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

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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.1–3 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.4,6,9 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),2,8,10–14 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.14 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 G0/G1 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 transcriptional factor that controls the acinar maturation program. Moreover, we show that Mist1 is required for the re-establishment of acinar gap junctions. Importantly, induction of E47 in vitro is 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), BxPC-3 (CRL-1687), MiaPaCa2 (CRL-1420) were cultured in Roswell Park Memorial Institute medium 1640 or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in 5% CO2 at 37°C. E47-inducible cell lines were generated by modeling data with ModFitLT software (Verity Software House). For flow cytometry, parental PDA cell lines were transfected (Lipofectamine 2000; Invitrogen) with green fluorescent protein (GFP) or inducible E47 plasmids and incubated with 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). 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 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-CKX3 (1:100, ab66613; Abcam), mouse anti-MIST1 (in-house antibody purified, 1:50), and mouse anti-actin (1:100, ab8224; Abcam). Secondary antibodies were conjugated to donkey antirabbit Alexa Fluor 488 (1:400, A21206; Invitrogen) or donkey antirabbit 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 (H2O2) 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 tetrahydrochloride) (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 RNasy 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 p21cip1/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]; Na3VO4, 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 E2-Alpha or anti-immunoglobulin G. Chromatin Immunoprecipitation (ChiP) amplimer 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 \( \text{width} \times \text{average radius of length} \times \text{average radius of width} \times \text{average radius of depth} \). 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 CO2 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 G0/G1 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. 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 E47MEr bound to ID3 in PDA/E47 cells. Using immunoprecipitation and western blots, we show that both endogenous E47 and ectopic E47MEr 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. 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 G0/G1, S, or G2/M, flow cytometry was employed to assess DNA content, revealing that E47 induced primarily G0/G1 arrest (Fig. 1C). The observed G0/G1 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
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 ($P = 1 \times 10^{-46}$), 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 (CDK4IP1/R104F/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 (p57Kip2) in PDA cells. However, reduction of p57Kip2 in PDA cells, using our previously validated short hairpin RNA,\(^{32}\) revealed that p57Kip2 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...
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,25,36 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 $0.58$ ($P = 0.0014$), 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. S8B, Supplemental Digital Content, http://links.lww.com/MPA/A363). 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 (PRSS2, 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 rapidly generated dramatic (111 fold-253,016 fold) increases in mRNA levels for digestive enzyme genes, including trypsin (PRSS2, trypsinogen, PRSS2), elastase 3 (ELA3), and carboxypeptidase A2 (CPA2), in all cell lines (Fig. 3D) that continued to rise for a 7-day time course (Fig. S6, Supplemental Digital Content, http://links.lww.com/MPA/A363). Therefore, we examined mRNA expression range among cell lines but were also below normal human exocrine tissue expression. In contrast, Sox9, Hnf1a, Gata6 and Rbpjl were low/absent in all 3 cell lines but were also below normal human exocrine tissue expression. In contrast, Rhyp38 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).
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).5,6,12,23 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 E47MER 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.

**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, 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, 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. Here, we report the extraordinary finding that E47 induces G0/G1 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 G0/G1 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. 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.

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, Pf1a 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 also reported low/absent Pf1a 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 Pf1a in the exocrine pancreas, Pf1a 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 Bpg1, which is required for Pf1a 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. The data also support our previous

**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 G0/G1 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.
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, is observed in PDA cells. We show that E47 complexes with ID3 expression acts in part as a sponge for the high levels of ID3 induced PDA cells to transition back towards a more normal acinar phenotype. These studies have employed immortalized human pancreatic cancer cell lines to provide a proof of concept for 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.

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