Multifunctional roles of urokinase plasminogen activator (uPA) in cancer stemness and chemoresistance of pancreatic cancer

Swapna Asuthkar\textsuperscript{a},* , Victoria Stepanova\textsuperscript{b},* , Tatiana Lebedeva\textsuperscript{b} , AiXuan L. Holterman\textsuperscript{c} , Norman Estes\textsuperscript{d} , Douglas B. Cines\textsuperscript{b} , Jasti S. Rao\textsuperscript{a} , and Christopher S. Gondi\textsuperscript{d}

\textsuperscript{a}\textsuperscript{a}Department of Cancer Biology and Pharmacology, \textsuperscript{b}\textsuperscript{b}Department of Surgery, and \textsuperscript{d}\textsuperscript{d}Department of Medicine, University of Illinois College of Medicine at Peoria, Peoria, IL 61605; \textsuperscript{c}\textsuperscript{c}Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104

ABSTRACT Pancreatic ductal adenocarcinoma (PDAC) is almost always lethal. One of the underlying reasons for this lethality is believed to be the presence of cancer stem cells (CSC), which impart chemoresistance and promote recurrence, but the mechanisms responsible are unclear. Recently the poor prognosis of PDAC has been correlated with increased expression of urokinase plasminogen activator (uPA). In the present study we examine the role of uPA in the generation of PDAC CSC. We observe a subset of cells identifiable as a side population (SP) when sorted by flow cytometry of MIA PaCa-2 and PANC-1 pancreatic cancer cells that possess the properties of CSC. A large fraction of these SP cells are CD44 and CD24 positive, are gemcitabine resistant, possess sphere-forming ability, and exhibit increased tumorigenicity, known characteristics of cancer stemness. Increased tumorigenicity and gemcitabine resistance decrease after suppression of uPA. We observe that uPA interacts directly with transcription factors LIM homeobox-2 (Lhx2), homeobox transcription factor A5 (HOXA5), and Hey to possibly promote cancer stemness. uPA regulates Lhx2 expression by suppressing expression of miR-124 and p53 expression by repressing its promoter by inactivating HOXA5. These results demonstrate that regulation of gene transcription by uPA contributes to cancer stemness and clinical lethality.

INTRODUCTION Pancreatic adenocarcinoma is the fourth-most-common cause of cancer deaths in the United States. Despite new insights into the molecular profile of pancreatic cancer and its precursor lesions and advances in diagnosis and therapy, survival rates have changed little over the past 40 yr. Major hallmarks of pancreatic cancer are extensive local tumor invasion, early systemic dissemination, and extremely poor response to chemotherapy and radiation treatment. The basis for these adverse features is not well understood. Emerging evidence suggests that the capability of tumors to grow, propagate, and recur may depend on an initially small subset of cells within a tumor, called cancer stem cells (CSC) or cancer-initiating cells. CSC, like normal stem cells, can both self-renew and produce differentiated progeny. The stem cell phenotype is associated with “en bloc” silencing of cell cycle–inhibitor genes (Nguyen et al., 2012). The resistance of pancreatic cancer to treatment and the high rate of recurrence have been attributed to a highly tumorigenic CSC subpopulation expressing cell surface CD44, CD24, CD133, and epithelial-specific antigen. Du et al. (2011) demonstrated that the chemoresistance of pancreatic cancer cells correlates with the expression of cell surface markers similar to those present on CSC that undergo epithelial–mesenchymal transition (EMT; Lonardo et al., 2010; Moriyama et al., 2010; Rausch et al., 2010). Urokinase plasminogen activator (uPA) expression correlates with increased number of EMTs in cancer cells, linking uPA to emergence of CSC and the
resultant chemoresistance (Chen et al., 2009). Down-regulating uPA expression by silencing Ets-1 transcription factors sensitizes pancreatic cancer cells to gemcitabine-induced apoptosis (Khanna et al., 2011). Furthermore, uPA and uPA receptor (uPAR) are strong independent prognostic indicators of cancer relapse after primary therapy and indicative of metastatic potential, advanced stage, and poor prognosis (Watabe et al., 1998; Kato et al., 2012). Serum levels of uPA are elevated in patients with pancreaticobiliary cancer (Harvey et al., 2003), and overexpression of uPA in tumors is associated with shorter survival times (Gibbs et al., 2009). We previously demonstrated that silencing uPA and uPAR inhibits the growth of pancreatic tumors in animal models (Gorantla et al., 2011). Inhibitors of uPAs catalytic activity and antagonists of its binding to uPAR have shown only partial success, however, in animal model studies and clinical trials (Mekkawy et al., 2009; Lund et al., 2011; Mazar et al., 2011). This outcome suggests that approaches targeting only the proteolytic or receptor-binding functions of uPA are suboptimal and critical functions of uPA in cancer progression have been overlooked. We also previously reported that uPA is internalized by proliferating cells and rapidly transported to cell nuclei (Stepanova et al., 2008; Gorantla et al., 2011). Within the nucleus of pancreatic cancer cells, uPA binds to a LIM homeobox-2 (Lhx2) transcription factor (Gorantla et al., 2011). Lhx2 helps to maintain stem/progenitor cell phenotype and EMT in vitro and in vivo (Pinto do et al., 2001; Richter et al., 2003; Tiede and Paus, 2006; Williams et al., 2006; Kim et al., 2012; Nadal et al., 2012). Lhx2 is up-regulated in several cancer transcriptome databases in mouse models of breast cancer (Chou and Yang, 2006) and neuroendocrine carcinoma (Zhao et al., 2010; Perez et al., 2012).

Together these data led us to investigate whether nuclear uPA contributes to maintenance of stemness in pancreatic cancer cells by binding and regulating Lhx2. Here we provide evidence that nuclear uPA promotes pancreatic cancer cell stemness by binding directly to specific homeobox transcription factors. We studied the mechanism of interplay between uPA and tumor-suppressive microRNA (miRNA) miR124 in regulating Lhx2 expression, which is linked to maintenance of pancreatic cancer cell stemness and chemoresistance. We demonstrate that uPA also down-regulates expression of the tumor suppressor p53 by binding to and interfering with the function of homeobox transcription factor AS (HOXAS), which further contributes to acquisition and maintenance of stemness in pancreatic cancer cells. Our data suggest that targeting nuclear uPA and its binding to the homeobox transcription factors may sensitize pancreatic cancer cells to chemotherapy-induced apoptosis and therefore has the potential to significantly improve treatment outcomes.

RESULTS
Side population of pancreatic cancer cells shows enhanced stem cell–like properties and uPA expression
Side-population (SP) cells play a crucial role in tumorigenesis and cancer recurrence (Zhang et al., 2013). We first determined whether the side populations of pancreatic cancer cells we studied contain subpopulations of stem-like cells. Because culturing cancer cells under serum-free condition promotes the growth of cancer stem cells (Gou et al., 2007), we cultured MIA PaCa-2 and PANC-1 cells in complete or serum-free media with appropriate growth factors. We then detached the cells with trypsin and sorted them for density and size by standard flow cytometry. Cells cultured under serum-free conditions showed a side population of cells (25–36%) with lower density and size (Figure 1A) that characterize the CSC phenotype (Gou et al., 2007). To confirm this inference, protein extracts from the sorted populations of MIA PaCa-2 and PANC-1 cells grown under serum-free conditions were immunoprobed for the known cancer stem cell markers CD44 and CD24 (Lonardo et al., 2010; Moriyama et al., 2010; Rausch et al., 2010). The SP cells were positive for both CD44 and CD24, whereas the “residual” cells were positive only for CD44 (Figure 1B). These data indicate that the SP cells possess the cancer stem cell surface phenotype (Supplemental Figure S1). To further validate the stem cell character of MIA PaCa-2 SP cells and SP–depleted cells (∆SP), we implanted these cells subcutaneously in nude mice (10,000 cells per mouse). The inoculates were allowed to grow for 40 d and then scored for the presence or absence of measurable (>1 mm in size) tumors. We observed that in 9 of 10 mice implanted with SP cells, tumors became visually evident within 40 d, whereas none of the mice implanted with CD24-negative cells (10,000 ∆SP cells) formed tumors over that time. When implanted with mixed population (MP) of MIA PaCa-2 cells (10,000 cells/mouse), 4 of 10 mice developed visually evident tumors (Figure 1C). Thus these in vivo studies indicate that the SP cells or cancer stem–like cells have a greater tumorigenicity potential than ∆SP or unseparated cancer cells. To obtain the orthotopic tumors derived from these subcutaneous tumors, we implanted naive nude mice orthotopically in the pancreas with fragments of these subcutaneous tumors as described previously (Fu et al., 1992) and allowed the tumors to develop for an additional 40 d. Forty days after implantation, pancreatic tissues were harvested and processed for paraffin sectioning and immunohistochemical analysis. Because increased expression of uPA is associated with higher “aggressiveness” for multiple tumor types, including pancreatic adenocarcinoma (Ceccarelli et al., 2010; Markl et al., 2010; Bekes et al., 2011; Provost et al., 2012), we studied expression levels of uPA in these orthotopic tumors using immunohistochemistry. We observed that orthotopic tumors grown from the implanted SP cell–derived subcutaneous tumors expressed uPA at much higher levels than those grown from implanted MP cells (Figure 1D). In contrast, normal pancreatic tissue expressed moderate-to-low levels of uPA (Figure 1D). To further assess the role of uPA in establishing the cancer stem cell phenotype, we overexpressed uPA in both SP and ∆SP MIA PaCa-2 cells (uPAOE-SP and uPAOE-∆SP, respectively) and compared their proliferation and growth patterns using the sphere formation assay. We observed that SP cells possessed greater sphere-forming ability (p < 0.001) than ∆SP cells. Overexpression of uPA induced sphere formation in ∆SP cells (Supplemental Figure S2). The sphere-forming ability of SP cells was attenuated when uPA expression was suppressed with uPA-specific short hairpin RNA (shRNA; Mia PaCa-2(uPA-)) and PANC-1(uPA-), which led to significant disintegration of the organoids (Figure 1E). Fluorescence-activated cell sorting analysis of the mixed populations of MIA PaCa-2 and PANC-1 cells revealed that uPA overexpression (uPAOE) increased the proportion of SP cells (Supplemental Figure S3). Together these data indicate that uPA promotes pancreatic cancer cell stemness.

Suppression of uPA expression sensitizes pancreatic CSC to gemcitabine
Human pancreatic CSC are highly tumorigenic and highly resistant to standard chemotherapy (Hermann et al., 2007), including gemcitabine (Hong et al., 2009). On the basis of our finding that uPA confers a stem-like phenotype to pancreatic cancer cells, we asked whether uPA contributes to chemoresistance and whether inhibition of uPA expression would sensitize these cells to gemcitabine. To do so, we expressed uPA-targeting or scrambled shRNA in SP and ∆SP MIA PaCa-2 and PANC-1 cells and added varying concentrations of gemcitabine (0–1000 nM). We observed that 1) SP cells are resistant...
FIGURE 1: Stem cell–like properties of the SP cells derived from pancreatic cancer cells. (A) Mixed populations of MIA PaCa-2 and PANC-1 cells ($2 \times 10^6$) were sorted by density-based flow cytometry (10,000 cells sorted per treatment condition, with three replications) to separate SP and ΔSP cells. Acquisition was performed on a FACSCalibur flow cytometer, and viable cells were analyzed with CellQuest software. (B) Cell lysates prepare from the sorted SP and ΔSP cells were immunblotted for CD24 and CD44 to elucidate expression of cancer stem cell markers. (C) SP, ΔSP, and MP cells were implanted subcutaneously in nude mice (10,000 cells/mouse), and the tumor volumes in treated groups were quantified and represented graphically (mean ± SD; n = 5 and p < 0.001). (D) Subcutaneous tumors grown as in C were implanted orthotopically in the pancreas of nude mice as described in Materials and Methods and allowed to grow for 40 d. At the end of this period, pancreatic tissues were harvested and processed for paraffin sectioning. Expression levels of uPA were determined by immunohistochemistry using anti-uPA and control immunoglobulin G. Brown color denotes uPA-antibody–positive reaction. Normal pancreatic tissue was also sectioned and immunoprobed for uPA. (E) Proliferation and formation of the neurospheres by untreated SP cells derived from MIA-PA Ca-2 and PANC-1 cells (left). Right, disintegration of the neurospheres after exposure to shRNA specific for uPA (puPA).
uPA activates stemness and chemoresistance

Volume 24 September 1, 2013 uPA activates stemness and chemoresistance

Suppression of uPA retards development of pancreatic cancer in nude mice and increases sensitization to gemcitabine

To determine whether tumorigenicity of MIA PaCa-2 cells was reduced after suppression of uPA expression, we orthotopically implanted nude mice with MIA PaCa-2 SP and ΔSP cells (100,000 cells initially) that had been pretreated with small interfering RNA (siRNA) specific for uPA (puPA), gemcitabine, or both, as described in Materials and Methods. Mice given puPA showed smaller tumor burdens than controls (p = 0.24). Mice implanted with ΔSP cells treated with gemcitabine alone showed the greatest reduction in tumor burden, whereas mice implanted with SP tumors did not respond to gemcitabine. The greatest reduction in tumor burden was seen in mice implanted with SP and ΔSP treated with both puPA and gemcitabine (Figure 2, B and C; p = 0.012 and 0.008, respectively).

FIGURE 2: uPA controls chemoresistance of pancreatic cancer cells to gemcitabine. (A) SP cells derived from MIA PaCa-2 and PANC-1 cells treated with shRNA specific for uPA (SP + puPA), untreated SP, and ΔSP cells derived from MIA PaCa-2 and PANC-1 cells were subjected to various concentrations of gemcitabine (0, 10, 100, 1000 nM), and cell cycle analysis was performed by fluorescence-activated cell sorting. (B) H&E staining of the paraffin sections of the tumors obtained from nude mice in which SP and ΔSP cells derived from MIA PaCa-2 cells that had been exposed to either puPA or gemcitabine or both as described in Materials and Methods were implanted orthotopically (100,000 cells/mouse). (C) Graphic representation of the tumor sizes in nude mice after orthotopic implantation of SP and ΔSP cells derived from MIA PaCa-2 cells (100,000 cells/mouse) that had been exposed to either puPA or gemcitabine (mean ± SD; n = 5; ΔSP, p = 0.01, and SP, p = 0.008).

uPA positively regulates Lhx2 expression in MIA PaCa-2 and PANC-1 pancreatic cancer cells and in human pancreatic tissues

We demonstrated previously that uPA is found within the nuclei of various types of proliferating cells (Stepanova et al., 2008). We therefore asked whether uPA localizes to the nuclei in pancreatic cancer cells. Immunocytochemical analysis of MIA PaCa-2, Capan-2, and PANC-1 cells revealed partial nuclear localization of uPA, which is significantly (p = 0.40) increased when recombinant uPA protein is added exogenously (Figure 3A and Supplemental Figure S4A). More recently, we reported that uPA binds to the transcription factor Lhx2 within the nuclei of pancreatic cancer cells and knockdown of uPA suppresses Lhx2 expression.
mutants on expression levels of Lhx2. PANC-1 (uPA-) cells overexpressed Lhx2 in response to exogenously added WT-uPA and ΔGFD-uPA, whereas levels were unchanged after addition of identical concentrations of ΔK-uPA (Supplemental Figure S4C).

miR-124 targets Lhx2 and is negatively regulated by uPA

We next investigated the mechanism by which uPA up-regulates the expression of Lhx2. We first performed miRNA target prediction analysis using the MiRanda (Enright et al., 2003) and PITA (Kertesz et al., 2007) algorithms. We found that Lhx2 mRNA is a potential target of hsa-mir-124 (miR-124) (Figure 4A). Specifically, we identified an 8-mer (CGUGCCUU) motif in the 3′ untranslated region (UTR) of Lhx2 that is highly conserved in multiple mammalian species as a potential binding site for miR-124 (Figure 4B). To validate this in silico prediction, we developed a reporter construct in which luciferase expression is controlled by the human Lhx2 3′-UTR DNA fragment containing the putative miR-124 interaction sequence. The Lhx2 3′-UTR reporter construct was transiently transfected into MIA PaCa-2 and PANC-1 cells alone or together with hsa-miR-124 again alone or in combination with the miR-124 inhibitor (anti–miR-124). Overexpression of miR-124 repressed the Lhx2 3′-UTR luciferase activity in both MIA PaCa-2 and PANC-1 cells, confirming the specificity of miR-124 toward the 3′-UTR region of Lhx2. We then examined expression levels of miR-124 in human pancreatic cancer tissues versus normal tissues. (Gorantla et al., 2011). Because Lhx2 is known to be involved in maintenance of stem/progenitor cell phenotype (Dahl et al., 2008; Tornqvist et al., 2010; Mardaryev et al., 2011; Nadal et al., 2012), we next investigated whether uPA-Lhx cross-talk regulates the maintenance of stem/progenitor cell phenotype in pancreatic cancer cells. To further decipher the role of nuclear uPA in the regulation of Lhx2 expression, we knocked down endogenous uPA expression in MIA PaCa-2 and PANC-1 cells using the puPA plasmid or uPA siRNA (MIA PaCa-2(uPA-) and PANC-1(uPA-) cells, respectively) and added wild-type (WT) uPA exogenously. We observed that addition of exogenous WT-uPA and overexpression of uPA (uPAOE) in MIA PaCa-2(uPA-) and PANC-1(uPA-) cells induced the expression of Lhx2 (Figure 3B). To determine whether uPA positively regulates Lhx2 expression in human tissues, we immunoprobed a human pancreatic tissue array for uPA and Lhx2. In malignant tissues, high levels of expression of both uPA with Lhx2 were observed. In contrast, normal pancreatic tissues showed no detectable expression of uPA and low expression levels of Lhx2 throughout (Figure 3C). Our previous studies indicated that uPAR is not essential for translocation of uPA to the nucleus. A uPA mutant lacking the uPAR-binding domain (ΔGFD-uPA) also translocates to cell nuclei, whereas a kringle-deficient uPA mutant (ΔK-uPA) did not, despite its ability to bind to uPAR (Stepanova et al., 2008). We found identical requirements for translocation of uPA in pancreatic cancer cells (Supplemental Figure S4, A and B). To address the role of uPAR in the regulation of Lhx2 expression by uPA specifically, we then studied the effect of these uPA deletion mutants on expression levels of Lhx2. PANC-1(uPA-) cells overexpressed Lhx2 in response to exogenously added WT-uPA and ΔGFD-uPA, whereas levels were unchanged after addition of identical concentrations of ΔK-uPA (Supplemental Figure S4C).

miR-124 targets Lhx2 and is negatively regulated by uPA

We next investigated the mechanism by which uPA up-regulates the expression of Lhx2. We first performed miRNA target prediction analysis using the MiRanda (Enright et al., 2003) and PITA (Kertesz et al., 2007) algorithms. We found that Lhx2 mRNA is a potential target of hsa-mir-124 (miR-124) (Figure 4A). Specifically, we identified an 8-mer (CGUGCCUU) motif in the 3′ untranslated region (UTR) of Lhx2 that is highly conserved in multiple mammalian species as a potential binding site for miR-124 (Figure 4B). To validate this in silico prediction, we developed a reporter construct in which luciferase expression is controlled by the human Lhx2 3′-UTR DNA fragment containing the putative miR-124 interaction sequence. The Lhx2 3′-UTR reporter construct was transiently transfected into MIA PaCa-2 and PANC-1 cells alone or together with hsa-miR-124 again alone or in combination with the miR-124 inhibitor (anti–miR-124). Overexpression of miR-124 repressed the Lhx2 3′-UTR luciferase activity in both MIA PaCa-2 and PANC-1 cells, confirming the specificity of miR-124 toward the 3′-UTR region of Lhx2. We then examined expression levels of miR-124 in human pancreatic cancer tissues versus normal
tissues did not express miR-124 (Figure 5C), in contrast to its expression in normal pancreatic tissues (Figure 5D). This suggests that overexpression of Lhx2 in pancreatic cancer tissues (Figure 3) might be due to suppression of miR-124. Because 1) enhanced miR-124 expression has inhibitory effects on cancer stem–like traits and invasiveness (Xia et al., 2012), 2) miR-124 targets Lhx2 transcript, and 3) uPA up-regulates Lhx2 in pancreatic cancer cells, we examined the relationship of uPA and miR-124. To determine whether uPA affects the expression of miR-124 and thereby regulates Lhx2, we used miRNA-specific stem loop PCR to examine expression of miR-124 in control MIA PaCa-2 and PANC-1, MIA PaCa-2 (uPA-), and PANC-1 (uPA-) cells cultured in absence or presence of exogenously added WT-uPA. We observed that down-regulation of uPA expression (MIA PaCa-2 (uPA-) and PANC-1 (uPA-) cells) induced expression of miR-124 compared with control MIA PaCa-2 and PANC-1 cells, whereas addition of WT-uPA inhibited miR-124 expression (Figure 5F). Furthermore, cells treated with shRNA specific for uPA (MIA PaCa-2 (uPA-) and PANC-1 (uPA-) cells) and transfected with the Lhx2 3′-UTR luciferase reporter construct showed significant decrease in luciferase activity, suggesting that uPA up-regulates Lhx2 by suppressing expression of miR-124. To determine whether down-regulation of uPA induces expression of miR-124 in vivo, we orthotopically implanted MIA PaCa-2 SP cells that exhibit cancer stem cell–like characteristics (Figure 1A and Supplemental Figures S2 and S4) into the pancreas of nude mice, which were then injected intraperitoneally with shRNA targeting uPA (puPA; plasmid expressing shRNA targeting uPA). In vivo suppression of uPA resulted in significant (p = 0.02) increase in expression of miR-124 in tumor tissue after 40 d but not in normal tissue (Figure 5E). Of interest, hsa-miR-124 also suppressed expression of both Lhx2 and uPA in MIA PaCa-2 and PANC-1 cells, whereas transfection of these cells with anti–miR-124 enhanced expression of Lhx2 and uPA (Figure 4D). Together these data suggest the existence of a negative feedback loop between uPA and miR-124, which may regulate expression of Lhx2 and pancreatic cancer cell stemness.

uPA complexes with HOXA5 and inhibits p53 promoter activity

To further elucidate the functions of uPA in the nucleus, we examined whether uPA binds directly to transcription factors (TFs), using a protein TF array. Supplemental Figure S5A shows that uPA binds to homeobox transcription factors Lhx2, HOXAS, and Hey. Binding of uPA to Lhx2 (Gorantla et al., 2011) and HOXAS (Figure 6A) was
confirmed by coimmunoprecipitation pull-down assays. HOXA5 up-regulates expression of the p53 tumor suppressor gene in breast cancer cells (Raman et al., 2000). It has been suggested that down-regulation of HOXA5 expression or loss of its function results in inhibition of p53 expression (Raman et al., 2000), which enhances tumorigenicity, EMT, and acquisition of stem-like phenotype by breast cancer cells (Mizuno et al., 2010). Loss of p53 function has also been linked to the induction of EMT and stemness in pancreatic cells (Keck and Brabletz, 2011; Pasi and Pelicci, 2011). Therefore we hypothesized that uPA further promotes stemness of pancreatic cancer cells by down-regulating p53 through interference with HOXA5. To assess whether uPA regulates activity of the p53 promoter, we stably transfected MIA PaCa-2 and PANC-1 cells with the human p53 promoter-driven luciferase reporter construct (Mia PaCa-2 (p53-luc) and PANC-1 (p53-luc) cells, respectively). Suppression of uPA expression in MIA PaCa-2 (p53-luc) and PANC-1 (p53-luc) cells caused activation of p53 promoter-driven luciferase expression, whereas uPA overexpression (uPAOE) inhibited p53 promoter-controlled luciferase activity (Figure 6B). Similar results were obtained using SP or ΔSP cells (Figure 6C). To determine whether uPA suppresses p53 promoter activity by interfering with the function of HOXA5, we used human embryonic kidney 293 cells (HEK293 cells), which express low levels of endogenous HOXA5 and uPA. HEK293 cells were cotransfected with the p53-luc construct and HOXA5- and/or uPA-encoding vectors. Figure 6D shows that coexpression of p53-luc and HOXA5 leads to activation of p53 promoter activity, whereas concomitant coexpression of uPA attenuates HOXA5-mediated activation of p53 promoter.

**FIGURE 5:** Down-regulation of uPA causes overexpression of miR-124 in pancreatic cancer cells. Human pancreatic cancer tissue arrays were subjected to (A) H&E staining or (B) miR-124 in situ hybridization. miR-124 expression was assessed in both PDAC (C) and normal pancreatic tissues (D). (E) Mice implanted subcutaneously with MIA PaCa-2 cells were treated with puPA as described in Materials and Methods, and expression levels of miR-124 in paraffin sections were assessed by in situ hybridization. (F) Total RNA was isolated from MIA PaCa-2 and PANC-1 cells treated with puPA, and expression levels of Lhx2 mRNA and miR-124 were determined.
These data suggest that binding of uPA to HOXA5 attenuates its DNA-binding capacity. To test this hypothesis, we examined whether uPA affects binding of HOXA5 to its target DNA sequence. We performed the electrophoretic mobility shift assay (EMSA) using p53 promoter-derived, double-stranded oligonucleotides that possess a HOXA5 consensus region (see Materials and Methods). We found that uPA does not bind the HOXA5 DNA consensus sequence but instead forms a complex with HOXA5 that binds p53 promoter-derived oligonucleotide less well than free HOXA5 (Figure 6E). Finally, we used pancreatic cancer cell line Capan-2, which expresses wild-type p53, to assess the role of p53, HOXA5, and uPA in chemoresistance. Exogenously added WT-uPA down-regulated p53 mRNA in Capan-2 cells by 1.6 ± 0.2 times (p < 0.05), and silencing uPA expression in Capan-2 cells up-regulated p53 mRNA (Supplemental Figure S5B). Finally, WT-uPA and ΔGFD-uPA but not ΔK-uPA increased resistance of uPA-targeting, shRNA-treated Capan-2 cells to gemcitabine-induced apoptosis (Supplemental Figure S5C). Together these data suggest that uPA regulates pancreatic cancer cell survival by down-regulating expression of p53 in addition to up-regulating expression of Lhx2.

DISCUSSION
Pancreatic ductal adenocarcinoma has a high mortality rate, which is attributable in part to delay in diagnosis but also to lack of effective treatment options. Gemcitabine, the cornerstone of adjuvant and metastatic therapy, delays the development of recurrent disease after “complete resection” (Oettle et al., 2007), but the estimated disease-free survival at 3 and 5 yr remains 23.5 and 16.5%, respectively, compared with
7.5 and 5.5% in untreated patients. Thus the intrinsic and acquired resistance of pancreatic tumors to gemcitabine and other treatments continues to be a major clinical problem. The goal of our study was to better understand the molecular basis underlying the drug resistance of pancreatic cancer cells. The rapid progression of pancreatic cancer is characterized by molecular changes within the tumor and the surrounding stromal cells (Bailey and Leach, 2012; Feig et al., 2012; Tod et al., 2013; Whatcott et al., 2013) that emerge from a side population of cells indicated by EMT and a cancer stem cell phenotype associated with drug resistance (Kabashima et al., 2009; Haque et al., 2011; Kabashima-Nibe et al., 2013). Pancreatic cancer cell lines (MIA PaCa-2 and PANC-1) cultured under serum-free conditions expressed stem cell markers CD44 and CD24 (Figure 1 and Supplemental Figure S1), had an increased proportion of SP cancer stem cell–like cells, formed pancreatospheres (Figure 1E and Supplemental Figure S2), and showed gemcitabine resistance, in contrast to mixed or ΔSP populations (Figure 2A). Down-regulation of uPA expression attenuated the stem cell phenotype (Hamada and Shimosegawa, 2012), suppressed formation of pancreatospheres (Bao et al., 2011), and restored sensitivity to gemcitabine. In contrast, overexpression of uPA increased drug resistance and formation of pancreatospheres by cultured pancreatic cancer cells in vitro (Figure 1E) and promoted tumor growth in vivo (Figure 1C). These outcomes provide new insight into the mechanisms underlying the well-established correlation between elevated tumor uPA, propensity to metastasize, and poor prognosis of this disease (reviewed in Ischenko et al., 2010; Rasheed and Matsui, 2012).

The adverse outcome associated with uPA expression has generally been attributed to its proteolytic activity and uPAR-dependent signaling mechanisms (Gupta et al., 2011; Andres et al., 2012; He et al., 2012), but uPAs role in maintaining cancer cell stemness, chemoresistance, and survival has not been addressed. Our studies identify a novel uPA-mediated pathway that contributes to the stem-like phenotype. We found that uPA is localized in the nucleus of pancreatic cancer cells (Figure 2A and Supplemental Figure S2B; Gorantla et al., 2011) and associates with the transcription factor Lhx2, which has been implicated in the maintenance of stemness (Pinto do et al., 2001; Tiede and Paus, 2006). We show that uPA up-regulates expression of Lhx2 in pancreatic cancer cells. Lhx2 expression was also seen in tumor-associated cells in an in vivo mouse model, suggesting that uPA may participate in reprogramming of these associated cells to facilitate tumor growth via Lhx2 (Gorantla et al., 2011). Overexpression of Lhx2 in response to uPA was associated with concomitant uPA-dependent down-regulation of miR-124, which, as we found, targets Lhx2 3′-UTR. In support of this proposed pathway, expression of miR-124 was inversely related to expression of uPA. Pancreatic tissue array analysis revealed that normal pancreatic tissues expressed miR-124, whereas tumor tissues did not. Furthermore, overexpression of uPA in orthotopic tumors in nude mice paralleled suppression of miR-124, whereas tumor tissues in which uPA were down-regulated expressed elevated levels of miR-124. The mechanism of miR-124 suppression by uPA requires further study. Our data suggest that a negative feedback loop links uPA and miR-124, which in turn increases expression of Lhx2, leading to pancreatic cancer cell stemness. uPA can also interact with stromal cells, in which it may activate Lhx2 and promote stemness through paracrine pathways. This paracrine regulation mediated by uPA may be one of the reasons uPA-overexpressing tumors are more resilient to conventional chemotherapies (Khanna et al., 2011).

uPA may up-regulate Lhx2 through several intracellular mechanisms that eventuate in cell survival and chemoresistance. For example, we found that uPA binds directly to several homeobox transcript factors, including HOXA5, which up-regulates p53 expression through direct transactivation of the p53 promoter (Raman et al., 2000; Gendronneau et al., 2010). Using EMSA and p53 promoter-driven luciferase reporter assays, we observed that uPA inhibits binding of HOXAS to its DNA consensus sequence, attenuates the ability of HOXAS to activate the p53 promoter, and inhibits p53 expression in the pancreatic cancer cell line Capan-2 (Figure 6 and Supplemental Figure S5). This is accompanied by increased chemoresistance of Capan-2 cells (Supplemental Figure S5), suggesting that the effect of uPA and HOXAS on p53 contributes to pancreatic cancer cell stemness. HOXAS-binding elements are present in Lhx2 and CD24 promoters (Qiagen, Valencia, CA; available at www.sabiosciences.com/chipqpcrsearch.php?app=TFBS). Whether and how HOXAS regulates activity of Lhx2 and CD24 promoters in clinical disease and whether uPA regulates expression of these genes in a HOXAS-dependent manner remain to be determined.

uPAR has been implicated in the progression of pancreatic and other types of cancer through uPA-dependent and uPA-independent pathways (Adachi et al., 2002; Blasi and Carmeliet, 2002; Gondi et al., 2003; de Bock and Wang, 2004; Kondraganti et al., 2006; Gorantla et al., 2011; Asuthkar et al., 2012). It is likely that uPA binds to uPAR on the surface of pancreatic cancer cells and uPAR binds to other receptors and/or ligands that might contribute to survival and progression. On the other hand, the incomplete response of pancreatic cancer to inhibition of uPA-proteolytic activity and incapacitation of the uPA-uPAR axis (Mekkawy et al., 2009; Hildenbrand et al., 2010; Boonstra et al., 2011; Carriero and Stopelli, 2011; Lund et al., 2011) provides compelling evidence that other pathways must be involved as well. Nucleolin, which we showed mediates nuclear translocation of uPA (Stepanova et al., 2008), associates with uPAR (Dumler et al., 1999) and participates in the nuclear translocation of endostatin (Song et al., 2012) and perhaps uPAR itself (Asuthkar et al., 2012). Whether nuclear translocation of uPAR in pancreatic cancer cells contributes to pancreatic cancer stemness and chemoresistance through its association with nucleolin remains to be determined. The uPA variant ΔGPD-uPA, however, which is unable to bind to uPAR but is capable of nuclear translocation, up-regulates Lhx2 expression in PANC-1 cells (Supplemental Figure S4) and increases chemoresistance of Capan-2 cells (Supplemental Figure S5) similar to WT-uPA, suggesting that uPAR is not an obligate carrier for uPA translocation to the nucleus and its effect on gene transcription. Thus, to our knowledge, this is the first evidence that links nuclear uPA to the maintenance of pancreatic cancer cell stemness and chemoresistance in an uPAR-independent manner. Further delineation of this cellular and genetic program might offer new therapeutic targets in this still devastating disease.

MATERIALS AND METHODS

Cell lines and culture conditions

Pancreatic cancer cells MIA PaCa-2 and PANC-1 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5.0% CO2 at 37.2°C with media changes every 48 h. To facilitate growth of cancer stem cells, MIA PaCa-2 and PANC-1 cells were cultured in serum-free DMEM supplemented with basal fibroblast growth factor (10 ng/ml), 1× B27 supplement, and epidermal growth factor (50 ng/ml; all from Life Technologies, Frederick, MD).

shRNA construct

shRNA-expressing plasmids targeting uPA (puPA) were constructed as described previously (Gondi et al., 2007a,b). Lentivirus-delivered,
pLKO-based, shRNA-targeting uPA mRNA in Capan-2 cells was obtained from Thermo Scientific (formerly Open Biosystems; West Palm Beach, FL). uPA siRNA was obtained from Santa Cruz Biotechnology (sc-36779; Santa Cruz, CA).

**Chemotherapy compound**

Gemcitabine (G6423) was purchased from Sigma-Aldrich (St. Louis, MO) and diluted in sterile water. MIA PaCa-2 and PANC-1 side-population cancer stem like cells were incubated with gemcitabine (0–1000 nM), and the cytotoxicity was determined after 48 h of incubation.

**Side-population sorting, cell cycle analysis, and sphere formation**

MIA PaCa-2 and PANC-1 cells (2 × 10⁴) resuspended in 0.5 ml of phosphate-buffered saline (PBS)–bovine serum albumin (BSA) were analyzed, and the SP cells were sorted by density-based flow cytometry (10,000 cells sorted per treatment condition, with three replications for each treatment). Acquisition was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), and viable cells were analyzed with CellQuest software. In separate experiments, SP cells were labeled with anti-CD44 and anti-CD24 antibodies using a similar staining protocol. The control for each sample was prepared identically, except that an isotype-specific antibody was used. Progression through different cell cycle phases by MIA PaCa-2 and PANC-1 cells alone and by puPA and after exposure to gemcitabine for 48 h was monitored by flow cytometric analysis of DNA content of cell populations stained with 50 μg/ml propidium iodide (BioSure, Grass Valley, CA). CSC neurospheres were obtained from SP cells sorted from parental MIA PaCa-2 and PANC-1 cells as described previously (Velpula et al., 2011).

**miRNA and anti-miRNA**

The pcDNA3.2/V5 hsa-miR-124 (plasmid 26306) was purchased from Addgene (Cambridge, MA), and hsa-miR-124 miRCURY LNA microRNA inhibitor (412512-00) was purchased from Exiqon (Woburn, MA).

**3′-UTR reporter assay**

3′-UTR mRNA sequences of Lhx2 (5′-GGTACCTTTTCAATGACT-CGCCAACC-3′; 5′-TAACAAAAAACAACCTCACAAGAAGATC-3′) were amplified and cloned into pIso (plasmid 12178) mammalian expression vector (Addgene). For the reporter gene assay, MIA PaCa-2 and PANC-1 cells were transiently transfected with the 200 ng/ml Lhx2 3′-UTR construct alone or in combination with either the 1 nmol hsa-miR-124 mimic or 5 nmol hsa-miR-124 inhibitor (anti-miR-124). After 48 h, cells were lysed to determine luciferase activity using a dual-luciferase reporter assay system (Promega, Madison, WI), and the relative luciferase units were measured in a luminometer (TD-20/20 DLReady).

**Stem-loop PCR (reverse transcriptase PCR)**

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA). For miRNA analysis, 100 ng of template RNA was reverse transcribed using universal first-strand cDNA synthesis kit and miR-124 specific reverse transcriptase (RT) primers from the miRCURY LNA® microRNA PCR system (Exiqon) following the supplied protocol. The miR-124 transcript levels were examined by RT-PCR using the CFX96 Real Time System (Bio-Rad, Hercules, CA) and the SYBR Green PCR Master Mix (Applied Biosystems, Framingham, MA). The following PCR conditions were used: 95°C for 10 min, followed by 50 cycles at 95°C/10 s and 60°C/30 s. The fold change was calculated using 2−ΔΔCT, where CT is the threshold cycle and ΔΔCT = ΔCT of treatment – ΔCT of control.

**RT-PCR analysis**

MIA PaCa-2 and PANC-1 cells were transfected with uPA-directed siRNA for 48 h. The cells were collected, and total cell RNA was isolated. RT-PCR was set up using primers specific for uPA (Table 1). The PCR cycle was 95°C/5 min, (95°C/30 s, 65°C/1 min, 72°C/1 min) × 30, 72°C/10 min. The PCR product was quantified and plotted relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as arbitrary units.

**Antibodies**

The following antibodies were obtained from Santa Cruz Biotechnology: anti-uPA (sc-14019), anti-Lhx2 (sc-81311), anti–HOXA5 (sc-13199), anti-P53 (sc-126), anti-p-P53 (Ser-15; sc-101762), and anti-GAPDH (sc-59541).

**Western blotting**

MIA PaCa-2 and PANC-1 cells were transfected with uPA-targeted siRNA for 48 h. Cells were collected, and total cell lysates were prepared in standard RIPA extraction buffer containing aprotonin and phenylmethlysulfonyl fluoride. Protein samples (40 μg) were separated under nonreducing conditions by 12% SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were immunoprobed using 1:500 dilutions of primary antibodies and 1:1000 dilutions of species-specific, horse-radish peroxidase (HRP)–conjugated secondary antibodies and then developed according to an enhanced chemiluminescence protocol (Amersham, Arlington Heights, IL).

**Fibrin zymography**

The enzymatic activity of electrophoretically separated forms of uPA in the conditioned media of MIA PaCa-2 and PANC-1 transfected with uPA-targeted siRNA for 48 h was determined by SDS–PAGE as described previously (Yamamoto et al., 1994; Mohanam et al., 1997). 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.

**Subcutaneous tumor growth and surgical orthotopic implantation of MIA PaCa-2 tumors**

We carried out subcutaneous implantation (10,000 or 100,000 cells) and orthotopic implantation of MIA PaCa-2 SP and ΔSP cells as described previously (Fu et al., 1992). Three days after implantation, mice were administered intraperitoneal injections of puPA five times at 150 μg/mouse every other day and or in combination with gemcitabine (0.33 mg/mouse; Koppe et al., 2006). Mice were monitored.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>uPA</td>
<td>TGGCTCCTGTGTCGAGCGAGCA</td>
<td>CAAGCGTGTCAGCGGTGAGTAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGGAGTCAACGGATTGGTCGTGTAAT</td>
<td>AGCGTCTCCATGGTGTGAACG</td>
</tr>
<tr>
<td>P53</td>
<td>TCAAAAGATGTTTTCGCAACTG</td>
<td>ATGTGCGTGACTGCGTAG</td>
</tr>
</tbody>
</table>

**TABLE 1:** Primers used for PCR and RT-PCR.
daily, and body weight was measured daily to ensure that weight loss did not exceed 20%. At 40 d after implantation, mice were killed, and pancreatic tissues were isolated and processed for paraffin embedding.

**Immunohistochemistry analysis**

Pancreatic tumors from control (untreated) and puPA- and puPA-treated mice were cut into thin sections (5–6 μm thick), which were deparaffinized in xylene and rehydrated in graded ethanol solutions. Antigen retrieval was carried out with 10 mM citrate buffer (pH 6) at boiling temperature for 60 min, with permeabilization in 0.1% Triton-X-100. Permeabilized sections were blocked for 1 h using 3% BSA in PBS. Cells were incubated with primary antibody (uPA or Lhx2) and Alexa Fluor– or HRP-conjugated secondary antibody for 60 min at room temperature. Before mounting, the slides were washed with PBS and incubated for 5 min with a 1:100 dilution of 4′,6-diamidino-2-phenylindole (DAPI) for fluorescence nuclear staining and analyzed using confocal microscopy (BX61 Fluoview; Olympus, Minneapolis, MN) at 40× magnification. If the HRP-conjugated secondary antibodies (Abs) were used, the sections were developed using diaminobenzidine substrate. Human pancreatic cancer tissue arrays were obtained from US Biomax (Rockville, MD). Tissue arrays were processed for immunohistochemistry using a standard protocol (Gondi et al., 2004). A control study was performed using a normal rabbit immunoglobulin fraction as the primary antibody (control Ab) in lieu of uPA or Lhx2. The tissues were counterstained with hematoxylin dye to visualize the nucleus.

**Transcription factor–binding array analysis**

TF protein–protein binding array analysis was performed using the TF Protein Array kit from Panomics (MA3501-08) as per the manufacturer’s instructions. Briefly, purified human wild-type uPA (American Diagnostics, Lexington, MA) was suspended in 1× blocking buffer at a concentration of 100 ng/ml. The TF membranes were incubated in 1× blocking buffer for 2 h at room temperature, followed by incubation with uPA in 1× blocking buffer for 2 h at room temperature. This was followed by washings and further incubation with anti-uPA antibody, followed by secondary HRP-conjugated antibody as per kit instructions. HRP was detected using the buffers provided, followed by exposure of membranes to x-ray film. Binding of uPA 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.

**ACKNOWLEDGMENTS**

We thank Peggy Mankin for assistance with flow cytometry analysis and Noorjahan Ali for histology processing. Support from the Department of Medicine, Department of Cancer Biology and Pharmacology, William E. McElroy Foundation, Springfield, IL (to C.S.G.), and the National Cancer Institute (1R21CA141228-01 to V.S.) for this research is acknowledged.

**REFERENCES**


