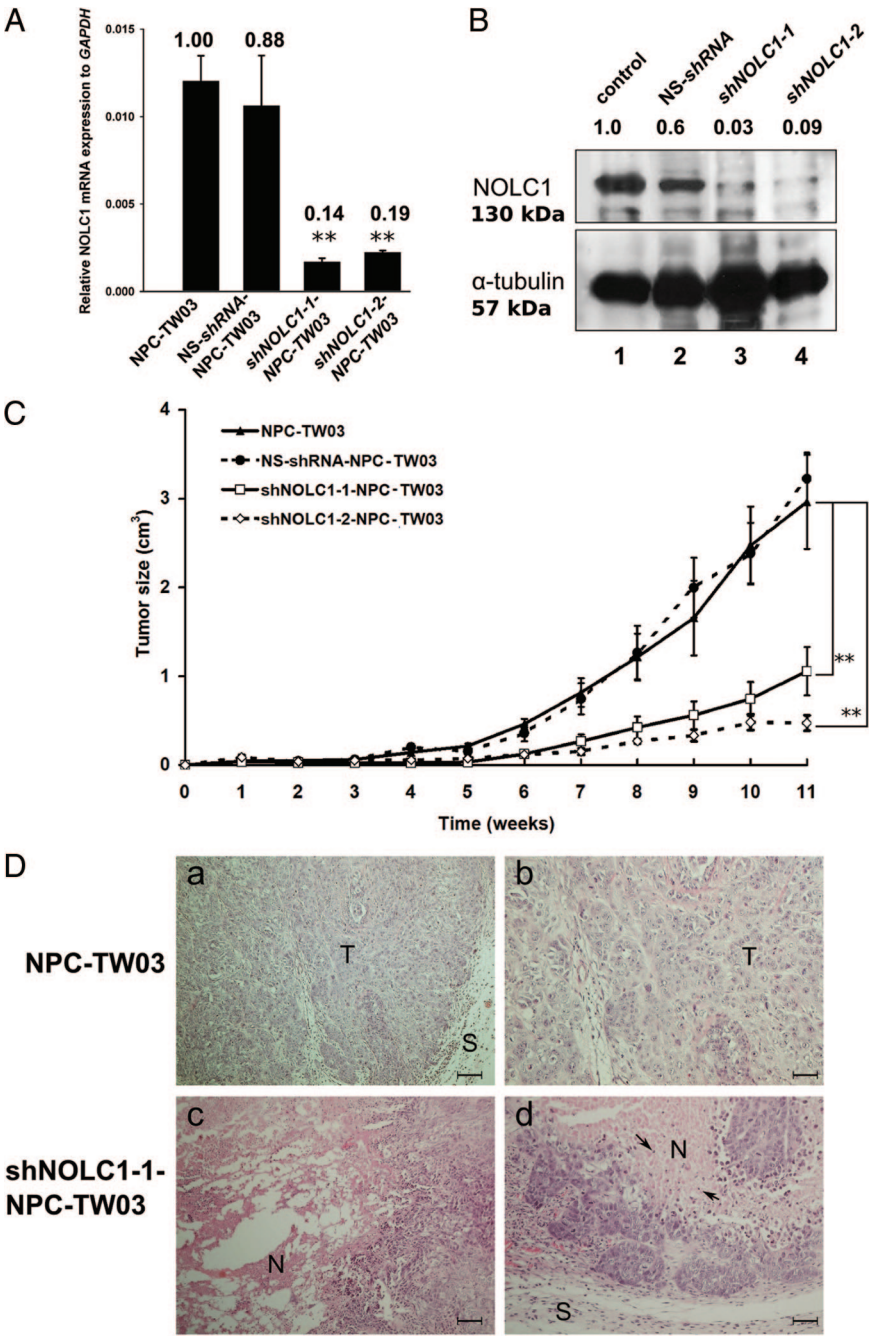

Figure 3. Verification of two shNOLC1-transfected NPC stable lines. **A:** After the transfection of two NOLC1 shRNAs plasmids (shNOLC1-1 and shNOLC1-2) separately into the NPC-TW03 cell lines, the total RNAs of each line were extracted and examined by QRT-PCR analysis. The data were obtained from triplicate experiments and normalized to *GAPDH* expression levels. Both shNOLC1-1 and shNOLC1-2 had a clear suppressive effect on *NOLC1* mRNA expression in the transfected NPC lines. Samples of mixed clone cells were measured, each point representing the mean value ± SEM ($n = 3$). $^{**}P < 0.01$. **B:** Western blot analysis of NOLC1 protein expression in NOLC1-shRNA-transfected NPC-TW03 cells. Lane 1, NPC-TW03 cells without transfection (control); lanes 2–4, stable lines transfected with NS-shRNA, shNOLC1-1, or shNOLC1-2, respectively. One 130-kDa band of NOLC1 protein was visible in lanes 1 and 2. The staining intensity of the same band was very weak in lane 3 and almost invisible in lane 4. α-tubulin a 57-kDa band was used as the internal control in each lane. **C:** The effect of NOLC1 shRNA transfection on NPC tumor growth *in vivo*. severe combined immunodeficient mice bearing xenografts of NPC-TW03 cells, including NS-shRNA-, NOLC1-shRNA (shNOLC1-1 and −2)-transfected and untransfected control NPC-TW03 cells, were observed for 11 weeks, and then the tumor masses from the four groups were removed and analyzed. Each point represents the mean value ± SEM. ($n = 6$) $^{**}P < 0.01$. **D:** Histological features of tumor sections from mice bearing NPC and shNOLC1-NPC xenografts. H&E staining from a paraffin section of an NPC-TW03 xenograft showed an undifferentiated carcinoma (**a,b**), whereas the shNOLC1-NPC xenograft sections revealed marked tumor cell necrosis (**c**) and apoptotic changes (**d**). T: tumor cells; N: necrosis; S: stroma; **arrows:** apoptotic cells. Scale bars = 50 μm (**a** and **c**); 100 μm (**b** and **d**).

cells were chosen for their higher NOLC1 expression (Figure 1B). Two days after transfection, we found reduced expression of *NOLC1* mRNA and protein in NOLC1- shRNA-transfected cells. The expression of *NOLC1* mRNA was found to be decreased to approximately 86% and 81% in shNOLC1-1 and shNOLC1-2 transfectants, compared with control (Figure 3A), and expression of protein was found to be decrease to 97% and 91% (Figure 3B) in shNOLC1-1 and shNOLC1-2 transfectants, compared with untransfected controls, respectively.

To determine the role of NOLC1 in NPC cell proliferation *in vivo*, we established an animal model using severe combined immunodeficient mice bearing NPC xenografts derived from either the shNOLC1-1- or the shNOLC1-2-transfected NPC-TW03 cell line. The tumor growth exhibited a clear reduction of tumor size when compared with that of mice bearing xenografts from untransfected NPC-TW03 cells. By 11 weeks, the tumor size of the shNOLC1-1-transfected xenografts was suppressed to 33% (67% inhibition) of the tumor sizes of the control group. In mice bearing the shNOLC1-2-NPC-TW03 xenograft, tumor growth had decreased to approximately 18% (82% inhibition) of that of the control group. The xenograft from the NS-shRNA transfectants did not exhibit any change in tumor size compared with the

control group (Figure 3C). Histopathological sections of the *shNOLC1-1*-NPC-TW03 (Figure 3D, c and d) and *shNOLC1-2*-NPC-TW03 (data not shown) xenograft tumors revealed massive tumor cell necrosis and apoptosis in the tumor region when compared with untransfected xenografts (Figure 3D, a and b). Taken together, the data show that depletion of NOLC1 results in a reduction of tumor growth and the induction of necrosis/apoptosis in NPC xenografts.

NOLC1 Regulates MDM2 *Gene Expression*

To clarify the molecular mechanisms underlying the effect of *NOLC1* expression on tumor behavior, we searched the previous studies of some genes involved in cell proliferation, angiogenesis, or apoptosis,[41,42] and then examined the expression of 26 genes (Table 1) by reverse transcription-PCR in shNOLC1-1-NPC-TW03 cells. The results showed that the expression of the *MDM2* oncogene (Figure 4A, bottom) was significantly suppressed (0.22-fold, 78% inhibition). To confirm the alteration of the mRNA expression pattern, we also determined the expression of protein by Western blotting. We found a reduced expression of MDM2 protein in shNOLC1-1-NPC-TW03 cells (Figure 4B). To further confirm this phenomenon *in vivo*, we checked the gene expressions in NPC-TW03 and shNOLC1-1-NPC-TW03 xenograft tissues. Expressions of the *NOLC1* and *MDM2* genes (Figure 4C) were also significantly suppressed (67% and 78% inhibition, respectively). Taken together, our *in vitro* and *in vivo* findings suggest that depletion of NOLC1 results in reduced expression of *MDM2*. Other NOLC1-regulated downstream genes, including *MMP9*, *VEGF*, *TNF-α, BAX*, and *CASP1,* are shown in the supplemental Figure S2 at *http://ajp.amjpathol.org*.

NOLC1 Acts Synergistically with TP53 to Up-Regulate MDM2 Expression

Since TP53 is a key activator of *MDM2* gene expression,[43] and TP53 can up-regulate MDM2 expression during NPC pathogenesis,[44,45] we used the luciferase reporter gene assay to analyze NOLC1's regulation of *MDM2* gene expression. The ability of NOLC1 to activate the transcription of *MDM2* was measured by the transient transfection of a reporter plasmid containing the luciferase gene under the *MDM2* p2 promoter (pGL2-MDM2-Luc) into NPC-TW03, shNOLC1-NPC-TW03, or NS-shRNA-NPC-TW03 cells, and cotransfection with a β-galactosidase expression construct for normalized transfection efficiencies (Figure 5A). When NOLC1 expression was inhibited, the *MDM2* promoter in the shNOLC1-NPC-TW03 cells showed reduced activity, resulting in a 0.39-fold decrease in the luciferase activity of the untransfected NPC-TW03 cells (Figure 5A, lanes 1 and 3). However, the *NS*-shRNA-transfected cells also displayed a reduction in luciferase activity to 0.68-fold that of the NPC-TW03 cells (Figure 5A, lane 2). These data support the hypothesis that normal *MDM2* promoter activity in NPC cells requires NOLC1 regulation, even in the presence of wild-type

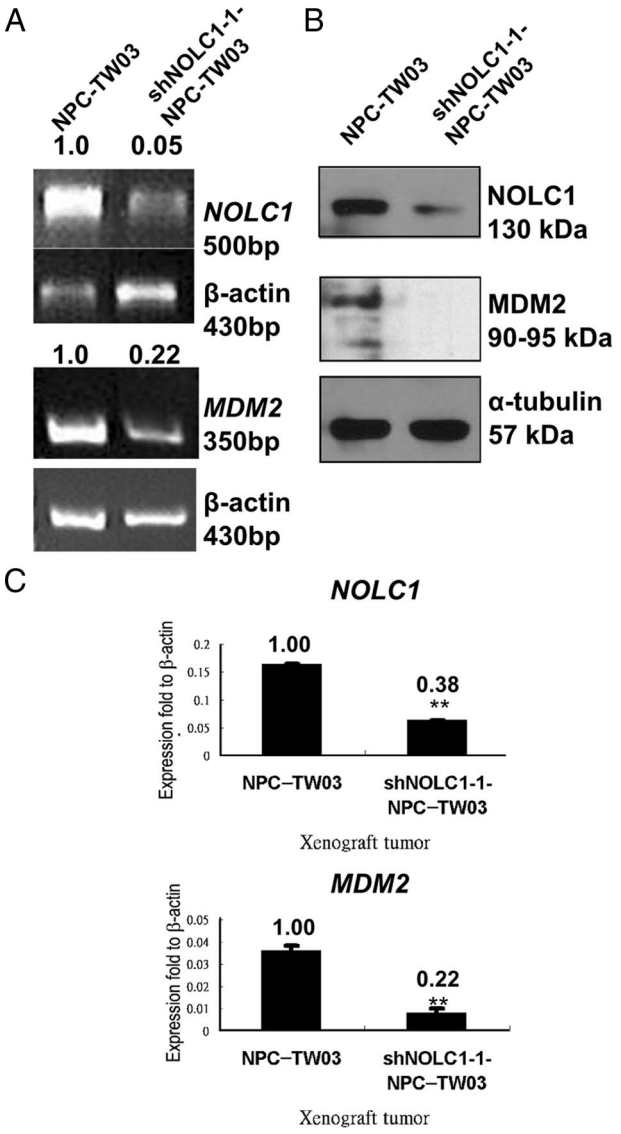

Figure 4. Depletion of NOLC1 suppressed MDM2 expression in NPC-TW03 cells. **A:** Knockdown of NOLC1 expression suppressed the MDM2 mRNA expression in NPC-TW03 cells. Total RNAs derived from NPC-TW03 and shNOLC1-1-NPC-TW03 cells were analyzed by reverse transcription-PCR. Both *NOLC1* and *MDM2* mRNA expression were suppressed. The β-actin cDNA was used as an internal control. **B:** Western blot analysis of NOLC1 and MDM2 protein expression in NPC-TW03 and shNOLC1–1-NPC-TW03 cells. α-tubulin was used as the internal control in each lane. **C:** QRT-PCR analysis of the expressions of the *NOLC1* (**top**) and *MDM2* (**bottom**) genes in NPC-TW03 and shNOLC1-1-NPC-TW03 xenograft tumors. The mRNA of *NOLC1* and *MDM2* expression in the shNOLC1-NPC xenograft was also markedly suppressed. The data were obtained from triplicate experiments and normalized to the β-actin cDNA as the control. $^{**}P < 0.01$.

TP53 (wtTP53). Therefore, *MDM2* transcription is not entirely dependent on the function of the TP53 transcription factor.

To examine the effects of increased TP53 expression on *MDM2* expression, we cotransfected the plasmid constructs pGL2-MDM2-Luc and pGL3-SV40-*TP53* into NPC-TW03, sh*NOLC1*-NPC-TW03, and NPC-TW06 cells (Figure 5B). In NPC-TW06 cells, the *TP53* gene contains a heterozygous point mutation causing the faulty TP53 protein to sequester any normal TP53 outside the nucleus, causing a lack of TP53 as a transcription factor.[35]

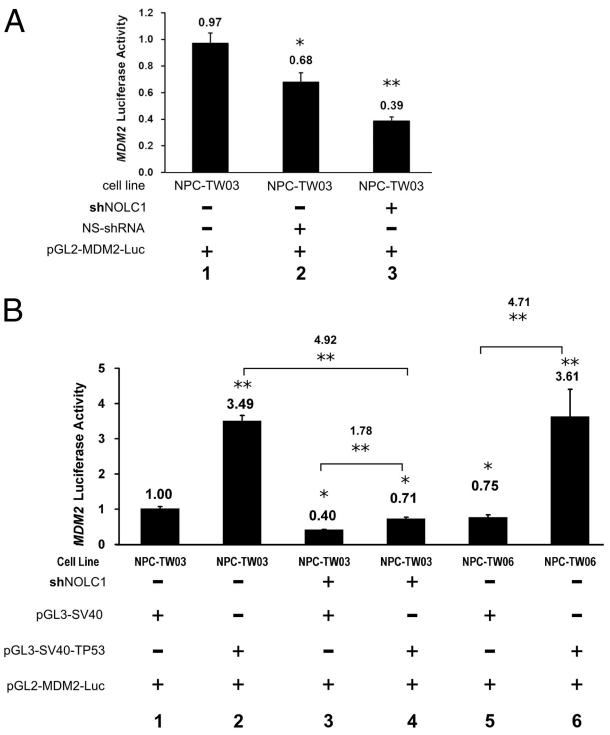

Figure 5. The relationship between TP53 and NOLC1 regulation of *MDM2* gene expression in NPC cells. **A:** Depletion of *NOLC1* gene expression resulted in down-regulation of *MDM2*-expression in NPC-TW03 cells. The *MDM2* promoter reporter (pGL2-MDM2-Luc) was cotransfected with the internal control pGL3-CMV-βgal into NPC-TW03 (lane 1), NS-shRNA-NPC-TW03 (lane 2) or shNOLC1-NPC-TW03 (lane 3) cells separately for 48 hours, and MDM2 P2 promoter expression was measured by luciferase assay. **B:** The regulation of *MDM2* gene expression by TP53 depended on NOLC1 co-activation. The *MDM2* P2 promoter reporter (pGL2-MDM2-Luc) was cotransfected with either pGL3-SV40-TP53 or pGL3-SV40 (control) and an internal control pGL3-CMV-βgal into NPC-TW03 (lane 1,2), shNOLC1-NPC-TW03 (lane 3,4) or NPC-TW06 (lane 5,6) cells for 48 hours, and the MDM2 P2 promoter expression was measured by luciferase assay. The luciferase activity was measured in each cell lysate and reported in arbitrary units. The same experiment was repeated three times. All data are reported as average values ± SEM; $n = 3$ for each condition. $^{*}P < 0.05$. $^{**}P < 0.01$.

In NPC-TW03 cells, which have wtTP53, exogenous TP53 brought about a 3.49-fold increase in *MDM2* promoter (Figure 5B, lanes 1 and 2). In the shNOLC1-NPC-TW03 cells, exogenous TP53 could only barely activate the *MDM2* promoter to regulate the luciferase activity (Figure 5B, lanes 3 and 4). There was 4.92-fold higher than that of the shNOLC1-NPC-TW03 cells while transfected with pGL3-SV40-TP53 (Figure 5B, lanes 2 and 4), indicating that the exogenous TP53 had a less effect on *MDM2* expression in shNOLC1 transfected cells. Thus, TP53 upregulation of the *MDM2* promoter activity in NPC-TW03 cells may require the presence of NOLC1 for full up-regulation (Figure 5B, lanes 2 and 4).

To test whether NOLC1 affected TP53's regulation of *MDM2* expression, we also performed shNOLC1 transfection experiments in a TW06 cell line, which did not have the TP53 transactivation activity.[35] We found that the cells with neither TP53 nor NOLC1 proteins could not survive (data not shown). However, in the NPC-TW06 cell line, which has normal NOLC1 but abnormal TP53 (Figure 5B, lane 5), we could still detect *MDM2* promoter activity, 0.75 times that found in NPC-TW03 cells (Figure 5B, lane 1). However, when the NPC-TW06 cells were cotransfected with pGL3-SV40-*TP53* and pGL2-*MDM2*-Luc, luciferase activity was up-regulated 4.71-fold times (Figure 5B, lanes 5 and 6). These results indicate the TP53 protein and NOLC1 protein work together synergistically upregulating *MDM2* promoter activity. However, in the cells little or no NOLC1 protein, the TP53 protein only mildly up-regulated *MDM2* promoter activity (Figure 5B, lanes 3 and 4). Taken together, these data indicate that increased levels of both wtTP53 and NOLC1 are necessary for the marked upregulation of *MDM2* promoter activity in NPC-TW03 and NPC-TW06 cells.

NOLC1 Interacts with the MDM2 *Promoter at the TP53 Binding Site*

Results of the luciferase activity assay revealed that inhibition of NOLC1 markedly down-regulated the expression of *MDM2* (Figure 5B, lanes 3 and 4). Therefore, we further analyzed the co-existence of the NOLC1 and the TP53 binding region of the *MDM2* promoter[46] using a ChIP assay in NPC cells with the NOLC1 antibody (Figure 6A and B, lane 1). The results showed that NOCL1 occupancy was specifically increased by 3 times at the TP53 binding region *of the MDM2* intron 1 promoter than the control downstream Exon 2 of the *MDM2* DNA sequence, which was measured by quantitative PCR (Figure 6B, lane 2). We also examined NOCL1 occupancies at the *GAPDH* and β-hemoglobulin (*HBB*) promoter as controls. Under normal physiological conditions, *GAPDH* was highly expressed; in contrast, *HBB* is strictly repressed in NPC cells (data not shown). We found no difference in NOCL1 occupancies between the two control regions and the no-antibody control (Figure 6B, lane 3 and 4). These data suggest that NOLC1 specifically targets the TP53 binding region of the *MDM2* promoter *in vivo*. To further address whether TP53 and NOCL1 cooperatively regulate *MDM2* transcription, we measured TP53 occupancies at the *MDM2* promoter intron 1.[46] TP53 was greatly enriched at *MDM2* intron 1 but not at exon 2 (Figure 6C, lanes 1 and 2), which was also found for NOCL1 binding at these two positions (Figure 6B, lanes 1 and 2). These data suggest that TP53, a DNA binding transcription factor, may recruit or cooperate with NOCL1 to bind *MDM2* promoter intron 1 and regulate *MDM2* transcription synergistically.

Discussion

Using SSH, we have identified two gene groups differentially expressed in NPC cells with high or low levels of expression. Of these genes, *NOLC1* was one of the most highly expressed genes in NPC cells, though it is comparatively very weakly expressed in normal cells (Figure 1A). NOLC1 protein was abundant in both NPC cell lines and tumor cells from NPC biopsy specimens (Figures 1, B and C, and 2). To determine the functional role of NOLC1 in NPC pathogenesis, we used interference RNA to knock down *NOLC1* gene expression and establish

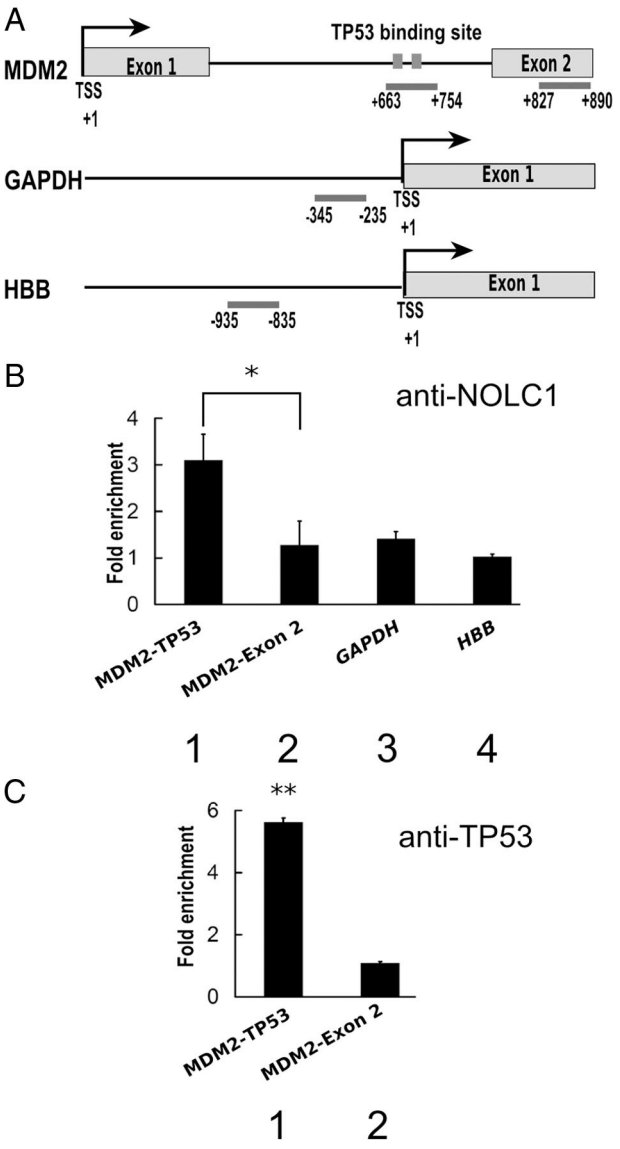

Figure 6. Identification of the interaction region of NOLC1 in the *MDM2* P2 promoter. **A:** Regions amplified by QRT-PCR are indicated by bars and nucleotide number relative to the transcription start site (TSS) of the internal promoter (the TP53 binding site in intron 1) of *MDM2*, and Exon 2 of the *MDM2*, *GAPDH*, and *HBB* promoter correspondingly. **B:** ChIP assay of the promoters by the NOLC1 antibody. QRT-PCR was used to evaluate the precipitation of the TP53 binding site and Exon 2 of the *MDM2* promoter, *GAPDH* and the *HBB* promoter region from chromatin prepared from NPC-TW04 cell line chromatin using an anti-NOLC1 monoclonal antibody. Relative enrichment was calculated by comparison with the threshold cycle value for 10% of input genomic DNA and data are expressed as the fold enrichment of DNA associated with immunoprecipitated NOLC1 relative to the no-antibody control chromatin. The error bars plotted represent the mean values ± SE of triplicate measurements. **C:** ChIP assay by the anti-TP53 antibody as described in **B**. HBB: hemoglobin β. $^{*}P < 0.05$, $^{**}P < 0.01$.

stable *shNOLC1* expression in NPC cell lines. To avoid the possibility of an inconsistent result induced by a single transfection clone, we used mixed shNOLC1 transfectant clones to test the cell growth rate *in vivo*. The shNOLC1 suppressed the growth of shNOLC1-NPC xenografts (Figure 3C) by inducing severe necrosis and apoptosis of the tumor cells (Figure 3D). This finding supports the hypothesis that *NOLC1* plays a role as an oncogene in NPC tumorigenesis. Although an increasing number of reports have shown that NOLC1 is a multiple functional protein,[47] no report has indicated that it has a function associated with tumorigenesis. Results from the present experiment demonstrate that NOLC1 plays a role in enhancing NPC tumorigenesis.

We found that when *NOLC1* expression was down-regulated in the shNOLC1-NPC-TW03 stable cell lines (Figure 4), the expression patterns of *MDM2* (Figure 4) and the tumor-invasion-related *MMP9* gene (see supplemental Figure S2 at *http://ajp.amjpathol.org*) were also suppressed while the expression of the apoptosis-related genes, such as *TNF-*α, *BAX*, and *CASP1,* were up-regulated (see supplemental Figure S3 at *http://ajp.amjpathol.org*). These findings suggest that NOLC1 may enhance NPC tumorigenesis by regulating of gene expression in NPC cells. The regulation of the *TNF-*α gene is complex at both the transcriptional and translational levels. Although TNF-α can activate caspase enzymes through its receptor, it can also mediate the activation of the transcription factor activity of NF-κB to protect the cells for survival.[48,49] However, there was no change in the expression of the general apoptosis inhibitors, such as *BCL2* mRNA (see supplemental Figure S4 at *http://ajp.amjpathol.org*), as well as other invasion related genes, such as *MMP2*, *TIMP1*, *TIMP2*, *TIMP4*, or *NME1*genes (see supplemental Figure S4 at *http://ajp.amjpathol.org*).

Several tumor growth related genes such as *MDM2*, *VEGF*, and *BAX* have been reported to be regulated by TP53, which itself does not undergo any changes at the mRNA level.[50] We found TP53 expression to be mildly suppressed by shNOLC1 treatment (Figures 4 and see supplemental Figure S2 at *http://ajp.amjpathol.org*). Furthermore, our study of *MDM2* promoter activation suggested that the up-regulation of *MDM2* expression by exogenous TP53 in NPC cells relied on the presence of NOLC1, with the two proteins acting synergistically (Figure 5B). NOLC1 protein cannot directly bind to DNA but it can promote gene expression in a manner similar to a transcription factor.[25] We used ChIP analysis to study the relationship between NOLC1 protein and the MDM2 promoter region, and found that NOLC1 may bind to certain factors that are able to cooperatively react with TP53 and bind to the *MDM2* intron promoter region and regulate the expression of *MDM2* (Figure 6).

EBV infection can enhance *MDM2* expression and NPC tumor growth.[29,44] Therefore, we also investigated the relationship between EBV infection and *NOLC1* expression in NPC cells. We found mild upregulation of NOLC1 in four EBV-infected NPC cell lines (see supplemental Figure S5 at *http://ajp.amjpathol.org*), suggesting that *NOLC1* expression may be regulated in the response of the EBV infected NPC cells. However, we did not observe any alteration in NOLC1 expression in the NPC-TW06 cell line (see supplemental Figure S5; lane 5 at *http://ajp.amjpathol.org*). It may result in the formation of a complex of wtTP53 and mutant TP53 (mtTP53) protein, which stays in the cytoplasm and loses TP53 transactivation activity.[35] Without TP53 protein in the NPC-TW06 nucleus, the *NOLC1* gene cannot be up-regulated by EBV infection, which suggests that TP53 plays some role in the regulation of *NOLC1* gene expression after EBV

infection in NPC cells. Previous studies have reported the development of NPC to be unlike other cancers. *TP53* is moderately up expressed and less mutated in different NPC specimen[21,23,44,51] and wtTP53 can act with BCL-2 synergistically to increase tumor cell growth.[22,54] However, the details of the mechanism between wtTP53 and EBV infection in NPC remain unclear. As mentioned above, EBV infection can induce *TP53* expression and also up-regulate *MDM2.*[44] In this report, we demonstrated that the NOLC1 protein can bind to the *MDM2* promoter of the TP53 binding region (Figure 6) and work synergistically with TP53 to regulate *MDM2* expression (Figure 5). Therefore, the *NOLC1* gene may play a role in regulation of NPC progression.

In our animal experiment, the xenograft from shNOLC1-NPC cells displayed marked shrinkage of tumor mass (about one sixth of the control xenografts, Figure 3C) with marked tumor necrosis and apoptosis (Figure 3D). This result suggests that it might be possible that NPC can be treated by inhibiting *NOLC1* gene expression in tumor cells.

ARF has been reported to be able to induce TP53 transcriptional activity by binding and inactivating MDM2,[52–54] and in NPC there is often decreased expression of ARF protein[23,54,55] We found no difference in ARF expression between the shNOLC1 transfected and non-transfected NPC-TW03 xenografts (data not shown). Therefore, we suggest that NOLC1 is a nucleolar protein that does not affect ARF-mediated *MDM2* regulation and has no effect on TP53 activity. Therefore, the regulation of *MDM2* expression by NOLC1 and TP53 is not affected by ARF expression.

In summary, although the NOLC1 protein has been studied for 15 years, it has not been previously reported to play a role in regulation of cancer progression , as our work has shown. A better understanding of NOLC1 regulation and its control of gene transcription as well as its co-activation with TP53 may provide greater insight into cancer cell behavior and may lead to the development of new therapeutic strategies.

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