

# HMGA1 Is a Determinant of Cellular Invasiveness and *In vivo* Metastatic Potential in Pancreatic Adenocarcinoma

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## Abstract

**HMGA1 proteins are architectural transcription factors that are overexpressed in a range of human malignancies, including pancreatic adenocarcinoma. We hypothesized that HMGA1 expression is a determinant of cellular invasiveness and metastasis in pancreatic cancer. Stable silencing of HMGA1 in MiaPaCa2 and PANC1 pancreatic adenocarcinoma cells was achieved by transfection of short hairpin RNA-generating vectors. Additionally, stable overexpression of HMGA1 in MiaPaCa2 cells (characterized by low levels of inherent HMGA1 expression) was achieved. HMGA1 silencing resulted in significant reductions in cellular invasiveness through Matrigel; in cellular matrix metalloproteinase-9 (MMP-9) activity, mRNA levels, and gene promoter activity; and in Akt phosphorylation at Ser<sup>473</sup>. Conversely, forced HMGA1 overexpression resulted in significant increases in cellular invasiveness; in cellular MMP-9 activity, mRNA levels, and promoter activity; and in Akt phosphorylation at Ser<sup>473</sup>. HMGA1 overexpression-induced increases in invasiveness were MMP-9 dependent. The role of phosphatidylinositol-3 kinase (PI3K)/Akt in mediating HMGA1-dependent invasiveness was elucidated by a specific PI3K inhibitor (LY294002) and constitutively active and dominant-negative Akt adenoviral constructs. Akt-dependent modulation of MMP-9 activity contributed significantly to HMGA1 overexpression-induced increases in invasive capacity. Furthermore, HMGA1 silencing resulted in reductions in metastatic potential and tumor growth *in vivo* and in tumoral MMP-9 activity. Our findings suggest that HMGA1 may be a novel molecular determinant of invasiveness and metastasis, as well as a potential therapeutic target, in pancreatic adenocarcinoma.** (Cancer Res 2006; 66(24): 11613-22)

## Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States (1). Its biology is characterized by the propensity for early and aggressive invasion and metastasis, such that <10% of patients have surgically resectable disease at the time of diagnosis (2). Even among patients able to undergo resection of all apparent disease, most are destined to succumb to locally recurrent and metastatic cancer.

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Hence, further understanding of the molecular mechanisms underlying pancreatic adenocarcinoma cellular invasion and metastasis is needed, as this information may facilitate the identification of novel molecular targets for the rational therapy of this deadly disease.

The human *HMGA1* gene, located on chromosomal locus 6p21, encodes two HMGA1 splice variants (HMGA1a and HMGA1b; ref. 3). These HMGA1 proteins are architectural transcription factors that play a role in both positive and negative transcriptional regulation of human gene expression *in vivo* (4, 5). They form stereo-specific, multiprotein complexes termed "enhanceosomes" on the promoter/enhancer regions of genes, where they are able to bind to the minor groove of AT-rich DNA sequences to induce DNA helix bending (4, 6). HMGA1 proteins are overexpressed in a range of human cancers, including pancreatic adenocarcinoma (7–11). Furthermore, HMGA1 protein overexpression has been reported to be associated with cancer metastasis (12–14) and transcriptional up-regulation of genes implicated in promoting metastasis (15).

The purpose of this study was to test the hypothesis that HMGA1 is a molecular determinant of cellular invasiveness and metastasis in pancreatic adenocarcinoma. Our findings indicate that HMGA1 promotes cellular invasiveness through phosphatidylinositol-3 kinase (PI3K)/Akt-dependent modulation of matrix metalloproteinase-9 (MMP-9) activity.

## Materials and Methods

**Cells and cell culture.** MiaPaCa2 and PANC1 human pancreatic ductal adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM containing 10% fetal bovine serum (FBS; Life Technologies, Inc., Gaithersburg, MD) and incubated in a humidified (37°C, 5% CO<sub>2</sub>) incubator, grown in 75-cm<sup>2</sup> culture flasks, and passaged on reaching 80% confluence.

**Reagents.** The PI3K inhibitor LY294002 was purchased from Calbiochem (San Diego, CA). Anti-HMGA1, anti-actin, anti-phospho-Akt (Ser<sup>473</sup>), anti-Akt1, anti-phospho-extracellular signal-regulated kinase 1/2 (anti-phospho-ERK1/2), anti-ERK1/2, and anti-hemagglutinin (anti-HA) tag antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-mammalian target of rapamycin (anti-phospho-mTOR; Ser<sup>2448</sup>) and anti-mTOR antibodies were obtained from Cell Signaling Technology (Danvers, MA).

**Dominant-negative and dominant-active Akt constructs.** Adenovirus carrying HA-tagged dominant-negative (Ad-DN-Akt), dominant-active (Ad-myr-Akt) murine Akt1, and control virus (Ad-CMV-null; all tittered at 1 × 10<sup>10</sup> plaque-forming units/mL) were purchased from Vector Biolabs (Philadelphia, PA). Adenoviral infection was done at multiplicity of infection of 10 in the presence of 6 μg/mL polybrene for 8 hours. Experiments were done on cells 48 hours following infection.

**Stable HMGA1 RNA interference.** Hairpin RNA interference plasmids (pLKO.1-HMGA1, TRCN0000018949), constructed as described previously (16), were obtained from The RNAi Consortium (Mission TRC Hs. 1.0, Sigma-Aldrich, St Louis, MO). The sequences of short hairpin RNA (shRNA) targeting the human *HMGA1* gene (Genbank accession no. NM\_002131)

were shHMGAI-1 plasmid, 5'-CAACTCCAGGAAGGAAACCAA-3' (coding region positions 446–466 of HMGAI mRNA transcript variant 2) and shHMGAI-2 plasmid, 5'-CCTTGGCCTCAAGCAGGAAA-3' (coding region positions 281–301 of HMGAI mRNA transcript variant 2). The control plasmid, which has a scrambled non-targeting shRNA sequence, was obtained from The RNAi Consortium. Each of these vectors had been sequence verified. Pooled stable transfectants were established using puromycin selection. Stable transfectant cells were maintained in medium containing 3 µg/mL puromycin (Invivogen, San Diego, CA).

**Expression vector and transfection.** The HMGAI coding sequence was PCR amplified from IMAGE clone 5399570 (Genbank accession no. BC063434) using gene-specific primers modified to include the appropriate restriction sites at their 5' end. The primers used were forward, 5'-TTTTGATATCATGAGTGAGTCGAGCTCGAAG-3' and backward, 5'-TTTTGAATTCTACTGCTCCTCTCCGAGGA-3'. Purified PCR products were digested with *EcoRV* and *EcoRI* before ligation into an *EcoRV/EcoRI*-digested pIRES-puro3 vector (Clontech, Palo Alto, CA). The expression plasmids were named pIRES-HMGAI. MiaPaCa2 cells were transfected with pIRES-HMGAI or empty pIRES-puro3, which acted as a control, using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. Stable clones were selected by exposure to incrementally increasing concentrations of puromycin (Invivogen), isolated using cloning cylinders, and maintained in medium containing 3 µg/mL puromycin (Invivogen). Clones pIRES-HMGAI.1 and pIRES-HMGAI.2, which expressed the highest levels of HMGAI, were used for further studies.

**MMP-9 gene promoter and luciferase assay.** The MMP-9 promoter reporter construct was custom-synthesized by Aviva Systems Biology (San Diego, CA). Briefly, the full-length human MMP-9 promoter region ranging from -992 to +304 bp, relative to the transcription initiation site, was amplified by PCR from human placental DNA, using proper primers designed according to the *MMP-9* gene sequence. The primers were forward, 5'-GGTACCTCTTCTGGGCTCAAGCAATC-3' and backward, 5'-CTCGAGCTAACCTGGACACCTCTGTTC-3'. The amplified promoter fragment with the addition of *XhoI* and *KpnI* restriction sites at each end was cloned into the pGL4.12 vector (Promega, Madison, WI) at the *XhoI/KpnI* site upstream of the firefly luciferase gene. By direct sequencing, the sequence of the cloned promoter region was confirmed. The empty pGL4.12 vector (pGL4e) was used as control.

Cells were plated onto a six-well cell culture plates at a concentration of  $5 \times 10^5$  per well and allowed to adhere for 12 hours in serum-containing medium. To assess the effects of modulating HMGAI expression on MMP-9 promoter activity, transient transfection experiments were done on MiaPaCa2 cells. For HMGAI suppression experiments, 5 µg shHMGAI or control shRNA vector was cotransfected with 5 µg pGL4-MMP9 or pGL4e and 0.5 µg pRLCMV vector (Promega), which contains a cytomegalovirus (CMV) promoter upstream of a *Renilla* luciferase gene. For HMGAI overexpression experiments, either pIRES-HMGAI or control vector pIRES-puro3 was used in cotransfection. After 48 hours, luciferase activity in lysates of the transfected cells was measured using the dual-luciferase reporter assay, according to the manufacturer's recommended protocol (Promega).

Relative luciferase activity was calculated as fold-induction of luciferase activity above the background (taken as activity associated with promoterless vector, pGL4.12). The activity of *Renilla* luciferase was used to normalize any variation in transfection efficiencies.

**Invasion assay.** Cellular invasion was quantified using a modified Matrigel Boyden chamber assay. The BD BioCoat Matrigel invasion chamber (BD Biosciences, Bedford, MA) was used according to the manufacturer's instructions. Pancreatic cancer cells ( $2.5 \times 10^4$ ) in serum-free media were seeded onto Matrigel-coated filters. In the lower chambers, 5% FBS was added as a chemoattractant. After 24 hours of incubation, the filters were stained using the Diff-Quik kit (BD Biosciences), and the number of cells that had invaded through the filter was counted under magnification (randomly selected high-power fields). The counting was done for 20 fields in each sample, and mean values from three independent experiments were calculated. In additional studies, invasion

assays were done in the presence of 10 mg/mL anti-MMP-9-neutralizing antibody (Santa Cruz, San Ramon, CA) or isotype-matched control (irrelevant) immunoglobulin (Santa Cruz).

**Proliferation assay.** Cell proliferation was quantified using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous One Solution Assay, Promega), in accordance to manufacturer's instructions and confirmed by cell counting. Logarithmically growing cells were seeded into 96-well plates at  $5 \times 10^3$  per well and allowed to adhere overnight in medium containing 10% or 2% FBS. Cell proliferation was determined after 48 hours. Plates were read with the use of the SpectraMax M5 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 490 nm. Ten samples were used for each experimental condition, and experiments were done in triplicate. At identical time points, cell counting was done. Cells were trypsinized to form a single-cell suspension. Viable cells, determined by trypan blue exclusion, were counted with the use of a Neubauer hemocytometer (Hausser Scientific, Horsham, PA).

**Fluorometric MMP-9 activity assay.** MMP-9 activities of cell or tumor lysates were assessed using the Enzolyte Plus 520 Enhanced Selectivity MMP-9 activity, in accordance with the manufacturer's instructions (AnaSpec, San Jose, CA). Fluorescence intensities were quantified using SpectraMax M5 microplate spectrophotometer (Molecular Devices) at excitation of 490 nm and emission of 520 nm.

**Quantitative real-time PCR.** Total RNA was extracted from cells using mirVana RNA isolation kit according to the manufacturer's recommendation (Ambion, TX). First-strand cDNA was synthesized from 5 µg of total RNA using the SuperScript III First-Strand Synthesis System, according to manufacturer's instructions (Invitrogen). Transcript analysis was done by quantitative real-time PCR using the Taqman assay, based on manufacturer's instructions (Applied Biosystems, Foster City, CA). Hybridization probe and primer sets for human MMP-9 (Hs00234579\_m1 MMP9) and  $\beta$ -actin were obtained from Applied Biosystems. Typically, thermal cycling was initiated with a denaturation step for 10 minutes at 95°C followed by 40 cycles done in two steps: for 15 seconds at 95°C and for 1 minute at 60°C. Reactions were done, and data were analyzed using the GeneAmp Sequence Detection System (Applied Biosystems). Results are expressed as the ratio of MMP9 to  $\beta$ -actin. Measured threshold cycles were converted to relative copy numbers using primer-specific standard curves.

**Western blotting.** Cells were harvested and rinsed twice with PBS. Cell extracts were prepared with lysis buffer [20 mmol/L Tris (pH 7.5), 0.1% Triton X-100, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 10 mg/mL leupeptin] and cleared by centrifugation at  $15,000 \times g$ , 4°C. Total protein concentration was measured using the bicinchoninic acid assay kit (Sigma, St Louis, MO) with bovine serum albumin as a standard, according to the manufacturer's instructions. Cell extracts containing 50 µg total protein were subjected to 10% SDS/PAGE, and the resolved proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Invitrogen). Equal protein loading was confirmed by Coomassie (Bio-Rad, Hercules, CA) staining of the gel. After blocking with PBS containing 3% bovine serum albumin for 1 hour at room temperature, membranes were incubated with 3 to 5 mg/mL antibody in PBS containing 0.1% Tween 20 overnight at 4°C. Chemiluminescent detection (Amersham Biosciences, Piscataway, NJ) was done in accordance with the manufacturer's instructions. The densitometric signal was quantified using ImagePro Plus software version 4.0 and normalized to that of actin. Blots were done in triplicate. Mean  $\pm$  SD densitometric values are shown.

**Nude mouse orthotopic xenograft model.** Male athymic *nu/nu* mice 5 weeks of age, weighing 20 to 22 g, and specific pathogen-free were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed in microisolator cages in a specific pathogen-free facility with 12-hour light/dark cycles. They received water and food *ad libitum*. Animals were observed for signs of tumor growth, activity, feeding, and pain in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals.

To determine the effect of *HMGAI* gene silencing on *in vivo* metastasis, MiaPaCa2 cells stably expressing the control or HMGAI shRNA

(shHMGA1-1) constructs were orthotopically implanted into the pancreata of nude mice. Mice were anesthetized with i.p. ketamine (200 mg/kg) and xylazine (10 mg/kg). In a laminar-flow hood, the abdomen was cleaned with isopropyl alcohol, and a left upper transverse incision was made. The pancreas was exposed, and  $1 \times 10^6$  cells suspended in 75  $\mu$ L PBS were slowly injected into the body of the pancreas. The pancreas was returned, and the abdomen was closed with 5-0 Vicryl (Ethicon, Somerville, NJ). Ten mice for each cell line were observed over 4 weeks and killed by overdose of ketamine (400 mg/kg) and xylazine (50 mg/kg). The liver of each animal was harvested, and metastatic foci were counted under a dissecting microscope (17). The primary tumors were excised, and the tumor lysates were assayed for HMGA1 expression and MMP-9 activity using Western blotting and MMP-9 activity assay, respectively.

**Immunohistochemistry.** Tumor sections (5  $\mu$ m) were deparaffinized, rehydrated through graded alcohol, and processed using a streptavidin-biotin-peroxidase complex method. Antigen retrieval was done by microwave heating sections in 10 mmol/L sodium citrate buffer (pH 6) for 10 minutes. Following quenching of endogenous peroxidase activity and blocking of nonspecific binding, sections were incubated with anti-HMGA1 (Santa Cruz) at 4°C overnight at a 1:50 dilution. The secondary antibody was biotinylated rabbit anti-goat antibody (DAKO, Carpinteria, CA) used at a dilution of 1:200 for 30 minutes at 37°C. After further washing with TBS, sections were incubated with StrepABCComplex/horseradish peroxidase (1:100; DAKO) for 30 minutes at 37°C. Immunolocalization was done by exposure to 0.05% 3,3'-diaminobenzidine tetrahydrochloride as the chromogen. Normal serum was used in place of the primary antibody as a negative control. Slides were counterstained with hematoxylin before dehydration and mounting. For H&E staining, standard procedures were used to stain the tumor sections.

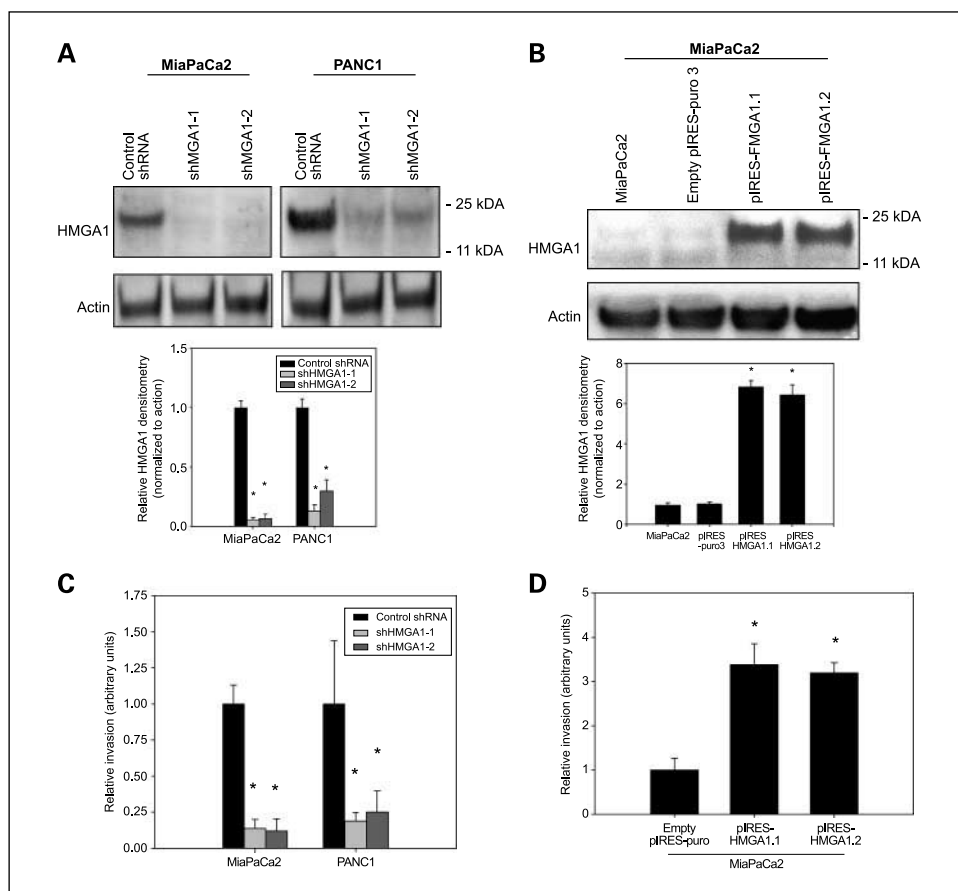
**Statistical analysis.** Differences between groups were analyzed using Student's *t* test, multifactorial ANOVA of initial measurements, and Mann-Whitney *U* test, for nonparametric data, as appropriate, using Statistica 5.5 software (StatSoft, Inc., Tulsa, OK). In cases in which averages were normalized to controls, the SDs of each nominator and denominator were taken into account in calculating the final SD. *P* < 0.05 was considered statistically significant.

## Results

**Effect of HMGA1 gene silencing on cellular invasiveness.** We analyzed HMGA1 gene expression using Western blot analysis. Cellular invasiveness was quantified using Matrigel-coated Boyden chambers. We used two shRNA-expressing plasmids with different target sequences to induce HMGA1 gene silencing. MiaPaCa2 and PANC1 cell lines were stably transfected with each of the HMGA1-targeting shRNA expression vectors (shHMGA1-1 and shHMGA1-2). The shRNA approach was associated with high efficacies in HMGA1 silencing (up to 90% silencing, as confirmed on Western blot analysis; Fig. 1A). Silencing of HMGA1 significantly attenuated cellular invasiveness in both MiaPaCa2 and PANC1 cells (Fig. 1C). For these experiments, controls were cells stably transfected with a vector encoding a non-targeting shRNA.

**Effect of HMGA1 overexpression on cellular invasiveness.** Next, we sought to determine the effect of HMGA1 overexpression on cellular invasiveness. Using MiaPaCa2 cells, which have relatively low levels of inherent HMGA1 expression, we developed the clones pIRES-HMGA1.1 and pIRES-HMGA1.2, which stably overexpress HMGA1. Overexpression of HMGA1 was confirmed on

**Figure 1.** A, stable silencing of HMGA1 expression using shRNA expression vectors with two independent shRNA target sequences (shHMGA1-1 and shHMGA1-2) was confirmed on Western blot analysis. Controls were cells stably transfected with non-targeting, scrambled shRNA. Greater suppression of HMGA1 expression was achieved in MiaPaCa2 cells (up to 90%) compared with PANC1 cells, which have HMGA1 silenced to a lesser degree. \*, *P* < 0.05 versus control shRNA. B, two clones of MiaPaCa2 cells were confirmed to stably overexpress HMGA1 (pIRES-HMGA1.1 and pIRES-HMGA1.2) on Western blot analysis. Controls were cells stably transfected with empty pIRES-puro3 vector. \*, *P* < 0.05 versus empty pIRES-puro3 vector and parental MiaPaCa2 cells. Representative blots of three independent experiments. Columns, mean densitometry values; bars, SD. C, cellular invasiveness was determined using Matrigel-coated Boyden chamber assays. Stable suppression of HMGA1 expression resulted in significant reductions in cellular invasiveness in both MiaPaCa2 and PANC1 cells. \*, *P* < 0.05 versus control shRNA. D, forced overexpression of HMGA1 in MiaPaCa2 cells (pIRES-HMGA1.1 and pIRES-HMGA1.2) resulted in ~3-fold increase in cellular invasive capacity when compared with empty pIRES-puro3 transfectants. \*, *P* < 0.05 versus empty pIRES-puro3 vector. Invasion assays were done in triplicate. Columns, mean from 20 randomly selected fields; bars, SD.



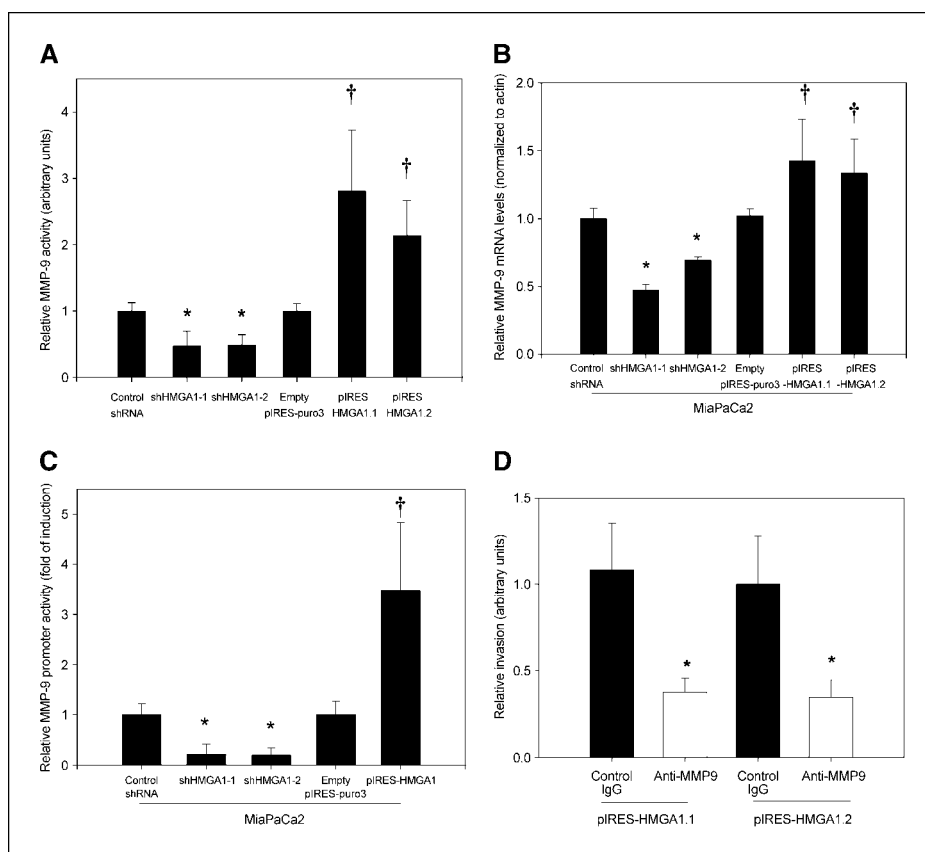
Western blot analysis (Fig. 1B). MiaPaCa2 cells stably transfected with empty pIRES-puro3 vector served as controls. HMGA1 expression in parental MiaPaCa2 cells and empty pIRES-puro3 transfectants did not differ (Fig. 1B). pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited ~3- to 3.5-fold increases in cellular invasiveness, compared with control cells (Fig. 1D).

**Effects of modulating HMGA1 expression on MMP-9 activity, mRNA expression, and promoter activity.** Given the importance of MMP-9 as a mediator of malignant cellular invasiveness and metastasis (18–20), we hypothesized that MMP-9 is a downstream effector that plays a critical role in HMGA1-dependent cellular invasiveness. HMGA1 silencing in MiaPaCa2 cells was found to be associated with a reduction in total MMP-9 activities (Fig. 2A). In addition, HMGA1 silencing led to reductions in MMP-9 mRNA levels as quantified by real-time quantitative reverse transcription-PCR (Fig. 2B). Conversely, MMP-9 activities and mRNA levels were significantly higher in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones than in controls (Fig. 2A and B). To assess the effect of modulating

HMGA1 expression on MMP-9 promoter activity, we did cotransfection experiments in which MiaPaCa2 cells were transiently transfected with either shHMGA1 or pIRES-HMGA1 vector together with MMP-9 promoter reporter vector. MMP-9 promoter activities were significantly lower with HMGA1 silencing, whereas MMP-9 promoter activity levels were markedly higher with HMGA1 overexpression, compared with controls (Fig. 2C).

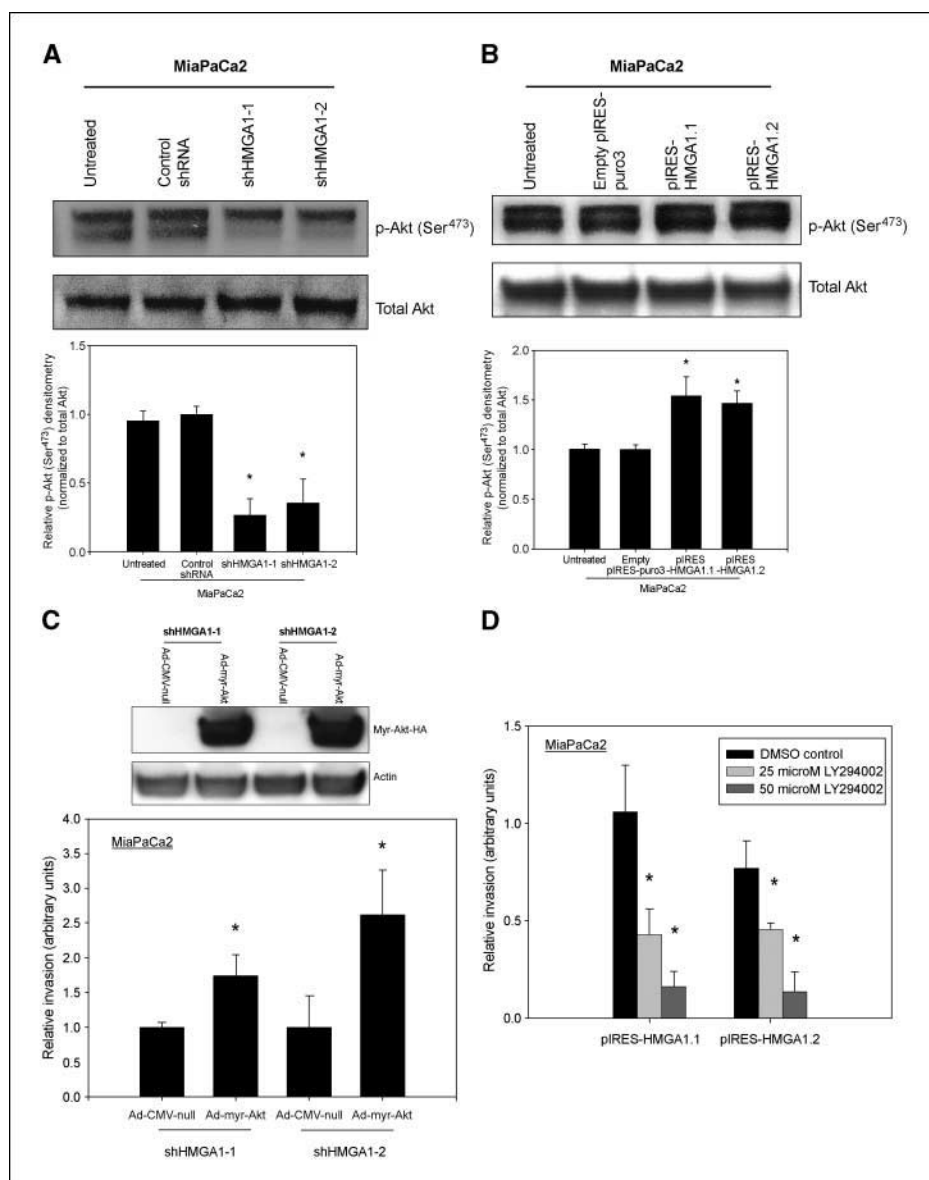
**MMP-9 is a mediator of HMGA1-dependent increases in cellular invasiveness.** The contribution of MMP-9 to the increased cellular invasiveness induced by HMGA1 overexpression was determined by performing the invasion assay in the presence of anti-MMP-9-neutralizing antibody. MMP-9 immunoneutralization reduced the cellular invasiveness of MiaPaCa2 pIRES-HMGA1.1 and pIRES-HMGA1.2 clones by ~70% (Fig. 2D).

**Effects of HMGA1 modulation on Akt activation.** Activation of the serine/threonine kinase Akt is common in pancreatic adenocarcinoma (21) and is an important determinant of malignant cellular invasiveness (22). The PI3K/Akt signaling



**Figure 2.** HMGA1 expression positively regulates MMP-9 activities, mRNA levels, and promoter activities. **A**, cellular MMP-9 activities were quantitated using a fluorometric MMP-9 activity assay. Targeted suppression of HMGA1 expression (shHMGA1-1 and shHMGA1-2) resulted in significant reductions in MMP-9 activity in MiaPaCa2 cells, whereas forced overexpression of HMGA1 led to increased cellular MMP-9 activity. Both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited ~2- to 2.5-fold increases in MMP-9 activity. \*,  $P < 0.05$  versus control shRNA; †,  $P < 0.05$  versus empty pIRES-puro3 transfectants. **B**, HMGA1 modulates MMP-9 mRNA levels. On quantitative real-time PCR, MMP-9 levels were significantly lower in shHMGA1-1 and shHMGA1-2 transfectants compared with control shRNA transfectants. Overexpression of HMGA1 led to increases in MMP-9 mRNA levels. \*,  $P < 0.05$  versus control shRNA; †,  $P < 0.05$  versus empty pIRES-puro3 transfectants. **C**, the effects of HMGA1 silencing and overexpression on MMP-9 promoter activities in MiaPaCa2 cells were determined using transient transfection and luciferase reporter assays. Cotransfection experiments were done. MiaPaCa2 cells were transiently transfected with shRNA (shHMGA1-1, shHMGA1-2, or control shRNA) or overexpression vectors (pIRES-HMGA1 or empty pIRES-puro3 control) together with the reporter plasmids. Reporter plasmids included pGL4e (empty firefly luciferase vector) or pGL4-MMP9, containing the full-length MMP9 promoter and pRL vector containing a *Renilla* luciferase gene. Forty-eight hours following transfection, luciferase activity in cell lysates was assayed. *Renilla* luciferase activities were used to normalize results for transfection efficiencies. Consistent with MMP-9 activity and mRNA studies, targeted suppression of HMGA1 expression using shRNA resulted in a significant reduction in MMP-9 promoter activities, whereas forced overexpression of HMGA1 in MiaPaCa2 cells led to significant increases in MMP-9 promoter activities. \*,  $P < 0.05$  versus control shRNA; †,  $P < 0.05$  versus empty pIRES-puro3 vectors. **D**, the contribution of MMP-9 to the increased cellular invasiveness induced by HMGA1 overexpression was determined by performing the invasion assay in the presence of anti-MMP-9-neutralizing antibody. MMP-9 immunoneutralization reduced the cellular invasiveness of pIRES-HMGA1.1 and pIRES-HMGA1.2 clones by ~70%.

**Figure 3.** The effects of HMGA1 modulation on Akt phosphorylation in MiaPaCa2 cells were confirmed on Western blot analysis using a phospho-specific antibody. *A*, in MiaPaCa2 cells, stable silencing of HMGA1 resulted in a decreased level of phosphorylation of Akt at Ser<sup>473</sup>, a marker of Akt activation. \*,  $P < 0.05$  versus MiaPaCa2 cells and control shRNA transfectants. *B*, overexpression of HMGA1 led to increased activation of Akt, as evident from increased levels of phospho-Akt (*p*-Akt). There was no difference in the level of expression of total Akt with suppression or overexpression of HMGA1. \*,  $P < 0.05$  versus MiaPaCa2 cells and empty pIRES-puro3 transfectants. Representative blots of three independent experiments. *Columns*, mean densitometry values; *bars*, SD. *C*, contribution of Akt to HMGA1-mediated invasiveness was assessed by transductions of MiaPaCa2 shHMGA1-1 and shHMGA1-2 transfectants with adenovirus expressing constitutively active Akt (Ad-myr-Akt). Constitutively active Akt was able to rescue the invasive phenotype in the MiaPaCa2 cells with HMGA1 silencing. Cell lysates were immunoblotted with anti-HA to detect expression of HA-tagged myristoylated Akt. \*,  $P < 0.05$  versus MiaPaCa2 transduced with control adenovirus (Ad-CMV-null). *D*, inhibition of PI3K with LY294002 attenuated the HMGA1-induced increases in invasiveness in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones. The effects of PI3K were dose dependent (25 and 50  $\mu\text{mol/L}$ ). \*,  $P < 0.05$  versus DMSO controls.



pathway has previously been reported to be important in the regulation of MMP-9 expression (23–25). Given our observations that HMGA1 expression regulates MMP-9 activity via transcriptional activation, we hypothesized that HMGA1 modulates Akt activation. We found that HMGA1 silencing results in a reduction in Akt phosphorylation at Ser<sup>473</sup> (Fig. 3A), whereas HMGA1 overexpression results in increased levels of phospho-Akt (Ser<sup>473</sup>; Fig. 3B). Modulating HMGA1 expression had no effect on total Akt levels.

**HMGA1-induced cellular invasiveness and MMP-9 activity is PI3K/Akt dependent.** Given our findings that HMGA1 modulates Akt activation, we tested whether cellular invasiveness mediated by HMGA1 is dependent on PI3K/Akt signaling. First, we determined if the invasive phenotype in MiaPaCa2 cells in which HMGA1 had been stably silenced could be rescued using constitutively active Akt. MiaPaCa2 stable transfectants shHMGA1-1 and shHMGA1-2 were infected with adenovirus expressing myristoylated Akt (Ad-myr-Akt). Constitutively active Akt was able to rescue the invasive phenotype in shHMGA1-1 and shHMGA1-2 transfectants resulting

in an increased invasiveness compared with cells infected with control adenovirus (Fig. 3C). Next, given that PI3K is an upstream regulator of Akt, we assessed the effects of PI3K inhibition on cellular invasiveness in MiaPaCa2 cells overexpressing HMGA1 (pIRES-HMGA1.1 and pIRES-HMGA1.2). Inhibition of PI3K activity using the PI3K inhibitor LY294002 resulted in dose-dependent reductions in cellular invasiveness in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Fig. 3D). Consistent with this finding, infection with adenovirus expressing dominant-negative Akt resulted in attenuation of HMGA1-induced invasiveness in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones. Importantly, dominant-negative Akt also induced reductions in cellular MMP-9 activity in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones, indicating that HMGA1-induced MMP-9-dependent cellular invasiveness is PI3K/Akt dependent (Fig. 4A).

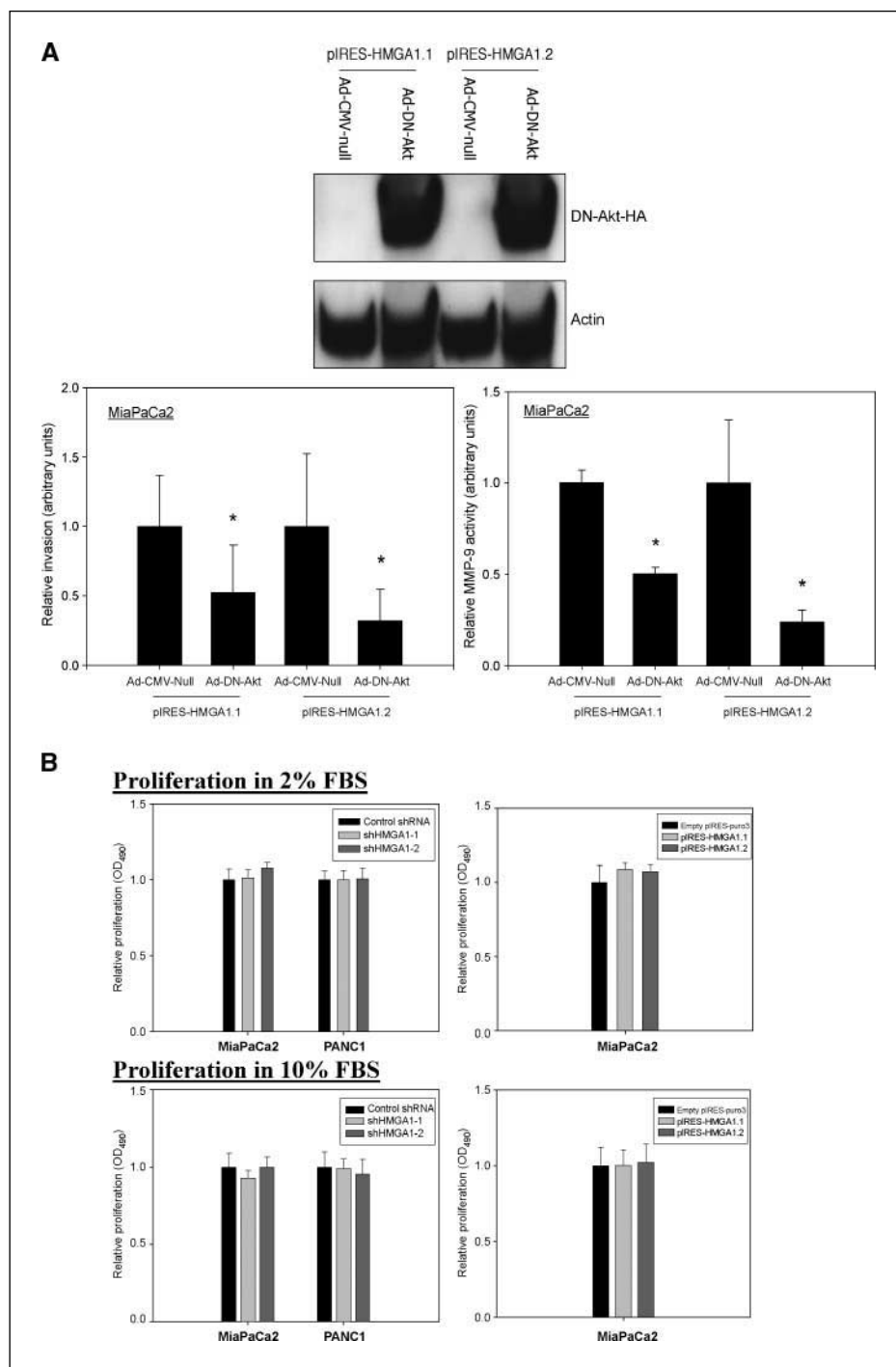
**Modulation of HMGA1 expression has no effect on cellular proliferation in monolayer culture.** The effects of modulating HMGA1 expression on cellular proliferation in monolayer culture were determined. Using the MTS assay, we observed no effect in

cellular proliferation in low (2% FBS) and high (10% FBS) serum conditions with either *HMG1A1* gene silencing or overexpression (Fig. 4B).

**Effects of HMG1A1 modulation on phosphorylation of ERK and mTOR.** Previous studies have shown that HMG1A1 modulates ERK activation (26). In our study, HMG1A1 silencing had no effect on ERK phosphorylation, whereas HMG1A1 overexpression resulted in increased ERK phosphorylation with no effects on the total ERK levels (Fig. 5A and B). Given that mTOR is a well-known downstream mediator of the PI3K/Akt pathway (27, 28), we sought to determine the effects of HMG1A1 modulation of mTOR

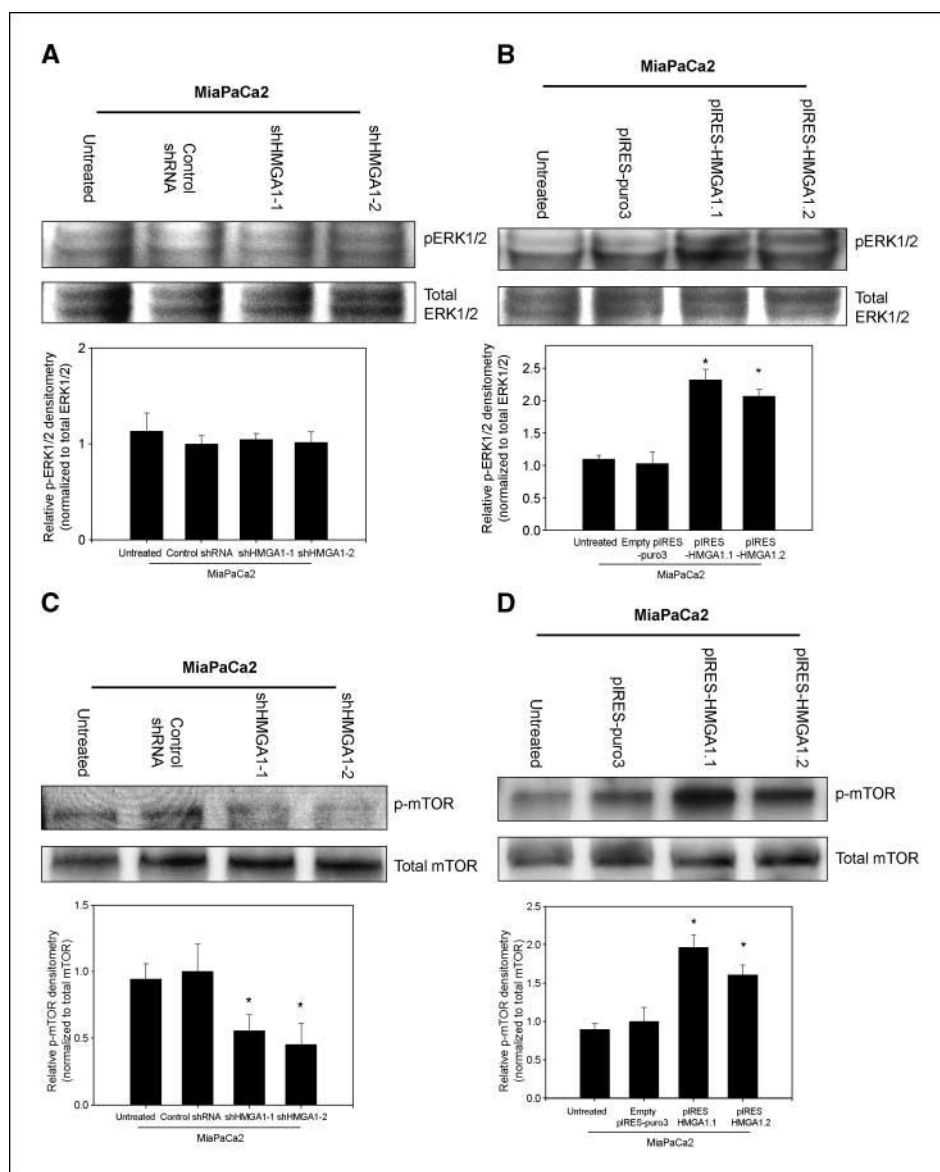
phosphorylation. HMG1A1 silencing resulted in reductions in mTOR phosphorylation at Ser<sup>2448</sup>, whereas HMG1A1 overexpression led to increases in mTOR phosphorylation (Fig. 5C and D). Modulation of HMG1A1 expression had no effect on levels of total mTOR expression.

**HMG1A1 silencing suppresses *in vivo* metastatic potential of pancreatic cancer cells and reduces tumoral growth and MMP-9 activity.** Given these *in vitro* findings, we sought to determine the effect of modulating HMG1A1 expression on metastatic potential *in vivo*. Four weeks following surgical orthotopic implantation of MiaPaCa2 stable transfectants expressing control



**Figure 4.** A, dominant-negative Akt attenuates HMG1A1-induced invasiveness and MMP-9 activity. MiaPaCa2 pIRES-HMGA1.1 and pIRES-HMGA1.2 clones were transduced with adenovirus expressing dominant-negative Akt (Ad-DN-Akt). Dominant-negative Akt resulted in reductions in invasiveness and in MMP-9 activities in both HMG1A1-overexpressing clones compared with control adenovirus. Cell lysates were immunoblotted with anti-HA to detect expression of HA-tagged DN Akt. \*,  $P < 0.05$  versus control adenovirus (Ad-CMV-null). B, based on MTS assay, modulation of HMG1A1 expression did not affect cellular proliferation in monolayer culture. Experiments were done at low (2% FBS) and high (10%) conditions.

**Figure 5.** The effects of HMGA1 modulation on ERK and mTOR phosphorylation in MiaPaCa2 cells were confirmed on Western blot analysis using a phospho-specific antibody. *A* and *B*, although HMGA1 silencing did not alter the levels of ERK phosphorylation, overexpression of HMGA1 increased ERK phosphorylation. There were no differences in the levels of total ERK. *C* and *D*, targeted suppression of HMGA1 expression resulted in reductions in phosphorylation of mTOR at Ser<sup>2448</sup> in both shHMGA1-1 and shHMGA1-2 transfectants, whereas overexpression of HMGA1 led to increases in phospho-mTOR levels. There was no difference in total mTOR levels among groups. \*,  $P < 0.05$  versus control shRNA or empty pIRES-puro3 transfectants, as appropriate.



shRNA ( $n = 10$ ) or shHMGA1 ( $n = 10$ ), necropsy was done. All mice in the control shRNA group were found to have histologically confirmed liver metastases, and six of these mice were found to have gross ascites. In the shHMGA1 group, only one mouse developed metastases (Table 1;  $P < 0.05$ ), and none of them had ascites. No lung metastases were detected in either group. There were no apparent differences in the histologic appearance of H&E-stained sections of xenografts harvested from animals in the shHMGA1 and control groups (Fig. 6A). Stable silencing of tumoral HMGA1 expression in the shHMGA1 group *in vivo* was confirmed by immunohistochemistry (Fig. 6A) and Western blotting (Fig. 6B). Additionally, HMGA1 silencing was associated with significant reductions in tumor growth and MMP-9 activity (Fig. 6C and D).

## Discussion

Pancreatic adenocarcinoma is among the most aggressive of human malignancies. The prognosis associated with this cancer remains dismal, despite the considerable advances in medical and

surgical management of patients diagnosed with pancreatic cancer. There is an urgent need to identify new therapeutic approaches. This study was designed to determine the role of HMGA1 in pancreatic adenocarcinoma cellular invasiveness and metastasis. We have shown that HMGA1 overexpression promotes cellular invasiveness, and that specific suppression of HMGA1 expression inhibits cellular invasiveness *in vitro* and metastasis *in vivo*. HMGA1-induced cellular invasiveness is in part due to PI3K/Akt-dependent modulation of MMP-9 activity. Together, these findings suggest that HMGA1 architectural transcriptional factors represent a molecular determinant of cellular invasiveness and a potential therapeutic target in pancreatic adenocarcinoma.

HMGA1 previously has been reported to be associated with malignant cellular behavior in a range of human cancers (11, 29, 30). HMGA1 has been shown to confer the ability of non-tumorigenic breast epithelial cells to grow under anchorage-independent conditions (31). Antisense oligonucleotide-mediated suppression of HMGA1 expression has been reported to inhibit neoplastic transformation in breast cancer (32) and in Burkitt's

**Table 1.** HMGA1 gene silencing by RNA interference suppresses the metastatic potential of pancreatic adenocarcinoma cells *in vivo*

	Control shRNA (n = 10)	shHMGA1 (n = 10)
Median no. metastases (range)	6 (1–20)	0* (0–3)
Mice with metastases	100%	10%*

NOTE: At 4 weeks following orthotopic implantation of  $1 \times 10^6$  MiaPaCa2 cells stably expressing control or shHMGA1 constructs, necropsy was done. Liver metastases were counted and confirmed histologically. HMGA1 silencing significantly inhibited metastasis in this nude mouse model.

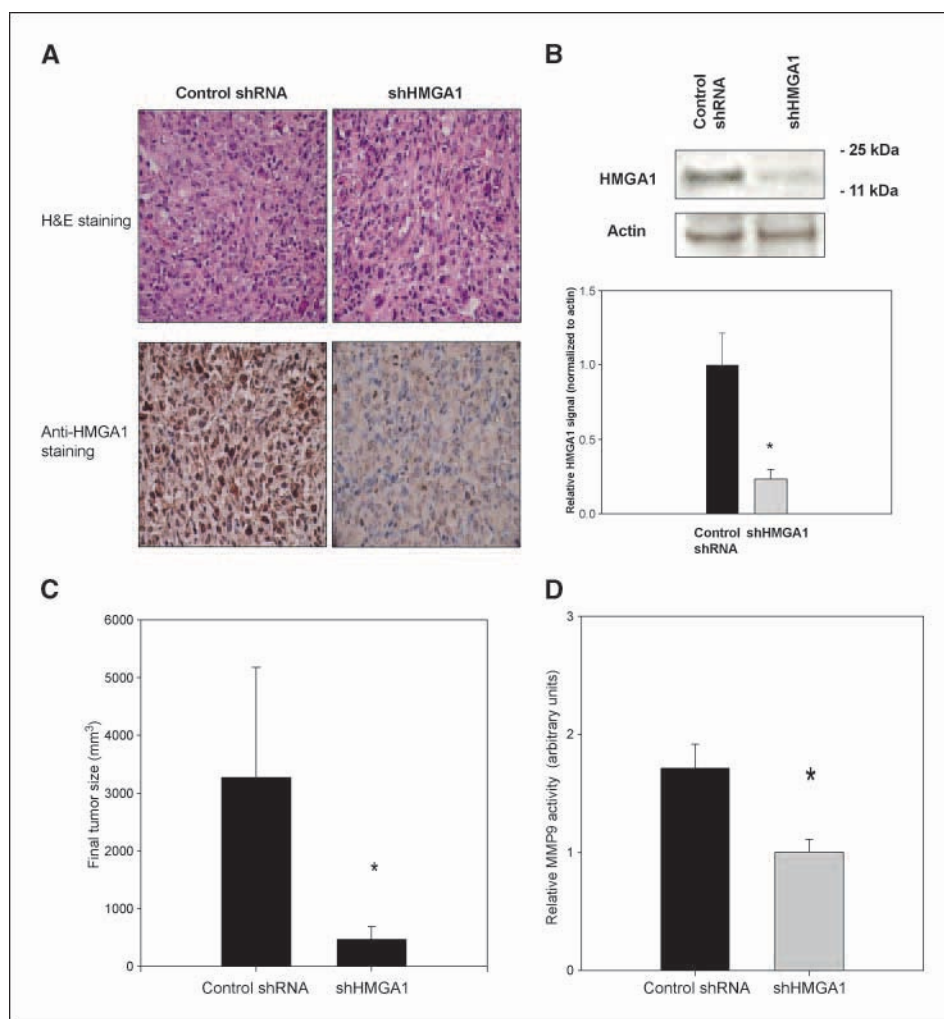
\* $P < 0.05$  versus control shRNA group.

lymphoma cells (33). Although there is extensive correlative evidence suggesting a role for HMGA1 in tumor metastasis (9, 34, 35), there have been few studies showing a direct functional link between HMGA1 expression and invasion and metastasis (14, 30, 31).

Our study is the first to show a role for HMGA1 in pancreatic adenocarcinoma cellular invasiveness and metastasis and provides

evidence that HMGA1 expression mediates cellular invasiveness through a PI3K/Akt/MMP-9–dependent pathway. Although MMP-9 is unlikely to be the sole effector of HMGA1-dependent invasiveness, this pathway represents a potential mechanism that may contribute to the decrease in cellular invasiveness induced by HMGA1 gene silencing. Our finding that PI3K/Akt signaling mediates regulatory actions of HMGA1 is novel. Our finding that HMGA1 promotes mTOR activation provides further support for the relationship between HMGA1 and PI3K/Akt signaling, as mTOR is a downstream target of this pathway (27, 28).

Our finding that HMGA1 overexpression results in increased ERK phosphorylation is consistent with findings of previously reported studies suggesting that HMGA1 is able to positively regulate the Ras/ERK mitogenic signaling pathway (26). The Ras/ERK signaling pathway is intrinsically linked to PI3K/Akt pathways. PI3K/Akt has been shown to be downstream of Ras/ERK signaling pathway (36, 37), and Ras can also directly activate the PI3K/Akt signaling pathway (38). Alternatively, HMGA1 has been shown to transcriptionally regulate the human insulin receptor gene (39, 40), and given that PI3K/Akt are downstream mediators of insulin signaling (41), it is not surprising that HMGA1 expression affects PI3K/Akt signaling. Both Ras/ERK and PI3K/Akt signaling pathways have been found to be critical in mediating cellular invasion in pancreatic cancer cells (42).



**Figure 6.** MiaPaCa2 stable transfectant cells expressing the control shRNA or shHMGA1 (shHMGA1-1) were orthotopically implanted into the pancreata of nude mice. Four weeks following implantation, necropsy was done, and the primary tumors were explanted. *A*, histologic appearance of primary tumors was examined at  $\times 400$  magnification following H&E staining of tumor sections. There was no apparent morphologic difference in tumors harvested from the two groups of animals. However, sections stained with anti-HMGA1 antibody revealed that tumors from shHMGA1 group exhibited reductions in nuclear staining for HMGA1. *B*, stable HMGA1 suppression in lysates of shHMGA1-derived tumors was further confirmed by Western blot. *C*, final volume of primary tumors harvested from shHMGA1 group was markedly reduced compared with those of the control shRNA group. *D*, MMP-9 activities in lysates obtained from HMGA1 shRNA transfectant-derived tumors were significantly lower than those of control shRNA transfectant-derived tumors. Columns, mean; bars, SD. \*,  $P < 0.05$  versus control shRNA transfectant-derived tumors.



Our observation that HMGA1 silencing suppresses Akt activity is significant, as Akt is now recognized as an important mediator of malignant cellular behavior, including the capacity for resisting apoptotic stimuli, in pancreatic adenocarcinoma (43–45). Trapasso et al. have reported that antisense-mediated suppression of HMGA1 expression results in an apoptotic response in three pancreatic cancer cell lines (46). Our findings that HMGA1 positively regulates PI3K/Akt signaling provides a possible mechanism through which HMGA1 suppression promotes apoptosis. Unlike Trapasso et al., we found that modulating HMGA1 expression has no effect on cellular proliferation in two-dimensional monolayer culture. However, our data indicate that HMA1 silencing does inhibit tumor growth *in vivo*.

Our study provides support to the hypothesis that the HMGA1 proteins are potential therapeutic targets for inhibiting the activation of the PI3K/Akt pathway in cancer cells. From a therapeutic standpoint, targeting HMGA1 is attractive in that it is overexpressed in a range of human malignancies. HMGA1 expression is absent or present at only very low levels in normal adult tissues (47). As such, targeting HMGA1 may have little or no effect on non-cancerous

tissues. Given our finding that RNA interference-mediated HMGA1 silencing inhibits invasive and metastatic potential, HMGA1 represents a rational molecular therapeutic target. The feasibility of *in vivo* gene silencing induced by the delivery of therapeutic small interfering RNA (siRNA) has already been shown (48). Technological advances, such as the development of improved delivery systems for siRNAs, will facilitate this approach.

In summary, our findings suggest that HMGA1 promotes pancreatic cancer cellular invasive and metastatic potential. Our findings also indicate that HMGA1 represents a potential therapeutic target for strategies designed to inhibit the progression of pancreatic cancer.

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## HMGA1 Is a Determinant of Cellular Invasiveness and *In vivo* Metastatic Potential in Pancreatic Adenocarcinoma

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