Analysis of mRNA Profiles after MEK1/2 Inhibition in Human Pancreatic Cancer Cell Lines Reveals Pathways Involved in Drug Sensitivity

Stephan Gysin, Jesse Paquette, and Martin McMahon

Abstract

Mutational activation of KRAS, detected in approximately 90% of pancreatic ductal adenocarcinomas (PDA), has proven an intractable pharmacologic target to date. Consequently, efforts to treat KRAS-mutated cancers are focused on targeting RAS-regulated signaling pathways. In mouse models, expression of BRAFV600E combined with dominant-negative TP53 elicits PDA, and pharmacologic blockade of mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibits proliferation of human PDA-derived cell lines. To better understand the role of RAF→MEK→ERK signaling on PDA cell proliferation, we assessed the consequences of MEK inhibition on global patterns of mRNA expression and tumor cell proliferation in a panel of human PDA-derived cell lines. This analysis revealed that RAF→MEK→ERK signaling regulates mRNAs involved in cell-cycle control as well as regulators of the immune system. Linear regression analysis of relative drug sensitivity and mRNA expression revealed mRNAs and pathways correlating with relative drug sensitivity of the cell lines. Mice carrying orthotopically implanted pancreas tumors that were treated with MEK inhibitor displayed reduced tumor growth, concomitant with a reduction of cells in S phase. Furthermore, analysis of tumor mRNA expression revealed PDA cell lines to display similar baseline and MEK inhibitor mRNA expression profiles in vitro and in vivo. Among the proteins subject to downregulation following MEK inhibition, we identified c-MYC as a key driver of cell proliferation downstream of RAF→MEK→ERK signaling. Indeed, in some PDA cell lines, RNA interference–mediated silencing of c-MYC expression had antiproliferative effects similar to that of MEK inhibition, thereby highlighting the importance of c-MYC in key aspects of pancreatic cancer cell maintenance.

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Introduction

Pancreatic ductal adenocarcinoma (PDA) is the fourth most common cancer in the United States and is estimated to be responsible for 37,660 deaths in the United States in 2011 (http://seer.cancer.gov/statfact/). The lack of early-stage diagnostic tools or effective regimens of systemic chemotherapy means that patients newly diagnosed with PDA have a poor prognosis (1, 2).

Despite the grim clinical situation, significant progress has been made in understanding how damage to tumor suppressors and oncogenes contributes to PDA initiation and progression. Furthermore, high-throughput microarray and DNA sequencing tools have revealed the genetic heterogeneity displayed by this disease (3, 4). Finally, mouse models that recapitulate key aspects of the genetics and pathophysiology of the human disease have been used to explore mechanisms of cancer initiation, progression, and maintenance (5–11).

The earliest and most common genetic alteration is mutational activation of KRAS, which is detected in 70% to 90% of pancreas cancer patients (12). However, in the absence of cooperating alterations, mutationally activated KRAS leads to development of premalignant pancreatic intra-epithelial neoplasias (a.k.a. PanINs). Hence, progression to malignancy is generally accompanied by silencing of tumor suppressors such as TP53, CDKN2A, and/or SMAD4 (13). Because mutationally activated KRAS has proven to be an intractable pharmacologic target, attention has shifted to key RAS-regulated signaling pathways as potential therapeutic targets, most notably the RAF-activated extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) and PI3-kinase-activated pathways. Importantly, expression of mutationally activated BRAFV600E, but not PIK3CAH1047R, is sufficient for PanIN development, and for PDAC when combined with dominant-negative TP53270H (11). Moreover, pharmacologic blockade of RAF→MEK→ERK signaling leads to arrest of the...
pancreatic cancer cell division cycle that is mediated by elevated expression of the cyclin-dependent kinase inhibitor p27Kip1 (14–16). Consequently, due to the importance of RAS-activated RAF→MEK→ERK signaling in PDA, we examined in more depth the effects of mitogen-activated protein/extracellular signal–regulated kinase 1/2 inhibition on patterns of mRNA expression in vitro and in vivo using a panel of PDA cell lines (17, 18). The results reveal a range of responsiveness of the various PDA cell lines to MEK1/2 inhibition that is reflected in the profiles of mRNAs that respond to drug treatment. On the basis of time course and RNA interference experiments, we identified the transcription factor c-MYC as a key mediator of the proliferative effects of MEK1/2 in pancreatic cancer cell lines.

Materials and Methods

Cell lines and culture conditions

MIA PaCa-2, Panc-1, CFPAC-1, HPAF II, Capan-2, Hs766T, and BxPC-3 cell lines were provided by Dr. Paul Kirschmeier (Scribing Pough Research Institute), SW1990, MPanc-96, Panc-02.03, Panc-08.13, PL45, and SU86.86 cell lines were purchased from American Type Culture Collection. Panc-02.13, Panc-03.27, Panc-05.04, Panc-06.03, and Panc-10.05 were provided by Dr. Elizabeth Debas (University of Virginia, Charlottesville, VA). COLO-357 and its derivatives L3.3, L3.6d, and L3.6pl were provided by Dr. Lance Tibbetts, (Brown University, Providence, RI; COLO-357) or Dr. Isaiah Fidler (MD Anderson Cancer Center, Houston, TX; L3.3, L3.6d, and L3.6pl; refs. 19, 20). Of the various PDA cell lines used in this study, all express mutationally activated KRAS, except for COLO-357, L3.3, and BxPC3 (21).

MIA PaCa-2, Panc-1, CFPAC-1, HPAF II, Capan-2, Hs766T, PL45, SW1990, COLO-357, L3.3, L3.6d, and L3.6pl cell lines were cultured in Dulbecco’s modified Eagle’s medium. BxPC-3, SU86.86, MPanc-96, Panc-02.03, Panc-02.13, Panc-03.27, Panc-05.04, Panc-06.03, Panc-10.05 cells were grown in RPMI including insulin/l-glutamine. Primary human pancreatic ductal epithelial cells were either snap frozen in liquid nitrogen or fixed in formalin.

RNA isolation, quantification, and amplification

mRNA for microarray analysis was isolated from cells cultured on 6 × 15 cm dishes to approximately 80% confluency. Three of the 6 dishes were treated for 24 hours with 2 μmol/L CI-1040 and 3 treated with dimethyl sulfoxide as solvent control. Each dish of cells was harvested and processed separately so that there were 3 independent biologic triplicates for each mRNA expression analysis. mRNA from cultured cells or orthotopic tumors was isolated using the RNeasy kit (Qiagen). Briefly, 2 μg of total RNA was converted to cDNA, amplified, and biotinylated using the Message Amp II mRNA amplification kit from Ambion. RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific/Pierce) and its quality assessed using a Bioanalyzer (Agilent). Fragmented amplified cDNA was hybridized to Affymetrix HG-U133A 2.0 gene chips according to manufacturer’s protocols (Affymetrix). Raw hybridization data was preprocessed using Affymetrix Expression Console software.

Protein extraction and immunoblot analysis

Proteins were extracted from cultured cells or orthotopic tumors by lysis in a buffer comprising 1% (v/v) NP40, 50 mmol/L HEPES (pH 7.5), 250 mmol/L NaCl plus protease inhibitors (1 mmol/L phenylmethylnsulfonylfluoride and 10 mmol/L pepstatin) and phosphatase inhibitors (1 mmol/L EGTA, 10 mmol/L NaF, 1 mmol/L tetrasodium pyrophosphate, 100 mmol/L β-glycerophosphate and 1 mmol/L sodium orthovanadate). Protein concentrations were measured using the bicinchoninic acid (BCA) assay (Pierce). Fifty-microgram aliquots of total protein were separated by SDS-PAGE, blotted onto nitrocellulose (Bio-Rad) and then incubated with the appropriate primary antibodies. Immunoblotts were probed with antibodies against Cyclin D1, Cyclin A, MAD2L1 or ETV4 (aka PEA3; Abcam), bromo-deoxyuridine (BrdUrd) or p27KIP1 (BD Pharmingen), Cytokeratin (CK)-19 (Dako), phospho-histone-H3 (Upstate Biotechnology), phospho-histone-H3 (Upstate Biotechnology) or SPC25 (a gift from Dr. Stukenberg, University of Virginia, Charlottesville, VA) as described in the text. Antigen–antibody complexes were detected using secondary antibodies linked to horseradish peroxidase and visualized using Supersignal chemiluminescence detection (Pierce).

Orthotopic pancreatic tumorigenesis and treatment of mice with P325901

Human PDA–derived cell lines mixed 1:1 with Matrigel were injected subcapsularly into the pancreas of 6-week-old female nu/nu mice (Taconic) as described previously (24). For real-time imaging of tumor growth, PDA cells were transduced with a lentivirus encoding firefly luciferase allowing weekly bioluminescent imaging to monitor tumor burden (25).

Tumor-bearing mice were treated with 15 mg/kg P325901 in HPMT [0.5% hydroxypropylmethylcellulose, 0.2% (v/v) Tween-80] once daily by oral gavage. Before isolation of tumor-derived mRNA, mice were drug dosed twice at 24 and then 12 hours before euthanasia. Orthotopic tumors were excised from euthanized mice and then portions were either snap frozen in liquid nitrogen or fixed in formalin.

Measuring GI50 and IC50 values for effects of CI-1040 on cellular proliferation or signaling

PDA cells were seeded onto 96-well plates and then treated with a range of concentrations of CI-1040 from 5 pmol/L to 5 μmol/L for 24 hours. Cell proliferation was
measured by BrdUrd incorporation (EMD, NJ), which was added to cells 4 hours before cell harvest. The effects of CI-1040 on phospho-ERK1/2 (pERK1/2) levels were measured by immunoblotting. Concentration needed to reduce the growth of treated cells to half that of untreated cells (GI50) values were derived from dose–response curves using GraphPad Prism Software (GraphPad Software Inc.).

Normalization and multiple regression analysis

For analysis of the effects of MEK1/2 inhibition on mRNA expression in cultured PDA cell lines, 141 .CEL files were normalized using the Affymetrix Power Tools (APT) utility (MAS5 algorithm). For analysis of MEK1/2 inhibition on orthotopic tumor-derived mRNA, 166 .CEL files were normalized using APT. Downstream data analysis used all of the probe sets. Multivariate regression models were produced in R using the package GEE (http://cran.r-project.org/web/packages/gee/index.html).

The one-way ANOVA including scatter plots for looking at differentially expressed mRNAs was carried out using Genespring software GX 11 trial version (Agilent Technologies Inc.). Cell lines Panc-10.05, Panc-5.04, Panc-1, Panc-2.03, and MIA PaCa-2 were classified as being in the sensitive group, whereas Panc-3.27, Panc-2.13, HPAF II, Panc-6.03, and BxPC-3 were classified as representative of the insensitive group. Untreated and treated mRNA expression levels were compared among the different groups. P values were calculated by carrying out 1,000 permutations and Benjamini–Hochberg was used for multiple correction testing. Heat maps were created using the software Permutmatrix (LIRMM).

Results

Effects of MEK1/2 inhibition on pancreatic cancer cells

To explore the importance of RAF→MEK→ERK signaling in the aberrant behavior of pancreatic cancer cells, we...
used a panel of 22 PDA-derived cell lines previously characterized by aCGH (21). To determine the relative sensitivity of PDA cells to the antiproliferative effects of MEK1/2 inhibition, PDA cells were treated with a range of CI-1040 (5 pmol/L–5 μmol/L) for 24 hours. GI50 values for inhibition of BrdUrd incorporation were calculated as described in Materials and Methods. GI50 values displayed approximately 43-fold variation ranging from 71 nmol/L for Panc-10.05 cells to 3.1 μmol/L for Panc-3.27 cells (Fig. 1A).

Using the most (Panc-10.05) or least (Panc-3.27) MEK1/2 inhibitor-sensitive PDA cell lines, we assessed the effects of MEK1/2 inhibitor dose on known targets of RAF → MEK → ERK signaling by immunoblotting (Fig. 1B). Panc-10.05 cells displayed an IC50 for pERK1/2 between 50 and 500 nmol/L CI-1040. Consistent with this, reduced expression of c-MYC, Cyclin D1 and FRA1, and elevated expression of p27KIP1 was detected within the same dose range. In the less sensitive Panc-3.27 cells, the IC50 for pERK1/2 was in the range of 500 nmol/L to 5 μmol/L. Consistent with this, expression of c-MYC, Cyclin D1, and FRA1 was less sensitive to CI-1040 in these cells. Moreover, we detected only modestly increased p27KIP1 expression in Panc-3.27 cells treated with 5 μmol/L CI-1040 (Fig. 1B). Hence, in these cells, there is a correlation between IC50 and GI50 for the effects on CI-1040 on PDA-derived cell lines.

To explore mRNA expression patterns correlating with the sensitivity of PDA cell lines to MEK1/2 inhibition, we assessed the effects of CI-1040 on mRNA expression using the panel of PDA cell lines and also in 3 primary cultures of normal human pancreatic ductal epithelial cells (hPDEC; ref. 22). Triplicate cultures of cells were treated with 2 μmol/L CI-1040 for 24 hours, previously determined as an optimal time point for inhibition of pERK1/2 (14), at which time mRNA was isolated and analyzed using Affymetrix U133 microarrays. Unsupervised hierarchical clustering of all mRNA samples with all probe sets on the arrays segregated normal hPDECs and the PDA-derived cell lines into 2 distinct groups (Fig. 1C). As expected, all biologic replicates clustered together. In addition, biologic replicates

![Figure 2](image-url)

**Figure 2.** Regression analysis between GI50 and mRNA expression and one-way ANOVA of differentially expressed mRNAs. A, linear regression graph of 7 selected mRNAs significantly correlated with GI50 for BrdUrd incorporation. B, heat map of the first 50 most significantly correlated mRNAs with GI50 (red: high expression; green: low expression). C, scatter plot of differentially expressed mRNAs in most sensitive PDA cell lines (diagonal cutoffs are 1.5-fold). D, scatter plot of differentially expressed mRNAs in least sensitive PDA cell lines (diagonal cutoffs are 1.5-fold).
of CI-1040–treated cells clustered together with the vehicle-treated samples from the same cells, indicating that the effects of CI-1040 were not so profound as to radically alter the patterns of mRNA expression in any given cell line (Fig. 1C). Finally, mRNA isolated from COLO-357, L3.3, L3.6sl, and L3.6pl cell lines, cell lines closely related to one another but with different metastatic potential, clustered together as a highly related group (Fig. 1C; refs. 19, 21).

Linear least square regressions were calculated to identify mRNAs that displayed high expression in cell lines that were more or less sensitive to the inhibitory effects of MEK1/2 inhibition by BrdUrd incorporation (GI50). This regression analysis yielded a set of linear regression coefficients that correlated mRNA expression values with the range of GI50 values. The set of coefficients was subject to a one class t test to test significance, yielding a P value that also indicated how well the mRNA expression levels fitted with a linear curve. A false discovery rate correction was not done because significance thresholds can produce misrepresentations of the analysis results. The linear relationship of the expression of 7 selected mRNAs (CTNNB1, RAF1, NRAS, SOS1, PIK3CA, PIK3R2, and CRKL) that positively correlated with GI50 values is presented (Fig. 2A). The heat map (Fig. 2B) shows the expression patterns of the top 50 mRNAs that correlated most closely with GI50 values (Gene list, Supplementary Table S1). Using these data, we generated a classifier using a support vector machine with the top 20 mRNAs by separating the 7 most sensitive and 10 least sensitive cell lines. The predictive value of the classifier was validated using a leave-one-out cross-validation. By this method, the classifier showed a 95.6% overall accuracy in predicting MEK inhibitor sensitivity (Supplementary Fig. S1). Regression coefficients and associated P values were analyzed using the exploratory gene association networks (EGAN) software package (26). Using EGAN we enriched for gene association terms in PDA cell lines that were more or less sensitive to MEK1/2 inhibition. We arbitrarily set a cut-off for the coefficients of 0.5 and a P value of 0.05 in the case of mRNAs that were positively correlated (399 genes) and −0.5/0.05 (coefficient/P) in the case of mRNAs that were negatively correlated (521 genes). Using these mRNA sets, we looked at enrichments of biochemical pathways represented in the molecular signature data base for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Broad Institute) using EGAN (Table 1).

mRNAs encoding components of the phosphatidylinositol signaling, insulin, VEGF, β-catenin, and Notch pathways as well as pathways involving immunoreceptors were expressed at higher levels in insensitive cell lines or at lower levels in sensitive cell lines. We also detected a highly

| Table 1. Gene-set enrichment analysis for mRNAs positively or negatively correlated with GI50 |
|---------------------------------|-----------------|-----------------|
| **MsigDB C2: KEGG-insensitive cell lines** | Neighbors | Enrichment |
| HSA00562_INOSITOL_PHOSPHATE_METABOLISM | 6 | 1.36E-03 |
| HSA04070_PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM | 7 | 3.83E-03 |
| HSA04810_REGULATION_OF_ACTIN_CYTOSKELETON | 12 | 9.66E-03 |
| HSA04370_VEGF_SIGNALING_PATHWAY | 6 | 1.22E-02 |
| HSA04910_INSULIN_SIGNALING_PATHWAY | 9 | 1.30E-02 |
| HSA04310_WNT_SIGNALING_PATHWAY | 9 | 1.43E-02 |
| HSA04530_TIGHT_JUNCTION | 8 | 2.16E-02 |
| HSA04330_NOTCH_SIGNALING_PATHWAY | 4 | 2.30E-02 |
| HSA04620_TOLLLIKE_RECEPTOR_SIGNALING_PATHWAY | 7 | 2.46E-02 |
| HSA04662_B_CELL_RECEPTOR_SIGNALING_PATHWAY | 5 | 2.98E-02 |
| HSA04670_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION | 7 | 3.13E-02 |
| HSA04012_ERBB_SIGNALING_PATHWAY | 6 | 3.24E-02 |
| HSA00310_LYSINE_DEGRADATION | 4 | 3.47E-02 |
| HSA04660_T_CELL_RECEPTOR_SIGNALING_PATHWAY | 6 | 4.79E-02 |

<table>
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<th><strong>MsigDB C2: KEGG-sensitive cell lines</strong></th>
<th>Neighbors</th>
<th>Enrichment</th>
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<tr>
<td>HSA03010_RIBOSOME</td>
<td>44</td>
<td>8.60E-47</td>
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<tr>
<td>HSA00190_OXIDATIVE_PHOSPHORYLATION</td>
<td>32</td>
<td>2.81E-21</td>
</tr>
<tr>
<td>HSA04120_UBIQUITIN_MEDIATED_PROTEOLYSIS</td>
<td>6</td>
<td>1.71E-03</td>
</tr>
<tr>
<td>HSA00620_PYRUVATE_METABOLISM</td>
<td>6</td>
<td>2.32E-03</td>
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<tr>
<td>HSA00230_PURINE_METABOLISM</td>
<td>12</td>
<td>2.63E-03</td>
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<tr>
<td>HSA03020_RNA_POLYMERASE</td>
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<td>4.35E-03</td>
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<tr>
<td>HSA00632_BENZOADE_DEGRADATION_VIA_COA_LIGATION</td>
<td>4</td>
<td>7.76E-03</td>
</tr>
<tr>
<td>HSA00240_PYRIMIDINE_METABOLISM</td>
<td>7</td>
<td>1.80E-02</td>
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| **a** | Msig DB enrichment analysis for mRNAs positively correlated with GI50 (P ≤ 0.05). |
| **b** | Enrichment analysis for mRNAs negatively correlated with GI50 (P ≤ 0.05). |
significant enrichment of mRNAs encoding ribosomal proteins expressed at higher levels in sensitive cell lines, suggesting that sensitive cell lines have elevated expression of proteins involved in protein translation.

mRNAs that responded to MEK1/2 inhibition

To identify mRNAs displaying differential expression following MEK1/2 inhibition in PDA cell lines, a one-way ANOVA analysis was carried out. mRNA expression levels under treated or untreated conditions were compared among the 5 most sensitive and least sensitive cell lines (see Materials and Methods). Among 2,420 mRNAs that were differentially expressed, 1,503 mRNAs were responsive to CI-1040 in the 5 most sensitive cell lines, and only 283 mRNAs were responsive to MEK inhibition in the 5 less sensitive cell lines. A total of 634 mRNAs were overlapping in the 5 most and 5 least sensitive cell lines. Perhaps as expected, this showed that MEK1/2 inhibition elicited more dramatic changes in mRNA expression in the more sensitive cell lines compared with the less sensitive group as indicated by scatter plots (Figs. 2C and D). Gene Ontology (GO) process enrichment analysis of those sets of mRNAs showed a significant decrease of mRNA-encoding proteins involved in DNA replication, DNA metabolic processes, mitotic phase, and the cell division cycle in general. In addition and perhaps surprisingly, mRNAs associated with inflammatory processes, defense responses, and nutrient levels were also elevated in the sensitive cell lines (Table 2 and gene list: Supplementary Table S2). We detected only a small number of enrichments in the less sensitive cell lines because of the overall low number of differentially expressed mRNAs. Downregulated mRNAs in the less sensitive cell lines were representative of processes involving cell communication, and upregulated mRNAs represented pathways involved in negative regulation of cell proliferation (Table 2 and gene list: Supplementary Table S3). These results indicated that increased drug

<table>
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<th>Table 2. Gene-set enrichment analysis for differentially expressed mRNAs in more versus less sensitive PDA cell lines</th>
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<td><strong>Sensitive cell lines</strong>a</td>
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<tr>
<td><strong>Downregulated Genes</strong></td>
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<tr>
<td>GO:0006260 DNA replication</td>
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<tr>
<td>GO:0006259 DNA metabolic process</td>
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<tr>
<td>GO:0006270 DNA replication initiation</td>
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<tr>
<td>GO:0007049 Cell cycle</td>
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<tr>
<td>GO:0006261 DNA-dependent DNA replication</td>
</tr>
<tr>
<td>GO:0022402 Cell-cycle process</td>
</tr>
<tr>
<td>GO:0022403 Cell-cycle phase</td>
</tr>
<tr>
<td>GO:0051301 Cell division</td>
</tr>
<tr>
<td>GO:0000279 M phase</td>
</tr>
<tr>
<td>GO:0000278 Mitotic cell cycle</td>
</tr>
<tr>
<td><strong>Upregulated Genes</strong></td>
</tr>
<tr>
<td>GO:0009605 Response to external stimulus</td>
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<tr>
<td>GO:0009611 Response to wounding</td>
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<tr>
<td>GO:0006954 Inflammatory response</td>
</tr>
<tr>
<td>GO:0006952 Defense response</td>
</tr>
<tr>
<td>GO:0048583 Regulation of response to stimulus</td>
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<td>GO:0031667 Response to nutrient levels</td>
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<td>GO:0007584 Response to nutrient</td>
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<tr>
<td>GO:0008285 Negative regulation of cell proliferation</td>
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<tr>
<td>GO:0050776 Regulation of immune response</td>
</tr>
<tr>
<td>GO:0009991 Response to extracellular stimulus</td>
</tr>
<tr>
<td>GO:0031347 Regulation of defense response</td>
</tr>
<tr>
<td>GO:0051187 Cofactor catabolic process</td>
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<td><strong>Insensitive cell lines</strong>b</td>
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<td><strong>Downregulated Genes</strong></td>
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<td>GO:0010647 Positive regulation of cell communication</td>
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<tr>
<td><strong>Upregulated Genes</strong></td>
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<tr>
<td>GO:0008285 Negative regulation of cell proliferation</td>
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aGene Ontology processes enrichment analysis for differentially expressed mRNAs in sensitive PDA cell lines (P ≤ 0.05).

bEnrichment analysis for differentially expressed mRNAs in less sensitive PDA cell lines (P ≤ 0.05).
sensitivity correlated with an increased response of mRNA encoding proteins involved in cell-cycle regulation.

To confirm the results of microarray-based analysis of mRNA expression, the expression of a small number of CI-1040–responsive mRNAs and/or their protein products was assessed by TaqMan reverse transcriptase PCR (RT-PCR) or by immunoblotting, respectively (Fig. 3). Asynchronously growing cultures of Panc-05.04 (sensitive) or Panc-06.03, (less sensitive) were treated with CI-1040 to inhibit RAF→MEK→ERK signaling for 48 hours. CI-1040 was then washed out and the cells re-fed fresh media for 6, 12, or 24 hours, at which time cell extracts were analyzed for pERK1/2, CDC45L, MAD2L1, CCNA2, SPC25, ETV4, MYC, and FRA1 mRNA or protein (Fig. 3A and B). The mRNAs of these proteins were significantly decreased after CI-1040 treatment in a large number of cell lines. Panc-05.04 and Panc-06.03 cells displayed readily detectable levels of pERK1/2 that were robustly inhibited following CI-1040 addition (Fig. 3B, pERK). Removal of CI-1040 led to pERK1/2 reactivation to the same level following CI-1040 addition (Fig. 3B, pERK). The mRNAs and proteins were reexpressed following ERK1/2 reactivation following wash-out of CI-1040 (14). As predicted by the microarray analysis, CDC45L, MAD2L1, PEA3, MYC, and FRA1 mRNA or protein were readily detectable at 6 hours following MEK1/2 inhibition. Moreover, all of these mRNAs and proteins were reexpressed following ERK1/2 reactivation following wash-out of CI-1040. Some mRNAs/proteins (MYC and FRA-1) displayed rapid kinetics of reinduction consistent with their genes being immediate-early targets of RAF→MEK→ERK signaling (27, 28). Others, such as CDC45L, MAD2L1, CCNA2, and SPC25, displayed delayed kinetics of reexpression, consistent with the fact that transcription of these genes is regulated downstream of E2F activation. At least for these mRNAs/proteins, results of the microarray analysis were borne out by direct analysis of mRNA and protein expression.

**Effects of MEK inhibition on pancreatic cancer proliferation and mRNA expression in vivo**

Because pharmacologic MEK1/2 inhibition elicited a G1 cell-cycle arrest and inhibition of PDA cell proliferation in vitro, we wished to determine the antitumor effects of MEK1/2 inhibition in vivo, using an orthotopic model of pancreatic cancer. To facilitate real-time monitoring of pancreatic tumor growth and the response of tumor cells to MEK1/2 inhibition, MIA PaCa-2, PL45, Hs766T, Panc-02.13, SU86.86, and L3.6pl PDA-derived cell lines were transduced with a retrovirus vector encoding a triple reporter composed of a fusion protein of Luciferase, red fluorescent protein, and HSV-TK (Luc-RFP-TK; ref. 25). In a pilot experiment, 10⁶ L3.6pl cells expressing the Luc-RFP-TK reporter were injected into the pancreas of each of 5 immunocompromised mice. Mice carrying orthotopic L3.6pl pancreatic tumors were dosed with PD0325901, an agent very similar to CI-1040 but with enhanced in vivo potency and specificity for MEK1/2 (29). As predicted, MEK inhibition led to striking inhibition of tumorigenesis that was readily detected by in vivo imaging of Luciferase activity at 7 or 14 days following drug administration (Fig. 4A). To evaluate short-term effects of MEK inhibition on S-phase progression, orthotopically implanted tumors were allowed to grow to an approximate size of 0.125 cm³ as assessed at

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**Figure 3.** Effects of MEK inhibition on selected mRNA and proteins in cultured PDAC cell lines. A, TaqMan RNA analysis (histogram) and immunoblotting analysis of selected mRNAs/proteins in Panc-5.04 or Panc-6.03 cells following treatment and then wash out of CI-1040. Cells were either untreated (−) or treated for 48 hours (+) with CI-1040. Drug was washed out and replaced with fresh medium for 6, 12, and 24 hours as indicated. B, immunoblot analysis of additional indicated proteins from the same experiment as in A.
euthanasia. Mice were then treated with (i) vehicle control; (ii) PD325901—single dose at 25 mg/kg 24 hours before euthanasia or; (iii) two doses of PD325901 at 25 mg/kg separated by 12 hours before euthanasia. All mice were administered BrdUrd 4 hours before euthanasia at which time tumors were resected for analysis.

As expected, treatment with PD325901 led to a striking inhibition of pERK1/2 that was readily detected by immunofluorescence analysis of either formalin-fixed or frozen tissue (Fig. 4B and C). Also, MEK1/2 inhibition led to a striking decrease in both BrdUrd incorporation (Fig. 4C) and phospho-Histone H3 staining consistent with inhibition of the cell division cycle. The effects of MEK1/2 inhibition on pERK and S-phase progression were more striking when mice were treated with 2 doses of PD325901, 24 and 12 hours prior to euthanasia rather than a single dose. Both RNA and protein was prepared from the frozen tumor samples (4 from control, 3 from 24 hours, and 4 from 12 + 12 hours of PD325901 treatment). TaqMan RT-PCR analysis revealed significant repression of PE3, CCNA2, MAD2L1, and SPC25 mRNAs by PD325901 regardless of the dosing schedule (Fig. 5A). Consistent with the mRNA analysis, immunoblotting confirmed reduced expression of PE3, CCNA2, MAD2L1, and SPC25 in PD325901-treated tumors consistent with decreased pERK1/2 (Fig. 5B). These experiments were also conducted with tumors grown from MIA PaCa-2, Panc-2.13, or SU86.86 with very similar results (Data not shown). Together, these data indicated that MEK inhibition in vivo leads to arrest of the cell division cycle in a manner consistent with the antitumor effects of this agent on PDA cell proliferation. Moreover, mRNAs repressed in response to MEK1/2 inhibition in vitro remain responsive to MEK1/2 inhibition in tumors orthotopically implanted into immunocompromised mice in vivo.

Effects of MEK1/2 inhibition on mRNA expression in orthotopically implanted tumors

To more generally profile the effects of MEK1/2 inhibition on mRNA expression in pancreatic tumors, RNA was prepared from a series of vehicle- versus PD325901-treated orthotopically implanted pancreas tumors generated by implantation of the following cell lines: MIA PaCa-2 (3 control and 2 PD325901), PL45 (1 control and 2 PD325901), Panc-02.13 (3 control and 3 PD325901), SU86.86 (2 control and 3 PD325901), L3.6pl (2 control and 2 PD325901), and Hs766T (1 control and 1 PD325901). Initially, we sought to determine whether orthotopic implantation of PDA cell lines would influence either baseline patterns of mRNA expression or those mRNAs previously identified as being sensitive to MEK1/2 inhibition in cells cultured in vitro. To do this, mRNA expression data of cultured cell lines (C), including the primary hPDECs, was compared with mRNA expression data from orthotopically implanted tumors (Fig. 6). As before, mRNA from cultured, nontumorigenic hPDECs clustered separately from mRNA isolated from orthotopic pancreas tumors generated by implantation of the following cell lines: MIA PaCa-2 (3 control and 2 PD325901), PL45 (1 control and 2 PD325901), Panc-02.13 (3 control and 3 PD325901), SU86.86 (2 control and 3 PD325901), L3.6pl (2 control and 2 PD325901), and Hs766T (1 control and 1 PD325901).
the various PDA cell lines (Fig. 6). Interestingly, mRNA expression in 4 of 6 of the orthotopic PDA tumors clustered together with their counterparts grown in tissue culture. These data suggest that, at least for these PDA cell lines, tumorigenic growth in the pancreas of immunodeficient mice did not dramatically alter the overall patterns of mRNA expression in these cells. By contrast, mRNA expression profiles of SU86.86 or Hs766T orthotopic tumors did not cluster together with their in vitro cultured counterparts and seemed to form a separate cluster distinct from the other PDA cell lines and more similar to nontumorigenic PDECs (Fig. 6). t tests confirmed that most of the mRNAs regulated by CI-1040 in tissue culture were also significantly regulated by PD0325901 in orthotopic tumors (not shown).
data indicated that analysis of mRNA expression in cultured PDA-derived cell lines is generally predictive of patterns of mRNA expression when the same cells are grown as orthotopically implanted tumors in mice.

**Regulation of c-MYC expression by RAF→MEK→ERK signaling in PDA cell lines**

One of our goals was to identify mRNAs/proteins regulated by ERK1/2 MAPK signaling that may be important mediators of PDA cell proliferation downstream of mutationally activated KRAS. We assessed mRNA expression of 4 transcription factors, MYC, FOSL1 (aka FRA1), ETV4 (aka PEA3), and ETV5 (aka ERM) that were sensitive to MEK1/2 inhibition in our microarray data. In MIA PaCa-2 cells, expression of MYC, FOSL1, and ETV5 was repressed following 6 hours of drug treatment, whereas ETV4 expression was repressed after 12 hours (Fig. 7A). Similar effects of MEK1/2 inhibition on these mRNAs were observed in Hs766T, PL45, Panc-2.13, SU86.86, and L3.6pl (not shown). Consequently, given the known importance of AP-1 and MYC in the regulation of the cancer cell proliferation, these proteins may be mediators of CI-1040–induced cell-cycle arrest. Hs766T or MIA PaCa2 cells were treated either with vehicle (−) or CI-1040 for 6, 12, or 24 hours as indicated at which time cell lysates were analyzed by immunoblotting (Fig. 7B). As expected, pERK1/2 was inhibited at all times analyzed. Consistent with the mRNA analysis, c-MYC was also decreased in both cell lines. To determine the importance of c-MYC in the control of PDA cell proliferation, Hs766T or MIA PaCa2 cells were treated either with vehicle, CI-1040, a control siRNA, or a validated siRNA-directed against c-MYC mRNA (Fig. 7C). As expected, both MEK1/2 inhibition and siRNA against c-MYC (siMYC) led to decreased c-MYC expression. However, whereas CI-1040 treatment inhibited pERK1/2, siMYC did not. Control siRNA was without any inhibitory effect on either c-MYC or pERK1/2. Parallel analysis of the cell division cycle revealed that MEK1/2 inhibition led to an increased percentage of cells in G1 and decreased percentages of cells in S and G2/M cell-cycle phases in both Hs766T and MIA PaCa2 cells. Similarly, siMYC also led to an increased percentage of cells in G1 and decreased percentages of cells in S and G2/M phases in both cell lines, which was almost as profound as that observed with MEK1/2 inhibition. We did not observe any consistent effects on PDA cell proliferation when we applied the same siRNA-mediated knockdown strategy with FOSL1 or ETV5 mRNAs. Taken together, these data indicated that c-MYC mRNA and protein are regulated by the RAF→MEK→ERK signaling in PDA cell lines. Moreover, the fact that RNAi-mediated inhibition of c-MYC expression had similar effects to CI-1040 on PDA cell lines suggests that regulation of c-MYC may be a key mechanism by which MEK1/2 inhibition exerts its inhibitory effects on PDA proliferation. The potency of the effects of siMYC on PDA cell cycling underscores the potential central significance of c-MYC as a mediator of the effects of RAF→MEK→ERK signaling on PDA cell proliferation. However, we found no correlation between c-MYC mRNA regulation after CI-1040 treatment and drug sensitivity (See Supplementary Fig. S2). Unfortunately, extensive efforts to

**Figure 7.** RNAi-mediated inhibition of c-MYC expression elicits cell-cycle arrest. A, TaqMan time course analysis of the effects of MEK inhibition on the expression of c-MYC, FOSL1, ETV5, and ETv4 mRNAs in MIA PaCa-2 cells. B, MIA PaCa-2 and Hs766T cells were either untreated or treated with CI-1040 for the indicated times at which point the expression of c-MYC, IERK, and ERK phosphorylation was assessed by immunoblotting. C, MIA PaCa-2 and Hs766T cells were either untreated or treated with CI-1040, an anti-MYC siRNA (MYC siRNA) or a control siRNA for 24 hours as indicated. The expression of c-MYC, IERK, and ERK phosphorylation was assessed by immunoblotting. D, MIA PaCa-2 and Hs766T cells were either untreated or treated with CI-1040, an anti-MYC siRNA (MYC siRNA) or a control siRNA for 24 hours as indicated at which time the cells were stained with propidium iodide to assess cell-cycle status as indicated.
render cells resistant to the effects of MEK1/2 inhibition by ectopic overexpression of either wild-type, T58A, or P57S forms of c-MYC under the control of the Moloney MuLV LTR, Mouse stem cell virus (MSCV) or the cytokeratin 19 promoter failed, as in each case, the ectopically expressed c-MYC remained sensitive to MEK1/2 inhibition (ref. 30; Supplementary Fig. S3). Moreover, because ERK1/2-mediated serine 62 phosphorylation is reported to be required for c-MYC stability, it is predicted to be difficult to sustain ectopic c-MYC expression in the face of MEK→ERK inhibition (30, 31).

Discussion

Pharmacologic blockade of RAF→MEK→ERK signaling leads to arrest of the pancreatic cancer cell division cycle in a manner dependent on elevated expression of the cyclin-dependent kinase inhibitor p27KIP1 (14). Here, we expand that initial analysis to a larger panel of 22 PDA cell lines treated with more specific, selective, and potent pharmacologic MEK1/2 inhibitors. In addition, we complemented analysis of the cell division cycle with extensive profiling of the effects of MEK1/2 inhibition on mRNA expression profiles in cultured cells and in orthotopically implanted tumors. These studies complement those of others who used mRNA expression profiling to identify potential biomarkers by comparing mRNAs overexpressed in cancer cell lines or tissues relative to normal cells (32, 33). Our observations confirm that many human PDA cell lines depend on the archetypal ERK1/2 MAPK pathway for their sustained proliferation. Among the PDA cell lines, we identified an approximately 43-fold difference in sensitivity of cell lines (GI50) to the antiproliferative effects of MEK1/2 inhibition on the S phase of the cell division cycle. Despite the differences in relative sensitivity, none of the PDA cell lines was formally resistant to MEK inhibition, consistent with the work of others (33). However, we were not able to determine a clear-cut molecular predictor of MEK inhibitor sensitivity in PDA cell lines by simply comparing the IC50 for inhibition of pERK1/2 with the GI50 for inhibition of S-phase progression, as IC50 values for pERK1/2 correlated only moderately with GI50 values for BrdUrd incorporation (data not shown). Consequently, we subjected the cell lines to Affymetrix microarray analysis of mRNA expression. As expected, unsupervised clustering of mRNA profiles from PDA cell lines and primary hPDECs showed that normal hPDEC mRNA profiles clustered together and were distinct from mRNA profiles of cancer cells. Although primary PDA specimens and PDA cell lines express markers (CK19, MUC1, and CFTR) suggestive of a ductal epithelial origin, recent data from mouse models suggests that PDA may emerge from a metaplastic transformation of acinar cells in response to tissue damage (34, 35) or from a poorly characterized exocrine pancreas stem cell. Because PDA is generally diagnosed late in its progression and many of the cell lines were isolated from metastatic lesions, it is unlikely that any of the PDA cell lines analyzed represent different stages of the disease. Therefore, by contrast to breast cancer mRNA profiling and the work of others in PDA, we were unable to discern subtypes within the PDA cell lines with regard to a specific pathologic state or clearly defined genetic subsets of PDA (23).

Because the biologic replicates of each CI-1040–treated sample clustered together with the replicates of the untreated samples for each cell line, this indicates that overall mRNA expression profiles are similar in control versus MEK1/2 inhibitor–treated samples. The overall pattern in differentially expressed mRNAs reflected a strong enrichment for cell-cycle–associated mRNAs that were downregulated after MEK inhibition, whereas there was enrichment for mRNAs involved in the immune response among the induced mRNAs. Consistent with the inhibitory effects of MEK1/2 inhibition on the cell cycle, many of the CI-1040–repressed mRNAs correspond to E2F target genes. Other transcription factor–binding motifs enriched in the list of mRNAs repressed by CI-1040 were those corresponding to genes regulated by the MYC family of transcription factors. However, the significance of the immune response pattern connected to innate immunity and inflammation in pancreatic cancer cells is unclear, although evidence suggests that RAF→MEK→ERK signaling controls the expression of many inflammatory cytokines and chemokines, such as interleukin-6 (IL-6), IL-8, and others (36).

Tumors expressing mutationally activated BRAFV600E are reported to be exquisitely sensitive to inhibition of RAF→MEK→ERK signaling. By contrast, tumors expressing mutationally activated RTKs or RAS are less sensitive to such agents (37, 38). Furthermore, acquired resistance of melanomas expressing BRAFV600E to PLX-4032 can be mediated either by RTK activation or mutational activation of NRAS (39, 40). Hence, in pancreatic cancers expressing mutated KRAS, it is possible that signaling through RAS-activated parallel signaling pathways might influence sensitivity to MEK1/2 inhibition. Indeed, PDA cells that were less sensitive to MEK1/2 inhibition displayed elevated expression of a set of mRNAs encoding the catalytic and regulatory subunits of PI3’-kinase, NRAS, RAF1, SOS1, CRKL, and CTNNB1 (Fig. 2). Although these pathways are regulated predominantly by posttranslational mechanisms not detectable by microarray analysis of mRNAs, it is likely that elevated pathway activation may result in changes in mRNAs encoding components of the pathway. Certainly, given the potential cooperation between RAF and PI3’-kinase signaling in cancer cells, it may be that a combination of inhibitors of components of these pathways may display synergistic antitumor activity against PDA. Indeed, it has been shown that PI3’-kinase activation can confer resistance to MEK inhibition in KRAS-mutant cancers (41). Moreover, KRASG12D-induced lung cancers seem to respond best to a combination of MEK1/2 plus combined PI3’-kinase/mTORc1 inhibition (42).

Although most PDA-derived cell lines tested were sensitive to the effects of MEK1/2 inhibition on BrdUrd incorporation, we were interested as to a possible general mediator of ERK1/2 signaling that might mediate effects on cellular proliferation. Statistical analysis of CI-1040 effects allowed us to define mRNAs regulated by RAF→MEK→ERK...
signaling in more versus less sensitive cell lines. Among those mRNAs were those encoding transcription factors such as FRA-1, PEA3, and ERM. In addition, the c-MYC transcription factor was also differentially expressed among our panel of 22 cell lines, although its mRNA level displayed no correlation with MEK inhibitor sensitivity. However, detailed time course experiments showed that both c-MYC mRNA and protein expression were decreased as soon as 6 hours after drug addition in at least 6 different cell lines. Given the important role of c-MYC in cancer cell proliferation, it represented a possible mediator of the effects of MEK1/2 inhibition on PDA cell proliferation. Interestingly, RNAi-mediated inhibition of c-MYC expression had potent inhibitory effects on the proliferation of PDA cell lines. Although pharmacologic MEK1/2 inhibition had more striking effects on PDA cell proliferation, indicating that additional targets of the pathway play a role in the behavior of PDA cell lines, the data suggest that suppression of c-MYC makes a substantial contribution to the antiproliferative effects of MEK1/2 inhibition on PDA cell proliferation.

It has been previously suggested that cultured cancer cells may behave differently from cells growing in an animal host in a more appropriate tissue microenvironment. However, analysis of orthotopic pancreatic tumors revealed that mRNA expression profiles were similar to those of the same cells in tissue culture. Furthermore, treatment of mice carrying orthotopic L3.6pl tumors led to a striking inhibition of tumor growth consistent with inhibitory effects on BrdUrd incorporation or phospho-Histone H3, markers of S and M phase, respectively. Analysis of mRNA profiles in PD325901-treated tumors revealed that many of the same mRNAs were regulated in vivo as were regulated in vitro, especially cell-cycle regulators and E2F target genes such as CCNA2 or CDC45L. However, the overall fold-change in mRNA expression was often larger in orthotopic tumors. These data suggest that, at least for these PDA cell lines, patterns of mRNA expression are not subject to substantial alterations when cells are transferred from tissue culture into the pancreas of immunocompromised mice.

Data presented here indicates the sensitivity of many PDA cell lines to the antiproliferative effects of pharmacologic blockade of RAF→MEK→ERK signaling. Furthermore, they underscore the complexity of the transcriptional program regulated by this pathway. Finally, because oncogenic BRAFV600E is sufficient for pancreatic cancer initiation (in mouse models) and RAF→MEK→ERK signaling is required for sustained proliferation of bona fide human pancreatic cancer cells, targeted blockade of this pathway either alone or in combination with other pathway-targeted therapeutics, may represent a tractable approach for the treatment of patients with this disease (11, 43).

Disclosure of Potential Conflicts of Interest

M. McMahon has received a commercial research grant from Novartis.

Authors’ Contributions

Conception and design: S. Gysin, M. McMahon
Development of methodology: S. Gysin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Gysin, J. Paquette, M. McMahon
Writing, review, and/or revision of the manuscript: S. Gysin, J. Paquette, M. McMahon
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Gysin
Study supervision: M. McMahon

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References

Analysis of RAF-MEK-ERK Signaling in Pancreas Cancer


Correction: Analysis of mRNA Profiles after MEK1/2 Inhibition in Human Pancreatic Cancer Cell Lines Reveals Pathways Involved in Drug Sensitivity

In this article (Mol Cancer Res 2012;10:1607–19), which appeared in the December 2012 issue of Molecular Cancer Research (1), the GEO accession number was not provided by the authors.

The accession number is #GSE45765. The authors regret this omission.

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