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Maspin Expression Is Transactivated by p63 and Is Critical for the Modulation of Lung Cancer Progression

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ABSTRACT

Maspin inhibits metastasis of some cancer cells, and clinical studies have identified correlations between maspin loss and poor prognosis in several cancer types. Maspin was found to be significantly overexpressed in lung cancer samples as compared with matched normal lung tissues. However, the regulatory mechanism of maspin expression remains unclear. We show here that differential expression of maspin in carcinoma-derived lung cancer cells is regulated at the transcriptional level. We found that p63 is a critical factor for the transcription of maspin, which is lost in highly invasive cancer cells such as NCI-H157, NCI-322, and NCI-358. No correlation was found between maspin expression and the previously associated transcription factors, p53, Ets1, and Pdef. Instead, maspin expression was strictly dependent on the presence of p63 in lung cancer tissues ($P < 0.001$) and in the tested cell lines. Transient expression of p63 transactivated the maspin promoter with remarkable fold changes in cells expressing the TAp63, suggesting that TAp63 might be a novel stimulator of the maspin promoter in lung cancer. We have also demonstrated the binding of p63 protein to a previously identified p53-binding site on the maspin promoter by gel shift and chromatin immunoprecipitation assays. In tumor tissues, maspin expression was associated with lymph node involvement ($P = 0.035$) and tumor stage ($P = 0.063$) in all tested cases, except squamous carcinoma. In terms of function, ectopic expression of maspin inhibited cell invasion in squamous carcinoma as well as adenocarcinoma. Taken together, these results define maspin as a new molecular target of p63 that eventually inhibits the invasion of lung cancer.

INTRODUCTION

Maspin is a M_r 42,000 protein with sequence homology to the serpin family protease inhibitors (1). In breast and prostate cancers, maspin acts as a tumor suppressor capable of inhibiting motility, invasion, and metastasis (2, 3). Maspin expression was found to decrease with increasing malignancy of primary tumors and was absent from the lymph nodes and distant metastases in breast cancer (4). Maspin was found to be significantly overexpressed in lung cancer samples as compared with matched normal lung tissues, particularly in squamous cell carcinoma, where this expression was associated with increased patient survival and longer remission duration (5). The transcriptional activators p53, Ets1, and Pdef have been reported to induce maspin expression in a variety of cancers (3, 6, 7). Therefore, observations of frequent and strong expression of maspin in lung malignancy, particularly in squamous cell carcinomas, are unusual considering the high proportion of p53 mutations in lung cancer (8). Here, we show that in lung cancer tissues and cell lines, maspin expression is independent of Ets1, p53, and Pdef and is instead strictly dependent on the presence of p63.

p63 is a homologue of p53. The gene for p63, located at chromosome 3q27-29, encodes six isoforms with transactivating and dominant negative activities. These are derived from two promoters (TAp63 and ΔNp63) and three splicing variants (α, β, and γ; refs. 9–11). Recent studies have shown that mutation of p63 is uncommon in human cancer cell lines and tissues (12, 13), but elevated expression of p63 was found in human squamous carcinomas and associated with better survival (14).

On the basis of these facts, we propose that p63 may activate maspin expression, thus contributing to modulation of lung cancer progression. Although maspin is expressed in lung malignancies (15), its role in lung cancer remains unknown. Here, we examined the regulatory mechanism of maspin expression and its functional consequences in lung malignancy. We found that (*a*) maspin expression is positively regulated by p63 in lung cancer, and (*b*) maspin expression is associated with tumor histology, stage, lymph node involvement, and lung cancer invasiveness. We found that differential maspin expression was controlled by the presence of p63 and that lack of maspin was correlated with cellular invasiveness. Expression of p63 remarkably transactivated the maspin promoter, and this transactivation was unaffected by the presence of mutant p53. In addition, ectopic expression of maspin inhibited cell invasion regardless of the histologic lung cancer type. Taken together, these results indicate for the first time that p63 modulates maspin and may therefore be a critical factor for lung cancer invasion and progression in the absence of wild-type p53 or in the presence of mutant p53.

MATERIALS AND METHODS

Cell Lines and Clinical Specimens. The following human lung cancer cell lines were studied A549, NCI-H23, NCI-H157, NCI-H322, NCI-H358, NCI-H520, and NCI-H661. All cancer cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 1 mmol/L $NaCO_3$, 2 mmol/L L-glutamine, penicillin-streptomycin with 5% CO_2 at 37°C. Resection specimens with lung cancer were obtained from a series of 49 patients. Sections of 4 μm were cut from a paraffin block, deparaffinized, and dehydrated. A total of 47 cases was informative in immunostaining analysis.

Plasmid Construction, Transfection, and Luciferase Assays. The p63 expression plasmids were obtained from Dr. Hyunsook Lee (Seoul National University, Seoul, Korea) and the full-length maspin open reading frame (GenBank accession number U04313) was cloned from normal breast tissue mRNA by reverse transcription-PCR for cloning into pCMVTaq4C (Invitrogen, Carlsbad, CA). The maspin promoter (−284 to +184) was amplified from genomic DNA and cloned into the *Xho*I-*Hin*dIII site of pGL3 (Promega, Mannheim, Germany) to generate pGLmas. All of the constructs were verified by sequence analysis. The maspin reporter plasmid, pGLmas, contains Ets-, AP1-, and p53-binding elements fused with luciferase reporter gene. All transfections were performed using effectine (Qiagen) according to the manufacturer's instructions. After 48 hours, the luciferase activity was determined using Luciferase Assay system (Promega). Transfections were performed in triplicate.

Reverse Transcription-PCR Analysis of Maspin Expression. Expression of maspin was evaluated by reverse transcription-PCR. Total RNA was isolated with using RNeasy mini kit (Qiagen, Hilden, Germany) and treated with DNase I (Qiagen). The primer sequences designated from the coding region of the human maspin cDNA are as follows: 5′-GGAGGCCACGTTCTGTAT-3′ (sense) and 5′-CCTGGCACCTCTATGGA-3′ (antisense). The PCR conditions were as follows: 35 cycles of 95°C for 90 seconds, 50°C for 90 seconds, and 72°C for 90 seconds, followed by a final incubation at 72°C for

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7 minutes. The length of primary PCR product was 413 bp. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Sense and antisense primers for the glyceraldehyde-3-phosphate dehydrogenase gene used as internal controls are as follows: 5′-AGTCAACGGATTTGGTCGTA-3′ and 5′-AAATGAGCCCCAGCCTTCT-3′, respectively. The PCR of glyceraldehyde-3-phosphate dehydrogenase is composed of denaturation at 95°C for 1 minute, annealing at 50°C for 90 seconds, and extension at 72°C for 90 seconds, followed by a final incubation at 72°C for 7 minutes. The amplified product was analyzed by electrophoresis on an agarose gel and staining with ethidium bromide.

Western Blot and Immunohistochemical Analysis. Western blot analysis was performed to detect the maspin and p63 expression in non–small-cell lung carcinoma cell lines. Briefly, cells were lysed in radioimmunoprecipitation assay buffer [20 mmol/L Na_2PO_4 (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 100 mmol/L NaF, and 2 mmol/L Na_3VO_4]. Equal amounts (70 μg) of cell lysates were resolved by 12% SDS-PAGE and subjected to Western blot analysis using enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL). Antibodies used were maspin (PharMingen, San Diego, CA) at dilution of 1:2000, p63 (Oncogene Research Products, San Diego, CA), and α-tubulin or β-actin (Sigma, St. Louis, MO) for loading control. Immunohistochemical staining was performed using a streptavidin peroxidase procedure. Briefly, tissue sections were deparaffinized and dehydrated in a graded series of alcohol. Antigenic epitopes were unmasked by autoclaving for 15 minutes in Target Retrieval Solution (DAKO, Carpinteria, CA). Maspin and p63 were detected with mouse monoclonal maspin antibody from PharMingen (1:300 dilution) and p63 antibody from Oncogene (1:100 dilution). A section of breast cancer tissue previously identified as strong staining was used as a positive control. Although cytoplasmic as well as nuclear staining was observed, only cytoplasmic staining was considered as positive maspin expression.

Preparation of Nuclear and Cytoplasmic Protein Fractions. For subcellular fractionation, total cell lysate was prepared by resuspending cells in a buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 0.5% Triton X-100]. Nuclear components were isolated by centrifugation at 15,000 rpm for 6 minutes at 4°C. The supernatants were separated for cytoplasmic protein fraction. The nuclear pellet was resuspended in a lysis buffer [20 mmol/L HEPES (pH 7.9), 20% glycerol, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 0.1% NP40], and left on ice for 20 minutes, followed by removal of debris by centrifugation at 15,000 rpm for 6 minutes. The protein fractions were stored at −70°C

Nuclear Extract Preparation and Gel Mobility Shift Assay. Nuclear extracts were prepared by lysing cells in 1 mL of lysis buffer [25 mmol/L Tris-HCl (pH 7.8), 1 mmol/L DTT, 1 mmol/L EDTA, 5% glycerol and 1% Triton X-100] supplemented with protease inhibitors and phosphatatase inhibitors. Intact nuclei were collected by gentle centrifugation and resuspended in hypotonic buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 5% glycerol]. Hypertonic buffer [10 mmol/L HEPES (pH 7.9), 1 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 5% glycerol] supplemented with protease and phosphatase inhibitors was added to a final NaCl concentration of 0.42 mol/L. Suspensions were mixed by inversion at 4°C for 30 minutes and cleared by centrifugation. The double-stranded oligonucleotides used for gel shift of p53 region were labeled and incubated with nuclear extracts at 4°C for 30 minutes. Electrophoresis was performed at 30 mA in a 0.5× Tris borate, EDTA on a native 5% polyacrylamide gel.

Chromatin Immunoprecipitation Assay. NCI-H23 cells expressing p63 were plated in a 100-mm dish. Approximately 80% confluent dishes were fixed in 1% formaldehyde. The cross-linked cells were washed with PBS, harvested by scraping, collected by centrifugation, and resuspended in a lysis buffer [1% SDS, 0.1 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.1)] containing protease inhibitors. After 10 minutes of incubation on ice, the lysate was sonicated, and the insoluble debris was removed by centrifugation. Soluble chromatin was diluted 1:10 in chromatin dilution buffer [0.01%SDS, 1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl (pH 8.1), and 16.7 mmol/L NaCl] containing protease inhibitors and precleared by incubation with protein-A Sepharose beads at 4°C. The supernatants were immunocleared by incubation with fresh beads and nonspecific murine IgG for 1 hour at 4°C. Recovered supernatants were incubated overnight with 2 μg of specific antibody and antibody-chromatin complexes were captured with preblocked protein-A magnetic beads. After immunoprecipitation and elution, the eluent was heated to 65°C for 4 hours to reverse the cross-link. After phenol/chloroform extraction and ethanol precipitation, the recovered DNA was subjected to PCR. Primers selected for the maspin promoter are as follows: p63 p53RE, ATGCATGTA-ACTCACAGCCC and ACGCCCACTGCCAGCCCAG.

Invasion Assay. Infected lung cancer cells were plated at 100,000 cells per well in RPMI medium with 1% serum in the upper chamber of a Transwell insert (8-μm pores; Chemicon) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber. After 24 hours, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained using CyQuant GR dye (Chemicon, Temecula, CA) to assess the number of cells.

Statistical Analyses. Either the χ^2 test or Fisher's exact test (two-sided) was performed to determine the correlation between the grade of maspin expression and the clinicopathological parameters such as age, sex, histologic grade, and Tumor-Node-Metastasis stage. The results were considered to be statistically significant at $P < 0.05$. All statistical analyses were conducted using the SPSS statistical software program (SPSS, Chicago, IL).

RESULTS

Expression of Maspin in Association with p63 in Human Lung Cancer Cell Lines. Maspin has been reported to be undetectable in normal lung tissues by Northern blot analysis (16). To examine the possible roles of maspin in human lung cancer, we first evaluated several lung cancer cell lines for expression of maspin mRNA and proteins. Reverse transcription-PCR and Western analysis demonstrated that maspin mRNA was expressed in noninvasive NCI-H23, NCI-H520, and A549 cell lines but not in highly invasive cell lines such as NCI-H157, NCI-H322, and NCI-H358 (Fig. 1). The endogenously expressed maspin in these cells had a molecular weight of M_r 42,000, identical to that found in breast cancer cells. The absence of

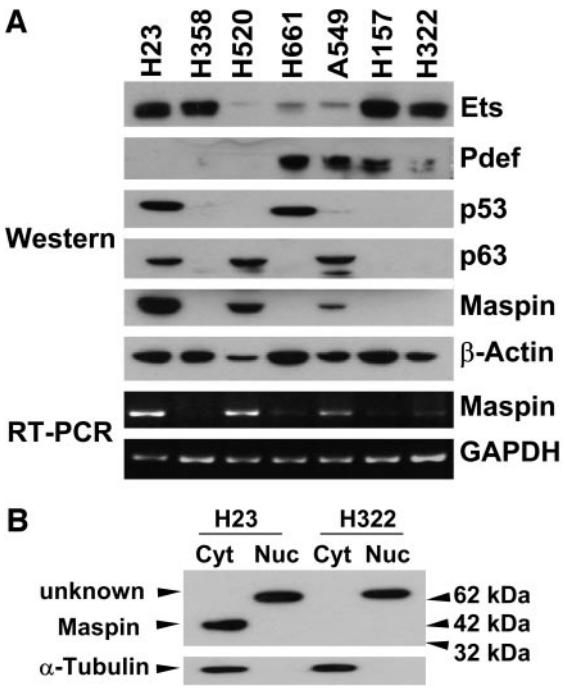


Fig. 1. Expression levels of maspin and related transcription factors in seven different lung cancer cell lines (A549, NCI-H23, NCI-H157, NCI-H322, NCI-H358, NCI-H520, and NCI-H661). *A*, expression of maspin and related transcription factors. *B*, immunoblot of nuclear and cytoplasmic fractions isolated from cells cultured from maspin expressing (NCI-H23) and nonexpressing (NCI-H322) lung cancer cells. α-Tubulin antibody reactivity was used as a cytoplasmic marker protein and β-actin is shown as a loading control. There was no nuclear maspin (M_r 42,000) detected on the Western blots. Note that the same antibody also recognizes an unknown high molecular weight (M_r 65,000) protein in the nuclear fraction of maspin-negative NCI-H322 cells.

Table 1 *Clinical properties of maspin and p63 in NSCLC tissues*

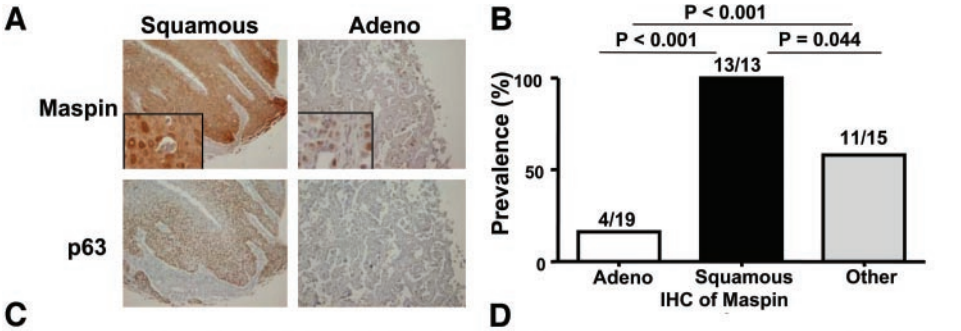
	Cytoplasmic maspin			Nuclear p63		
	Present (n = 28)	Absent (n = 19)	P*	Present (n = 18)	Absent (n = 29)	P*
Stage			0.186			0.482
1	16	8		9	15	
2	5	8		6	7	
3	7	3		6	4	
Histology			<0.001			<0.001
Adenocarcinoma	4	15		2	17	
Squamous cell carcinoma	13	0		13	0	
Others	11	4		6	9	

* χ^2 test.

maspin in these cells may be caused by the absence of critical transcription factor for maspin transcription. Previous studies have shown that maspin expression may be modulated by a variety of upstream effectors, including the transcription factors Ets1, p53, and Pdef. However, as shown in Fig. 1, the expression levels of Ets1, p53, and Pdef were not correlated with maspin expression. Instead, expression of p63, a p53 analogue, was well correlated with maspin expression in the presence of mutant p53 (NCI-H23) or in the absence of p53 (NCI-H520, A549).

Localization of Maspin in the Cytoplasm of Lung Cancer Cells. To confirm the correlation between maspin and lung cancer progression, we used immunohistochemical staining to evaluate maspin protein expression in human lung cancer tissue sections. Maspin protein was found in both the nucleus and cytoplasm of tumor cells in all of the 13 tested squamous cell carcinomas (Table 1; Fig. 2*A*). In contrast, adenocarcinomas showed strong nuclear staining, but only a few of them (4 of 19, 21%) demonstrated cytoplasmic immunoreactivity. Maspin has previously been reported in both the nucleus and cytoplasm by immunohistochemical staining (3, 16–18). These previous findings suggested that the presence of maspin in specific cell compartments may have different biological and clinical implications. However, these previous reports lacked nuclear and cytoplasmic markers indicative of proper subcellular fractionation. Here, we fractionated cells into cytoplasmic and nuclear fractions and performed Western blot analysis of maspin expression using the same PharMingen anti-maspin antibody used by the previous investigators. These blots were then stripped and reprobed with α-tubulin antibody as an internal purity control. Surprisingly, a higher molecular weight (M_r ~65,000) unknown protein was detected in the nuclear fraction of all tested cells irrespective of the presence of maspin protein or mRNA, whereas the M_r 42,000 band was detected only in the cytoplasm of cells with maspin mRNA expression (Fig. 1). To verify and clarify the cytoplasmic localization of maspin, we separated the nuclear and cytoplasmic fractions of cell lysates from maspin expressing and nonexpressing cell lines (NCI-H23 and NCI-H322 respectively) and subjected them to Western blot analysis using an anti-maspin antibody; α-tubulin was used as a marker for the cytoplasmic fraction and also as an internal test for the purity of subcellular fractionation. Our results showed that the proteins from the two compartments were successfully separated and that maspin was found only in the cytoplasm (Fig. 1). Interestingly, a higher molecular weight unknown protein was present in the nuclear fraction, perhaps accounting for the nuclear staining in our immunohistochemical data and in other previous reports.

Correlation between Cytoplasmic Maspin Immunopositivity and Clinicopathological Parameters. To additionally investigate the association of maspin and p63 that we observed in the cell lines, we investigated 47 non–small-cell lung cancer specimens by immunohistochemistry and compared their clinicopathological characteristics with evidence of cytoplasmic maspin expression. As shown in Table 1, positive associations were found between histology ($P < 0.001$) and cytoplasmic maspin immunoreactivity. Maspin was expressed in all squamous cell carcinomas (13 of 13) but in fewer adenocarcinomas (4 of 19), demonstrating differential expression in terms of histology (Fig. 2; $P < 0.001$). All squamous carcinomas showed positive immunoreactivity to maspin, so expression of maspin



C
Association of Maspin and p63 or p53 in NSCLC

P53 / p63		Maspin +	Maspin -	P value
p63	+	17	1	<.001
	-	9	20	
p53	+	13	6	.309
	-	15	13	

D
Maspin expression according to p63 and p53

P63 / p53	Maspin +	Maspin -
P63+ / p53+	10	1
P63+ / p53-	10	0
P63- / p53+	5	3
P63- / p53-	5	13
Total	P < 0.001	

Fig. 2. Immunohistochemical staining of sections from formalin-fixed, paraffin-embedded patient samples. *A*. Intense cytoplasmic and nuclear maspin immunoreactivity is seen in tumor cells from squamous cell carcinomas. Only nuclear staining is observed in a maspin negative adenocarcinoma sample. Maspin and p63 colocalize in squamous carcinoma. *B*, histologic distribution of maspin immunoreactivity. *C*, significant association between maspin and p63 expression. *D*, the relationship of maspin expression to the presence of p63 and/or p53 in 47 lung cancer tissues.

Table 2 *Properties of maspin expression in NSCLC tissues except squamous carcinomas*

	Cytoplasmic maspin		
	Present (n = 15)	Absent (n = 19)	P*
Stage			0.063
1	11	8	
2	1	8	
3	3	3	
Lymph node			0.035
No	13	10	
Yes	2	9	

* χ^2 test.

was not significantly correlated to clinical parameters in squamous cell carcinoma. However, in other tumor tissues (Table 2), we observed a significant association between maspin expression and parameters such as lymph node involvement (P = 0.035) and tumor stage (P = 0.063). In lung cancers other than squamous carcinoma, the presence of cytoplasmic maspin was correlated with lower tumor stage and less lymph node involvement.

p63 Regulates the Expression of Maspin. Maspin is up-regulated in lung and pancreatic cancers (5, 16), but the molecular mechanism is currently unknown. Because both p63 and maspin were highly expressed in squamous carcinoma tissues, the correlation of p63 expression with differential maspin expression in lung cancer tissues was examined by immunohistochemical analysis using a p63 monoclonal antibody that recognized all six splice variants. As shown in Table 1, significant association was demonstrated between maspin expression and p63 expression. Interestingly, there was clear tendency of cell type-specific maspin expression. For example, in adenocarcinoma, mixed nuclear and cytoplasmic maspin staining was demonstrated for 4 of 19 samples. The staining was nuclear for large cell carcinoma (3 of 3), and both nuclear and cytoplasmic for mucoepidermoid carcinoma (6 of 6), and adenoid cystic carcinoma (2 of 2). However, because of the few maspin-expressing samples, cell type-specific association between maspin expression and p63 expression was not statistically significant in nonsquamous cell carcinomas. Because we found that a majority of p63 immunopositive cancer cells (17 of 18, 94%) stained for maspin, we examined whether maspin is under the control of p63 *in vitro*. Our data demonstrate that expression of some of p63 splice variants transactivated the maspin promoter (Fig. 3). Among the tested p63 splice variants, TAp63 demonstrated remarkable maspin promoter transactivation. Because a p63 splice variant-specific antibody was not available, we could not immunohistochemically confirm the predominant expression of a specific splice variant in lung tumors. However, our data on the concordant localization of p63 and maspin suggests that TAp63 may play a direct role in the modulation of maspin expression.

p63 Protein Binds to the p53 Consensus Sequence of the Maspin Promoter. To ascertain whether p63 directly interact with the maspin promoter, we performed gel shift assays using previously defined p53-binding site on the maspin promoter (6). As shown in Fig. 3*B*, p63 protein exhibited binding to the oligonucleotide with p53 consensus sequence. Anti-p63 antibody caused reduction in the intensity of the binding, thus indicating the presence of p63 in the binding complex (Fig. 3*B*). The lack of reduced binding with anti-p53 antibody correlates well with the absence of p53 in NCI-H322. Emerging evidence suggests regulation of maspin expression by DNA methylation *in vivo* (19–21). To examine whether the methylation affects the binding of p63 to p53 binding site of maspin promoter, a gel shift assay was carried out using a methylated probe. A p53-binding site located 103 bp upstream from the transcriptional start site of the maspin promoter is the functional site for maspin transcription (6). Although it is not within the p53 consensus sequence, a CpG (bold) is located proximal to the p53 consensus sequence (CC**CG**AACATGTTGGAGGCCTTTTGA). As shown in Fig. 3*B*, p63 exhibited binding to the methylated probe (mp53), indicating that the methylation of the maspin promoter may not directly inhibit the binding of p63 to the p53-binding site.

To assess *in vivo* physical interaction between p63 and the maspin promoter, we investigated whether p63 associate on the chromatin of endogenous maspin promoter using the chromatin immunoprecipitation assay. We immunoprecipitated chromatin from NCI-H23 cells using specific antibody against p63 or no antibody at all as a control. Genomic DNA fragments bound to p63 were analyzed by PCR using primers representing p53-binding site on the maspin promoter. Analysis of genomic DNA immunoprecipitated with p63 revealed p53-binding region of maspin promoter in NCI-H23 cells (Fig. 3*C*). Chromatin immunoprecipitation with anti-p63 antibody, subjected to PCR using primers representing a non-p63-binding site on the maspin promoter, did not amplify any DNA, thus demonstrating that p63 specifically bind to the p53-binding site on the maspin promoter. The

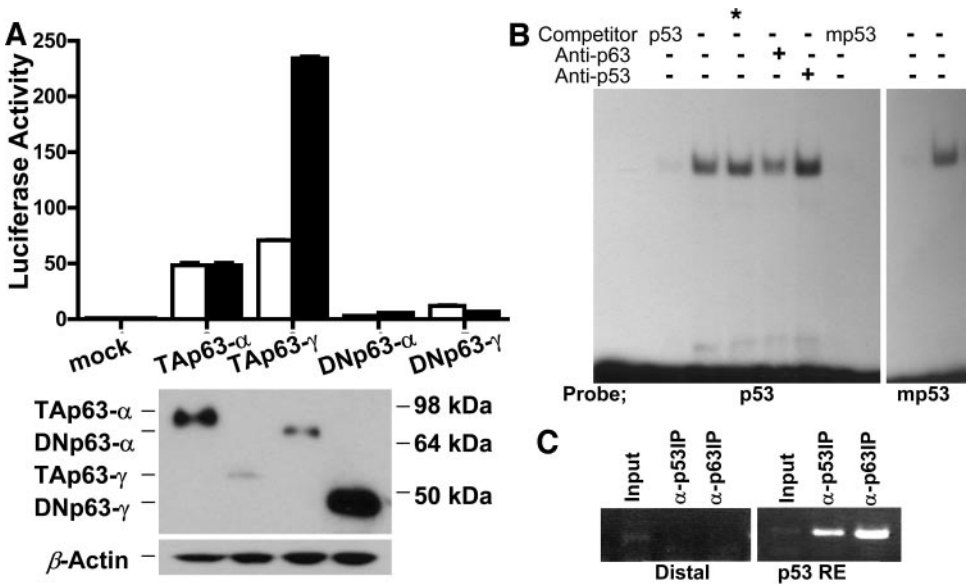


Fig. 3. p63 regulates maspin transcription. *A.* p63 enhances maspin promoter activity. NCI-H322 cells were transfected with a luciferase reporter construct containing the maspin promoter and a p63 splice variant plasmid or an empty mock plasmid. Relative activities were derived from arbitrary light units of luciferase activity normalized for Renilla luciferase activity (*white*) or after additional normalization with the expressed protein level of the p63 splice variant (*black*). The value obtained by transfecting the mock plasmid was arbitrarily designated as 1. The results are presented as fold change; *bars*, ± SD. *B*, specific binding of p63 to the p53 binding site on maspin promoter. The gel mobility shift assay was carried out as described under Materials and Methods. p53 consensus oligonucleotides from the maspin promoter (5′-CC**CG**AACATGTTGGAGGCCTTTTGGA-3′) was used as a probe. Same oligonucleotide with a methylated CpG (mp53) was used to demonstrate the p63 binding equally well to the methylated p53 site. Nuclear extract from p63-transfected NCI-H322 was used except * (NCI-H23). *C*, demonstration of *in vivo* binding of p63 to the p53-binding site on the maspin promoter by chromatin immunoprecipitation assay. Chromatin lysate from NCI-H23 cells were immunoprecipitated with antibody against p63. Samples were processed as described in Materials and Methods.

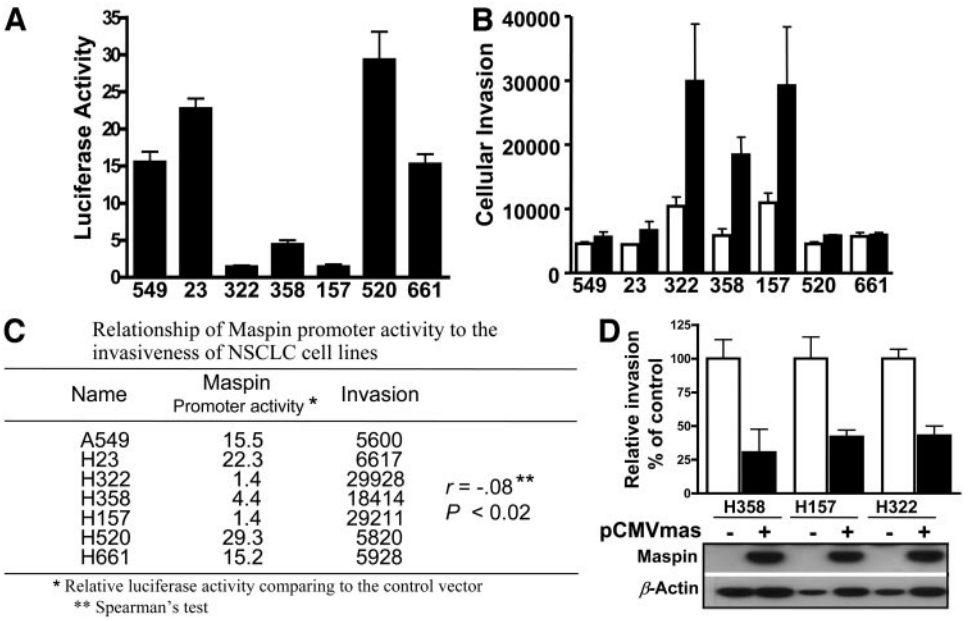


Fig. 4. Maspin affects the invasiveness of lung cancer cells. *A*, maspin promoter activity in various cell lines. Expression of a luciferase reporter gene under the control of the maspin promoter was analyzed in lung cancer cell lines 48 hours after transfection. Luciferase activity was normalized to transfection efficiency using a Renilla luciferase control vector. *B*, the relative invasion of lung cancer cells in the presence (■) or absence (□) of serum. *C*, the relationship of maspin promoter activity to the invasiveness of lung cancer cell lines. *D*. The expression of maspin reduces the invasion of invasive lung cancer cells. Cells were transiently transfected with mock plasmid pCMVflag or maspin-expressing pCMVmas and plated onto Matrigel-coated membranes; after 24 hours, invasive cells were counted. Ectopic maspin expression is shown by Western blotting, with β-actin as a loading control. ■, maspin +; □, maspin −.

binding with anti-p53 antibody correlates well with the presence of mutant p53 in NCI-H23, which is mutated at codon 246 but has intact DNA binding activity. Taken together, these results provide clear evidences that p63 interact with a previously defined p53-binding site on the maspin promoter.

Maspin Overexpression Inhibits Lung Cancer Cell Invasiveness. To explore the importance of differential maspin induction in lung cancer, we investigated the transcriptional activity of the maspin promoter in seven lung cancer cell lines using a reporter gene fused to the maspin promoter. Maspin promoter activity was almost negligible in NCI-H322, NCI-H358, and NCI-H157 cells, whereas it was quite high in NCI-H23, NCI-H520, and A549 cells (Fig. 4). Furthermore, cells with cytoplasmic maspin (NCI-H23, NCI-H520, and A549) demonstrated much less invasiveness than cells without maspin (NCI-H157, NCI-H322, and NCI-H358; Fig. 4). Intriguingly, a comparison between maspin promoter activity and the relative invasiveness of the seven cell lines (Fig. 4) revealed a statistically significant inverse correlation (Spearman's test, $r = -0.8$, $P = 0.013$). Thus, we transfected cells with a maspin-expressing construct, then tested invasiveness to determine whether ectopic expression of maspin could inhibit the invasion of NCI-H157, NCI-H322, and NCI-H358 cells. Maspin transfectants showed a >50% reduction in the number of invading cells compared with controls (Fig. 4), indicating that maspin expression inhibited invasion *in vitro*.

DISCUSSION

In breast and prostate cancers, maspin acts as a tumor suppressor capable of inhibiting motility, invasion, and metastasis (2, 3). To date, few studies have investigated the role of maspin in lung cancer, likely because it is not expressed in normal lung tissues. The experiments presented here demonstrate two important findings. First, maspin expression is positively regulated by p63 in lung cancer. Second, maspin expression is associated with tumor histology, stage, lymph node involvement, and (most importantly) invasiveness of lung cancer.

Maspin is up-regulated in lung cancer (5), but the molecular mechanism is not yet understood. Maspin is known to be under the control of p53 and Ets, and a recent article reported that the Pdef transcription factor is also capable of activating the maspin promoter (3, 4, 7). Unexpectedly, the expression levels of Ets1, p53, and Pdef were not correlated with maspin expression in lung cancer cells. Instead, maspin was expressed in close association with p63. Additionally, the experiments presented here demonstrate that transfer of the gene for p63 induced the binding of p63 to the p53 consensus DNA element and transactivation of maspin promoter.

To our knowledge, this is the first study analyzing the target of p63 in relation to cellular invasion or tumor progression. The product of the tumor suppressor p53 has been well known to influence the progression of cancer. Unlike p53, the p63 gene encodes multiple isotypes with remarkably divergent abilities to transactivate p53 reporter genes and induce apoptosis (9). Importantly, the predominant p63 isotypes in many epithelial tissues lack an acidic NH_2 terminus corresponding to the transactivation domain of p53. These truncated p63 variants can act as dominant-negative agents toward transactivation by p53 (9), and this could be an important determinant of tumor progression.

Because it has been reported that squamous lung carcinomas showed strong maspin immunoreactivity (5) in a pattern similar to that of p63 (14), we examined the correlation of p63 expression with differential maspin expression in lung cancer tissues by immunohistochemical analysis using a p63 monoclonal antibody that recognized all six splice variants. Because it has been suggested that maspin expression may be directly induced by p53 (6) and p53 and p63 bind to the same DNA element (9, 22), we additionally checked for association among p53, p63, and maspin. Mutant p53 is seen in 35 to 50% of lung cancers (23) and can function as an oncogene; under these conditions, its accumulation in the nucleus can be detected by immunohistochemical staining. Therefore, we tested whether p53 mutation correlated with expression of p63 or maspin. Here, we observed that p63 and mutant p53 were simultaneously expressed in 69% of squamous carcinomas and that p63 and maspin expression were strongly correlated in the presence or absence of mutant p53 ($P < 0.001$). However, no significant correlation was found between maspin and p53 staining in the tumors ($P = 0.309$).

Although the mechanism by which p63 participates in tumor progression remains unclear, sequence homology with p53 suggested that p63 may have similar expression profiles and roles in tumors. There is no available data correlating p63 immunostaining with its activity in

human tumors, so our ability to make a functional interpretation of the association between maspin and p63 is somewhat limited. Although immunostaining is an imperfect predictor of p63 mutation status, the observed overexpression appears not to be the result of a p63 mutation because these mutations are rare in human cancer cell lines and tissues (12, 13).

Interestingly, the level of maspin promoter activity correlated well with invasiveness of lung cancer cells (Spearman's test, $r = -0.8$, $P = 0.013$). In addition, transiently overexpressed maspin inhibited cell invasion *in vitro*. The invasion in NCI-H322 cells was not completely blocked by maspin gene transfer; perhaps because not all cells were expressing maspin after transient transfection (Fig. 4). Recently, Massion *et al.* (14) examined the percentage of p63-positive cells in lung cancer and demonstrated a prolonged survival in patients with p63 expression. Our analysis of lung cancer tissues revealed that the majority of p63 immunopositive cells stained positive for maspin (17 of 18, 94%). Because all squamous carcinomas showed positive immunoreactivity to maspin, expression of maspin could not be correlated to clinical parameters in squamous cell carcinoma. However, in nonsquamous cell carcinoma, the presence of cytoplasmic maspin was correlated with lower tumor stage ($P = 0.063$) and less lymph node involvement ($P = 0.035$).

Previous articles often reported significant relationship between maspin expression and clinical aggressiveness in pancreatic, ovarian, and gastric cancers (16, 24, 25), a finding contradictory to the tumor-suppressive function of maspin. However, unlike breast and prostatic cancer (26, 27), the *in vivo* functions of maspin have never been explored in these cancers. Although the precise mechanism of maspin action is unknown, maspin has been reported to interact with other proteins (28). Differential function of maspin may be determined by the interaction with these proteins, which may often result in a critical effect on tumor progression depending on a specific tumor microenvironment *in vivo*. Therefore, additional studies may be necessary to clarify the contribution of maspin in these cancers, as well as lung cancer.

In summary, our results show for the first time that p63 transactivates maspin expression, which may lead to inhibition of invasion; this is a new function for p63 in addition to its previously known functions in cell cycle and/or apoptosis. This newly emerging p63 function may provide a mechanistic explanation for the better prognosis of lung cancers that overexpress p63 (5).

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