

1 **Title:** Mesothelin overexpression promotes mesothelioma cell invasion and MMP-9
2 secretion in an orthotopic mouse model and in epithelioid pleural mesothelioma
3 patients
4

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19

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1 **Abstract**

2 **Purpose:** Mesothelin (MSLN) is a tumor-associated antigen, being investigated as a
3 biomarker and therapeutic target in malignant pleural mesothelioma (MPM). The
4 biological function of MSLN overexpression in MPM is unknown. We hypothesized that
5 MSLN may promote tumor invasion in MPM, a tumor characterized primarily by regional
6 aggressiveness and rare distant metastases.

7 **Experimental Design:** Human and murine MPM cells with MSLN forced expression
8 and shRNA knockdown were examined for proliferation, invasion, and matrix
9 metalloproteinase (MMP) secretion. The influence of MSLN overexpression on MPM
10 cell invasion was assessed in an orthotopic mouse model and in patient samples.

11 **Results:** MSLN expression promotes MPM cell invasion and MMP secretion in both
12 human and murine MPM cells. In an orthotopic MPM mouse model characterized by our
13 laboratory, MPM cells with MSLN overexpression preferentially localized to the tumor
14 invading edge, co-localized with MMP-9 expression, and promoted decreased survival
15 without an increase in tumor burden progression. In a tissue microarray from epithelioid
16 MPM patients (n=139, 729 cores), MSLN overexpression correlated with higher MMP-9
17 expression at individual core level. Among stage III MPM patients (n=72), high MSLN
18 expression was observed in 26% of T2 tumors and 51% of T3 tumors.

19 **Conclusions:** Our data provide evidence elucidating a biological role for MSLN as a
20 factor promoting tumor invasion and MMP-9 expression in MSLN-expressing MPM. As
21 regional invasion is the characteristic feature in MSLN-expressing solid cancers (MPM,
22 pancreas, and ovarian), our observations add rationale to studies investigating MSLN
23 as a therapeutic target.

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Translational relevance

Mesothelin (MSLN) is a cancer-associated antigen overexpressed in malignant pleural mesothelioma (MPM) and other regionally invasive malignancies. We hypothesized that MSLN expression promotes MPM cell invasion. In human and murine MPM cells with MSLN overexpression and knockdown, we show that MSLN promotes MPM cell invasion. In an orthotopic MPM mouse model characterized by our laboratory, we demonstrate that MSLN overexpressing MPM cells preferentially localize to the tumor invading edge and decrease survival without an increase in tumor burden progression. Both *in vitro* and *in vivo*, and in MPM patient samples, we demonstrate an association between MSLN overexpression, tumor invasion and matrix metalloproteinases (MMPs), a family of enzymes that facilitate tumor cell invasion and known to be upregulated in MPM. As regional invasion is a characteristic feature in MSLN-expressing solid cancers (MPM, pancreas, and ovarian), these findings add support for studies investigating MSLN as a therapeutic target.

1 **Introduction**

2 Mesothelin (MSLN) is a 40-kDa glycoprotein overexpressed in mesothelioma,
3 pancreatic and ovarian cancers – malignancies characterized by regionally aggressive
4 phenotypes and poor prognosis(1-3). Although MSLN is being investigated as a tumor
5 biomarker and therapeutic target in MPM, the biological role of MSLN remains
6 unexplored(4-9).

7
8 MSLN is highly expressed in epithelioid MPM, the most common MPM subtype
9 constituting 80% of patients. Only 3% of epithelioid MPM patients demonstrated distant
10 metastases in multiple series published by our group(10-12). Despite an absence of
11 distant metastases, a majority of epithelioid MPM patients are not eligible for surgical
12 resection due to advanced T-stage (T3 tumors are unresectable due to local invasion
13 into the endothoracic fascia, mediastinal fat, chest wall or pericardium compared to T2
14 tumors without invasion)(13-15). Matrix metalloproteinases (MMPs), a family of
15 endopeptidases capable of degrading extracellular matrix, have been shown to be
16 elevated in MPM and are known to increase invasive potential in MPM cells(16).
17 Furthermore, asbestos exposure, a causative agent in MPM, is known to upregulate
18 both MSLN and MMP-9 secretion in experimental models of asbestosis(17).

19
20 In this study, we explored the role of MSLN in tumor invasion and its relationship to
21 MMP-9 secretion using human and murine mesothelioma cells both *in vitro* and *in vivo*
22 as well as in clinical specimens from epithelioid MPM patients, known to overexpress
23 MSLN. We demonstrate for the first time that MSLN promotes MMP-9 expression as

1 well as tumor invasion shown by MSLN forced overexpression and confirmed by shRNA
2 knockdown experiments in mesothelioma cells. To further elucidate MSLN biology in an
3 appropriate tumor microenvironment, we developed and characterized an orthotopic
4 MPM mouse model. With this model, we demonstrate that MSLN-expressing MPM
5 cells are invasive, express MMP-9 on the invasive tumor edge, and decrease overall
6 survival independent of tumor cell proliferation or metastasis. Furthermore, our clinical
7 observations from a large cohort of epithelioid MPM patients demonstrate that MSLN
8 expression correlates with MMP-9 expression. The results reported herein provide
9 evidence that MSLN also plays an important role in MPM biology and suggest the MMP
10 pathway as a mediator of invasiveness in MSLN-expressing MPM.

11

12 **Materials and Methods**

13 ***Cell lines and culture***

14 MSTO-211H (human pleural mesothelioma) and AB12 (murine mesothelioma line) were
15 obtained from American Type Culture Collection and CellBank Australia, respectively.
16 MSTO-211H cells were maintained in RPMI-1640 media and AB12 cells in DMEM in a
17 5% CO₂ humidified incubator at 37°C – all media was supplemented with 10% fetal
18 bovine serum(FBS), 100 units/mL penicillin, and 100 ug/mL streptomycin.

19

20 ***Establishment of stably transduced cell lines***

21 Green fluorescent protein-firefly luciferase fusion was cloned into a SFG retroviral
22 vector and transfected into H29 cells with calcium phosphate. MSTO-211H were plated
23 in 24-well plates 24 hours prior to transduction. Filtered virus was added to cells

1 permeablized with 8µg/mL polybrene(Sigma-Aldrich, MO) and reinfected 24 hours later.
2 The human MSLN-variant 1 was isolated from a human ovarian cancer cell line
3 (OVCAR-3). RT-PCR synthesis of full-length cDNA of human MSLN was performed
4 using SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity Kit.
5 Plasmid DNA was isolated, subcloned into a SFG retroviral vector, confirmed by
6 sequencing, and used to stably transduce MSLN. For experiments comparing MSLN-
7 transduced cells to MSLN-negative cells, transduction control was performed with a
8 GFP-Luciferase vector. For all experiments, a stably-transduced population of cells was
9 used with confirmation of unchanged MSLN expression by flow cytometry and western
10 blot analysis.

11

12 ***Mesothelin knockdown with MSLN specific shRNA***

13 To obtain a stable cell line with decreased murine MSLN expression, three predesigned
14 siRNA oligonucleotides and complementary murine MSLN shRNA sequences were
15 obtained(Ambion, TX), ligated into the pSilencer 2.1-U6 hygro plasmid(Ambion, TX),
16 and transfected into the AB12 cell line with calcium phosphate. After 2 week selection
17 with 500µg/ml hygromycin(Invitrogen, CA) the AB12 cell line demonstrating greatest
18 murine MSLN silencing by flow cytometry, qPCR analysis, and western blot was
19 selected for subsequent experiments and is denoted by AB12shRNA. AB12 cells were
20 also transfected with scramble shRNA as a control.

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1 **Flow Cytometry**

2 Fluorescence activated cell sorting(FACS) was performed following retroviral
3 transductions using a FACSAria(BD Biosciences) cytometer to sort for a pool of highly-
4 transduced cells. Human MSLN expression was detected using a PE-conjugated or
5 APC-conjugated anti-human MSLN rat IgG_{2a}(R&D systems, MN). Murine MSLN
6 expression was detected with an anti-mouse MSLN rat IgG_{2a} primary antibody(MBL,
7 Japan). Subsequent flow cytometry for GFP and MSLN expression analysis was
8 performed on either FACSCaliber or LSRII cytometers(BD Biosciences) and analyzed
9 using FlowJo(TreeStar) analysis software.

10

11 **Cell proliferation assays**

12 MSTO-211H cells with or without MSLN expression were plated in 6-well tissue culture
13 plates at a density of 1×10^5 cells/3 mL/well and were counted on days 1, 2, 4, and 6. At
14 each time point cells were counted in triplicate using trypan exclusion after brief
15 trypsinization. Cell number versus time was plotted for each cell line and compared at
16 time points using a Student's T-test.

17

18 **Invasion and migration assays**

19 BD BioCoat™ Matrigel™ Invasion Chamber(BD Biosciences) assays were performed
20 in 24-well plates using polyethylene terephthalate inserts(8μm pores) without matrigel
21 (for migration) and with matrigel coating (for invasion assays). Cells were seeded in the
22 upper well of the chamber (5×10^4 - 1×10^5) in 0.5 mL RPMI-1640 without FBS and 0.75
23 mL RPMI-1640 with 10% FBS in the bottom chamber. Migration was assessed at 12

1 hours and invasion at 24 hours for MSTO-211H. For AB12 cells, migration was
2 assessed at 6 hours and invasion at 20 hours. Nonmigrating or noninvading cells were
3 removed from the upper membrane surface of the insert using a cotton swab. The
4 insert membrane was fixed and stained using the Diff-Quik™ staining system and
5 mounted on glass slides. Stained cells were counted in 10 high-power fields in
6 predetermined areas of the membrane.

7 8 ***Orthotopic pleural mesothelioma animal model***

9 To develop the orthotopic mouse model of pleural mesothelioma, female SCID/beige or
10 BALB/c mice(Taconic, NY) at 6-10 weeks of age were utilized. All procedures were
11 performed under approved Institutional Animal Care and Use Committee protocols.
12 Mice were anesthetized using inhaled isoflurane and oxygen. Direct intrapleural
13 injection of 1×10^5 tumor cells in 200 μ L serum-free media via a right thoracic incision
14 was performed to establish orthotopic MPM tumors as previously described(18-21). For
15 experiments to establish heterogeneously MSLN expressing tumors, mice were
16 inoculated by direct pleural injection with a mixture of 1×10^5 MSTO-211H expressing
17 and deficient in MSLN.

18 19 ***Histology and immunostaining***

20 Histopathological evaluation of tumors was performed following hematoxylin and eosin
21 staining of paraffin-embedded, 4% paraformaldehyde fixed tissue samples. For
22 angiogenesis, CD34 rat monoclonal antibody (5ug/ml, eBioscience) was incubated for 7
23 hours, followed by 16 minutes with (1:200) biotinylated rabbit anti-rat IgG (Vector Labs,

1 Cat. #BA-4000). Rat IgG_{2a} (5ug/ml) was used as an appropriate isotype negative
2 control. For lymphangiogenesis, goat polyclonal LYVE-1 antibody (1µg/ml; R&D
3 Systems) was incubated for 3 hours, followed by 60 minutes with biotinylated rabbit
4 anti-goat IgG (ABC kit from Vector labs). The protocols for immunofluorescence
5 detection using Tyramide-Alexa Fluor 488(Invitrogen) or Tyramide-Alexa Fluor
6 568(Invitrogen) for CD34 and LYVE-1, respectively, were established and performed at
7 the MSKCC Molecular Cytology Core Facility using a Discovery XT automatic processor
8 (Ventana Medical Systems). Immunohistochemistry for human MSLN was performed
9 with a mouse anti-human MSLN IgG(1:100, Vector Labs, CA) using the Ventana
10 platform. Immunohistochemistry for MMP-9 was performed by Premier
11 Laboratories(Boulder, CO) using polyclonal rabbit anti-human MMP-9(Dako, CA).
12 Immunohistochemistry for MMP-7 was performed utilizing a mouse IgG_{2b} anti-human
13 MMP-7 clone ID2 (abcam, Cambridge, MA) in a 1:100 dilution. Placenta with
14 cytoplasmic staining in the villi was used as the MMP-7 positive control.

15

16 ***Quantitative bioluminescence imaging***

17 *In vitro* standardization was performed using GFP-Firefly Luciferase expressing MSTO-
18 211H cells with and without MSLN expression. Serially diluted cells were plated in 96-
19 well tissue culture plates (1.6x10⁶ to 2.5x10⁴ cells/100µL/well). Twenty minutes after
20 the addition of 100µL D-Luciferin (15mg/mL; Caliper Lifesciences, MA), plates were
21 imaged using the Xenogen IVIS 100 Imaging System(Caliper Lifesciences). Cell
22 number versus total BLI flux(photon/s) was evaluated by Pearson's correlation.

1 *In vivo* BLI in tumor-bearing mice was performed using a single intraperitoneal dose of
2 150mg/kg D-Luciferin. Mice were imaged with the Xenogen IVIS 100 Imaging System
3 20 minutes following D-Luciferin injection. Images were acquired for 5-30 seconds
4 depending on signal strength. BLI data were analyzed using Living Image 2.60
5 software and BLI signal reported as total flux(photons/s).

6

7 ***Magnetic resonance imaging***

8 Magnetic resonance imaging(MRI) was performed in a Bruker 4.7T USR
9 scanner(Bruker Biospin Inc., Ettlingen, Germany) equipped with a 400 mT/m gradient
10 coil and a 32 mm ID custom build birdcage resonator. Thoracic axial MRI images were
11 acquired using a RARE fast spin-echo sequence (repetition time(TR)=1.7s, echo
12 time(TE)=40 ms, and 12 averages). The slice thickness was 0.7 mm and the in-plane
13 image resolution was 117 x 156 mm. Image acquisition was triggered by animal
14 respiration and tumor volumes (mm³) were calculated from tracing tumor boundaries in
15 each slice using Bruker ParaVision Xtip software(Bruker Biospin Inc., Ettlingen,
16 Germany).

17

18 ***Gene expression analysis***

19 Gene expression profiles were compared between MSLN-expressing and non-
20 expressing MSTO-211H cells. Cell pellets were collected in triplicate and snap frozen
21 from 3 separate plates for each cell line. The Genomics Core Laboratory at MSKCC
22 collected, processed, and hybridized RNA samples to Illumina Humanref-6 BeadChips.
23 Using Bioconductor package LIMMA, sample probesets were normalized to control

1 probesets to minimize inter-beadchip variability. Individual gene expression was
2 screened for significance using a false discovery rate (FDR) threshold of <0.05 and a
3 fold change threshold of 1.5. Gene set enrichment analysis (GSEA)(22) was performed
4 to determine biological pathways related to MSLN overexpression.

6 ***Matrix metalloproteinase assays***

7 MMP secretion by tumor cells *in vitro* was quantified by multiplex bead assays
8 (Millipore, MA) for MMP-1, MMP-2, MMP-7, and MMP-9 on a Luminex 100 xMAP with
9 internal quality control standards for each analyte. 1×10^5 cells in 1mL media were
10 plated in 24-well tissue culture plates and allowed to grow for 24 hours, media was then
11 removed, washed with PBS, and media replaced with 0.5mL RPMI-1640 without FBS.
12 Cell supernatant MMP-9 secretion was quantified by multiplex bead assay for MMP-9
13 (Millipore, MA). Supernatant was collected 12, 24, and 36 hours after media exchange
14 and stored at -80°C until analyzed.

16 ***Epithelioid MPM tissue microarray and immunohistochemistry***

17 Patients diagnosed with epithelioid MPM between 1989 and 2009 at Memorial Sloan-
18 Kettering Cancer Center were included. For each of the 139 patients with available
19 specimens, all H&E slides (median 9, range 1-43) were reviewed. Representative
20 blocks were selected to construct a tissue microarray (TMA) by taking nine
21 representative cores(0.6 mm) from each patient tumor block and ensuring at least six
22 complete tumor cores. Five- μ m sections were cut from the TMA and stained by specific
23 antibodies (MSLN:Vector, 1:200 dilution; MMP-9:Oncogene Science, 1:200 dilution).

1 Grading of MSLN and MMP-9 intensity was performed on separate occasions by a
2 pathologist who was blinded to the clinical data as follows: 0(absent stain), 1(weak
3 expression), 2(moderate expression), and 3(strong expression). The distribution of
4 MSLN-positive tumor cells from all tumor cells found in a single core was graded as
5 0(absent), 1(1-50%), and 2(51-100%). The sum of the MSLN stain intensity and
6 distribution grades was used to determine a total MSLN score ranging from 0 to 5 at the
7 core level. MSLN score for each patient was then determined using the average of all
8 tumor cores.

9
10 We examined the correlation between MMP-9 and MSLN expression using Mantel-
11 Haenszel correlation statistic. The p-values were adjusted through bootstrap to account
12 for the fact that each of the 139 patients can contribute with more than one core to the
13 analysis, leading to a total of 721 cores. The correlation between increasing MSLN
14 expression and T-stage was determined using the Fisher's exact test.

15 16 **Results**

17 ***Mesothelin expression promotes invasion and migration in vitro independent of*** 18 ***tumor cell proliferation***

19 We initially investigated the effect of MSLN overexpression on MPM by comparing
20 MSTO-211H cells without MSLN expression to those stably transduced to overexpress
21 MSLN(Fig. 1A) and AB12 cells with natural MSLN expression or knockdown(Fig. 1B).
22 We noted no morphological differences in cultured cells overexpressing MSLN and cell-
23 counting assays demonstrated no effect of MSLN expression on MSTO-211H

1 proliferation (Fig. 1C). Similarly, we observed no differences in morphology or log-phase
2 growth rates between AB12 cells transfected with control scramble shRNA or MSLN-
3 specific shRNA (data not shown). In order to examine if MSLN expression promoted
4 changes in MSTO-211H proliferation during periods of cellular stress, experiments were
5 repeated with serum-starved(2% FCS) media; no differences were observed (data not
6 shown). Since local invasion is the characteristic clinical feature of mesothelioma, we
7 evaluated *in vitro* cell migration and invasion using a standard Boyden chamber assay.
8 MSLN overexpression in MSTO-211H significantly increased cell migration(2.56-fold,
9 $p<0.001$) and invasion(1.54-fold, $p<0.001$) compared to non-MSLN expressing MPM
10 cells (Fig. 1D & 1E (left panel)). To confirm our observations of MSLN influence on
11 invasion, we next examined the effect of MSLN-knockdown in the AB12 cell line that
12 natively expresses MSLN(Fig. 1B). Decreased MSLN expression resulted in decreased
13 migration(2.14-fold, $p<0.001$) and invasion (2.35-fold, $p<0.0001$)(Fig. 1E (right panel)).
14 These results demonstrate that MSLN overexpression promotes invasion in MPM cells.

15

16 ***Development and characterization of a clinically relevant orthotopic mouse model*** 17 ***of MPM to investigate mesothelin biology***

18 Having noticed that MSLN overexpression promotes MPM invasion and migration *in*
19 *vitro*, we next sought to investigate the biology of MSLN within an appropriate tumor
20 microenvironment. In order to evaluate local pleural and chest wall invasion, an
21 important clinical factor in the progression of MPM, which cannot be fully simulated in
22 subcutaneous or intraperitoneal mouse models, we developed an orthotopic mouse
23 model by directly inoculating MPM cells into the pleural space(18, 20, 23, 24).

1 Resultant tumors mimicked human MPM as demonstrated by: (a) gross pleural and
2 mediastinal tumor distribution seen on necropsy and magnetic resonance imaging (MRI)
3 (Fig. 2A); (b) histology demonstrating tumors growing along visceral and parietal pleural
4 surfaces, frequent chest wall and diaphragmatic invasion, and rare lung invasion (Fig.
5 2B); (c) frequent metastases to mediastinal lymph nodes confirmed by histology (Fig.
6 2C) without distant metastases; (d) retention of characteristic MPM tumor markers
7 including WT-1, Calretinin, and MSLN even at late stages of disease while TTF-1, a
8 marker of lung adenocarcinoma, remained negative (Fig. 2D); and (e) extensive neo-
9 lymphangiogenesis throughout the MPM tumor specimens by CD34 and LYVE-1
10 immunofluorescence for angio- and lymphangiogenesis, respectively (Fig. 2E) (MPM is
11 known to be a well vascularized tumor with high levels of VEGF secretion (25, 26)).
12 These findings not only confirm that our orthotopic pleural mesothelioma mouse model
13 resembles human MPM, but also provides an appropriate *in vivo* tumor
14 microenvironment to investigate MPM tumor cell invasion.

15

16 ***Quantitative bioimaging monitors the effect of mesothelin on tumor progression***
17 ***in vivo***

18 Using our orthotopic MPM mouse model, we first sought to evaluate whether MSLN
19 imparts a growth advantage *in vivo*. Unlike subcutaneous flank tumors, which are easily
20 accessible for serial monitoring, evaluating the effect of MSLN expression on pleural
21 tumors required an accurate noninvasive method to serially evaluate tumor burden and
22 progression. To do so, we validated bioluminescent imaging (BLI) as a quantitative
23 modality to assess tumors in the pleural model. First we performed an *in vitro* BLI

1 standardization with MSLN-expressing and non-expressing MPM cells stably
2 transduced express a GFP-firefly luciferase fusion gene (Fig. 3A). Our results
3 demonstrated that BLI signal correlated with number of cells for both MSLN-negative
4 (Pearson $r = 0.99$, $p < 0.0001$) and positive (Pearson $r = 0.99$, $p < 0.0001$) cell lines (Fig.
5 3B). We next validated BLI as an accurate method to monitor tumor progression within
6 the orthotopic MPM mouse model by serially comparing BLI signal to MRI tumor volume
7 averaging, the gold standard for tumor volume assessment (27). BLI signal from the
8 engrafted pleural tumors correlated with MRI tumor volume over a wide range of tumor
9 burdens ($r = 0.86$, $p < 0.0001$; Fig. 3C and D). Thus, this orthotopic MPM mouse model
10 allows for quantifying tumor burden and monitoring tumor progression in the pleural
11 tumor microenvironment (28).

12

13 ***Mesothelin expression promotes tumor invasion in vivo and decreases survival***
14 ***without affecting tumor proliferation***

15 Mice inoculated with equivalent intrapleural MSLN-expressing and non-expressing MPM
16 cells were compared for *in vivo* tumor progression. Serial BLI was used to monitor
17 tumors in mice and revealed no differences in tumor burden between tumors with or
18 without MSLN expression at any point in disease progression (Fig. 4A). Despite
19 equivalent tumor progression rates and tumor burden, mice with MSLN-expressing
20 MPM experienced significantly decreased survival compared to mice with MSLN-
21 negative tumors (29 vs. 37 days, $p = 0.001$; Fig. 4B). These results of decreased survival
22 with MSLN expression were reproduced in our syngeneic orthotopic MPM model using
23 BALB/c mice with murine AB12 MPM cells. Mice inoculated with AB12 wild type

1 (MSLN-expressing) tumors had a median survival of 30 days compared to AB12 shRNA
2 (MSLN-knockdown), which survived the 60-day study period ($p < 0.001$). We further
3 evaluated AB12 survival by transducing MSLN-expressing AB12 cells with mouse
4 MSLN vector to express MSLN at even higher levels (AB12 M). We observed
5 decreased survival of mice inoculated with these high MSLN expressing AB12 cells
6 compared to AB12 wild-type cells (24 vs 30 days, $p = 0.005$; Supplemental Figure 1).

7
8 Systematic histologic evaluation of necropsy specimens demonstrated MSLN-
9 expressing tumors to have qualitatively increased local tumor invasion of chest wall,
10 diaphragm, and mediastinal structures as compared to tumors without MSLN. To
11 further investigate the association between MSLN expression and local tumor invasion
12 *in vivo*, we evaluated mice with orthotopic MPM tumors heterogeneously expressing
13 MSLN. Heterogeneously expressing tumors were established by inoculating mice with
14 a mixture of MSLN-expressing and MSLN-deficient MSTO-211H cells. These tumors
15 uniformly demonstrated clustering of MSLN expression at the advancing (invading)
16 edge as determined by MSLN IHC (Fig. 4C and D upper panel).

17
18 ***Mesothelin expression promotes MMP secretion, which co-localizes at the***
19 ***invading tumor edge***

20 Having observed MSLN expression at the invasive edge of MPM tumors, we next
21 investigated if pathways known to cause tumor invasion were upregulated in MPM
22 cells overexpressing MSLN. Gene set enrichment analysis (GSEA)(22) of gene array
23 data comparing human MPM cells (MSTO-211H) with or without MSLN expression

1 demonstrated that MSLN expression increased the expression of genes known to
2 regulate MMP secretion (“SA-MMP-cytokine connection”; hypergeometric $p < 0.001$)
3 (Supplemental Table 1). To investigate these findings, we evaluated MMP secretion in
4 cultured human MSTO-211H and murine AB-12 MPM cells. Using multiplex bead
5 assays for human and murine MMPs, we found that MSLN-overexpressing MSTO-211H
6 cells produced significantly increased MMP-1, 2, 7, and 9 compared to MSLN-deficient
7 MPM cells ($p < 0.01$, Fig. 5A). To confirm that MSLN expression has a direct relationship
8 to MMP secretion, we examined the effect of MSLN knockdown by shRNA using the
9 MSLN-expressing murine MPM cell line AB12; decreased MSLN expression was
10 confirmed by flow cytometry (Fig. 1B) and qPCR (relative-fold MSLN expression $0.483 \pm$
11 0.29). We found that cell supernatant secretion of MMP-9 was significantly decreased
12 following MSLN knockdown in the AB12 cell line ($p < 0.0001$, Fig. 5B).

13
14 We then evaluated expression of MMP-9 in MPM tumors *in vivo*. We performed IHC for
15 MMP-9 on heterogeneously MSLN-expressing tumors and found increased intensity of
16 MMP-9 staining at the invasive edge co-localizing with MSLN staining compared to the
17 baseline level of MMP-9 staining of non-MSLN expressing cells (Fig. 4D lower panel).

18 These results suggest an association between MMP-9 and MSLN expression *in vivo*.

19

20 ***Mesothelin is overexpressed in 90% of epithelioid MPM patients***

21 As MSLN IHC is not routinely performed in MPM patients, the only MSLN expression
22 data available to date is from small case series (33–49 patients) comprised of varying
23 histological subtypes(29, 30). Therefore, we first evaluated MSLN expression in a large

1 uniform cohort of epithelioid MPM patients (n=139). MSLN is overexpressed in 90% of
2 epithelioid MPM patients (score 1-5), with strong expression present in 29% of
3 epithelioid MPM patients (score 4-5) (Fig. 6A).

4
5 ***Mesothelin overexpression is associated with high MMP-9 expression in human***
6 ***epithelioid MPM specimens***

7 To determine the relationship between MSLN and MMP-9 expression in human tumor
8 samples, a tissue microarray (6 tumor cores/patient) derived from 139 surgically
9 resected epithelioid MPM tumors(Supplemental Table 2) was examined by IHC(Fig 6A).
10 We detected a strong correlation between MMP-9 expression and MSLN intensity at
11 individual core level – observing increased levels of MMP-9 expression with increasing
12 MSLN expression(Fig. 6B, $p<0.001$). These clinical findings support our preclinical
13 observations associating MSLN expression with increased MMP levels.

14
15 ***Mesothelin intensity and distribution in epithelioid MPM patients***

16 We examined MSLN expression score (sum of intensity and distribution) in stage III
17 epithelioid MPM patients. To avoid confounding effects due to heterogeneous clinical
18 stages and neo-adjuvant therapy, we selected a uniform cohort of 72 stage III patients
19 with no prior therapy (T1=1 (excluded), T2=17 and T3=54 patients). In this cohort, a
20 high MSLN score (4-5) is observed in 26% vs. 51% for T2 and T3 patients, respectively
21 (Fig. 6C, $p=0.05$). These data are notable since increasing T-stage represents an
22 important clinical distinction of tumor invasion.

23

1 **Discussion**

2 Tumors that are known to express MSLN including MPM, pancreatic, and serous
3 ovarian cancers are characterized by locoregional aggressiveness(31). Local tumor
4 invasion is the primary cause of morbidity in patients with MPM and the factor most
5 often preventing tumor resectability(11, 14). The majority of studies in MPM and other
6 regionally aggressive malignancies have focused on MSLN as a tumor biomarker(8, 9)
7 and therapeutic target, while none have evaluated the influence of MSLN on tumor
8 biology. Our study provides evidence demonstrating for the first time a correlation
9 between MSLN and MMP expression as well as regional tumor aggressiveness and
10 invasion in MPM cells, in an orthotopic MPM model, and in epithelioid MPM patients

11
12 It is well documented that MMP-mediated degradation of extracellular matrix proteins
13 facilitates cancer cell migration and invasion(32, 33). MMPs have been implicated in
14 the tumor progression of several cancers including breast(34, 35), pancreatic(36),
15 lung(37), ovarian(38), and mesothelioma (39, 40). In a recent publication, Chang et al.
16 demonstrate an associated between MMP-7 and MSLN expression in ovarian cancer
17 using murine cell lines and human tumor specimens(41). In this study, we have shown
18 that MSLN overexpression increases tumor cell MMP secretion, whereas shRNA
19 knockdown of MSLN decreases MMP secretion in mesothelioma(Fig. 2A and B; Fig. 3).
20 These data provide evidence that MSLN promotes MMP secretion contributing to tumor
21 aggressiveness. A recent report further highlights the role of MMP-9-mediated invasion
22 in MPM and the beneficial therapeutic efficacy of suppressing MMP-9 expression(42).
23 In addition to MMP-9, we have observed increased *in vitro* secretion of MMP 1, 2, and 7

1 with MSLN overexpression – consistent with published observations that in promoting
2 cancer cell invasion MMPs can influence each other directly(41, 43). In an orthotopic
3 MPM mouse model, we showed that MSLN and MMP-9 co-localize along the invasive
4 edge of pleural tumor nodules. Although we report for the first time that MSLN is
5 expressed along the invading tumor edge in MPM, other investigators have reported
6 similar observations in rats following exposure to multi-wall carbon nanotubes, which
7 are hypothesized to cause MPM strengthening our observation(44). Furthermore, in
8 colorectal cancer, >50% of patient specimens demonstrated MSLN expression along
9 the invading edge of tumors(45). These data correlate with clinical MPM tumor
10 specimens where we have demonstrated an association between MSLN and MMP-9
11 expression. It is notable that, in contrast to data reported for ovarian cancer (41), we
12 found no correlation in human MPM tumor specimens between MMP-7 and either
13 MSLN expression or tumor stage (data not shown). Our data suggest that while
14 secretion of multiple MMPs is increased in association with MSLN *in vitro*, MMP-9 plays
15 a predominant role in the *in vivo* human MPM tumor. The results reported herein
16 highlight MMP-9 as a potential pathway for MSLN-mediated MPM invasiveness.
17
18 MSLN is a glycoposphoinositol (GPI)-anchored cell-surface protein, for which signal
19 transduction depends upon associating with accessory proteins. GPI-anchored proteins
20 such as T-cadherin can transduce signals that modulate cell migration and invasion by
21 mechanisms mediated through direct interactions with integrin-linked kinase(46) and
22 integrin β 3(47). Data from our gene array studies show an association between integrin
23 β 4 and MSLN overexpression in MPM (Supplemental Table 1). We have confirmed the

1 expression of integrin β 4 in MSLN overexpressing MPM cells by flow cytometry
2 (Supplemental Figure 2). Integrin β 4 is expressed by epithelioid MPM (48), and
3 published literature implicates integrin β 4 expression with tumor cell migration, invasion,
4 and MMP expression(49). Further studies to better define the association between
5 MSLN and integrin β 4 may ultimately help elucidate this pathway's relationship to MPM
6 tumor cell migration, invasion, and MMP expression.

7
8 Review of the literature reveals other putative mechanisms for MSLN's influence on
9 tumor phenotype besides the association with MMP expression that we have
10 demonstrated. Functionally, investigators have shown high-affinity binding between
11 MSLN and CA-125 present on mesothelial cells(50) – an interaction thought to
12 contribute to peritoneal migration of ovarian cancer. Uehara and colleagues (51) have
13 found MSLN overexpression to promote anchorage-independent growth and resistance
14 to anoikis in association with suppression of pro-apoptotic Bim. Chang *et al*(52) have
15 implicated the PI3K pathway in MSLN-associated anti-apoptosis. Perturbations in
16 Wnt1/ β -catenin or Ras pathways lead to the expression of MSLN in mammary(53) and
17 pancreatic adenocarcinomas(54). Overexpression of Wnt1 in colorectal cancer cells,
18 which is also often constitutively activated in mesothelioma, ovarian, and pancreatic
19 cancers, was associated with MSLN expression(45). However, these mechanisms
20 neither explain nor provide a pathway for the specific observation of MSLN-associated
21 local tumor invasion, a clinically important characteristic of MSLN-expressing cancers.

22

1 Tumor invasion has typically been studied using the Boyden chamber and scratch
2 assays *in vitro*. We have established a novel orthotopic MPM mouse model wherein
3 tumor invasion into diaphragm, mediastinal fat, and pericardium mimic human MPM and
4 have demonstrated that MSLN overexpressing cells localized to the invading edge.
5 Furthermore, to demonstrate that MSLN is indeed associated with invasion, we have
6 utilized a uniform cohort of stage III epithelioid MPM patients differing by T-stage and
7 confirmed our preclinical observations of MSLN and MMP-9 expression at tumor core
8 level. In the experience of our group and others, MPM local invasion and regional
9 aggressiveness is a major factor preventing surgical resection with curative intent(14,
10 55); 40% of patients are deemed unresectable on the operating table in spite of
11 extensive pre-operative imaging studies. Based on our observations, we are currently
12 investigating the role of MSLN level in both tumor tissue and patient serum in order to
13 predict resectability for patients with a low clinical 'T' stage based. In addition, recent
14 cancer vaccine clinical trials in patients with pancreatic cancer have demonstrated the
15 beneficial effects of MSLN-specific immune responses in prolonging survival(56, 57) – a
16 feature currently being explored for the development of MSLN-targeted
17 immunotherapies by our group and others(58-60). This study provides further rationale
18 for investigating MSLN-targeted therapies in MPM, as MSLN provides a molecular
19 target that is commonly expressed in human epithelioid MPM and promotes tumor
20 invasion.

21
22 In summary, this study is the first to demonstrate an association between MSLN
23 expression and tumor MMP-9 secretion in MPM. We also illustrate the association

1 between MSLN expression and local tumor invasion in MPM. These results are
2 consistent with the fact that MSLN-expressing solid tumors such as MPM, pancreatic
3 cancer and serous ovarian carcinoma uniformly display regionally aggressive features
4 and dismal outcomes. We have demonstrated our findings not only *in vitro*, but also *in*
5 *vivo*. The strength of these observations is highlighted by the use of a clinically relevant
6 orthotopic mouse model, which accurately recapitulates human disease and provides
7 an appropriate pleural tumor microenvironment to investigate tumor cell invasion and
8 MSLN biology. Our observations are reproduced with both human and murine MSLN
9 cells and have been verified in samples from 139 human epithelioid MPM tumors. This
10 study provides a better understanding of the locoregional aggressiveness of MSLN-
11 expressing tumors, demonstrates MSLN's impact on tumor MMP, and provides
12 rationale for further clinical study of MSLN as a potential therapeutic target.

13

14

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19
20

1 **Figure Legends**

2 **Figure 1.** MSLN expression promotes invasion and migration *in vitro* without affecting
3 proliferation. **(A)** Flow cytometry histogram showing MSTO-211H mesothelioma cells
4 transduced with human MSLN by use of retroviral vector to stably overexpress MSLN
5 (dark shading) compared to isotype control (light shading). **(B)** Flow cytometric analysis
6 of murine MSLN expression in AB12 (dark shading), AB12shRNA (dotted line), and
7 isotype control (light shading). **(C)** Cell counting assay demonstrates equivalent cell
8 proliferation *in vitro* comparing MSTO-211H MSLN-positive and MSLN- negative MPM
9 cells. **(D)** Cellular migration and invasion of MSTO-211H was determined by plating
10 equivalent cell number of MSLN-positive or negative MPM cells in standardized
11 transwell Boyden chambers. **(E)** MSLN expression significantly increased both cell
12 migration and invasion *in vitro* ($p<0.001$) in MSTO-211H (left panel). Migration and
13 invasion of AB12 cells (right panel) with naturally expressing MSLN (AB12) and MSLN-
14 knockdown (AB12shRNA) demonstrates a correlation between increased MSLN-
15 expression and increased migration and invasion ($p<0.001$).

16
17 **Figure 2.** Characterization of a clinically relevant orthotopic mouse model of MPM for
18 investigating MSLN biology. **(A)** MPM tumor in the orthotopic mouse model resembles
19 human disease grossly (top panel) and by MRI (lower panel) as tumor growing along
20 pleural surface and encasing mediastinal structures. In this mouse model: **(B)**
21 Histologically, tumor is distributed along visceral pleural surfaces (left panel,
22 arrowhead), the interlobar fissures (right panel, arrowhead), and parietal pleural
23 surfaces with frequent chest wall invasion (bottom panel, arrow). **(C)** MPM metastases

1 to mediastinal lymph nodes (circle) confirmed by H&E (top right) and MSLN IHC
2 staining (bottom right). **(D)** Engrafted tumors maintain characteristic IHC tumor markers
3 for MPM even in advanced stages (TTF-1⁻; WT-1⁺, Calretinin⁺, Mesothelin⁺). **(E)**
4 Immunofluorescence (LYVE-1 (red) – lymphatics; CD34 (green)– vascular endothelium)
5 demonstrates lymphangiogenesis throughout pleural tumor nodules (arrow shows chest
6 wall, arrowhead tumor nodule) with intratumoral vessels (inset).

7
8 **Figure 3.** Validation of bioluminescence imaging (BLI) in the mouse model to
9 quantitatively monitor MSLN effect on tumor burden and progression. **(A)** Flow
10 cytometry dot plots showing human MPM cells (MSTO-211H) stably transduced to
11 express GFP-Luciferase fusion gene (x-axis) in both MSLN-negative (left plot) and
12 positive (right plot) cells. **(B)** *In vitro* validation of BLI shows that BLI signal correlates
13 with cell number over a wide range for both MSLN-negative ($r=0.99$, $p<0.0001$) and
14 MSLN-positive ($r=0.99$, $p<0.0001$) cells. **(C)** *In vivo* BLI validation by correlating BLI
15 signal (left column) to tumor volume by MRI volume averaging (right column) in mice
16 with orthotopic MPM tumors. **(D)** BLI signal correlated strongly with tumor volume over
17 a wide range of tumor progression in mice with pleural mesothelioma with and without
18 MSLN expression.

19
20 **Figure 4.** Mesothelin expression promotes tumor invasion *in vivo* and decreases
21 survival without affecting tumor proliferation. **(A)** BLI monitors tumor proliferation in mice
22 with orthotopic MPM and reveals no difference in tumor progression between MSLN-
23 positive (n=12, red line) and negative (n=12, blue line) tumors. **(B)** Kaplan-Meier

1 survival analysis demonstrated mice with MSLN-positive tumors (n=12, red line) have
2 decreased survival compared to MSLN-negative (n=12, blue line) tumors (29 vs. 37
3 days, $p=0.001$). Results confirmed on multiple repeat experiments. (C) Heterogeneous
4 MSLN-expressing MPM tumors in mice show increased MSLN staining at the advancing
5 tumor edge in tumor nodules on both diaphragm (upper image asterisk) and chest wall
6 (lower image arrowhead) (D) Mice engrafted with tumors of heterogeneous MSLN
7 expression (arrowhead shows area of absent MSLN expression, asterisk shows area of
8 heterogeneous MSLN expression) demonstrate consistent MSLN positivity at areas of
9 local tumor invasion (upper image arrow). MMP-9 IHC shows increased MMP-9
10 expression at the leading invasive edge (lower image arrow) compared to baseline
11 MMP-9 expression in the areas of tumor with low levels of MSLN expression (lower
12 image asterisk).

13

14 **Figure 5.** MSLN expression upregulates tumor cell MMP secretion. (A) Human MPM
15 cells (MSTO-211H) with and without MSLN expression were compared *in vitro* for MMP
16 secretion by multiplex bead array assay of cell culture supernatant. MMP-1, 2, 7, and 9
17 were all significantly increased with MSLN expression. (B) MSLN knockdown
18 correlated with decreased tumor cell secretion of MMP-9 as determined by multiplex
19 bead assay (* $p<0.01$) in AB12 MPM cells.

20

21 **Figure 6.** Tissue Microarray (TMA) constructed from 139 epithelioid MPM tumors
22 reveals an association between MSLN expression and MMP-9 overexpression. (A)
23 Representative TMA cores depicting strong or low MSLN and MMP-9 expression (bar =

1 50 μ m). **(B)** MMP-9 expression increases as the proportion of tumor cores showing
2 strong (black) and moderate (grey) expression of MSLN increases as compared to
3 weak (hatched) or absent (white) MSLN expression ($p < 0.001$). **(C)** In patients with
4 invasion into the endothoracic fascia, mediastinal fat, chest wall or pericardium (T3-
5 tumors) there is a trend towards increased strong MSLN expression (black; score 4-5)
6 compared to absent-moderate expression (grey; score 0-3) when compared to T-2
7 tumors ($p=0.05$).

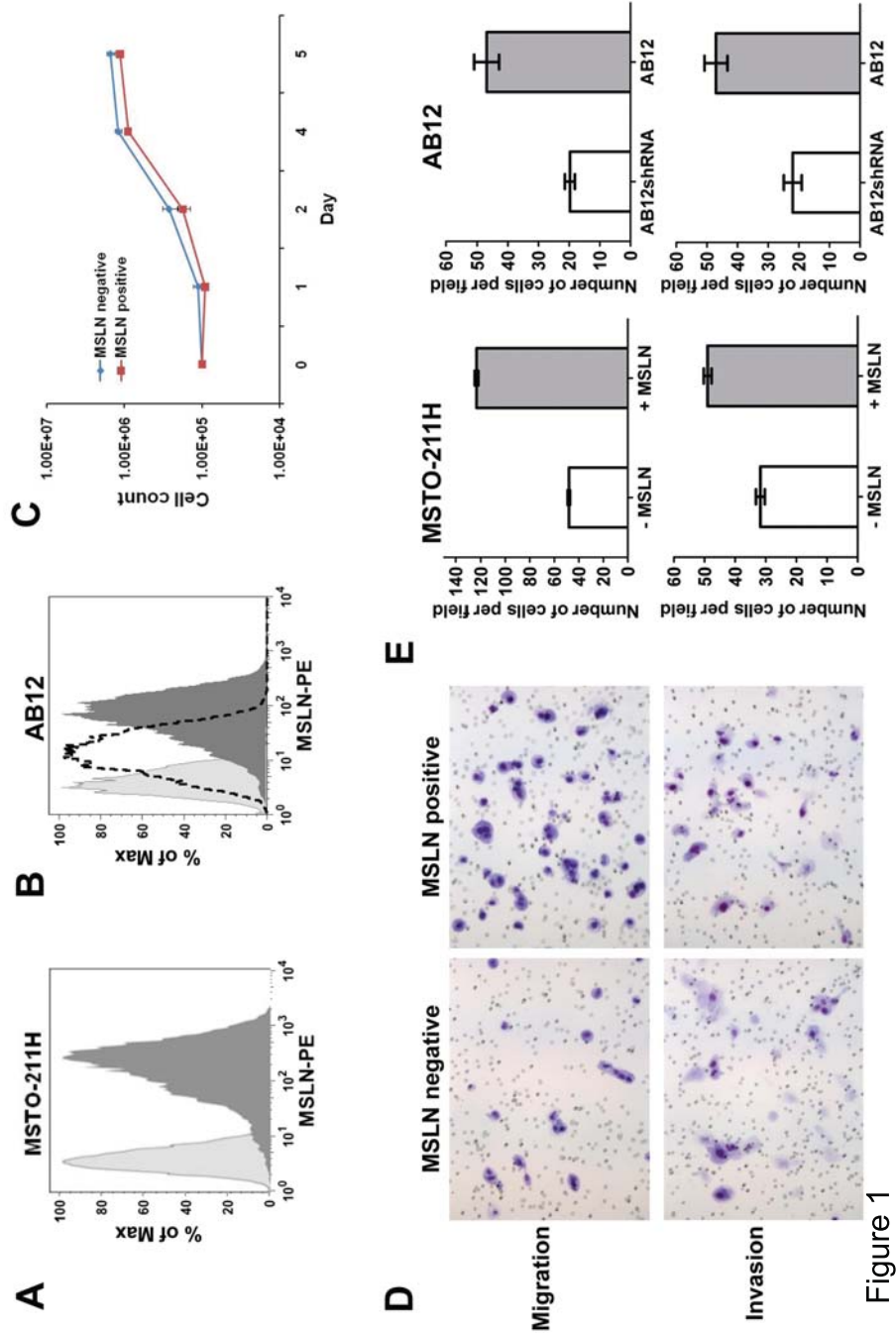
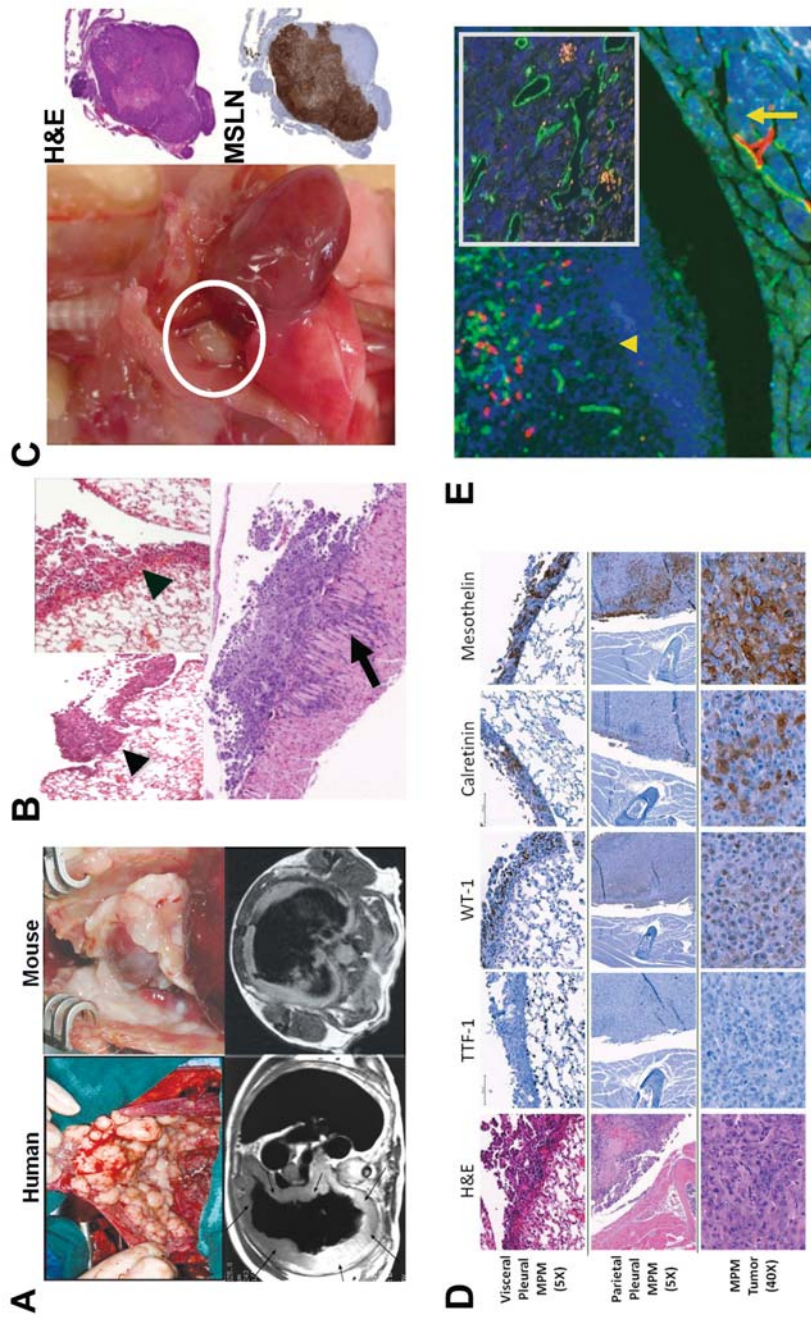
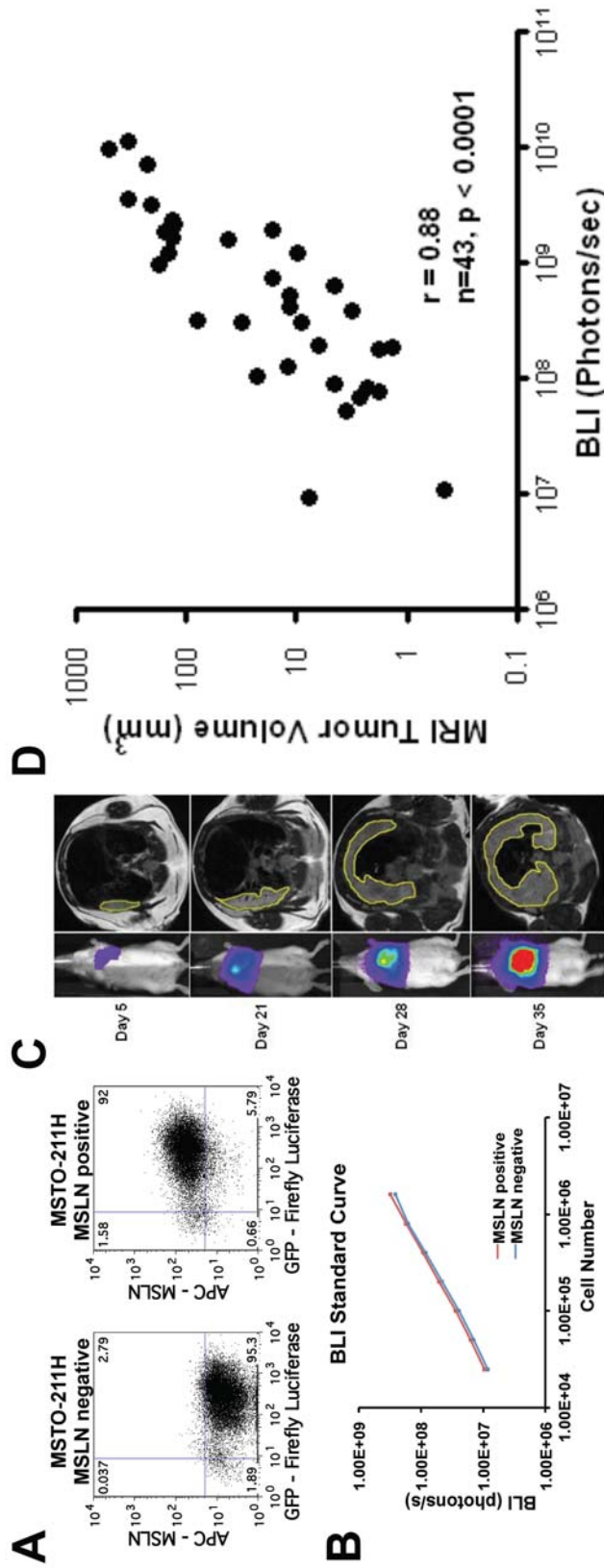


Figure 1





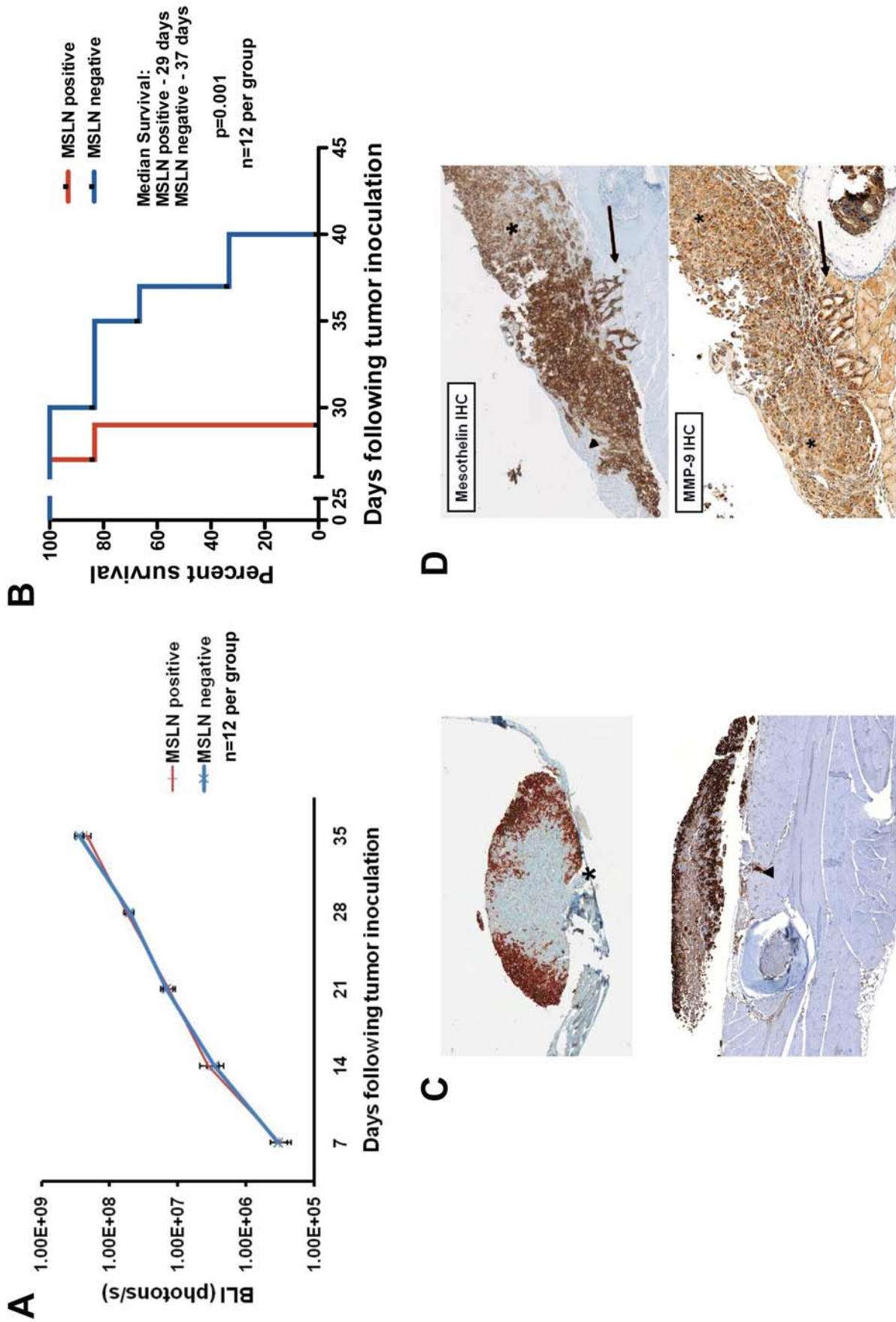


Figure 4

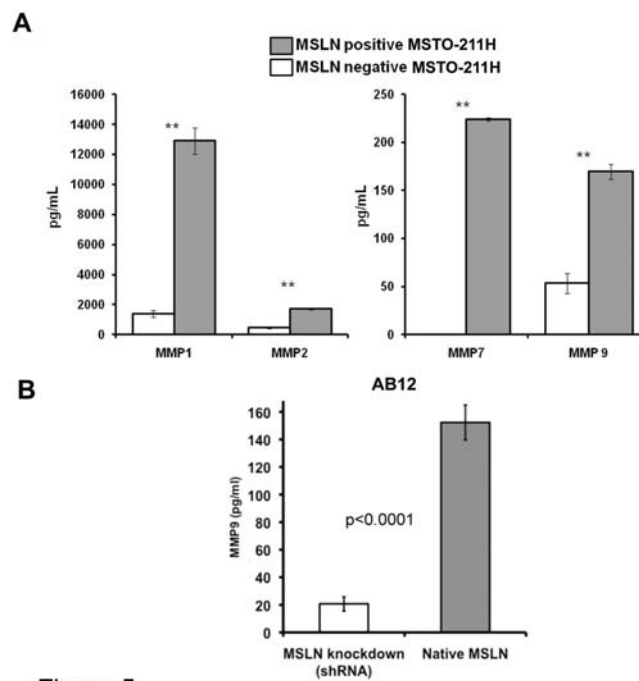


Figure 5

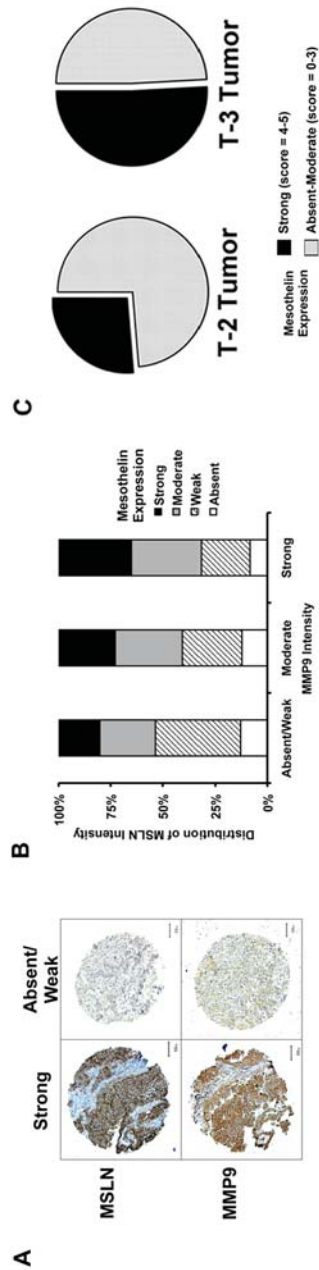


Figure 6

Clinical Cancer Research

Mesothelin overexpression promotes mesothelioma cell invasion and MMP-9 secretion in an orthotopic mouse model and in epithelioid pleural mesothelioma patients

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