OPEN

The Basic Helix-Loop-Helix Transcription Factor E47 Reprograms Human Pancreatic Cancer Cells to a Quiescent Acinar State With Reduced Tumorigenic Potential

SangWun Kim, MD,†‡ Reyhaneh Lahmy, PhD,*‡ Chelsea Riha, BS,*‡ Challeng Yang, BS,*‡ Brad L. Jakubison, BS,§ Jaco van Niekerk, BS,*‡ Claudio Staub, BS,*‡ Yifan Wu, BS,*‡ Keith Gates, PhD,|| Duc Si Dong, PhD,|| Stephen F. Konieczny, PhD,§ and Pamela Itkin-Ansari, PhD*‡*

Objectives: Pancreatic ductal adenocarcinoma (PDA) initiates from quiescent acinar cells that attain a *Kras* mutation, lose signaling from basic helix-loop-helix (bHLH) transcription factors, undergo acinar-ductal metaplasia, and rapidly acquire increased growth potential. We queried whether PDA cells can be reprogrammed to revert to their original quiescent acinar cell state by shifting key transcription programs.

Methods: Human PDA cell lines were engineered to express an inducible form of the bHLH protein E47. Gene expression, growth, and functional studies were investigated using microarray, quantitative polymerase chain reaction, immunoblots, immunohistochemistry, small interfering RNA, chromatin immunoprecipitation analyses, and cell transplantation into mice.

Results: In human PDA cells, E47 activity triggers stable G_0/G_1 arrest, which requires the cyclin-dependent kinase inhibitor p21 and the stress response protein TP53INP1. Concurrently, E47 induces high level expression of acinar digestive enzymes and feed forward activation of the acinar maturation network regulated by the bHLH factor MIST1. Moreover, induction of E47 in human PDA cells in vitro is sufficient to inhibit tumorigenesis.

Conclusions: Human PDA cells retain a high degree of plasticity, which can be exploited to induce a quiescent acinar cell state with reduced tumorigenic potential. Moreover, bHLH activity is a critical node coordinately regulating human PDA cell growth versus cell fate.

Key Words: pancreatic ductal adenocarcinoma, ID3, bHLH signaling, E47, reprogramming

(*Pancreas* 2015;44: 718–727)

From the *Development and Aging Program, Neuroscience, Aging, and Stem Cell Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA; †Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Institute of Women's Life Medical Science, Yonsei University College of Medicine, Seoul, Republic of Korea; ‡Department of Pediatrics, University of California, San Diego, La Jolla, CA; §Department of Biological Sciences, Center for Cancer Research, the Bindley Bioscience Center, Purdue University, West Lafayette, IN; and ||Sanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA.

Received for publication January 29, 2015; accepted February 9, 2015.

Reprints: Pamela Itkin-Ansari, PhD, Development and Aging Program, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Rd, La Jolla, CA 92037 (e-mail: pitkin@sanfordburnham.org).

This study was supported by the National Cancer Institute under award number 5P30CA030199 (P.I.A. and D.S.D.) from The Hartwell Foundation and The Hirshberg Foundation (P.I.A.), by DK55489 and CA124586 from the National Institutes of Health (S.F.K.), by the Korean government (Ministry of Education, Science and Technology) number 2011-0013127 from the National Research Foundation (NRF) of Korea, and a faculty research grant 6-2012-0078 from Yonsei University College of Medicine for 2012 (S.W.K.). For the remaining authors, no funding sources were declared.

The authors declare no conflicts of interest.

Supplemental digital contents are available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.pancreasjournal.com).

Pancreatic ductal adenocarcinoma (PDA) arises from the exocrine pancreas, which is composed of 2 major cell populations—acinar cells that produce digestive enzymes and duct cells that are tasked with transporting acinar enzymes to the duodenum. Despite the ductal morphology of PDA, lineage tracing studies have revealed that the tumors arise from the acinar compartment.[1–9] In response to oncogenic *Kras*, quiescent acinar cells enter the cell cycle and undergo acinar-ductal metaplasia (ADM) where the cells silence acinar transcription programs. The cells acquire a duct-like transcriptome that drives the formation of precancerous pancreatic intraepithelial neoplasia (PanIN) lesions.[5,6,9] Subsequent mutations in additional oncogenes or tumor-suppressor genes fuel disease progression, leading to aggressive PDA. Whether this process is reversible is not known, but if tumor cells could be induced to enter a quiescent, differentiated acinar cell state, this would provide an important new strategy for combating this deadly disease.

During ADM, tumor-initiating cells repress expression of the acinar transcription factors MIST1 and pancreas-specific transcription factor 1a (PTF1a).[7,8,10–14] leading to loss of acinar cell polarity, gap junction complexes, and key acinar gene products, including digestive enzymes. Because PTF1a and MIST1 belong to the basic helix-loop-helix (bHLH) family of transcription factors, they must form bHLH dimer complexes to bind to cognate E-box DNA sites found in target genes.[15] The bHLH family members assemble as either homodimer or heterodimer complexes with other bHLH proteins such as E47.[16–21] The bHLH family is also subject to negative regulation by the inhibitor of DNA binding (ID) family of proteins (ID 1–4 in humans). IDs contain an α-helical HLH dimerization motif but lack a basic domain, resulting in dominant-negative bHLH/ID heterodimers.[15]

We and others have shown that ID3 protein levels are greatly elevated in PDA tumors.[18–20] Moreover, we showed that upregulation of ID3 is sufficient to induce cell cycle entry in quiescent human exocrine tissue.[18] High ID3, as well as attenuated expression of bHLH proteins, suggests that bHLH transcriptional networks are profoundly dysregulated in PDA and that restoring normally high bHLH activity might promote pancreatic cellular homeostasis. The misregulation of bHLH and HLH factors in PDA provides a testable model to determine whether shifting these regulatory networks would revert cells towards their original acinar cell state. To this end, we generated PDA lines expressing an inducible form of E47. E47 was chosen because of its versatility in binding to client bHLH proteins, its known regulation of acinar gene expression in concert with PTF1a,[20] and its activity as a homodimer. Moreover, E47 efficiently binds and thus sequesters ID3.[18,22] Initially, we found that induced E47 activity in PANC-1 cells led to reduced Ki67 expression.[18] Here, we report

that E47 causes rapid and stable G_0/G_1 arrest in all cell lines tested and that, mechanistically, this requires upregulation of $p21^{CIP1/WAF1}$ and the stress response protein TP53INP1. Remarkably, cell cycle exit is accompanied by reactivation of the acinar cell differentiation program, including robust expression of acinar digestive enzyme genes. In addition, E47-induced cells re-express *Mist1*, a key bHLH transcription factor that controls the acinar maturation program.[14,23,24] Moreover, we show that MIST1 is required for the re-establishment of acinar gap junctions. Importantly, induction of E47 in vitro was sufficient to inhibit PDA cell tumorigenesis, because E47-expressing cells maintained growth arrest and acinar gene expression following transplantation. The data suggest that changes to the acinar cell transcriptome can have a dominant role in PDA pathogenesis. Collectively, the results demonstrate that PDA cells maintain a previously unrecognized level of cellular plasticity that is amenable to restoring a quiescent acinar cell state. We propose that bHLH transcriptional activity serves as the fulcrum dictating the growth and differentiation status of acinar cells undergoing oncogenic insults, findings which should support novel avenues for therapeutic intervention.

MATERIALS AND METHODS

Cell Culture

Human pancreatic adenocarcinoma cell lines from the ATCC (Rockville, Md), PANC-1 (CRL-1469),[25] BxPC-3 (CRL-1687),[26] and MiaPaCa2 (CRL-1420)[27] were cultured in Roswell Park Memorial Institute medium 1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C. E47-inducible cell lines were generated by infection with a retroviral vector expressing E47 fused to a tamoxifen-inducible modified estrogen receptor (MER). This vector also expresses CD25, which was used to isolate stable lines by fluorescence-activated cell sorting (FACS) as previously described.[18,19] E47 activity was induced by incubating cells with tamoxifen for 48 to 96 hours unless otherwise noted.

Transfections

For flow cytometry, parental PDA cell lines were transfected (Lipofectamine 2000; Invitrogen) with green fluorescent protein or inducible E47 plasmids and incubated with tamoxifen for 48 to 72 hours. For knockdown studies, PDA cells were transfected with small interfering RNA (siRNA) to $p21^{CIP1/WAF1}$ (AM51331; Applied Biosystems and J-003471–12; Dharmacon), *TP53INP1* (J-016159–05–0005, J-016159–05; Dharmacon), *Id3* (J-009905–07, J-009905–08; Dharmacon), or *Mist1* (J-009045–15, J-009045–16; Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) and incubated for 96 hours. For each gene, at least 2 independent siRNAs were employed.

Flow Cytometry

Live sorting: cells were immunostained with fluorescein isothiocyanate-conjugated mouse antihuman CD25 (1:100, BD Biosciences) as previously described.[18] Cell cycle analysis: cells were fixed with 100% ethanol, incubated with antihuman CD25 and propidium iodide (Invitrogen) for analysis on a FACS Canto cytometer (BD Biosciences). G_0/G_1, S, and G_2/M phase estimates were generated by modeling data with ModFitLT software (Verity Software House).

Microarray Analysis

Four biological replicates of PANC-1/E47 cells were harvested from each of the following 3 treatment groups: untreated controls and 2 different doses of tamoxifen for 48 hours. Data are deposited in gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo), accession number GSE55999. Briefly, RNA was labeled with biotin-16-UTP and hybridized to HumanHT-12 v4 Expression BeadChip (Illumina, Inc). BeadChips were scanned and normalized with a BeadArray Reader. The resulting data were collected by Scanner software and preprocessed by GenomeStudio software (Illumina, Inc). Principal component analysis of differential gene detection was performed with Partek Genomics Suite (Partek, Inc). Hierarchical clustering and other statistical analyses were performed using R/Bioconductor software package (www.bioconductor.org). A change in gene expression of at least 1.5-fold at the 99% confidence level was considered significant. Pathway analyses were performed with Ingenuity Pathway Analysis software (Ingenuity Systems, Inc). GSE16515 and GSE15471 datasets were used to identify genes, which are highly expressed in human PDA tumors relative to normal pancreas tissue, and to determine the correlation between *Id3* and *TP53INP1* expression in PDA-tumor samples relative to control tissue (statistically analyzed by Pearson coefficient).[28,29] Gene Set Enrichment Analysis (GSEA) was used to compare the E47-induced gene set versus GSE1133 and GSE2361 gene sets comparing normal human pancreas with other tissues[30,31] using the NCBI GEO2R tool. The final pancreas-enriched gene sets were further defined by the following criteria: (1) greater than 2.0-fold change in expression, compared with other tissues; (2) $P < 0.05$; (3) greater than 50 expression signals ("Present") from detection calls in pancreas samples; and (4) absent genes in the islet-enriched gene set.

Immunostaining

Cultured cells were fixed in 4% paraformaldehyde (USB Corp), permeabilized with 0.3% Triton X-100, and incubated with the following primary antibodies: mouse anti-E47 (1:100, 554077; BD Pharmingen), mouse anti-Ki67 (1:100, 550609; BD Biosciences), rabbit anti-$p21^{CIP1/WAF1}$ (1:100, ab7960; Abcam), rabbit anti-ZO.1 (1:100, 402200; Invitrogen), mouse anti-PRSS2 (1:100, SAB140022; Sigma), rabbit anti-CX32 (1:100, ab66613; Abcam), mouse anti-MIST1 (in-house affinity purified, 1:50), and mouse antiactin (1:100, ab8224; Abcam). Secondary antibodies were conjugated to donkey antirabbit Alexa Fluor 488 (1:400, A21206; Invitrogen) or donkey antimouse rhodamine (1:400, 100185–414; Jackson ImmunoResearch). Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; AppliChem). Digital images were acquired with fluorescence or confocal microscopes equipped with a digital camera (Nikon). Paraffin sections (5 μm) of explanted tumors were subjected to antigen retrieval (Dako: pH, 6.0). Endogenous peroxidase was blocked with 3% hydrogen peroxide (H_2O_2) and methanol followed by nonspecific blocking with Block M (BioCare) for 25 minutes, primary antibody at 4°C, and MM-HRP-Polymer (Biocare) at room temperature. The color reaction was developed using DAB (3,3′-diaminobenzidine tetrahydrochoride) (Sigma-Aldrich), counterstained with Mayer's hematoxylin (Sigma-Aldrich), and mounted in Cytoseal 60 (Thermo Scientific).

Real-Time qPCR Analysis

Ribonucleic acid was extracted with an RNeasy Mini Kit (Qiagen) and reverse transcribed with qScript cDNA Supermix (Quanta). Real-time qPCR was performed using the LightCycler 480 II system with SYBR Green I (Roche), and gene expression was normalized to 18S rRNA. Primer sequences are provided in Table S1, Supplemental Digital Content, http://links.lww.com/MPA/A363.

Immunoblotting

PANC-1/E47 cell lysates were blotted with antibodies to $p21^{CIP1/WAF1}$, Ki67 (Leica Biosystems), α/β HSP90 (Santa Cruz), MIST1 (in-house affinity purified), as well as those listed in immunofluorescence studies, resolved on 4% to 12% SDS-PAGE gels (Thermo Scientific), and transferred to nitrocellulose membranes (LI-COR Biosciences). Membranes were incubated with infrared dye-conjugated secondary antibodies (LI-COR) and imaged with a LI-COR Odyssey v 3.0 imaging system.

Immunoprecipitation

The PANC-1 cells were transfected with Id3-Flag plasmids using Reagent (Invitrogen). After 6 hours of incubation at 37 °C, growth media were changed. Tamoxifen was added to concentration of 4 μm. After incubation with tamoxifen for 48 hours, cells were harvested using cell lysis buffer (TrisHCl, 20 mM [pH, 7.5]; Triton X-100, 1%; glycerol, 10%; NaCl, 300 mM; EDTA, 0.5 mM [pH8.0]; Na_3VO_4, 1 mM; protease inhibitors] for immunoprecipitation. The FLAG fusion proteins were extracted using Anti-FLAG M2 Magnetic Beads, proteins were electrophoretically separated on SDS-4–12% polyacrylamide gels (Thermo Scientific), transferred onto nitrocellulose membranes (LI-COR Biosciences), blocked with Odyssey Blocking Buffer (LI-COR Biosciences), and incubated at RT with anti-FLAG (Abcam) and anti-β-actin (Abcam) as control. Secondary antibody detection was performed with infrared dye conjugates (LI-COR Biosciences).

Chromatin Immunoprecipitation

The PANC-1/E47 cells were induced with 1-μm tamoxifen or EtOH at 70% confluence. After 48-hour tamoxifen treatment, the cells were fixed in a 1% formaldehyde-phosphate-buffered saline solution. Formaldehyde was neutralized using glycine. The cells were lysed, and DNA was fragmented via sonication using 15-second pulses 5 times per sample. Thirty micrograms of chromatin was incubated with 4 μg of either anti-transcription factor E2-Alpha or anti-immunoglobulin G. Chromatin was extracted using Sepharose A beads, and crosslinks were reversed using NaCl. Deoxyribonucleic acid was extracted and purified using Proteinase K digestion and the QIAquick PCR purification kit. Chromatin immunoprecipitation (ChIP) amplicon primer sequences are provided in Table S2, Supplemental Digital Content, http://links.lww.com/MPA/A363.

Animal Studies

Forty-eight Severe Combined Immunodeficiency (SCID)/beige mice were purchased from The Jackson Laboratory. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Sanford-Burnham Medical Research Institute, institutional animal welfare assurance number A3053–01. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering. The PANC-1/E47 cells or parental PANC-1 cells were treated with tamoxifen for 8 days in vitro before transplantation. The BxPC-3/E47 cells were untreated or treated with tamoxifen for 48 hours in vitro before transplantation. Three million live cells (cells excluding Trypan Blue) were injected into the flank of SCID/beige mice. The condition of the mice was examined daily. On a weekly basis, the animals were weighed and imaged by ultrasound to measure tumor volumes. The formula used to calculate tumor volume is 4/3 × 3.14 × average radius of length × average radius of width × average radius of depth. The animals were humanely killed when they showed clinical signs of distress or pain; these included hunched posture, ruffled coat, open sores, slow breathing, or reduced response to external stimuli. The mice were euthanized by CO_2 asphyxiation. All aspects of the animal protocol were approved by Institutional Animal Care and Use Committee.

Statistical Analyses

Data are expressed as mean (SD) or standard error of the mean (SEM). Groups were compared using Student *t* test. Pearson coefficient was used when indicated.

RESULTS

Induction of E47 Causes Stable G_0/G_1 Arrest in Human PDA Cells

In an effort to increase bHLH activity in aggressively growing human PDA cells, we stably transduced 3 human PDA lines with a tamoxifen-inducible form of E47 fused to a MER, generating the PDA/E47 lines PANC-1/E47, BxPC-3/E47, and MiaPaCa2/E47.[18,19,32] All PDA/E47 cell lines express high levels of E47, and nuclear localization of E47 is induced by tamoxifen treatment (Fig. S1A, Supplemental Digital Content, http://links.lww.com/MPA/A363). Because the rationale for increasing E47 expression was in part to sequester ID3 in PDA cells, we next investigated whether $E47^{MER}$ bound to ID3 in PDA/E47 cells. Using immunoprecipitation and western blots, we show that both endogenous E47 and ectopic $E47^{MER}$ form protein complexes with ID3 (Fig. S1B, Supplemental Digital Content, http://links.lww.com/MPA/A363).

Recently, we showed that upon induction of E47 in PANC-1 cells, Ki67 expression was diminished.[18] Here, we queried whether this response was universal among different PDA cell lines. As shown in Figure 1A, induction of E47 activity produced rapid loss of Ki67 expression in all cell lines. Importantly, tamoxifen treatment of parental PDA cells lacking ectopic E47 had no effect on Ki67 expression, indicating that cell cycle exit was due to induced E47 activity, not to tamoxifen (Fig. S2, Supplemental Digital Content, http://links.lww.com/MPA/A363). Remarkably, for an 8-day culture period, the number of tamoxifen-induced PANC-1/E47 cells remained static or declined, compared with uninduced PANC-1/E47 cells that expanded 68-fold (Fig. 1B). To determine whether E47 arrested PDA cells in G_0/G_1, S, or G_2/M, flow cytometry was employed to assess DNA content, revealing that E47 induced primarily G_0/G_1 arrest (Fig. 1C). The observed G_0/G_1 arrest suggested that E47 might generate stable changes in the cell cycle. To test this hypothesis, E47 was induced with tamoxifen for 8 days, followed by culture for an additional 8 days in the absence of tamoxifen. Notably, tamoxifen-treated cells maintained a nonproliferative state even when switched to normal growth medium (Fig. S3, Supplemental Digital Content, http://links.lww.com/MPA/A363), revealing that temporary induction of E47 activity was sufficient to arrest cells for prolonged periods.

E47 Induces Global Gene Expression Changes Associated With Cell Cycle, Cancer, and Tissue Differentiation

To systematically probe the alterations in gene expression promoted by E47, we performed microarray analyses of PANC-1/E47 cells with and without tamoxifen treatment (GSE55999). Principal component analysis of microarray data, which measures variance between the sample sets, revealed that E47 induced highly

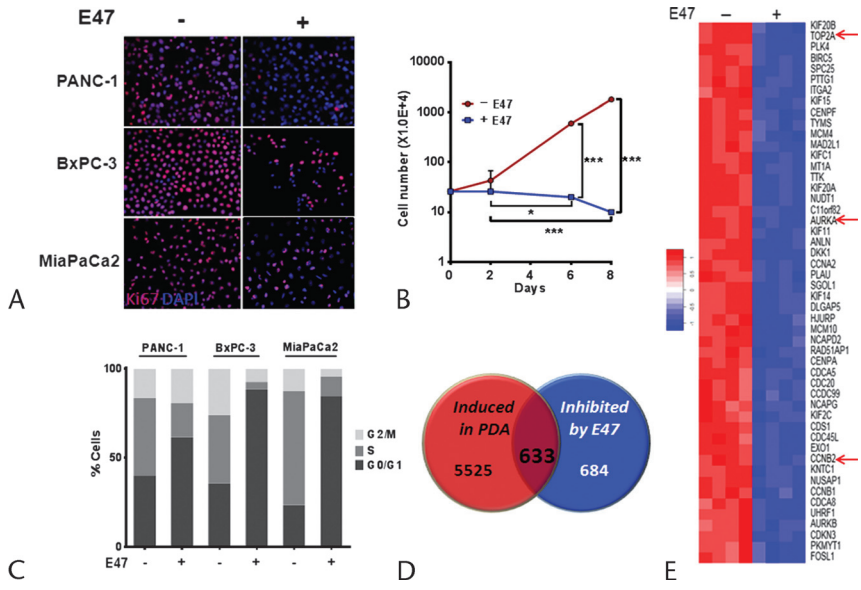

FIGURE 1. E47 induces G_0/G_1 arrest and significantly downregulates cancer-associated genes in PDA cells. A, Immunostaining for the replication marker Ki67 (red) and DAPI (blue), ×200. B, Growth curves (log scale) for PANC-1/E47 cells. *$P < 0.05$, ***$P < 0.001$, n = 3 per group. C, Percentage of cells in individual cell cycle phases determined by flow cytometry. D, Venn diagram depicts genes upregulated in human PDA tumors relative to adjacent tissue (GSE16515, red) versus genes downregulated by E47 activity in PANC-1/E47 cells (dataset deposited-GSE55999, blue), overlap $P = 1.1E^{-40}$. E, Heat map from microarray data showing 50 genes most profoundly downregulated by E47 activity, n = 4 biological replicates each. Arrows depict examples of genes validated by quantitative polymerase chain reaction (qPCR) in 3 PDA/E47 cell lines (Fig. S4, Supplemental Digital Content, http://links.lww.com/MPA/A363). (Note: for simplicity, $E47^{ER}$ cells treated with or without tamoxifen are labeled with the name of the parental cell line +/− E47 activity).

significant changes in gene expression in PDA cells (Fig. S4A, Supplemental Digital Content, http://links.lww.com/MPA/A363). Unbiased bioinformatics analysis identified gene sets clustering within key biological themes of "Cell Cycle," "Cancer," and "Tissue Development" (Fig. S4B, Supplemental Digital Content, http://links.lww.com/MPA/A363). Interestingly, when the microarray data were compared with datasets from PDA tumors, we found that 47% of the genes downregulated in the E47 groups were those commonly upregulated in PDA ($P = 1 \times 10^{-40}$), suggesting that E47 activity promotes a return towards tissue homeostasis (Fig. 1D, E, S4C, Supplemental Digital Content, http://links.lww.com/MPA/A363). Among the genes, most profoundly inhibited by E47 were the PDA-associated cell cycle activators *topoisomerase2A* (*Top2A*), *aurora kinase A* (*AURKA*), and *Cyclin B2* (*CCNB2*) (Fig. 1E). Importantly, PCR analysis of these genes confirmed that they were significantly downregulated by E47 in all cell lines tested (Fig. S5, Supplemental Digital Content, http://links.lww.com/MPA/A363), revealing universally conserved effects of E47 in PDA cells.

E47 Dependent Growth Arrest Requires Induction of *p21* and *TP53INP1* Expression

To probe the mechanism by which E47 activity mediates cell cycle arrest, we considered that E47 might induce expression of cell cycle inhibitors, which often exhibit pleiotropic effects. Indeed, one of the genes highly upregulated by E47 in the microarray profile was the cyclin-dependent kinase inhibitor (CDKI) $CDKN1A^{CIP1/WAF1/Kip1}$ (*p21*) (Fig. S4B, Supplemental Digital Content, http://links.lww.com/MPA/A363). The *p21* was of particular interest because its expression is greatly diminished in PDA and enforced expression halts PDA cell growth.[33] Validation by Real-Time qPCR confirmed that E47-induced robust increases in *p21* mRNA in all cell lines tested. Moreover, p21 was also induced at the protein level (Figs. 2A, S6, Supplemental Digital Content, http://links.lww.com/MPA/A363). To determine whether p21 is essential for E47-mediated growth arrest, we performed siRNA-mediated knockdown, achieving a 90% reduction in *p21* mRNA, as well as reduced protein levels (Figs. S7A, B, Supplemental Digital Content, http://links.lww.com/MPA/A363). Inhibition of *p21* led to a significant increase in the number of Ki67-expressing PDA cells (9.1%–28.6%) (Figs. 2B, C), supporting a required role for p21 in E47-mediated cell cycle arrest. Chromatin immunoprecipitation studies further established that E47 binds directly to the *CDKN1A* promoter and that binding is greatly elevated in tamoxifen-treated cells (Fig. 2D). Interestingly, microarray and PCR analyses also showed that E47 induced high level expression of an additional member of the Cip/Kip CDKI family, *CDKN1C* ($p57^{Kip2}$) in PDA cells. However, reduction of $p57^{Kip2}$ in PDA cells, using our previously validated short hairpin RNA,[32] revealed that $p57^{Kip2}$ did not attenuate E47-mediated arrest. Thus, the function of the Cip/Kip CDKI family members in PDA is highly context specific.

Because reversal of E47-mediated growth arrest by *p21* knockdown was not complete, we searched for additional effectors of cell cycle exit. Microarray (Fig. S4B, Supplemental Digital Content, http://links.lww.com/MPA/A363) and confirmatory qRT-PCR studies revealed that E47 activity also markedly induced expression of the stress response protein *TP53INP1* (tumor protein p53 inducible nuclear protein 1) in all PDA lines tested (Fig. 2E). Interestingly, like *p21*, *TP53INP1* expression is diminished in PDA and its forced expression inhibits PDA cell growth.[34] Remarkably, loss of *TP53INP1* by siRNA knockdown completely reversed E47-mediated growth arrest in PDA cells (Figs. 2E, S7C, Supplemental Digital Content, http://links.lww.com/MPA/A363), revealing for the first time that TP53INP1 is an essential component of an E47-induced transcriptional network. The finding that bHLH activity and *TP53INP1* expression are linked in the same pathway in PDA cells led us to predict that tumors expressing the highest

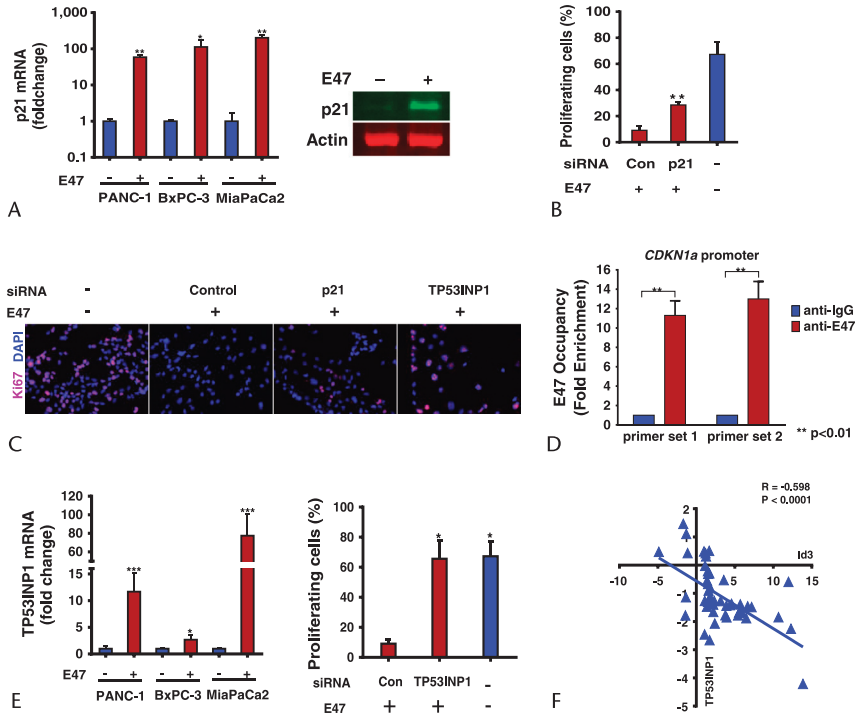

FIGURE 2. E47-induced cell cycle arrest requires p21 and TP53INP1. A, RT-qPCR analysis of *p21* mRNA levels in PDA/E47 cells and immunoblot of p21 protein levels in PANC-1/E47 cells. B, Percentage of Ki67$^+$ cells after treatment with control (Con) or *p21*-specific siRNA. C, Immunostaining for Ki67 (red) and DAPI (blue) in PANC-1/E47 cells, untransfected (–) or transfected with control siRNA (CON), *p21*-specific siRNA, or TP53INP1-specific siRNA, ×200. D, ChIP assays confirm that E47 accumulates at the *CDKN1a* promoter in Panc-1/E47 cells induced with tamoxifen. E, RT-qPCR analysis of *TP53INP1* expression in PDA/E47 cells and percentage of Ki67$^+$ cells after *TP53INP1* knockdown. F, Correlation between *Id3* and *TP53INP1* expression in individual human PDA tumors using published GEO datasets.[25,26] Note: negative values on axes denote lower expression in tumors relative to normal pancreas tissue. The negative R value denotes an inverse relationship between *Id3* (high) and *TP53INP1* (low) expression. A-E, Results are representative of at least 3 independent experiments.

levels of the bHLH inhibitor ID3 would exhibit the lowest levels of TP53INP1 expression. Therefore, we examined mRNA expression data from 122 human PDA tumors and controls,[28,29] finding consistent upregulation of Id3 in PDA relative to controls. Indeed, as hypothesized, the analysis revealed a striking inverse correlation between *Id3* and *TP53INP1* levels ($P < 0.0001$) (Fig. 2F), suggesting loss of bHLH signaling as a novel mechanism for *TP53INP1* dysregulation in PDA.

Induction of Acinar Cell Reprogramming by E47

Given the important role of bHLH proteins in acinar cell development and homeostasis,[35,36] we next examined whether changes in transcriptional networks, via induced E47 activity, could instruct PDA cells to regain a differentiated acinar cell state. The first evidence that PDA cells could indeed revert to an acinar identity was obtained through computational GSEA comparison of our microarray data with a set of human pancreas-enriched genes (GSE1133).[30] As shown in Figure 3A, E47 significantly induced an acinar gene signature profile, achieving an enrichment score of 0.58 ($P < 0.001$), compared with a normalized enrichment score of 1.8021667. Notably, similar results were obtained using a second pancreatic gene set ($P = 0.0014$), normalized enrichment score of 1.6041193 (Fig. S8A, Supplemental Digital Content, http://links.lww.com/MPA/A363).[31] Strikingly, E47 induced 28% of the most highly specific acinar genes (genes enriched ≥ 50-fold in primary acinar tissue vs other primary tissues) (Fig. 3B).

To validate the GSEA results, we next measured transcript levels of acinar gene products in PANC-1/E47, BxPC-3/E47, and MiaPaCa2/E47 cells by qRT-PCR. Induction of E47 activity rapidly generated dramatic (111 fold-253,016 fold) increases in mRNA levels for digestive enzyme genes, including *trypsin* (*trypsin 2*, *trypsinogen*, *PRSS2*), *elastase 3* (*ELA3*), and *carboxypeptidase A2* (*CPA2*), in all cell lines (Fig. 3C). Moreover, upon removal of tamoxifen, enzyme expression was stable for at least 8 days (the longest period tested). Importantly, tamoxifen alone did not induce enzyme gene expression in parental PDA cells lacking inducible E47 (Fig. S8B, Supplemental Digital Content, http://links.lww.com/MPA/A363). Immunostaining of *trypsin* (*PRSS2*) expression revealed that E47 also increased protein levels (Fig. 3D) that continued to rise for a 7-day time course (Fig. S6, Supplemental Digital Content, http://links.lww.com/MPA/A363). Interestingly, although *PRSS2*, *ELA3*, and *CPA2* are known transcriptional targets of the bHLH protein PTF1a,[37] *Ptf1a* mRNA expression was below the level of detection in PDA cells, and its expression did not reach significant values in response to E47 induction. Similar to *Ptf1a*, expression of *Rbpjl*, a member of the PTF1 protein complex,[38] and *Hnf1a*[39] were low/absent in all 3 cell lines ± tamoxifen, whereas *Nr5a2*[40] and *Gata6*[41] exhibited a wider expression range among cell lines but were also below normal human exocrine tissue expression. In contrast, *Rbpj*[38] was expressed in PDA/E47 lines at levels similar to normal exocrine tissue levels but again its expression was not altered by E47. Similarly, ductal gene transcripts for *Sox9*, *Hnf1b*, and *Hnf6* were also relatively unchanged by E47 (Fig. S8C, Supplemental Digital Content, http://links.lww.com/MPA/A363).

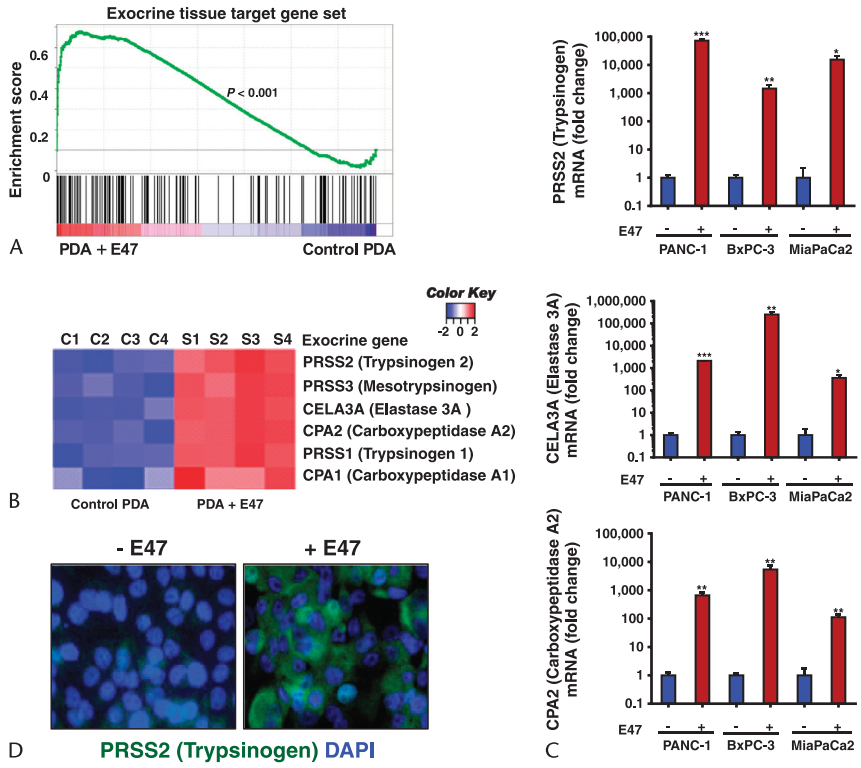

FIGURE 3. E47 induces acinar cell differentiation in PDA cells. A, GSEA analysis of microarray data for E47-induced genes in PANC-1/E47 cells (GSE55999) compared with human exocrine pancreas-enriched genes (GSE1133). The green curve is the running enrichment score ($P < 0.001$). B, E47 induces seven of the 25 most highly specific exocrine pancreas genes identified in GSE1133; S1-S4, samples +E47 activity; C1-C4, uninduced control samples. C, qRT-PCR for *trypsinogen*, *carboxypeptidase A2* (*CPA2*), and *elastase 3* (*ELA3*) in PDA/E47 cells with and without E47-induced activity. D, Immunostaining for trypsinogen in PANC-1/E47 cells with induced E47 activity for 7 days, ×400 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n = 3 per group). Results are representative of at least 3 independent experiments.

We next investigated whether the induction of acinar gene products included activation of the acinar maturation pathways normally regulated by the tissue-restricted bHLH protein MIST1. Similar to PTF1a, downregulation of MIST1 occurs during ADM. Intriguingly, E47 activity rapidly led to significant induction of *Mist1* expression in all PDA/E47 lines (Figs. 4A, B, S6, Supplemental Digital Content, http://links.lww.com/MPA/A363). Moreover, ChIP analysis revealed that E47 directly targets the *Mist1* promoter (Fig. 4C). MIST1 functions as an acinar scaling/maturation factor, ensuring in part, proper cell-cell junctions, and intracellular organization.[23] To determine whether induced MIST1 protein was functional in these cells, we first examined expression of the MIST1 target genes *Foxp2*, *Copz2*, and *Rab3d*.[42] As predicted, expression of each MIST1 target was significantly increased within 48-hour post-E47 activity (Fig. 4D). Moreover, PDA/E47 cells also acquired MIST1-dependent acinar cell characteristics, including the generation of ZO.1-containing tight junctions and accumulation of an extensive intracellular actin network (Fig. 4E).[43] We previously showed that MIST1 is required for the formation of *Cx32*-expressing gap junctions, a hallmark of differentiated acinar tissue (Fig. S9A, Supplemental Digital Content, http://links.lww.com/MPA/A363).[6,7,14,23] Consistent with this observation, E47-induced PDA cells accumulated both *Cx32* mRNA and gap junction complexes (Figs. 4F, G). To directly test whether MIST1 expression was essential for E47-induced *Cx32* expression, we next inhibited *Mist1* expression by siRNA strategies. As predicted, loss of MIST1 significantly reduced E47-induced *Cx32* expression (Fig. S9, Supplemental Digital Content, http://links.lww.com/MPA/A363). Thus, E47 induces feed-forward activation of the bHLH gene *Mist1*, which is required for acinar maturation in PDA cells.

Because we had previously shown that, similar to increased E47 expression, *Id3* knockdown reduced Ki67 expression in PDA cells,[18] we considered that decreasing *Id3* levels might also induce acinar-specific gene expression. Indeed, inhibition of *Id3* by siRNA significantly induced *PRSS2* transcripts ($P = 0.003$), recapitulating the observed results with E47 upregulation (Fig. S10, Supplemental Digital Content, http://links.lww.com/MPA/A363). These data support a model in which E47 shifts the balance between functional bHLH/bHLH complexes and nonfunctional bHLH/ID3 interactions, which is also consistent with our studies showing direct interactions between E47 and ID3 in PDA cells (Fig. S1B, Supplemental Digital Content, http://links.lww.com/MPA/A363).

E47 Inhibits PDA-Tumor Formation In Vivo

Because induced E47 activity was sufficient to convert PDA cells to a stable quiescent and differentiated acinar cell state in vitro, we hypothesized that altering the bHLH transcription networks might also reduce the tumor-forming potential of PDA cells in vivo. Therefore, to begin to address the effects of E47 on tumor growth dynamics, control PANC-1 cells and experimental PANC-1/E47 cells were treated with tamoxifen in vitro for 8 days before transplantation into immunocompromised mice. As shown in Figure 5, during the course of 35 days, control parental PANC-1 cells treated with tamoxifen produced large tumors, whereas tumors in mice transplanted with tamoxifen-induced PANC-1/E47

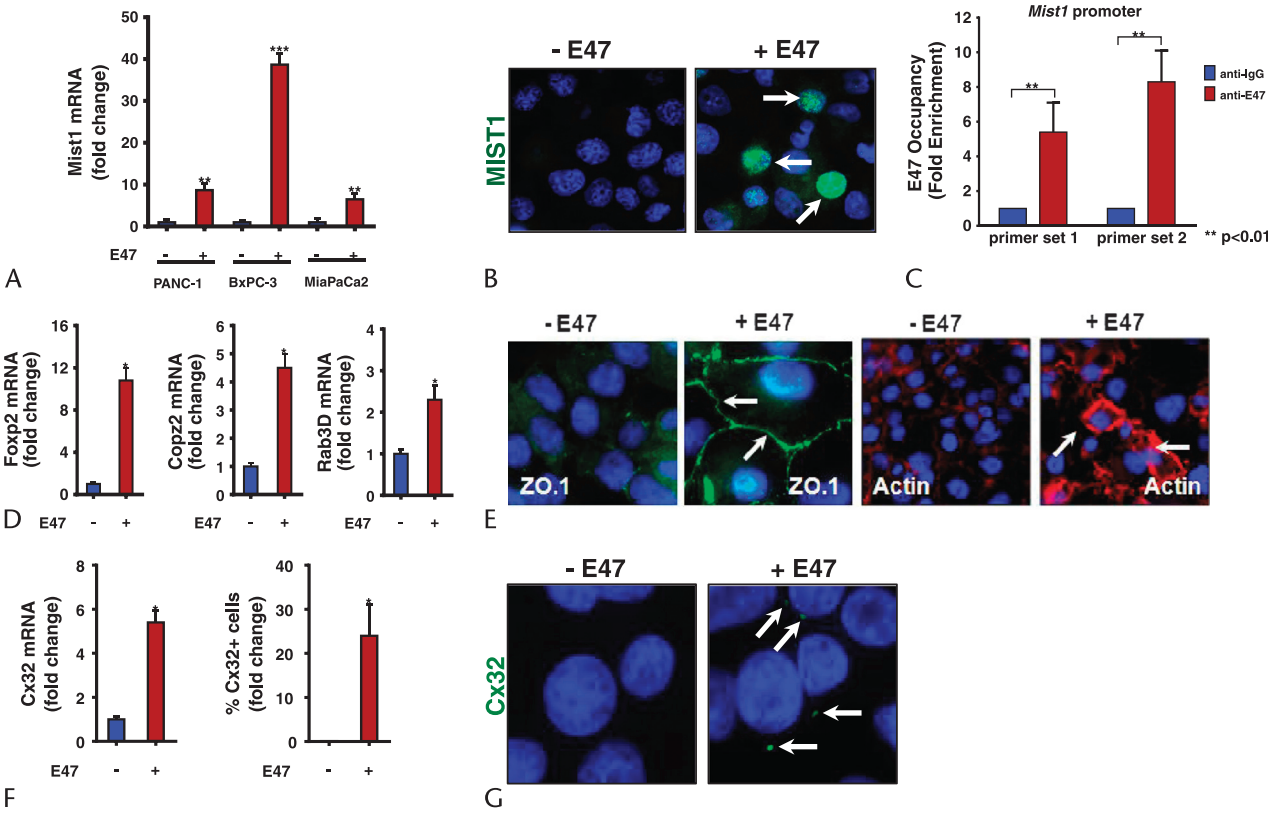

FIGURE 4. E47 induces expression of *Mist1* and MIST1 target genes in PDA cells. A, qRT-PCR for *Mist1* transcripts in all PDA/E47 lines. B, Immunostaining for MIST1 in PANC-1/E47 cells, ×800. C, E47 ChIP assay on the *Mist1* promoter. E47 binding is enriched on the *Mist1* promoter in Panc1/E47 cells induced with tamoxifen. D, qRT-PCR for MIST1 target genes *Foxp2*, *Copz2*, and *Rab3D*. E, Immunostaining for ZO.1, ×800, and actin, ×400. F, qRT-PCR for the gap junction protein connexin 32 (CX32) and quantification of the % CX32-expressing cells in PANC-1/E47 cells. G, Immunostaining for CX32-containing gap junctions, ×1200. White arrows in B, D, and F identify examples of positive expression. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n = 3 per group.

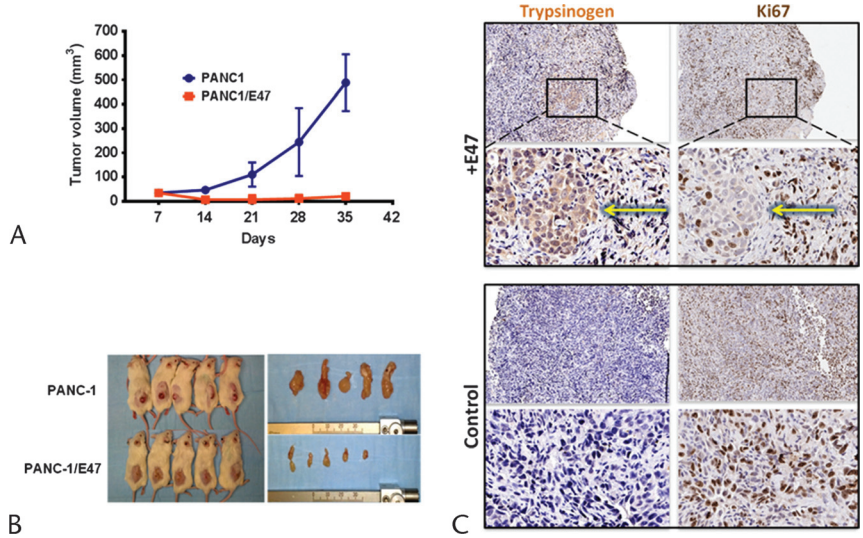

FIGURE 5. E47 in vitro inhibits PDA cell tumorigenesis in vivo. PANC-1/E47 and parental PANC-1 cells were treated with tamoxifen for 8 days in vitro before transplantation into SCID/beige mice. A, Tumor growth as measured by ultrasound. B, Comparison of animals and excised tumors at 35 days posttransplant. Note that large tumors formed in tamoxifen-treated parental PANC-1 cells lacking ectopic E47, indicating that E47, not tamoxifen, is responsible for reduced tumor growth. Representative of 3 independent experiments. C, Immunostaining for trypsinogen (brown/orange cytoplasm), Ki67 (dark brown nuclei), and hemotoxylin nuclear counterstain in slides from explanted tumors. Images are ×100 and ×400 original.

cells were significantly reduced (~18-fold) relative to controls ($P = 0.00011$) (Figs. 5A, B). To further examine the phenotype of individual PANC-1 and PANC-1/E47 cells, tumors were explanted after 35 days and processed for immunohistochemistry. Remarkably, the small tumors arising from the PANC-1/E47 cells treated with tamoxifen in vitro contained clusters of cells-expressing trypsinogen (Fig. 5C). Moreover, these cells exhibited reduced Ki67 expression relative to the control PANC-1 cell-derived tumors. Thus, the acinar phenotype induced by E47 in vitro remained stable in the absence of tamoxifen for at least 35 days in vivo. Because we have shown that growth arrested, reprogrammed, PDA cells eventually die in vitro (Fig. S3, Supplemental Digital Content, http://links.lww.com/MPA/A363), the in vivo findings are most consistent with a model in which a minority of cells, which do not contain the E47^{MER} vector (Fig. S1, Supplemental Digital Content, http://links.lww.com/MPA/A363) form small tumors. Additional studies in the BxPC-3/E47 line revealed that 2 days of tamoxifen treatment in vitro was sufficient to significantly inhibit tumor formation in vivo (Fig. S11, Supplemental Digital Content, http://links.lww.com/MPA/A363). Thus, the data establish that E47 reprogramming of PDA cells in vitro reduces tumorigenesis in vivo.

Collectively, our data establish for the first time that E47 reprograms aggressive PDA cells to a quiescent acinar state by restoring expression of p21, TP53INP1, acinar enzymes, and MIST1 (Fig. 6). Importantly, reprogramming greatly diminishes the tumor promoting potential of PDA cells, a finding with broad implications for therapeutic advancement for this highly lethal disease.

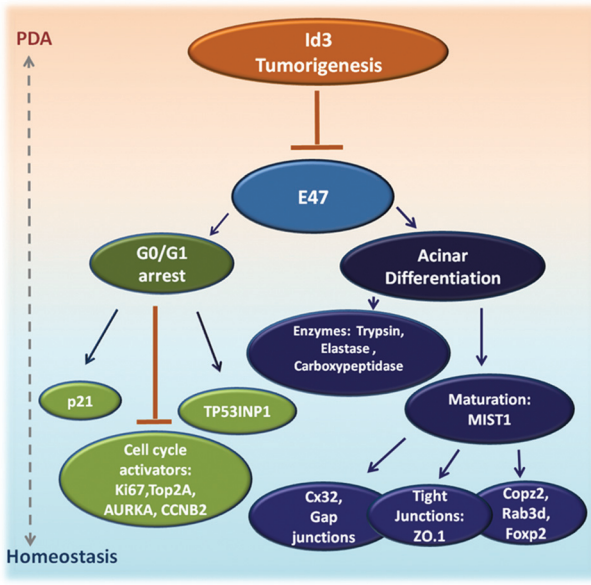

FIGURE 6. Proposed model of mechanisms underlying E47/ID3 regulation of growth, acinar differentiation, and tumorigenicity in human PDA. Our working model is that high levels of ID3 contribute to tumorigenicity by inhibiting the bHLH transcriptional network. Conversely, E47 induces G_0/G_1 arrest via *p21* and *TP53INP1* induction and downregulation of cell cycle activators (eg, *aurora kinase*, *topoisomerase*, *cyclinb2*). Concurrently, E47 induces an acinar differentiation program that is characterized by re-expression of digestive enzymes, upregulation of *Mist1*, and its target genes and re-establishment of cell-cell junctions. Together, the combined activities of E47 constrain tumor growth.

DISCUSSION

Given the multitude of genome-wide alterations in PDA and the fact that many of the commonly studied human PDA cell lines have been cultured in laboratories for more than 30 years,[44] it seemed doubtful that highly tumorigenic cells could retain the capacity to revert to a quiescent, differentiated acinar cell state. Yet, because we had previously uncovered powerful roles for bHLH and HLH transcription factors in the pancreas,[7,12,14,32] we hypothesized that dysregulated bHLH/HLH activity could be integral to PDA pathogenesis. Although E47 expression is not downregulated in PDA relative to control tissue, an inhibitor E47 activity, ID3, becomes highly expressed in PDA, suggesting loss of E47 function in PDA. The first evidence that this was true was our finding that either downregulation of ID3 or induced expression of E47 lowered Ki67 expression in the PANC-1 cell line.[18] Here, we report the extraordinary finding that E47 induces G_0/G_1 arrest, promotes a high degree of acinar differentiation, and profoundly reduces the tumor-promoting potential of PDA cells.

All PDA cell lines tested responded to E47 with upregulation of *p21* and *TP53INP1* gene expression associated with G_0/G_1 arrest. It has been shown that *p21* and *TP53INP1* are each downregulated in PDA and that their re-expression in PDA cells results in growth arrest.[33,34] To our knowledge, however, this is the first demonstration that a common mechanism (bHLH activity) coordinately controls both genes. Further, we show that the action of E47 on the *p21* promoter is direct, consistent with several reports showing that E47 and other bHLH factors bind to and regulate the *p21* promoter.[34,45–47]

An additional link between the bHLH/ID axis and *TP53INP1* expression was revealed by our finding of a significant inverse relationship between *Id3* and *TP53INP1* expression in RNA from human PDA tumors. Thus, PDA tumors with the highest expression of *Id3* mRNA harbored the lowest level of *TP53INP1* transcripts. Together, our data suggest a novel regulatory network in PDA whereby elevated ID3 may be responsible for the loss of both *p21* and *TP53INP1* gene expression through inhibition of bHLH factor activity.

Remarkably, we find that concurrent with growth arrest, E47 expression promotes acinar differentiation of PDA cells. All of the PDA cell lines tested exhibited robust induction of digestive enzyme gene expression. Interestingly, although digestive enzyme genes are known targets of the acinar-restricted bHLH factor PTF1a,[17,20,37] *Ptf1a* expression was generally below the limit of detection in PDA/E47 induced for 2 to 3 days as in parental PDA cells. Consistent with the latter, Dufresne et al[20] also reported low/absent PTF1a expression in human and murine PDA tissue. Therefore, our data are most consistent with a model in which E47 is capable of substituting for PTF1a to induce the level of enzyme observed here. The E47-induced shift towards acinar differentiation is not complete, however, as the level of most acinar genes tested remains below normal tissue levels. Therefore, given the important role of *Ptf1a* in the exocrine pancreas, PTF1a may be required to promote enzyme expression to the levels expressed by primary cells and/or to induce the full complement of acinar genes. We also found that expression of *Rbpjl*, which is required for PTF1a function in mature cells, is below the limits of detection in the 3 PDA/E47 cell lines.

The MIST1 bHLH factor and its downstream transcription network, which are responsible for acinar cell maturation and formation of acinar cell junctions, were clearly elevated by E47 activity, indicating that E47 amplifies bHLH signaling in a feed-forward fashion. Our findings are consistent with reports demonstrating that *Mist1* is influential in converting embryonic stem cells to an acinar cell fate.[48] The data also support our previous

in vivo studies showing that loss of MIST1 activity dramatically enhances ADM/PanIN formation in *Kras*^{G12D}-expressing acinar cells, whereas sustained *Mist1* expression produces a greatly attenuated *Kras*^{G12D}-induced transformation response.[7,8] Interestingly, we find that E47 binds directly to the MIST1 promoter as it does to the p21 promoter, suggesting that bHLH signaling may be the final common denominator between growth and differentiation. Future studies will address whether E47 binds to the *p21* and *Mist1* promoters as homodimers or as heterodimers with other bHLH partners (eg, E12, HEB, MIST1) and whether E47 is unique in its ability to reprogram PDA cells. In contrast to acinar genes, the ductal genes we analyzed were not highly influenced by E47, although a more exhaustive survey of ductal gene expression will be needed to precisely define the degree to which E47 induced PDA cells to transition back towards a more normal acinar cell state.

The data are consistent with a mechanism in which E47 overexpression acts in part as a sponge for the high levels of ID3 observed in PDA cells. We show that E47 complexes with ID3 in PDA cells and that ID3 knockdown, like E47 upregulation, induces growth arrest[18] and trypsinogen expression. Thus, targeting ID3 to shift the bHLH/ID balance may also be a promising approach to treating PDA. Indeed, strategies based on inhibition of other ID family members are currently in clinical trials for ovarian cancer and glioma.[49,50]

To our knowledge, this is the first report showing that ectopic expression of a single gene (E47) is sufficient to cause G_0/G_1 quiescence, acinar differentiation, and activation of the MIST1 network in PDA cells. Further, the stable nature of E47-induced reprogramming to quiescent cells with acinar characteristics in vitro was striking. These findings prompted us to ask whether the growth arrest and acinar gene expression induced in vitro might be sufficient to diminish the tumor-forming potential of these aggressively growing cells. Indeed, temporary induction of E47 for 2 to 8 days in vitro produced stable cell cycle arrest and trypsinogen expression in transplanted human PDA cells. It will now be of interest to investigate the effects of E47 on the growth dynamics of established tumors.

Our observation that PDA cells retain the plasticity to be forced back towards an acinar-like differentiated state despite their many genomic alterations is consistent with recent studies[1,2] showing that inactivation of *Kras*^{G12D} expression in established acinar-derived PanINs is sufficient to revert transformed cells back to their previous differentiated acinar phenotype. Thus, perturbation of at least 2 pathways, *Kras* signaling[2] or shifting the bHLH/HLH axis, can have a dramatic effect on PDA cell growth and differentiation status. These multiple lines of evidence establish that PDA cells clearly retain memory of their acinar cell lineage. How the bHLH transcription network inhibits the oncogenic activity of *Kras*^{G12D} to promote acinar differentiation properties remains as yet unknown. Importantly, however, we have generated a rich microarray dataset (GSE55999), which is generating testable hypotheses regarding the intersections between E47 and oncogenic signaling pathways in PDA.

Presently, PDA is treated with cytotoxic agents, yet the average survival for PDA patients postdiagnosis is merely 6 months, and improvements in therapies are measured in days.[51] Thus, there is an urgent need to devise novel strategies to combat this disease. We conclude that the bHLH/ID axis acts as a pivotal hub through which oncogenes and tumor suppressors exert control over acinar cellular homeostasis. These studies have employed immortalized human pancreatic cancer cell lines to provide a proof of concept and to investigate mechanisms of action. In future studies, it will be important to investigate the role of E47 in primary patient-derived tumor tissue to determine whether targeting the bHLH/ID axis can provide a novel therapeutic approach to combating this highly lethal disease.

ACKNOWLEDGMENTS

We thank Ashley Russell, Kaitlyn Kirk, Chiara Leroy, Li Huang, Tarek Almaleh, and Quinn McNichols for technical assistance; Cornelis Murre for the E47^{MER} *vector; Antonio Iavarone for Id3-Flag; and the Bioinformatics, Functional Genomics, Histochemistry, and FACS core facilities at the Sanford-Burnham Medical Research Institute for their assistance.*

REFERENCES

1. Collins MA, Bednar F, Zhang Y, et al. Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice. *J Clin Invest*. 2012;122:639–653.
2. Collins MA, Yan W, Sebolt-Leopold JS, et al. MAPK signaling is required for dedifferentiation of acinar cells and development of pancreatic intraepithelial neoplasia in mice. *Gastroenterology*. 2014;146:822–834.
3. Hingorani SR, Petricoin EF, Maitra A, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell*. 2003;4:437–450.
4. Guerra C, Schuhmacher AJ, Cañamero M, et al. Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer Cell*. 2007;11:291–302.
5. Habbe N, Shi G, Meguid RA, et al. Spontaneous induction of murine pancreatic intraepithelial neoplasia (mPanIN) by acinar cell targeting of oncogenic Kras in adult mice. *Proc Natl Acad Sci U S A*. 2008;105:18913–18918.
6. De La O JP, Emerson LL, Goodman JL, et al. Notch and Kras reprogram pancreatic acinar cells to ductal intraepithelial neoplasia. *Proc Natl Acad Sci U S A*. 2008;105:18907–18912.
7. Shi G, DiRenzo D, Qu C, et al. Maintenance of acinar cell organization is critical to preventing Kras-induced acinar-ductal metaplasia. *Oncogene*. 2013;32:1950–1958.
8. Shi G, Zhu L, Sun Y, et al. Loss of the acinar-restricted transcription factor Mist1 accelerates Kras-induced pancreatic intraepithelial neoplasia. *Gastroenterology*. 2009;136:1368–1378.
9. Kopp JL, von Figura G, Mayes E, et al. Identification of Sox9-dependent acinar-to-ductal reprogramming as the principal mechanism for initiation of pancreatic ductal adenocarcinoma. *Cancer Cell*. 2012;22:737–750.
10. Adell T, Gómez-Cuadrado A, Skoudy A, et al. Role of the basic helix-loop-helix transcription factor p48 in the differentiation phenotype of exocrine pancreas cancer cells. *Cell Growth Differ*. 2000;11:137–147.
11. Krapp A, Knöfler M, Ledermann B, et al. The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. *Genes Dev*. 1998;12:3752–3763.
12. Dong PD, Provost E, Leach SD, et al. Graded levels of Ptf1a differentially regulate endocrine and exocrine fates in the developing pancreas. *Genes Dev*. 2008;22:1445–1450.
13. Rodolosse A, Chalaux E, Adell T, et al. PTF1alpha/p48 transcription factor couples proliferation and differentiation in the exocrine pancreas [corrected]. *Gastroenterology*. 2004;127:937–949.
14. Direnzo D, Hess DA, Damsz B, et al. Induced Mist1 expression promotes remodeling of mouse pancreatic acinar cells. *Gastroenterology*. 2012;143:469–480.
15. Massari ME, Murre C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol*. 2000;20:429–440.
16. Zhu L, Tran T, Rukstalis JM, et al. Inhibition of Mist1 homodimer formation induces pancreatic acinar-to-ductal metaplasia. *Mol Cell Biol*. 2004;24:2673–2681.

17. Roux E, Strubin M, Hagenbüchle O, et al. The cell-specific transcription factor PTF1 contains two different subunits that interact with the DNA. *Genes Dev*. 1989;3:1613–1624.
18. Lee SH, Hao E, Kiselyuk A, et al. The Id3/E47 axis mediates cell-cycle control in human pancreatic ducts and adenocarcinoma. *Mol Cancer Res*. 2011;9:282–790.
19. Schwartz R, Engel I, Fallahi-Sichani M, et al. Gene expression patterns define novel roles for E47 in cell cycle progression, cytokine-mediated signaling, and T lineage development. *Proc Natl Acad Sci U S A*. 2006;103:9976–9981.
20. Dufresne M, Clerc P, Dieng M, et al. Id3 modulates cellular localization of bHLH Ptf1-p48 protein. *Int J Cancer*. 2011;129:295–306.
21. Lemercier C, To RQ, Carrasco RA, et al. The basic helix-loop-helix transcription factor Mist1 functions as a transcriptional repressor of myoD. *EMBO J*. 1998;17:1412–1422.
22. Teachenor R, Beck K, Wright LY, et al. Biochemical and phosphoproteomic analysis of the helix-loop-helix protein E47. *Mol Cell Biol*. 2012;32:1671–1682.
23. Mills JC, Taghert PH. Scaling factors: transcription factors regulating subcellular domains. *Bioessays*. 2012;34:10–16.
24. Rukstalis JM, Kowalik A, Zhu L, et al. Exocrine specific expression of Connexin32 is dependent on the basic helix-loop-helix transcription factor Mist1. *J Cell Sci*. 2003;116:3315–3325.
25. Lieber M, Mazzetta J, Nelson-Rees W, et al. Establishment of a continuous tumor-cell line (panc-1) from a human carcinoma of the exocrine pancreas. *Int J Cancer*. 1975;15:741–747.
26. Loor R, Nowak NJ, Manzo ML, et al. Use of pancreas-specific antigen in immunodiagnosis of pancreatic cancer. *Clin Lab Med*. 1982;2:567–578.
27. Wu M, Arimura GK, Yunis AA. Purification and characterization of a plasminogen activator secreted by cultured human pancreatic carcinoma cells. *Biochemistry*. 1977;16:1908–1913.
28. Collisson EA, Sadanandam A, Olson P, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med*. 2011;17:500–503.
29. Pei H, Li L, Fridley BL, et al. FKBP51 affects cancer cell response to chemotherapy by negatively regulating Akt. *Cancer Cell*. 2009;16: 259–266.
30. Su AI, Wiltshire T, Batalov S, et al. A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci U S A*. 2004;101: 6062–6067.
31. Ge X, Yamamoto S, Tsutsumi S, et al. Interpreting expression profiles of cancers by genome-wide survey of breadth of expression in normal tissues. *Genomics*. 2005;86:127–141.
32. Kiselyuk A, Farber-Katz S, Cohen T, et al. Phenothiazine neuroleptics signal to the human insulin promoter as revealed by a novel high-throughput screen. *J Biomol Screen*. 2010;15:663–670.
33. Joshi US, Dergham ST, Chen YQ, et al. Inhibition of pancreatic tumor cell growth in culture by p21WAF1 recombinant adenovirus. *Pancreas*. 1998; 16:107–113.
34. Gironella M, Seux M, Xie MJ, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci U S A*. 2007;104: 16170–16175.
35. Arda HE, Benitez CM, Kim SK. Gene regulatory networks governing pancreas development. *Dev Cell*. 2013;25:5–13.
36. Seymour PA, Sander M. Historical perspective: beginnings of the beta-cell: current perspectives in beta-cell development. *Diabetes*. 2011;60: 364–376.
37. Rose SD, Swift GH, Peyton MJ, et al. The role of PTF1-P48 in pancreatic acinar gene expression. *J Biol Chem*. 2001;276:44018–44026.
38. Masui T, Long Q, Beres TM, et al. Early pancreatic development requires the vertebrate Suppressor of Hairless (RBPJ) in the PTF1 bHLH complex. *Genes Dev*. 2007;21:2629–2643.
39. Boj SF, Parrizas M, Maestro MA, et al. A transcription factor regulatory circuit in differentiated pancreatic cells. *Proc Natl Acad Sci U S A*. 2001;98:14481–14486.
40. von Figura G, Morris JP, Wright CV, et al. Nr5a2 maintains acinar cell differentiation and constrains oncogenic Kras-mediated pancreatic neoplastic initiation. *Gut*. 2014;63:656–664.
41. Martinelli P, Cañamero M, del Pozo N, et al. Gata6 is required for complete acinar differentiation and maintenance of the exocrine pancreas in adult mice. *Gut*. 2013;62:1481–1488.
42. Tian X, Jin RU, Bredemeyer AJ, et al. RAB26 and RAB3D are direct transcriptional targets of MIST1 that regulate exocrine granule maturation. *Mol Cell Biol*. 2010;30:1269–1284.
43. Pin CL, Rukstalis JM, Johnson C, et al. The bHLH transcription factor Mist1 is required to maintain exocrine pancreas cell organization and acinar cell identity. *J Cell Biol*. 2001;155:519–530.
44. Moore PS, Sipos B, Orlandini S, et al. Genetic profile of 22 pancreatic carcinoma cell lines. Analysis of K-ras, p53, p16 and DPC4/Smad4. *Virchows Arch*. 2001;439:798–802.
45. Prabhu S, Ignatova A, Park ST, et al. Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins. *Mol Cell Biol*. 1997;17:5888–5896.
46. Ciarrocchi A, Jankovic V, Shaked Y, et al. Id1 restrains p21 expression to control endothelial progenitor cell formation. *PLoS One*. 2007;2:e1338.
47. Jia D, Sun Y, Konieczny SF. Mist1 regulates pancreatic acinar cell proliferation through p21 CIP1/WAF1. *Gastroenterology*. 2008;135: 1687–1697.
48. Rovira M, Delaspre F, Massumi M, et al. Murine embryonic stem cell-derived pancreatic acinar cells recapitulate features of early pancreatic differentiation. *Gastroenterology*. 2008;135:1301–1310.
49. Ren Y, Cheung HW, von Maltzhan G, et al. Targeted tumor-penetrating siRNA nanocomplexes for credentialing the ovarian cancer oncogene ID4. *Sci Transl Med*. 2012;4:147ra112.
50. Lasorella A, Boldrini R, Dominici C, et al. Id2 is critical for cellular proliferation and is the oncogenic effector of N-myc in human neuroblastoma. *Cancer Res*. 2002;62:301–306.
51. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007;25:1960–1966.