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KLF6/Sp1 Initiates Transcription of the *tmsg*-1 Gene in Human Prostate Carcinoma Cells: An Exon Involved Mechanism

Miaozi Gong,[1] Wenjuan Yu,[1] Fei Pei,[1] Jiangfeng You,[1] Xianglin Cui,[1] Michael A. McNutt,[1] Gang Li,[2*] and Jie Zheng[1**]

[1] *Department of Pathology, Peking University Health Science Center, Beijing 100191, China*

[2] *Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100191, China*

ABSTRACT

The tumor metastasis suppressor gene-1 (*tmsg*-1) was first cloned as a new tumor suppressor gene in our laboratory several years ago. Since then, however, despite the substantial progression that has been made in investigation of the biologic roles played by this gene, the manner in which it exerts its regulatory influence is still unknown. With transfection of various deletion or mutation constructs, we identified a potential enhancer and three potential silencers in the 5′-flanking region. However, it was particularly interesting to find that a region (+59 to +123 bp) of exon 1 exhibited a strong role in initiation of *tmsg*-1 gene transcription. Deletion of this region led to essentially complete loss of driving activity of exon-1 sequence on luciferase. Further analysis showed that transcription factors KLF6 and Sp1 are able to interact with each other and bind to their elements in this region. Co-transfection of pGL3-114/+123 with KLF6- and/or Sp1-expressing plasmids resulted in an elevation of luciferase activity and transcription level of *tmsg*-1, which was abolished by knockdown of KLF6 or Sp1. Analysis of metastastic capacity showed that cells with high metastatic capability exhibited a lower level of KLF6/TMSG-1 proteins with higher invasive capability and vice versa. Thus, we concluded that interaction of KLF6 and Sp1, together with their binding of elements in exon 1 are critical events in initiation of transcription of the *tmsg*-1 gene. These results reveal a hitherto unreported mechanism for initiation of transcription of the tmsg-1 gene. J. Cell. Biochem. 113: 329–339, 2012.

KEY WORDS: *tmsg*-1; TUMOR SUPPRESSOR GENE; TRANSCRIPTIONAL REGULATION; TRANSCRIPTION FACTOR; EXON

Since discovery of the first tumor suppressor gene Rb in 1987, dozens of genes which exhibit tumor-suppressing properties have been reported. The tumor metastasis suppressor gene-1 (*tmsg*-1) was first cloned as a new tumor suppressor gene (GenBank accession number AF189062) in our laboratory from a human prostate cancer cell line in 1999 and was designated as LASS2 (homo sapiens longevity assurance homologue 2 of yeast LAG1) in 2001 [Pan et al., 2001; Xu et al., 2010]. The *tmsg*-1 gene is located on human chromosome 1, 1q21.3, and transcribed as two variants (No.NM_181746 and No.NM_022075) which both encode an identical protein of 380 amino acids of 45 kDa molecular weight (accession number of AAH10032) [Pei et al., 2004]. The TMSG-1 protein has six protein kinase phosphorylation sites, a homeodomain (amino acids 71–128) and a TLC (amino acids 131–332) structural domain, which forms five transmembrane domains [Xu et al., 2010]. Evaluation with monoclonal antibody has demonstrated that TMSG-1 protein is located in the endoplasmic reticulum of cells in the liver and kidney, and in the cytoplasm and membranes of tumor cells [Mizutani et al., 2005; Laviad et al., 2008].

In addition to its fundamental function as a synthase of long-chain ceramide, the TMSG-1 protein plays a prominent role in suppression of tumor invasion and metastasis [Mizutani et al., 2005].

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Miaozi Gong's present address is Department of Pathology, Peking University Shougang Hospital, Beijing, China.

*Correspondence to: Prof. Gang Li, PhD, Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100191, China. E-mail: ligang55@bjmu.edu.cn

**Correspondence to: Jie Zheng, PhD, Department of Pathology, Peking University Health Science Center, Beijing 100191, China. E-mail: zhengjie@bjmu.edu.cn

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It has been found that TMSG-1 is highly expressed in the low metastatic potential prostate cancer cell line PC-3M-2B4 and shows low expression in the highly metastatic prostate cancer cell line PC-3M-1E8, demonstrating a negative correlation with tumor metastatic potential. The roles played by TMSG-1 in suppression of tumor invasion and metastasis have been reported to be achieved through various mechanisms. TMSG-1 has been shown to directly bind to C subunit of V-ATPase [Pan et al., 2001; Xu et al., 2010]. V-ATPase is a newly identified ATP-dependent hydrogenion pump, which can pump H^+ out of the cell through transmembrane transportation and thus play an important role in formation and maintenance of the extracellular acidic microenvironment and tumor metastasis [Sennoune et al., 2004]. These mechanisms were involved in activation of V-ATPase as well as consequent increase of cell movement and migration, the release of signal molecules attributing to degradation of extracellular matrix and the increase of V-ATPase-mediated sensitivity of cells to these molecules [Liotta and Kohn, 2001; Chen et al., 2004]. In addition, TMSG-1 also promotes cell apoptosis through the synthesis of ceramide, which in turn induces cell cycle arrest and affects the activity of telomerase as well as cell life span [Pettus et al., 2002; Ruvolo, 2003; Mizutani et al., 2005; Rossi et al., 2005; Su et al., 2008].

However, very little subsequent progress has been made in determining the mechanism of regulation of *tmsg*-1 gene transcription. The multifaceted roles of TMSG-1 in relation to tumor metastasis are intimately correlated with its manner of regulatory expression that is being worked out only slowly. Primary studies showed that the expression of ceramide synthase is regulated by the LAG1 promoter, which is structurally and functionally related to the gene [Yu et al., 2006]. A high level of expression was detected in human kidney and liver, and there were low levels of expression in the brain, heart, placenta, and lung [Pan et al., 2001]. These results reflect differential expression of the *tmsg*-1 gene in various tissues, which is highly correlated to the metastatic potential of tumor from these tissues [Su et al., 2008]. However, the limited data from these studies has fallen far short of complete explanation of the mechanisms involved. Accordingly, it was of considerable interest to us to undertake exploration of the mechanism underlying transcriptional regulation of the *tmsg*-1 gene in this study.

MATERIALS AND METHODS

CELL CULTURE

Human prostate carcinoma cell sublines PC-3M-1E8 (1E8) and PC-3M-2B4 (2B4), and human pulmonary giant cell carcinoma cell sublines PG-BE1 (BE1) and PG-LH7 (LH7) were isolated in the Department of Pathology, Peking University and were cultured in RPMI 1640 medium supplemented with 10% FCS [Zhu et al., 1995; Liu et al., 1999].

PLASMID CONSTRUCTS AND SITE-DIRECTED MUTAGENESIS

A series of 5′ deleted *tmsg*-1 gene regulatory region-Luc fusion constructs were created by using a deletional and PCR strategy. Products amplified from −2,182 bp to +19 bp of the *tmsg*-1 gene were cloned into the pGL3-basic vector containing a firefly luciferase reporter gene, resulting in pGL3-2182/+19(primers P8/P12), −1190/+19(primers P7/P12), −981/+19(primers P6/P12), −847/+19(primers P5/P12), −669/+19(primersP4/P12), −429/+19(primers P3/P12), −271/+19(primers P2/P12), and −114/+19(primers P1/P12). Constructs pGL3-156/+19(primers P9/P12), −203/+19(primers P10/P12), and −243/+19(primers P11/P12) potentially containing the promoter were created by further amplification starting from +19 bp. Considering the potential impact of the exons on activity of the promoter, oligonucleotides of different lengths amplified starting from +303 bp of exon 1 of the *tmsg*-1 gene were inserted into a pGL3-basic vector to obtain constructs pGL3-271/+303(primers P2/P17) and −114/+303(pri-P1/P17). For a detailed analysis of the role of exon 1, oligonucleotides of different lengths were amplified starting from −114 bp and were inserted into a pGL3-basic vector to create pGL3-114/+59(primers P1/P13), −114/+123(primers P1/P14), −114/+161 (primers P1/P15), and −114/+249(primers P1/P16). All primers containing *Nhe*I/*Hin*dIII restriction endonuclease recognition sequences at the 5′-end were designed and synthesized by the Augct Co. (China) (Table I). The reaction system for PCR included preheating at 95°C for 2 min followed by 98°C for 15 s, 68°C for 30 s for 30 cycles, and 68°C for 5 min.

To verify the interaction of transcription factors and their *cis*-elements in exon 1, Luc-constructs pGL3-114/+123m1, and pGL3-114/+123m2, which were mutated at potential binding sites of KLF6 and Sp1 as analyzed by software MatInspector and amplified from pGL3-114/+123 using the mutation primers mut1 sense/antisense and mut2 sense/antisense (Table I), were created by DpnI digestion and transformation of competent DMT cells. pGL3-114/+123m2 was further amplified with primers mut1 sense and mut1/2 antisense to create pGL3-114/+123m1/2, which contained mutated bases at different sites within the binding region of KLF6 and Sp1. The PCR reaction conditions consisted of 95°C for 2 min, 98°C for 15 s, and 68°C for 2 min and 30 s for 25 cycles.

Expressional constructs containing the open-reading frame of KLF6, splice variant KLF6-SV2 and Sp1 were generated by PCR strategy. Primers containing *Eco*RI and *Xho*I restriction endonuclease recognition sequences at the 5′-end of upstream and downstream primers, respectively, were designed (Table I). The PCR reaction conditions consisted of 95°C for 1 min followed by 95°C for 20 s, 56°C for 20 s, and 68°C for 2 min for 30 cycles. Products were subjected to *Eco*RI/*Xho*I digestion and inserted into a pcDNA3 vector to create expressional plasmids pcDNA3-KLF6, pcDNA3-KLF6-SV2, and pcDNA3-Sp1. All of these constructs were verified by DNA sequencing (data not shown).

To further observe the effects of KLF6 and Sp1, we employed small interference oligoduplexes as listed in Table I targeting against KLF6 and Sp1 mRNA. After PC-3M-2B4 cells were transfected with expressional constructs (2 μg) or si-RNA (50 pmol) with Lipofectamin 2000 (Invitrogen) for 24 or 48 h, the efficiency of reduction of expression of KLF6 and Sp1 was examined with real-time PCR.

TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER GENE ASSAY

All plasmids used in these transfection experiments were prepared by the PureYield™ Plasmid Miniprep System (Promega) following

TABLE I. Sequences of Oligonucleotides Used as Primers or Probes for Plasmid Constructs, Site-Directed Mutagenesis, EMSA/super-EMSA, ChIP, Real-Time PCR, and RNA Interference Assay

Primers or probes	Sequences
Deletion plasmids	
Sense	
P1	5′-CTAGCTAGCAGACCTCCCACCCCGAAAAGA-3′
P2	5′-CTAGCTAGCGGGGCTGGAAGTGGGAATGTG-3′
P3	5′-CTAGCTAGCTTTTACGGCGGGGTAGGCGA-3′
P4	5′-CTAGCTAGCCGCCTCGGCCTCCCAAAGT-3′
P5	5′-CTAGCTAGCCGGCTCACTGCAAACTCCACCT-3′
P6	5′-CTAGCTAGCACCTCGGCCACCCAAAGTGC-3′
P7	5′-CTAGCTAGCCATCTTAGTCACCACTCGTTCACG-3′
P8	5′-CTAGCTAGCAGGGACTACAGGAAAGCCTGGCA-3′
P9	5′-CTAGCTAGCGGACCCCGCGGCATCCAG-3′
P10	5′-CTAGCTAGCGGGGTGGGTAGGAGTAAGGGGA-3′
P11	5′-CTAGCTAGCTGGCGGGGACCGGGGG-3′
Antisense	
P12	5′-CCCAAGCTTAGACCCCAACCCTCCGCTCC-3′
P13	5′-CCCAAGCTTTCTGCGCTGCGGCTGCCCCTT-3′
P14	5′-CCCAAGCTTAAGGCCGCGAGCAGCCCAA-3′
P15	5′-CCCAAGCTTAGCCCAGTCGAGCTGAGCC-3′
P16	5′-CCCAAGCTTTCCTCCCTCTTCCGCCCG-3′
P17	5′-CCCAAGCTTTCCGTCTGCTCGGGTGGTTG-3′
Site-directed mutagenesis	
mut1	
Sense	5′-CGCAGAGGCCCGTTTCGCCCTCCCCT-3′
Antisense	5′-GCGAAACGGGCCTCTGCGCTGCGG-3′
mut2	
Sense	5′-GCCCTAAACTCCCGTCACGCCCAGC-3′
Antisense	5′-GAGTTTAGGGCGGGGCGGGCCTCTG-3′
mut1/2	
Antisense	5′-GCGTGACGGGAGTTTAGGGCGAAACGGGCC-3′
Expressional plasmids	
pcDNA3-KLF6/pcDNA3-KLF6-SV2	
Sense	5′-GGAATTCGCCGCCACCATGGACGTGCTCCCCATGTG-3′
Antisense	5′-CCGCTCGAGTCAGAGGTGCCTCTTCATGTGCA-3′
pcDNA3-Sp1	
Sense	5′-GGAATTCGCCACCATGAGCGACCAAGATCACTC-3′
Antisense	5′-CCGCTCGAGTCAGAAGCCATTGCCACTGA-3′
EMSA	
P30B	
Sense	5′biotin-GGCCCGCCCCCGCCCTCCCCTCCCGTCACGC-3′
Antisense	5′biotin-GCGTGACGGGAGGGGAGGGC*GGGGC*GGGCC-3′
P30	
Sense	5′-GGCCCGCCCCCGCCCTCCCCTCCCGTCACGC-3′
Antisense	5′-GCGTGACGGGAGGGGAGGGC*GGGGC*GGGCC-3′
P30m	
Sense	5′-GGCCCGTTTCGCCCTAAACTCCCGTCACGC-3′
Antisense	5′-GCGTGACGGGAGTTTAGGGCGAAACGGGCC-3′
P-KLF6	
Sense	5′-GATCAGGTCACCCACAGGCCC-3′
Antisense	5′-GGGCCTGTGGGTGACCTGATC-3′
P-Sp1	
Sense	5′-ATTCGATC*GGGGCGGGGC*GAG-3′
Antisense	5′-CTCGCCCCGCCCCGATCGAAT-3′
P-Smad3	
Sense	5′-TCGAGAGCCAGACAAAAAGCCAGACATTTAGCCAGACAC-3′
Antisense	5′-GTGTCTGGCTAAATGTCTGGCTTTTTGTCTGGCTCTCGA-3′
ChIP assay	
Sense	5′-CTAGCTAGCAGACCTCCCACCCCGAAAAGA-3′
Antisense	5′-CCCAAGCTTAAGGCCGCGAGCAGCCCAA-3′
Real-time PCR	
GAPDH	
Sense	5′-GAAGGTGAAGGTCGGAGTC-3′
Antisense	5′-GAAGATGGTGATGGGATTTC-3′
TMSG-1	
Sense	5′-TCCTGCCTTCTTTGGCTATTACTT-3′
Antisense	5′-TGCGTTCATCTTCTACCAGCTTTC-3′
wt-KLF6	
Sense	5′-CGGACGCACACAGGAGAAAA-3′
Antisense	5′-CGGTGTGCTTTCGGAAGTG-3′

(*Continued*)

TABLE I. (*Continued*)

Primers or probes	Sequences
Sp1	
Sense	5′-CACGTTCGGATGAGCTACAGAGG-3′
Antisense	5′-TACACCTGGGCCTCCCTTCTTAT-3′
Oligonucleotides for RNA interference	
si-KLF6	
Sense	5′-CACGGCCAAGUUUACCUCCGATT-3′
Antisense	5′-UCGGAGGUAAACUUGGCCGUGTT-3′
si-Sp1	
Sense	5′-UUGGGUAAGUGUGUUGUUUAATT-3′
Antisense	5′-UUAAACAACACACUUACCCAATT-3′

The mutated bases are in boldface and underlined. The core sequences for binding of Sp1 (italic) or KLF6 are in boldface.

the manufacturer's recommended protocol. Transfection efficiency was monitored by co-transfection of the pRL-SV40 (Promega) containing renilla luciferase reporter gene driven by the SV40 promoter. Transfection of PC-3M-2B4 cells with Lipofectamine 2000 Reagent (Invitrogen) was performed as described in our previous report [Wei et al., 2005]. The relative luciferase activity was expressed as the ratio of firefly luciferase activity to renilla luciferase activity. The empty vector (pGL3-basic) served as the negative control.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) AND SUPERSHIFT EMSA (sEMSA).

Nuclear extracts from PC-3M-2B4 cells were prepared by the method as described by Li et al. [2008]. All double strand oligonucleotides used as probes for EMSA were listed in Table I. EMSA and sEMSA were performed by using biotin labeled probes P30B(+60/+89 bp) and unlabeled probes P-KLF6, P-Sp1 which contained transcription factor KLF6 and Sp1 consensus binding sites. Competition experiments included the addition of a 50-fold excess of unlabeled oligonucleotide, while supershift analysis included the addition of 2 μg antibody against either KLF6 or Sp1 (Santa Cruz Biotechnology) to the reaction mixture for 30 min at room temperature before the addition of probe. Probe P-Smad3 was used as a non-specific competition control. Probe P30m mutated in the binding sequences of KLF6 and Sp1 was used for further verification of the binding of proteins to their *cis*-element.

CHROMATIN IMMUNOPRECIPITATION

The chromatin immunoprecipitation (ChIP) assay was performed in PC-3M-2B4 and PC-3M-1E8 cells as described previously to determine the binding capacity of KLF6 and Sp1 to their *cis*-elements [Li et al., 2008]. DNA fragments in DNA–protein complexes precipitated by antibodies against KLF6 or Sp1 were purified with the ZYMO ChIP DNA Clean & Concentrator™ Kit (ZYMO RESEARCH) and subjected to PCR assay with primers as listed in Table I. The reaction conditions were as follows: 95°C for 2 min, followed by 30 cycles at 98°C for 15 s (denaturation); and 68°C for 30 s (annealing and elongation). Products of PCR were analyzed by 2% agarose gel electrophoreses.

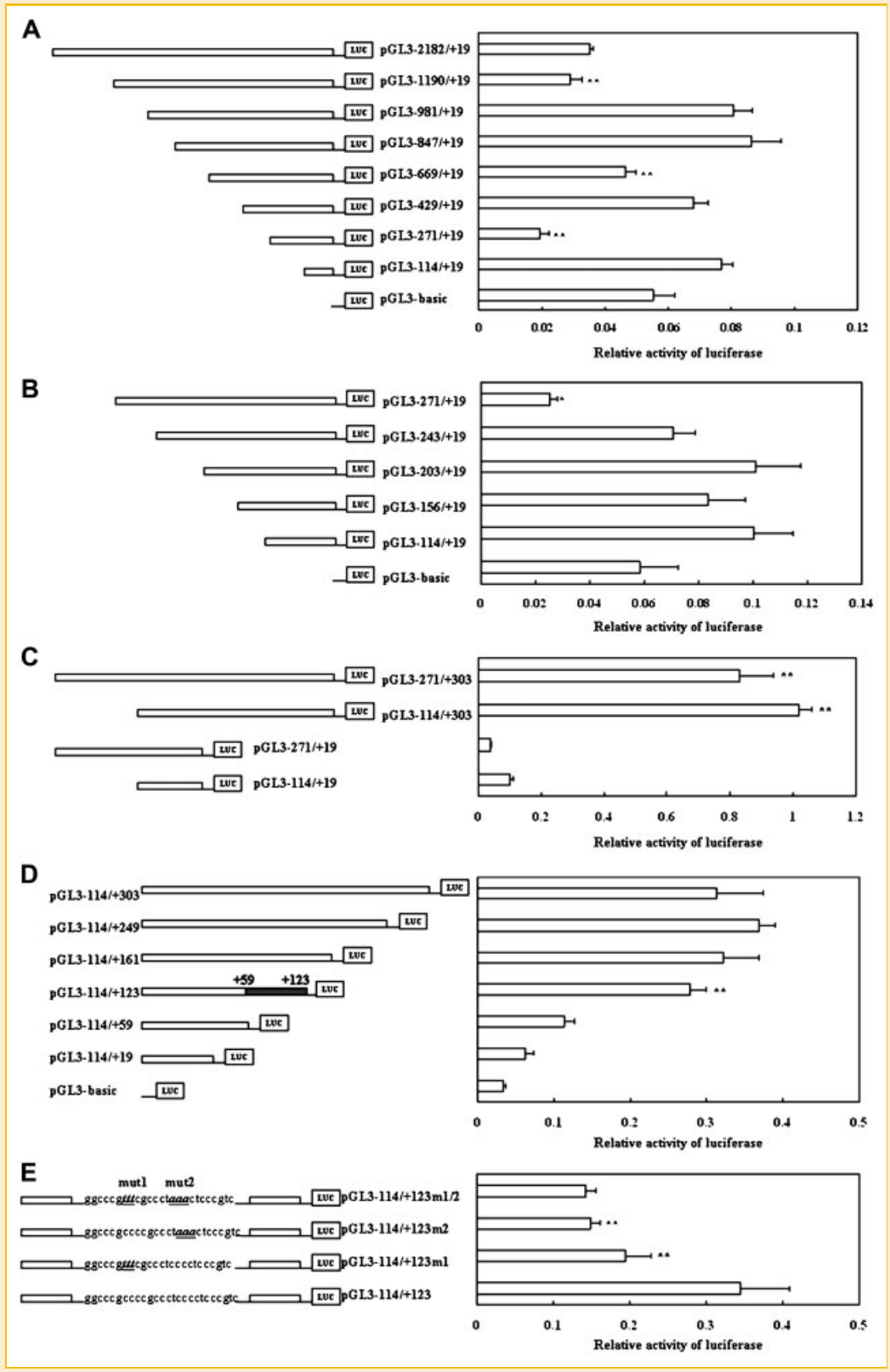
A
pGL3-2182/+19
pGL3-1190/+19
pGL3-981/+19
pGL3-847/+19
pGL3-669/+19
pGL3-429/+19
pGL3-271/+19
pGL3-114/+19
pGL3-basic
0 0.02 0.04 0.06 0.08 0.1 0.12
Relative activity of luciferase
B
pGL3-271/+19
pGL3-243/+19
pGL3-203/+19
pGL3-156/+19
pGL3-114/+19
pGL3-basic
0 0.02 0.04 0.06 0.08 0.1 0.12 0.14
Relative activity of luciferase
C
pGL3-271/+303
pGL3-114/+303
pGL3-271/+19
pGL3-114/+19
0 0.2 0.4 0.6 0.8 1 1.2
Relative activity of luciferase
D
pGL3-114/+303
pGL3-114/+249
pGL3-114/+161
+59 +123
pGL3-114/+123
pGL3-114/+59
pGL3-114/+19
pGL3-basic
0 0.1 0.2 0.3 0.4 0.5
Relative activity of luciferase
E
mut1 mut2
pGL3-114/+123m1/2
pGL3-114/+123m2
pGL3-114/+123m1
pGL3-114/+123
0 0.1 0.2 0.3 0.4 0.5
Relative activity of luciferase

Fig. 1.

COIMMUNOPRECIPITATION (CoIP)

CoIP experiments were routinely performed to evaluate the interaction of KLF6 and Sp1 proteins [Liu et al., 2009]. Antibodies against KLF6 and Sp1 used for CoIP assays were purchased from Santa Cruz Biotechnology.

QUANTITATIVE REAL-TIME PCR AND WESTERN BLOT ANALYSIS

The expression of the *tmsg*-1 gene was determined by reverse transcription and quantitative real-time PCR (RT-qPCR) assay, and by Western blotting. Primers for real-time PCR were synthesized by the Augct Co. (China) and are listed in Table I. The reaction conditions for qPCR were as follows: 95°C for 3 min, followed by 40 cycles at 95°C for 30 s (denaturation); and 60°C for 1 min (annealing and elongation). Threshold cycle numbers (CT) were determined with the ABI PRISM 7000 sequence detection system (Version 1.1 software). The mRNA levels from the qPCR assay were calculated as: ratio $= 2^{-CT(sample)}/2^{-CT(GAPDH)}$, where CT represents the threshold cycle number of the sample and GAPDH. The relative concentration of qPCR products is presented as the fold change of mRNA level of samples relative to GAPDH. Western blotting was routinely performed by using anti-KLF6 and -Sp1 antibodies purchased from Santa Cruz Biotechnology. RT-qPCR and Western blotting were also carried out for analysis of the relationship between KLF6 and TMSG-1 mRNA/protein levels, in which cells with highly metastatic properties (1E8 and BE1) and non-metastatic properties (2B4 and LH7) were used.

MATRIGEL INVASION ASSAY

In vitro invasion assay was performed as previously described by Albini et al. [1987]. Briefly, PC-3M-2B4 cells were transfected with pcDNA3-KLF6 or si-KLF6. Twenty-four hours after transfection, cells were harvested and seeded in the upper compartment. Cells which had invaded found on the underside of the filters were examined under a light microscope with 200× magnification. Ten random fields were counted on each filter. These experiments were repeated three times.

RESULTS

TRANSCRIPTIONAL ACTIVITY OF THE PUTATIVE *tmsg*-1 GENE PROMOTER

To analyze the function of the putative *tmsg*-1 regulatory region, a series of 5′-deleted *tmsg*-1-luc plasmids were constructed with the 3′ends terminating at +19 bp or +303 bp. The *tmsg*-1-luc constructs were transiently transfected into PC-3M-2B4 cells to analyze the function of the regulatory regions of the *tmsg*-1 gene. As shown in Figure 1, the fold changes of pGL3-2182/+19, −1190/+19, −981/+19, −847/+19, −669/+19, −429/+19, −271/+19 and −114/+19 as compared to pGL3-basic were 0.6, 0.5, 1.5, 1.6, 0.8, 1.2, 0.3, and 1.4, respectively (Fig. 1A). pGL3-981/+19, −429/+19 and −114/+19 deleted, respectively, in the 5′-region from −1190 bp to −981 bp, −669 bp to −429 bp and −271 bp to −114 bp resulted in an enhancement of activity as compared to pGL3-1190/+19, −669/+19 and −271/+19 ($P < 0.01$), indicating there were potential silencer elements in these regions. Reduced activity occurred, respectively, in pGL3 − 669/+19 and pGL3−271/+19 upon deletion of the 5′-region from −847 bp to −669 bp and from −429 bp to −271 bp as compared to pGL3-847/+19 and pGL3-429/+19 ($P < 0.05$, $P < 0.01$), indicating the presence of potential enhancer elements in these regions. Further analysis by using deleted constructs pGL3-156/+19, −203/+19, and −243/+19 showed that the silencer elements were located in the region from −271 to −243 bp (Fig. 1B).

It was of particular interest that the highest activities were detected in pGL3-271/+303 and −114/+303 in transfected cells. Deletion of partial sequences from +19 to +303 bp in exon 1 caused a remarkable loss of activity as compared to pGL3-271/+19 and −114/+19 ($P < 0.01$) (Fig. 1C). This result strongly indicated involvement of exon 1 in transcriptional regulation of the *tmsg*-1 gene. Further analysis by creation of pGL3-114/+59, −114/+123, −114/+161, and −114/+249 which covered different lengths of the region of exon 1 showed that deletion of a sequence from +59 to +123 bp led to apparent loss of driving activity of the exon-1 sequence on luciferase, indicating it is an enhancer region (Fig. 1D). Analysis of the sequence showed that there were binding sites for transcription factors KLF6 and Sp1 in the region by using software MatInspector. It was therefore inferred that the involvement of exon 1 in transcriptional regulation of the *tmsg*-1 gene is achieved through interaction of trans-factors with their elements.

We further introduced mutation (mut1 or/and mut2) into plasmids to analyze the underlying role of *cis*-elements of exon 1 in the transcription of the *tmsg*-1 gene (Fig. 1E). Constructs with wild-type and mutated sites for KLF6/Sp1 were transfected into PC-3M-2B4 cells. Data showed that pGL3-114/+123 with wild-type KLF6/Sp1 binding sites expressed a higher level of luciferase activity. However, activity of luciferase in the construct containing a

Fig. 1. Impact of the 5′ untranslated region and exon-1 of the *tmsg*-1 gene on luciferase activity. A: A series of 5′-deleted *tmsg*-1-luc plasmids with the 3′ end terminating at +19 bp were prepared and introduced into PC-3M-2B4 cells. After culture for 24 h, cells were harvested for luciferase activity assay. B: Further analysis for a potential silencer between −271 and −114 bp using deletion constructs pGL3-156/+19, −203/+19, and −243/+19. The driving role on luciferase activity of each construct was standardized by cotransfection of the internal control plasmid expressing renilla luciferase and was expressed as the ratio of firefly luciferase activity to renilla luciferase activity. $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant in comparing construct pGL3-basic and all other constructs. C: Constructs pGL3-271/+303 and −114/+303 with the 3′end terminating at +303 bp were prepared and introduced into PC-3M-2B4 cells. pGL3-271/+19 and −114/+19 were used for comparison with driving roles of pGL3-271/+303 and −114/+303. $^{**}P < 0.01$ was considered statistically significant in comparing construct pGL3-114/+19 and −114/+303 or pGL3-271/+19 and −271/+303. D: Further analysis for effect of the exon-1 sequence between +19 and +303 bp using deletion constructs pGL3-114/+303, −114/+249, −114/+161, −114/+123, and −114/+59. A potential enhancer in panel D is indicated by solid box. $^{**}P < 0.01$ was considered statistically significant in comparing constructs pGL3-114/+59 and −114/+123. E. Constructs (pGL3-114/+123m1, m2, and m1/2) containing the exon-1 region mutated at KLF6 or/and Sp1-binding sites were used for evaluation of the impact of mutation. The sequences represent potential KLF6/Sp1 *cis*-acting elements. Mutated bases are in boldface and are italicized. mut-1 and mut-2 represent mutation site 1 and site 2. $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant in comparing construct pGL3-114/+123 and all other constructs. The bars in each panel show the mean ± sd of the results from three independent transfection experiments.

mutation (mut1 or/and mut2) in the KLF6/Sp1 element was significantly lower than that of the wild-type construct ($P < 0.01$). It was noted that the construct pGL3-114/+123m2 gave rise to the lowest activity even though no further decline occurred with pGL3-114/+123m1/2.

IDENTIFICATION OF THE PUTATIVE KLF6 AND Sp1-BINDING REGION IN THE EXON 1 OF THE *tmsg*-1 GENE

EMSA and sEMSA were carried out to verify the existence of binding sites of KLF6 and Sp1 in exon 1 of the *tmsg*-1 gene. The results showed that a slow migrating band was visualized with probe P30B, which could be specifically abolished by competition with a 50-fold molar excess of unlabeled oligonucleotide P30, P-Sp1, and P-KLF6, but not by the non-specific probe P-Smad3 (Fig. 2A). The mutated probe P30m did not result in competitive inhibition. These results confirmed the existence of binding sites for KLF6 and Sp1 proteins in exon 1. The specificity of this binding was further verified by a super shift band using antibody against KLF6 or Sp1 in the sEMSA assay (Fig. 2A).

EVALUATION OF THE CAPACITY OF KLF6 AND Sp1-BINDING TO EXON 1 OF THE *tmsg*-1 GENE

The ChIP assay was performed to analyze the capacity of KLF6 and Sp1 for binding to exon 1 of the *tmsg*-1 gene. As shown in Figure 2B, both transcription factors KLF6 and Sp1 had the capability of binding to their elements in exon 1 (Fig. 2B). The signal in non-metastatic PC-3M-2B4 cells was much stronger than in the highly metastatic PC-3M-1E8 cells. The results further demonstrated not only the involvement of exon 1 in transcriptional regulation of the *tmsg*-1 gene, but also the functional relationship between the regulation of KLF6/Sp1 in *tmsg*-1 gene transcription and potential for tumor metastasis.

ANALYSIS OF THE INTERACTION BETWEEN TRANSCRIPTION FACTOR KLF6 AND Sp1 PROTEINS

CoIP analysis was performed to analyze the possible interaction between KLF6 and Sp1 proteins. The result showed that immunoprecipitation with anti-KLF6 led to the detection of a Sp1 band and vice versa (Fig. 2C, lanes 2 and 3). However, the KLF6-Sp1 complex was undetectable when a non-immune rabbit IgG was used as a control (Fig. 2C, lane 4), indicating the specificity of the interaction.

THE ROLE OF KLF6 AND Sp1 IN PROMOTION OF TRANSCRIPTION OF THE *tmsg*-1 GENE

To further confirm the role of KLF6 and Sp1 in promotion of transcription of the *tmsg*-1 gene, we co-transfected pGL3-114/+123 carrying KLF6 and Sp1-binding sites of exon 1 and constructs encoding KLF6 or/and Sp1 into PC-3M-2B4 cells. Data showed that the luciferase activity was promoted by construct pcDNA3-KLF6, but not by splice variant KLF6-SV2 (Fig. 3A). Co-transfection of pGL3-114/+123 with pcDNA3-Sp1 or pcDNA3-Sp1/pcDNA3-KLF6-SV2 resulted in moderate elevation of enzyme activity. The most apparent increase was observed by co-transfection of

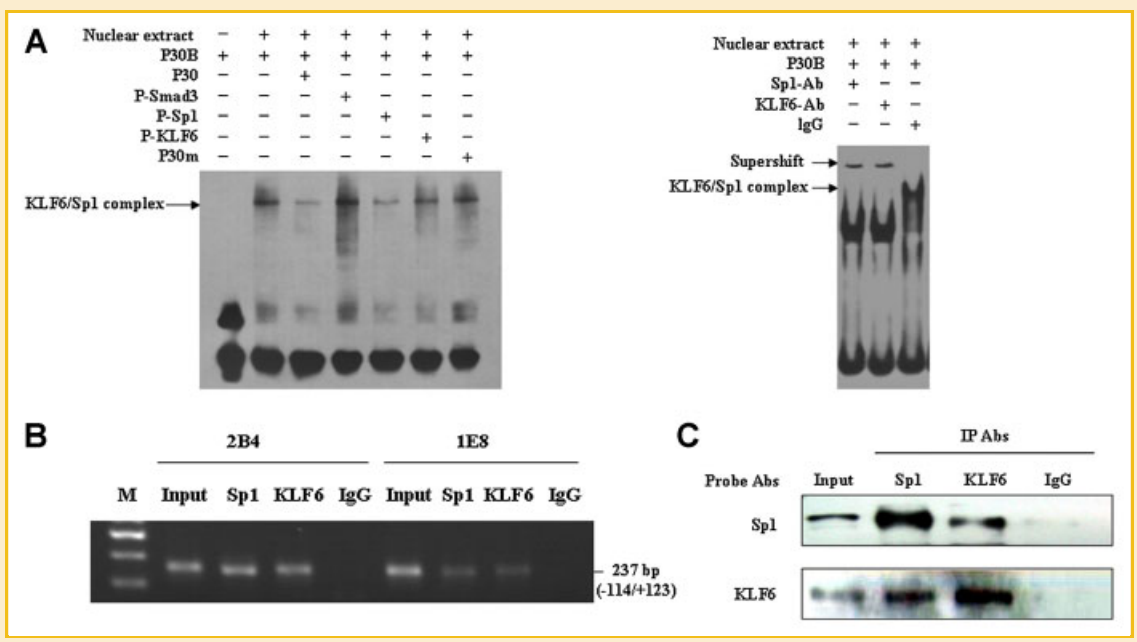

Fig. 2. Interaction of transcription factors and their capability for binding to elements in exon-1. A: EMSA (left image) and sEMSA (right image) were used to analyze the binding of KLF6 and Sp1 to their elements. Each complex is indicated by an arrow. The image is representative of an experiment that was repeated at least three times. B: ChIP was used for confirming binding of KLF6/Sp1 to their element in exon-1 and for comparison of binding capacity in tumor cell lines with various metastatic potentials in PC-3M-2B4 and PC-3M-1E8 cells. C: CoIP was used for evaluation of the interaction between KLF6 and Sp1. Lysates from PC-3M-2B4 cells were immunoprecipitated (IP) with antibodies against KLF6 and Sp1 protein, or with non-immune rabbit IgG (as indicated above each lane) and separated on an SDS/PAGE gel. Coimmunoprecipitated complexes were transferred to a nitrocellulose membrane, immunoblotted with anti-KLF6 or -Sp1 antibody and X-ray film was exposed. Input represents total cellular protein without immunoprecipitation. Images are representative of three repetitions of each experiment.

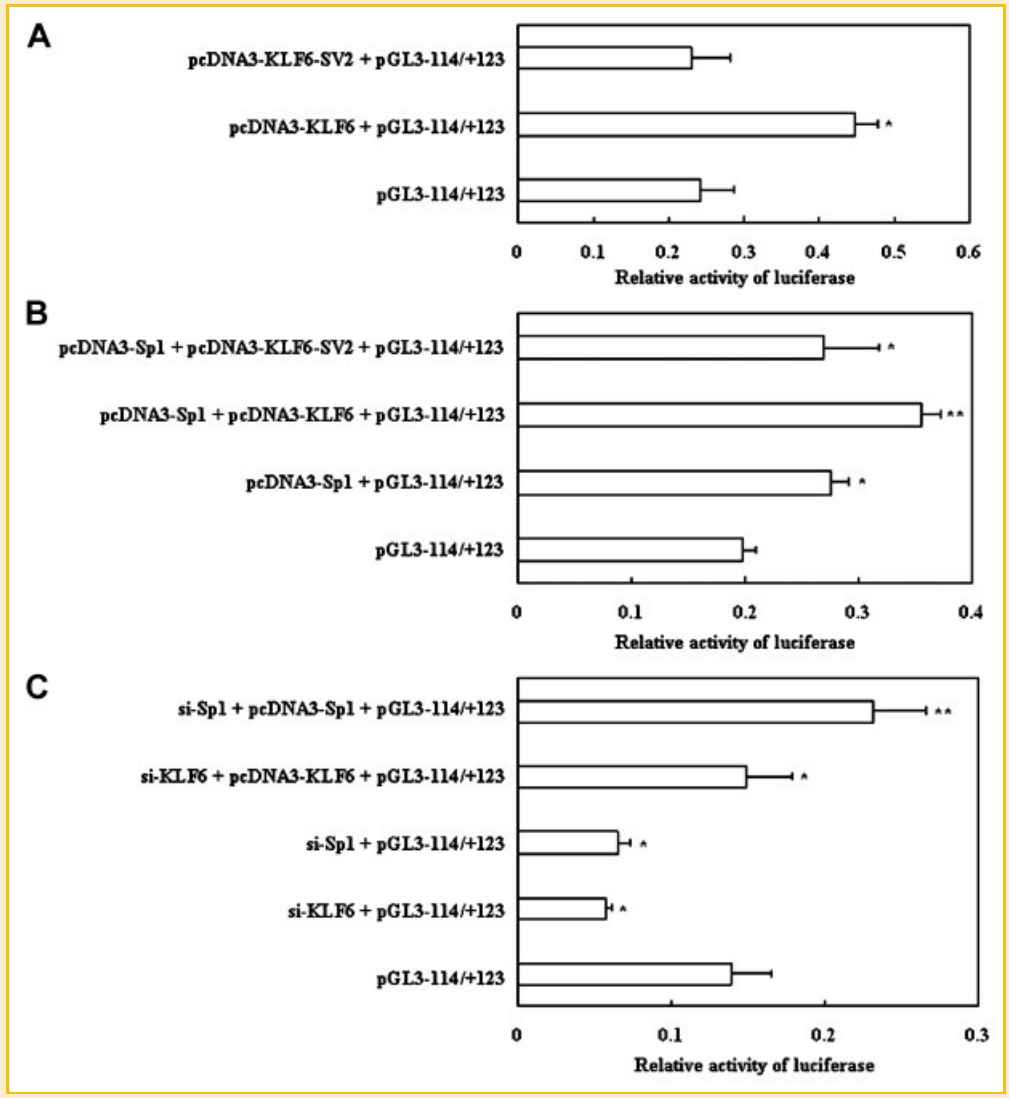

Fig. 3. Effects of over expression of transcription factor KLF6 or/and Sp1 on luciferase activity driven by the sequence of exon-1 containing KLF6 or/and Sp1-binding sites. A: Effect of over expression of KLF6 or KLF6 splice variant on activity of luciferase. B: Effect of over expression of Sp1, Sp1/KLF6, Sp1/ KLF6 splice variant on activity of luciferase. C: Effect of KLF6 or Sp1 knockdown on activity of luciferase. Data represent mean ± SD of results from three independent transfection experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant.

pGL3-114/+123 with pcDNA3-Sp1/pcDNA3-KLF6 as compared with pGL3-114/+123 ($P < 0.01$) (Fig. 3B).

The effect of KLF6 and Sp1 in promotion of transcription of the *tmsg*-1 gene was further verified with the RNA interference technique. As shown in Figure 3C, enzyme activity was not affected by co-transfection of pcDNA3-KLF6 with si-KLF6 (Fig. 3C). The effects of knockdown became remarkable in the absence of over-expressed KLF6 or Sp1 even though si-Sp1 alone was not able to abolish the effect of pcDNA3-Sp1 on incremental enzyme activity.

THE ROLE OF KLF6 AND Sp1 IN EXPRESSION OF THE *tmsg*-1 GENE

The effect of transfection of pcDNA3-KLF6 or pcDNA3-Sp1 on expression of the *tmsg*-1 gene was verified with the qPCR assay. As shown in Figure 4A, the elevation of KLF6 level by transfection of pcDNA3-KLF6 was associated with elevation of TMSG-1 in comparison with the empty vector control ($P < 0.01$) (Fig. 4A). This effect was not observed in the case of transfection of splice variant pcDNA3-KLF6-SV2. On the other hand, the effect was abolished by knockdown of KLF6 (Fig. 4B). Coincidently, the effects of transfection of pcDNA3-Sp1 were apparent and were reversed by interference RNA ($P < 0.01$) (Fig. 4C,D). These results further emphasize the roles of KLF6 and Sp1 in expression of the *tmsg*-1 gene.

DETERMINATION OF THE EXPRESSION OF KLF6 AND TMSG-1 IN CELLS WITH DIFFERENT METASTATIC CAPACITY

The qPCR assay was used to explore the relationship of KLF6 and expression of the *tmsg*-1 gene through analysis of the differences of expression of wt-KLF6 and TMSG-1 in cells with different metastatic capacity. It was intriguing that the human prostate carcinoma cell

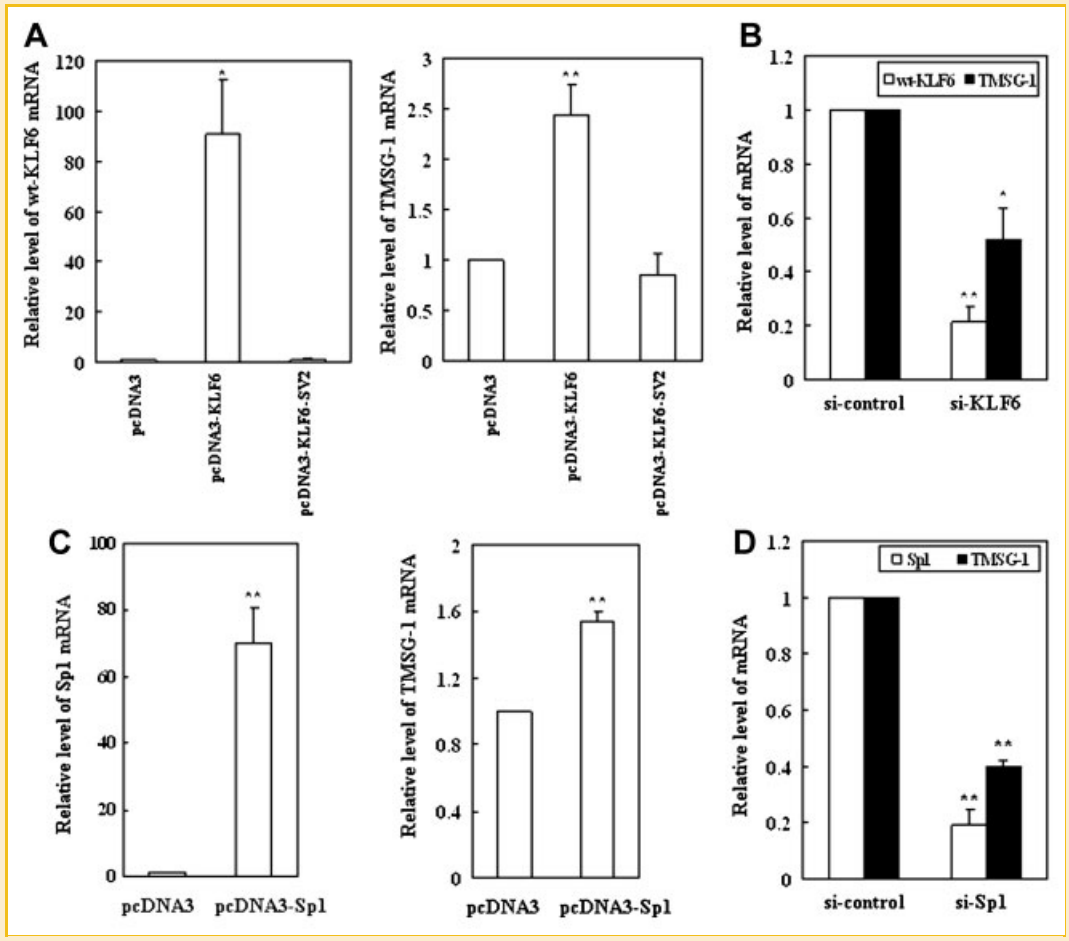

Fig. 4. Analysis for a relationship between KLF6/Sp1 and TMSG-1 transcripts in transfected PC-3M-2B4 cells. RT-qPCR was performed to evaluate the relationship of elevated levels of transcription factor with expression of TMSG-1. A: Levels of wild-type KLF6(wt-KLF6) and TMSG-1 mRNA after transfection with KLF6- (pcDNA3-KLF6) or KLF6 splice variant-expressing vector (pcDNA3-KLF6-SV2). B: Levels of wild-type KLF6 (wt-KLF6) and TMSG-1 mRNA after KLF6 knockdown. C: Levels of Sp1 and TMSG-1 mRNA after transfection with Sp1-expressing vector (pcDNA3- Sp1). D: Levels of Sp1 and TMSG-1 mRNA after Sp1 knockdown. Data represent mean ± SD of three samples. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison with empty vector or control.

line PC-3M-1E8 and pulmonary giant cell carcinoma cell line PG-BE1 exhibited a lower level of wt-KLF6 and TMSG-1 and demonstrated highly metastatic properties (Fig. 5A,B) [Zhu et al., 1995; Liu et al., 1999]. In contrast, human prostate carcinoma cell line PC-3M-2B4 and pulmonary giant cell carcinoma cell line PG-LH7 characterized by non-metastatic properties showed a higher level of wt-KLF6 and TMSG-1. Western blotting showed the same tendency (Fig. 5C). The results showed an apparent relationship between wt-KLF6/TMSG-1 proteins and the suppression of tumor metastasis.

EVALUATION OF THE ROLE OF KLF6 IN TUMOR INVASION

The role of KLF6 was also evaluated by observation of invasive capability of tumor cells with over-expressed KLF6. The results showed that transfection of pcDNA3-KLF6 led to over-expression of KLF6 as well as reduction of invasive capability of PC-3M-2B4 cells, which was reversed by knockdown of small interference RNA targeting the KLF6 gene (Fig. 5D,E). These results further suggested the impact of KLF6 levels on the invasive capability of tumor cells.

DISCUSSION

In the course of carcinogenesis a factor critical to the onset of metastasis is the inability of the regulatory region of tumor suppressor gene(s) to interact with their transcription factors and initiate the transcriptional process. Although there has been progress in understanding the properties of the TMSG-1 protein, as well as considerable data demonstrating the close relationship of tumor metastasis to *tmsg*-1 expression in carcinogenesis, questions have remained as to how triggering of this gene is involved in the pathological progression of tumor metastasis. More than 10 years after its discovery and functional evaluation, the transcriptional mechanism of the tumor suppressor gene *tmsg*-1 was entirely unknown. Only in recent years have we begun to gain insight into how transcription of the *tmsg*-1 gene is initiated with molecular biology approaches. Bioinformation analysis showed that the structure of the *tmsg*-1 promoter region lacks classic TATA (consensus initiator) and CAAT motifs in the expected region 25–30 bases upstream of the transcription initiation site (TIS). The

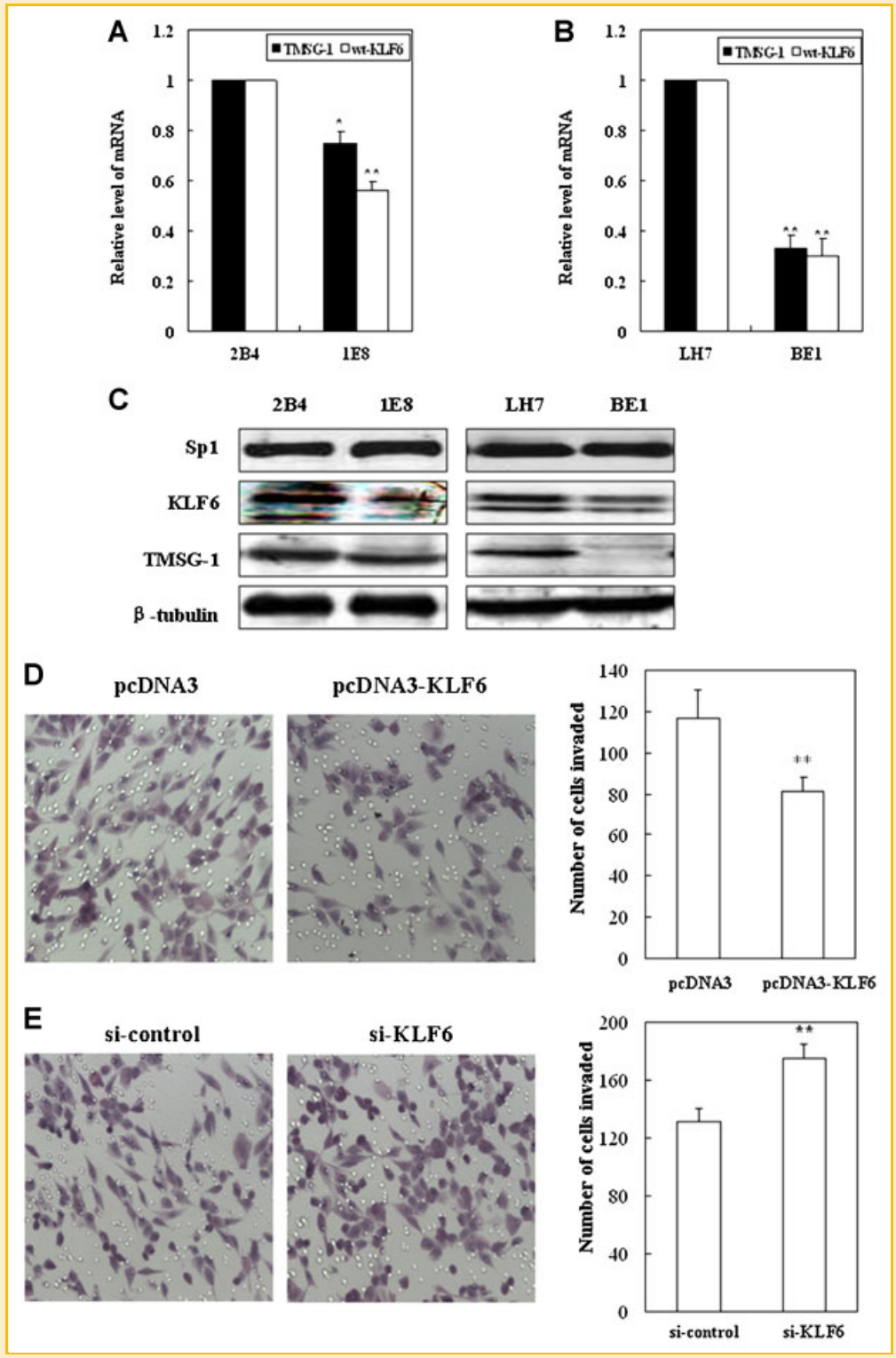

Fig. 5. Determination of expression of KLF6 and TMSG-1 in cells with different metastatic capacity. RT-qPCR analysis for expression of KLF6 and TMSG-1 in human prostate carcinoma cell lines 2B4 and 1E8 (A) and in human pulmonary giant cell carcinoma cell lines BE1 and LH7 (B). C: Western blot analysis for expression of KLF6 and TMSG-1 in cells with different metastatic capacity. Images are representative of at least three repetitions of each experiment. D: With transfection of KLF6-expressing vector in PC-3M-2B4 cells, invaded cells on the underside of the filters were examined under light microscopy with 200× magnification and imaged (left panel). Ten random fields were counted on each filter and statistically analyzed (right panel). E: Effect of KLF6 knockdown on invasive capability of tumor cells (left panel). Right panel show the statistical analysis. All experiments were repeated at least three times. $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant in comparison with the control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

tmsg-1 gene was also shown to have a GC-rich region (~80%) in exon 1 and a translational start site in exon 2. Further analysis of the sequence of the exon 1 showed that the region within approximate 300 bp downstream of the TIS possesses canonical KLF6 and Sp1-binding sites. It is well known that the Sp1 protein can initiate transcription through binding its element in a TATA-less promoter, and it was therefore of considerable interest to determine whether exon 1 with binding sites for KLF6 and Sp1 play a regulatory role in the transcription of the tumor suppressor gene [Gao et al., 2005; Papadodima et al., 2005; Xia et al., 2005; Li et al., 2008].

In this study, we present evidence to support the role of exon 1 in gene expression. Although the transcription of *tmsg*-1 is initiated in a synergistic manner, in which various trans-factors bind to their *cis*-elements in different regulatory regions, the interplay of KLF6 and Sp1 with exon 1 is a critical event that underlies initiation of the transcription process of the *tmsg*-1 gene. It has been documented that the transcription factor KLF6 is a product of the tumor-suppressor gene and is mutated in human prostate cancer. The expression of the KLF6 gene is attenuated in a variety of glial tumor cell lines, and this is correlated with its transactivation of some promoters [Kimmelman et al., 2004]. The impact of interaction of KLF and Sp1 on target genes has been reported, in which KLF activates p21(WAF1/Cip1) through a specific Sp1-like *cis*-element in the p21(WAF1/Cip1) proximal promoter [Zhang et al., 2000].

The involvement of an exon in expression of a gene has been previously documented. It was reported that the fibroblast growth factor-4 gene found in embryonal carcinoma cells is regulated by a powerful distal enhancer located 3 kb downstream of the transcription start site in the last exon of the gene [Lamb and Rizzino, 1998]. The mechanism involved transcription factor-mediated chromatin remodification allowing the promoter access to the basal transcription machinery. A recent report addressed the fact there is a nuclear factor-binding domain between position +72 and +115 of the 5′-UTR of the amyloid protein precursor gene and noted that its deletion might result in reduced expression of the gene [Vostrov et al., 2010]. Another study revealed that the glutamate decarboxylase 1 gene contained the TIS within a large CpG island that spans a region extending from upstream through the first exon containing *cis*-acting regulatory elements [Chen et al., 2010]. In agreement with these results, our data further emphasize the interplay of transcription factors with their *cis*-elements within a region of xon 1 and emphasize the consequent biological effects.

In summary, the results presented here outline the the mechanism underlying transcription of the *tmsg*-1 gene. It now appears that exon 1 of the *tmsg*-1 gene is not only a structural fragment participating in transcription, but is also a part of the regulatory machinery of the gene. Thus, understanding of this mechanism is of great potential importance for explanation of the tumor invasion and metastatic properties that have been ascribed to dysregulation of expression of the *tmsg*-1 gene, and may have significance for development of new clinical therapeutic strategies.

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