Author's manuscript

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MUSASHI 2 (MSI2) REGULATES TUMOR GROWTH FACTOR BETA (TGF- β) SIGNALING AND CLAUDINS TO PROMOTE LUNG ADENOCARCINOMA METASTASIS

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PhD. Thesis Abstract

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Ph.D. thesis is located at Scientific library named after N.I. Lobachevsky at Kazan Federal University.

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The Scientific secretary of dissertation committee, Associate professor Z.I. Abramova, Ph.D.

INTRODUCTION

Background. Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths in the world (Siegel et al., 2013). The five-year survival rate for lung cancer is around 16% of diagnosed cases (Howlader et al., 2013). Much of the lethality of lung cancer is due to frequent diagnosis of the malignancy at the metastatic stage, when fundamental changes in tumor biology cause the disease to be refractory to many treatments.

The leading cause of non-small cell lung cancer (NSCLC) is activating mutation of *K-Ras* (approx. 30%) (Mitsudomi et al., 1991), and *p53* allele loss (approx. 60% cases) (Takahashi et al., 1989). Tumors arising from transgenic *K-Ras/p53* knockout mice resemble ones from human adenocarcinoma (Zheng et al., 2007). These features make the KP murine model a useful tool with which to evaluate factors that underlie NSCLC metastasis, in particular – processes of epithelial-mesenchymal transformation (EMT) of cancer cells.

EMT plays essential role in metastatic process development (Hay et al., 1995; Kalluri, Weinberg et al., 2009). Often it is mediated by changes in normal functioning of intracellular protein signaling pathways, such as tumor growth factor beta (TGF-β) pathway and because of phenotype change from epithelial to mesenchymal and vice versa (Zavadil et al., 2005; Taylor et al., 2010).

These features make the KP murine model a useful tool with which to evaluate factors that underlie NSCLC metastasis. Among these factors are evolutionally conserved proteins Musashi (Msi1 and Msi2). They are coded by *Msi1* and *Msi2* genes and have high percent of homology in RNA binding sequences. Musashi proteins are described as regulators of mRNA of numerous protein targets, therefore regulating multiple biological processes, including stem cells divisions (Sakakibara et al., 2001; Siddal et al., 2006) and cell cycle (Park et al., 2014). MSI2 is less studied than its homolog MSI1 and is well described in the context of hematologic malignancies (Park et al., 2014; Ito et al., 2010; Kharas et al., 2011) and several solid tumors. All cases of MSI2 elevated expression were

associated with worsening of clinical prognosis, metastasis development and increase in overall malignancy of the disease.

The goal of this project was to reveal the mechanism of MSI2-mediated regulation of growth and metastasis of lung adenocarcinoma.

According to the long-term goal we had specific **tasks**:

1. Conduct the screening and measure the levels of stem cell-related markers in NSCLC cell lines with high metastatic capacity.
2. Assess the levels of MSI2 protein in the tissue samples from patients with lung adenocarcinoma.
3. Investigate the biological role of MSI2 using murine lung cancer model.
4. Reveal signaling pathways, related to biological effects of MSI2.

The novelty. This work for the first time describes the MSI2-dediated mechanism of lung adenocarcinoma metastasis. It is demonstrated, that levels of this translation regulator are elevated in patients with NSCLC. This leads to the reprogramming of intracellular signaling pathways and increase of adenocarcinoma metastasis. We have revealed the mechanism of this increase and determined protein targets of MSI2 – claudins-3, -5 and -7, TGF-βR1 and SMAD3. MSI2 drives lung adenocarcinoma metastasis via pathological activation of TGF-β/SMAD signaling and downregulation of claudins, therefore leading to malignant spread of NSCLC cells.

Significance. The results obtained in this study promote better understanding of the intracellular processes underlying invasion and metastases of lung cancer and leading to the increase of therapeutical resistance. This opens opportunities for development of new diagnosis methods, improving the therapeutical response prognosis and therapy of lung cancer in comprehensive medical centers, such as Russian oncologic center named after N.N. Blockhin, Moscow scientific oncologic institute named after P.A. Khertsen and oncologic hospitals.

The mechanism described is of the great interest for better understanding the intracellular signaling pathways. Thus, the data obtained is very important and

have great significance both from practical point of view (for a new strategy of therapeutic drug development) and from theoretical point of view (for molecular biology and biochemistry).

The dissertation statements:

1. MSI2 expression is elevated in NSCLC tumor cell lines derived from K-Ras/p53 mice.
2. MSI2 is progressively elevated in lung cancer patient samples at metastasis stage.
3. MSI2 knockdown decreases invasion of NSCLC cells with high metastatic capacity 2 – 4-fold *in vitro* ($p<0.001$) and metastasis rate 5-fold *in vivo* ($p<0.0001$).
4. Mechanistically MSI2 regulates invasion and metastasis of lung adenocarcinoma cells via activation of TGF-β/SMAD signaling and decreased claudins expression.

Study approbation. The results of this study were presented at four international scientific conferences: two Annual Meetings of American Association of Cancer Research (Philadelphia, USA, 2012 and New Orleans, USA 2016), Annual meeting of American Association of Clinical Oncology (Chicago, USA, 2015) and Annual meeting of American Association of Cell Biology (Philadelphia, USA, 2014). Results were presented at on the 19th, 20th, 21th Annual conferences at Fox Chase Cancer Center (Philadelphia, USA, 2013 and 2014) and VII Russian Scientific Conference "Human Health in XXI Century" (Kazan, Russia, 2016).

Related publications. There were 13 papers published related to the topic of dissertation. Within them, 2 manuscripts were published in the peer reviewed journals, accredited by The Dissertation Examination Committee and included in SCOPUS database.

Structure and contents. The thesis include introduction, review of the current known data about the topic, materials and methods, results, discussion,

conclusions and references. The manuscript consists of 99 pages, includes 33 figures and 2 tables. 153 references are listed.

SHORT SUMMARY OF THE STUDY

The study objects. Mouse cell lines (344SQ, 531LN2, 393p) from $p^{53R172H\Delta g}$/$K\text{-}ras^{LA1/+}$ mice were derived from tumor tissues at necropsy from different mice as previously described (Gibbons et al, 2009). Human alveolar basal epithelial adenocarcinoma cell lines A549, H358 were obtained were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). To prepare lentivirus for introduction of shRNAs into NSCLC cells, HEK-293T cells were transfected with shRNA lentivirus prepared in the pLKO.1 system (Addgene, Cambridge, MA), *In vivo* experiment were conducted on 129Sv black mice and C.B17 SCID mice. All experiments involving mice were performed according to protocols approved by Institutional Animal Care and Use Committees (IACUCs) at M.D. Anderson Cancer Center or the Fox Chase Cancer Center.

Tissue preparation, Histology, Quantitative Analysis. Lungs and tumors were collected. Tissues were collected and fixed in 10% phosphate-buffered formaldehyde (formalin) 24-48 hrs, dehydrated and embedded in paraffin. Prepared specimens were analyzed by hematoxylin and eosin (H&E) staining (Sigma-Aldrich, St. Louis, MO). Tumor sections were immunostained with antibodies to Ki-67 (DAKO, Carpinteria, CA) to allow quantitation of proliferation, and with antibodies to cleaved caspase (Cell Signaling, #9661) to allow quantitation of apoptosis. Immunohistochemistry and H&E were performed by standard protocols. Immunostained slides were scanned using Vectra Automated Quantitative Pathology Imaging System (Perkin Elmer, Waltham, MA). Expression levels of the proliferative index marker Ki-67 or the cleaved caspase indicator of apoptosis were quantified using Vectra Automated Quantitative Pathology Imaging System specific protocols and algorithms.

Quantitative RT–PCR analysis. Gene expression levels were determined by using quantitative reverse-transcription polymerase chain reaction. Total RNA from

the cells was isolated using a RNeasy Mini Kit and using TRIzol from the tumor lysates, according to the manufacturer's specific protocols.

siRNA transfection. NSCLC cells at 50% confluence were transfected with siRNA at final concentrations of 10 nmol/L using the HiPerfect transfection reagent (Qiagen, Waltham, MA) according to the manufacturer's instructions.

shRNA Targeting Sequences and lentivirus production. Short hairpin RNAs (shRNAs) were obtained from SIGMA-ALDRICH (St Louis, MO). See Supplementary Table S4 for sequences used. To prepare lentivirus for introduction of shRNAs into NSCLC cells, HEK-293T cells were transfected with shRNA lentivirus prepared in the pLKO.1 system (Addgene, Cambridge, MA), with the psPAX2 and pMD2.G packaging plasmids. Media containing lentiviral particles was collected on day 4. Subsequently, lung cancer cells were infected with lentivirus and selected by growth in RPMI 1640 with 10% FBS and puromycin, using standard methods.

SDS-PAGE and Western Blots. For Western blotting, cells were disrupted in T-Per lysis buffer (Sigma-Aldrich, St. Louis, MO) supplemented with protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Whole cell lysates were used directly for SDS–PAGE and Western blotting, using standard procedures. Image analysis was done using ImageJ (National Institutes of Health, Bethesda, MD), with signal intensity normalized to β-actin or total level of detected proteins. Data was analyzed in Excel by paired t-test to determine statistical significance.

Cell invasion assay. The upper chambers of growth factor–reduced Matrigel invasion chambers (354483; BD Biosciences, Franklin Lakes, NJ or 3483-024-01, Trevigen, Gaithersburg, MD) were seeded with cells (1 × 105 cells per well) in triplicate wells. Medium (RPMI 1640), with or without 10% FBS, was placed in the lower and upper chambers, respectively. Mitomycin C at a concentration of 10 μg/ml was added to the upper chamber to inhibit cell proliferation. Cells were incubated at 37°C for 24 hours. Cells that had invaded through Matrigel were visualized with crystal violet. Three microscopic fields (original magnification,

10X) were photographed and counted per chamber, and the results were expressed as the mean ± SEM of invaded cells from replicate wells and multiple independent experiments. Data was analyzed in Excel by paired t-test to determine statistical significance.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde (10 min) and then cold methanol (5 min), permeabilized with 1% Triton X-100 in PBS and blocked with 3–5% BSA in PBS. Samples were then incubated with the primary antibodies overnight at 4°C. Following rinse in PBS + 0.01% Tween, samples were incubated for 1 hour with an Alexafluor 488, 568 or 647 tagged donkey anti-rabbit, mouse, or goat secondary antibody (Life Technologies, Eugene, OR), and counterstained with a 2 μmol/L 4′, 6-diamidino-2-phenylindole (DAPI) (Life Technologies, #1652731, Eugene, OR) solution. Samples were imaged using a Nikon C1 Spectral confocal microscope (Nikon, Melville, NY) equipped with a numerical aperture (NA) 1.40, oil immersion, 63x Plan Apo objective (Nikon). Images were acquired at room temperature using EZ-C1 3.8 (Nikon) software and analyzed with MetaMorph (Molecular Devices, Union City, CA).

Overexpression studies. For overexpression of MSI2, a full-length cDNA encoding MSI2 (for sequence information, please refer to NM_138962.2) or empty vector was obtained from the human ORFeome collection and transferred to the following viral vectors via Gateway recombination and virus production following manufacturer's recommendations: pLenti63/V5 DEST (Thermo Fisher Scientific, Waltham, MA). All overexpression studies were performed using newly transduced stable cell lines generated by single clone selection.

Statistical analysis. We used nonparametric Mann-Whitney t-test to calculate p-values to compare differences between markers expressions. We used regression analysis to assess growth curves. $p<0.05$ was considered as statistically significant in all of the analyses. All statistical analysis was performed in «Microsoft Excel» и «Graphpad Prism» software.

RESULTS AND DISCUSSION

Investigation of *Msi2* gene expression in highly metastatic lung adenocarcinoma

Using qRT-PCR, we compared the mRNA expression of a candidate set of stem cell marker genes in a highly metastatic (344SQ) versus a non-metastatic (393P) NSCLC cell line, obtained from *K-Ras*$^{LA1/+}$/*p53*$^{R172HG/+}$ mice (Figure 1)

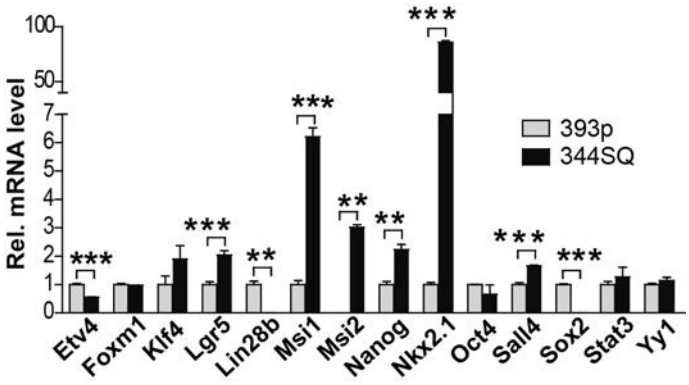

Figure 1. Genes assessed for differential expression in 344SQ high metastatic cell line versus 393P low metastatic cell line; graphs show quantification of qRT-CPR data. Notable increase in *Msi1* and *Msi2* expression. **, p<0.01; ***, p<0.001

(Gibbons et al., 2009). Genes with significant difference in expression between these two cell lines were further assessed in a panel of 7 metastatic and 7 non-metastatic cell lines derived from additional independent K-Ras/p53 tumors. Msi2 expression was consistently elevated in metastatic cell lines at both the mRNA and the protein level (Figs. 2 A and B). Msi1, a paralogue of Msi2, also displayed a trend towards elevated expression in metastatic murine cell lines at the mRNA level, but this was not observed at the protein level (Fig. 2 C, D).

To assess whether MSI2 overexpression is physiologically relevant in the context of human NSCLC, we first used Automated Quantitative Analysis (AQUA) to analyze the protein expression of MSI2 in tissue microarrays (TMAs) containing 123 primary human NSCLC tumors and normal lung tissue. This analysis indicated highly significant elevation of MSI2 in tumors compared to normal tissue. In contrast, parallel assessment of MSI1 expression did not reveal differences in expression between normal lung tissue and primary NSCLC tumor specimens.

The progressive increase in MSI2 expression as human lung tumors metastasized from primary site, combined with data from the K-Ras/p53 model,

suggested a potential functional and clinical relevance for MSI2 in regulating tumor progression.

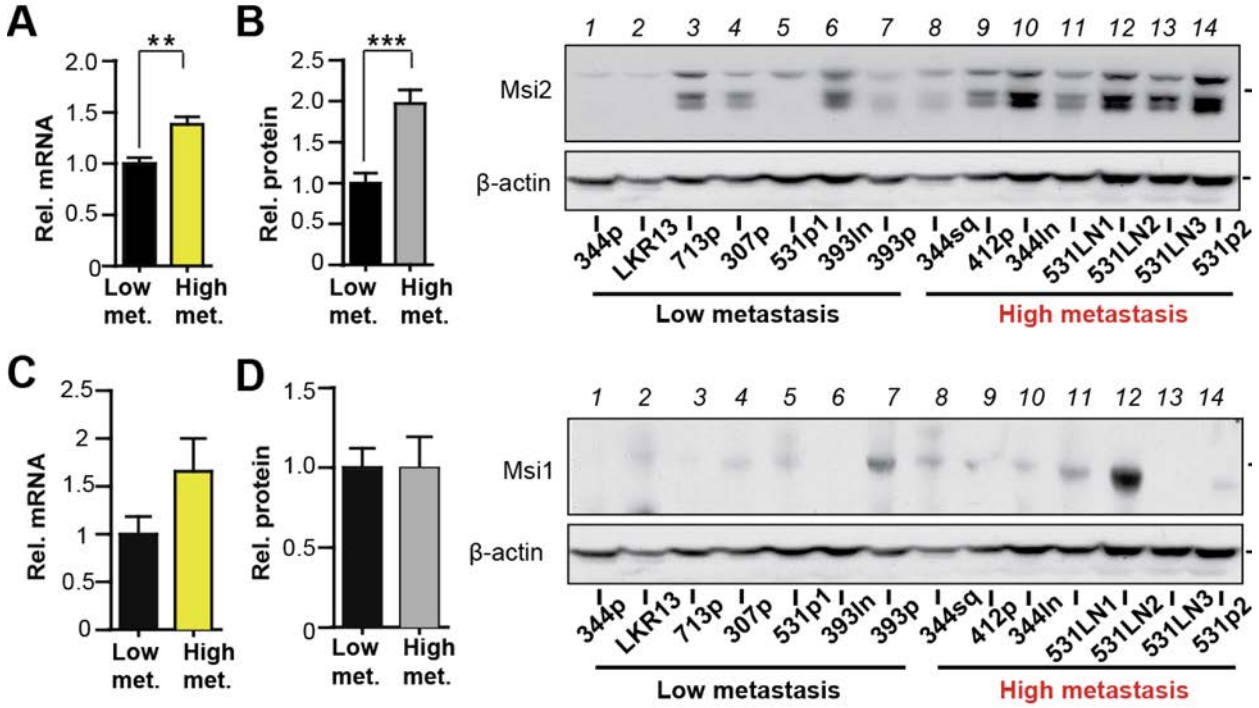

Figure 2. qRT-PCR (A, C,) and Western blot (B, D) of MSI2 (A, B) and MSI1 expression in 7 non-invasive versus 7 metastatic murine cell lines. **, p<0.01; ***, p<0.001

Investigation of role of Msi2 on *in vitro* invasion of tumor cells with high metastatic capacity

Based on expression profiles of NSCLC cell lines available through the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012), we identified the human NSCLC cell lines A549 (K-Ras^{mut}) and H358 (K-Ras^{mut}; p53$^{-/-}$) and two murine cell lines (344SQ и 531LN2), derived from *K-Ras/p53* knockout mice as metastasis-competent adenocarcinoma cell lines with high expression of MSI2.

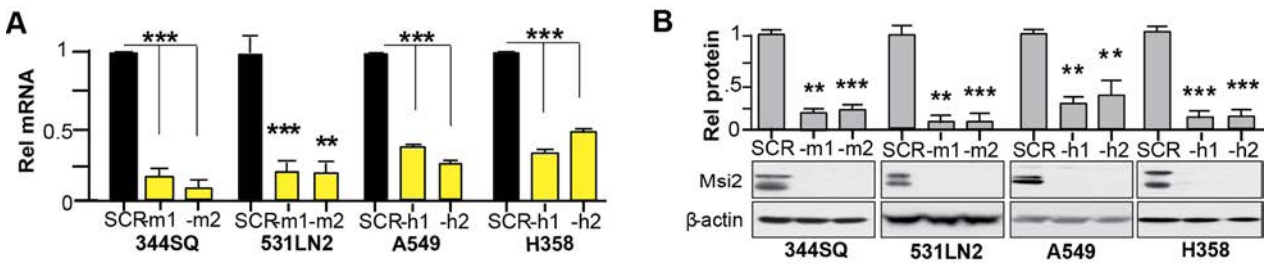

Figure 3. qRT-PCR (A) and Western (B) validation of MSI2 depletion with two independent shRNAs (-m1, -m2, -h1, -h2) in indicated cell lines, referenced to control shRNA (SCR) depleted cells. **, p<0.01; ***, p<0.001

We used shRNA depletion of these NSCLC cell lines to further study the role of MSI2 in metastasis. Further: SCR – control; -m1/-m2 – murine cell lines with sh-depleted MSI2; -h1/-h2 – human cell lines with sh-depleted MSI2. Effective MSI2 mRNA and protein depletion were confirmed for four MSI2-depleted cell lines, in reference to matching scrambled shRNA control cell lines on qRT-PCR and Western blot (Figure 3).

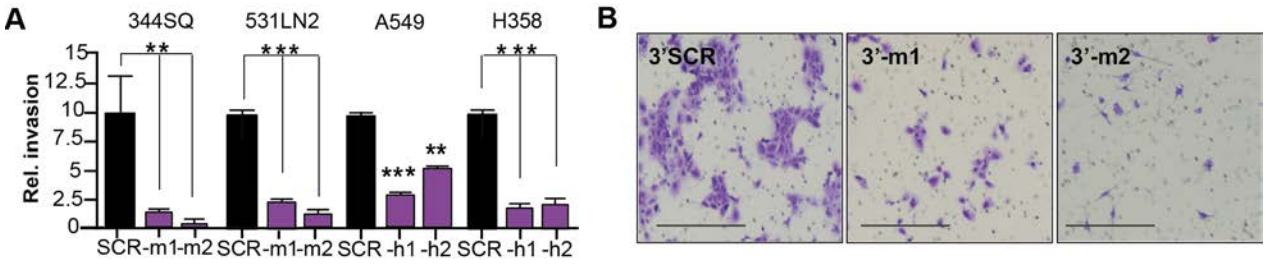

Figure 4. Quantified (A) and representative (B; for 344SQ cells) data for Matrigel invasion. Scale bar – 50um. **, $p<0.01$; ***, $p<0.001$

MSI2 depletion consistently and significantly reduced invasion through Matrigel for all lines (Figure 4). Similar results were obtained using transient siRNA transfections to deplete MSI2. Therefore, it was proposed that MSI2 knockdown leads to decrease of NSCLC cell invasion capacity *in vitro.*

In reciprocal experiment, MSI2 was overexpressed in non-metastasizing 393P cell line with low endogenous MSI2 levels, obtained from K-Ras/p53 mice model. Lentiviral overexpression of MSI2 resulted in increased invasive capacity of NSCLC cells in Matrigel.

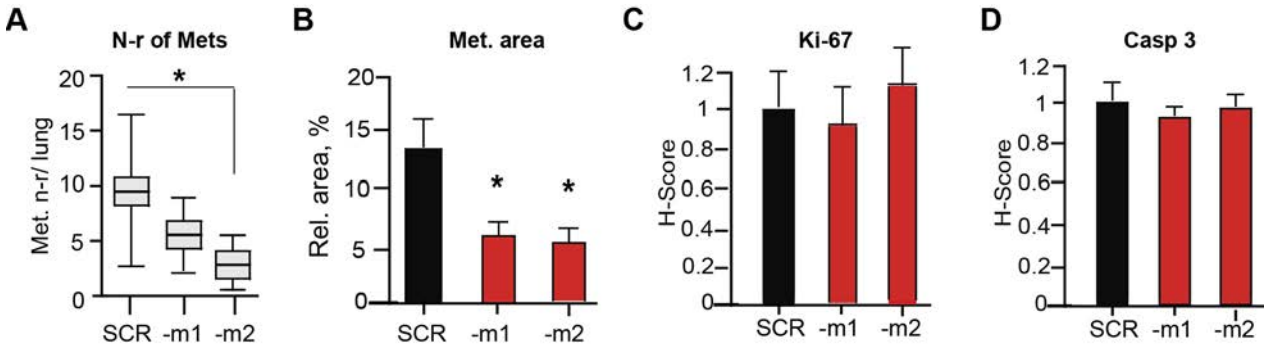

Figure 5. Quantified number of independent metastases per lung (A), and relative area of lung metastases (B) 28 days after injection of orthotopic 344SQ xenografts in 129Sv immunocompetent mice. Quantification of Ki-67 (C) and cleaved caspase (D) in orthotopic xenografts; caspase levels were low in all specimens. All graphs: *, $p<0.05$ relative to controls.

We therefore focused our subsequent studies on mechanistic analysis of invasion signaling.

Tumors with low MSI2 levels demonstrate decreased metastatic activity *in vivo*

To support *in vitro* results we performed several *in vivo* experiments using murine models.

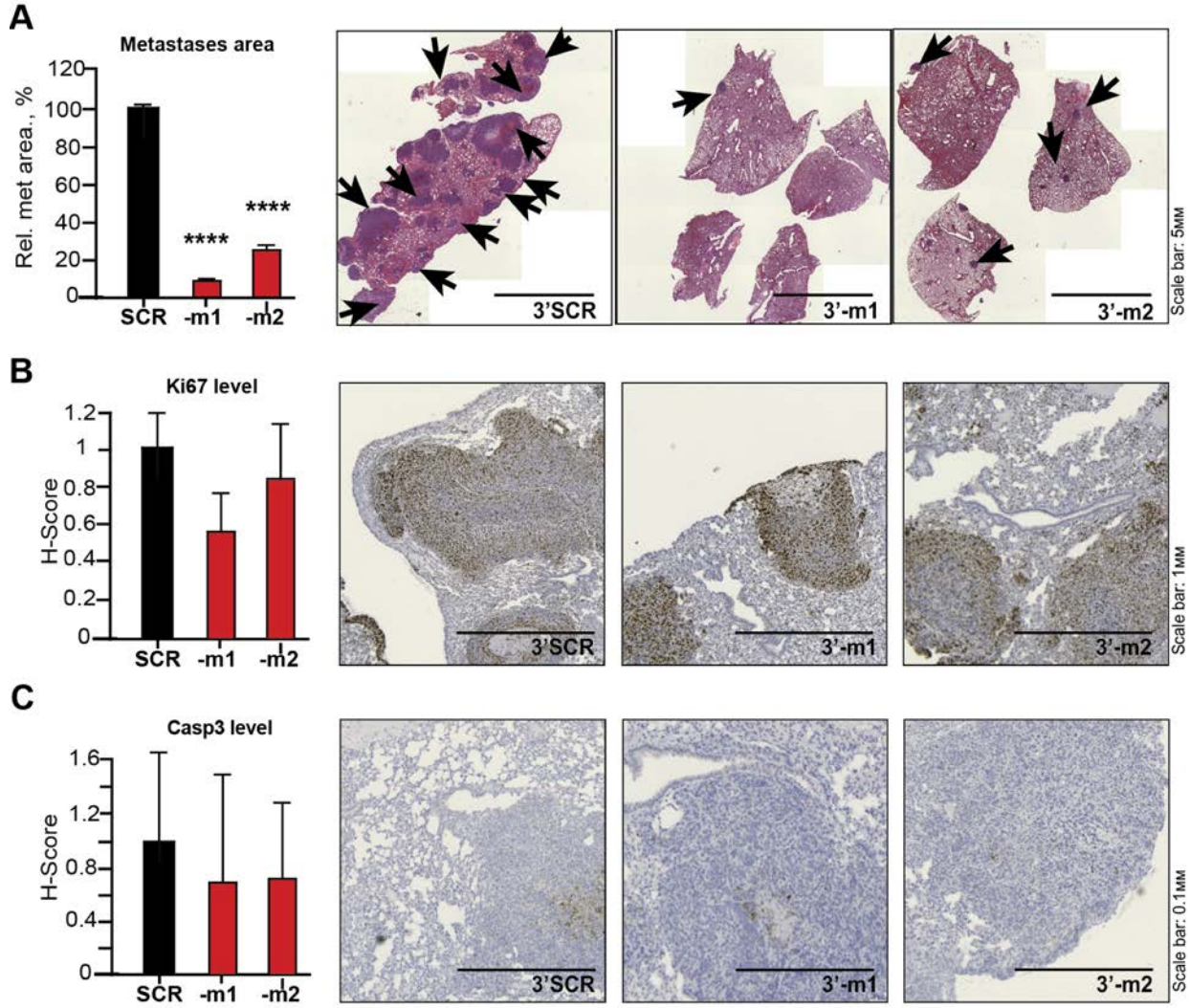

Figure 6. Primary and metastatic 344SQ Tumor Growth in Subcutaneous Xenografts. Analysis (left) and representative images (right) of metastatic burden (A), Ki-67 proliferation marker (B) and cleaved caspase apoptotic marker (C) in SCID mice with 344SQ control (SCR) and MSI2 depleted (-m1/- m2) subcutaneous xenografts. All graphs: ****, $p \leq 0.0001$ relative to controls.

We performed orthotopic lung injections of metastasis-competent 344SQ cells with shRNA vector control or shRNA targeting Msi2 into syngeneic, immunocompetent 129Sv mice. Msi2 depletion significantly reduced the burden of

lung metastases following injection, predominantly affecting the total number of metastases and to a lesser extent the size of individual metastases (Fig. 5A, B), while no statistically significant difference in Ki-67 or cleaved caspase staining were observed (Fig. 5C, D).

In a complementary experiment, subcutaneous xenograft of 344SQ in immunocompromised SCID mice also indicated Msi2 depletion induced no statistically significant difference in the growth of primary xenografts, or associated Ki-67 or cleaved caspase staining (Fig 6B). However, there was a significantly higher metastatic burden in the lungs of mice bearing control versus Msi2-depleted tumors (Fig. 6A). In this case, the difference in metastatic area predominantly reflected differences in numbers of metastatic foci, with Ki-67 and caspase staining comparable between control and MSI2-depletion groups (Fig 6B, C).

Obtained results indicate that increased MSI2 expression characterizes NSCLC tumors with high metastatic capacity and supports invasion and metastasis *in vitro* and *in vivo*.

Investigation of MSI2-regulated signaling targets

We used Reverse Protein Phase Array (RPPA) (Iadevaia et al., 2010; Tibes et al., 2006) to query 171 total and phospho-proteins for expression changes

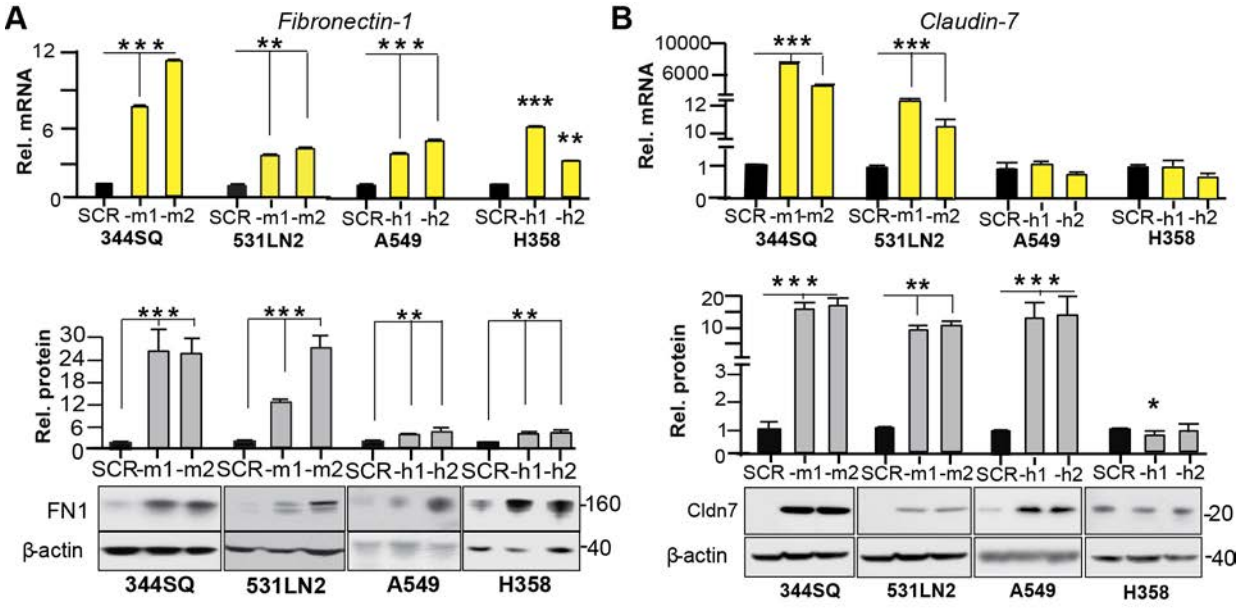

Figure 7. MSI2 depletion controls the expression of indirect and direct targets relevant to invasion and EMT. Western analysis of FN1 (A) and CLDN7 (B) in murine and human NSCLC cell lines in the context of MSI2 depletion with independent targeting shRNAs (-m1/-m2, -h1/-h2). SCR, control scrambled shRNA. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

associated with Msi2 knockdown using control shRNA and Msi2-targeted shRNA derivatives of 344SQ cells. This work suggested a number of novel candidates associated with Msi2 expression and relevant to control of EMT and invasion.

Proteins with the greatest magnitude of response to Msi2 depletion that were subsequently validated by low throughput Western analysis included the tight junction (TJ)-associated protein claudin 7 (CLDN7) (Lu et al., 2011; Bruna et al., 2012; Prat et al., 2013), elevated 19.4-fold, and the ECM protein fibronectin (FN1) (Liu et al., 2008; Urtreger et al., 2006; Zhang et al., 2009; Yi and Rouslahti et al., 2001), elevated 2.5-fold. Subsequent independent evaluation using qRT-PCR and Western confirmed these RPPA results, as MSI2 depletion significantly elevated FN1 mRNA (4.0-9.4-fold) and protein (2.4-23 fold) in all 4 cell lines, and elevated CLDN7 protein (2.5-28 -fold) in 3 of the 4 cell lines (Figure 7 A, B). Results were independently confirmed using transient siRNAs to deplete MSI2.

MSI2 translationally regulates invasion related proteins

NSCLC cells have been shown to express multiple claudins with partially redundant function (Kwon et al., 2013), most not represented in the RPPA panel. In direct testing, we found MSI2 depletion also induced CLDN3 and CLDN5 in all 4 cell lines at the protein level (3.8 - 22-fold for CLDN3 and 3.4 - 4.1-fold for

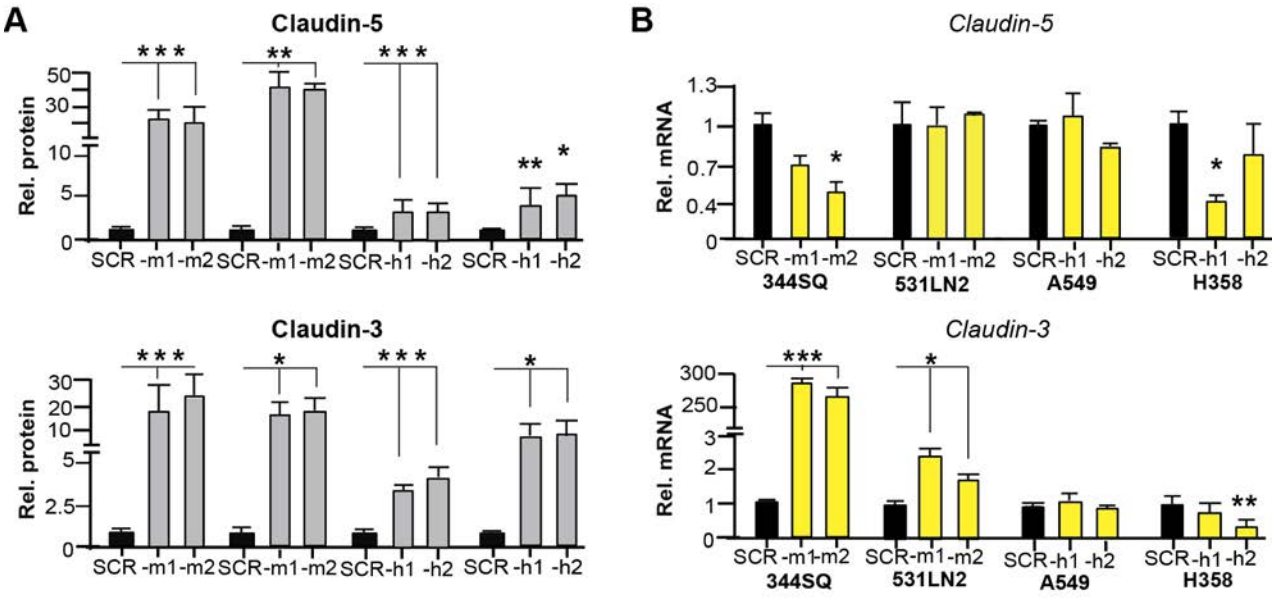

Figure 8. *Msi2* knockdown leads to increase of the Claudin-3, -5 levels the protein, not mRNA. Western (A) and qRT-PCR analysis of Claudin-3, -5 levels in murine and human NSCLC cell lines in the context of MSI2 depletion with independent targeting shRNAs (-m1/-m2, -h1/-h2). SCR, control scrambled shRNA. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

CLDN5) (Fig. 8A), making restraint of claudin expression a consistent feature of MSI2 function.

Studies of the MSI proteins (predominantly focused on MSI1) have defined these proteins as RNA-binding proteins that regulate mRNA translation (Sakakibara et al., 2001; Rezza et al., 2010; Park et al., 2014). The induction of claudins may reflect a combination of transcriptional and post-transcriptional consequences of MSI2 depletion, as the mRNA level shows induction less marked than at the protein level (Fig 8B). However, the claudin mRNAs lack [(G/A)U(n)AGU (n = 1–3)] consensus motifs for MSI2 binding described in Wang et al (Wang et al., 2015), suggesting direct regulation of translation is not involved. Further studies need to be conducted to investigate the mechanism of this regulation.

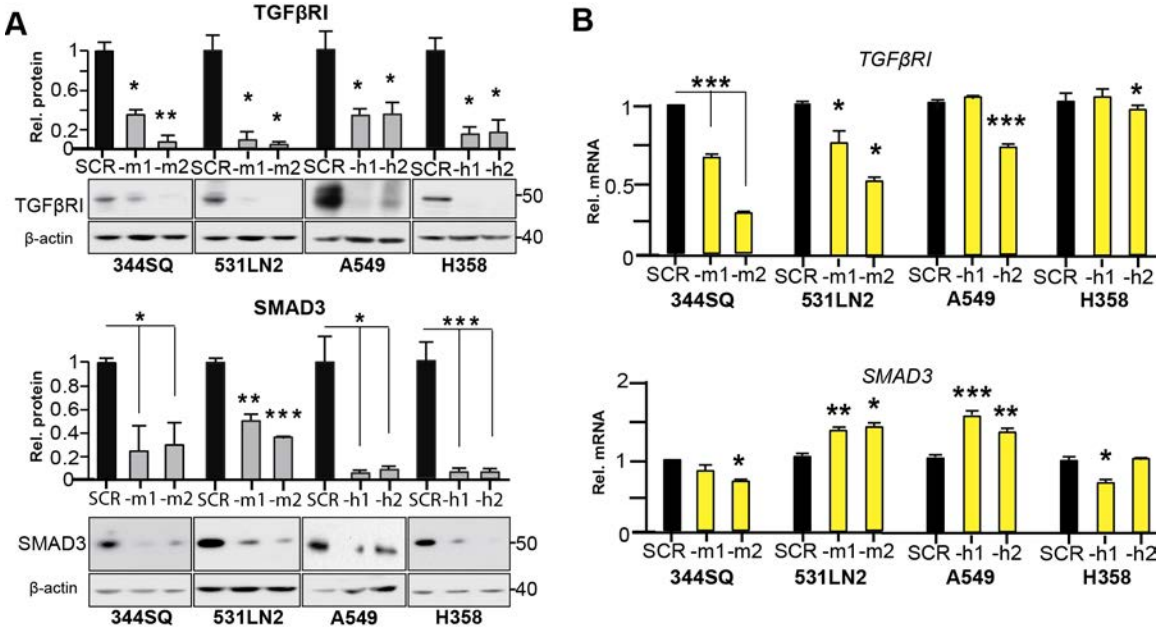

Figure 9. *Msi2* knockdown leads to decreased levels of TGF-βR1 and SMAD3. Western (A) and qRT-PCR analysis of TGF-βR1 and SMAD3 levels in murine and human NSCLC cell lines in the context of MSI2 depletion with independent targeting shRNAs (-m1/-m2, -h1/-h2). SCR, control scrambled shRNA. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

Direct MSI2 translational targets defined in other cell types that might be relevant to the invasiveness of NSCLC cells and tumors include the TGF-β receptor (TGFβR1) and its effector SMAD3 (Park et al., 2014), which promote epithelial-

mesenchymal transition by downregulating E-cadherin (CDH1) and inducing other transcriptional changes (Massague et al., 2012).

We found that stable or transient MSI2 knockdown caused strong downregulation of TGFβR1 and SMAD3, predominantly at the protein level, in all 4 models (Figure 9).

Reciprocally, exogenous overexpression of MSI2 induced TGFβR1 and SMAD3 expression and caused loss of CLDN3, CLDN5 and CLDN7 expression in the 393P cell line with low endogenous MSI2 levels.

Depletion of MSI2 affects the composition of cell-cell junctions and causes partial EMT

Based on the action of MSI2 in supporting the expression of TGFβR1 and SMAD3, while repressing CLDN3, CLDN5, CLDN7, and FN1, we hypothesized

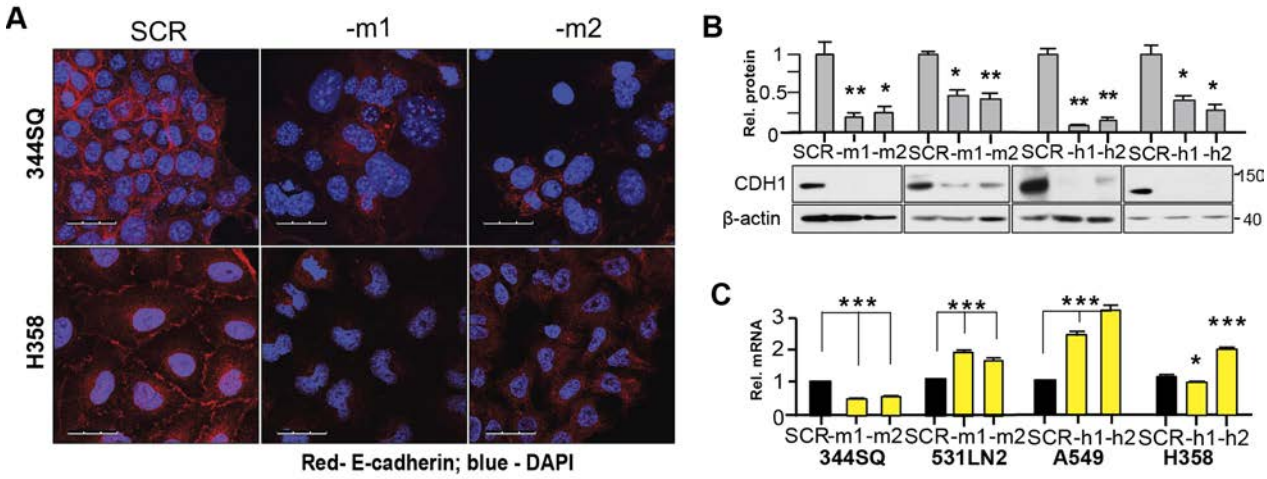

Figure 10. Immunofluorescence analysis of E-Cadherin staining in 344SQ murine NSCLC cell line with or without depleted MSI2 (A). Red - E-Cadherin; Blue – DAPI. Scale bars: 30μm. Western (B) and qRT-PCR (C) analysis of E-Cadherin expression in 4 NSCLC cell line models with or without depleted MSI2. Graphs represent data from three independent runs. All graphs: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ relative to SCR (scrambled shRNA) controls.

that the reduced invasiveness of MSI2-depleted cells might reflect changes involving TJs and reduced EMT, associated with elevated E-cadherin (CDH1).

Unexpectedly, immunofluorescence analysis demonstrated expression of epithelial protein E-cadherin at cell-cell junctions was much reduced by MSI2 depletion, as was total E-cadherin protein expression, while mRNA levels were not consistently affected (Figure 10). In contrast, there was a significant increase in

CLDN3 and CLDN7 staining at cell-cell contact points, while ZO-1, which localizes to the cytoplasmic surface of tight junctions, was unaffected.

We examined expression of additional proteins associated with mesenchymal identity (Bertolini et al., 2009). MSI2 depletion upregulated the pro-EMT factors ZEB1, ZEB2 and FOXC2 but downregulated VIM (vimentin), SLUG and SNAIL. Conversely, MSI2 overexpression induced CDH1, VIM, SNAIL and SLUG (Hassan et al., 2013) (Figure 11).

MSI2 overexpression experiments resulted in opposing effect. Collectively, these data indicated a mixed effect of MSI2 depletion on EMT.

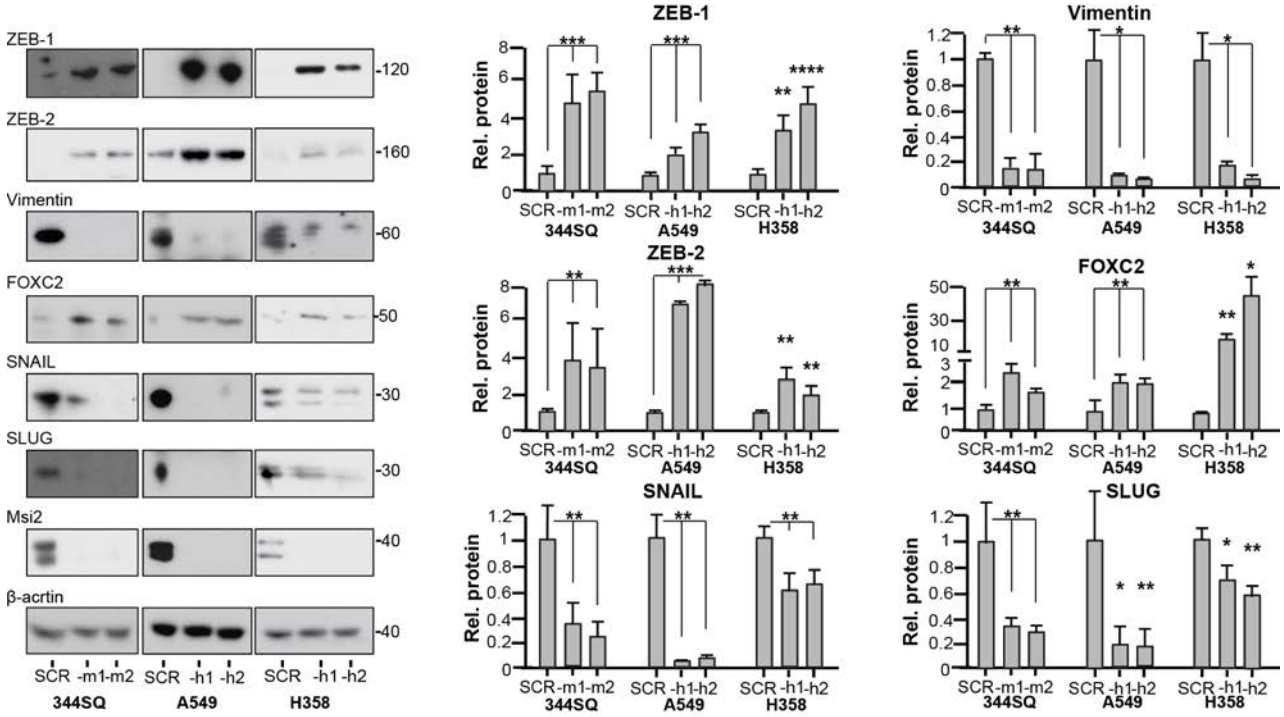

Figure 11. Western blot analysis of EMT markers in MSI2 overexpressing or depleted cells. Western blot analysis (left) and quantification (right) of MSI2, ZEB-1, ZEB-2, FOXC2, SNAIL, SLUG, VMN (vimentin) versus β-actin loading control in 344SQ, A549 and H358 cell lines expressing MSI2 (SCR) or depleted of MSI2 (-m1, -m2, -h1, -h2). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

MSI2 regulation of invasion via TGFβR1 and claudins

To assess the functional interaction between MSI2, its direct targets TGFβR1 and SMAD3, and claudins, we depleted SMAD3 or TGFβR1 in MSI2-depleted versus control cell lines.

SMAD3 knockdown reduced CDH1 expression levels in both parental and MSI2-depleted lines (Fig 12 A). By contrast, the relationship between TGFβR1 and CDH1 expression was modulated by MSI2 status, with the TGFβR1 knockdown

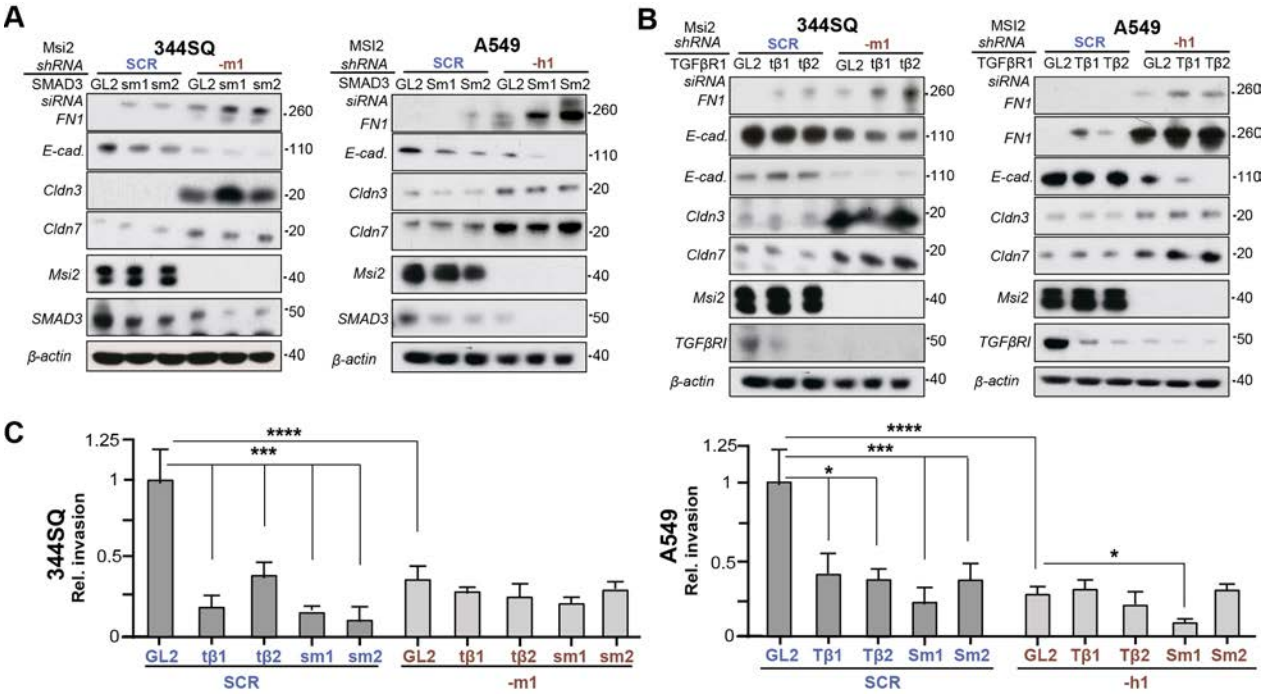

Figure 12. Functional interaction of MSI2 with TGFβR1, SMAD3, FN1, E-Cadherin and Claudins 3, 7. A. Western analysis for expression of indicated proteins in the 344SQ and A549 cell lines with (m1 and h1) or without (SCR) shRNA depletion of MSI2, and with TGFβR1 (-tβ1, - tβ2 and -Tβ1, - Tβ2) or without (GL2) siRNA depletion of TGFβR1. B. Western analysis for expression of indicated proteins in the 344SQ and A549 cell lines with (-m1 and -h1) or without (SCR) shRNA depletion of MSI2, and with (-sm1, -sm2 and -Sm1, - Sm2) or without (GL2) siRNA depletion of SMAD3. C. Quantification of results from three independent Matrigel invasion assays for 344SQ and A549 with (-m1 and -h1) or without (SCR) shRNA depletion of MSI2, in the context of additional siRNA depletion of TGFβR1 (-tβ1, -tβ-2 and -Tβ1, -Tβ2) or SMAD3 (- sm1, -sm2 and -Sm1, -Sm2) versus siRNA negative control (GL2). ***, $p<0.001$ relative to Gl2 siRNA controls.

elevating CDH1 expression in the parental cell lines, but reducing it in MSI2-depleted cell lines (Fig 12 B).

Importantly, depletion of TGFβR1 or SMAD3 caused a statistically significant decrease in invasion in SCR-depleted NSCLC cell lines, but not in those with depleted MSI2 (Fig 12 C). Conversely, overexpression of TGFβR1 partially but incompletely rescued the decrease in invasion seen in MSI2-depleted cells.

The profile of mixed pro- and anti-EMT changes, and incomplete rescue by TGFβR1 overexpression, suggested a possible important role for claudin-associated

TJs in limiting NSCLC invasion induced by MSI2-dependent TGFβR1/SMAD3 signaling. Exploring the relationship between these proteins, we found that siRNA depletion of TGFβR1 or SMAD3 did not significantly affect the expression of CLDN3, CLDN7, or MSI2. This indicated that MSI2 regulates CLDN3/CLDN7 expression independently of TGFβR1 and SMAD3.

Functionally, overexpression of CLDN7 significantly decreased invasion in cells expressing high levels of endogenous MSI2 (Fig 13 A - D), therefore representing important functional role of this protein (and possibly other claudins) in regulation of NSCLC metastasis. Conversely, siRNA depletion of CDLN7 significantly increased invasion in 344SQ cells with depleted MSI2, but had no effect in cells with significant endogenous MSI2 (Fig 13 E – G).

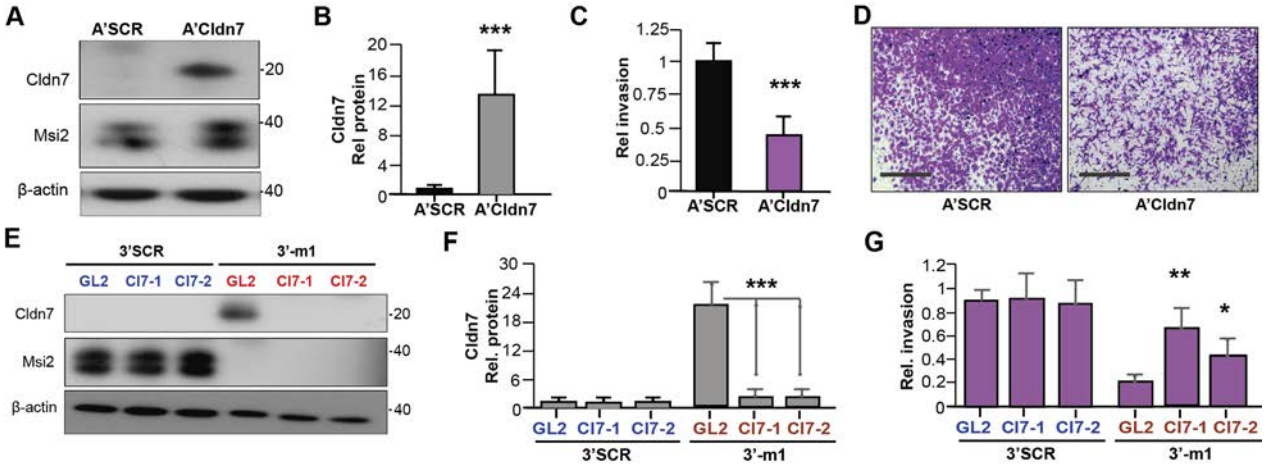

Figure 13. CLDN7 regulation of invasion. A., B. Representative Western blot analysis of indicated proteins (A) and quantification of CLDN7 expression (B) in negative control A'SCR and A'CLDN7 stably transfected A549 cells. C., D. Quantification (C) and representative images (D) of Matrigel invasion for A'SCR and A'CLDN7 stably transfected A549 cells. E., F. Western blot analysis (E) and quantification of CLDN7 expression (F) in 344SQ cells expressing MSI2 (SCR) or stably MSI2-depleted (-m1) transiently transfected with GL2 control or Cl7 siRNAs (Cl7-1, Cl7-2). G. Quantification of Matrigel invasion assay for cells in E, F., based on three experiments. All graphs: *, $p \leq 0.05$; *, $p \leq 0.01$; ***, $p \leq 0.001$ relative to controls.

Taken together, we conclude that MSI2 stimulates invasion in lung cancer in part by sustaining TGFβR1 signaling and suppressing the expression of CLDN7 and potentially other claudins.

RESULT STATEMENTS

1. MSI2 expression is elevated in NSCLC tumor cell lines derived from K-Ras/p53 mice.

2. MSI2 is progressively elevated in lung cancer patient samples at metastasis stage.

3. MSI2 knockdown decreases invasion of NSCLC cells with high metastatic capacity 2 – 4-fold *in vitro* ($p<0.001$) and metastasis rate 5-fold *in vivo* ($p<0.0001$).

4. Mechanistically MSI2 regulates invasion and metastasis of lung adenocarcinoma cells via activation of TGF-β/SMAD signaling and decreased claudins expression.

CONCLUDING REMARKS

In summary, our study for the first time shows that elevation of MSI2 expression progressing NSCLC supports tumor cell invasion and metastasis. We screened for stem cell identity related genes preferentially expressed in a panel of cell lines with high versus low metastatic potential, derived from NSCLC tumors of K-Ras/p53 mice. The MSI2 protein, a regulator of mRNA translation, was consistently elevated in metastasis-competent cell lines. MSI2 was overexpressed in human NSCLC tumor specimens versus normal lung, while higher expression was associated with disease progression. Depletion of MSI2 in multiple independent metastatic murine and human NSCLC cell lines reduced invasion potential of tumor cells *in vitro* and metastatic potential in subsequent *in vivo* experiments in a murine model. We have determined biological role of MSI2 as a driver of invasion and metastasis via regulation EMT-related protein targets. It was confirmed that MSI2 regulates invasion and metastasis of lung adenocarcinoma cells via activation of TGF-β/SMAD signaling and decreased claudins expression.

Further we established that therapeutical resistance in NSCLC might be mediated by numerous processes, such as EMT, changes in DNA reparation systems and global changes in protein and kinase signaling pathways in cancer cells (Gaponova et al., 2016).

Further study of MSI2 function in normal lung development, cellular transformation, and NSCLC drug resistance is clearly warranted, aiming to develop new methods of targeted cancer therapy, including MSI2 as a novel therapeutic target. Furthermore, this protein might be considered as a biochemical marker oa tumor malignancy in the lung. This conclusion is supported by the fact that its expression is elevated in lung adenocarcinoma drives the metastasis.

Ph.D. Thesis related publications list

1. Kudinov A. Musashi-2 (MSI2) supports TGF-β signaling and inhibits claudins to promote non-small cell lung cancer (NSCLC) metastasis / A. Kudinov, **A. Deneka**, A.S. Nikonova, et al. // Proceedings of the National Academy of Sciences. – 2016. – Vol. 113, №25. – P. 6955–6960, authors 0.2 p.p. (VAK list).

2. Gaponova A. V. A novel HSP90 inhibitor-drug conjugate to SN38 is highly effective in small cell lung cancer. / A. Gaponova, A. Nikonova, **A. Deneka**, et al. // Clinical Cancer Research. – 2016. – Vol. 22 (20). – P. 5120 – 5129, authors 0.2 p.p. (VAK list).

3. **Deneka A.** [The Role of translational inhibitor Musashi2 in metastatic lung adenocarcinoma]. / A. Deneka, IG Serebriiskii, ZI Abramova. Article for the VIII Russian conference with international participation «Human Health in XXI Century». Book of articles under prof. S.S. Ksembaev edition. Kazan, Brig, 2016. – 376-380., authors 0.04 p.p.

4. Kudinov A. Musashi-2 (MSI2) drives TGFBR1/SMAD3 dependent partial EMT and supports VEGFR2 expression and metastasis of human and mouse NSCLC cells / A. Kudinov, **A. Deneka**, A. Nikonova et al. // Cancer Research. – 2016. - V. 30. - P. 1584, authors 0.04 p.p.

5. Kudinov A. Musashi-2 (MSI2) activates TGF-β signaling and inhibits CLDN7 to promote non-small cell lung cancer (NSCLC) metastasis / A. Kudinov**, A. Deneka**, A.S., Nikonova et al. // Clinical Cancer Research. – 2015. - Vol. 75 (15 Supplement). - P. 4098, authors 0.04 p.p.

6. Gaponova A. Preclinical testing demonstrates strong activity of STA-12-8666, an HSP90 inhibitor-SN-38 conjugate, in small cell lung cancer (SCLC) / A. Gaponova, **A. Deneka**, A. Nikonova et al. // Cancer Research. – 2015. – Vol. 75 (15 Supplement). – P. 1731, authors 0.04 p.p.

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10.Gaponova A. STA-12-8666, an HSP90 inhibitor-SN-38 conjugate, demonstrates sriking efficacy in small cell lung cancer (SCLC) / A. Gaponova, A. Nikonova, **A. Deneka**, et al. // 20th Annual Postdoctoral and Graduate Student Research Conference: Book of abstracts. Philadelphia, PA. – 2015. – P. 46, authors 0.04 p.p.

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