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Cancer Research

The Noncoding RNA *MALAT1* Is a Critical Regulator of the Metastasis Phenotype of Lung Cancer Cells

Tony Gutschner[1], Monika Hämmerle[1,2], Moritz Eißmann[3], Jeff Hsu[4], Youngsoo Kim[4], Gene Hung[4], Alexey Revenko[4], Gayatri Arun[5], Marion Stentrup[1], Matthias Groß[1], Martin Zörnig[3], A. Robert MacLeod[4], David L. Spector[5], and Sven Diederichs[1]

Abstract

The long noncoding RNA *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1), also known as *MALAT-1* or *NEAT2* (nuclear-enriched abundant transcript 2), is a highly conserved nuclear noncoding RNA (ncRNA) and a predictive marker for metastasis development in lung cancer. To uncover its functional importance, we developed a *MALAT1* knockout model in human lung tumor cells by genomically integrating RNA destabilizing elements using zinc finger nucleases. The achieved 1,000-fold *MALAT1* silencing provides a unique loss-of-function model. Proposed mechanisms of action include regulation of splicing or gene expression. In lung cancer, *MALAT1* does not alter alternative splicing but actively regulates gene expression including a set of metastasis-associated genes. Consequently, *MALAT1*-deficient cells are impaired in migration and form fewer tumor nodules in a mouse xenograft. Antisense oligonucleotides (ASO) blocking *MALAT1* prevent metastasis formation after tumor implantation. Thus, targeting *MALAT1* with ASOs provides a potential therapeutic approach to prevent lung cancer metastasis with this ncRNA serving as both predictive marker and therapeutic target. Finally, regulating gene expression, but not alternative splicing, is the critical function of *MALAT1* in lung cancer metastasis. In summary, 10 years after the discovery of the lncRNA *MALAT1* as a biomarker for lung cancer metastasis, our loss-of-function model unravels the active function of *MALAT1* as a regulator of gene expression governing hallmarks of lung cancer metastasis. *Cancer Res; 73(3); 1180–9. ©2012 AACR.*

Introduction

Activation of migration, invasion, and metastasis is a crucial characteristic of malignancy as one of the hallmark capabilities of cancer (1). Metastasis is the major cause of cancer recurrence and tumor-related death (2, 3). Most studies investigating metastasis mechanisms focused on protein-coding genes, although recent transcriptome-wide analyses have revealed an overwhelming amount of transcribed but not translated genes (4, 5). Hence, transcription generates many long noncoding RNAs, abbreviated as ncRNA or lncRNA, capable of influencing diverse cellular processes such as proliferation, cell-cycle progression, apoptosis, or cell growth (reviewed in ref. 6). LncRNAs can apply diverse modes of action to regulate these processes and most lncRNAs studied so far regulate gene expression (7–12). Consequently, lncRNAs are deregulated in diverse human cancers and associated with disease progression (13–16). For example, the ncRNA *HOTAIR* (Hox transcript antisense RNA) is highly expressed in breast cancer and is a predictor for metastasis formation and associated with a poor prognosis (17).

One of the first cancer-associated lncRNAs discovered was *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1; ref. 18), also referred to as *NEAT2* (nuclear-enriched abundant transcript 2). *MALAT1* is extremely abundant in many human cell types and highly conserved over its full length (~8 kb) across mammalian species underscoring its functional importance. Its 3′-end can be modified by RNase P and RNase Z cleavage, which yields an additional tRNA-like ncRNA, the cytoplasmic *MALAT1*-associated small cytoplasmic RNA (*mascRNA*; ref. 19). The longer form of *MALAT1* is retained in the nucleus and specifically localizes to nuclear speckles (20). These structures are regions enriched in pre-mRNA splicing factors and could serve as storage, assembly, or modification sites (21). *MALAT1* might regulate alternative splicing of a subset of pre-mRNAs by modulating serine/arginine splicing factor activity (22), which regulates tissue- or cell-type–specific alternative splicing in a phosphorylation-dependent manner (23). However, splicing alterations were not found after *Malat1* ablation in mice (24). In contrast, alternative functions for *MALAT1* were recently identified (25): *MALAT1* could interact with the demethylated form of CBX4

Authors' Affiliations: [1]Helmholtz-University-Group "Molecular RNA Biology & Cancer," German Cancer Research Center DKFZ & Institute of Pathology; [2]Institute of Pathology, University Hospital Heidelberg, Heidelberg; [3]Georg-Speyer-Haus, Frankfurt am Main, Germany; [4]ISIS Pharmaceuticals, Carlsbad, California; and [5]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

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Corresponding Author: Sven Diederichs, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280 (B150), D-69120 Heidelberg, Germany. Phone: 49-6221-424383; Fax: 49-6221-424384; E-mail: s.diederichs@dkfz.de

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(chromobox homolog 4), also referred to as Pc2 (polycomb 2), a component of the polycomb repressive complex 1 (PRC1). This interaction controls the relocalization of growth control genes between polycomb bodies and interchromatin granules, areas of silent or active gene expression, respectively. *MALAT1* resides in these subnuclear structures and acts as an activator of gene expression potentially by mediating the assembly of coactivator complexes (25).

Given these two alternative proposed mechanisms of action for *MALAT1*, the exact function of *MALAT1* is still unknown. In addition, it remains to be elucidated whether the ubiquitously expressed *MALAT1* has one universal function or whether its mechanisms of action might be tissue-specifically different.

MALAT1 was originally identified as a prognostic marker for metastasis and patient survival in non–small cell lung carcinoma (NSCLC), specifically in early stages of lung adenocarcinoma (18). In lung squamous cell carcinoma, high *MALAT1* expression is also associated with poor prognosis. *MALAT1* might impact growth and colony formation of NSCLC cells *in vitro* (26). Upon injection into nude mice, cells with moderately decreased *MALAT1* expression show reduced tumor growth. Reduced *MALAT1* levels impair cell motility *in vitro* (27).

A potential active role of *MALAT1* in metastasis as well as its specific functions remains unknown. Thus, to clarify the function of *MALAT1* at the cellular and molecular level—more precisely to determine its functional importance in metastasis and the regulation level affected by *MALAT1*—we have developed a methodology to establish a comprehensive loss-of-function model for *MALAT1* using zinc finger nucleases (ZFN) to stably integrate RNA destabilizing elements into the human genome (28). This approach resulted in a specific and more than 1,000-fold silencing of *MALAT1* and allowed specific and effective loss-of-function studies of this abundant ncRNA in human cancer cells.

Here, we report that loss of *MALAT1* deregulates gene expression but not alternative splicing in lung cancer. *MALAT1*-deficient lung cancer cells are impaired in migration and form significantly fewer and smaller lung tumor nodules than their wild-type (WT) counterparts in a mouse xenograft assay. Beyond its value as a prognostic marker for metastasis development (18, 26), these findings uncover *MALAT1* as an active player in lung cancer metastasis and establish gene regulation of metastasis-associated genes rather than alternative splicing as the critical *MALAT1* mechanism affiliated with metastasis. Consequently, targeting *MALAT1* expression in established human xenograft tumors with free-uptake antisense oligonucleotides (ASO) drastically reduces lung cancer metastasis formation *in vivo* and validates *MALAT1* as a potential therapeutic target in lung cancer.

Materials and Methods

Cell culture

A549 lung adenocarcinoma cells were purchased from American Type Culture Collection (ATCC; CCL-185) in 2010 and cultivated at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium + 10% FBS; 0.2 mmol/L glutamine and antibiotics. A549 *MALAT1* knockout cells were generated as previously published (28). The EBC-1 lung squamous cell carcinoma line was obtained in 2010 from the Health Sciences Foundation (Tokyo, Japan) and maintained in RPMI-1640 media containing 10% FBS in a humidified incubator with 5% CO_2 at 37°C. Cell lines were authenticated by ATCC or by the Health Science Foundation Japan via short tandem repeat (STR) DNA profiling. No further cell line authentication was conducted, but the A549 WT and knockout cells were tested for *MALAT1* expression and mycoplasma negativity every six months. All cell lines were maintained in culture for a maximum of 20 passages (two months).

Scratch/wound healing assay

The CytoSelect 24-well Wound Healing Assay (Cell Biolabs) was used to analyze migration of A549 WT and *MALAT1* knockout cells. The assay was done according to the manufacturer's recommendations using 2.0×10^5 cells per well. Image acquisition of wound fields was done after removal of inserts (0 hours) and wound closure documentation was done after 24 and 48 hours with a phase-contrast microscope (Leica DM IL; Leica Microsystems) equipped with a digital camera (Leica DFC300FX). Image analysis was conducted with Adobe Photoshop CS3 software.

A549 xenograft lung cancer mouse model

A549 have previously been used to establish lung tumor nodules in mice (29). Two months after intravenous injection into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, mice were sacrificed and the lungs were isolated, fixed, and tumor nodule numbers and areas were analyzed in a blinded fashion.

EBC-1 xenograft assay

Exponentially growing EBC-1 cells were collected by trypsin–EDTA and washed once with PBS. The cell pellet was suspended in PBS and 10 million cells were implanted by subcutaneous injection into BALB/c nude mice. Two weeks after implantation, the mice were randomly divided into two treatment groups. The first treatment group was injected with 250 mg/kg/wk of *MALAT1* ASO, administered subcutaneously for five weeks. The second treatment group was injected with 250 mg/kg/wk of control oligonucleotide. In week seven, the subcutaneous tumor was surgically removed and the wound closed with a 4-0 suture. Mice were euthanized in week 12 after the start of ASO treatment. Lung tissues were collected and processed for further analysis. The tumor multiplicity was counted using a light microscope.

Additional details on Materials and Methods can be found in the Supplementary Data.

Results

Efficient silencing of the highly abundant *MALAT1* in human lung cancer cells

To enable this study, we recently developed a new method for highly effective and specific silencing of protein-coding as well as nonprotein-coding genes (28). The functional knockout of *MALAT1* was achieved by ZFN-mediated site-specific integration of RNA-destabilizing elements into the genome of human cancer cells. Stable knockout clones showed on average

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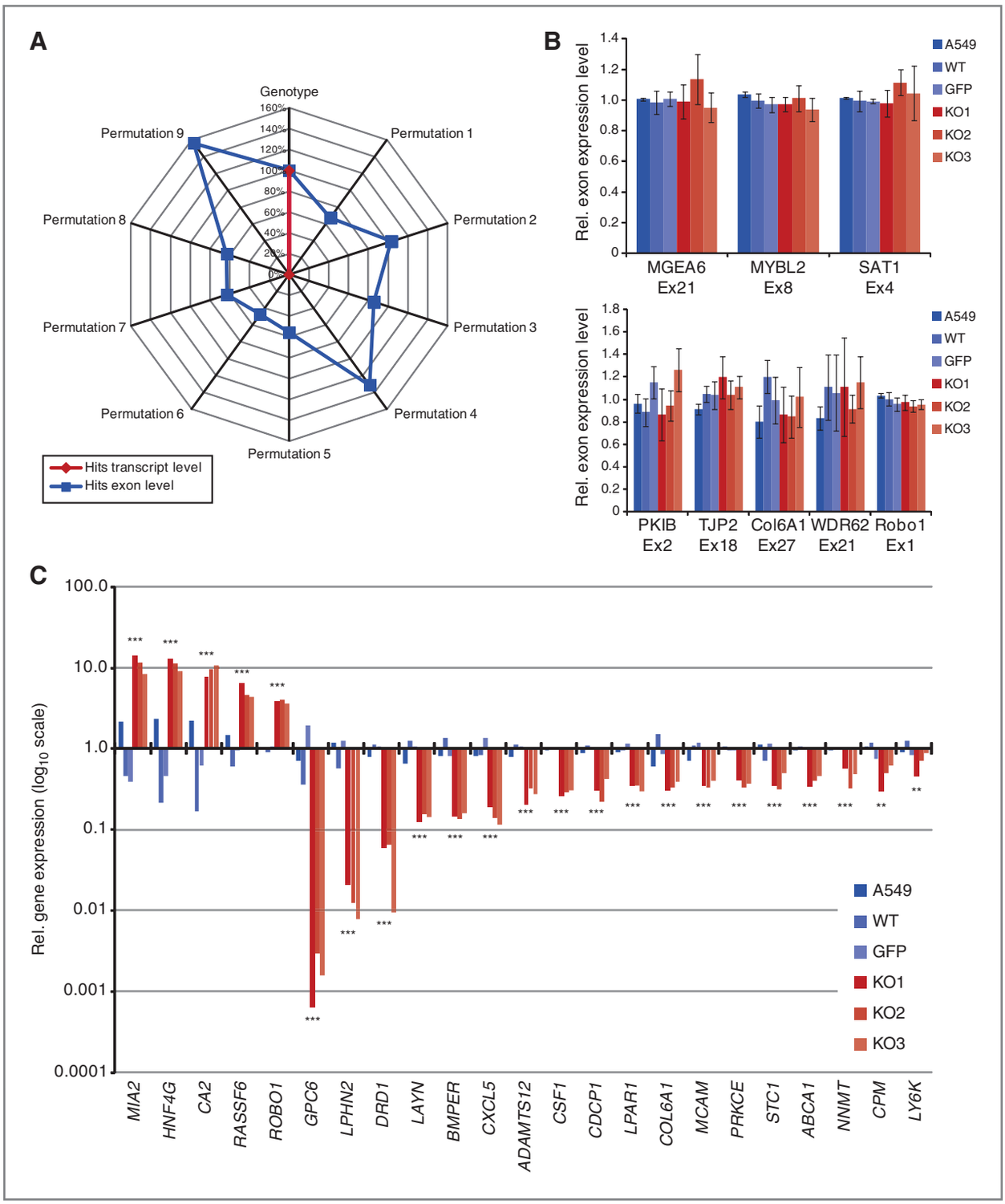

Figure 1. *MALAT1* specifically regulates gene expression but not splicing. The impact of *MALAT1* on genome-wide gene expression and alternative splicing was analyzed by exon microarrays. Three WT (A549, WT, and GFP) and three knockout (KO1–3) lines were analyzed in biologic duplicates. A, spider web chart for the specificity of gene regulation (red) or alternative splicing (blue) alterations by loss of *MALAT1*. The exon array analysis revealed 459 significantly regulated transcripts and 3,025 differentially spliced exons. To assess the specificity of the uncovered regulations, we permutated the dataset and repeated the analysis: first, the six cell lines were grouped into two groups according to their genotype (WT vs. knockout). Then, the six samples were randomly divided into two groups in all nine possible permutations. The genotype-based grouping yielded significant hits both at the exon and the transcript level (each set as 100%). At the exon level, all nine random sample permutations gave rise to significant hits. In stark contrast, none of the nine sample permutations gave

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1,000-fold less *MALAT1* expression than their WT counterparts (28, 30). To exclude clone-specific effects, we selected a panel of 6 different cell lines derived from A549 lung cancer cells to analyze their phenotype. The loss of *MALAT1* expression was previously validated (30). The expression level of *MALAT1* was comparable in all control WT cells and not affected by clonal selection (A549 vs. WT and GFP; ref. 30).

For the validation of our data and to prove that the obtained results were neither clone-specific nor dependent on the integration site within the *MALAT1* gene, we have generated a second ZFN-targeting *MALAT1* downstream of the first ZFN (Supplementary Fig. S2A). With this ZFN-2, we created two additional knockout (KO) clones (KO4, KO5) that also lost *MALAT1* expression (Supplementary Fig. S2B).

Alternative splicing is not affected by loss of *MALAT1*

MALAT1 had been previously linked to two different molecular mechanisms: regulating alternative splicing (22) and regulating gene expression (25). Therefore, we started to analyze its effect on gene expression and alternative splicing in our quantitative loss-of-function system. We investigated both possible mechanisms using microarrays to analyze gene expression at the exon and the transcript level in all six cell lines (A549, WT, GFP, KO1-3). Statistical analysis of the exon array data revealed that *MALAT1* seemed to regulate both, gene expression and splicing resulting in 459 significantly regulated transcripts (adj. $P < 0.05$; Supplementary Table S1) and 215 or 3,025, respectively, significantly regulated exons (splice index >1.0/>0.5). To determine the specificity of the observed regulations, we conducted dataset permutations. First, the six cell lines were grouped according to their genotype annotation comparing the three *MALAT1* WT samples with the three *MALAT1* knockout samples. This analysis based on the genotypes yielded both, significant hits at the exon and the transcript level (Fig. 1A). In addition, the six samples were divided randomly into two groups in all nine possible permutations. Surprisingly, all nine different sample permutations gave rise to significant hits at the exon level—two permutations even with larger numbers of regulated exons than the correct sample grouping. In stark contrast, none of the nine sample permutations gave rise to any significantly regulated transcript (Fig. 1A). This indicated that the differences at the transcript level were highly significant and specifically associated with the loss of *MALAT1*, whereas the detected differences at the exon level were not specific. The permutation analysis was repeated with groups containing equal numbers of WT and knockout datasets (32 permutations of the technical replicates) yielding the same result (Supplementary Fig. S1A). Hence, *MALAT1* specifically regulated gene expression but not alternative splicing in lung cancer cells.

To expand this analysis, we tested the alternative splicing pattern of genes whose splicing pattern had previously been shown to be affected by knockdown of *MALAT1* (22). However, none of the exons tested was differentially spliced upon complete loss of *MALAT1* in the A549 lung cancer model (Fig. 1B, top). In addition, splicing analysis by quantitative real-time PCR (qRT-PCR) of a set of candidate cassette exons regulated in the exon array screen in three independent replicates did not verify any differential splicing (Fig. 1B, bottom). Thus, neither the previously published nor the new candidate exons from our microarray screen were reproducibly altered upon loss of *MALAT1* indicating that *MALAT1* does not impact alternative splicing in this system.

In addition, neither the expression nor the phosphorylation of selected splicing factors was changed between *MALAT1*-expressing and *MALAT1*-deficient cells (Supplementary Fig. S1B) further corroborating that splicing remains unaffected after loss of *MALAT1* in lung cancer cells.

MALAT1 regulates gene expression including metastasis-associated transcripts

Next, we focused on the deregulated genes at the transcript level whose expression levels were altered after *MALAT1* depletion. An in-depth literature search revealed that several of the *MALAT1* target genes contribute to or are associated with the metastatic potential of cancer cells (Supplementary Table S2). We were able to validate the differential expression of all 23 of these metastasis-associated target genes via qRT-PCR (Fig. 1C). Notably, these differential gene expression patterns were verified in additional knockout clones generated with an independent ZFN (Supplementary Fig. S2D). Expression levels were highly consistent within each group (WT or knockout), but differed strongly between the two genotypes. For example, *MIA2* (melanoma inhibitory activity 2), a negative regulator of tumor growth and invasion (31), or *ROBO1* (roundabout 1), an inhibitor of glioma migration and invasion (32, 33), were increased in all knockout cells compared with all WT cells. In turn, *GPC6* (glypican 6), a promoter of breast cancer metastasis (34), *LPHN2* (latrophilin 2), an important factor for the epithelial-to-mesenchmyal rransition (EMT) of endothelial cells in the atrioventricular canal of the heart and involved in cancer cell invasion (35, 36), *CDCP1* (CUB domain containing protein 1), a promoter of lung adenocarcinoma metastasis in a mouse model (37), and *ABCA1* (ATP-binding cassette, subfamily A, member 1), an important factor for prostate cancer cell migration and EMT (38, 39), were strongly reduced by loss of *MALAT1*. The expression of several other *MALAT1* target genes was associated with metastasis (e.g., *DRD1*, *COL6A1*, *STC1*, etc.) or they represented critical regulators of metastasis formation (e.g., *GPC6*, *MCAM*, *PRKCE*, etc.).

rise to any significantly regulated transcripts, indicating the specificity of the gene regulation. B, analysis of alternative splicing. *MALAT1*-dependent, alternatively spliced exons were analyzed by qRT-PCR ($n = 3$; mean ± SEM). Alternative splicing alterations were detected neither for previously identified cassette exons (22; top) nor for cassette exons from our own exon array analysis (bottom). C, validation of differentially expressed genes after loss of *MALAT1*. *MALAT1* knockout cells showed a significant deregulation of 459 genes (Supplementary Table S1). The differential expression of 23 metastasis-associated target genes was confirmed using qRT-PCR. All validation experiments were carried out in three independent RNA panels. Given is the relative expression of genes normalized to the mean of the WT cells. *PPIA* (cyclophilin A) served as reference gene.

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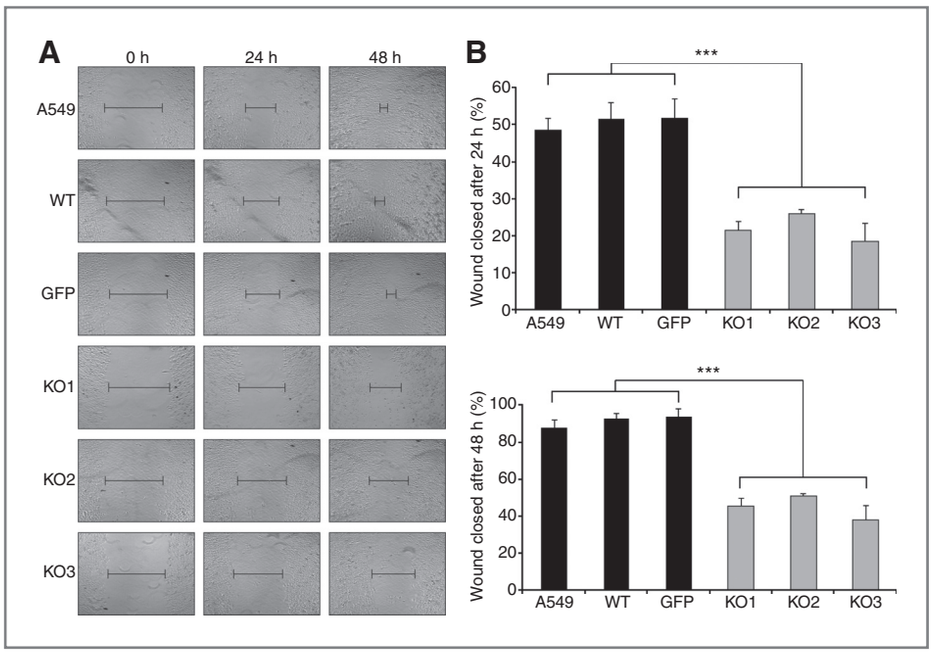

Figure 2. *MALAT1* is important for cell migration *in vitro*. Scratch/wound healing assays were conducted with *MALAT1* WT and knockout A549 cells. Shown are representative figures from three independent experiments. A, wound fields were observed directly after removal of inserts (0 hours) and cell migration was followed for 24 and 48 hours. An obvious impact of *MALAT1* loss on cell migration was detected at both time points. B, statistical analysis of wound closure. Gap size at 0 hours was set to 100% and percentage of closed wound was calculated after 24 and 48 hours after image analysis. WT clones efficiently migrated into the gap, whereas knockout clones uniformly displayed a significantly impaired wound closure ($P \leq 0.001$).

Notably, no gene ontology term "metastasis" exists—most likely due to the complexity of the underlying multistep process. However, a gene ontology analysis of the deregulated genes revealed a significant enrichment (all: $P < 10^{-7}$) of the biologic processes "cell communication," "signal transduction," and "cell adhesion," which are all related to the metastatic cascade but also making it possible that loss of *MALAT1* has an impact on other cellular processes than metastasis (Supplementary Fig. S1C).

Taken together, our microarray analysis revealed that *MALAT1* may act as a regulator of gene expression inducing among others a set of genes previously associated with metastasis, that is, suppressing the expression of metastasis inhibitors and activating the expression of metastasis promoters. These could act in concert determining the metastatic capacity to human A549 lung cancer cells (Supplementary Table S2) leading to the hypothesis that *MALAT1* itself could function as a prometastatic factor.

Cell motility depends on *MALAT1 in vitro*

Given the impact of *MALAT1* depletion on the expression of motility-relevant genes, we conducted scratch assays with the six *MALAT1* WT and knockout cell lines to study this phenotype *in vitro* (Fig. 2A). Already after 24 hours, the *MALAT1* knockout cells showed a significant reduction of cell motility (Fig. 2B). After 48 hours, the WT cells showed an almost complete closure of the gap, whereas knockout cells reduced the gap by only approximately 50% (Fig. 2A and B). Loss of *MALAT1* in the additional clones generated with an independent ZFN also caused impaired migration validating these results (Supplementary Fig. S3). Of note, we could not detect differences in A549 cell proliferation in the knockout clones KO1–KO3 (30) or KO4–KO5 (Supplementary Fig. S2C) further associating *MALAT1* not with tumor growth but a metastatic phenotype.

MALAT1 is critical for important steps of the metastatic cascade in human lung cancer *in vivo*

MALAT1 was originally discovered as a prognostic marker for lung cancer metastasis (18). Although the migratory capacity might impact the metastatic potential of a cancer cell, the complex metastatic phenotype has not been analyzed before *in vivo* and a functional link between *MALAT1* and cancer metastasis has not been established so far. Therefore, we conducted a mouse xenograft assay by injecting either A549 *MALAT1* WT cells or the two knockout cell lines with the lowest *MALAT1* expression (KO2 and KO3) into the tail vein of nude mice and analyzed the formation of lung tumor nodules after two months. While this assay did not recapitulate all steps of the complex metastatic cascade, it mimics the important steps of extravasation and tumor nodule formation in the lung. The results showed uniformly that *MALAT1* was required for effective tumor nodule formation *in vivo*, and thus played an active role in the metastatic process (Fig. 3A). A549 WT cells were able to establish numerous tumor nodules in the lung with an average number of 136 per lung (Fig. 3B). In contrast, two independent cell lines lacking *MALAT1* (KO2 and KO3) showed a significantly reduced number of metastases [24 (83% reduction) and 12 (91% reduction), respectively].

For mice injected with WT cells, lungs stained with hematoxylin and eosin (H&E) showed an extensive and diffuse growth of tumor cells, which destroyed the normal alveolar structure of the lung (Fig. 3C, left). Lungs of mice injected with A549 *MALAT1* knockout cells either developed very small lung tumor nodules or tumor cells resided inside blood vessels (Fig. 3C, mid and right). Magnified inlets of representative tumor nodules are shown. Staining of consecutive lung sections with an antibody detecting human pan-cytokeratin confirmed the human origin of cancer cells invading mouse lung tissue (Fig. 3C, bottom). Thus, *MALAT1* seems to act as an

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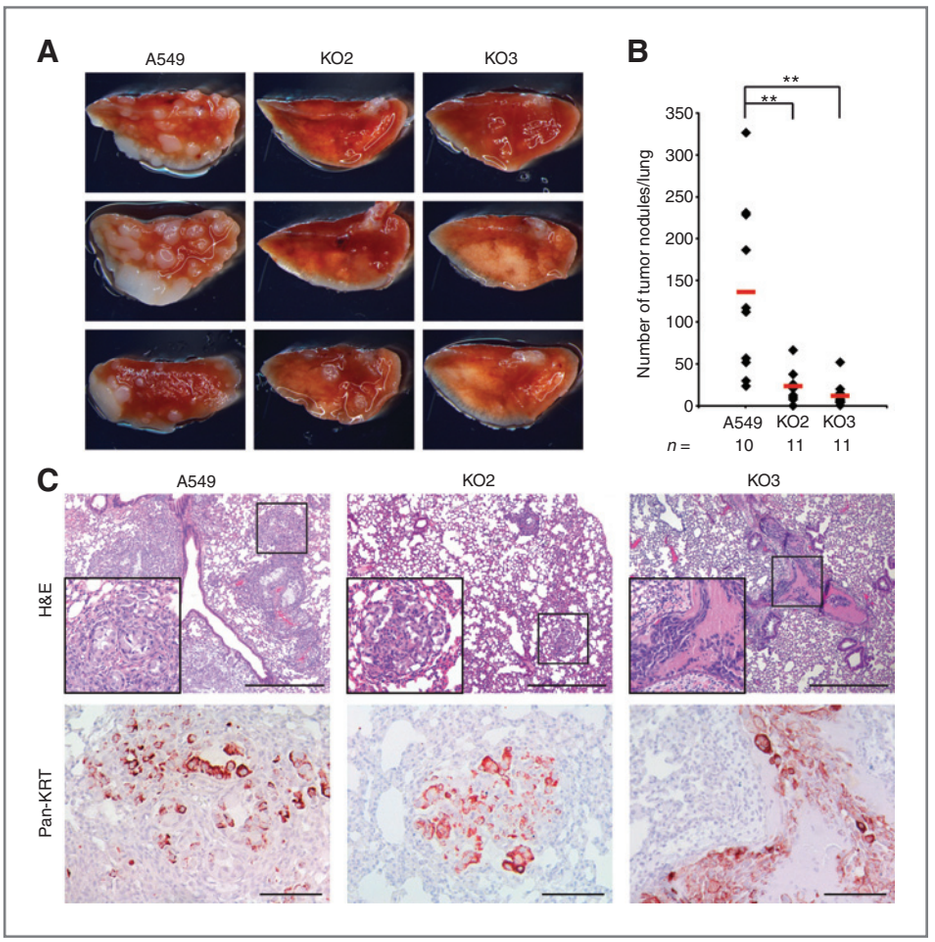

Figure 3. *MALAT1* is a critical factor for lung cancer homing and nodule formation *in vivo*. A549 lung cancer cells or *MALAT1* knockout cells (KO2 and KO3) were analyzed for the metastatic potential in a mouse xenograft assay recapitulating major steps of metastasis development. Ten to 11 animals were analyzed per cell line. A, lung cancer tumor nodule formation. After tail vein injection of *MALAT1* WT or knockout cells, formation of lung tumor nodules was analyzed. The lungs were resected, fixed, and the total number of tumors and the tumor area were determined. The tumor burden was drastically reduced in *MALAT1* knockout cells (KO2 and KO3) compared with WT control cells. Representative pictures from the left lobe are shown. B, statistical analysis. On average, 136 distinct nodules were counted in mice injected with A549 WT cells, whereas only 24 and 12 metastases were found in knockout cells (*t* test: $P = 0.007$ and $P = 0.004$, respectively). C, histology of lung tumor nodules. H&E staining (top) shows an extensive growth of *MALAT1* WT tumor cells, which destroyed the normal alveolar structure of the lung (left). *MALAT1* knockout cells either developed micrometastases (magnified inlet, mid) or tumor cells resided inside blood vessels (right). Staining of a consecutive section against human pan-cytokeratin (clone KL-1) proves the human origin of tumor cells that invaded into the mouse lung tissue (magnified inlets, bottom). Scale bar, 200 μm for ×4 magnification and 50 μm for ×20 magnification.

important regulator of the metastatic cascade of lung cancer *in vivo*.

ASOs can effectively target *MALAT1 in vivo*

Cancer metastasis is the major cause of cancer recurrence and tumor-related death. After establishing a critical role for *MALAT1* in this process, we hypothesized that *MALAT1* might be a potential therapeutic target. Therefore, we developed an approach to target *MALAT1* effectively *in vivo* relying on the administration of free-uptake ASOs, which were designed to potently target both human and mouse *MALAT1*. These second generation ASOs are effective at low nanomolar concentrations and are efficiently taken up by the cells, avoiding any toxic effects of lipofection. First, we analyzed the effectiveness of this approach *in vitro* and treated human EBC-1 lung cancer cells with increasing amounts of *MALAT1* ASOs. These knocked down *MALAT1* up to 20-fold (Supplementary Fig. S4A).

Next, we injected human NSCLC EBC-1 cells into mice and applied ASOs by subcutaneous injection at 50 mg/kg, five times a week for five weeks. We could observe an accumulation of *MALAT1* ASO in both tumor and tumor-associated stromal cells (Fig. 4A). In these tissues, ASOs effectively reduced both human and mouse *MALAT1* expression compared with control ASO (Fig. 4B), which was also validated in tumor and surrounding stromal cells by the ViewRNA *in situ* hybridization method using species-specific probes (Fig. 4C). Thus, ASOs knocked down *MALAT1* effectively *in vitro* and *in vivo*.

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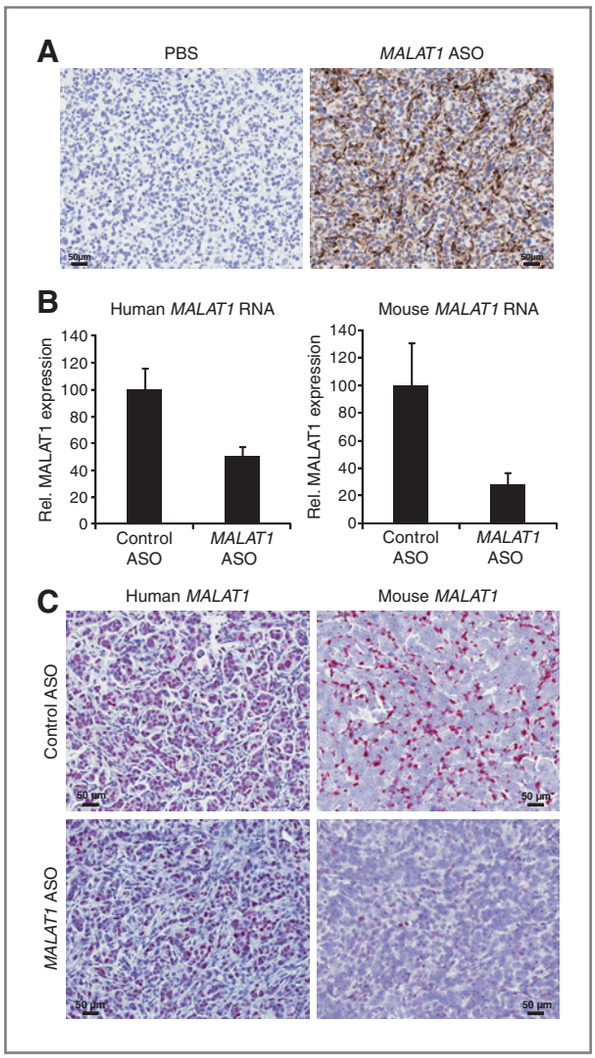

Figure 4. Downregulation of *MALAT1* in EBC-1 lung cancer cells and tumor stroma. Mice bearing human NSCLC EBC-1 cells were treated with ASOs by subcutaneous injection at 50 mg/kg, five times a week for five weeks. A, accumulation of *MALAT1* ASO in both tumor and tumor-associated stromal cells was shown by immunohistochemistry using an antibody specific for ASOs. B, human and mouse *MALAT1* RNA levels in EBC-1 tumor were measured by qRT-PCR using species-specific probe/primer sets and verified the downregulation of *MALAT1* in human tumor and murine normal tissue after ASO application *in vivo* in the animal model. C, reduction in *MALAT1* RNA levels in both tumor and its surrounding stromal cells was visualized by the ViewRNA *in situ* hybridization method using species-specific probes.

MALAT1 ASO inhibits EBC-1 tumor metastasis to the lung

As our ASO approach proved to be effective in targeting *MALAT1* in solid tumors, we took advantage of a well-established pulmonary metastatic model of human NSCLC (40) to analyze the effect of *MALAT1* depletion on the spread of EBC-1 cells. In this assay, EBC-1–derived primary tumors were induced by subcutaneous injection of tumor cells and their ability to form distant tumor nodules in the lung was determined recapitulating all major steps of the metastastic cascade *in vivo* including invasion and intravasation not assayed in the A549 xenografts. While the depletion of *MALAT1* in EBC-1 cells had no effect on cell proliferation *in vitro* (Supplementary Fig. S4B), the application of ASO had a minor impact on EBC-1 tumor growth *in vivo* (data not shown). To avoid any influence on tumor metastasis, EBC-1 tumors were controlled for their size and were surgically excised from their primary sites after five weeks of ASO treatment. Animals were kept for additional seven weeks without ASO treatment. At week 12, the lung tissues were collected and analyzed for tumor burden and histology. Indeed, significantly fewer and smaller tumor nodules were found in the lung in the *MALAT1* ASO group compared with control ASO (Fig. 5A). A significant decrease in tumor volume in the *MALAT1* ASO-treated group compared with the control ASO group was also revealed by microCT scanning for primary tumors of similar size (Fig. 5B and C).

Thus, the knockdown of *MALAT1 in vivo* was effective possibly providing a therapeutic option for metastasis prevention.

Discussion

Dysregulation of the highly conserved, nuclear long noncoding RNA *MALAT1* has been linked to many cancer entities (for review see ref. 6), but it was originally discovered as a marker for metastasis development in early stages of lung adenocarcinoma (18) and more recently in squamous cell carcinoma of the lung (26). However, its functional role in this process was only beginning to emerge by virtue of its link to cell migration (27).

At the functional level, our A549 knockout model corroborated the strong influence of *MALAT1* on A549 cell migration, as described previously (26, 27), and migration of other malignant cells (41–43).

One hurdle in elucidating *MALAT1* function was its high expression and its localization to the nucleus making it a less efficient target for conventional RNA interference (RNAi)-based gene silencing. Previous loss-of-function studies using short hairpin RNAs (shRNA) or siRNAs decreased *MALAT1* expression two- to four-fold (26, 27), which left a high remaining concentration of the abundant *MALAT1*. Despite overlapping findings about cell migration, both studies differ for other phenotypes: while one study found an impact of *MALAT1* on tumor cell growth and colony formation, the other study could not establish a function for *MALAT1* in cellular proliferation, adhesion, or anchorage-independent growth. This contradiction might be due to inadequate loss-of-function models using shRNAs or siRNAs moderately reducing *MALAT1* and prone to different off-target effects. Our ZFN-based approach reduces *MALAT1* expression more than 1,000-fold, and thus allows a quantitative loss-of-function analysis of its cellular and molecular impact. The treatment of cultured cells with ASOs also effectively silenced *MALAT1* up to 20-fold (Supplementary Fig. S4A), and hence was more effective than standard RNAi-based approaches. Importantly, the A549 approach based on genetically modified cancer cell clones was complemented and validated by the use of highly potent ASOs in bulk cell culture and xenograft models independent of clonal selection.

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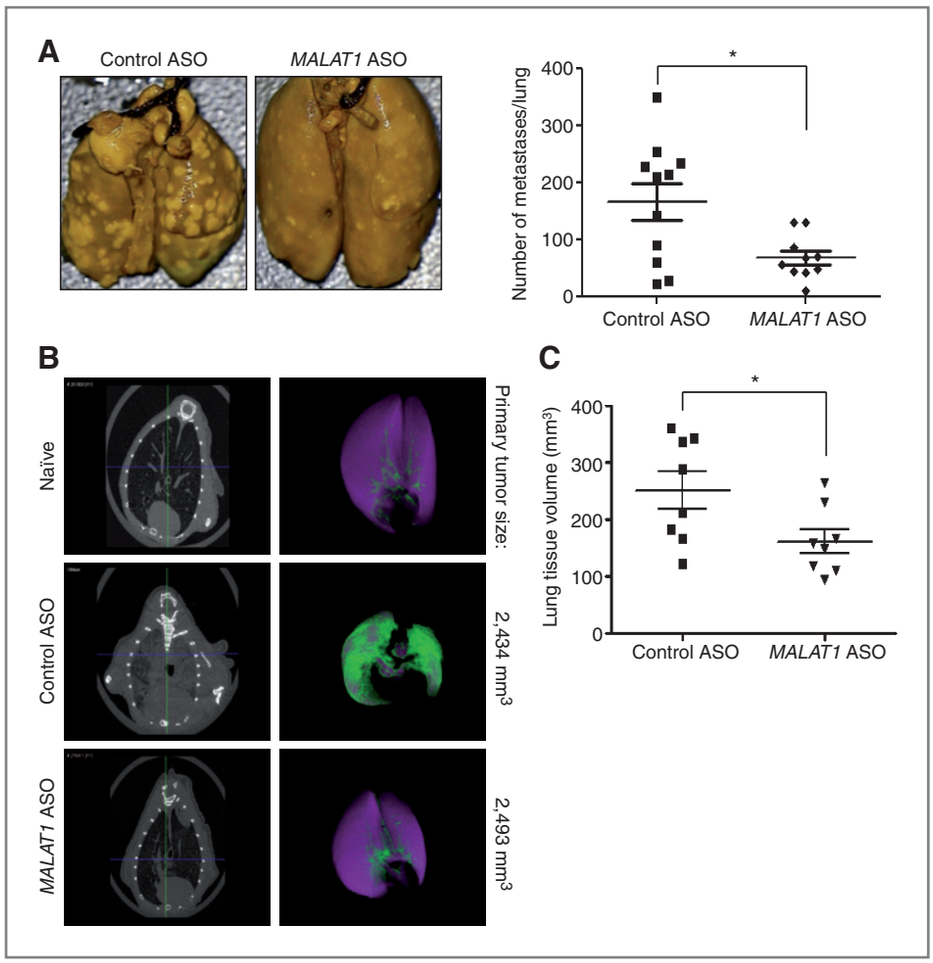

Figure 5. *MALAT1* ASO inhibits the metastatic spread of EBC-1 tumors to the lung. EBC-1–derived primary tumors were induced by flank injections into nude mice. The animals were then treated with *MALAT1* ASO. After five weeks, tumors were surgically excised from their primary sites and the animals were kept for the following seven weeks without ASO treatment. At week 12, lung tissues were collected and analyzed for tumor burden and histology. A, animals treated with *MALAT1* ASO had significantly fewer tumor nodules in the lung compared with control ASO-treated animals ($P = 0.038$). B and C, a significant decrease in tumor volume in the *MALAT1* ASO-treated group compared with the control ASO group was shown by microCT scanning ($P = 0.038$). Purple indicates airway volumes and green represents lung tissue volumes including tumor mass.

The molecular mechanism of *MALAT1* action is currently under debate: previous studies identified *MALAT1* as a regulator of alternative splicing of a subset of genes (22), whereas others suggested a mechanism of gene regulation (25). Our data strongly indicate that *MALAT1* has no major impact on alternative splicing in lung cancer cells—matching most recent data in mouse models (24, 44), whereas we find the critical and specific function of *MALAT1* in regulating the expression of several target genes. Our study identified a set of genes that might act in concert to promote lung cancer metastasis and whose expression depends on *MALAT1*. However, as only a subset of the deregulated genes have a proven connection to metastasis without a significant enrichment, *MALAT1* could also regulate other important processes.

In contrast to previous studies in HeLa cells, our knockout strategy for *MALAT1* did not lead to differential expression of growth-control genes in our microarray analysis or altered growth of A549 knockout cells. This might indicate cell-type–specific functions for *MALAT1* and future studies will uncover the underlying principles, for example, cell-type–specific coactivator molecules recruited by *MALAT1*. This lack of regulation of major growth regulatory genes is also underscored by the lack of a growth or proliferation phenotype after loss of *MALAT1* in mouse models (24, 30, 44).

On the basis of the gene regulation of several metastasis-associated genes and its impact on cell migration, we went one step further and hypothesized that *MALAT1* could be an active player in the metastatic process, as this *in vivo* phenotype had not been analyzed before. Therefore, we used two model systems recapitulating hallmark steps of the metastatic cascade *in vivo*: intravenous A549 xenografts revealed the important role of *MALAT1* in lung cancer cell extravasation and formation of new tumor nodules in the lung. The EBC-1 metastasis model mimicked the process of metastasis formation from a primary subcutaneous tumor. In summary, these data indicate for the first time that *MALAT1* is not only a prognostic biomarker for metastasis development but also a major player for disease progression by regulating a metastatic gene expression program.

The knockdown efficiency of the ASO approach *in vivo* (Fig. 4B) was significantly weaker than the knockdown efficiency *in vitro* (Supplementary Fig. S4A) or in our A549 knockout model (28). While the strong silencing *in vitro* was enabling unequivocal determination of the impact of *MALAT1* on lung cancer cell proliferation or migration, the metastasis formation *in vivo* seems to be dependent on higher levels of *MALAT1* and thus was already impaired at these comparatively weaker knockdown levels. On the one hand, one could

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speculate that *MALAT1* could impact multiple steps in the metastatic cascade, and hence the sensitivity toward its knockdown was larger *in vivo* than *in vitro*. On the other hand, this result is encouraging for the development of *MALAT1* knockdown strategies as an antimetastatic therapy approach, as this effective level of downregulation could be achievable also in human tumors.

The metastatic cascade is a complex process and, as exemplified here for *MALAT1*, lncRNAs can significantly contribute to this malignant cancer phenotype. Hence, identification and functional analysis of other involved lncRNAs might add another layer of complexity and may even foster novel therapeutic options. In a mouse xenograft model, we established that the application of ASOs against *MALAT1* proved to be effective in blocking lung cancer spreading. These data put *MALAT1* forward as a valid therapeutic target to be further tested *in vivo*. Thus, *MALAT1* is uncovered as a valuable prognostic marker, an important active player and a promising therapeutic target in lung cancer metastasis.

Disclosure of Potential Conflicts of Interest

D.L. Spector is a consultant for and has minor ownership interest in Isis Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: T. Gutschner, J. Hsu, Y. Kim, S. Diederichs
Development of methodology: T. Gutschner, M. Eißmann, J. Hsu, G. Hung, M. Stentrup, M. Zörnig, S. Diederichs
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Hämmerle, M. Eißmann, J. Hsu, G. Hung, A. Revenko, G. Arun, M. Stentrup, M. Groß, M. Zörnig
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Gutschner, M. Hämmerle, J. Hsu, Y. Kim, G. Hung, A. Revenko, M. Zörnig, D.L. Spector, S. Diederichs
Writing, review, and/or revision of the manuscript: T. Gutschner, M. Hämmerle, J. Hsu, Y. Kim, G. Hung, A.R. MacLeod, D.L. Spector, S. Diederichs
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Hsu
Study supervision: T. Gutschner, J. Hsu, Y. Kim, A.R. MacLeod, S. Diederichs

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