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Loss of Med1/TRAP220 promotes the invasion and metastasis of human non-small-cell lung cancer cells by modulating the expression of metastasis-related genes

Hyun-Ju Kim [a,b,1], Mee Sook Roh [c,1], Choon Hee Son [d], Ae Jeong Kim [a,b], Hye Jin Jee [a,b], Naree Song [a,b], Minjee Kim [a,b], Su-Young Seo [e], Young Hyun Yoo [b,f], Jeanho Yun [a,b,*]

[a] Department of Biochemistry, College of Medicine, Dong-A University, Busan 602-714, South Korea
[b] Mitochondria Hub Regulation Center, College of Medicine, Dong-A University, Busan 602-714, South Korea
[c] Department of Pathology, College of Medicine, Dong-A University, Busan 602-714, South Korea
[d] Department of Internal Medicine, College of Medicine, Dong-A University, Busan 602-714, South Korea
[e] Department of Microbiology, College of Medicine, Dong-A University, Busan 602-714, South Korea
[f] Department of Anatomy and Cell Biology, College of Medicine, Dong-A University, Busan 602-714, South Korea

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ABSTRACT

Med1/TRAP220 is an essential component of the TRAP/Mediator complex. In this study, we present a novel function of Med1 in human non-small-cell lung cancer (NSCLC) progression. We found that the loss of Med1 expression was strongly associated with increased rates of invasion and metastasis in NSCLC patients. Consistent with lung cancer patient data, the knockdown of Med1 in NSCLC cell lines led to an increase in cell migration and invasion. Med1-depleted cells displayed an increase in metastasis in a xenograft tumor model and in an *in vivo* metastasis assay. Moreover, a microarray analysis revealed that the mRNA levels of the metastasis-related genes *uPAR*, *ID2*, *ID4*, *PTP4A1*, *PKP3*, *TGM2*, *PLD1*, *TIMP2*, *RGS2*, and *HOXA4* were altered upon Med1 knockdown. Collectively, these results suggest that the loss of Med1 increases the invasive potential of human NSCLC cells by modulating the expression of metastasis-related genes.

1. Introduction

Lung cancer is the leading cause of cancer-related death for both men and women. Primary lung cancer can be classified as small-cell lung cancer (SCLC) or non-small-cell lung cancer (NSCLC). Approximately 85% of lung cancers are categorized as NSCLC, and the most common types of NSCLCs are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma [1]. Because NSCLCs are relatively resistant to chemotherapy and radiation therapy compared to SCLCs, a multi-faceted treatment regimen is often required for patients with NSCLC [2]. Despite recent advances in NSCLC treatment, the overall 5-year survival rate remains less than 14% [2,3]. Metastasis is the most common cause of treatment failure and death in NSCLC patients. Therefore, identifying the critical regulators of NSCLC invasion and metastasis is important in order to understand the pathogenesis of NSCLC and improve its clinical treatment.

Med1 (also known as PBP/RB18A/TRAP220/DRIP205) is a component of the human TRAP/Mediator complex that plays an important role in the transcriptional control of various genes [4,5]. In addition to its essential role as a transcriptional activator of several nuclear receptors, such as estrogen receptor (ER), thyroid receptor (TR), and androgen receptor (AR) [6,7], recent studies have suggested that Med1 function is associated with tumorigenesis. Med1 interacts directly with the tumor suppressors p53 and Brca1 [8,9]. The overexpression of Med1 has been reported in breast and prostate cancers [10,11]; however, the downregulation of Med1 has also been observed in metastatic melanoma [12]. Although an increasing number of studies have suggested that Med1 is involved in tumorigenesis, the exact mechanistic role of Med1 in lung cancer progression remains unclear. We have previously shown that the loss of Med1 expression is strongly correlated with increased invasion, metastasis, and poor survival in human lung adenocarcinoma patients [13]. In this study, we investigate the role of Med1 in human NSCLC and show that reduced Med1 expression is strongly associated with an increased rate of invasion and metastasis in all of the NSCLC subtypes analyzed. We also demonstrate that the loss of Med1 promotes the invasive and metastatic potential of human

* Corresponding author at: Department of Biochemistry, College of Medicine, Dong-A University, Busan 602-714, South Korea. Tel.: +82 51 240 2919; fax: +82 51 241 6940.
E-mail address: yunj@dau.ac.kr (J. Yun).
[1] These authors equally contributed to this work.

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NSCLC cells. Finally, we propose that Med1 may be a potent new prognostic marker and treatment target for NSCLC.

2. Materials and methods

2.1. Clinical samples and Med1 immunohistochemistry

Tissue samples were obtained from 173 Korean patients who underwent surgical resection for primary NSCLC at Dong-A University Medical Center. The collection and use of these samples was approved by the Institutional Ethical Review Board (approval number 09-10-20). Three biopsy cores from different areas of each tumor were removed from each sample to produce a tissue microarray, and 4 μm-thick sections were taken for immunohistochemical staining. Immunohistochemical staining for Med1 was performed on the tissue microarray slides using the avidin–biotin-peroxidase complex method with a rabbit polyclonal antibody directed against Med1 (CRSP1; Novus Biologicals, Littleton, CO) as described previously [13]. All of the slides were independently evaluated by an experienced pathologist and by one of the authors (M.S.R.), who were blinded to the clinicopathologic data. The percentage and intensity of the immunoreactive tumor cells in each core were recorded, and the mean number of positive-staining tumor cells in the three cores of each tumor was calculated, as described previously [13].

2.2. Cell culture and generation of Med1 knockdown cells

H1299 and A549 human NSCLC cells were grown in DMEM containing 10% fetal bovine serum (FBS). To generate expression plasmids encoding shRNAs targeting Med1 (shMed1), we subcloned two annealed shMed1 oligonucleotides (shMed1 #1 and #2) or negative control shRNA (NC) into the pU6-puro vector. The pU6-shMed1 or pU6-NC plasmid was transfected into H1299 and A549 cells using the Lipofectin reagent (Invitrogen, Carlsbad, CA), and the cells were selected with 1 μg/μl of puromycin (Sigma) for 1–2 days. The resulting colonies were pooled (for transfection into H1299) or isolated individually (for A549) for subsequent experiments. The sequences of the shRNAs against Med1 were as follows: Med1 #1 sense, 5′-ATCCGGGGATCCGAATCCTAAATGGCATGGCTTCAAGAGAGCCATGCCATTTAGGATTCTTTTTTGGAAAAGCTTGGCACT-3′, antisense, 5′-TAGGCCCCTAGGCTTAGGATTTACCGTACCGAAGTTCTCTCGGTACGGTAAATCCTAAGAAAAAACCTTTTCGAACCGTGA-3′; Med1 #2 sense, 5′-ATCCGGGGATCCGAGCAAGGCTTATATGGTGTTCAAGAGACACCATATAAGCCTTGCTCTTTTTTGGAAAAGCTTGGCACT-3′, antisense, 5′- TAGGCCCCTAGGCTCGTTCCGAATATACCACAAGTTCTCTGTGGTATATTCGGAACGAGAAAAAACCTTTTCGAACCGTGA-3'.

2.3. Western blot analysis and antibodies

Cells were lysed in RIPA buffer and subjected to Western blot analysis, as described previously [14]. Med1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Actin was probed as a loading control using an anti-actin (Sigma) antibody. Three independent Western blot analyses were performed.

2.4. Cell proliferation assay

Cells were seeded at a density of 1×10^4 cells per well in 6-well plates and allowed to proliferate for 6 days. The media was changed every 2 days. After 6 days, the cells were trypsinized, stained with trypan blue, and counted to measure proliferation. To measure growth factor-dependent proliferation, the cells were cultured in DMEM containing 0.1% FBS and 50 ng/ml of epidermal growth factor (EGF; Sigma). The culture medium was changed every 2 days, and the cells were counted after 6 days via trypan blue staining. The experiments was repeated three times and performed in triplicate each time.

2.5. In vitro migration and invasion assays

Cell migration was analyzed using multi-chambered wells. Cells were detached by incubation with trypsin-EDTA, washed three times with serum-free medium, and resuspended. A 25 μl aliquot of serum-free medium containing 1×10^5 resuspended cells was transferred to the upper chamber, which was separated from the complete culture medium in the bottom chamber by a polyethylene terephthalate membrane filter (8 μm pore size) (Neuro Probe, Inc., Gaithersburg, MD). After 18 h of incubation, non-migrated cells were carefully removed using a cotton swab. The cells that had migrated to the undersurface of the filter were fixed and stained with hematoxylin (Sigma–Aldrich). The stained cells in three randomly chosen fields (100× magnification) were photographed and counted.

The cell invasion assay was performed using Boyden chambers with Matrigel-pre-coated filter inserts (8 μm pore size) (Costar, Corning, NY). A suspension of 1×10^5 cells in 100 μl of medium was added to the upper chamber. After an 18 h incubation, the cells that had invaded the membrane were fixed and stained with hematoxylin and eosin (H&E). The stained cells in three randomly chosen fields (100× magnification) were photographed and counted.

2.6. In vivo mouse studies

Five- to six-week-old BALB/c NOD/SCID mice were purchased from the Animal Laboratory of the Korean Research Institute of Bioscience and Biotechnology (Daejeon, South Korea) and maintained under pathogen-free conditions. All procedures were performed according to a protocol (DIACUC-09-19) approved by the Dong-A Institutional Animal Care and Use Committee. Suspensions of 2×10^6 A549 or A549-shMed1 cells in 100 μl of PBS were injected subcutaneously into 5 mice per group. Tumor size was measured using a digital caliper once a week for 17 weeks. At the end of the experiment, the mice were sacrificed, and tissues were harvested, embedded in paraffin, sectioned, and stained with H&E and anti-Med1 antibody as described previously [13].

For the *in vivo* metastasis assay, 10 mice were divided into two groups, and 2×10^6 A549 or A549-shMed1 cells were injected into each mouse via the tail vein. After 8 weeks, the lungs were harvested, fixed, and embedded in paraffin. The number of metastatic lung nodules was counted in serial histological sections stained with H&E. The areas of metastatic lung nodules were measured in scanned images of the H&E-stained tumor sections using Paint.NET software.

2.7. Microarray analysis

Total RNA was isolated from A549 and A549-shMed1 cells using the RNeasy Micro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The gene expression profiles of each sample were analyzed using Affymetrix U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA), and genes whose expression differed by a factor of at least 2 in A549-shMed1 with respect to the A549 control were selected for further analysis. An unsupervised hierarchical clustering method was used to group the genes based on expression pattern, and the resulting data were visualized.

2.8. Quantitative RT–PCR

To assess mRNA levels, total RNA was extracted from 80% to 90% confluent cells using the TRIzol reagent (Ambion), and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and oligo-dT primers (Promega, Madison, WI). Real-time PCR was performed in triplicate using SYBR Green PCR Master Mix (Takara Bio Inc., Shiga, Japan) and an ABI Prism 7500 Real-Time PCR system (Applied Biosystems). The β-actin transcript was used as an internal control for all samples, and the gene-specific mRNA level was normalized to the level of β-actin mRNA. The expression of each mRNA was determined using the $2^{-\Delta CT}$-threshold cycle method. The primers used for PCR are listed in Supplementary Table 1.

2.9. Statistical analysis

All data were presented as the arithmetic mean ± standard deviation. The statistical analyses were performed using SPSS statistical software. The *p*-values were obtained using an unpaired two-tailed *t*-test, and a p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Med1 expression is decreased in human NSCLC patients

To investigate the role of Med1 in lung cancer and to dissect the function of Med1 in promoting lung cancer metastasis, we first extended our analysis of Med1 expression to 171 non-small-cell lung cancer (NSCLC) patient tissue samples. These tissue samples represented all NSCLC subtypes: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. Med1 expression was analyzed by immunohistochemistry (IHC). Strikingly, we found that 120 of the 171 tumor samples (70%) showed low Med1 expression (Table 1). Similarly low levels of Med1 expression were observed in all NSCLC subtypes, including squamous cell carcinomas (66%), adenocarcinomas (71%), and large cell carcinomas (88%). Importantly, reduced Med1 expression was significantly associated with an increase in lymphovascular invasion and lymph node metastasis ($p < 0.001$) (Table 1), consistent with previous reports of poor clinical outcomes and advanced tumor progression in Med1-negative lung adenocarcinomas [13]. Interestingly, however, tumor size was not significantly correlated with the level of Med1 expression ($p = 0.314$). These results demonstrate that the loss of Med1 is common in all subtypes of human NSCLC and suggest that Med1 plays a role in the progression of human NSCLC.

Table 1
The relationship between MED1 expression and clinicopathological characteristics in tumor tissues from 171 NSCLC patients.

Characteristics	Expression of MED1		*p* value
	Negative	Positive	
	120 (70%)	51 (30%)	
Gender			0.342
Male	88	41	
Female	32	10	
Age	59.11 ± 9.59	62.38 ± 7.46	0.094
Tumor type			0.231
Squamous cell carcinomas	46	24	
Adenomas carcinomas	60	25	
Large cell carcinomas	14	2	
Margin			0.201
Clear	112	43	
Involve	7	7	
Tumor size			
	4.07 ± 1.74	3.44 ± 1.38	0.314
Size grad			0.273
⩽2 cm	5	3	
2 cm < size ⩾ 3 cm	29	14	
>3 cm	86	34	
Lymphovascular invasion			0.001
Negative	79	45	
Positive	44	7	
Lymph node metastasis			<0.0001
Negative	53	40	
Positive	67	12	□

The level of Med1 expression was determined by immunohistochemical staining of 171 NSCLC patient samples. The correlation between Med1 expression and each clinicopathological characteristic was analyzed using the χ^2 test or the Fisher's exact test.

3.2. Knockdown of Med1 inhibits NSCLC cell proliferation

To address the role of Med1 in human NSCLC progression, we generated Med1-depleted cell lines by stably transfecting an expression plasmid encoding an shRNA against Med1 (shMed1) into the human NSCLC cell lines H1299 and A549. To control for possible artifactual effects of an individual short hairpin sequence, two different shMed1 expression plasmids (shMed1 #1 and #2) were used. To control for clonal variation, two independent clones were isolated after introducing shMed1 #2 into A549 cells. Western blotting analysis confirmed a significant reduction of Med1 expression in both H1299 and A549 cells stably expressing shMed1 (Fig. 1A and B).

Because previous studies have shown that the knockdown of Med1 results in the decreased proliferation of prostate and breast cancer cells [10,11], we first examined the effect of Med1 knockdown on cellular proliferation. The depletion of Med1 suppressed H1299 and A549 cell proliferation relative to controls (Fig. 1C and D). Moreover, the epidermal growth factor (EGF)-induced growth of H1299 and A549 was abolished after Med1 knockdown (Fig. 1E and F). However, in contrast with a previous report on prostate cancer [10], defects in the cell cycle and in apoptosis were not observed after Med1 knockdown (Supplementary Fig. 1). These results suggest that the loss of Med1 mildly reduces the proliferative potential of human NSCLC cells.

3.3. The loss of Med1 increases the invasive potential of NSCLC cells

Because we observed that the reduced expression of Med1 was associated with an increase in tumor invasion and metastasis in NSCLC patients, we examined whether the loss of Med1 expression could increase the invasive potential of human NSCLC cells. To this end, we performed *in vitro* migration and invasion assays comparing Med1-depleted H1299 and A549 cells with control cells. As shown in Fig. 2A, the migration capacity of both Med1-depleted H1299 and A549 cells (H1299- and A549-shMed1) was increased by 2.5-fold and 1.5-fold respectively compared with non-transfected H1299 or A549 cells (Fig. 2A and B; $^*p < 0.05$); cells expressing a negative control shRNA (H1299 and A549 NC) had migration rates similar to those of non-transfected cells. Moreover, Med1 knockdown increased the invasion activity of both H1299 and A549 cells in trans-well invasion assays (Fig. 2C and D; $^*p < 0.05$). These results indicate that the loss of Med1 confers increased migratory and invasive potential in human NSCLC cells, which may contribute to cancer cell metastasis.

3.4. The loss of Med1 promotes the metastasis of NSCLC cells in vivo

To validate the relevance of the *in vitro* results described above, we further extended our studies to tumor xenograft mouse models. NOD/SCID mice were subcutaneously inoculated with either Med1-depleted A549 cells (A549-shMed1) or non-transfected A549 control cells. Initially, the growth rate of tumors derived from the A549-shMed1 cells was slightly slower than that of the tumors derived from control cells. However, the tumor growth rates were similar by 13 weeks post-implantation, and the tumors derived from the A549-shMed1 cells were growing slightly faster than the control tumors by week 16 (Fig. 3A). Upon termination of the study, the tumors in both groups were of comparable size ($p = 0.16$) (Fig. 3B). Immunohistochemical analysis confirmed the low expression of Med1 in the tumors of mice inoculated with A549-shMed1 (Fig. 3C). Notably, secondary tumors, which are characteristic of local invasion and metastasis [15], were observed in four out of five mice injected with A549-shMed1 cells (Fig. 3D), but not in any mice inoculated with the control A549 cells. These data suggest that the loss of Med1 increases the *in vivo* invasive potential of A549 cells without significantly affecting initial tumor formation.

To further confirm that the loss of Med1 promotes metastasis *in vivo*, we performed an *in vivo* metastasis assay. Either A549-shMed1 or non-transfected A549 cells were injected into the tail veins of NOD/SCID mice, and the rate of lung metastasis was examined 8 weeks later. The number of metastatic lung nodules formed in mice injected with the A549-shMed1 cells was approximately 5-fold higher than that in mice injected with the control A549 cells (Fig. 4A). The average size of the metastatic nodules in A549-shMed1 was also approximately 1.5-fold larger than that of the metastatic nodules derived from the control A549 cells (Fig. 4B). The low expression of Med1 in the metastatic nodules derived from A549-shMed1 cells was confirmed by immunohistochemistry (Fig. 4C). Collectively, these results further support the notion that the loss of Med1 promotes the metastasis of human NSCLC *in vivo*. Therefore, we conclude that the loss of Med1 increases the invasive potential of human NSCLC cells.

3.5. Metastasis-related gene expression is upregulated upon Med1 loss

Because Med1 is a transcriptional mediator, we investigated whether Med1 influences invasion and metastasis in human NSCLC via the modulation of a specific gene expression program. To characterize the changes in gene expression upon Med1 loss and to define specific gene targets that may be involved in the subsequent pro-metastatic effect, we compared the gene expression profiles of Med1-depleted A549 cells and control A549 cells using Affymetrix oligonucleotide microarrays. The microarray analysis revealed that the knockdown of Med1 resulted in a greater than 2-fold upregulation of 603 probe sets and a greater than 2-fold downregulation of 472 probe sets (Supplementary Tables 2 and 3).

While many genes showed expression changes upon Med1 knockdown, we focused on metastasis-related genes in our search for mediators of the increase in the invasive potential of NSCLC cells upon Med1 loss (Fig. 5A and B). Several of the genes identified

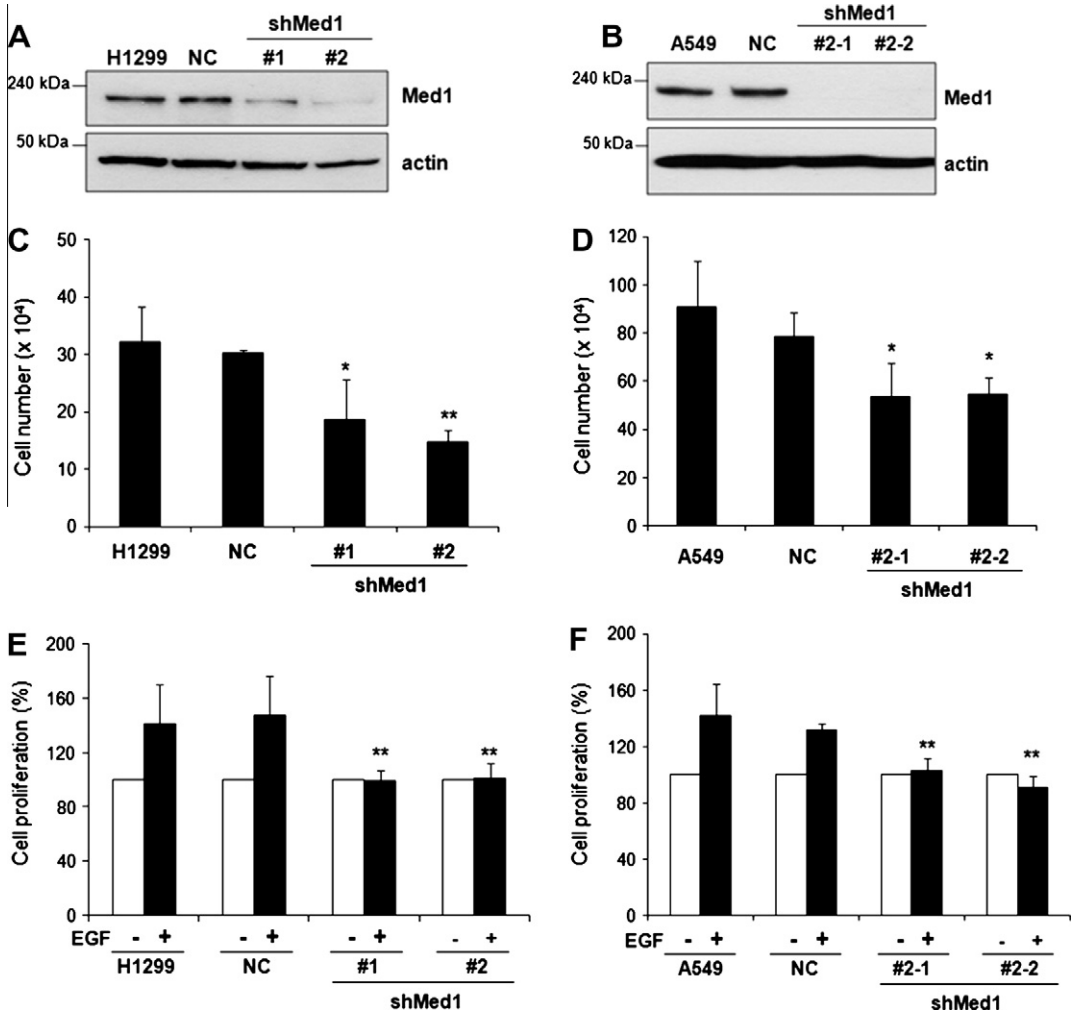


Fig. 1. The loss of Med1 expression decreases the proliferation of human NSCLC cells. (A and B) Stable Med1 knockdown in H1299 (A) and A549 (B) cells was achieved by introducing a Med1-specific shRNA expression vector. The reduction in Med1 expression was confirmed by Western blotting to compare the levels of Med1 in the transfected cells with that in cells expressing nonspecific shRNA (H1299 and A549 NC) and in non-transfected H1299 and A549 cells. (C and D) The proliferation of Med1 knockdown H1299 (C) and A549 (D) cells was measured and compared with that of the respective control cells. Cell proliferation was evaluated by counting the numbers of trypan blue-staining cells present after 6 days in culture. (E and F) The cell proliferation stimulated by EGF (50 ng/ml) was measured and compared with that of control cells, as described in (C and D), and normalized to the proliferation in unstimulated cells. All cell proliferation assays were repeated three times, and the presented data are the means ± standard deviation (SD). $^{*}p < 0.05$; $^{**}p < 0.01$ compared with H1299 or A549 control cells using Student's *t*-test.

as upregulated in our analysis have been previously reported to promote metastasis, including *uPAR*, *ID2*, *ID4*, *PTP4A1*, *PKP3*, *TGM2*, and *PLD1* (Fig. 5A and C). In addition, several of the genes identified as downregulated have been previously reported to suppress metastasis, including *TIMP2*, *RGS2*, and *HOXA4*. Real-time PCR analysis confirmed the upregulation of *uPAR*, *ID2*, *ID4*, *PTP4A1*, *PKP3*, *TGM2*, and *PLD1* and the downregulation of *TIMP2*, *RGS2*, and *HOXA4* in the Med1-depleted A549 cells compared to the non-transfected A549 cells (Fig. 5D). These results suggest that Med1 modulates the invasive potential of human NSCLC cells by regulating the expression of various metastasis-related genes.

4. Discussion

In this study, we demonstrate that Med1 is an important regulator of metastasis in human NSCLC. In human NSCLC samples, the loss of Med1 was significantly associated with an increase in the invasive and metastatic potential of these tumors. Consistent with this observation, the knockdown of Med1 increased the migratory and invasive potential of human NSCLC cells *in vitro*. Moreover, the knockdown of Med1 increased the metastatic capacity of H1299 and A549 cells in a mouse xenograft model and in an *in vivo* metastasis assay. In addition, by analyzing the gene expression profile of Med1-depleted cells, we found that the expression of many metastasis-related genes was modulated upon Med1 knockdown. Therefore, our results suggest that the loss of Med1 plays a critical role in the progression of human NSCLC cells to malignant metastatic carcinoma.

In previous studies, Med1 has been shown to participate in the regulation of cell cycle progression and cell proliferation. Med1-deficient mouse embryonic fibroblasts (MEFs) have been shown to exhibit impaired cell cycle progression and growth [16]. Consistent with these findings, Mastumoto et al. reported that Med1-deficient hepatocytes display impaired proliferative potential [17]. The knockdown of Med1 expression in prostate cancer cells also results in cell cycle arrest, decreased proliferation, and increased cell death [10]. Based on reports of Med1 amplification or overexpression in breast cancer, hepatocellular carcinoma, and prostate cancer [10,11,17], Med1 has been proposed to potentially promote tumorigenesis through a coregulatory role in cancer cell proliferation. Interestingly, Ndong et al. recently showed that Med1 expression is reduced in highly metastatic human melanoma

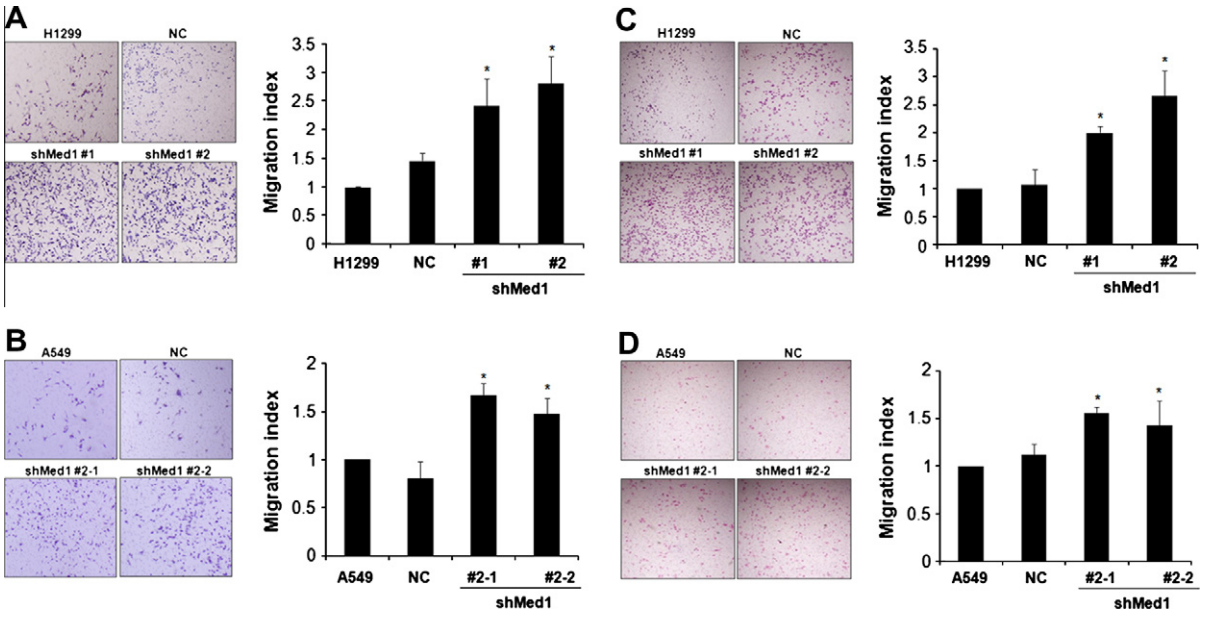


Fig. 2. The loss of Med1 increases the migration and invasion of human NSCLC cells. (A and B) A trans-well cell migration assay was performed using Med1-depleted H1299 (A) or A549 (B) cells, using cells expressing nonspecific shRNA (H1299 and A549 NC) and non-transfected H1299 and A549 cells as controls. The cells that migrated through the membrane were stained with hematoxylin and photographed (100×; left panel). The migration of non-transfected H1299 or A549 cells was defined as 1 and the relative cell migration of the Med1-depleted H1299 or A549 was calculated (right panel). (C and D) The Matrigel cell invasion assay was performed using Med1-depleted H1299 (C) or A549 (D) cells and compared with the respective control cells, as described in (A and B). After incubating the cells with Matrigel-coated filters for 18 h, the cells on the undersurface of the filter were stained with hematoxylin and eosin (H&E) and photographed (100×; left panel). The cell invasion of non-transfected H1299 or A549 cells was defined as 1 and the relative cell invasion of the Med1-depleted H1299 or A549 cells was calculated. The values shown are the means ± SD of three independent experiments. $^{*}p < 0.05$ compared with H1299 or A549 cells using Student's *t*-test.

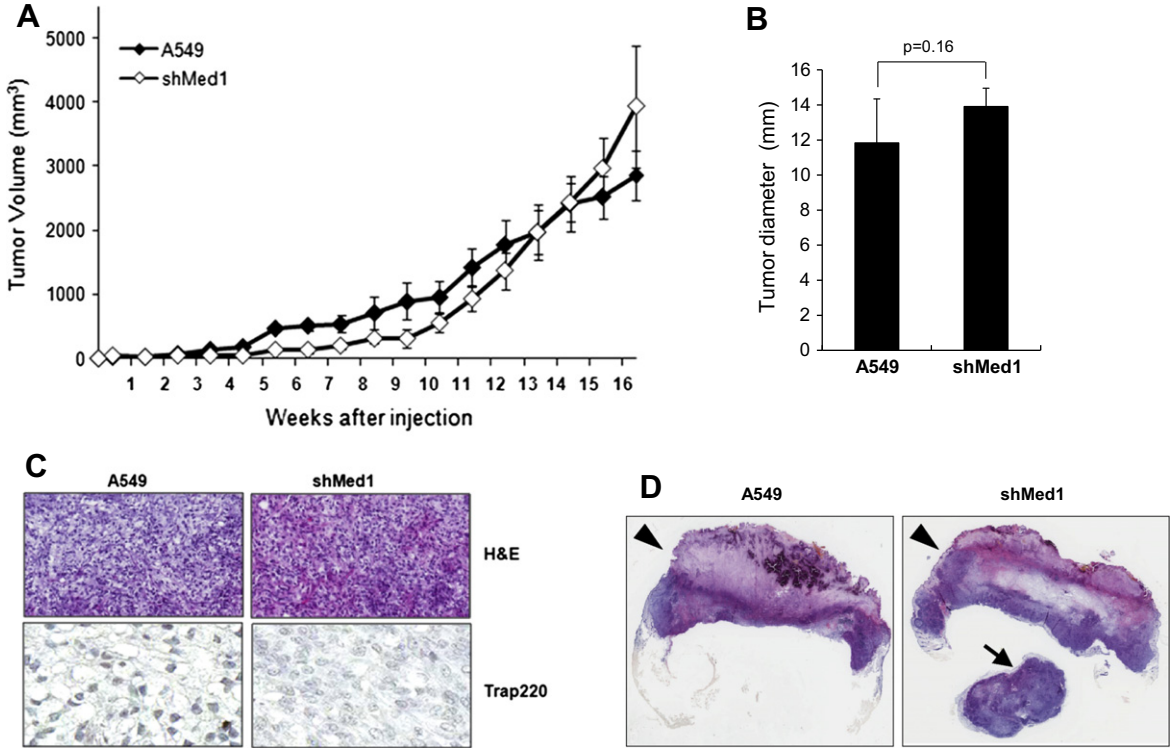


Fig. 3. The effect of Med1 loss on tumor development *in vivo*. (A) The primary tumor growth in NOD/SCID mice injected subcutaneously with 2×10^6 A549 cells ($n = 5$) or A549-shMed1 cells ($n = 5$) was measured once per week for 17 weeks. The data provided represent the mean values ± SD calculated for each group. (B) After week 17, the mice were sacrificed, and the diameters of the tumors derived from the A549 or the A549-shMed1 cells were measured and plotted as mean ± SD. (C) Histological analysis of the primary tumors derived from the injection of A549 or A549-shMed1 cells [upper panel, H&E staining (100×); lower panel, Med1 immunohistochemistry (200×)]. (D) A representative view of the tumors formed in mice injected with A549 or the A549-shMed1 cells. Arrowheads indicate the primary tumor and the arrow indicates a secondary tumor.

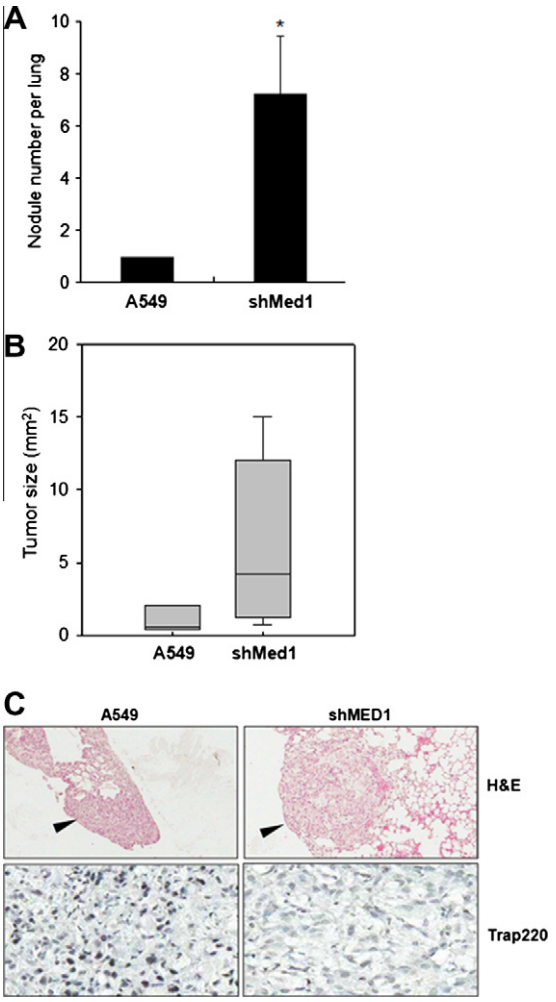
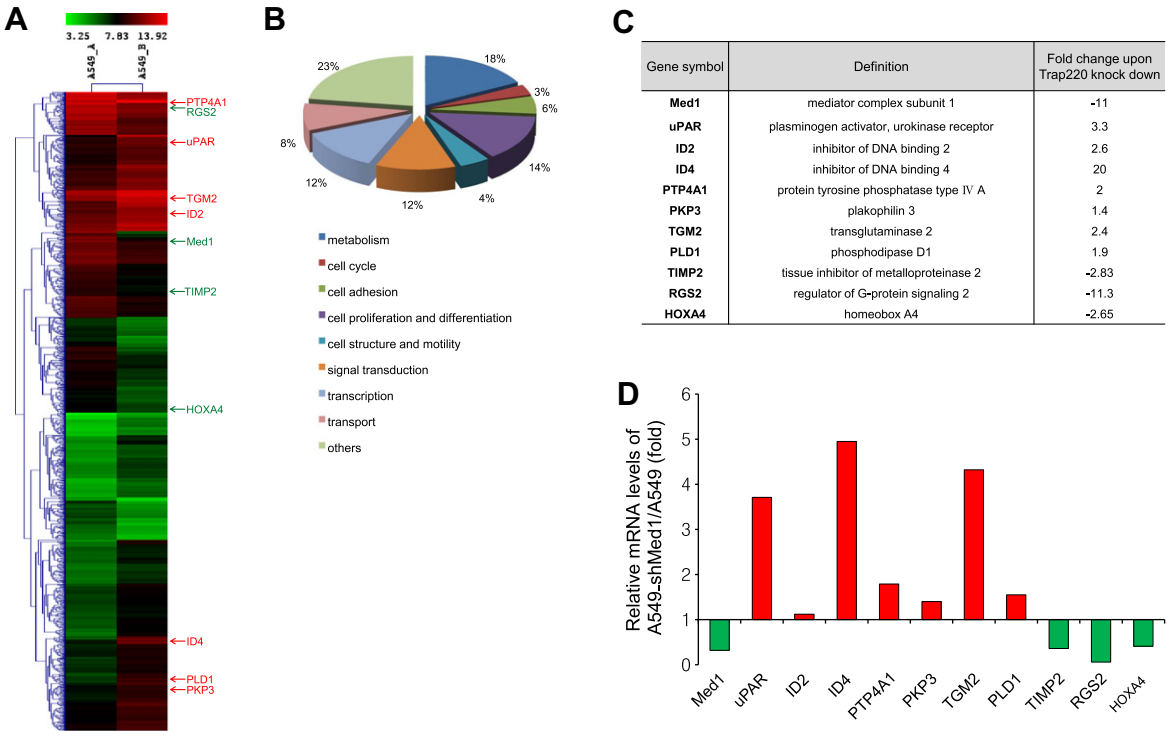


Fig. 4. The loss of Med1 promotes metastasis in an *in vivo* metastasis assay. (A) Lung metastases were measured after injecting either A549 cells ($n = 5$) or the A549-shMed1 cells ($n = 5$) via the tail vein. After 8 weeks, the total number of metastatic lung nodules in individual mice was counted in serial histological sections. The data shown represent the means ± SD. (B) The areas of metastatic lung nodules were measured in scanned images of the H&E-stained tumor sections. The data were plotted as means ± SD. (C) Representative H&E staining (upper panel) and Med1 immunohistochemistry (200×; lower panel) of lung sections prepared from mice injected with either A549 cells or A549-shMed1 cells. Arrowheads indicate metastatic lung nodules.

cells, and that the knockdown of Med1 expression increases the invasive properties of melanoma cells *in vitro* and *in vivo* without impairing cell proliferation [12]. These contradictory results suggest that Med1 is involved in various cellular processes and that changes in Med1 expression may result in opposing effects depending on the cellular context. Consistent with this notion, Ge et al. used Med1$^{-/-}$ MEFs to show that Med1 is essential for PPARγ-stimulated adipogenesis but not for MyoD-stimulated myogenesis [18]. In the present study, we observed that the depletion of Med1 in human NSCLC H1299 and A549 cells increased their migratory capacity and invasive potential, while basal and growth factor-induced proliferation was reduced. Interestingly, the size of tumors derived from Med1-depleted A549 and control A549 cells was comparable in a NOD/SCID mouse xenograft model, suggesting that the expression level of Med1 does not have a significant effect on the initiation and establishment of human NSCLC. Consistent with this result, the average sizes of Med1-positive and Med1-negative NSCLC tumors were not significantly different. These results indicate that the loss of Med1 contributes primarily to an increase in the invasive properties of human NSCLC, rather than to tumor growth per se.

Cancer invasion and metastasis are multifactorial processes that require the coordinated transcriptional regulation of many groups of genes [19]. Importantly, gene expression analysis of Med1-depleted and control A549 cells revealed that the loss of Med1 leads to the induction of many metastasis-promoting genes, including *uPAR*, *ID2*, *ID4*, *PTP4A1*, *PKP3*, *TGM2*, and *PLD1*. Previous studies have shown that the expression of these genes promotes migration and invasion in various human cancers [20–26]. Urokinase-type plasminogen activator receptor (uPAR) plays an essential role in the proteolytic degradation of the extracellular matrix (ECM) and the basement membrane [27]. Interestingly, Ndong et al. have previously shown that the knockdown of Med1 increases uPAR expression in melanoma cells [12]. ID2 (inhibitor of DNA binding 2) and ID4 (inhibitor of DNA binding 4) are inhibitors of basic helix-loop-helix (HLH) transcription factors. Recent studies have shown that an increase in the expression of ID2 and ID4 stimulates tumor cell migration and invasion either by modulating specific target gene expression [21,28] or through a transcription-independent mechanism [29]. PTP4A1 (protein tyrosine phosphatase 4A1), also known as PRL-1 (phosphatase of regenerating liver-1), has been found to increase Rho family GTPase activity, effectively promoting cell motility and invasion [30]. The overexpression of PKP3 (plakophilin 3) is observed in most lung cancer patients and is correlated with increased invasion and metastasis [24]. TGM2 (transglutaminase 2) is involved in the modulation of the ECM and its cellular interactions. TGM2 was recently reported to promote cell migration by upregulating the expression of MMP-9 [31]. PLD1 (phospholipase D1) modulates cell adhesion and migration by reorganizing the actin cytoskeleton [32]. Our gene expression analysis showed that the loss of Med1 also leads to the reduction of many metastasis-suppressing genes, including *TIMP2*, *RGS2*, and *HOXA4*. These genes have previously been shown to suppress the migration and invasion of human cancer cells [33–36]. TIMP2 (tissue inhibitor of metalloproteinase 2) is a member of the TIMP family of proteins, which directly bind and inhibit metalloproteinases (MMPs) and thus inhibit tumor invasion and metastasis [37]. Previous studies have shown that the overexpression of TIMP2 suppresses invasion and metastasis in pancreatic cancer and bladder carcinoma cells [34,36]. The downregulation of RGS2 (regulator of G-protein signaling 2) has recently been observed in metastatic colorectal cancer cell lines and has been implicated in colon cancer metastasis [33]. HOXA4 (homeobox A4), which belongs to the HOX family of transcription factors, has been implicated in ovarian cancer [35], and knockdown of HOXA4 was recently shown to increase ovarian cancer cell migration, possibly through a modulation of β1 integrin [38]. More studies are needed to establish how the loss of Med1 modulates the expression of these genes and how these genes contribute to the progression of human NSCLC. Importantly, Gade et al. previously reported the loss of Med1 in several human lung cancers and showed that this was associated with a reduced expression of the metastasis suppressor DAPK1 [39]. Thus, our study, along with results from other groups, demonstrates that Med1 plays a critical role in human lung cancer progression by controlling the expression of genes that are important to various aspects of cancer cell metastasis.

In conclusion, our findings support a novel mechanism by which the loss of Med1 promotes the metastatic progression of human NSCLC. Our results show that many important regulators of cancer cell invasion and metastasis are induced upon Med1 loss, suggesting that Med1 may act as a suppressor of human lung cancer metastasis. Whether the loss of Med1 expression is involved in the development of malignancy in other types of human cancers should be addressed in future studies. Further investigation is also necessary to determine whether Med1 is a bona fide suppressor of



Gene symbol	Definition	Fold change upon Trap220 knock down
Med1	mediator complex subunit 1	-11
uPAR	plasminogen activator, urokinase receptor	3.3
ID2	inhibitor of DNA binding 2	2.6
ID4	inhibitor of DNA binding 4	20
PTP4A1	protein tyrosine phosphatase type Ⅳ A	2
PKP3	plakophilin 3	1.4
TGM2	transglutaminase 2	2.4
PLD1	phosphodipase D1	1.9
TIMP2	tissue inhibitor of metalloproteinase 2	-2.83
RGS2	regulator of G-protein signaling 2	-11.3
HOXA4	homeobox A4	-2.65

Fig. 5. Metastasis-related gene expression increases upon Med1 loss. (A) This dendrogram shows the unsupervised cluster analysis of the expression patterns detected in microarrays for A549-shMed1 cells (A549_B) relative to control A549 cells (A549_A). (B) The functional classification of genes regulated by Med1 in A549 cells. Individual functional categories are indicated by a color code, and the percentages shown indicate their representation among all genes whose expression changed by more than twofold upon Med1 knockdown. (C) A list of metastasis-related genes that are changed upon Med1 depletion. The fold changes in the expression of metastasis-related genes upon Med1 depletion are indicated. (D) Quantitative RT–PCR (qPCR) analysis was performed using gene-specific primers in A549 and A549-shMed1 cells to confirm the observed alteration of metastasis-related genes. After the mRNA level of each gene was normalized to the level of β-actin mRNA, the relative mRNA level in A549-shMed1 cells was compared with that in A549 cells.

metastasis in human NSCLC and to identify the mechanisms by which Med1 expression is decreased during human NSCLC progression. This study establishes Med1 as an important regulator of NSCLC metastatic progression and identifies novel molecular targets for NSCLC treatment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2012.02.009.

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