Constitutive \( K-Ras^{G12D} \) Activation of ERK2 Specifically Regulates 3D Invasion of Human Pancreatic Cancer Cells via MMP-1

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Abstract

Pancreatic ductal adenocarcinomas (PDAC) are highly invasive and metastatic neoplasms commonly unresponsive to current drug therapy. Overwhelmingly, PDAC harbors early constitutive, oncogenic mutations in \( K-Ras^{G12D} \) that exist prior to invasion. Histologic and genetic analyses of human PDAC biopsies also exhibit increased expression of extracellular signal-regulated kinase (ERK) 1/2 and proinvasive matrix metalloproteinases (MMP), indicators of poor prognosis. However, the distinct molecular mechanisms necessary for \( K-Ras \)/ERK1/2 signaling and its influence on MMP-directed stromal invasion in primary human pancreatic ductal epithelial cells (PDEC) have yet to be elucidated in three-dimensions. Expression of oncogenic \( K-Ras^{G12D} \) alone in genetically defined PDECs reveals increased invadopodia and epithelial-to-mesenchymal transition markers, but only when cultured in a three-dimensional model incorporating a basement membrane analog. Activation of ERK2, but not ERK1, also occurs only in \( K-Ras^{G12D} \)-mutated PDECs cultured in three-dimensions and is a necessary intracellular signaling event for invasion based upon pharmacologic and short hairpin RNA (shRNA) inhibition. Increased active invasion of \( K-Ras^{G12D} \) PDECs through the basement membrane model is associated with a specific microarray gene expression signature and induction of MMP endopeptidases. Specifically, MMP-1 RNA, its secreted protein, and its proteolytic cleavage activity are amplified in \( K-Ras^{G12D} \) PDECs when assayed by real-time quantitative PCR, ELISA, and fluorescence resonance energy transfer (FRET). Importantly, shRNA silencing of MMP-1 mimics ERK2 inhibition and disrupts active, vertical PDEC invasion. ERK2 isoform and MMP-1 targeting are shown to be viable strategies to attenuate invasion of \( K-Ras^{G12D} \)-mutated human pancreatic cancer cells in a three-dimensional tumor microenvironment. Mol Cancer Res; 10(2); 183–96. © 2011 AACR.

Introduction

Pancreatic cancer is fatal in 95% of patients within 6 months of diagnosis. This dismal prognosis is attributed to early tumor invasion, impalpable metastatic progression, and a lack of response to current therapeutic modalities (1, 2). Pancreatic ductal adenocarcinoma (PDAC) accounts for the vast majority of exocrine pancreatic tumors, and of these, 90% harbor early mutations in the \( K-Ras \) oncogene (3). The common single-nucleotide \( K-Ras \) mutation of G(G)X to G (A)X (glycine, G, to aspartate, D, respectively) at codon 12 (G12D) causes the membrane-associated GTPase \( K-Ras \) to remain locked in a constitutively GTP-bound state. In most epithelial cells, the activated \( K-Ras^{G12D} \) mutation constitutively signals through the mitogen-activated protein kinase (MAPK) cascade, increasing transcription of downstream genes. Many of these upregulated genes and their protein products enhance neoplastic growth, cytoskeletal arrangement, and metastasis (4, 5). Considerable effort has been made to pharmacologically inhibit upstream effectors of this active \( K-Ras \) pathway, such as Raf and MAP/ERK kinase (MEK), as well as Ras itself (6, 7). Unfortunately, the complex signaling networks activated by mutant \( K-Ras \) differ between epithelial cell types. Thus, upstream effector targeting has limited downstream specificity resulting in toxicity and/or ineffectiveness (8, 9). Considering that constitutive \( K-Ras^{G12D} \) is the most frequent mutation in pancreatic cancer and that invasion and metastasis of PDAC is early, insidious, and fatal, clinical advances will need clarification of the role of \( K-Ras \) in influencing invasion in a human model.

Genetically engineered mouse models of PDAC provide suitable systems capable of recapitulating the full progression of the human disease (10). These powerful models have furthered the understanding of \( K-Ras \)-induced oncogenic...
effects but harbor deficits common to human translatability (10, 11). Thus, groups have generated genetically engineered primary human pancreatic ductal epithelial cells (PDEC) through a series of defined, stepwise genetic alterations which cause transformation and result in anchorage-independent growth, augmented motility, hyperproliferation, and xenograftable tumors (12, 13). These cell progression series incorporate human telomerase reverse transcriptase (hTERT), inactivation of Rb and p53 by the human papillomavirus E6 and E7 proteins, mutant K-Ras<sup>G12D</sup>, and either SV40 small t or large T antigen. Although these in vitro human PDEC systems are capable of completely delineating the K-Ras–controlled phosphoinositide 3-kinase (PI3K) and Ral pathways, they have been unable to unequivocally show nonstimulated, constitutive K-Ras–induced MAPK/ERK1/2 activation and transcriptional regulation in 2-dimensions (13, 14). A need remains to determine the proteins, genes, and gene products under the influence of the mutated K-Ras<sup>G12D</sup>/MAPK/ERK1/2 pathway in more physiologically relevant 3-dimensional (3D) basement membrane systems (15–17). The basement membrane and extracellular matrix (ECM) along with their inherent 3-dimensionality greatly influence cell–cell contact, cell–matrix connections, cellular organization, intracellular signaling cascades, and gene expression in many ductal epithelial cells (15–20). Indeed, a highly inducible K-Ras/MAPK/ERK1/2–regulated gene expression pathway may also exist in pancreatic ductal cells and influence invasion when cultured in a 3D model (5).

Matrix metalloproteinases (MMP) are a family of activated endopeptidases capable of enzymatically degrading ECM proteins that compose the tumor microenvironment. MMP signaling and remodeling of the local environment allow inherently motile transformed cells the ability to permissively invade into the surrounding tissue, eventually leading to metastasis (1, 19–24). Human pancreatic cancer is consistently associated with a desmoplastic reaction incorporating increased levels of stromal and basement membrane ECM proteins (laminin, collagen IV), all of which are capable of degradation by MMPs (15, 22–24). Specifically, the secreted collagenase, MMP-1, is found upregulated in preeoplastic pancreas and constitutively expressed within in vitro pancreatic cell lines (24, 25). Catalytically active MMP-1 is capable of assisting in cell invasion either by cleavage of a G-protein–coupled receptor, PAR-1 (protease-activated receptor-1), resulting in Rho cytoskeletal changes or by a mesenchymal type of invasion via connective tissue collagen and basement membrane degradation at a leading invadopodial edge, opening routes for metastasis through the ECM (21, 22). Although signaling pathways controlling MMPs and their relative invasive importance in pancreatic cancer have been hypothesized, the exact molecular methods and genes necessary for K-Ras<sup>G12D</sup>–driven ECM invasion in human pancreatic cancer cells remain to be understood (26).

To identify the role of the K-Ras<sup>G12D</sup> mutation in the morphology and invasion of pancreatic cancer, we characterized the mutation’s morphologic and invasive effects after addition into PDECs cultured within a 3D basement membrane model. Compared with untransformed PDECs, the K-Ras<sup>G12D</sup>–mutated cells exhibit increased invadopodia, epithelial-to-mesenchymal markers, and active, vertical basement membrane invasion in the synthetic stroma. To understand the mechanism of this invasiveness, we show that only mutated K-Ras<sup>G12D</sup> PDECs signal specifically through extracellular signal-regulated kinase (ERK) 2, not ERK1, in 3D. The K-Ras<sup>G12D</sup>/ERK2 signaling axis regulates invadopodia and active basement membrane invasion as well as a unique microarray gene expression signature distinct from nonmutated PDECs. Specifically, the constitutive activation of ERK2 by K-Ras<sup>G12D</sup> is necessary for the upregulation of MMP-1 RNA, its secreted protein, and its proteolytic activity. Relevant to attenuating the early invasion and metastasis of PDACs, pharmaceutical inhibition or RNA silencing of either ERK2 or MMP-1 disrupts the vertical invasion of pancreatic cells into the 3D basement membrane.

Materials and Methods

Cell lines

Immortalized primary human PDECs were a generous gift from Dr. Michel Ouellette (University of Nebraska, Lincoln, NE). All populations were maintained in conventional 2D cultures in T-75 flasks and a humidified incubator (95% air/5% CO<sub>2</sub>) at 37°C. All PDECs were cultured in a pancreatic-specific growth medium: 4 parts low-glucose Dulbecco’s Modified Eagles’ Media (DMEM; Cellgro) to one part M3 base culture medium (INCELL) supplemented with 5% FBS + 1% penicillin–streptomycin. Cells were passaged every 48 hours by 0.05% trypsin detachment over 5 minutes.

Microarray analysis

One microgram of isolated RNA was reverse transcribed to cDNA using the Qiagen First Strand Kit reaction assay on an Eppendorf Mastercycler Pro S (Hauppauge). The cDNA was aliquoted with Qiagen Mastermix and added to the wells of a SABiosciences Extracellular Matrix Microarray Plate (PAHS-0013, SABiosciences) at 25 μL per well. The cDNA plate was analyzed on an aluminum block Eppendorf ep Realplex II calibrated with an SABiosciences ‘A’ Plate using a 520-nm filter and a SYBR Green probe. The program contained a heat activation cycle followed by 40 cycles of PCR at a 35% ramp rate followed by one cycle of melting curve analysis to verify purity. Bioinformatic analysis by the Web-based RT<sup>2</sup> Profiler PCR Array Data Analyzer (SABiosciences) was conducted after logarithmic transformation of C<sub>T</sub> amplification values against internal controls, whereas the threshold was consistently set. Data are expressed as ΔΔC<sub>T</sub> as well as fold changes with ±2-fold considered significant.

MMP activity assay

PDEC medium was collected after 24 hours of addition of U0126, PD98059, LY294002, AEMT, or short hairpin RNA (shRNA; ERK1, ERK2, MMP-1) lentiviral particles
after 24 hours in 3D culture. The activity of MMP-1 was assayed using a fluorescence resonance energy transfer (FRET)-based assay (71150; AnaSpec) according to the manufacturer’s instructions: Briefly, a 5-carboxyfluorescein (5-FAM) fluorescent molecule bound to a QXL 520 quencher by a cross-link capable of enzymatic cleavage specifically by MMP-1 was added to 25 μL of the collected PDEC medium. Upon separation from the quencher explicitly by MMP-1 enzymatic cleavage, the 5-FAM molecule fluoresces at 520 nm and the intensity correlates with MMP-1 activity. Pro-MMP-1 was activated immediately before the FRET MMP activity assay by incubating the collected PDEC medium with 5 μL of 2-amino-3-hydroxy-5-methyl-4-isoaxazolepropionic acid (AMPA) and kept on ice. Working solutions were prepared incorporating the MMP-1 substrate solution (a 5-FAM/QXL 520 peptide) as well as the assay buffer. The enzymatic reaction was set up with 2 controls: (i) a substrate control containing assay buffer and (ii) a positive control containing MMP-1 diluent.

### Statistical analysis

All experiments were run in triplicate and repeated as stated. Where possible, the data are expressed as the average ± SEM. Significance was based upon P < 0.05 and 0.01 and clarified in each figure whereas error bars represent SEM. Significance between 2 data sets was determined using a two-tailed Student t test whereas differences between multiple experimental groups were determined by ANOVA with a Bonferroni post hoc test. Microarray data sets were initially filtered for genes with more than a 2-fold change in expression. Additional materials and methods are presented in the Supplementary Material.

### Results

#### PDECs harboring a K-RasG12D mutation exhibit invasive morphology in 3D

Studies in various epithelial cells have shown that morphologic behavior depends on the dimensionality and signals provided by the surrounding ECM; thus a 3D model may more naturally mimic the endogenous milieu (15–17). In line with previous studies, all PDECs of the hTERT, hTERT plus E6 and E7 (E6/E7), and hTERT/E6/E7 plus mutated K-RasG12D (E6/E7/Rat) lineage exhibit similar fibroblast-like morphology in 2D, plastic culture, despite progressive inhibition of p53 and p65 or a constitutively active K-RasG12D mutation (12, 13). Fluorescence microscopy shows similar F-actin cytoskeletal arrangements in all cell types maintained in 2D culture (Fig. 1A and Supplementary Fig. S1).

Interestingly, only culture in a 3D ECM basement membrane (Matrigel) recapitulating pancreatic ductal architecture and elasticity (380 ± 63 Pa) reveals significant morphologic differences between the 3 PDEC clones (Fig. 1B and C). The hTERT and E6/E7 PDECs formed rounded, pseudo-organized multicellular aggregates, exhibiting a perimembranal web of F-actin. Moreover, the basolateral membrane of the hTERT or E6/7 PDEC clusters stained positive for integrin-α6, a laminin receptor (Fig. 1D). In contrast, E6/E7/Rat PDECs cultured in 3D displayed invasive morphology (stellate, invadopodia) characterized by similar cytoplasmic extensions found in metastatic epithelial cells (27–29). Individual invadopodia were composed of a single migrating cell extension (Fig. 1B and C, single arrow) or multicellular invadopodial aggregates extending from a central proliferative mass (Fig. 1B and C, double arrow and Supplementary Movie S1A). Phalloidin staining of the E6/E7/Rat PDECs exhibited an elongated F-actin distribution reminiscent of a mesenchymal phenotype. Furthermore, while E6/E7 PDECs lacked nucleating F-actin markers of invadopodia, E6/E7/Rat PDECs were highly positive for the invadopodial marker cortactin (Fig. 1B and C). Magnification of individual invadopodia exhibit F-actin staining along the entire extension whereas parallel cortactin is found under the cell membrane as well as within developing invadopodial “buds” (Fig. 1C, see inset). Interestingly, these cells lacked elaborate stress fibers and the surrounding basolateral membranes lacked integrin-α6 labeling (Fig. 1B and D). In addition to the presence of actin-rich invadopodial protrusions, vimentin, a mesenchymal intermediate filament necessary for invadopodial elongation and marker for a migratory epithelial cell phenotype was prominently upregulated in E6/E7/Rat PDECs over that of E6/E7 PDECs (Fig. 1D and E; refs. 27–29). Taken together, these results show that a 3D ECM model composed of a basement membrane mimic is capable of revealing invasive morphologic and phenotypic differences between normal (E6/E7) and tumorigenic (E6/E7/Rat) pancreatic cancer cells.

#### Invasive phenotype of K-RasG12D PDECs is regulated by ERK2

We next attempted to identify downstream effector proteins involved in PDEC invasiveness and mediated by the K-RasG12D mutation. Neither the E6/E7 nor the E6/E7/Rat PDECs constitutively phosphorylated ERK1 or ERK2 proteins in 2D culture, despite the E6/E7/Rat PDECs possessing the constitutively active K-RasG12D mutation (Supplementary Fig. S2). Addition of epidermal growth factor (EGF) transiently induced ERK1/2 phosphorylation, specifically only ERK2 in the E6/E7/Rat PDECs, which peaked after 30 minutes in 2D culture and returned to baseline levels within 1 hour (Supplementary Fig. S3).

As only the E6/E7/Rat PDECs exhibited invasive morphology in 3D, we hypothesized that dimensionality might facilitate constitutive activation of downstream targets of mutated K-RasG12D. In contrast to 2D culture, ERK1 was phosphorylated in both cell types for at least 24 hours in 3D culture even in the absence of EGF. Interestingly,
constitutive phosphorylation of ERK2 occurred only in E6/E7/Ras PDECs when cultured in 3D (Fig. 2A). A specific inhibitor of ERK’s upstream activator MEK (MAP2K), UO126, inhibited this constitutive ERK2 phosphorylation in 3D culture (Supplementary Fig. S4). As seen in Fig. 2B, ELK-1 (ets like gene-1) and RSK-1 (ribosomal S6 kinase-1), 2 downstream ERK substrates previously implicated in invasive gene transcription, were phosphorylated in E6/E7/Ras PDECs cultured in 3D (30–32). Phosphorylation of these 2 targets, but not of ERK2, was inhibited by 3-(2-aminoethyl)-5-((4-ethoxyphenyl) methylene)-2, 4-thiazolidinedione hydrochloride (AEMT), a small-molecule drug which preferentially antagonizes the ability of ERK2 to phosphorylate these effectors (33). Importantly, E6/E7/Ras phosphorylation of RSK-1 could be reduced by 2.5-fold to the low-level, basal RSK-1 phosphorylation of E6/E7 PDECs by AEMT addition (Supplementary Fig. S5). Taken together, these experiments propose that 3D alone enables mutant K-RasG12D to specifically activate ERK2, not ERK1, signaling in PDECs.

To assess whether other activated K-Ras pathways are involved in 3D PDEC invasiveness, we examined phosphorylation of the PI3K-specific protein, Akt (T308), a known regulator of cell migration in PDECs (13). There was little
difference in total or phosphorylated protein between E6/E7 and K-RasG12D-mutated PDECs (Fig. 2C). The addition of EGF marginally upregulated E6/E7/Ras PDEC Akt phosphorylation but was not significant, implying that K-RasG12D has greater influence on MEK/ERK2 signaling.

Morphologic characteristics were examined after treating the 3D PDECs with a panel of small-molecule inhibitors that are known to attenuate downstream Ras signaling pathways associated with transcription, motility, and cytoskeletal rearrangement (4). Strikingly, MEK inhibition with UO126 and PD98059 or ERK2-specific inhibition with AEMT drastically attenuated (in a dose- and time-dependent manner) invadopodia and proliferation of the E6/E7/RasPDECs in 3D (Fig. 3A and B). The PI3K inhibitor LY294002 added at a concentration of 10 μg/mL and GAPDH was used as an internal normalization control for densitometric quantification. There was no significant difference in the abundance of phosphorylated Akt before or after addition of EGF in either the E6/E7 or E6/E7/Ras PDECs. See also Supplementary Figs. S2–S4. All experiments: SEM and *, P < 0.05. DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ERK2 is necessary for an invasive PDEC phenotype

Recent publications implicate ERK2, not ERK1, as a potent inducer of epithelial-to-mesenchymal transition (EMT) and suggest that its specific, active phosphorylation affects morphology, survival, and proliferation (30–35). To
validate the necessity of ERK2 in the invasive phenotype of K-Ras\(^{G12D}\)-mutated PDECs in 3D, we separately silenced endogenous ERK1 and ERK2 with 2 different shRNA lentiviral constructs (Fig. 4A). Silencing yielded at least a 70% decrease in both ERK isoforms in the E6/E7/Ras\(^{PDECs}\) by either lentiviral construct. In agreement with the pharmacologic inhibition studies, specific silencing of ERK2, but not ERK1, caused a significant reduction in the number of invadopodia that developed after drug application was counted and normalized to E6/E7/Ras plus DMSO at 48 hours. Each count is the combination of 5 areas of 3 independent experiments. Vertical bars represent SEM. B, E6/E7/Ras PDECs were cultured in 3D with or without the ERK2-specific inhibitor AEMT and imaged at 0 and 48 hours by phase-contrast microscopy. Left scale bars, 50 \(\mu m\); right scale bars, 100 \(\mu m\); inset = 10 \(\mu m\). The number of invadopodia that developed after drug application was counted and normalized to E6/E7/Ras plus DMSO at 48 hours. Each count is the combination of 5 areas of 3 independent experiments. Vertical bars represent SEM. C, E6/E7/Ras PDECs were cultured upon growth factor–reduced (GFR) Matrigel Transwell invasion chambers for 48 hours with DMSO (control), 20 \(\mu\)mol/L AEMT, 10 \(\mu\)mol/L UO126, 15 \(\mu\)mol/L PD98059, or 20 \(\mu\)mol/L LY294002. After, the bottom of each well’s membrane was fluorescently observed for the nuclei of invading cells. The number of invading E6/E7/Ras PDECs with DMSO addition was used as a normalization control for quantification. D, MiaPaCa-2 cells, another human pancreatic cancer cell line harboring the K-Ras mutation, had 20 \(\mu\)mol/L of the ERK2-specific inhibitor, AEMT, applied for 48 hours on GFR Matrigel Transwell invasion chambers and similarly analyzed for invasion. The number of invading MiaPaCa-2 cells with DMSO addition was used as a normalization control for quantification. See also Supplementary Fig. S6. All experiments: SEM and *; \(P < 0.05\). DMSO, dimethyl sulfoxide.

MMP-1 RNA expression is regulated by oncogenic K-Ras\(^{G12D}/MAPK/ERK2\) signaling

To identify genes stimulated by the activated K-Ras/MAPK/ERK2 signaling pathway, differential gene expression profiles of 3D-cultured E6/E7 and E6/E7/Ras PDECs were generated by ECM-focused cDNA microarrays (Fig. 5A). While many genes were altered between the K-Ras\(^{G12D}\)-transformed and untransformed E6/E7 PDECs, a specific subgroup of these genes was regulated exclusively by the MAPK pathway, as determined by microarray analysis after MAPK inhibition (MAPKi). Those genes which originally exhibited upregulation by K-Ras\(^{G12D}\) and then were subsequently downregulated following MAPKi were associated with a specific gene signature (Fig. 5B, left and right clustergram). The PDEC RNA microarray changes following MAPK pathway inhibition were validated by qRT-PCR

Figure 3. ERK2 regulates K-Ras\(^{G12D}\)-mutated PDEC invasiveness. A, E6/E7/Ras PDECs were cultured in 3D for 24 hours with the addition of DMSO (control), 20 \(\mu\)mol/L of the PI3K pathway inhibitor (LY294002), 10 or 15 \(\mu\)mol/L of MEK inhibitors (UO126 or PD98059, respectively), or 20 \(\mu\)mol/L of the ERK2-specific inhibitor (AEMT). After applying the drugs for 24 hours, each sample was observed by phase-contrast microscopy. Scale bars, 20 \(\mu m\). The number of invadopodia that developed after drug application was counted and normalized to E6/E7/Ras plus DMSO at 48 hours. Each count is the combination of 5 areas of 3 independent experiments. Vertical bars represent SEM. B, E6/E7/Ras PDECs were cultured in 3D with or without the ERK2-specific inhibitor AEMT or MEK inhibitor UO126 and imaged at 0 and 48 hours by phase-contrast microscopy. Left scale bars, 50 \(\mu m\); right scale bars, 100 \(\mu m\); inset = 10 \(\mu m\). The number of invadopodia that developed after drug application was counted and normalized to E6/E7/Ras plus DMSO at 48 hours. Each count is the combination of 5 areas of 3 independent experiments. Vertical bars represent SEM. C, E6/E7/Ras PDECs were cultured upon growth factor–reduced (GFR) Matrigel Transwell invasion chambers for 48 hours with DMSO (control), 20 \(\mu\)mol/L AEMT, 10 \(\mu\)mol/L UO126, 15 \(\mu\)mol/L PD98059, or 20 \(\mu\)mol/L LY294002. After, the bottom of each well’s membrane was fluorescently observed for the nuclei of invading cells. The number of invading E6/E7/Ras PDECs with DMSO addition was used as a normalization control for quantification. D, MiaPaCa-2 cells, another human pancreatic cancer cell line harboring the K-Ras mutation, had 20 \(\mu\)mol/L of the ERK2-specific inhibitor, AEMT, applied for 48 hours on GFR Matrigel Transwell invasion chambers and similarly analyzed for invasion. The number of invading MiaPaCa-2 cells with DMSO addition was used as a normalization control for quantification. See also Supplementary Fig. S6. All experiments: SEM and *; \(P < 0.05\). DMSO, dimethyl sulfoxide.
(Fig. 5C). Many of the MAPK pathway–regulated genes were of the MMP family: MMP-1, MMP-3, MMP-10, and tissue inhibitor of MMP-1 (TIMP-1). Unbiased hierarchical analysis–clustered E6/E7/Ras plus MAPKi with nonmutated E6/E7 PDECs and MMP-1, MMP-3, and MMP-10 within a closely linked node. Oncomine data mining of K-Ras–mutated human pancreatic adenocarcinoma biopsy samples indicate similar upregulation of these MMPs after meta-analysis and Z-score calculation (Supplementary Fig. S7). Combined with previous data, these results support the notion that the K-Ras/MAPK/ERK2 pathway has a specific ECM invasion gene expression signature and upregulates specific MMP RNA in 3D.

MMP-1 protein and invasive activity are specifically regulated by the K-Ras/ERK2 pathway

To ascertain which of the transcriptionally upregulated MMPs contribute to the invasiveness of E6/E7/Ras PDECs in 3D, the effects of mutated K-RasG12D on MMP-1, MMP-3, and MMP-10 zymogen release were studied. Incorporating ELISA, medium from E6/E7 and E6/E7/Ras PDECs was analyzed for the concentration of specific MMPs after 48 hours in 3D culture. Although RNA for each of the 3 MMPs

Figure 4. ERK2, not ERK1, silencing specifically decreases invasion of E6/E7/Ras PDECs in 3D. A, two distinct shRNA expression constructs (A or B) for either ERK1 or ERK2 were introduced by lentiviral infection into E6/E7/Ras PDECs over 24 hours. Scramble shRNA was introduced as a negative control whereas a GFP construct was added as a positive transfection control. At 24 and 48 hours postinfection, RIPA lysis of all cells was conducted followed by protein isolation and Western blot analysis. Lysates were probed for both total ERK1 and total ERK2 and quantified for protein abundance. GAPDH was used as an internal normalization control. At least, a 70% reduction in expression of ERK1 or ERK2 was observed in both the A and B silencing constructs. **, P < 0.01 for ERK1 and ERK2 and vertical bars represent SEM. B, subpopulations of each E6/E7/Ras PDEC after silencing with sh-ERK1 or sh-ERK2 construct B were cultured in 3D for 48 hours. Representative images of the populations after 2 independent experiments were observed by phase-contrast microscopy. Scale bars, 20 μm. A minimum of 15 cell aggregates in 5 different images were counted and averaged for each condition after 3 independent experiments. C, E6/E7/Ras PDECs were infected with lentiviral particles harboring scramble shRNA, sh-ERK1 B, or sh-ERK2 B for 24 hours. PDECs were cultured inside Transwell invasion plates covered in growth factor–reduced (GFR) Matrigel for 48 hours and nuclei were labeled with DAPI. Five individual areas for each shRNA construct of 3 separate experiments were counted for cells that invaded through the Matrigel to the opposite side of the membrane and compared with control. DMSO-treated E6/E7/Ras PDECs were used as the normalized control of invasion. All experiments: SEM and *, P < 0.05 unless noted. DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.
had increased (Fig. 5) and all are extracellularly secreted, only MMP-1 protein was significantly detectable by ELISA (Fig. 6A). The abundance of MMP-1 protein was significantly higher (3-fold) in the E6/E7/Ras cells than in the nonmutated PDECs (Fig. 6B and Supplementary Fig. S8). Addition of EGF lead to ERK2 phosphorylation and increased MMP-1 secretion in E6/E7/Ras but not in nonmutated E6/E7 PDECs, mimicking both 2D plus EGF and
3D-only culture conditions (Fig. 6B and Supplementary Fig. S3). Conversely, inhibition of MEK or ERK2 signaling with U0126 or AEMT, respectively, significantly decreased MMP-1 secretion in K-Ras<sup>G12D</sup>–mutated PDECs (Fig. 6B). Finally, shRNA silencing of ERK2, but not ERK1, also significantly decreased MMP-1 zymogen secretion from 3D-cultured PDECs (Fig. 6C).

Because ELISA quantifies total secreted proteases (inactive and active) and considering that only activated MMPs are capable of proteolytic PDAC ECM degradation, zymography and FRET were used to quantify the enzymatic activity rate of MMP-1 secreted from 3D PDECs (22). In line with MMP-1 ELISA results, kinetic analysis verified that MMP-1 proteolysis was augmented in E6/E7/Ras medium compared with E6/E7 PDECs and was readily reversed following MEK or ERK2 inhibition (Fig. 6D). E6/E7/Ras PDECs exhibited an average MMP-1 enzymatic activity rate of 33.4 ± 0.25 arbitrary units per minute (AU/min), whereas the E6/E7 PDEC activity rate was 5-fold lower (6.0 ± 0.3 AU/min). Treatment of 3D E6/E7/Ras PDECs with the MEK- or ERK2-specific inhibitors (U0126 or AEMT) significantly lowered their protease activity rate to that of E6/E7 PDECs over 1 hour, whereas silencing of MMP-1 showed a proteolytic rate that was inherently indistinguishable from the E6/E7 PDECs (Fig. 6D). Thus, the K-Ras<sup>G12D</sup> mutation increases MMP-1 transcription, protein secretion, enzymatic activity, and invasion of 3D-cultured PDECs by a MEK/ERK2 signaling axis.

### Discussion

The following 3 main messages emerge from this study: (i) 3D culture of human PDECs reveals a distinct invasive phenotype regulated by mutated K-Ras<sup>G12D</sup> alone, which is not detected in conventional 2D culture. (ii) Invasion is specifically mediated by an active ERK2 signaling pathway and is detected only in 3D and only in K-Ras–mutated pancreatic cancer cells. (iii) Constitutive activation of MAPK/ERK2 increases transcription, protein abundance, and activity of MMP-1 which is necessary for pancreatic cancer cell invasion in 3D. Taken together, this set of data is important as the significance of the K-Ras/MAPK/ERK2/MMP pathway in mutated K-Ras<sup>G12D</sup> pancreatic cancer cells has not been previously shown. Prior 2D studies found that constitutively active K-Ras phosphorylated MEK but not

**Figure 5.** (Continued) C, RNA from E6/E7/Ras PDECs was isolated after 48 hours of culture in 3D conditions, purified, and mixed with MMP-1, MMP-3, or MMP-10 TaqMan gene expression probes. qRT-PCR was used to validate the microarray results by also determining the increased expression of these RNA transcripts in E6/E7/Ras PDECs. GAPDH was used as an internal housekeeping control and all C<sub>t</sub> values were normalized to its expression. Analysis of significant gene expression increases was determined by the Pfaffl method and ANOVA with a Bonferroni post hoc test. All experiments: SEM and *P < 0.05.

**Table 1.** MMP-1 is necessary for K-Ras<sup>G12D</sup>–mutated PDEC invasion

To determine the K-Ras–regulated MMP-1 influence on basement membrane invasion, E6/E7/Ras PDEC MMP-1 RNA was silenced by shRNA lentivirus. As seen in Fig. 7A, the E6/E7/Ras PDECs with the addition of 5 pooled sh-MMP-1 constructs exhibited increased horizontal spreading across the ECM when compared with control over 30 minutes. Fluorescent phalloidin imaging shows multicellular extensions along the length of trabecular structures terminating in laminin V rich areas, common to migratory pancreatic cells (36). qRT-PCR analysis established that MMP-1 was approximately 80% silenced compared with control (Fig. 7B). Importantly, MMP-1 silencing in E6/E7/Ras PDECs by shRNA reduced its protease activity to that of E6/E7 PDECs over 1 hour (Fig. 6D). Furthermore, basement membrane invasion assays determined that the sh-MMP-1 E6/E7/Ras PDECs had an approximately 80% reduction in vertical invasion through the Matrigel basement membrane, as would be expected with its decreased MMP-1 expression and protease activity (Fig. 7C). In agreement, live, individual cell motion tracking of E6/E7/Ras PDECs with and without sh-MMP-1 lentiviral particle infection determined a significant increase in the total horizontal distance traveled by E6/E7/Ras PDECs when MMP-1 was silenced (Fig. 7D and E and Supplementary Movies S2A and S3A). Therefore, specific inhibition of MMP-1 impedes active vertical invasion into the ECM and subsequently facilitates the horizontal spreading of inherently migratory K-Ras<sup>G12D</sup> PDECs upon the basement membrane.

Overall, these data indicate that the specific addition of a K-Ras<sup>G12D</sup> mutation to human PDECs increases invasive characteristics via activation of the MAPK/ERK2 pathway. In turn, the constitutive activation of the ERK2-specific signaling axis regulates a unique gene expression signature in PDECs and increases MMP RNA transcripts. Distinct increases in MMP-1 gene expression correlates with amplified secretion of enzymatically active MMP-1 protein which is necessary for invasion into the basement membrane by human pancreatic cancer cells.
ERK unless growth factors were added to pancreatic cells (13, 14, 37). This study underscores the idea that proper assessment of signaling pathways, specifically through ERK2, in cultured cells requires emulation of the appropriate 3D physiologic environment of the pancreas, namely, the addition of basement membrane components (5, 38, 39).

E6/E7/Ras PDECs harboring the constitutively active K-RasG12D mutation exhibit invadopodia and increased cell numbers per aggregate similar to transformed prostate and mammary epithelial cells in 3D (Fig. 1 and Supplementary Movie S1A; refs. 39–41). Importantly, preliminary data suggest that this morphologic differentiation is only possible in Matrigel and not in collagen type I. Insertion of a K-RasG12D mutation alone in hTERT PDECs results in senescence (13). Therefore, immortalized PDECs require p53 ubiquitin-mediated degradation and p16INK4a/Rb inactivation (by E6 and E7 genes, respectively) in addition to the K-RasG12D mutation for this transformation. Nonmutated
E6/E7 PDECs exhibit a well-developed system of stress fibers and peripheral integrin-α6 expression in 3D, whereas the K-Ras/ERK2 mutation causes aggregate disorganization and decreased expression of this laminin-binding integrin, common characteristics of invasive pancreatic cells (42).

Correlatively, the invadopodia-specific markers cortactin and vimentin exhibited increased fluorescent expression in E6/E7/Ras PDECs, underlining true invadopodial development. In addition to increased cortactin and vimentin labeling, intercellular vimentin protein also increased in the
E6/E7/Ras PDECs, all of which are seen in EMT and pancreatic cell invasion (27–29, 41, 43). Evaluation of the ECM microarrays of the different PDEC clones indicates upregulation of several other EMT-associated genes besides those in E6/E7/Ras PDECs (Supplementary Fig. S9). CD44, a hyaluronan receptor associated with cancer stem cells and malignancy, was upregulated along with the EMT markers fibronectin, integrin-β2, tenascin-c, and vitronectin (43, 44). As addition of a K-Ras G12D mutation is the sole genetic distinction between E6/E7 and E6/E7/Ras PDECs and mutated cells have significant 3D invasive characteristics, we conclude that constitutively active K-Ras is the key factor regulating these invasion-inducing downstream genes.

Ras regulates gene expression pathways controlling motility, invasion, and proliferation via downstream effector pathways. Considering the influence of K-Ras on the MAPK pathway, phosphorylated ERK1/2 is upregulated in surgically resected K-Ras–mutated pancreatic cancer specimens (43, 45). In 2D culture, EGF induces ERK1 phosphorylation in PDECs regardless of their K-Ras G12D mutational status. Interestingly, EGF causes phosphorylation of ERK2 only in K-Ras G12D–mutated PDECs (Supplementary Fig. S3). Remarkably, 3D culture (in the absence of exogenous EGF) is the only factor necessary to mimic these results in E6/E7/Ras PDECs (Fig. 2). Specific inhibition of ERK2 by AEMT or shRNA (Figs. 3 and 4 and Supplementary Fig. S5) decreases K-Ras G12D downstream signaling as well as E6/E7/Ras and MiRPaCa-2 invasiveness suggesting that 3D plus the iterative K-Ras G12D mutation is sufficient to induce ERK2 phosphorylation/activation and invasion.

Various Ras mutants (H-Ras G12V, K-Ras G12V, K-Ras G13D) constitutively activate MAPK pathway signaling and subsequently increase genes that mediate epithelial cell invasion (30, 32). Specific regulation of RSK/FRA-1 (fos-related antigen-1) interactions by the D-domain of ERK2 has been shown in kidney cells and breast and colon cancer cells (32). In addition, ERK2 is recognized as an isoform-specific regulator of EMT through DEF-motif–docking sites in breast cancer cells (30). DEF-domain EMT is characterized by invasive morphologic changes, decreased E-cadherin expression, and increased expression of vimentin, N-cadherin, and fibronectin—similarly determined in the pancreatic cancer cells of this study (Fig. 1C and D and Supplementary Fig. S9; refs. 30, 43). Both the ERK2 D-domain and DEF-domain substrates seem significant to K-Ras G12D–mutated PDEC signaling in 3D, as neither ELK-1 (DEFand D-domains) nor RSK-1 (DEF-domain) were significantly phosphorylated in E6/E7 PDECs or following ERK2 inhibition in E6/E7/Ras PDECs by AEMT (an inhibitor of both motifs, Fig. 2B and Supplementary Fig. S5). As ERK1-knockout mice survive past birth whereas ERK2-knockout mice die en utero, it follows that ERK2 may direct unique signaling traits over ERK1 (35).

Specific ERK2 signaling in 3D may derive from focal adhesion stabilization of a unique intercellular scaffold protein. The scaffolding proteins focal adhesion kinase (FAK), KSR (kinase suppressor of Ras), Sef [similar expression to fibroblast growth factor (FGF) genes], and IQGAP1 (IQ motif–containing GTPase-activating protein 1) increase MAPK signaling efficiency by facilitating proper gathering and orientation of effectors (46, 47). A preferential ERK2-binding scaffold is plausible considering the discovery of the preferential MEK1/ERK1 binding scaffold, MEK partner 1 (MP1; ref. 48). Differences seen in F-actin stress fiber formation and cortactin localization within 3D-cultured PDECs support this hypothesis (Fig. 1A; refs. 46–48).

Microarray analysis identified distinct ECM-related genes that are increased in K-Ras G12D PDECs over E6/E7 when cultured in 3D (Fig. 5). When E6/E7/Ras PDECs are then inhibited by MAPKi, we observed decreased expression of genes that are specifically controlled by this pathway: MMP-1, MMP-3, MMP-10, and TIMP-1. Unbiased clustering analysis more similarly separates the E6/E7 and E6/E7/Ras plus MAPKi PDEC gene expression profiles apart from E6/E7/Ras, signifying their similar genetic regulation (Fig. 5B).

Meta-analysis of data mined from human pancreatic adenocarcinoma biopsy samples agrees with the upregulation of these MMPs within K-Ras–mutated tumors, further pinpointing their influence (Supplementary Fig. S7). Interestingly, MMP-1, MMP-3, and MMP-10 all reside on chromosome 11 and have similar promoter elements enriched in activator protein 1 (AP-1) and polypoma-enhancing activator 3 (PEA-3)-binding sites approximately 180 bp upstream from the transcription start site (49). Coincidently, MMP-1 expression is regulated by MAPK-related transcription factors (c-fos and c-jun) at these sites in gastric cancers (50). MMP-7 and MMP-14 (MT-MMP-1) have been implicated in the invasion of pancreatic cancer cells and PDACs (51, 52). Interestingly, the expression of these MMPs is higher in E6/E7 and in E6/E7/Ras plus MAPKi PDECs at 48 hours, implying later upregulation in K-Ras G12D PDECs.

Broad-spectrum MMP inhibitors were clinically withdrawn due to unforeseen complexities in the influence of MMPs on tumor progression and inhibition (53). It is necessary to understand the tissue specificity and the proper balance of MMPs in various pathologies, as many MMPs are effective tumor suppressors and their inhibition can expedite neoplastic development. In this study, increasing levels of MMP-1 RNA correlated to increased secreted MMP-1 protein and activity but only in 3D E6/E7/Ras PDECs and not when MMP-1 RNA was silenced (Fig. 6 and Supplementary Fig. S8). Although MMP-3 and MMP-10 transcripts were also increased in the medium of E6/E7/Ras PDECs, their protein secretion was not measurable by ELISA signifying post-transcriptional attenuation or extracellular neutralization, possibly by TIMPs. Kinetic analysis of MMP-1 protease activity using a FRET assay show that MMP-1 secretion/activity could be reduced to non–K-Ras G12D–mutated levels with pharmacologic and shRNA inhibition of ERK2, but not ERK1. Furthermore, shRNA silencing of MMP-1 in E6/E7/Ras PDECs by 80% reduced its protease activity to that of the E6/E7 PDECs without the K-Ras G12D mutation, thereby underlining this mutation’s influence in increasing the abundance of mRNA, protein, and enzymatic activity of MMP-1 (Figs. 5 and 6). Silencing MMP-1 also disrupted the ability of the cells to vertically

Published OnlineFirst December 8, 2011; DOI: 10.1158/1541-7786.MCR-11-0399
invade into the basement membrane and instead significantly redirected their intrinsic motility across the Matrigel surface horizontally (Fig. 7 and Supplementary Movie S2A and S2B). Thus, the upregulation of MMP-1 by K-Ras$^{G12D}$ as well as its downregulation after ERK2 and shRNA inhibition directly link the mutated K-Ras$^{G12D}$/ERK2 signaling pathway to the MMP-1-mediated invasiveness of PDECs in 3D. MMP-1 expression may be increased because of interactions with the laminin, proteoglycans, and collagen IV present in the human basement membrane, aiding in their degradation as seen in Matrigel models (54–57). Further examination of collagen IV proteolysis by MMP-1 seems appropriate. Although silencing MMP-1 in E6/E7/Ras PDECs reduces their invasion into the ECM and unveils their inherent horizontal motility rate compared with the same clone without sh-MMP-1, we cannot definitively rule out the possibility that MMP-1 may be cleaving PAR-1, assisting in invasion (21). The positive F-actin, vimentin, and contactin labeling as well as elongated morphology, invadopodia extension, and increased protease activity of E6/E7/Ras PDECs imply the necessity of the Arp2/3 complex in directing mesenchymal, invadopodial invasion and run contrary to an amoeboid migration model of PDECs through Matrigel (Fig. 1 and Supplementary Movie S2A and S2B; ref. 58). Overall, the results of this study substantiate that the K-Ras$^{G12D}$-regulated ERK2 pathway and subsequent MMP-1 induction are specific therapeutic targets potentially capable of decreasing the stromal invasiveness of pancreatic adenocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Drs. Michel Ouellette (University of Nebraska, Omaha, Nebraska); Jane Clifford, Gianluca Gallo, Boris Poljak, and Shimmon Lecht (Drexel Medicine, Philadelphia, PA); Maureen Murphy (Fox Chase Cancer Institute Philadelphia, PA); John Blenis (Harvard Medical School, Boston, MA), and their laboratories, the Penn Pancreatic Cancer Group, and the entire Drexel iCTERM Center for their assistance.

Grant Support

This work was supported in part by the NIDDK: F30 DK088402-01 to G.P. Botta; the NCI: R01 CA155413-01 to M.J. Regnagato; the NIH: R01 DK060694 to A. K. Rustgi, and the NTI to P.I. Lelkes.

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Received August 29, 2011; revised November 28, 2011; accepted November 29, 2011; published OnlineFirst December 8, 2011.

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