Subtype-Specific MEK-PI3 Kinase Feedback as a Therapeutic Target in Pancreatic Adenocarcinoma

Olga K. Mirzoeva1, Eric A. Collisson2,3, Peter M. Schaefer1, Byron Hann3, Yun K. Hom3, Andrew H. Ko2,3, and W. Michael Korn1,2,3

Abstract

Mutations in the KRAS oncogene are dominant features in pancreatic ductal adenocarcinoma (PDA). Because KRAS itself is considered "undruggable," targeting pathways downstream of KRAS are being explored as a rational therapeutic strategy. We investigated the consequences of MAP–ERK kinase (MEK) inhibition in a large PDA cell line panel. Inhibition of MEK activated phosphoinositide 3-kinase in an EGF receptor (EGFR)-dependent fashion and combinations of MEK and EGFR inhibitors synergistically induced apoptosis. This combinatorial effect was observed in the epithelial but not mesenchymal subtype of PDA. RNA expression analysis revealed predictors of susceptibility to the combination, including E-cadherin, HER3, and the miR200-family of microRNAs, whereas expression of the transcription factor ZEB1 was associated with resistance to the drug combination. Knockdown of HER3 in epithelial-type and ZEB1 in mesenchymal-type PDA cell lines resulted in sensitization to the combination of MEK and EGFR inhibitors. Thus, our findings suggest a new, subtype-specific, and personalized therapeutic strategy for pancreatic cancer. Mol Cancer Ther; 12(10); 1–13. ©2013 AACR.

Introduction

The prognosis for patients with pancreatic ductal adenocarcinoma (PDA) remains dismal despite recent modest improvements resulting from optimized use of systemic chemotherapy (1, 2). Effective therapeutic targeting of key pathways involved in the pathogenesis of this disease could lead to substantial improvements in outcome. The presence of mutations in the KRAS oncogene in more than 90% of PDAs has long been documented (3). Genetically engineered mouse models of pancreatic cancer have proved the crucial role of this mutation for tumorigenesis and tumor maintenance (4). Activating mutations in KRAS result in constitutive signaling through effector pathways (5). Indeed, the presence of phosphorylated ERK (p-ERK) and AKT in PDA is associated with poor prognosis (6). Because mutant KRAS is considered “undruggable” (7), pathways up- and downstream of RAS have emerged as attractive therapeutic targets (8). However, clinical results with specific inhibitors of these pathways have been disappointing. For example, the EGFR receptor (EGFR) inhibitor, erlotinib, was approved by the U.S. Food and Drug Administration for use in PDA in combination with gemcitabine despite only a very modest improvement in median overall survival (9). Available efficacy data on single-agent activity of inhibitors of MAP–ERK kinase (MEK) and phosphoinositide 3-kinase (PI3K) have also shown limited tumor activity (10, 11). Recent data from our institution show the existence of molecularly defined subtypes of PDA with differential sensitivity to conventional chemotherapy (12). Thus, inefficacy of targeted therapies for this disease might be a consequence of failure to understand the subtype-dependent complexities of molecular networks associated with the therapeutic target. Signaling in response to treatment with targeted agents has been shown to result in feedback activation of antiapoptotic and other important cellular pathways. For example, S6K1-mediated feedback activation of AKT and eIF4E in response to pharmacologic inhibition of mTOR resulting in activation of cell survival pathways has been well documented (13, 14). Similarly, we discovered a novel, EGFR-dependent, feedback loop resulting in PI3K activation in response to inhibition of MEK (15).

The relevance of this mechanism is highlighted by our finding that interruption of this feedback, for example, by combining MEK inhibitors with inhibitors of EGFR or PI3K, results in synergistic induction of cell-cycle arrest or apoptosis in breast cancer (15, 16) models. In addition, Diep and colleagues reported similar findings exclusively in PDA with wild-type KRAS (17). Herein, we examine both the frequency and determinants of this feedback loop.
in addition to examining the consequences of interrupting the feedback loop in cell line and in vivo models of pancreatic cancer. We identify synergistic interactions between targeted agents within this feedback loop and identify molecular predictors of this synergy within specific FDA subtypes. These studies will further inform sensitivity enrichment strategies with MEK/EGFR inhibitor combinations in this disease.

Materials and Methods

Reagents

The following reagents were used: EGF (Millipore), CI1040 (PD 184352) was from Tocris, PD0325901 and erlotinib were from Selleck. All drugs were diluted in dimethyl sulfoxide (DMSO). The following antibodies were from Cell Signaling Technology: p-AKT (S473), total AKT, p-EGFR (Y1068), p-HER3 (Y1197, Y1289), total HER3, p-PRAS40 (T246), p-GSK3 (S21/9), and p-ERK (T202/Y204); β-actin antibody was from Sigma, and ZEB1 (H-102) and E-cadherin (sc-7870) antibodies were from Santa Cruz. Protease inhibitors cocktail set III and phosphatase inhibitor cocktail set II were from EMD Millipore.

Structures of erlotinib, CI1040, PD0325901 are presented in Supplementary Fig. S1.

Cell culture

Pancreatic cancer cell lines used in this study were described previously (12, 18). MiaPaCa-2, Panc1, CFPAC-1, HPAF-II, Capan-2, Hu6766T, and BxPC-3 cell lines were provided by Dr. Paul Kirschmeier (Schering Plough Research Institute, Kenilworth, NJ). SW1990, Panc2.03, and Su86.86 cell lines were purchased from American Type Culture Collection. Panc2.13, Panc3.27, Panc5.04, Panc6.03, and Panc10.05 were provided by Dr. Elizabeth Jaffee (Johns Hopkins University, Baltimore, MD). Colo357 was provided by Dr. Lance Tibbetts, Brown University, Providence, RI). Panc8.13 and YAPC-3 were from J. Settleman, (Massachusetts General Hospital, Boston, MA). Suit2, HcG25, Capan-1, and T3M4 were from S.K. Batra (University of Nebraska, Lincoln, NE). HupT3, HPAC, DanG, PA-TU8902, PA-TU89885, PA-TU8988T, and ASPC-1 were from Lynda Chin, (MD Anderson, Houston, TX). All cell lines were genotyped by Affymetrix SNP6.0 for definitive future disambiguation of provenance. No additional authentication was done by the authors. All cells were cultured in high glucose Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS (US Origin). All PDA cell lines used in this study had activating KRAS mutations, except one, BxPC3, which harbors wild-type KRAS. RAS mutational status was verified by sequencing at University of California, San Francisco (UCSF, San Francisco, CA) Genomics Core Facility.

Cell growth inhibition assays

Cells were plated in 96-well plates at density from 3,000 to 12,000/well depending on the cell line so they are in logarithmic growth at the time of assay. Cells were allowed to attach overnight before being exposed to the drugs for 72 hours. Increasing doses of each drug were added in triplicate wells. The final DMSO concentration was 0.2% or less. Cell viability was determined using the CellTiter-Glo assay (Promega). Drug combination studies were designed according to Chou and Talalay (19). Combined drugs were used at fixed molar ratios. Equitoxic drug doses that produced approximately 50% of growth inhibition in single-agent experiments were chosen to determine an appropriate fixed molar ratio of 2 combined drugs. For most of the cell lines, IC50 was not reached with one or both single agents, therefore the CalcuSyn software (Biosoft), commonly used for drug combination studies, was not applicable. The enhancement effect was designated as potentiation when the IC50 from the drug combination treatment was at least 3-fold lower than the lowest IC50 of the single agent.

Apoptosis analysis

Cells were treated with drugs 24 hours after plating and were harvested for apoptosis assay at 3 days after drug treatment. Apoptosis was measured in live cells by Annexin V-fluorescein isothiocyanate and propidium iodide (PI) labeling using Apoptosis Detection Kit I (BD Pharmingen) and quantified by Flow Cytometry (FACS Calibur) with FlowJo software. The experiments were done in duplicates, and at least 40,000 cells were acquired from every sample for each duplicate. The results were reproduced in 3 independent experiments.

siRNA transfection

Cells were plated at 20 × 10^4/mL in 6-well plates and transfected with siRNAs using reverse transfection protocol with 20 nmol/L siRNA and 2.5 μL RNAiMax transfection reagent (Invitrogen). Forty-eight hours after transfection, the cells were trypsinized and replated at a required density for the specific assay of drug sensitivity (cell viability or apoptosis.) Drug treatment was started 72 hours posttransfection (24 hours postreplating). Seventy-two hours after drug treatment, cells were analyzed for apoptosis or for cell viability. The following siRNAs were used in this work: nontargeting smartpool siRNA (ON-TARGETplus, Dharmacon #D-001810-10-05) and HER3-targeting smartpool siRNA (ON-TARGETplus, Dharmacon #L003127-00-0005). For ZEB1 siRNA experiments, we used two negative controls: Ctrl NT, siControl ON-TARGETplus Nontargeting siRNA (#1 (Dharmacon) and Ctrl FF, firefly luciferase-targeting siRNA. ZEB1 siRNA #1: 5’-CAGUGAAAGAGAAGGGAAUUU-3’/5’-AUUCCCUUUCUCUUCUACAGUCUGUUU-3’; siRNA #2: 5’-AACUGUGACCGAUAAUUU-3’/5’-AUAAUCCACAGGUCAGUUU-3’.

Preparation of protein lysates and Western blot analysis

The cells were treated with drugs either in low serum or in full serum conditions as indicated in the figures.
For the low serum conditions, cells were plated in medium containing 0.1% FBS 24 hours after plating and incubated for 24 hours in this medium. Drugs or DMSO (control) were added followed by addition of EGF at a final dose of 10 ng/mL 30 minutes later. Cells were harvested at 4 hours post-EGF stimulation. For full serum conditions, cells were grown in their established growth medium (DMEM with 10% FBS), at 48 hours after plating were treated with the drugs and harvested at 4 hours postdrug exposure. Protein lysates were prepared from cells at 70% to 90% confluency. The cells were washed in ice-cold PBS, and then extracted in radioimmunoprecipitation assay lysis buffer (Boston Bioproducts) containing protease and phosphatase inhibitor cocktails. The lysates were clarified by centrifugation at 13,000 rpm for 10 minutes on ice and frozen at –80°C. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology). Twenty microgram of protein extract per lane was electrophoresed, transferred to nitrocellulose membranes (Bio-Rad), and probed with specific antisera using standard techniques. Bound antibodies on immunoblot analyses were detected and quantitated by fluorescent imaging (Odyssey imager, Li-COR).

miRNA expression
To analyze whether miRNA were differentially expressed in cell lines sensitive or resistant to a combination of EGFRi/MEKi, we analyzed miRNA expression in 6 sensitive and 6 resistant cell lines. Total RNA was isolated using mirVana kit (Ambion). miRNA assays were conducted with 100 ng total RNA by Nanostring Technologies based on nCounter Analysis System using digital color-coded barcode technology, as well as by HTG Molecular Inc, using quantitative nuclease protection assay (qNPA) technology. Out of 733 miRNA screened by Nanostring Tech, 8 miRNAs were highly expressed in sensitive but not in resistant cell lines. Data are presented as direct counts proportional to miRNA expression in each cell line. HTG Molecular assay showed consistent results with the same miRNAs significantly higher expressed in sensitive cell lines.

In vivo xenograft tumor studies
Female 7- to 9-week-old Nu/Nu mice (Harlan, FoxN1/nude) were inoculated subcutaneously with 10⁷ HPAF-II cells. Mice were monitored according to the protocol approved by the UCSF Institutional Animal Care and Use Committee. When tumors reached about 100 mm², mice were randomly assigned to one of the 4 treatment groups: vehicle control, erlotinib 35 mg/kg, PD0325901 7.5 mg/kg, or the drug combination. The drugs were administered by oral gavage once a day 5 times per week. Each treatment regimen was tested in cohorts of 8 mice, and the dosing was continued for 34 days. Tumor sizes were measured twice weekly in 2 dimensions using a caliper, tumor size (mm³) was calculated as (length × width²)/2. The doses for drug treatments were chosen as maximum-tolerated dose according to the preceding toxicity study in mice bearing HPAF-II xenografts. Animals were monitored for the absence of drug toxicity by measuring the body weight twice weekly and monitoring the overall activity. To assess for target inhibition, 2 mice from each treatment group were sacrificed at day 3 of treatment, 3 hours postdosing. Tumors were excised and protein lysates prepared and subjected to Western blot analysis as described above for cell lines.

Statistical analysis
Statistical analysis was conducted using GraphPad Prism software. An unpaired two-tailed t test was used to determine the significance of change in the levels of cell viability and apoptosis between different treatment groups. Data are expressed as mean ± SD. Statistical analysis to compare tumor sizes in xenograft-bearing mice was done with ANOVA with Bonferroni posttests. P values of <0.05 were considered significant.

Correlation analysis of drug sensitivity
mRNA expression data of PDA cell lines panel were described previously (12). The data were normalized by robust multi-array average (RMA) method. Low performing probesets (those with intensity values below a threshold across all samples, the threshold was taken to be the global lowest 25th percentile of intensity values) were excluded from analysis. Differential gene expression for dichotomous EGFRi + MEKi sensitivity were obtained from moderated t statistics using the limma package (20) in Bioconductor (21). q values (22) were calculated to control the false discovery rate and q values < 0.01 were considered significant. The expression data were also classified to predict EGFRi + MEKi sensitivity (sensitive or not) by applying nearest shrunken centroid classification using Pam (23) with 10-fold cross-validation and obtaining the top genes with low misclassification error. We obtained the list of 263 and 29 predictors of sensitivity and resistance, respectively. All calculations were conducted using the R language (24).

Results
MEK inhibition results in negative feedback activation of PI3K mediated by the EGFR
In our previous work, using the models of triple-negative breast cancer, we showed that MEK inhibition induces feedback activation of PI3K mediated by the EGFR (15). To investigate whether pathway interactions occur in pancreatic cancer, we treated KRAS-mutant (HPAF-II and Panc 10.05) and KRAS-wt (BxPC-3) PDA cell lines with the MEK inhibitor CI1040, the EGFR inhibitor erlotinib, and their combination at low serum conditions (cell culture medium containing 0.1% FBS) in the presence of EGF. As expected, a strong reduction in p-ERK levels in response to MEK inhibitor treatment...
was observed (Fig. 1A). However, MEK inhibition led to enhanced phosphorylation of EGFR and activation of the PI3K pathway, as determined by markedly enhanced protein levels of phosphorylated AKT (p-AKT, pS473), PRAS40 (pT246), and GSK3α/β (pS21/9). MEK inhibitor-induced activation of AKT was fully abolished by the EGFR inhibitor erlotinib (Fig. 1A). Thus, a negative regulatory feedback loop between MEK and the PI3K pathway is operative in PDA cells and depends on activation of the EGFR.
**Interruption of MEK-PI3K feedback results in synergistic inhibition of cell viability and induction of apoptosis in PDA cell lines and mouse xenograft tumors**

Because MEK inhibition resulted in feedback activation of the EGFR pathway in PDA cell lines, we asked whether a combination of MEK with EGFR inhibitors would elicit synergistic effects on cell survival in PDA cell lines. Indeed, combination of the MEK inhibitors, CI1040 or PD0325901, with the EGFR inhibitor erlotinib resulted in synergistic cell growth inhibition in PDA cell lines harboring wild-type (BxPC-3) or mutant KRAS (HPAF-II; Fig. 1B). This synergistic effect on cell growth was due to highly increased rate of apoptosis as determined by fluorescence-activated cell sorting (FACS) analysis of annexin V/PI-positive cells (Fig. 1C). To test whether the combination of MEK and EGFR inhibitors induced enhanced tumor growth inhibition in vivo, we generated subcutaneous xenograft tumors using the human PDA cell line HPAF-II. Tumors were grown to a size of approximately 100 mm³ and mice were treated with the specific, orally bioavailable MEK inhibitor, PD0325901 and erlotinib, either as single agents or in combination, for up to 34 days by oral gavage.

At daily doses of 35 mg/kg of erlotinib and 7.5 mg/kg of PD0325901, an additive effect of both drugs was observed (Fig. 1D). The combination of both drugs administered at higher doses (50 mg/kg of erlotinib and 12.5 mg/kg of PD0325901) resulted in strong synergistic induction of tumor shrinkage. However, increased toxicity was observed at this dose level and the study had to be stopped after 18 days of treatment. Western blot analysis of tumors confirmed inhibition of p-ERK by PD0325901 and phosphorylated EGFR (p-EGFR) by erlotinib (Fig. 1E). The feedback of MEKi-induced activation of p-EGFR, p-AKT, p-PRAS40, and p-GSK3 was also evident in vitro, similar to in vivo observations in cell lines. However, in contrast to in vitro effect, combination of erlotinib and PD0325901 did not result in complete p-AKT and p-EGFR inhibition in vivo, suggesting that additional pathway inputs are operational.

Because molecular profiling of clinical PDA samples from our institution revealed up to 3 different subtypes of the disease (12), we set out to assess possible subtype-dependent differences between PDA cell lines with respect to their response to the MEK/EGFRi combination. We treated a panel of 29 pancreatic cancer cell lines with increasing concentrations of PD0325901 and erlotinib. Compounds were administered as single agents or in combination at fixed molar ratios. Because the cell sensitivity to PD0325901 varied greatly, we did the assay in the dose range of 0.01 to 1 μmol/L; for the combination study at fixed molar ratio, we selected the dose which was close to IC₅₀. For each cell line the experiments were done 3 to 4 times at different dose ratios to ensure consistency in response pattern and IC₅₀ values. Figure 2A shows representative examples of 4 different response patterns; the complete dataset of all cell lines responses is presented in Supplementary Fig. S2. In 11 cell lines, single drug treatment had only limited effects on cell viability, but treatment with the drug combination resulted in strong inhibition of cell viability and apparent cell killing, indicating potentiation of activity of one or both drugs (e.g., Panc3.27; Fig. 2A). In 12 cell lines, the potentiation effect was moderate (e.g., Panc10.05; Fig. 2A). In 3 cell lines, cell viability was only inhibited by the MEK inhibitor, and its combination with EGFR inhibitor had no additional effect (e.g., MiaPaCa-2 cells; Fig. 2A). Finally, 3 cell lines were resistant to both inhibitors as single agents and to their combination (e.g., Panc2.13; Fig. 2A). The IC₅₀ was calculated for each drug and for their combination using the GraphPad Prism software. If the combined IC₅₀ was at least 7-fold lower (such as for Panc3.27) or 3- to 6-fold lower (such as for Panc10.05) compared with the lowest IC₅₀ of the single agent, the response was classified as strong synergy/potentiation or as moderate potentiation, respectively. When the combined IC₅₀ was less than 3-fold different than the lowest IC₅₀ of a single drug, the response was classified as "no combinatorial effect" (such as for MiaPaCa-2 or Panc2.13).

In summary, these data clearly show that the combination of MEK and EGFR inhibitors results in strongly enhanced inhibition of cell and tumor growth. Although these effects are independent of the KRAS mutation status, there was marked heterogeneity in cell line responses to treatment with the drug combination.

**RNA profiling of PDA cell lines reveals genes predictive of synergistic response to the MEK/EGFR inhibitor combination**

To elucidate molecular determinants associated with the different response patterns to treatment with the combination of MEK and EGFR inhibitors, we generated mRNA expression profiles for all cell lines in our panel. Correlation analysis between gene expression and drug responses identified probes that were significantly (q < 0.01, and difference in expression >2-fold) correlated with cellular sensitivity (263 probes) or resistance (29 probes) to the combination of MEK and EGFR inhibitors in our PDA cell line panel. The complete list of sensitivity and resistance predictors is presented in Supplementary Table S1. Figure 2B illustrates a short list of 32 probes associated with sensitivity (q < 0.00001) and 10 probes associated with resistance (q < 0.01), which show a difference in mRNA expression level of more than 2.5-fold (fold change (FC) > 2.5). Cell lines were hierarchically clustered according to their expression of response predictors. This analysis revealed that genes known to be markers of epithelial phenotype were highly expressed in the sensitive cell lines, including CDH1 (q = 10⁻¹²), ST14 (q = 10⁻⁶), KR7 (q = 7 x 10⁻⁹), and KRT19 (q = 6 x 10⁻⁸). In contrast, all 6 resistant cell lines displayed low expression of these genes but showed high levels of ZEB1 (q = 0.001) expression, in agreement with their mesenchymal phenotype. Almost all moderately responsive cell lines clustered to...
the interface between the sensitive and resistant cell line clusters (Fig. 2B). In contrast to data from human primary PDAs, there was no apparent acinar subtype (characterized by expression of chymotrypsin-like elastase family members, and regenerating islet-derived genes, among others; ref. 12).

Three genes (FOXQ1, HER3, and ELF3) identified by us as associated with sensitivity were part of the gene set characterizing the epithelial (classical) subtype of primary human PDAs (12), representing significant overrepresentation ($P < 0.0004$; Fisher exact test). These findings indicate that clinically relevant subtypes of PDA respond differently to the combination of MEK/EGFR inhibition. This has implications for further understanding of underlying molecular mechanisms as well as for patient selection strategies in future trials.

Feedback activation of the PI3K pathway resulting from MEK inhibition in PDA subtypes

We next asked whether the differences in drug sensitivity between epithelial and mesenchymal subtypes were reflected in differential behavior of signal transduction pathways following MEK inhibition. In all cell lines, inhibition of MEK with CI1040 resulted consistently in inhibition of phosphorylation of its effector, ERK. However, in the epithelial-type PDA lines, increases of p-AKT levels were detected in cells grown in medium containing 10% FBS following treatment with CI1040 (Fig. 3), although this effect was less evident compared with low serum conditions with EGF stimulation. In epithelial-type cell lines, pAKT was strongly inhibited by erlotinib treatment and completely suppressed by its combination with CI1040. In contrast, the mesenchymal-type PDA lines showed no induction of pAKT following MEK inhibition. In these cells, high basal levels of pAKT remained unchanged following treatment with single inhibitors and their combination. Thus, these data indicate that there are differences in receptor tyrosine kinase (RTK) signal transduction between epithelial and mesenchymal-type pancreatic cancer cells, which are consistent with differential responses to MEK and EGFR inhibitors and their combination.

Inhibition of HER3 enhances effects of MEK inhibition in epithelial-type PDA

The RTK HER3 (ERBB3) was found to be among the genes significantly associated with the sensitivity of PDA...
cell lines to MEKi/EGFRi combination. Because HER3 is well known to mediate signals from EGFR and HER2 to PI3K, we ascertained whether feedback activation of EGFR following MEK inhibition resulted in HER3 activation. As shown in Fig. 3, this was indeed the case as we observed a clear increase in HER3 phosphorylation at Y1197 and Y1289 in response to treatment with CI1040 in epithelial-type cells. In contrast, mesenchymal type cells, as expected, had low basal expression of HER3, and did not show any increase in HER3 phosphorylation in response to MEK inhibition.

The phosphorylation of HER3 of both Y1197 and Y1289 was also induced in vivo, in tumor xenograft tissues of mice treated with MEK inhibitor (Fig. 1E). Taken together, these findings suggested that HER3 might represent a meaningful therapeutic target in combination with MEK inhibitors specific to epithelial subtype PDA. To explore this possibility, we used siRNAs targeting HER3 that effectively reduced HER3 protein levels when transfected into various PDA lines. As determined by quantitative Western blot analysis, HER3 expression decreased 2- to 5-fold at 72 hours posttransfection with siRNA (pool of 4), depending on cell line (Fig. 4A). Mesenchymal cell lines, such as Panc2.13, had very low basal expression of HER3 protein that was not changed after siRNA knockdown.

In agreement with our hypothesis, epithelial PDA cell lines in which HER3 had been knocked down showed significantly higher rates of apoptosis in response to single inhibitors or to MEK/EGFR-combined inhibition (Fig. 4B). The drug-induced apoptosis rate in cells treated with triple HER3/MEK/EGFR suppression reached 50% to 80%
depending on the cell line. Knockdown of HER3 in addition to EGFR inhibition might increase apoptosis induction due to a greater degree of inhibition of signal flow from EGFR to PI3K. Indeed, we observed residual phosphorylation of HER3 following treatment of cells with erlotinib (Fig. 3). Furthermore, signals from additional RTKs might contribute to this phenomenon. Mesenchymal cell lines (such as Panc2.13), characterized by low levels of HER3, also displayed apoptosis increase, although the overall apoptotic rate was below 10% (Fig. 4B). Therefore, knockdown of HER3 did not sensitize mesenchymal cells to the combination of MEK and EGFR inhibitors. These data suggest that combinations of MEK and EGFR as well as HER3 inhibitors may be particularly effective in epithelial subtype of PDA cells.

**miRNA expression of miR-200 family is significantly downregulated in resistant PDA cells**

In cancer cells, epithelial and mesenchymal subtypes are maintained through molecular circuits including transcription factors, such as ZEB1, SIP1, SNAIL, and SLUG and microRNAs (miRNA), in particular those belonging to the miR-200 family. We therefore assessed expression of miRNAs using commercially available technology provided by Nanostring Technologies based on their nCounter Analysis System with digital color-coded barcode technology. We selected 6 sensitive and 6 resistant cell lines based on their response to the PD0325901/erlotinib combination and analyzed global expression of their miRNAs (733 miRNAs in total). We found that all 5 members of the miRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and in addition, miR-155, -139-5p, and 135b, were markedly upregulated in sensitive, but almost not expressed in resistant cell lines (Fig. 5). We compared these results with the data obtained by a different technology available through HTG Molecular using qNPA screening of 1,250 miRNAs, and obtained very similar results (data not shown). In addition, we validated miR-200 family expression levels by quantitative PCR conducted on total Trizol-isolated RNA. Because miR-200 family is known to be crucially involved in regulating epithelial-to-mesenchymal transition (EMT; refs. 25, 26), our findings support the notion that molecular programs regulating differentiation status of PDA cells determine susceptibility to combinations of MEK and EGFR inhibitors.
Suppression of ZEB1 results in sensitization to MEK/EGFR inhibitor combination

Our analysis of molecular predictors of response to the MEK/EGFR inhibitor combination clearly showed that epithelial features significantly correlated with sensitivity. High E-cadherin expression was found to be the strongest predictor of sensitivity, whereas ZEB1 expression was found to be a significant predictor of resistance to the MEK/EGFR inhibitor combination. ZEB1 is a well-characterized repressor of E-cadherin transcription and promoter of EMT (27). ZEB1 also regulates and is regulated by, as part of a double-negative feedback loop, expression of miRNAs of the miR-200 family (28). In agreement with this, we found that miR-200 family levels were downregulated in resistant cells lines. Therefore, we asked whether knockdown of ZEB1 would result in mesenchymal-to-epithelial transition and in sensitization to the inhibitor combination. We used 2 ZEB1 siRNAs that we optimized and validated previously (29) and compared their effect with 2 controls: nontargeting and Firefly luciferase-targeting siRNAs. Indeed, knockdown of ZEB1 in mesenchymal-type PDA cells (Panc2.13, HS766T, and PA-TU8988T) resulted in more than 90% inhibition of ZEB1 protein expression (Fig. 6A). Following knockdown,
noticeable changes in cell morphology resulting in less predominant spindle cell phenotype were observed (data not shown). This was accompanied by a marked upregulation of E-cadherin protein expression (Fig. 6A), and activation of pAKT and pHER3 above the background levels in mesenchymal cells (Supplementary Fig. S3). In contrast, knockdown of ZEB1 in epithelial cells (which have very low ZEB1 expression) had no effect on E-cadherin levels (Panc10.05 cells; Fig. 6A; HPAF-II cells; Supplementary Fig. S3). In agreement with these biochemical findings, knockdown of ZEB1 in mesenchymal-type PDA cells resulted in moderate increases in apoptosis rates in cells treated with single-agent erlotinib or CI1040, whereas a significant increase in apoptosis rates was observed in cells treated with the combination of both (Fig. 6B). These phenomena were strongly contrasted by observations in epithelial-type PDA cells that did not show differences in apoptosis rates with or without knockdown of ZEB1.

Discussion

The RAS oncogene, which is mutated in the vast majority of pancreatic cancers (30), has resisted direct therapeutic targeting with small-molecule inhibitors. Thus, inhibition of RAS effector pathways such as the RAF-MEK-ERK pathway represents a rational alternative strategy. However, initial clinical experience with second-generation MEK inhibitors has been disappointing, with the exception of BRAF-mutant melanoma (30). Previously, we showed that MEK inhibition leads to EGFR-dependent feedback activation of the PI3K pathway in basal-type breast cancer, which uses the RAF-MEK-ERK pathway preferentially (15). Here, we show that MEK inhibition in pancreatic cancer also leads to EGFR-mediated PI3K activation and, as a result, profound sensitivity to combinations of MEK and EGFR inhibitors in PDA cell line models. These findings occur independent of the KRAS mutation status, in contrast with observations published by others (17). Our study expands on the published literature substantially as we identify phenotypic differences as crucial predictors of sensitivity to the drug combination, thus setting the stage for patient selection strategies in future trials of this combination.

The KRAS oncogene is predicted to result in consistent activation of the RAF-MEK-ERK signal transduction cascade. In agreement with this hypothesis, expression of mutant $BRAF^{V600E}$ but not $PIK3CA^{H1047R}$ is sufficient to induce PanIN formation in conjunction with mutant $p53^{R270H}$ in transgenic mouse models (31). In addition, studies of the effects of MEK inhibitors in pancreatic cancer cell lines show the dependence of these cells on the RAF-MEK-ERK pathway for their proliferation (32). Consistent with these data, expression levels of p-ERK in pancreaticectomy specimens were found to be correlated with poor survival (6). Thus, targeting this pathway therapeutically and understanding mechanisms of de novo resistance is of particular biologic and clinical significance.

Cellular signal transduction networks are complex functional units that are regulated in their activity by feedback and feed-forward mechanisms resulting in frequently nonintuitive network behaviors in response to targeted inhibitors. Increasing evidence suggests that feedback mechanisms indeed confer de novo resistance to inhibitors of the broader RTK signal transduction network (reviewed in ref. 33). For example, upregulation of the RTK HER3 contributes to resistance to AKT inhibitors (34), whereas feedback activation of PI3K mediates resistance to mTOR inhibitors (14). Our current study expands on these findings by showing that MEK-dependent feedback activation occurs despite the presence of KRAS mutations and highlights a role of HER3 in mediating PI3K activation in this context. HER3 is a kinase-deficient member of the HER kinase family containing 6 tyrosines in its intracellular domain that, when phosphorylated, mediate high-affinity binding to the p85 subunit of PI3K (35). In our experiments, inhibition of MEK resulted in increased phosphorylation of HER3. Underscoring the functional relevance of this effect, siRNA-mediated inhibition of HER3 in combination with MEK inhibitors resulted in significantly increased apoptosis rates, in particular, in epithelial-type PDA cell lines. Addition of erlotinib further enhanced this effect. Our findings are consistent with previous studies showing that persistence of HER3 activation in cells treated with EGFR or HER2 kinase inhibitors is a crucial factor in mediating resistance to these inhibitors (36). Thus, combinations of compounds targeting MEK and HER3 with or without EGFR inhibitors might have substantial therapeutic potential.

Recent analyses of molecular profiles of human pancreatic cancers revealed the existence of at least 3 distinct subtypes: classical (epithelial-like), quasimesenchymal, and exocrine-like (12). Of those, epithelial-like and quasimesenchymal subtypes are present in cell line models of pancreatic cancer, whereas the exocrine-like subtype is not represented in this setting. These subtypes are strongly associated with differential susceptibility to therapeutics. For example, the quasimesenchymal subtype lacks responsiveness to erlotinib (12). Here, we expand on this theme by showing that synergistically enhanced cell killing by the combination of MEK and EGFR inhibitors is restricted to cell lines with epithelial characteristics as determined by mRNA and miRNA profiling. We identified subsets of genes and miRNAs (in particular the miR-200 family) that are strongly predictive of synergistic cell killing activity of the drug combination. Strong evidence suggests that the miR-200 family plays an important role in mediating epithelial versus mesenchymal differentiation in tumor cells by targeting the repressors of CDH1, in particular ZEB1 and ZEB2 (Sip1; refs. 25, 26). Interestingly, an inverse relationship between ZEB proteins and miR-200 family has been found, revealing a dual-negative feedback loop regulating and maintaining the differentiation state of tumor cells (25, 26). Previously, it was shown that mesenchymal differentiation of tumor
cells promotes resistance to EGFR inhibitors (37, 38). In agreement with these findings, miRNA signature predictive of sensitivity to EGFR inhibitors is strongly enriched for miRNAs involved in maintaining epithelial differentiation (39). Here, we show that induction of mesenchymal-to-epithelial transition by inhibition of ZEB1 expression results in marked sensitization to the combination of MEK and EGFR inhibitors. Re-expression of CDH1 has been shown before to restore sensitivity to EGFR inhibitors (40). Thus, it is conceivable that reversal of EMT by repression of ZEB1 restores molecular circuits critical for EGFR activation of antiapoptotic pathways following MEK inhibition.

The results of these studies hold significant clinical relevance in the treatment of pancreatic cancer, which continues to be a devastating disease for the majority of patients. On the basis of our findings, we have completed a clinical phase II trial that tested the efficacy of a combination of the MEK inhibitor AZD6244 in combination with erlotinib in patients with advanced, gemcitabine-refractory pancreatic cancer (Protocol ID #NCT01222689 in clinicaltrials.gov). Tissue samples obtained from patients before enrollment into the study will allow us to further validate candidate predictive markers identified in the cell line panel. Future studies will use patient selection strategies based on the results of these studies. In addition, based on our findings, exploration of novel combinations involving MEK and HER3 inhibitors, as well as agents capable of reversing EMT, is warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: O.K. Mirzoeva, W.M. Korn
Development of methodology: O.K. Mirzoeva, B. Hann
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O.K. Mirzoeva, E.A. Collisson, P.M. Schaefer, B. Hann, Y.K. Hom
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O.K. Mirzoeva, P.M. Schaefer, W.M. Korn
Writing, review, and/or revision of the manuscript: O.K. Mirzoeva, E.A. Collisson, A.H. Ko, W.M. Korn
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F.M. Schaefer, B. Hann
Study supervision: W.M. Korn

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