Inhibition of the long non-coding RNA MALAT1 suppresses tumorigenicity and induces apoptosis in the human ovarian cancer SKOV3 cell line

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Abstract. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a 8,000 nucleotide-long, spliced non-coding RNA, which has been reported to be deregulated in several tumors. However, to the best of our knowledge, the role of MALAT1 in ovarian cancer has not been previously investigated. The aim of the present study was to investigate the effect of MALAT1 inhibition on the tumorigenity of SKOV3 cells. First, stable MALAT1-knockdown ovarian cancer cells and control cells were established using lentivirus-mediated artificial micro RNA interference in order to investigate the effect of MALAT1 inhibition on cell viability, clonability, migration, invasion and apoptosis *in vitro*. In addition, the effect of MALAT1 on cell growth in nude mice was assessed. To identify the possible targets of MALAT1, total RNA was extracted from MALAT1-knockdown cells and control cells and a microarray analysis was performed. The results showed that MALAT1 inhibition significantly suppressed tumorigenity *in vitro* and *in vivo* (P<0.01). Compared with the control cells, 921 genes in the MALAT1-knockdown cells were deregulated by at least two-fold. The results of the reverse transcription-quantitative polymerase chain reaction showed that 19 of the 20 genes selected for validation confirmed the deregulation indicated by the microarray analysis. The findings define a major oncogenic role for MALAT1, which may offer an attractive novel target for therapeutic intervention in ovarian cancer.

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Introduction

Ovarian cancer is the most lethal gynecological malignancy, and <40% of patients with ovarian cancer are cured. In 2015, it is estimated that 21,290 new cases and 14,180 mortalities will occur in association with this disease in the United States (1). Epidemiological studies have identified several risk factors of ovarian cancer, including nulliparity, hormone therapy and pelvic inflammatory disease (2-4). Initial treatment for ovarian cancer consists of surgical staging and cytoreduction, followed by systemic chemotherapy in the majority of patients. Given the high mortality rate of ovarian cancer, the identification of novel biomarkers and therapeutic targets has been a major research focus for a number of years.

Long non-coding RNA (lncRNA) is an RNA molecule that is >200 nucleotides long and is not translated into a protein. Although the current understanding of the role of lncRNAs is limited, increasing numbers of studies have indicated that lncRNAs may regulate gene expression at various levels, including chromatin modification, transcription and post-transcriptional processing (5-7). lncRNAs have been reported to control various cellular processes, including proliferation, apoptosis and invasion, and are implicated in human diseases, including various types of tumors.

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known as non-coding nuclear-enriched abundant transcript 2, is a 8,000 nucleotide-long, spliced non-coding RNA, which is highly conserved in mammals. MALAT1 has been reported to be deregulated in several tumors, including non-small cell lung cancer, hepatocellular carcinoma, cervical cancer, bladder cancer and colorectal cancer (8-12). However, the role of MALAT1 in ovarian cancer has not been previously investigated.

The present study indicated that MALAT1 inhibition significantly suppressed tumorigenicity and induced apoptosis in SKOV3 cells. In addition, the knockdown of MALAT1 was indicated to alter the expression of a number of genes associated with cell proliferation, metastasis and apoptosis. To the best of our knowledge, the present study is the first to report an oncogenic role for MALAT1 in ovarian cancer.

Table I. Sequences of the four microRNA oligonucleotides.

Oligonucleotide	Sequence
KD1	
Forward	5'-TGCTGTCCACTTGATCCCAACTCATCGTTTTGGCCACTGACTGACGATGAGTTGATCAAGTGGA-3'
Reverse	5-CCTGTCCACTTGATCAACTCATCGTCAGTCAGTGGCCAAAACGATGAGTTGGGATCAAGTGGAC-3'
KD2	
Forward	5'-TGCTGCTTATCTGTTAACAGCTGCCTGTTTTGGCCACTGACTGACAGGCAGCTTAACAGATAAG-3'
Reverse	5'-CCTGCTTATCTGTTAAGCTGCCTGTCAGTCAGTGGCCAAAACAGGCAGCTGTTAACAGATAAGC-3'
KD3	
Forward	5'-TGCTGTGTACTATCCCATCACTGAAGGTTTTGGCCACTGACTGACCTTCAGTGGGGATAGTACA-3'
Reverse	5'-CCTGTGTACTATCCCCACTGAAGGTCAGTCAGTGGCCAAAACCTTCAGTGATGGGATAGTACAC-3'
KD4	
Forward	5'-TGCTGTTCCTTAGTTGGCATCAAGGCGTTTTGGCCACTGACTGACGCCTTGATCAACTAAGGAA-3'
Reverse	5'-CCTGTTCCTTAGTTGATCAAGGCGTCAGTCAGTGGCCAAAACGCCTTGATGCCAACTAAGGAAC-3'

Materials and methods

Cell lines and cell culture. The human ovarian cancer SKOV3 cell line and 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The two cell lines were cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in standard culture conditions of 5% CO_2 at 37°C.

Establishment of stable MALAT1-knockdown (KD) and negative control (NC) cells. Four microRNA (miRNA) oligonucleotides targeting MALAT1 (KD1, KD2, KD3 and KD4; Table I) were synthesized and inserted into the pcDNA6.2-GW/EmGFP-miR vector (Invitrogen; Thermo Fisher Scientific, Inc.) and used for transient transfections. The pcDNA6.2-GW/EmGFP-miR-Neg vector (Invitrogen; Thermo Fisher Scientific, Inc.) was used as negative control. Cells were maintained in 5% CO_2 at 37°C and harvested at 2 days post-infection. Reverse transcription-quantitative polymerase chain reactive (RT-qPCR) was performed in order to determine the efficiency of MALAT1 knockdown and to screen for the miRNA with the greatest knockdown efficiency, which was then used for subsequent experiments. pLenti6.3-MCS/V5DEST lentiviral vector was purchased from Shanghai R&S Biotechnology Co., Ltd. (Shanghai, China). The pLenti6.3-EmGFP-MALAT1-miR and plenti-EmGFP-miR-Neg vectors were constructed using the pcDNA6.2-GW/EmGFP-miR and pcDNA6.2-GW/EmGFP-miR-Neg vectors with the greatest knockdown efficiency, respectively. To produce recombinant lentiviruses, the 293T cells were co-transfected with lentivirus expression plasmids and packaging plasmid mix (Invitrogen; Thermo Fisher Scientific, Inc.) using POLOdeliverer™ 3000 Transfection Reagent (Ruisai Inc., Shanghai, China). The SKOV3 cells were then infected with these lentiviruses at a multiplicity of infection of 30, and selected with blasticidin (Invitrogen; Thermo Fisher Scientific, Inc.). At 2 weeks post-infection, cells were harvested and qRT-PCR was performed to determine the expression levels of MALAT1 in KD and NC cells.

RNA extraction and RT-qPCR. Total RNA was extracted from the cell samples using the Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). Each RT reaction consisted of 0.5 μg RNA, 2 μl PrimerScript Buffer, 0.5 μl oligo(dT), 0.5 μl random primers and 0.5 μl PrimerScript RT Enzyme mix I (Takara, Otsu, Japan), in a total volume of 10 μl. Reactions were performed in a GeneAmp® PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.) for 15 min at 37°C, followed by heat inactivation of reverse transcriptase for 5 sec at 85°C. The 10-μl RT reaction mixture was then diluted 10 times in nuclease-free water and held at -20°C. qPCR was performed using the LightCycler® 480 II Real-Time PCR instrument (Roche Diagnostics, Basel, Switzerland) with a total volume including 1 μl cDNA, 5 μl 2X

Table II. Primers used in reverse transcription-quantitative polymerase chain reaction.

Gene symbol	Forward primer	Reverse primer
MALAT1	ATCAGACCACCACAGGTTTACAG	GACCATCCCAAAATGCTTCA
WISP2	GCTTTCTCTCCGACTTCC	TCTGTGTGCCTTCTCTTCA
MUC16	ATCATCAGCTATGACATCGAC	TCTTTCTCAGTGGATAGGCTTA
ZEB2	TTGGTGTACCAAGAGGCAA	GCTAAGCCTTCAGTCTGAAT
MMP2	ACAGAAGGACTCAGGTTG	GTGGAGAAGAGACTCGGT
MMP11	CTGAGCAACTGGGCTGTA	CTGGATTCTGGAGAACAGATTT
VEGFA	GGGCAAATATGACCCAGT	TGTACCTGTGATCTGTCTTTCT
PGF	CCTTGGAGGAGAGAGACC	CAGGGAAACAGTTGGCTAA
H19	TTTCATCCTTCTGTCTCTTTGT	CAACCAGTGCAAATGACTTAG
ERBB4	AATAAATGCTAAACCAAGCGT	TAACAACTAGAAGCTGATGCAC
SNAI2	TGCAGACCCATTCTGATGTAA	GCGTCACTCAGTGTGCTA
E2F8	CCCAAAGGGTCACAATTAGT	GGGACAAAGAGAGTTCCTAAG
CDK2	CCCTTGTTTGTCCCTTCTAC	CATCTCTCACCTGCCTCATA
CDC20	TGGATCAAAGAGGGCAACT	GTGAACCACTGGACAGGATA
ADRA1B	AAGAGAACCACCAAGAACCTA	AAGGGTGTCCTCGTGAAA
RASGRP1	GTTCCAAACTAAAGCCAATAGC	ATGGGAACTGATCTTTCATCAA
FGF1	TTTCAACTTTAGAACCGGGTC	AAACAGAGCAGGGAACTAC
BAX	AGATGTGGTCTATAATGCGTT	TCAAGTCAAGGTCACAGTGAG
FN1	AAGATTCCCGAGAGTAAATCAT	TCTAAGCTGGGTCTGCTAAC
MTBP	GAAAGCCACAAACAGAACG	TTGACCTGAACTGGTCTTTGTA
ECT2	ACAACTCATTTGATATGAAGCG	AGCTTTCACCAAGTGCTAA
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG

MALAT1, metastasis-associated lung adenocarcinoma transcript 1; WISP2, WNT1 inducible signaling pathway protein 2; MUC16, mucin 16, cell surface associated; ZEB2, zinc finger E-box binding homeobox 2; MMP2, matrix metallopeptidase 2; MMP11, matrix metallopeptidase 11; VEGFA, vascular endothelial growth factor A; PGF, placental growth factor; H19, H19, imprinted maternally expressed transcript (non-protein coding); ERBB4, erb-b2 receptor tyrosine kinase 4; SNAI2, snail family zinc finger 2; E2F8, E2F transcription factor 8; CDK2, cyclin-dependent kinase 2; CDC20, cell division cycle 20; ADRA1B, adrenoceptor α 1B; RASGRP1, RAS guanyl releasing protein 1; FGF1, fibroblast growth factor 1; BAX, Bcl-2-associated X protein; FN1, fibronectin 1; MTBP, MDM2 binding protein; ECT2, epithelial cell transforming 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

LightCycler® 480 SYBR Green I Master (Roche Diagnostics), 0.2 μl of forward primer, 0.2 μl of reverse primer and 3.6 μl of nuclease-free water. Reactions were incubated in a 384-well optical plate (Roche Diagnostics) at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec and 60°C for 30 sec. At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. Three independent experiments were performed, with each sample run in triplicate. The primers were synthesized by Generay Biotech Co., Ltd. (Shanghai, China) and are listed in Table II. The expression levels of the genes were normalized with regard to GAPDH and were calculated using the $2^{-\Delta\Delta Cq}$ method (13).

Cell proliferation, colony formation, invasion and motility assays. Cell viability was determined at 24, 48, 72 and 96 h using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's protocol. For the colony formation assay, cells were counted, seeded at low density (1,000, 1,500 and 2,000 cells/plate), and allowed to grow until visible colonies appeared. Cells were then stained with crystal violet (GenMed ,Shanghai, China) and colonies were counted by eye. *In vitro* invasion assay was performed using 24-well Transwell units (Corning Life Science, Tewksbury, MA, USA) with polycarbonate filters coated on the upper side with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The cells were harvested, and 4×10^4 cells were placed in the upper part of the Transwell unit and were allowed to invade the membrane for 72 h at 37°C. Successfully penetrating cells were fixed, stained and quantified at an optical density of 570 nm. The motility assay was conducted in a similar fashion without coating with Matrigel. In total, 8×10^4 cells were seeded per well and incubated in 5% CO_2 at 37°C for 5 h prior to detection. Successfully penetrating cells were quantified at an optical density of 570 nm using a microplate reader (Infinite 200 Pro; Tecan Schweiz AG, Männedorf, Switzerland). Each experiment was performed in triplicate.

Apoptosis assay. Cell apoptosis was determined by flow cytometric analysis. Briefly, floating and attached cells were collected, resuspended, stained with
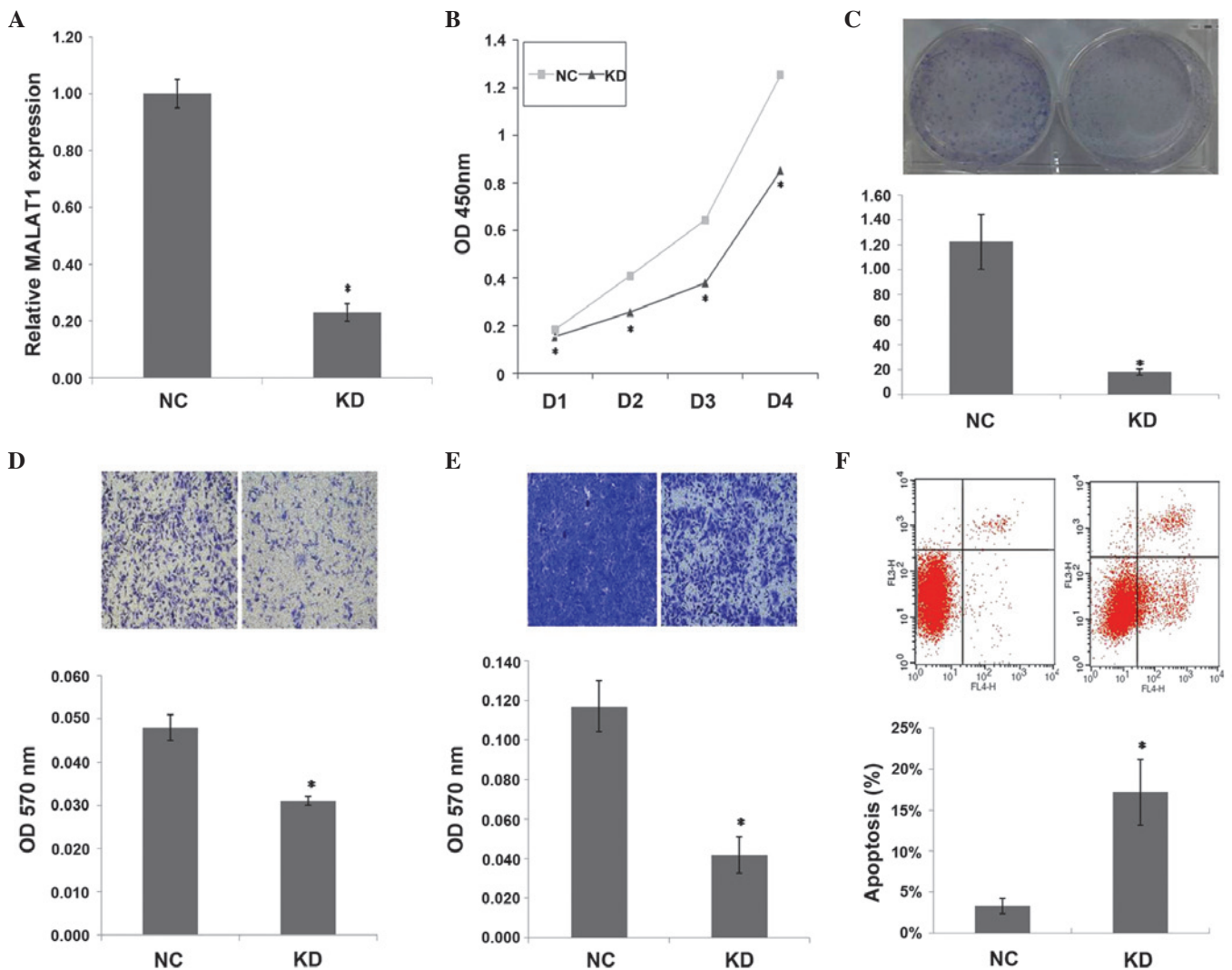
Figure 1. Inhibition of MALAT1 expression suppresses tumorigenicity and induces apoptosis *in vitro*. (A) Relative MALAT1 expression (*P=0.000), (B) cell proliferation assay (*P<0.01), (C) colony formation assay (*P=0.001), (D) Transwell-migration assay (*P=0.002), (E) invasion assay (*P=0.001) and (F) apoptosis assay (*P=0.004) in KD cells and NC cells. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; NC, normal control cells; KD, stable MALAT1-knockdown cells; OD, optical density; D1, day 1; D2, day 2; D3, day 3; D4, day 4.

Annexin V-allophycocyanin (BD Biosciences) and incubated for 30 min in the dark at 20°C. The analysis was then performed using a BD FACSAria II flow cytometer (BD Biosciences).

In vivo tumorigenic assay. Mice were housed and maintained in a specific pathogen-free facility under controlled environmental conditions (temperature, 22±2°C; humidity, 55±10%) with *ad libitum* access to rodent chow and water. KD cells or NC cells (5x10^6) were injected subcutaneously into the left armpit of nude mice (6 to 7 weeks old; n=7/group). Once palpable tumors developed, caliper measurements were taken twice a week and tumor volume was calculated on the basis of width (x) and length (y): x^{2}y/2, where x<y. All mice were sacrificed by cervical dislocation when tumors reached 2 cm^3 in size, and tumors were collected.

Microarray analysis and verification of selected genes by RT-qPCR. Microarray analysis was performed by Shanghai OeBiotech. Co., Ltd. (Shanghai, China), using SurePrint G3

Human Gene Expression 8x60 K version 2 software (Agilent Technologies, Inc.). Briefly, total RNAs from KD cells and control cells were used to synthesize complementary DNA, from which labeled complementary RNA was then synthesized and hybridized to SurePrint G3 Human Gene Expression 8x60K v2 (Agilent Technologies, Inc.). Subsequent to hybridization, processed slides were washed using the Gene Expression Wash Pack (Agilent Technologies, Inc.) and scanned using the Agilent Microarray Scanner (Agilent Technologies, Inc.). The acquired array images were analyzed using Agilent Feature Extraction software (version 10.7; Agilent Technologies, Inc.), which performs background subtractions. Quantile normalization and subsequent data processing were performed using the GeneSpringGX version 11.0 software package (Agilent Technologies, Inc.). A threshold of a >2-fold change was used to screen upregulated or downregulated genes. A total of 20 differentially-expressed genes, which have been reported to be associated with cell proliferation, metastasis and apoptosis, were subsequently selected for validation using RT-qPCR.

Table III. Deregulated genes in metastasis-associated lung adenocarcinoma transcript 1-knockdown cells compared with negative control cells.

Genbank access number	Gene symbol	Microarray fold change	qPCR fold change
NM_003881	WISP2	-17.73	-10.38
NM_024690	MUC16	4.81	3.34
NM_014795	ZEB2	-5.87	-2.66
NM_004530	MMP2	-5.17	-3.43
NM_005940	MMP11	-4.32	-5.07
NM_001025370	VEGFA	-7.93	-4.92
NM_002632	PGF	-4.87	-8.38
NR_002196	H19	-4.47	-3.35
NM_005235	ERBB4	-20.57	-12.87
NM_003068	SNAI2	9.01	3.40
NM_024680	E2F8	4.63	7.98
NM_001798	CDK2	5.67	8.54
NM_001255	CDC20	4.52	5.67
NM_000679	ADRA1B	4.26	7.82
NM_000800	FGF1	4.68	5.14
NM_138764	BAX	4.48	2.05
NM_054034	FN1	-23.30	-3.79
NM_022045	MTBP	4.70	5.55
NM_018098	ECT2	4.29	3.87

qPCR, quantitative polmerase chain reaction; WISP2, WNT1 inducible signaling pathway protein 2; MUC16, mucin 16, cell surface associated; ZEB2, zinc finger E-box binding homeobox 2; MMP2, matrix metallopeptidase 2; MMP11, matrix metallopeptidase 11; VEGFA, vascular endothelial growth factor A; PGF, placental growth factor; H19, H19, imprinted maternally expressed transcript (non-protein coding); ERBB4, erb-b2 receptor tyrosine kinase 4; SNAI2, snail family zinc finger 2; E2F8, E2F transcription factor 8; CDK2, cyclin-dependent kinase 2; CDC20, cell division cycle 20; ADRA1B, adrenoceptor α 1B; FGF1, fibroblast growth factor 1; BAX, Bcl-2-associated X protein; FN1, fibronectin 1; MTBP, MDM2 binding protein; ECT2, epithelial cell transforming 2.

Statistical analyses. All statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences between means were analyzed using a two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

KD and NC cells were successfully established. The four pcDNA6.2-GW/EmGFP-miR vectors harboring various miRNAs (KD1, KD2, KD3 and KD4) were used to transiently infect 293T cells. In parallel, an NC experiment was performed. The highest knockdown efficiency was achieved using KD3 (71% relative to the NC group), which was then used for producing recombinant lentiviruses. The results of the RT-qPCR showed that the expression of MALAT1 was decreased by 77% in KD cells, compared with NC cells (P=0.000) (Fig. 1A).

MALAT1 inhibition suppresses tumorigenicity in vitro. As shown in Fig. 1B, a significant decrease in cell proliferation was observed over time in KD cells compared with NC cells (P<0.01). MALAT1 inhibition also decreased the clonogenicity of KD cells compared with NC cells (P=0.001) (Fig. 1C). Transwell migration and invasion assays showed

that MALAT1 inhibition decreased the migration (P=0.002) and invasion (P=0.001) of KD cells (Fig. 1D and E). These observations suggest that MALAT1 knockdown suppresses the tumorigenicity of ovarian cancer cells *in vitro*.

MALAT1 inhibition induces apoptosis in KD cells. Apoptosis in KD and NC cells was measured by flow cytometry analysis. The mean apoptotic cell fractions (early apoptotic+apoptotic) were significantly increased upon MALAT1 inhibition compared with the negative control (P=0.004), with a concomitant decrease in the viable cell population (Fig. 1F). This finding suggests an anti-apoptotic role of MALAT1 in SKOV3 cells.

MALAT1 inhibition suppresses tumor growth in vivo. Since the *in vitro* data revealed the antitumorigenic role of MALAT1 inhibition in ovarian cancer cells, the effect of MALAT1 inhibition on tumor growth was examined *in vivo*. As shown in Fig. 2, the mean volume of the tumors in the KD group was significantly smaller compared with the tumors in the NC group (P<0.01), suggesting that the inhibition of MALAT1 significantly suppressed the tumorigenicity of SKOV3 cells *in vivo*.

Genes identified during the knockdown of MALAT1 expression. In order to additionally characterize the function of
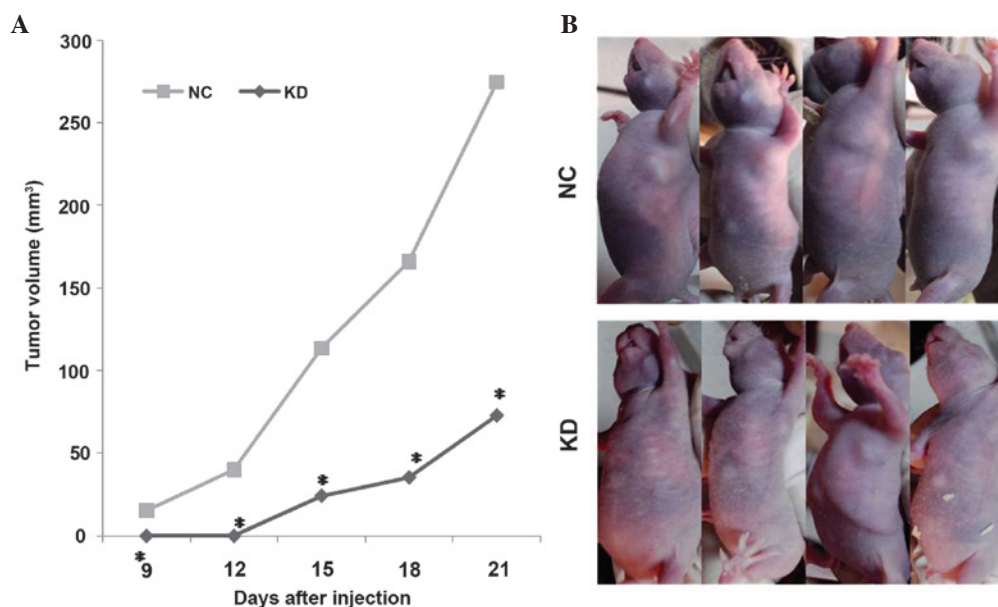
Figure 2. Inhibition of MALAT1 expression suppresses tumor growth *in vivo*. (A) Mean volume of tumors in the KD group was significantly smaller compared with the NC group. *P<0.01, KD vs. NC. (B) Representative images of mice from the two groups. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; NC, normal control cells; KD, stable MALAT1-knockdown cells.

MALAT1 within cells, RNA isolated from the KD cells was hybridized to the Agilent gene expression microarray and compared with the RNA isolated from the NC group. Data analysis showed that, when compared with the NC cells, a >2-fold deregulation was indicated in 921 genes in the KD cells. Since MALAT1 inhibition suppresses tumorigenicity and induces apoptosis in ovarian cancer cells, 20 genes, which have previously been reported to be associated with cell proliferation, metastasis and apoptosis, were selected for validation. As shown in Table III, 19 of these genes confirmed the deregulation that was indicated by the microarray analysis.

Discussion

MALAT1 was one of the first cancer-associated lncRNAs to be identified, and is widely expressed in normal human tissues. In 2003, Ji *et al* reported for the first time that MALAT1 was upregulated in non-small cell lung cancer and was a prognostic parameter for patient survival (8). Subsequent studies showed that MALAT1 was also associated with the formation and progression of several other types of tumors (9-12,14-18). However, the role of MALAT1 in ovarian cancer remains unknown.

The present study first investigated the effect of MALAT1 inhibition on SKOV3 cells, and the *in vitro* and *in vivo* data suggested that MALAT1 inhibition suppressed tumorigenicity in SKOV3 cells. A limitation to the present study was the lack of clinical samples available. Additional studies with clinical samples are warranted.

The gene expression in KD cells was then compared with control cells, and a large number of genes that were altered in association with decreased MALAT1 expression were identified. The results of the RT-qPCR validation showed that MALAT1 inhibition altered the expression levels of

genes that have been reported to be associated with cell proliferation (placental growth factor, cyclin-dependent kinase 2, cell division cycle 20, adrenoceptor α 1B and fibroblast growth factor 1), metastasis [WNT1 inducible signaling pathway protein 2, mucin 16, cell surface associated, zinc finger E-box binding homeobox 2 (ZEB2), matrix metallopeptidase 2, matrix metallopeptidase 11, vascular endothelial growth factor A, H19, imprinted maternally expressed transcript (non-protein coding), snail family zinc finger 2 (SNAI2) and fibronectin 1], and apoptosis [erb-b2 receptor tyrosine kinase 4 and Bcl-2-associated X protein (BAX)]. Among these genes, ZEB2, SNAI2 and BAX have been reported to be possible targets of MALAT1 (10,11). The mechanism of regulation of gene expression by MALAT1 remains unclear. Tripathi *et al* reported that MALAT1 regulated alternative splicing by modulating SR splicing factor phosphorylation (19). However, this phenomenon was not observed in lung cancer cells (20). Additional studies are required in order to clarify the mechanisms underlying MALAT1.

In conclusion, MALAT1 inhibition significantly suppressed tumorigenity in ovarian cancer cells and the inhibition of MALAT1 altered the expression of a number of genes associated with cell proliferation, metastasis and apoptosis. Overall, the findings of the present study define a major oncogenic role for MALAT1, which may provide an attractive novel target for therapeutic intervention in ovarian cancer.

Acknowledgements

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