

# Mesothelin is a malignant factor and therapeutic vaccine target for pancreatic cancer

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## Abstract

Given the high fatality rate of pancreatic cancer, an effective treatment for this devastating disease is urgently needed. We have shown that mesothelin expression was higher in human pancreatic cancer cells than in human pancreatic duct epithelial cells, and mesothelin mRNA was substantially overexpressed in 18 of 21 (86%) clinical pancreatic adenocarcinoma specimens when compared with the surrounding normal tissues. However, the biological functions of mesothelin in tumor progression are not clearly understood. Here we studied the effects of mesothelin overexpression in pancreatic cancer cell proliferation and migration *in vitro* and pancreatic cancer progression *in vivo*. We found that forced expression of mesothelin significantly increased tumor cell proliferation and migration by 90% and 300%, respectively, and increased tumor volume by 4-fold in the nude mice xenograft model when compared with the vector control cell line. Silencing of mesothelin inhibited cell proliferation and migration in pancreatic cancer cells and ablated tumor progression *in vivo*. Vaccination with chimeric virus-like particles that contain human mesothelin substantially inhibited tumor progression in C57BL/6J mice. The increases in mesothelin-specific antibodies and CTL activity and the decrease in regulatory T cells correlated with reduced tumor progression and prolonged survival. This study revealed novel functions of mesothelin and suggested a new therapeutic vaccine strategy whereby

mesothelin is targeted to control pancreatic cancer progression. [Mol Cancer Ther 2008;7(2):286–96]

## Introduction

Pancreatic cancer is the deadliest cancer and ranks as the fourth leading cause of cancer death in North America. Survival statistics are poor because there are no reliable tests for early diagnosis and no effective therapies for the metastatic form (1, 2). Clearly, there is a need to understand more about the molecular mechanisms of pancreatic cancer tumorigenesis and to develop effective treatment strategies for pancreatic cancer. Global gene expression profile studies have identified genes that are overexpressed in pancreatic cancer that might be used for the development of histologic diagnostic markers. Many genetic alterations in pancreatic cancer progression reside in oncogenes and tumor suppressor genes such as *K-ras*, *HER-2/neu*, *p16*, *p53*, *Smad4/DPC4*, and *BRCA2*. Point mutations in the *K-ras* oncogene and overexpression of *HER-2/neu* are thought to be the earliest genetic alterations in pancreatic cancer (3–7). Many other molecules such as mesothelin, CEA-CAM-1, CEACAM-6, S100P, and Rap1GAP are also listed as potential candidates for early detection markers in pancreatic cancer and are heavily involved in pancreatic cancer pathogenesis (8–11). However, biological functions and molecular mechanisms that contribute to the tumor progression caused by the overexpressed genes remain largely unknown. Therefore, understanding of genes that are specifically expressed in pancreatic cancer would be critical in the development of new diagnostic and therapeutic strategies for pancreatic cancer.

The mesothelin (*MSLN*) gene encodes a 69-kDa precursor protein that is proteolytically cleaved into an NH<sub>2</sub>-terminal secreted form and a COOH-terminal membrane-bound form, 40-kDa mesothelin, which is a glycosylphosphatidylinositol-linked glycoprotein (12). mesothelin-knockout mice do not show any pathologic characteristics, so the biological functions of mesothelin are not clearly understood. Previous studies have suggested a role of mesothelin in tumor adhesion and dissemination (13, 14). A recent study has shown that interaction of mesothelin and mucin 16 facilitates peritoneal metastasis of ovarian cancer cells (15). Moreover, mesothelin has been shown to be overexpressed in several cancer types, including mesothelioma, ovarian cancer, and pancreatic cancer (12, 16–20). Recently, high expressions of mesothelin were detected in lung cancer (21), uterine serous carcinoma (22), and acute myeloid leukemia (23). Little or no expression of mesothelin is seen in other cancer types and normal tissues (12, 17, 24). Argani et al. (18) found that mesothelin staining was positive in all 60 resected primary pancreatic adenocarcinomas, but negative or weak in adjacent normal pancreatic tissues. That finding has been confirmed by

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**Note:** M. Li, U. Bharadwaj, and R. Zhang contributed equally to this work.

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many other studies with microarray, serial analysis of gene expression, U133 oligonucleotide array, and immunohistochemical staining (25–27). These latest studies indicate that mesothelin may play some role in tumorigenesis.

Immunotherapy for pancreatic cancer is promising (8). Several studies have used whole-cell vaccine, peptide vaccine, and monoclonal antibody targeting key signaling pathways such as epidermal growth factor receptor, vascular endothelial growth factor, and K-ras (28). An effective cancer vaccine must be able to elicit strong CTL responses and antibody responses with little toxicity. Vaccination with virus-like particles (VLP) has the unique property of inducing protective antiviral immune responses without carrying the infectious and replicative capacities of the original virus. Therefore, VLPs constitute a major advantage in the design of efficient and safe vaccines. In addition, VLPs have been shown to induce maturation of dendritic cells directly to enhance innate immune responses (29). VLPs are also potent inducers of B-cell responses and are capable of breaking self-tolerance (30). In light of the overexpression of mesothelin in pancreatic cancer, it is intriguing to incorporate mesothelin into VLPs to make a chimeric VLP-MSLN and to test the efficacy of VLP vaccination against pancreatic cancer. In this study, we first investigated the biological functions of mesothelin in human pancreatic cancer cells *in vitro* and *in vivo*. We then investigated the effect and mechanism of chimeric VLPs that contain human mesothelin (VLP-hMSLN) as a potent candidate vaccine for controlling pancreatic cancer progression in an orthotopic pancreatic cancer mouse model.

## Materials and Methods

### Cells, Chemicals, Antibodies, and Human Tissue Specimens

Human pancreatic cancer cell lines Panc-1, MIA PaCa-2, BxPC-3, and Capan-1 and mouse endothelial cell line SVEC4-10 were purchased from the American Type Culture Collection. The human pancreatic duct epithelial cells were provided as a generous gift from Dr. Ming-Sound Tsao (Division of Applied Molecular Oncology, Ontario Cancer Institute, Toronto, Ontario, Canada; ref. 31). Panc02 was originally established by Corbett et al. (32), and NS47 was provided by A. Takashima (University of Texas, Dallas, TX). All cells were cultured as previously described (33, 34). Rabbit anti-mesothelin polyclonal antibody was generated from Genemed, and anti-mesothelin monoclonal antibody was purchased from Abcam. Phycoerythrin-conjugated anti-mouse/rat Foxp3 staining antibody was purchased from eBioscience. Other antibodies and chemicals were from Sigma. Human pancreatic adenocarcinoma specimens were collected from patients who underwent surgery according to an approved human protocol at Baylor College of Medicine (Houston, TX).

### Real-time RT-PCR, Immunoblot, and Immunohistochemical Analysis

The mesothelin mRNA was analyzed by real-time RT-PCR as previously described (33, 34). The primer sequences

for human *MSLN* gene are 5'-CTCAACCCAGATGCGT TCTCG-3' (sense) and 5'-AGGTCCACATTGGCCTTCGT-3' (antisense). Immunoblot and immunohistochemical staining were done as previously described (34) and the mesothelin protein was detected with anti-mesothelin antibodies.

### Stable Cell Line Selection

Cells with mesothelin overexpression or siRNA silencing were selected in MIA PaCa-2 and BxPC-3 cells, respectively, with retrovirus vectors pBabe and pSuper (Clontech) following the manufacturer's instructions. Briefly, full-length human mesothelin cDNA (NM\_005823) and siRNA sequences for mesothelin (5'-GAAGAATGTCAAGCTCTCA-3') were cloned into pBabe or pSuper vectors, and the recombinant plasmid was cotransfected into 293T cells with plasmid PegPam3 and RDF. Viral supernatants were collected and transduced into the target cells. Stable cell lines were selected by adding 0.5 to 1  $\mu\text{g}/\text{mL}$  of puromycin. At least three individual clones were selected for each stable cell line. The overexpression and silencing of mesothelin in each stable cell lines were confirmed by both real-time RT-PCR and Western blot.

### Cell Proliferation Assay

Stable MIA PaCa-2 or BxPC-3 cells were seeded in 96-well plates ( $2 \times 10^3$  per well) and serum starved (0% fetal bovine serum) for 24 h. For 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, cell growth was assessed 6 days after releasing from starvation. Twenty microliters of MTS reagent mixed with 100  $\mu\text{L}$  of growth medium were added to each well and incubated at 37°C for 2 h. Absorbance was recorded at 490 nm with an EL-800 universal microplate reader (Bio-Tek Instruments).

### *In vitro* Migration Assays

Cell migration was determined with a modified Boyden chamber assay. Briefly, cells ( $10^5/500 \mu\text{L}$ ) were added into the upper compartment of a migration chamber. After 24 h, the cells were incubated with calcein-AM (Molecular Probes) before fixation. Cells in the upper chamber were then removed, and cells that had migrated to the lower surface of the membrane were quantified. The migration rate was presented as the ratio of the mean fluorescence reading after scraping of the cells divided by the reading before removing the top cells. A monolayer wound healing assay was also done. Wounds were created in confluent monolayer cells with a sterile pipette tip and wound healing was observed at 0, 24, 48, and 72 h within the scrape line; representative fields for each cell line were photographed.

### Subcutaneous and Orthotopic Pancreatic Cancer Mouse Models

Subconfluent stable pancreatic cancer cells with mesothelin overexpression or siRNA silencing were harvested by trypsinization and resuspended in DMEM. Cells ( $2 \times 10^6$ ) were inoculated either into the right flank (s.c. tumor model) or into the pancreas (orthotopic tumor model) of 5- to 6-week-old male nude mice (National Cancer Institute-Charles River) as previously described

(35). For intrapancreatic injection, mice were anesthetized with 2.5% Avertin and a 0.5- to 1-cm incision was made in the left subcostal region. Tumor cells ( $2 \times 10^6$ ) in a volume of 50  $\mu\text{L}$  were injected into the body of the pancreas. For the s.c. tumor model, tumor size was measured weekly using a digital caliper (VWR International) and tumor volume was determined with the following formula: tumor volume ( $\text{mm}^3$ ) = [length (mm)]  $\times$  [width (mm)]<sup>2</sup>  $\times$  0.52. For the orthotopic tumor model, after 4 weeks, all surviving mice were euthanized by overdose of CO<sub>2</sub> exposure and evaluated macroscopically for the presence of orthotopic tumor and metastases in the abdominal cavity. The orthotopic and metastatic tumor nodules were then explanted, counted, and measured. For both s.c. and orthotopic experiments, the animals were euthanized when their tumor size reached 2 cm in diameter or the animals became moribund during the observation period, and the time of euthanization was recorded as the time of mortality.

#### VLP Preparation and Vaccination

The production and purification of VLP-hMSLN were done similarly as SHIV VLP production, which was previously described (36). For a fixed-time study, three groups of C57BL/6J mice (The Jackson Laboratory;  $n = 10$ ) were implanted orthotopically with  $5 \times 10^5$  Panc02 cells on day 0. On day 3, 50  $\mu\text{g}$  of VLP-hMSLN, VLP-Gag, or PBS were vaccinated i.p. into mice, followed by day 7 and day 14 boosts with 100  $\mu\text{g}$  of VLP-hMSLN, VLP-Gag, or PBS. On days 0, 7, 14, 21, and 28, mice were bled to obtain

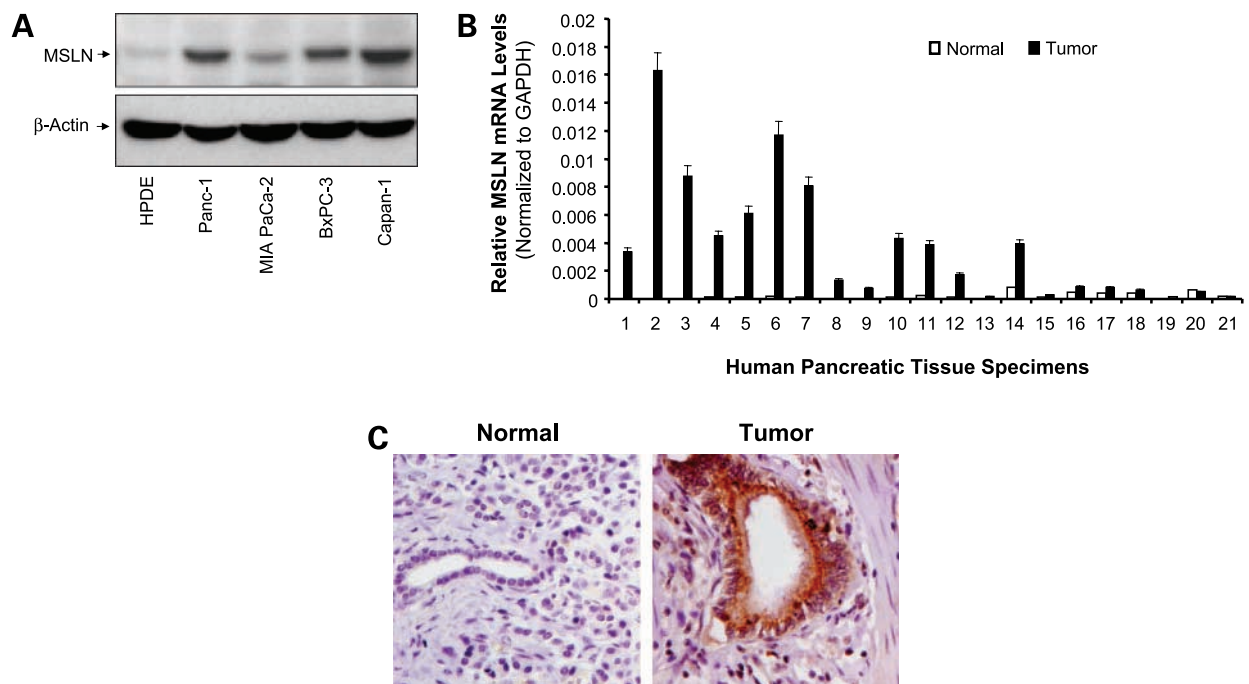
serum. On day 28, mice were sacrificed and splenocytes were collected for cellular immune response assays. For a survival study, the three groups of mice received the same treatment as above with VLPs. The survival of each mouse in each group was recorded until 9 weeks after tumor implantation.

#### ELISA and ELISpot Assay

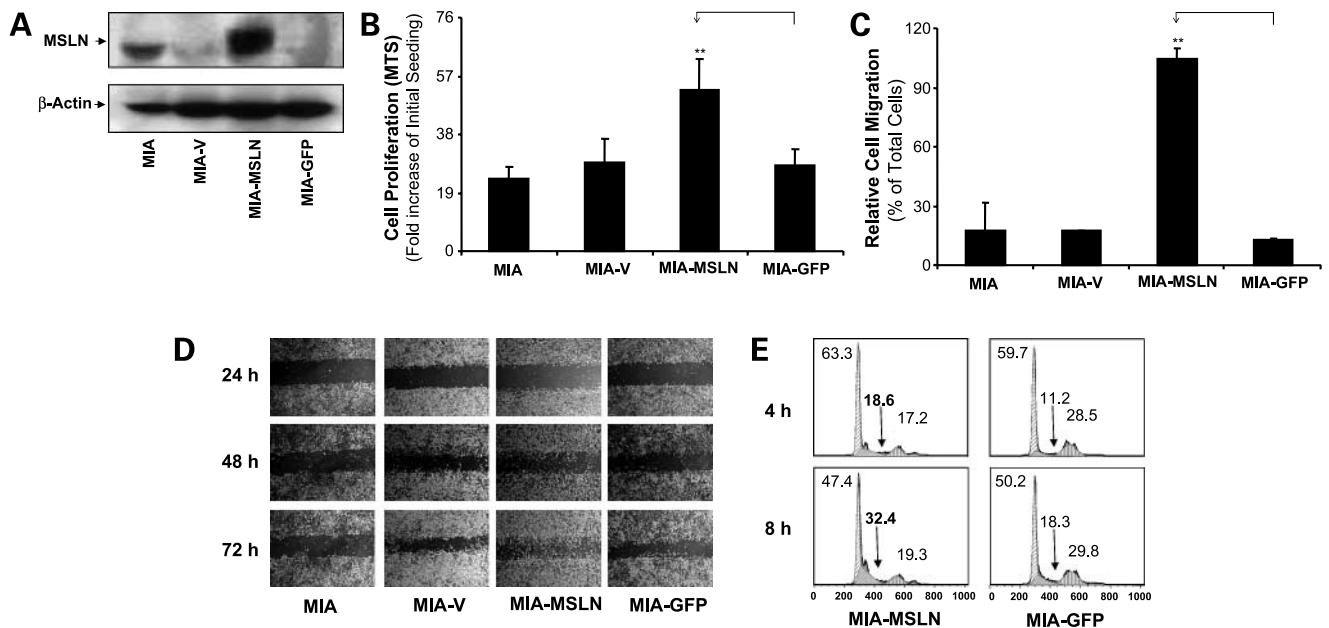
All serum samples were individually collected, and titers of IgG against mesothelin were determined by ELISA. Immunlon-4 HBX 96-well microtiter plates were coated with 100  $\mu\text{L}$  of purified mesothelin protein (Abnova Corporation) at 2  $\mu\text{g}/\text{mL}$ . Plates were blocked with PBS containing 1% BSA, and 100-fold diluted serum was added to the wells and incubated at 4°C overnight. After four washes, the wells were treated with goat anti-mouse IgG-peroxidase conjugates (Sigma-Aldrich) for 1 h at room temperature. After removal of the unbound conjugates, the substrate solution prepared in ABTS (Sigma) and hydrogen peroxide were added to the plates. Absorbances were read at 405 nm with an ELISA reader. IFN- $\gamma$ -producing mesothelin-specific T cells were determined by an ELISpot assay as previously described (37).

#### CTL Activity Assay

CTL activity was measured by using the CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions (Promega). Splenocytes were freshly isolated and stimulated with mesothelin-specific peptide (GQKMNAQAI) at 2  $\mu\text{mol}/\text{L}$  for 5 days. These cells



**Figure 1.** Expression of mesothelin in pancreatic cancer cell lines and pancreatic cancer tissues. **A**, mesothelin protein expression in pancreatic cancer cell lines and human pancreatic ductal epithelium (HPDE) cells by Western blot analysis. **B**, mesothelin mRNA in pancreatic tissues detected by real-time RT-PCR. Twenty-one paired tumor and adjacent normal tissues were studied. Relative mRNA concentrations of mesothelin were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and presented as  $2^{[Ct(GAPDH) - Ct(mesothelin)]}$ . **C**, immunohistochemical staining of mesothelin expression in pancreatic cancer tissues.



**Figure 2.** Effects of mesothelin overexpression in MIA PaCa-2 cells on cell proliferation and migration. **A**, mesothelin expression in stable cells by Western blot analysis. **B**, cell proliferation by MTS assay. Cells were seeded in 96-well plates ( $2 \times 10^3$  per well) and serum starved for 24 h before changing to growth medium with 2% fetal bovine serum. Absorbance was recorded on day 6 after releasing from starvation at 490 nm. **C**, cell migration by modified Boyden chamber assay. Stable cells were trypsinized and resuspended in growth medium ( $10^5/500 \mu\text{L}$ ) and added into the upper compartment of a migration chamber for 24 h. The cells were stained with Calcein-AM. The mean fluorescence reading after scraping of the cells at the top was divided by the reading before removal of the top cells, and the ratio was plotted. **D**, cell migration by wound healing assay. Confluent stable cells were scraped with a pipette tip and cultured in DMEM for 3 d. Pictures were taken daily. **E**, cell cycle analysis by flow cytometry. Confluent cells were serum starved (0% fetal bovine serum) for 24 h before changing to growth medium with 2% fetal bovine serum. Cells were collected at 4 and 8 h, permeabilized, stained with propidium iodide, and analyzed by flow cytometry. \*\*,  $P < 0.001$ .

were used as effector cells in the CTL assay. EL-4 cells ( $H-2^b$ ) pulsed with mesothelin-specific peptide ( $2 \mu\text{mol/L}$ ) for 1 h were used as target cells. EL-4 cells pulsed with no peptides were used as negative control target cells. Five different effector-to-target cell ratios (81:1, 27:1, 9:1, 3:1, and 1:1) were tested in the assay with  $1 \times 10^4$  target cells for 4 h. The specific lysis of target cells was measured by lactate dehydrogenase release and calculated using the formula indicated in the CytoTox 96 assay kit (Promega).

#### Regulatory T-Cell Assay

Freshly prepared splenocytes were collected on day 28 after tumor implantation in different vaccination groups and were used for staining surface markers CD4, CD25, and intracellular regulatory T-cell (Treg) specific marker Foxp3 followed by flow cytometric analysis. Phycoerythrin-conjugated anti-mouse/rat Foxp3 Staining Set was purchased from eBioscience. All experiments were repeated at least thrice. For immunohistochemical staining of Foxp3<sup>+</sup> cells in the frozen tumor sections, phycoerythrin-conjugated anti-mouse/rat Foxp3 Staining Set (eBioscience) was also used.

#### Statistical Analysis

Quantitative results are shown as mean  $\pm$  SD. The statistical analysis was done by Student's *t* test for paired data between control and treated groups or one-way ANOVA for data from multiple groups. A log-rank test was done for comparing the survival curves between the treatment and control groups.  $P < 0.05$  was considered significant.

## Results

### mesothelin Is Overexpressed in Human Pancreatic Cancer Cell Lines and Tissues

We examined mesothelin expression in 4 human pancreatic cancer cell lines, 21 pairs of human pancreatic cancer tissues with the surrounding normal tissues, and 5 pancreatitis tissues. mesothelin expression was differentially increased in all four cell lines tested compared with that in human pancreatic duct epithelial cells (Fig. 1A). mesothelin mRNA was substantially overexpressed in 18 of 21 (86%) clinical pancreatic-adenocarcinoma samples compared with that in their surrounding normal tissues (Fig. 1B; Supplementary Table S1).<sup>1</sup> In all five pancreatitis tissues, mesothelin mRNA was as low as that in the surrounding normal tissues (data not shown). Overall mRNA expression in pancreatic cancer tissue was 17.5 times that in the surrounding normal tissues. The tumor samples also showed strong immunoreactivity to mesothelin antibody (Fig. 1C). Thus, human pancreatic cancer is associated with increased expression of mesothelin.

### Overexpression of mesothelin Increases Cell Proliferation and Migration in Pancreatic Cancer Cells

To study the potential functions of mesothelin in pancreatic cancer, we established three stably mesothelin-overexpressing

<sup>1</sup> Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

cell lines in MIA PaCa-2 cells (MIA-MSLN) because parental MIA PaCa-2 cells express mesothelin less than the other pancreatic cancer cell lines (Fig. 1A). Stable cells containing empty vectors (MIA-V) or nonrelated *GFP* gene (MIA-GFP) were also established in MIA PaCa-2 cells as controls. Overexpression of mesothelin in all three MIA-MSLN cells at both mRNA and protein levels was confirmed when we compared them with MIA-V and MIA-GFP controls. Mesothelin overexpression in a representative MIA-MSLN stable cell line is shown in Fig. 2A and Supplementary Fig. S1<sup>1</sup> ( $P < 0.01$ ). Mesothelin overexpression in MIA-MSLN cells was associated with increases in cell proliferation and migration of 90% and 300%, respectively, compared with those in MIA-GFP cells ( $P < 0.01$ ; Fig. 2B and C). In the monolayer wound healing assay, MIA-MSLN cells showed much greater migration potential than the parental MIA PaCa-2, MIA-V, and MIA-GFP cells (Fig. 2D). Overexpression of mesothelin is also associated with an increased S-phase cell population in a cell cycle analysis. MIA-MSLN cells included 18.6% and 32.4% entering the S phase 4 and 8 h after release from serum starvation, respectively. That is 66% and 77% more than MIA-GFP cells, which had 11.2% and 18.3% entering the S phase, respectively, at those time points (Fig. 2E). Thus, overexpression of mesothelin in MIA PaCa-2 cells is associated with increased cell proliferation and migration. We also constructed mesothelin-overexpressing Panc-1 stable cells and found that forced expression of mesothelin increased cell proliferation in those cells (data not shown).

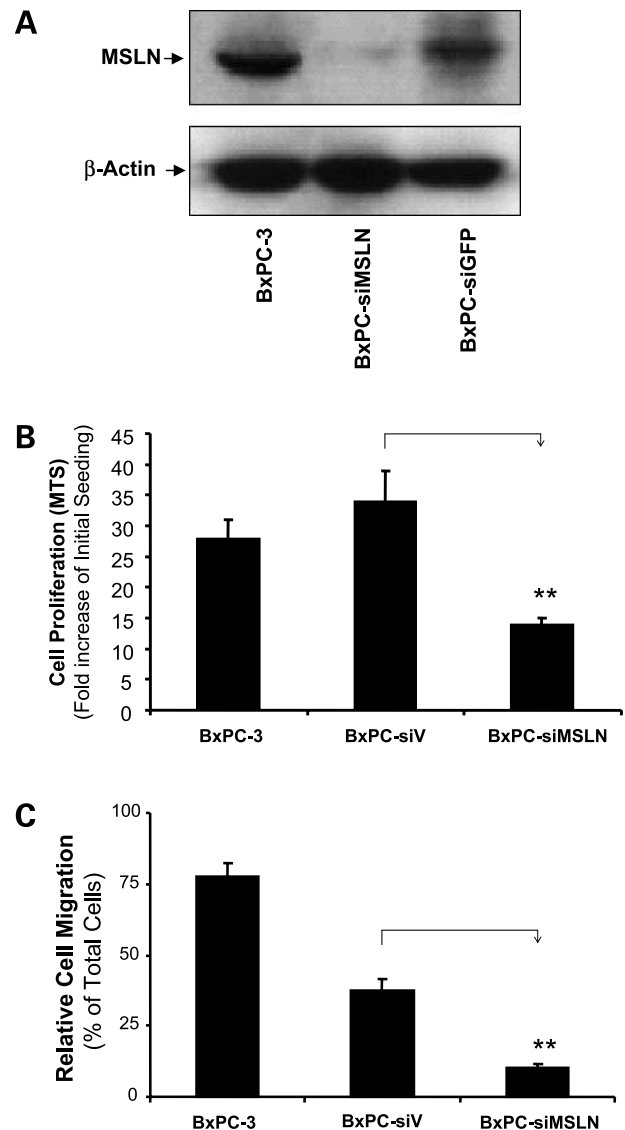
#### Silencing of mesothelin Decreases Cell Proliferation and Migration in Pancreatic Cancer Cells

We established three stably mesothelin-silenced cell lines in BxPC-3 cells (BxPC-siMSLN) because parental BxPC-3 cells express mesothelin more than other pancreatic cancer cell lines (Fig. 1A). Cells containing empty vector (BxPC-siV) were included as controls. A reduction in expression of mesothelin in three BxPC-siMSLN cells at the mRNA and protein levels was confirmed when we compared them with BxPC-siV cells. Silencing of mesothelin in a representative BxPC-siMSLN stable cell line is shown in Fig. 3A and Supplementary Fig. S2<sup>1</sup> ( $P < 0.01$ ). The decrease in mesothelin in BxPC-siMSLN cells was associated with decreases in cell proliferation and migration of 50% and 70%, respectively, compared with BxPC-siV cells ( $P < 0.01$ ; Fig. 3B and C).

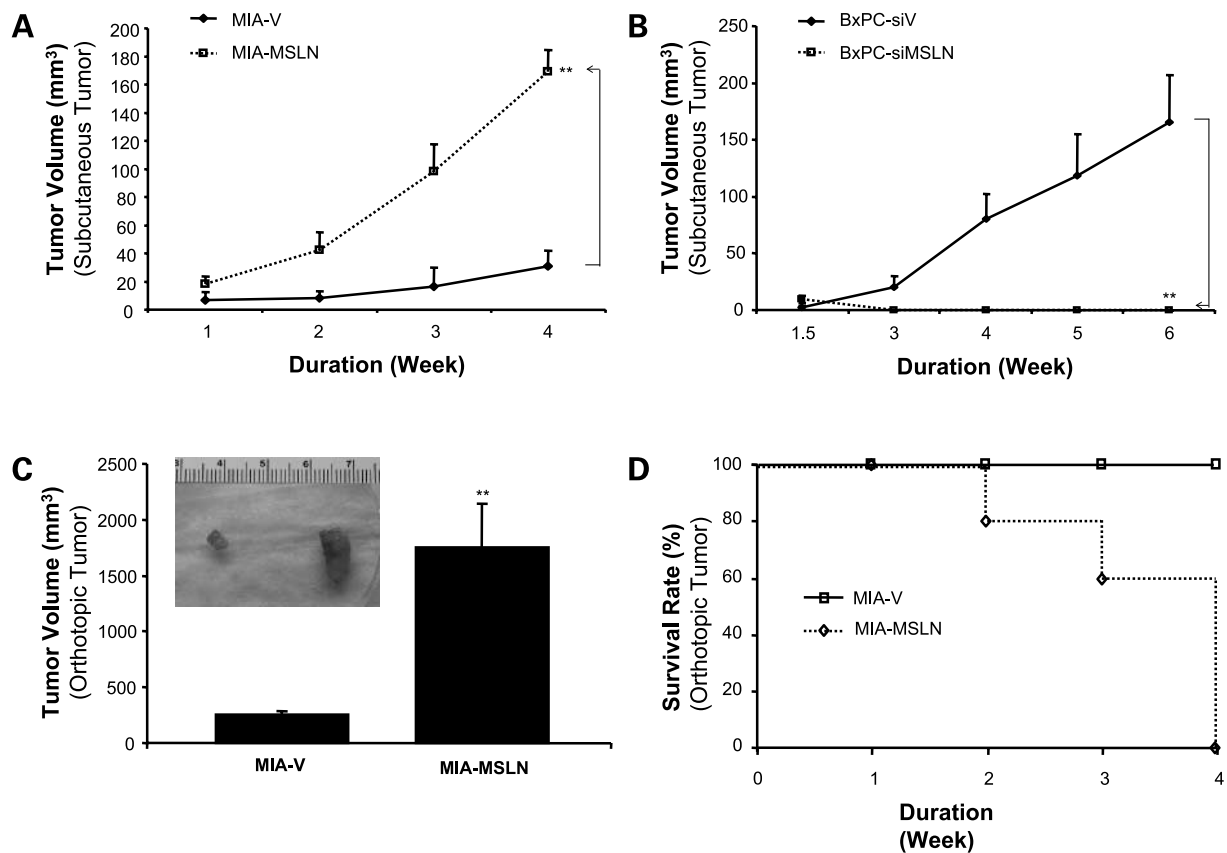
#### Mesothelin Contributes to Pancreatic Cancer Progression in the Nude Mouse Xenograft Model

On the basis of the effects of mesothelin on pancreatic cancer cell proliferation and migration *in vitro*, we analyzed the role of mesothelin in tumor progression *in vivo* with an immunodeficient nude mouse model. MIA-MSLN cells showed a dramatic increase (4.3-fold) in tumor volume over MIA-V control cells in the s.c. tumor model ( $P < 0.01$ ; Fig. 4A; Supplementary Table S2).<sup>1</sup> In contrast, BxPC-siMSLN cells with reduced mesothelin expression showed a significant reduction in tumor volume compared with BxPC-siV control cells ( $P < 0.01$ ; Fig. 4B; Supplementary Table S2).<sup>1</sup> Similarly, MIA-MSLN cells significantly

increased tumor size by 6.8-fold after 4 weeks compared with MIA-V control cells in the orthotopic model ( $P < 0.01$ ; Fig. 4C). Furthermore, mice given injections of MIA-MSLN cells showed jaundice (60%), local metastasis (50%), liver metastasis (60%), and abdominal ascitic fluid (60%), whereas none of the MIA-V control mice showed any of these symptoms (Supplementary Table S3).<sup>1</sup> A log-rank test was done for comparing the survival curves between the MIA-MSLN group and the MIA-V group. The log-rank test showed that the difference of the survival curves between the two groups is statistically significant ( $P < 0.00001$ ).



**Figure 3.** Effects of mesothelin silencing in BxPC-3 cells on cell proliferation and migration. **A**, mesothelin expression in stable cells by Western blot analysis. **B**, cell proliferation by MTS assay. Cells were seeded in 96-well plates ( $2 \times 10^3$  per well) and serum starved (0% fetal bovine serum). Cell growth was assessed with the MTS assay on day 6 after releasing from starvation. **C**, cell migration by modified Boyden chamber assay with calcein-AM labeling. \*\*,  $P < 0.001$ .



**Figure 4.** Effects of mesothelin on pancreatic cancer growth in the xenograft nude mouse model. **A**, s.c. tumor volume of MIA-MSLN cells. MIA-MSLN or MIA-V cells ( $2 \times 10^6$ ) were s.c. inoculated into nude mice (10 per treatment group). Tumor size was measured weekly for 4 wks. \*\*,  $P < 0.01$ . **B**, s.c. tumor volume of BxPC-siMSLN cells. BxPC-siMSLN cells ( $2 \times 10^6$ ) were injected into the right flank of nude mice (eight per treatment group). Tumor size was measured weekly for 6 wks. \*\*,  $P < 0.01$ . **C**, orthotopic tumor volume of MIA-MSLN cells. MIA-MSLN or MIA-V cells ( $2 \times 10^6$ ) were implanted into the body of pancreases in nude mice (10 in each group). The mice were euthanized at different times. Orthotopic tumor volume was measured. Tumor volume in mice inoculated with MIA-MSLN was significantly higher than that in mice inoculated with MIA-V (\*\*,  $P < 0.01$ ). **D**, survival rate of nude mice with orthotopically inoculated tumors. MIA-MSLN or MIA-V cells ( $2 \times 10^6$ ) were implanted into the body of pancreases in nude mice (10 in each group). Death was recorded up to 4 wks after tumor inoculation. Survival of mice with MIA-MSLN inoculations was substantially lower than that of mice with MIA-V inoculations ( $P < 0.00001$ ).

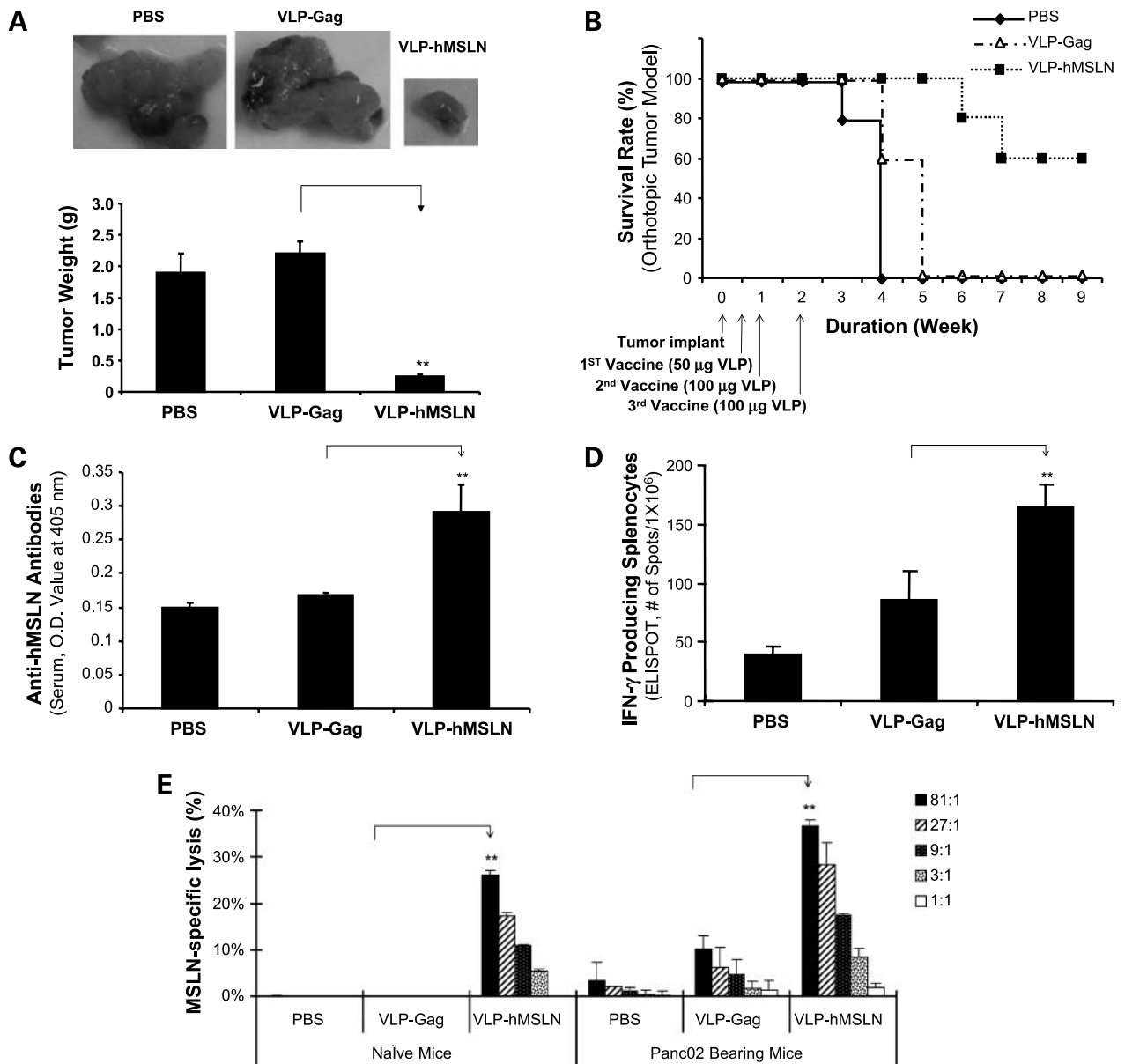
Nude mice with MIA-mesothelin cells had a substantially lower survival rate over 4 weeks than mice with MIA-V control cells (Fig. 4D). These results indicate that mesothelin is a malignant factor that significantly contributes to pancreatic cancer progression *in vivo*.

#### VLP-hMSLN Constitutes an Efficient Therapeutic Vaccine against Pancreatic Cancer Growth in the Mouse Model

Because surface expression of mesothelin played an important role in pancreatic cancer growth and pathogenesis, we tested the hypothesis that vaccination against mesothelin could be an effective therapeutic approach to pancreatic cancer. Higher expression of mouse mesothelin in Panc02 cells than in normal mouse pancreas, mouse fibroblast cells NS47, and mouse endothelial cells SVEC4-10 was confirmed with real-time RT-PCR ( $P < 0.01$ ; Supplementary Fig. S3).<sup>1</sup> Our previous studies have shown that VLP is a strong immunogen and vaccine candidate that elicits both cellular and humoral immune responses to incorporated surface protein. Therefore, we constructed a

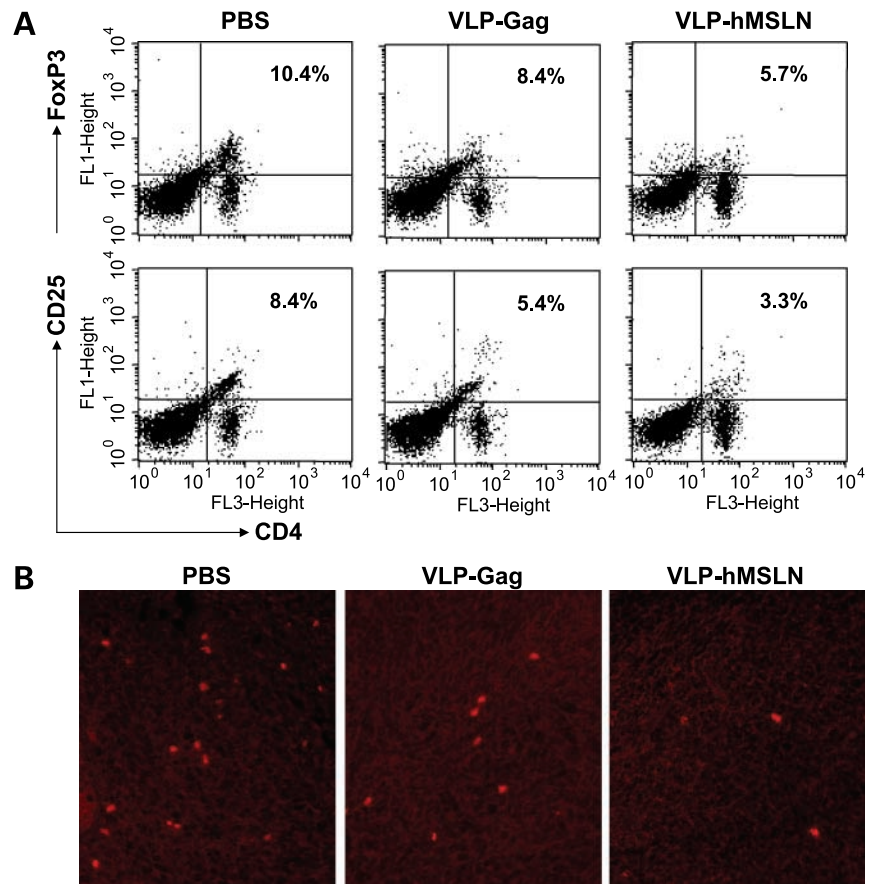
chimeric VLP-hMSLN in which the human mesothelin protein was incorporated into the SHIV VLPs. The expression of mesothelin on the VLPs was confirmed, as shown in Supplementary Fig. S4.<sup>1</sup>

To test the therapeutic efficacy of VLP-hMSLN, we implanted Panc02 cells orthotopically in C57BL/6J wild-type mice, which were then vaccinated i.p. with 50  $\mu$ g of VLP-hMSLN on day 3 and with 100  $\mu$ g of VLP-hMSLN on days 7 and 14. We monitored tumor progression in C57BL/6J mice 4 weeks after tumor implantation. The VLP-hMSLN-vaccinated group showed significantly lower tumor progression and smaller tumor mass than the VLP-Gag and PBS groups ( $P < 0.01$ ; Fig. 5A). In addition, some of the VLP-hMSLN-vaccinated mice showed mild peritoneal dissemination and little abdominal ascitic fluid, whereas all the control (VLP-Gag and PBS) mice showed multiple peritoneal dissemination and severe abdominal ascitic fluid (Supplementary Table S4).<sup>1</sup> Survival of the VLP-hMSLN-vaccinated mice was significantly increased: 60% of them were still alive 9 weeks after tumor



**Figure 5.** Effects of therapeutic vaccine against mesothelin on pancreatic cancer growth and pathogenesis in the orthotopic mouse model. **A**, effects of VLP-hMSLN vaccination on orthotopic tumor growth in mice. Panc02 cells ( $5 \times 10^5$ ) were implanted into the body of pancreases of C57BL/6J wild-type mice. On day 3, 50  $\mu$ g of VLP-hMSLN, VLP-Gag, or PBS were injected i.p. (10 mice in each group); this was followed on days 7 and 14 with two 100- $\mu$ g VLP-hMSLN, VLP-Gag, or PBS boosts. Tumor growth was analyzed 4 wks after implantation. Photo images show a representative tumor size in each group at the same distance. Columns, mean tumor mass; bars, SD. \*\*,  $P < 0.01$ . **B**, effects of VLP-hMSLN vaccination on the survival of mice. The same groups of animals as in **A** were studied. Death was recorded up to 9 wks after tumor inoculation. Survival of VLP-hMSLN-vaccinated mice was substantially greater than that of VLP-Gag- or PBS-treated mice ( $P = 0.006$ ). **C**, effects of VLP-hMSLN vaccination on serum anti-mesothelin antibody production. The same groups of animals as in **A** were studied. Two weeks after the final VLP boost, serum samples were individually collected, and titers of IgG against mesothelin were determined by ELISA. Serum anti-mesothelin antibodies in the VLP-hMSLN-vaccinated mice were substantially increased compared with those in VLP-Gag- or PBS-treated mice. \*\*,  $P < 0.01$ , compared with the VLP-Gag group. **D**, effects of VLP-hMSLN vaccination on mesothelin-specific IFN- $\gamma$ -producing cells by ELISPOT assay. The same groups of animals as in **A** were studied. Two weeks after the final VLP boost, mouse spleen cells were collected and stimulated with recombinant vaccinia virus expressing mesothelin *in vitro*. Spleen IFN- $\gamma$ -producing T cells in the VLP-hMSLN-vaccinated mice were substantially increased compared with those in VLP-Gag- or PBS-treated mice. \*\*,  $P < 0.01$ , compared with the VLP-Gag group. **E**, increased mesothelin-specific CTL activity elicited by VLP-hMSLN immunization. Three groups of naïve mice and three groups of Panc02-implanted mice were immunized with PBS, VLP-Gag, or VLP-hMSLN at days 3, 7, and 14. At 28 days post-immunization, splenocytes isolated from PBS, VLP-Gag, or VLP-hMSLN-immunized groups were stimulated with a mesothelin-specific peptide at 2  $\mu$ mol/L for 5 d to generate effector cells. EL-4 cells pulsed with mesothelin-specific peptide (2  $\mu$ mol/L) for 1 h were used as target cells. Different effector-to-target cell ratios (81:1, 27:1, 9:1, 3:1, and 1:1) were mixed for 4 h. The specific lysis of target cells was measured by lactate dehydrogenase (LDH) release and calculated using formula indicated in the CytoTox 96 assay kit (Promega). Columns, mean; bars, SD. The data represent one of five independent experiments with different mice. \*\*,  $P < 0.01$ .

**Figure 6.** Reduced numbers of Tregs in VLP-hMSLN-vaccinated mice. About  $5 \times 10^5$  Panc02 cells were implanted into the body of pancreases of C57BL/6J wild-type mice. On day 3, 50  $\mu$ g of VLP-hMSLN, VLP-Gag, or PBS were injected i.p. (10 mice in each group); this was followed on days 7 and 14 with two 100- $\mu$ g VLP-hMSLN, VLP-Gag, or PBS boosts. Two weeks after the final VLP boost, mouse splenocytes were collected and stained for CD4, CD25, and Foxp3 expression by flow cytometry (A), or mouse Panc02 tumor sections were stained with Foxp3 antibody to visualize number of Treg cells (red cells) in tumors with PBS, VLP-Gag, or VLP-hmesothelin immunization (B). The number of Tregs was lower in VLP-hMSLN-vaccinated mice. All data shown are representative of three separate experiments.



implantation, but all the PBS and VLP-Gag mice died 4 and 5 weeks after tumor implantation, respectively (Fig. 5B). The log-rank test showed that the survival of VLP-hMSLN treatment is significantly higher than that of VLP-Gag treatment ( $P = 0.006$ ).

To determine human mesothelin-specific immune responses elicited by VLP-hmesothelin vaccination, we examined serum human mesothelin-specific antibody production and splenocyte human mesothelin-specific cellular immune responses. As shown in Fig. 5C, higher concentrations of specific antihuman mesothelin antibodies were detected in the serum of VLP-hMSLN-vaccinated mice than in control mice (10 in each group;  $P < 0.01$ ). To examine human mesothelin-specific T cells, we collected spleens from mice sacrificed 4 weeks after tumor implantation. Splenocytes were activated by a recombinant vaccinia virus that expresses human mesothelin or by vaccinia virus vector controls, and IFN- $\gamma$  production by T cells was determined with the ELISpot assay. More IFN- $\gamma$ -producing human mesothelin-specific T cells were detected in VLP-hMSLN-vaccinated mice (10 in each group;  $P < 0.01$ ; Fig. 5D) than in other control mice that received PBS or VLP-Gag. Thus, increased human mesothelin-specific antibody production and increased number of T cells correlated well with inhibition of tumor growth and increase in survival in animals vaccinated with VLP-hMSLN.

To determine and compare the human mesothelin-specific immune responses elicited by VLP-hMSLN immunization in naïve mice and Panc02-implanted mice, three groups of naïve mice and three groups of Panc02-implanted mice were immunized with PBS, VLP-Gag, or VLP-hMSLN. At 28 days post-immunization, human mesothelin-specific CTL activity was analyzed and compared in the two groups. As shown in Fig. 5E, VLP-hMSLN immunization in naïve mice can stimulate strong CTL responses against mesothelin; VLP-hMSLN immunization in Panc02 tumor cell-bearing mice can stimulate even higher CTL responses but with high-baseline mesothelin-specific CTL activity in PBS and VLP-Gag immunized animals. These data indicate that although Panc02 itself can induce weak mesothelin-specific immune responses, VLP-hMSLN immunization can stimulate much stronger specific CTL responses, which contribute to controlling pancreatic cancer progression.

To further study the immune response in the VLP-hMSLN-vaccinated mice, we examined the populations of Tregs in these mice and in the PBS and VLP-Gag control mice by flow cytometric analysis. CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs were decreased to 5.7% in VLP-hMSLN-vaccinated mice compared with 10.4% in PBS mice and 8.4% in VLP-Gag mice, representing a reduction of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs by 45% and 32%, respectively. In addition, CD4<sup>+</sup>CD25<sup>+</sup> Tregs were also decreased in VLP-hMSLN-vaccinated mice to 3.3%



compared with 8.4% in PBS mice and 5.4% in VLP-Gag mice, or 61% and 39% lower, respectively (Fig. 6A). To determine whether Treg is affected by the different treatments in Panc02 tumors, immunohistochemical staining of Foxp3<sup>+</sup> cells in the tumor tissues was done. As shown in Fig. 6B, the amount of Treg in Panc02 tumor tissue was slightly reduced in VLP-Gag and dramatically reduced in VLP-hMSLN-immunized mouse tumor tissue compared with that in the PBS group. Our data indicate that VLP-hMSLN-induced immune responses may be involved in the reduction in inhibitory Tregs and thereby enhance the immune response against tumor progression.

## Discussion

We have shown that mesothelin was overexpressed in human pancreatic cancer cells and clinical specimens. Increased mesothelin is associated with increased cell proliferation and migration of pancreatic cancer cells *in vitro* and contributes to tumor progression in the nude mouse xenograft model. Silencing of mesothelin significantly decreased cell proliferation and migration in pancreatic cancer cells and inhibited tumor growth *in vivo*. Vaccination with chimeric VLP-hMSLN significantly inhibited tumor progression in C57BL/6J mice. The increases in mesothelin-specific antibodies and T-cell responses and the decrease in Tregs correlated with reduced tumor progression and prolonged survival.

Mesothelin overexpression was reported to be present in all of 60 resected primary pancreatic adenocarcinomas but not in the adjacent normal pancreas or benign pancreatic ducts (18, 19). Significantly more ( $P < 0.05$ ) metastatic pancreatic adenocarcinomas were found to be positive for mesothelin (64%) than bile duct adenomas (0%) and hepatic hamartomas (0%; ref. 38). We found that mesothelin was significantly increased in all pancreatic cancer cell lines tested compared with human pancreatic duct epithelial cells and was substantially overexpressed in 86% of clinical pancreatic adenocarcinoma specimens compared with surrounding normal tissues and pancreatitis tissues. These results confirmed that mesothelin can serve as a marker of pancreatic adenocarcinoma. Therefore, detection of mesothelin could be valuable in diagnosing pancreatic adenocarcinoma, particularly in small biopsy or cytopathology samples (39). It might be possible to use soluble mesothelin-related proteins as biomarkers of pancreatic carcinoma with previously used markers, such as CA19-9 and CEA, to increase the sensitivity of diagnosis (39).

Mesothelin is a surface glycoprotein that is attached to the cell membrane by a glycosylphosphatidylinositol anchor and is postulated to function in cell adhesion (12). However, the details of the biological functions of mesothelin are not clear. Methylation-specific PCR revealed that the *MSLN* gene displayed a high prevalence of hypomethylation in pancreatic cancer cell lines and in primary pancreatic carcinomas (40). The identification of

novel CTL epitopes of mesothelin that could efficiently activate human T cells to lyse human tumors supports the hypothesis that mesothelin is a potential target for immune-based therapy for pancreatic cancer (41).

In the study reported here, we chose MIA PaCa-2 cells, which expressed relatively little endogenous mesothelin, and overexpressed mesothelin by selecting several stable cell lines. We found that mesothelin overexpression in MIA PaCa-2 cells is associated with increased cell proliferation, migration, and S-phase cell population. In contrast, in BxPC-3, a pancreatic cancer cell line that expressed more endogenous mesothelin, reduction in expression of mesothelin by siRNA stable silencing resulted in decreased cell proliferation and migration. In the cell cycle study, as shown in Fig. 2E, we observed a second peak in proximity of the G<sub>0</sub>-G<sub>1</sub> peak, which we believe are cells in mitosis that are going into S phase but not an aneuploid peak because of the following possible reasons: it might be that these cells have different levels of mesothelin expression and hence some cells showed faster cell cycle progression than the rest of the cells. We further found that when we blocked the cell proliferation with STAT3 siRNA, we do not see the peak or see a very low and shifted peak almost merging with the dominant G<sub>0</sub>-G<sub>1</sub> peak.<sup>2</sup> These results indicate that mesothelin is an important factor in pancreatic cancer growth and a potential target for pancreatic cancer treatment. The significant reduction in pancreatic cancer growth by mesothelin siRNA indicated the importance of siRNA blockage and opened the door for siRNA pancreatic cancer therapy that targets mesothelin.

Toxicity and lack of tumor specificity are the most obvious limitations of conventional approaches such as chemotherapy and radiation therapy in cancer treatment. The therapeutic vaccine approach, which would exploit a naturally occurring defense system, embodies an ideal nontoxic treatment capable of evoking tumor-specific immune responses. Vaccination immunotherapy is being tested for many types of cancers, but no current vaccination regimen has been shown in randomized controlled trials. Identification of novel CTL epitopes of mesothelin that could efficiently activate human T cells to lyse human tumors would shed light on the hypothesis that mesothelin is a potential vaccine candidate for immune-based therapy for pancreatic cancer (41). We have shown that vaccination with VLP-hMSLN significantly inhibited pancreatic cancer progression in C57BL/6J mice by increasing concentrations of human mesothelin-specific antibodies, increasing CD8<sup>+</sup> T-cell responses, and decreasing Tregs. We found that high-baseline mesothelin-specific antibody and CTL responses were observed in the Panc02-bearing mouse model. It led us to further study whether Panc02 tumor cell implantation by itself can stimulate immune response against human mesothelin. As shown in Fig. 5, although Panc02 itself can induce weak mesothelin-specific

<sup>2</sup> Unpublished data.

immune responses, VLP-hMSLN immunization can stimulate much stronger mesothelin-specific CTL responses, which contribute to controlling pancreatic cancer progression.

Because of their size and structure, VLPs seem to be actively taken up by antigen-presenting cells; this leads to antigen delivery to cell compartments that are inaccessible to soluble protein antigens. Some VLPs reportedly include papilloma virus-like particles, and SHIV VLPs have intrinsic adjuvanticity, which avoids the requirement for adjuvants to stimulate strong T-helper and CTL responses (36, 37, 42). Our study has extended the VLP-based vaccine to pancreatic cancer and showed a great potential of VLP-hMSLN as a therapeutic pancreatic cancer vaccine because of its excellent immunogenic property, safety, and feasibility for multiple vaccinations. The i.p. route of immunization was chosen initially to prove the efficacy of VLP-hMSLN immunization in the mouse pancreatic cancer orthotopic model. Because i.p. route approach limits the clinical applicability of this finding, we plan to further compare the immunotherapeutic efficacy among different routes of immunization such as s.c., i.d., or i.m., which are more practical for the clinical use.

We found from our *in vitro* data that MIA-MSLN cells had higher cell migration rate than MIA-V cells. This correlates well with the *in vivo* migration and *in vivo* metastasis in our MIA PaCa-2 xenograft mouse pancreatic cancer model. We found a good correlation of higher metastasis in MIA-MSLN than in MIA-V group. In our orthotopic pancreatic cancer wild-type mouse model, Panc02 cells grow aggressively in a short period of time to reach the tumor burden big enough for sacrifice, and thus metastasis is hard to establish in this model. We observed that Panc02 cells predominantly grow in local implant area and we did not observe any distal metastasis in the Panc02 orthotopic pancreatic cancer model. Therefore, it was inconclusive whether VLP vaccine could affect metastasis of Panc02 cells. In our future study, we plan to use a highly metastatic mouse pancreatic cancer cell line, Panc H7, to investigate if the VLP vaccine affects metastasis.

One reason for the ineffectiveness of tumor immune response is the acquisition of tolerance of tumor antigen during the development of the immune system as though it is self-antigen. The breaking of immune tolerance of autologous tumor antigen should be a useful approach for a cancer vaccine. We have shown here suppression of tumor progression by vaccination of the xenogeneic homologue VLP-hMSLN in the mouse model. Previous studies have shown successful immunotherapy for tumors in a cross-reaction between the xenogeneic homologues and self-molecules in a mouse model (43). In a recent study, mice vaccinated with Ad-hEp-CAM (epithelial cell adhesion molecule) or Ad-rhEp-CAM were protected from a tumor challenge with MC38-hEp-CAM cells; this showed that the immune response induced against hEp-CAM with both vaccines is sufficient to produce a biological antitumor effect (44). VLPs have the capacity to break self-tolerance to

produce antibody responses against self-proteins expressed on them (45). Therefore, human mesothelin is used in this study to evaluate the vaccine efficacy against mouse mesothelin. Human mesothelin (NM\_005823, 622 amino acid residues) and mouse mesothelin (BC023753, 625 amino acid residues) are 58% homologous in amino acid sequence. Our study proves the effectiveness of using xenogeneic antigen as a candidate tumor vaccine.

Tregs protect the host from autoimmune disease by suppressing self-reactive immune cells. Therefore, Tregs may also block antitumor immune responses (46). Recent reports have shown that the prevalence of Tregs is increased in several solid tumors, particularly in the tumor environment. Those Tregs are usually CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> (in some circumstances, CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup>). One study in a mouse pancreatic cancer model suggests that the tumor actively promotes the accrual of Tregs through several mechanisms involving both activation of naturally occurring Tregs and conversion of non-Tregs to Tregs (46). However, little is known about the molecular mechanisms responsible for increases in Tregs in cancer. For the first time, we have found that vaccination with VLP-hMSLN could significantly decrease the population of Tregs in both systemic immune organ (mouse spleen) and local tumor site. CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs and CD4<sup>+</sup>CD25<sup>+</sup> Tregs were significantly decreased in VLP-hMSLN-vaccinated mice compared with PBS- and VLP-Gag-treated groups, respectively. Therefore, VLP-hMSLN-induced decrease in the Treg population could be the important mechanism of enhanced immune responses and inhibition of pancreatic cancer growth in VLP-hMSLN-vaccinated mice.

In conclusion, VLP-based vaccination immunotherapy for pancreatic cancer shows hope of not only attacking the tumor but also dealing with important regulatory mechanisms that may have adversely influenced clinical efficacy. This novel strategy has potential clinical applications in treatment of human pancreatic cancer and other cancers with high expression of mesothelin.

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