Suppression of the uPAR-uPA System Retards Angiogenesis, Invasion and in vivo Tumor Development in Pancreatic Cancer Cells

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Running Title: uPAR-uPA suppression retards pancreatic cancer progression

Key words: uPA, uPAR, Lhx-2, angiogenesis, invasion, pancreatic cancer, transcription factor

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This work was supported by Grant #09-28 from the American Cancer Society, Illinois Division, Inc. to CSG.
ABSTRACT

Despite existing chemotherapy and surgical resection strategies, pancreatic cancer is one of the major causes of mortality in the United States with a 5-year mean survival of less than 5%. The activation of the uPAR-uPA system in the development of pancreatic ductal adenocarcinoma has been well established. In the present study, we used two pancreatic cancer cell lines, MIA PaCa-2 and PANC-1 to demonstrate the effects of uPAR and uPA down regulation. From the results, we observed that RNAi expressing plasmids efficiently down regulated mRNA and protein expression of uPAR and uPA. *In vitro* and *in vivo* angiogenic assays revealed a significant decrease in the angiogenic potential of MIA PaCa-2 and PANC-1 cells that were down regulated for both uPAR and uPA. From the angiogenesis antibody array analysis, we observed that the simultaneous down regulation of uPAR and uPA resulted in the down regulation of angiogenin and over expression of RANTES. Further, FACS analysis demonstrated that the simultaneous down regulation of uPAR and uPA caused the accumulation of cells in the subG₀/₁ phase in both MIA PaCa-2 and PANC-1 cells. In addition, western blot analysis revealed that down regulation of uPAR and uPA caused the activation of caspase 8 and CAD, which is indicative of apoptosis, *in vivo* TUNEL assay confirmed these results. Finally, we observed the nuclear localization of uPA and that uPA interacts with the transcription factor Lhx-2. Taken together, the results of the present study demonstrate that the targeting of the uPAR-uPA system has therapeutic potential.
INTRODUCTION

Each year about 37,000 individuals in the United States are diagnosed with pancreatic cancer, and more than 30,000 die from the disease. Depending on the size of the tumor at the time of diagnosis, prognosis is generally poor with less than 5 percent still alive five years after diagnosis, and complete remission is extremely rare (1). Treatment of pancreatic cancer depends on the stage of the cancer. Gemcitabine was approved by the FDA in 1998 after a clinical trial reported improvements in quality of life in patients with advanced pancreatic cancer; this marked the first FDA approval of a chemotherapy drug for a non-survival clinical trial endpoint (2). Patients diagnosed with pancreatic cancer typically have a poor prognosis partly because the cancer usually does not cause symptoms at earlier stages, which leads to locally advanced or metastatic disease by the time of diagnosis. Median survival is 3 to 6 months after diagnosis and as mentioned earlier, the five-year survival rate is less than 5 percent (3). Although it accounts for only 2.5 percent of new cases, pancreatic cancer is responsible for 6 percent of cancer deaths each year (4). The survival rate (3) and high recurrence after surgery clearly demonstrate the need for novel therapies (5).

Importance of the uPAR-uPA system in pancreatic cancers

Recent studies have suggested that the expression levels of uPA and fibroblastic uPAR are correlated with liver metastasis of human pancreatic cancer (6), indicating the relevance of specifically targeting the uPAR-uPA system. Other studies have demonstrated similar results and have shown the importance of the uPAR-uPA system in pancreatic carcinoma cell migration (7). Invasion studies also suggest that uPAR is a potential target for therapy in patients with pancreatic cancer (1, 6-10). In addition, studies have demonstrated the influence of the IGF (insulin-like growth factor) system on tumor progression from benign mucinous cystadenomas.
(BMC) to pancreatic ductal adenocarcinoma (PDAC) (11). However, the uPAR-uPA system had a greater influence on survival of PDAC (11), which highlights the importance of targeting the uPAR-uPA system.

**uPAR-uPA associated complex**

Malignant tumors have the capacity to degrade the extracellular matrix (ECM) by controlled proteolysis. One proteolytic system involved in these processes is the urokinase-type plasminogen activator (uPA) system, which consists of uPA, the uPA receptor (uPAR) and uPA inhibitors 1 and 2 (PAI-1 and PAI-2). A growing body of evidence suggests that the uPA system promotes tumor metastasis by several different mechanisms and not solely through the breakdown of the ECM (12). Initially, uPAR was thought to function simply as a mechanism to concentrate the urokinase/plasmin system near the cell surface. However, extensive evidence has since shown that this glycolipid-anchored receptor also functions in both the adhesive and signaling pathways of many migratory cells. One mechanism through which uPAR directs these actions is by complexing with other membrane proteins (e.g., integrins) for signal transduction. There are three basic steps involved in invasion and intracellular signaling: (1) uPAR-uPA promotes extracellular proteolysis by regulating plasminogen activation, (2) uPAR-uPA regulates cell(ECM) interactions as an adhesion receptor for vitronectin (Vn) and through its capacity to modulate integrin function, and (3) uPAR-uPA regulates cell migration as a signal transduction molecule and by its intrinsic chemotactic activity (13).

Recent studies point to important structural features of uPAR-integrin interactions, suggesting uPAR is a cis-acting integrin ligand (14). Although uPAR transcripts are induced by adhesion, rapid synthesis of the protein uses constitutive mRNA without a requirement for new transcription and is regulated by mammalian target of rapamycin, demonstrating new biologic
roles for the signal-dependent translation pathway controlled by this intracellular kinase (15). Integrins are known to activate PI3K and MEK pathway, and higher activation of Akt is associated with increased phosphorylation of glycogen synthase kinase 3β (GSK3β), FKHR and mTOR (16), indicating a possible feedback mechanism. Researchers have demonstrated that urokinase-induced signaling in human vascular smooth muscle cells is mediated by PDGFR-β (17), which demonstrates its multifaceted role in signaling. In the present study, we further delineated the complex role of uPAR and its associated molecules in activating apoptosis in pancreatic cancer.

MATERIALS AND METHODS

Cell lines and culture conditions: MIA PaCa-2 and PANC-1 cells were obtained from ATCC and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS in a humidified 5.0% CO2 atmosphere at 37.2°C with media changes every 48 hrs.

Tissue array: Human pancreatic cancer tissue arrays were obtained from US Biomax, Inc. (Rockville, MD). The tissue arrays consisted of pancreatic cancer and normal tissues with stage and grade information of 101 cases, consisting of 208 cores (PA2082-024 and -025). H&E stained core images are available from the manufacturer at [http://www.biomax.us/tissue-arrays/Pancreas/PA2082](http://www.biomax.us/tissue-arrays/Pancreas/PA2082). Tissue arrays were processed for immuno-histochemistry as per standard protocol. Briefly, slides were deparaffinized and endogenous peroxidases activity was blocked at room temperature by 5-10 min incubation in 0.3% H2O2 in PBS (pH 7.7). The slides were then rinsed in PBS for 5 min followed by antigen retrieval by heating to about 95°C in citrate buffer (0.01M sodium citrate buffer, pH 6.0) for 10 min. The tissues were blocked using normal goat serum for 20 min at room temperature and followed by addition of primary antibody
(anti-uPA or anti-uPAR) for 60 min at room temperature. The slides were then rinsed in PBS followed by the addition of a HRP-conjugated secondary antibody for 20 min at room temperature. HRP substrate DAB (3,3’-diaminobenzidine tetrahydrochloride) at 1 mg/mL in 50 mM Tris, pH 7.2 and 0.3% H₂O₂ was then added for the development of DAB substrate. Next, the slides were dehydrated and mounted with cover slips. Transmitted light images were obtained after hematoxylin staining, to determine uPAR and uPA expression levels.

**Construction of shRNA expressing plasmid:** shRNA expressing plasmids were constructed as described previously (18, 19).

**RT-PCR analysis:** MIA PaCa-2 and PANC-1 cells were transfected with pSV, puPA, puPAR, or pU2 for 72 hrs. The cells were collected and total cell RNA was isolated. Reverse transcriptase PCR was set up using primers specific for uPAR and uPA (Table 1). The PCR cycle was: 95°C-5', (95°C-30 sec, 65°C-1' 72°C-1') x 30, 72°C-10'. The PCR product was quantified and plotted relative to GAPDH expression as arbitrary units.

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**Antibodies:** The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): anti-uPA (sc-14019), anti-uPAR (sc-13522), anti-Lhx-2 (sc-81311), anti-GAPDH
(sc-59541), anti-CAD (caspase activated DNase) (sc-8342), and anti-Lamin-B (sc-6216). We purchased anti-cleaved caspase 8 (9496) from Cell Signaling Technology (Boston, MA).

**Western blotting:** MIA PaCa-2 and PANC-1 cells were transfected with pSV, puPA, puPAR, or pU2 for 72 hrs. Then, cells were collected, and total cell lysates were prepared in standard RIPA extraction buffer containing aprotinin and phenyl-methyl-sulfonyl-fluoride. 20 μg of protein from these samples were separated under non-reducing conditions by 12% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were immunoprobed for 2 hrs with antibodies against uPAR, cleaved caspase 8, CAD and GAPDH as per standard protocols. The membranes were treated with the appropriate HRP-conjugated secondary antibody and then developed according to enhanced chemiluminescence protocol (Amersham, Arlington Heights, IL). Similarly, nuclear extracts were immunoprobed for uPA and Lhx-2; for controls, membranes were stripped and probed with monoclonal antibodies for Lamin B as per standard protocol.

**Fibrin zymography:** The enzymatic activity and molecular weight of electrophoretically separated forms of uPA in the conditioned media of MIA PaCa-2 and PANC-1 cells transfected with pSV, puPA, puPAR, or pU2 were determined by SDS-PAGE as described previously (20, 21). As stated, the acrylamide gels were enriched with purified plasminogen and fibrinogen before polymerization. Equal amounts of sample proteins were electrophoresed, and the gels were washed and stained to determine enzymatic activity as per standard protocols.

**In vitro angiogenic assay:** MIA PaCa-2 and PANC-1 cells (2×10^6/well) were seeded and transfected with pSV, puPAR, puPA, or pU2. After a 24-hr incubation period, the conditioned
medium was removed and added to a $4 \times 10^4$ human dermal endothelial cell in 8-well chamber slides. Cells were allowed to grow for 72 hrs. Cells were then fixed in 3.7% formaldehyde and H&E stained to visualize capillary network formation. The degree of angiogenesis was quantified based on the numerical value for the product of the number of branches and number of branch points as an average of 10 fields. The results are graphically represented.

**In vivo angiogenic assay**: The implantation technique of the dorsal skin-fold chamber model has been described previously (22). Sterile small animal surgical techniques were followed. Diffusion chambers were filled with $2 \times 10^6$ MIA PaCa-2 and PANC-1 cells, which were transfected with pSV, puPAR, puPA, or pU2 and suspended in 100-150 μL of sterile PBS. Mice were anesthetized using isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane)-saturated oxygen gas. Once the animal was anesthetized completely, a dorsal air sac was made in the mouse by injecting 10 mL of air. A 1.5-2 cm superficial incision was made horizontally along the edge of the dorsal air sac, and the air sac was opened. With the help of forceps, the chambers were placed underneath the skin and sutured carefully. After 10 days, the animals were anesthetized with ketamine/xylazine and sacrificed by intracardiac perfusion with saline (10 mL) followed by a 10 mL of 10% formalin in 0.1 M standard phosphate buffer. The animals were carefully skinned around the implanted chambers and the implanted chambers were removed from the air sac fascia. The skin-fold covering the chambers was photographed under visible light. The number of blood vessels within the chamber in the area of the air sac fascia was counted and their lengths measured.

**Angiogenesis antibody array**: Angiogenic antibody array was performed as per the manufacturer’s instructions (Ray Bio Human Antibody array C series, Norcross GA). Briefly,
MIA PaCa-2 and PANC-1 cells (2×10^6/well) were seeded and transfected with pU2. After a 24-hr incubation period, the conditioned medium was removed and membranes were incubated with conditioned media. Following incubation, the membranes were washed and 1 mL of diluted biotin-conjugated antibody mix was added to the membranes and incubated at room temperature for 1-2 hours, followed by washing and addition of diluted HRP-conjugated streptavidin. Detection was based on ECL-protocols where membranes were exposed to X-ray films to visualize binding of angiogenic factors to membranes, which are seen as spots of varying intensities. Positive controls were used to normalize the results from different membranes being compared. Spot intensities were quantified using Image J software, and angiogenic molecules showing significant change in expression levels were identified.

Matrigel invasion assay: The in vitro invasiveness of MIA PaCa-2 and PANC-1 cells in the presence of the vector expressing siRNA for uPAR and uPA was assessed using a modified Boyden chamber assay. MIA PaCa-2 and PANC-1 cells were transfected with pSV, puPAR, puPA, or pU2 for 48 hrs. 1×10^6 cells were suspended in 200 μL of serum-free medium supplemented with 0.2% BSA and placed in the upper compartment of transwell chambers (Corning Costar Fischer Scientific Catalog #07-200-158, Pittsburgh, PA) coated with Matrigel (0.7 mg/mL). The lower compartment of the chamber was filled with 700 μL of serum-free medium, and the cells were allowed to migrate for 24 hrs. After incubation, the cells that passed through to the lower chamber were fixed and stained with Hema-3 and quantified as previously described (23).

Cell cycle analysis: MIA PaCa-2 and PANC-1 cells (2×10^6) were transfected with pSV, puPAR, puPA, or pU2 for 48 hrs. Cells were then trypsinized and treated with 50 μg/mL propidium
iodide + 0.001% RNAse A solution as per standard protocol. The cells were sorted on a fluorescence-activated cell sorter and quantified (10,000 cells sorted per treatment condition with 3 replications).

Orthotopic tumor model

Subcutaneous tumor growth: We carried out subcutaneous implantation as previously described (24). Briefly, MIA-PaCa-2 cells were harvested by trypsinization and washed with PBS. Cells were injected subcutaneously into mice in a total volume of 0.2 mL within 30 min of harvesting. The subcutaneous tumors were used as the source of tissue for orthotopic implantation of tissue onto the pancreas.

Surgical orthotopic implantation of MIA-PaCa-2 tumors: The following procedure was performed as described by Katz et al. (25). MIA-PaCa-2 tumors in the exponential growth phase, grown subcutaneously in nude mice, were resected aseptically. Necrotic tissues were cut away, and the remaining healthy tumor tissues were cut with scissors and minced into 1 mm³ pieces in Dulbecco’s Modified Eagle’s Medium (DMEM). Mice were then anesthetized using isoflurane (2-chloro-2-(difluoromethoxy)-1, 1, 1-trifluoro-ethane)-saturated oxygen gas, and their abdomens were sterilized with alcohol. An incision was created through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed, and 2 tumor pieces were transplanted onto the middle of the gland using a single surgical suture. The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed in 2 layers using 6-0 surgical suture. Three days after implantation, mice were given ip injections of pU2 five times at 150μg/mouse every other day. Thirty days after implantation, mice were sacrificed and pancreatic tissues were isolated and processed for paraffin embedding.
**Immunohistochemical analysis:** Pancreatic tissues with tumors of control (untreated), pSV - and pU2-treated mice implanted with MIA PaCa-2 tumors were fixed in formaldehyde and embedded in paraffin as per standard protocols. Sections were deparaffinized as per standard protocol, pretreated with 0.3% H$_2$O$_2$ to inactivate native peroxidases followed by incubation in 1% BSA in PBS for 1 hr, and subsequently transferred to primary antibody (uPAR, uPA or Lhx-2) diluted in 1% BSA in PBS (1:500). Sections were allowed to incubate in the primary antibody solution for 2 hrs at room temperature in a humidified chamber, followed by washes (3 at 10 min each) in 1% BSA in PBS and placed in a solution with the appropriate HRP-conjugated secondary antibody. The sections were allowed to incubate with the secondary antibody for 1 hr followed by washes in PBS (5 for 5 min each). HRP substrate DAB (3,3’-diaminobenzidine tetrahydrochloride) at 1 mg/mL in 50 mM Tris (pH 7.2) and 0.3% H$_2$O$_2$ was then added for the development of DAB substrate. Transmitted light images were obtained after hematoxylin staining as per standard protocol to visualize the morphology of the sections. A control study was performed using a normal rabbit immunoglobulin fraction as the primary antibody (control Ab) instead of uPAR, uPA or Lhx-2.

**In situ terminal-deoxy-transferase mediated dUTP nick end labeling (TUNEL) assay:** A TUNEL apoptosis detection kit (Upstate Biotechnology Inc, Lake Placid, NY) was used for DNA fragmentation fluorescence staining and carried out according to the manufacturer's protocol. Briefly, MIA PaCa-2 tumor sections from control, pSV - and pU2-treated mice were incubated with a reaction mix containing biotin-dUTP and terminal deoxynucleotidyl transferase for 60 min. Fluorescein-conjugated avidin was applied to the sample, which was then incubated in the dark for 30 min. Positively stained fluorescein-labeled cells were visualized after DAPI
staining using fluorescence microscopy and quantified as percent positive cells relative to cell density.

**Immunofluorescence:** MIA PaCa-2 and PANC-1 cells grown on chamber slides were transfected with pSV, puPAR, puPA or pU2. After 72 hrs, cells were fixed with 3.7% formaldehyde and incubated with 1% BSA in PBS at room temperature for 1 hr for blocking. Then, the slides were washed with PBS and incubated with IgG anti-uPA (rabbit) and IgG anti-Lhx-2 (mouse) at a concentration of 1:200. The slides were incubated at room temperature for 4 hrs and washed three times with PBS to remove excess primary antibody. Cells were then incubated with anti-rabbit FITC conjugated IgG (1:500 dilution) and anti-mouse Texas-Red conjugated IgG (1:500 dilution). Next, the slides were washed three times, covered with glass cover slips using a DAPI containing mounting media and fluorescent photomicrographs were obtained using a triple filter cube (RGB).

**Transcription factor binding array analysis:** Transcription Factor (TF) protein-protein binding array was done using the TF Protein Array kit from Panomics (Catalog #MA3501-08, Redwood City, CA) as per the manufacturer’s instructions. Briefly, recombinant human uPA (huPA) protein (American Diagnostics) was suspended in 1X blocking buffer at a concentration of 100 ng/mL. The TF membranes were incubated in 1X blocking buffer for 2 hrs at room temperature (RT) followed by incubation with huPA in 1X blocking buffer for 2 hrs at RT. This was followed by washings and further incubation with anti-uPA antibody followed by secondary HRP-conjugated antibody as per kit instructions. Detection for HRP was done using the provided detection buffers followed by exposure of membranes to x-ray film. Binding of huPA to TFs was observed as spots on the x-ray film.
**Statistical analysis:** All experiments were repeated in triplicate with the exception of the human pancreatic tissue array. A \( p \) value of 0.01 or less was considered significant.

**RESULTS**

*Human pancreatic carcinoma and adenocarcinoma show increased expression of uPAR and uPA*

We analyzed human pancreatic tissue arrays to determine expression levels of both uPAR and uPA by immunohistochemistry. Identical tissue arrays obtained by serial sectioning were used for this study. We observed that, expression of both uPAR and uPA was co-localized in most cases (Fig. 1A). uPA expression was seen uniformly throughout the tumor tissue with little expression in the large excretory ducts, whereas uPAR expression was observed in the tumor tissues as well as in the excretory ducts (Figs. 1B-C). In most tissues, overexpression of uPA was accompanied by overexpression of uPAR. Expression of uPAR and uPA with grade information is provided in the supplementary data.

*Plasmids expressing shRNA targeting uPAR and uPA suppress uPAR and uPA mRNA and protein levels in MIA PaCa-2 and PANC-1 cells*

MIA PaCa-2 and PANC-1 cells were transfected with plasmids expressing shRNA targeting uPAR (puPAR), uPA (puPA), and uPAR-uPA simultaneously (pU2). Cell lysates were used to determine uPAR expression levels and the conditioned media was used to determine uPA activity by fibrin zymography. Figure 2A shows a two and four fold decrease in the mRNA levels of uPAR in puPAR- and pU2-transfected cells when compared to pSV-transfected and control cells respectively. Similarly, we observed a significant (>2fold) decrease in the mRNA
levels of uPA in puPA- and pU2-transfected cells as compared to pSV-transfected and control cells (Fig. 2A). From the fibrin zymography results, we observed a decrease in uPA activity in puPA and pU2-transfected cells as compared to pSV-transfected and control cells by more than two and four fold in both cell lines respectively (Fig. 2B&2C). uPAR protein expression decreased by 3-fold in puPAR-transfected cells and 4-fold in pU2-transfected cells as compared to the controls (Figs. 2B-D). Cells transfected with puPA had decreased uPA activity but no significant change in uPAR expression levels (Fig. 2B-D).

Suppression of uPAR and uPA expression in MIA PaCa-2 and PANC-1 cells retards their invasive potential, causes the accumulation of cells in the sub G₀/₁ phase, and activates caspase 8-mediated apoptosis

To study the effect of pU2 on pancreatic cancer cell invasion, we used the Matrigel invasion model. MIA PaCa-2 and PANC-1 cells were transfected with puPAR, puPA or pU2 and were allowed to invade through Matrigel for 24 hrs. No significant difference was observed in control and pSV-transfected cells, while decreased invasion was observed in transfection with puPAR, puPA and pU2 (Fig. 3A). Quantitative analysis revealed that invasion decreased by 20±3% in MIA PaCa-2 cells and by 47±1% in PANC-1 cells after puPAR transfection. Cells transfected with puPA showed similar decreases in invasion (MIA PaCa-2: 18±2% and PANC-1: 45±4%). Cells transfected with pU2 showed the maximum decrease in invasion (MIA PaCa-2: 57±2% and PANC-1: 63±4%) when compared to all other treatment conditions (Fig. 3A). To further determine whether the downregulation of uPA caused changes in cell cycle progression, MIA PaCa-2 and PANC-1 cells were transfected with pSV, puPAR, puPA or pU2. From the results of the FACS analysis, we observed that simultaneous downregulation of uPAR and uPA caused a higher accumulation of cells in the sub-G₀/₁ phase in both MIA PaCa-2 and PANC-1 cells as
compared to downregulation of either uPAR or uPA alone. Controls and pSV-transfected cells had very little accumulation of cells in the sub- $G_{0/1}$ phase (Fig. 3B). Quantitative analysis indicated that MIA PaCa-2 and PANC-1 cells transfected with puPAR (MIA PaCa-2: 34±3% and PANC-1: 36±3%) or puPA (MIA PaCa-2: 28±32% and PANC-1: 30±2%) had increased accumulation of cells in the sub- $G_{0/1}$ phase. Cell death in pU2-transfected cells (MIA PaCa-2: 57±2% and PANC-1: 58±3%) was found to be more than 50% (Fig. 3B). We next sought to determine whether the accumulation of cells in the sub- $G_{0/1}$ phase was indicative of apoptosis. MIA PaCa-2 and PANC-1 cells were transfected with siRNA expressing plasmids (puPAR, puPA and pU2) and proteins were isolated. Western blot analysis showed that control and pSV-treated MIA PaCa-2 and PANC-1 cells did not have increased cleavage of caspase 8 or CAD expression. In contrast, cells transfected with puPAR and puPA had increased caspase 8 cleavage (MIA PaCa-2: 12±2% and PANC-1: 15±3%) and CAD expression (MIA PaCa-2: 6±2% and PANC-1: 9±3%). We observed the most significant increase in CAD expression in pU2-transfected cells (MIA PaCa-2: 65±2% and PANC-1: 72±3%) (Fig 3C).

**Suppression of uPAR and uPA expression in MIA PaCa-2 and PANC-1 cells retards their angiogenic potential in vitro and in vivo**

To assess whether downregulation of uPAR and uPA can influence tumor-induced angiogenesis in an in vitro system, we cultured human endothelial cells with conditioned media from MIA PaCa-2 or PANC-1 cells transfected with pSV, puPAR, puPA or pU2. Endothelial cells formed dense capillary-like structures when cultured with conditioned media from untransfected or pSV-transfected MIA PaCa-2 or PANC-1 cells. Endothelial cells grown in conditioned media of MIA PaCa-2 or PANC-1 cells transfected with puPAR or puPA showed a decrease in the density and completeness of the capillary network structure as compared to the controls. We observed broken
and rudimentary network formation in endothelial cells grown in conditioned media from pU2-transfected MIA PaCa-2 or PANC-1 cells (Fig. 4A). Quantitative analysis indicated that transfection with puPAR (MIA PaCa-2: 37±3% and PANC-1: 55±4%) or puPA (MIA PaCa-2: 36±2% and PANC-1: 41±2%) decreased capillary network formation (controls considered as 100% angiogenic). Transfection with pU2 (MIA PaCa-2: 68±2% and PANC-1: 78±4%) caused the most significant decrease in endothelial cell network formation (Fig. 4A).

To further demonstrate the effect of uPAR and uPA downregulation on tumor angiogenesis, we used the dorsal skin-fold chamber assay in vivo model system. Implantation of a chamber containing MIA PaCa-2 or PANC-1 cells pre-transfected with pSV, puPAR, puPA or pU2 resulted in the development of microvessels with curved thin structures. MIA PaCa-2 or PANC-1 cells transfected with pU2 showed in no new development of vasculature, whereas puPAR- and puPA-transfected cells did result in formation of neovasculature but was less than that of control or pSV-transfected cells where maximum neovasculature formation was observed (Fig. 4B). Quantitative analysis of neovasculature from the in vivo angiogenesis assay indicated that controls and pSV-treated cells had similar increases in vasculature, whereas puPAR-treated cells (MIA PaCa-2: 28±6% and PANC-1: 36±3%) and puPA-treated cells (MIA PaCa-2: 37±3% and PANC-1: 15±3%) had decreased angiogenesis. pU2-treated cells (MIA PaCa-2: 88±3% and PANC-1: 85±3%) exhibited little increase in vasculature (Fig. 4B).

Simultaneous downregulation of uPAR and uPA in MIA PaCa-2 and PANC-1 cells regulates expression of angiogenic factors

To further analyze the effect of pU2 on angiogenic inhibition, we used an antibody array specific for angiogenic factors. We used conditioned media from untransfected and pU2-transfected MIA
PaCa-2 and PANC-1 cells. We observed that the expression level of angiogenin reduced significantly (2- to 3-fold decrease) in both MIA PaCa-2 and PANC-1 cells. TIMP-2 expression levels also decreased 1 to 2-fold. MCP-1 expression level decreased by 4-fold in PANC-1 cells but not in MIA PaCa-2 cells. Similarly, a 2-fold decrease in ENA-78 expression levels was observed in MIA PaCa-2 cells but not in PANC-1 cells. Expression levels of the angiogenic inhibitor RANTES increased 2 to 3-fold in both cell lines (Figs. 4C).

**Intraperitoneal injections of plasmids expressing shRNA targeting uPAR and uPA induce cell death in MIA PaCa-2 orthotopic tumors**

To determine the *in vivo* effect of pU2 on pancreatic cancer cells, we used an orthotopic tumor model. Untreated mice pancreas showed normal pancreatic morphology with well-defined acini and islets of langerhans. In the control and pSV-treated mice, pancreatic tissues were infiltrated with tumor cells. However, in pU2-treated mice, we observed necrotic tumor tissue and decreased tumor cell density when compared to controls. Immunohistochemical analysis for uPAR and uPA expression indicated that normal pancreatic tissue expressed little to no uPAR or uPA, whereas mice with orthotopic pancreatic tumors (control and pSV-treated) showed strong expression of both uPAR and uPA (expression was stronger at the invasive fronts) (Figure 5A). Mice treated with pU2 showed very low expression levels of both uPAR and uPA (Figure 5A).

*In situ* TUNEL assay showed that pU2-treated orthotopic tumors showed presence of DNA damage (green fluorescence) (Fig. 5A). Determination of percent TUNEL expression indicated that 63±9% cells in pU2-treated tumors had DNA damage whereas no DNA damage was observed in control or pSV-treated mice. We measured tumor volumes by serial sectioning, and we observed that control (550±50 mm³) and pSV-treated (620±100 mm³) mice had the largest
tumor volumes while pU2-treated mice had the smallest tumor volume (268±35 mm³); normal mice were used as negative controls (Fig. 5B).

**MIA PaCa-2 and PANC-1 cells show co-localization of Lhx-2 and uPA, and simultaneous downregulation of uPAR and uPA in MIA PaCa-2 tumors causes suppression of Lhx-2 in vivo but not in vitro**

Fluorescent immunohistochemical analysis for the expression of uPA and Lhx-2 in MIA PaCa-2 and PANC-1 cells revealed that uPA was present in the cytoplasm, cell membrane and the nucleus with uPA and Lhx-2 co-localized in the nucleus and cytoplasm (Fig. 6A). Immunohistochemical analysis of Lhx-2 in pancreatic tumor tissues revealed that Lhx-2 is strongly expressed at the invasive front of tumors surrounding the acinar tissues (Fig. 6B). To determine the role of uPA in the nucleus, we performed a transcription factor binding assay using a protein array of known transcription factors. The results demonstrate that uPA is bound to the transcription factor Lhx-2 (Fig. 6C). We performed western blot analysis of nuclear extracts of MIA PaCa-2 and PANC-1 cells transfected with pSV, puPAR, puPA or pU2 for uPA and Lhx-2 expression. In MIA PaCa-2 cells, transfection with puPAR, puPA or pU2 caused a decrease in nuclear localization of uPA with a corresponding decrease in the expression level of Lhx-2. In PANC-1 cells, transfection with puPAR, puPA or pU2 caused a decrease in nuclear uPA similar to MIA PaCa-2 cells but did not result a decrease in the levels of nuclear Lhx-2 (Fig. 6D). We present a rudimentary theoretical model for the possible role of uPA and Lhx-2 in maintenance of an invasive phenotype and the possible role of Lhx-2 in initiating differentiation (Fig. 6D).
DISCUSSION

In the present study, we have attempted to retard the invasive ability and angiogenic potential of pancreatic cancer cells both in vitro and in vivo. From the human tissue array analysis, we observed that both uPAR and uPA were highly expressed in human pancreatic cancer tissues, and the expression of uPAR was always correlated with the expression of its ligand uPA. Researchers have demonstrated that activation of the uPAR-uPA system is an early event in the development of pancreatic ductal adenocarcinoma and that uPAR gene amplifications identify a subgroup of particularly aggressive tumors, thereby making the uPAR-uPA system a critical and highly promising target for therapeutic interventions (10).

To target the uPAR-uPA system, we used plasmids expressing shRNA targeting uPAR and uPA (19, 26-30). Using plasmid-mediated RNAi induction did not cause non-specific inhibition, which indicates that no off-target effects were present. From the Matrigel invasion assay, we observed that the simultaneous downregulation of uPAR and uPA caused a significant decrease in invasive potential as compared to cells downregulated for either uPAR or uPA. Recent research using papillary thyroid carcinoma (PTC) cells provide new functional evidence of the uPAR-uPA system's role in PTC invasion/metastasis (31). Researchers have also demonstrated that inhibiting the activity of the uPAR and associated molecules like integrins (32) causes a significant decrease of migration and intracellular signaling in cancer cells. The uPAR-uPA system is known to be localized at the invasive front of tumor cells, activating plasmin to plasminogen and further enabling the activation of various proteases (33). Interestingly, the downregulation of the uPAR-uPA system also caused the activation of pro-apoptotic molecules like caspase 8 and CAD. From our FACS analysis, we did observe the accumulation of cells in the sub- G₀/₁ phase in cells downregulated for uPAR and uPA either alone or in combination. Western blot analysis for CAD activation demonstrated that cells
simultaneously downregulated for uPAR and uPA had strong CAD activation, indicating that both uPAR and uPA are necessary for maintenance of a viable invasive phenotype.

The classical role of the uPAR-uPA system is the activation of plasmin, and plasmin-overproducing cancer cells are known to also overexpress tPA, uPA and uPAR. Plasmin is also known to promote angiogenesis and metastasis (34). The mechanisms by which plasmin promotes angiogenesis and metastasis remain poorly understood. We hypothesize that suppressing the plasmin activating system could essentially inhibit angiogenic activation. Our results demonstrate that suppression of both uPAR and uPA retarded angiogenic activation under \textit{in vitro} and \textit{in vivo} conditions. Our results also demonstrate that simultaneous downregulation of uPAR and uPA suppressed angiogenin, a pro-angiogenic molecule. Recently, researchers have demonstrated that the recombinant kringle domain (UK1) of uPA had anti-angiogenic activity by causing the suppression of various pro-angiogenic molecules, including angiogenin, in glioma cells (35). Our results confirm the previous findings that interference with the uPAR-uPA system retards angiogenesis.

We also observed that the suppression of uPAR-uPA system caused the overexpression of RANTES in both MIA PaCa-2 and PANC-1 cells. Studies have shown that the cleaved form of suPAR binds and activates the fMLP-Rs and regulates the activity of MCP-1, RANTES and SDF1 receptors (36). Hence, overexpression of uPAR may suppress RANTES expression. RANTES [also known as Chemokine (C-C motif) ligand 5 (CCL5)] is a protein that is encoded by the \textit{CCL5} gene in humans. Studies have demonstrated that RANTES plays a role in chemoattraction and activation of immune cells and its potential clinical application as an adjuvant for boosting anti-tumor immunity has been suggested (37). As our \textit{in vivo} studies were performed in an immune-compromised setting, further experimentation in a normal setting would shed light on the relevance of RANTES. The regulation of RANTES is not clearly understood and the role
of the uPAR-uPA system in modulating RANTES expression has not been reported. It is known that uPAR functionally interacts with integrins, fMLP-receptors (fMLP-Rs) and growth factor receptors, thereby regulating cell adhesion, migration and proliferation. Similarly, interaction of RANTES with uPAR (37) may localize RANTES at the cell surface and not shed into the ECM. Removal of uPAR from the cell surface retards this binding force and allows the shedding of RANTES into the ECM, facilitating the recruitment of immune cells. *In vivo* experimentation using normal mice (versus immune-compromised mice) would shed light on whether suppression of uPAR would actually recruit immune reaction via RANTES.

In the present study, MCP-1 was also downregulated but only in PANC-1 cells; expression of MCP-1 was not observed in MIA PaCa-1 cells. Similar to RANTES, MCP-1 [monocyte chemotactic protein-1; also known as Chemokine (C-C motif) ligand 2 (CCL2)] is a small cytokine belonging to the CC chemokine family and is known to recruit monocytes (38). The upregulation of RANTES and the downregulation of MCP-1 would indicate the absence of a global cellular immune reaction. Generally, such responses are accompanied by the expression of OAS1, which was not observed in the present study (supplementary data).

In MIA PaCa-2 cells, ENA-78 expression levels were reduced after uPAR/uPA downregulation but not in PANC-1 cells. ENA-78 (also known as CXCL-5) is produced following stimulation of cells with inflammatory cytokines, such as interleukin-1 or tumor necrosis factor-alpha, and is a known pro-angiogenic molecule (39-41). Hence, the suppression of ENA-78 would retard angiogenic induction. The link between the uPAR-uPA system and ENA-78 is rudimentary and has not been investigated thoroughly so we only have preliminary evidence indicating the influence of uPAR-uPA system on ENA-78. Similarly, the mechanism by which TIMP-2 levels decrease as a result of suppression of the uPAR-uPA system is not clear. Our Western blot analysis suggests this decrease may be due to the overall shut down of
cellular processes and an indicator of apoptosis. Our *in vivo* studies demonstrate that the simultaneous downregulation of uPAR and uPA caused the activation of DNA damage as shown by TUNEL assay. The tumor tissues had decreased tumor cell density and fragmented nuclei, which are both indicative of apoptosis.

Next, to determine the *in vitro* presence of uPA, we performed immunocytochemistry on both MIA PaCa-2 and PANC-1 cells and looked for the presence of uPA in the nucleus. Upon further investigation using western blot analysis, we confirmed its presence in the nucleus of MIA PaCa-2 and PANC-1 cells. To determine whether uPA has any regulatory role, we performed a protein-protein binding array and observed that uPA bound strongly to Lhx-2, a transcription factor. To our knowledge, this is the first report to demonstrate the presence of uPA in the nucleus as well as the interaction of uPA with Lhx-2. Modeling and protein-protein docking studies did not indicate any possible interactions, whereas immuno-precipitation studies indicates that Lhx-2 was immuno-precipitated with uPA (see supplementary data). Recently, researchers have identified Lhx2 as a transcription factor functionally positioned downstream of p63 and NF-κB, but upstream of signals like Wnt/β-catenin, Bmp and Shh that are required to drive activated stem cells towards terminal differentiation (42). This would indicate that Lhx-2 may behave like a gatekeeper molecule mediating the activation of cancer stem cells. Immunohistochemical analysis of tumor sections revealed that Lhx-2 was overexpressed at the invasive front of tumors while completely absent in tumors downregulated for both uPAR and uPA. As such, uPA may have certain hitherto unknown regulatory functions, and further study to determine if uPA has any regulatory function is warranted. Taken together, our results demonstrate that the targeting of the uPAR-uPA system has significant therapeutic potential for the treatment of pancreatic cancer.
ACKNOWLEDGMENTS:

We thank Shellee Abraham for manuscript preparation. We thank Diana Meister and Sushma Jasti for manuscript review.
Reference List


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Figure Legends

Figure 1: Human pancreatic cancer tissues show overexpression of both uPAR and uPA. Tissue arrays consisted of pancreatic cancer and normal tissues with stage and grade info of 101 cases consisting of 208 cores (PA2082-024 and -025). Tissue arrays were processed for immunohistochemistry as per standard protocols. Expression of uPAR and uPA was observed as brown coloration of DAB reaction (A). Microscopic examination at 4X and 10X magnifications was done to observe tumor tissue morphology (B). Microscopic examination of the large excretory ducts was done to determine invasion of tumor tissue (C).

Figure 2: Effects of puPA-, puPAR- and pU2-mediated downregulation on mRNA and protein levels. Pancreatic cell lines (MIA PaCa-2 and PANC-1) were transfected with scrambled vector (pSV) or constructs encoding siRNA for uPAR (puPAR) or uPA (puPA) singly or in combination (pU2). 72 hrs after transfection, total RNA extracted from control (un-transfected) and transfected cells were subjected to RT-PCR using primers specific for uPAR and uPA. GAPDH primers were used to verify that equal amounts of total RNA were used during cDNA synthesis (A). The amplified (RT-PCR) fragments were quantified by densitometry analysis (A). Enzymatic activity of uPA was determined by fractionating equal amount of conditioned media (CM) collected from control and transfected cells on fibrin gels (B). For western blot analysis, total cells lysates were immunoblotted with antibodies specific for uPAR and uPA. GAPDH was used as a loading control. Bands or signals detected by autoradiography were quantified using densitometry analysis (C-D). Values in all the experiments are mean ± SD from three independent experiments (p<0.05).
Figure 3: Simultaneous downregulation of uPAR and uPA retards invasion and causes accumulation of cells in the sub G₀/₁ phase. MIA PaCa-2 and PANC-1 cells were transfected with pSV, puPAR, puPA or pU2 as described in Materials and Methods. These cells were then allowed to invade Matrigel in the Boyden’s chamber for 24 hrs followed by Hema-3 staining (A). Percent cell invasion was determined (A). To determine changes in cell cycle, MIA PaCa-2 and PANC-1 cells transfected with pSV, puPAR, puPA or pU2 were trypsinized and stained with propidium iodide as per standard protocols. To determine cellular DNA content, the cells were sorted using a flow cytometer. 10,000 cells were sorted per experiment with three replications (B). Quantitative analysis for sub- G₀/₁ phase was done and graphically represented as percentage of controls (B). To determine activation of apoptosis, cell lysates from MIA PaCa-2 and PANC-1 cells transfected with pSV, puPAR, puPA or pU2 were immunoblotted with antibodies specific for cleaved caspase 8 and CAD; GAPDH was used as a loading control (C). Quantitative analysis was done by optical densitometry as per standard protocols (C). Values in all the experiments are mean ± SD from three independent experiments (p<0.05).

Figure 4: Simultaneous downregulation of uPAR and uPA decreases angiogenesis in pancreatic cancer cell lines both in vitro and in vivo and inhibits angiogenin and activates RANTES. In vitro angiogenesis: MIA PaCa-2 and PANC-1 cells were transfected with puPAR, puPA or pU2. After 48 hrs of incubation, serum-free medium was added, and cells were incubated overnight. Tumor conditioned medium (TCM) was transferred into 96-well plates, which were seeded with human dermal microvascular endothelial cells (HMEC) (2×10⁴ cells/well). After 72 hrs of incubation at 37°C, cells were stained with Hema-2 and observed under the bright field microscope for capillary-like structure formation (A). The degree of angiogenic induction by control cells and cells transfected with pSV, puPAR, puPA or pU2 was
quantified (the numerical value of the product of the relative capillary length and number of branch points per field) (A). MIA PaCa-2 and PANC-1 cells were transfected with puPAR, puPA or pU2 and incubated for 48 hrs. After the incubation period, $1 \times 10^6$ cells from each treatment were injected into diffuse chambers, and placed under the dorsal skin cavity. The chamber was removed, observed for neovascularization, and photographed (B). The mean number of neovasculature (>20 μm length) per field was quantified (B). MIA PaCa-2 and PANC-1 cells were transfected with pU2. After 48 hrs of incubation, serum-free medium was added, and cells were incubated overnight. Conditioned medium was used to immunoprobe various angiogenesis-related molecules using an angiogenic-specific antibody array (Ray-Biotech) as per the manufacturer’s protocol (C). Quantitative analysis of spot density was measured and represented as fold change (C).

Figure 5: Intraperitoneal injection of a plasmid expressing shRNA targeting both uPAR and uPA retards pancreatic tumor development and induces apoptosis. MIA PaCa-2 tumors, which were grown subcutaneously in nude mice and in the exponential growth phase, were resected aseptically and transplanted onto the middle of the pancreatic gland as described in Materials and Methods. For each set of treatments 8 animals were used. Paraffin sections were used for immunohistochemical analysis for uPA or uPAR. To determine apoptotic induction, TUNEL assay was performed on the tumor sections and compared to DAPI-stained sections. Standard H&E staining was also done to determine tumor and pancreatic morphology (A). Tumor volume was measured by serial sectioning and assessment of tumor area. The percentage of TUNEL-positive cells was determined by counting the number of green florescent cells per field (B).
Figure 6: Lhx-2 and uPA are co-localized in the nucleus of both MIA PaCa-2 and PANC-1 cells. MIA PaCa-2 and PANC-1 cells grown on chamber slides were immunoprobed for uPA and Lhx-2 and incubated with IgG anti-uPA and IgG anti-Lhx-2 at a concentration of 1:200. The slides were covered with glass cover slips using DAPI-containing mounting media. Fluorescent photomicrographs were obtained using a triple filter cube (A). MIA PaCa-2 tumors from mice left untreated or treated with pU2 were paraffin sectioned and immunoprobed for Lhx-2 using HRP-conjugated secondary antibody and visualized using DAB reagent (B). Interaction of uPA with transcription factors was done by using a Transcription Factor (TF) protein-protein binding array (C). To determine nuclear interaction of uPA with Lhx-2, nuclear extracts from MIA PaCa-2 and PANC-1 cells transfected with puPAR, puPA or pU2 were western blotted for uPA and Lhx-2; Lamin B served as a loading control (D). Schematic representation showing the possible role of uPA and Lhx-2 in maintenance of a tumor phenotype (D).
# Molecular Cancer Research

## Suppression of the uPAR-uPA System Retards Angiogenesis, Invasion and in vivo Tumor Development in Pancreatic Cancer Cells


*Mol Cancer Res* Published OnlineFirst March 9, 2011.

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