

# Multiple Mitogenic Pathways in Pancreatic Cancer Cells Are Blocked by a Truncated Epidermal Growth Factor Receptor<sup>1</sup>

Kei Matsuda, Takenao Idezawa, Xue Juan You, Nayantara H. Kothari, Hung Fan, and Murray Korc<sup>2</sup>

Division of Endocrinology, Diabetes and Metabolism, Departments of Medicine, Biological Chemistry, and Pharmacology [K. M., T. I., M. K.], and Department of Molecular Biology and Biochemistry [X. J. Y., N. H. K., H. F.], University of California, Irvine, California 92697

## ABSTRACT

The epidermal growth factor (EGF) receptor (EGFR) family consists of four transmembrane tyrosine kinases that undergo homodimerization and heterodimerization. Pancreatic cancers overexpress these receptors. To examine the effects of EGFR blockade on pancreatic cancer cell mitogenesis in relation to activation of specific signaling pathways, four pancreatic cancer cell lines were infected with an adenoviral vector encoding a truncated EGFR (AdtrEGFR), and activation of signaling was assessed with the mitogen-activated protein kinase (MAPK) kinase inhibitors PD98059 and U0126, the p38 MAPK inhibitor SB203580, and the c-Jun NH<sub>2</sub>-terminal kinase inhibitor SP600125. In all four cell lines, AdtrEGFR markedly attenuated EGF and heparin-binding EGF-dependent cell growth, EGFR family tyrosine phosphorylation, and phosphorylation of MAPK, c-Jun NH<sub>2</sub>-terminal kinase, p38 MAPK, and activating transcription factor 2. AdtrEGFR did not alter fibroblast growth factor 2 actions on mitogenesis. In ASPC-1, PANC-1, and T3M4 cells, PD98059 and U0126 inhibited MAPK kinase activation but not EGF-stimulated mitogenesis, whereas SB203580 inhibited EGF-stimulated mitogenesis, p38 MAPK activation, and MAPK-activated protein kinase 2 activation, without attenuating the mitogenic effect of insulin-like growth factor 1. In contrast, in COLO-357 cells, PD98059, and U0126, but not SB203580, inhibited EGF-stimulated mitogenesis, whereas SP600125 did not alter the mitogenic actions of EGF in any of the cell lines. Thus, EGF promotes proliferation via the MAPK in COLO-357 cells but via p38 MAPK in ASPC-1, PANC-1, and T3M4 cells, and whereas EGFR activation leads to the activation of all four members of the EGFR family in these cells, downstream signaling is efficiently blocked by AdtrEGFR.

## INTRODUCTION

PDAC<sup>3</sup> is the fourth to fifth leading cause of cancer-related mortality in the Western world (1). It afflicts 9–10/100,000 people every year with a mortality rate that virtually equals its incidence rate (1). Its aggressive growth behavior is due, in part, to difficulties in establishing the diagnosis before the development of metastases and to its unresponsiveness to standard chemotherapy (1). There is currently an improved understanding of the molecular alterations that occur in PDAC, and it is likely that these alterations also contribute in a significant manner to the biological aggressiveness of this disease. These alterations include a high frequency of mutations in the *K-ras* oncogene (75–90%), the *p53* (50–80%) and *DPC4* (50%) tumor

suppressor gene, and the p16 gene (2, 3). In addition, there is overexpression of many mitogenic growth factors and their receptors, including the EGFR and related receptors (2).

The EGFR family consists of four transmembrane tyrosine kinases: EGFR (erbB-1, human EGFR; HER); erbB-2 (HER-2); erbB-3 (HER-3); and erbB-4 (HER-4; Ref. 4). After ligand binding, EGFR undergoes homodimerization, as well as heterodimerization with other members of the EGFR family (4). EGFR is then autophosphorylated and transphosphorylated on tyrosine residues leading to the association with adapter and signaling molecules and activation of a network of pathways that promote mitogenesis. One major pathway is represented by the Ras/Raf/MAPK cascade (4). Activated MAPK translocates to the nucleus and induces the phosphorylation of the protein products of *jun* and *fos* nuclear proto-oncogenes (4). Another major pathway is represented by the Rac/JNK/p38 MAPK (5). Additional pathways may be activated after EGFR heterodimerization with other members of this family. For example, HER-3 undergoes tyrosine phosphorylation after heterodimerization with activated EGFR despite being devoid of intrinsic tyrosine kinase activity (6). Phosphorylated HER-3 then associates efficiently with phosphatidylinositol 3'-kinase, thereby enhancing the signaling potential of EGFR, which cannot efficiently associate with phosphatidylinositol 3'-kinase directly.

A significant proportion of PDACs express high levels of EGFR, HER-2, HER-3, and, to a more limited extent, HER-4 (2, 7). These cancers also express high levels of the EGF family of ligands. Thus, the cancer cells in PDACs express high levels of EGF, TGF- $\alpha$ , HB-EGF, betacellulin, and epiregulin (2, 8). Furthermore, the presence of EGFR in the cancer cells is associated with decreased patient survival when these cells also coexpress either EGF or TGF- $\alpha$  (9). Together, these observations suggest that excessive activation of EGFR-dependent pathways may have an important role in the biological aggressiveness of PDAC.

Previously, we reported that expression of a trEGFR in PANC-1 cells, after transfection or retroviral infection, leads to abrogation of EGF-dependent tyrosine phosphorylation of EGFR and attenuated anchorage-dependent and -independent cell growth (10). However, EGFR can heterodimerize with HER-2, HER-3, or HER-4 (4), and pancreatic cancer cells express variable levels of these receptors. It is therefore not clear whether expression of a trEGFR will impede the growth of multiple pancreatic cancer cell lines. The importance of these questions is underscored by recent studies that have suggested that it is feasible to target EGFR for therapy in PDAC. Thus, EGFR blockade with an anti-EGFR antibody attenuates pancreatic tumor growth, and inhibition of EGFR tyrosine kinase activity suppresses pancreatic tumor angiogenesis (11–13). In this study, therefore, we infected four pancreatic cancer cell lines with an AdtrEGFR. Because EGF binds directly to EGFR whereas HB-EGF binds to both EGFR and erbB-4, we sought to determine whether AdtrEGFR would block signaling by both ligands. We now report that AdtrEGFR markedly attenuates EGF and HB-EGF-mediated tyrosine phosphorylation of EGFR and related receptors, as well as EGF-dependent cell growth and mitogenic signaling pathways. We also demonstrate that mitogenesis in pancreatic cancer cells is effected via distinct signaling pathways downstream of EGFR.

Received 5/29/02; accepted 7/30/02.

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<sup>1</sup> This work was supported by Public Health Science Grant CA-40162 awarded by the National Cancer Institute (to M. K.) and by Grant 6RT-0266 from the California Tobacco-related Disease Research Program (to H. F., M. K.).

<sup>2</sup> To whom requests for reprints should be addressed, at Division of Endocrinology, Diabetes and Metabolism, Medical Science I, C240, University of California, Irvine, CA 92697. Fax: (949) 824-1035; E-mail: mkorc@uci.edu.

<sup>3</sup> The abbreviations used are: PDAC, pancreatic ductal adenocarcinoma; EGF, epidermal growth factor; EGFR, EGF receptor; HER, human EGF receptor; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; TGF, transforming growth factor; HB-EGF, heparin-binding EGF-like growth factor; trEGFR, truncated EGFR; AdtrEGFR, adenoviral vector encoding the trEGFR; ATF-2, activating transcription factor 2; MAPKAP, MAPK-activated protein; ERK-2, extracellular signal-regulated kinase isoform 2; Hsp27, heat shock protein 27; FGF-2, fibroblast growth factor 2; IGF-1, insulin-like growth factor 1; MTT, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; MEK, MAP/ERK kinase.

## MATERIALS AND METHODS

**Materials.** The following materials were purchased: fetal bovine serum from Omega Scientific (Tarzana, CA); DMEM, RPMI 1640, trypsin solution, and penicillin-streptomycin solution from Irvine Scientific (Santa Ana, CA); Genescreen membranes from New England Nuclear (Boston, MA); pBlue-script IISK+ vector from Stratagene (La Jolla, CA); restriction enzymes and random-primed labeling kit from Boehringer Mannheim (Indianapolis, IN); [ $\alpha$ - $^{32}$ P]dCTP and [ $\gamma$ - $^{32}$ P]ATP from Amersham (Arlington Heights, IL); intensifying screens and XAR-5-film from Eastman Kodak Co. (Rochester, NY); PY-20 antiphosphotyrosine antibody from Transduction Laboratories (Lexington, KY); rabbit polyclonal antiactive MAPK antibody, rabbit polyclonal antiactive JNK antibody, and rabbit polyclonal antiactive p38 MAPK antibody from Promega (Madison, WI); rabbit polyclonal antiphospho-ATF-2 (Thr<sup>71</sup>) antibody, rabbit polyclonal anti-ATF-2 antibody, rabbit polyclonal antiphospho-c-Jun (Ser<sup>73</sup>) antibody, and rabbit polyclonal anti-c-Jun antibody from Cell Signaling Technology (Beverly, MA); mouse monoclonal anti-EGFR (R1) antibody and sheep polyclonal anti-MAPKAP kinase 2 antibody from Upstate Biotechnology, Inc. (Lake Placid, NY); mouse monoclonal anti-c-neu (Ab-3) antibody from Oncogene Research Products (Cambridge, MA); rabbit polyclonal anti-ErbB-3 (C-17) antibody, rabbit polyclonal anti-ErbB-4 (C-18) antibody, rabbit polyclonal C-14 anti-ERK-2 antibody, and protein A/G agarose from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); horseradish peroxidase-conjugated antirabbit antibody from Bio-Rad Laboratories (Hercules, CA); Immobilon-P polyvinylidene difluoride transfer membrane from Millipore Corp. (Bedford, MA); enhanced chemiluminescence blotting kit from Pierce (Rockford, IL); PD98059, SB203580, and U0126 from Alexis Biochemicals (San Diego, CA); SP600125 from Calbiochem-Novabiochem Corp. (San Diego, CA); human recombinant HB-EGF from R&D systems (Minneapolis, MN); recombinant human Hsp27 from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada); and all other reagents were from Sigma Chemical Co. (St. Louis, MO). Human recombinant EGF was a gift from Chiron (Emeryville, CA); human recombinant FGF-2 was a gift from Dr. J. Abraham at Scios Nova, Inc. (Mountain View, CA); human recombinant IGF-1 was a gift from Genentech, Inc. (South San Francisco, CA). COLO-357 and T3M4 human pancreatic cancer cell lines were obtained from R. S. Metzgar at Duke University (Durham, NC). ASPC-1 and PANC-1 human pancreatic cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA).

**Construction of Vectors.** A trEGFR cDNA corresponding to the first 653 amino acids of human EGFR (HER653) was cloned into the pCMVpXCJL.2 shuttle plasmid (14). The resulting plasmid drives expression from the cytomegalovirus immediate early gene promoter. Cotransfection of the HER653 shuttle plasmid along with pJV-17 DNA into 293 cells yielded adenoviral vector expressing the extracellular and transmembrane domains of EGFR (residues 1–653). Initial experiments revealed that infection with the control adenoviral vector devoid of HER653 sequences resulted in enhanced degradation of EGFR. This observation is in agreement with previous reports that the adenovirus E3 region (present in our original vector) down-regulates EGFR (15). To eliminate E3-induced EGFR degradation, we prepared a second adenoviral vector from which the E3 region was removed. All subsequent experiments in this study were performed with either the E3-vector control (AdLacZ) or the E3-vector encoding HER653 (AdtrEGFR). The recombinant adenoviral vectors were propagated on a permissive 293 cell line, followed by purification and determination of MOI according to standard techniques.

**RNA Extraction and Northern Blot Analysis.** Total RNA was extracted, size fractionated, electrotransferred onto nylon membranes, prehybridized and hybridized with cDNA probes, and washed under high stringency conditions as previously reported (7). Blots were then exposed at  $-80^{\circ}\text{C}$  to XAR-5 films, and the resulting autoradiographs were scanned to quantify the intensity of the radiographic bands. An 860-bp *EcoRI* fragment of human EGFR cDNA was used to assess EGFR mRNA expression, and a *BamHI* 190-bp fragment of mouse 7S cDNA that cross-hybridized with human cytoplasmic RNA was used to confirm equal RNA loading and transfer (7).

**Cell Culture and Growth Assay.** Human pancreatic cancer cells were grown in DMEM (COLO-357 and PANC-1) or RPMI (ASPC-1 and T3M4) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu\text{g/ml}$  streptomycin (complete medium). Adenoviral vectors (AdLacZ control or AdtrEGFR) were diluted in serum-free medium (medium containing 0.1%

BSA, 5  $\mu\text{g/ml}$  transferrin, 5 ng/ml sodium selenite, and antibiotics), and 3 ml of the diluted virus solution were added/10-cm dish. Subsequently, cells were incubated at  $37^{\circ}\text{C}$  in humidified air with 5%  $\text{CO}_2$  for 1 h with gentle agitation every 15 min before incubation in complete medium for 24 h. To perform growth assays, cells were incubated overnight at a density of 5000 cells/well in 96-well plates, washed in HBSS, and subsequently in serum-free medium in the absence or presence of the specified additions. After 48 h, they were incubated with MTT (62.5  $\mu\text{g/well}$ ) for 4 h. Cellular MTT was solubilized with acidic isopropanol, and absorbance was measured at 570 nm with an ELISA plate reader (Molecular Devices, Menlo Park, CA). In pancreatic cancer cells, the results of the MTT assay correlate with results obtained by cell counting with a hemocytometer and by monitoring [ $^3\text{H}$ ]thymidine incorporation (16). Data were expressed as percent growth above the corresponding untreated controls. Results were plotted as means  $\pm$  SE of three separate experiments for eight determinations/experiment at each test point.

**Immunoblotting and Immunoprecipitation.** Immunoblotting was performed as previously reported using Immobilon-P membranes (17). After blocking for 2 h at  $23^{\circ}\text{C}$ , the membranes were incubated overnight at  $4^{\circ}\text{C}$  with the indicated primary antibodies and for 60 min with the corresponding horseradish-conjugated secondary antibody. After washing, bound antibody was visualized using enhanced chemiluminescence. For immunoprecipitation with anti-EGFR or anti-erbB antibodies, cells were grown to 50% confluency in complete medium and then incubated for 24 h in serum-free medium before stimulation with EGF or HB-EGF for 5 min and cell lysis. Cell lysates (500  $\mu\text{g}$  in 500  $\mu\text{l}$  of lysis buffer) were incubated overnight at  $4^{\circ}\text{C}$  with anti-EGFR or anti-erbB antibody (2  $\mu\text{g/sample}$ ), followed by 2-h incubation with protein A or G agarose (30  $\mu\text{l}$ ) at  $4^{\circ}\text{C}$ . Precipitates were washed with ice-cold PBS, resuspended in loading buffer, and boiled for 5 min at  $100^{\circ}\text{C}$ . After centrifugation, the supernatants were subjected to immunoblotting. For reprobing, membranes were incubated for 30 min at  $50^{\circ}\text{C}$  in buffer containing 2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM 2-mercaptoethanol.

**MAPKAP Kinase 2 Assay.** Cells were serum-starved by overnight incubation and then stimulated with EGF for 5 min in the presence or absence of 10  $\mu\text{M}$  SB203580, which was added 30 min before EGF. The cells were then lysed in phosphorylation lysis buffer (1% Triton X-100, 150 mM NaCl, 200  $\mu\text{M}$  sodium orthovanadate, 10 mM Na PP<sub>i</sub>, 100 mM sodium fluoride, 1 mM EDTA, 50 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu\text{g/ml}$  aprotinin). Cell lysates (500  $\mu\text{g}$  in 500  $\mu\text{l}$ ) were initially incubated overnight at  $4^{\circ}\text{C}$  with anti-MAPKAP kinase 2 antibody (2  $\mu\text{g/sample}$ ) and then for 2 h with protein G agarose (20  $\mu\text{l}$ ). Precipitates were washed three times with phosphorylation lysis buffer and twice with kinase buffer [25 mM HEPES (pH 7.4), 25 mM MgCl<sub>2</sub>, 25 mM  $\beta$ -glycerophosphate, 100  $\mu\text{M}$  sodium orthovanadate, 2 mM DTT, and 20  $\mu\text{M}$  ATP] before resuspension for 30 min at room temperature in 20  $\mu\text{l}$  of kinase buffer containing 5  $\mu\text{g}$  of Hsp27 protein and 10  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}$ P]ATP. The reaction was terminated by the addition of SDS-sample buffer. Proteins were subsequently analyzed by SDS-PAGE, and the phosphorylated form of Hsp27 was detected by autoradiography.

**Statistics.** Student *t* test was used for statistical analysis of the experiments.  $P < 0.05$  was taken as the level of significance. Results of MTT cell growth assays are expressed as SE of at least three separate experiments.

## RESULTS

**Effects of trEGFR on Cell Proliferation.** Pancreatic cancer cells were infected with of AdLacZ (control) or AdtrEGFR at multiplicity of infections of 70 (ASPC-1) or 30 (COLO-357, PANC-1, and T3M4). Northern blot analysis 48 h later revealed the presence of a major mRNA transcript ( $\sim 3$  kb) in the four AdtrEGFR-infected cells (Fig. 1). This transcript, corresponding to the trEGFR (Fig. 1), was not present in the control cells infected with AdLacZ (Fig. 1). The endogenous 10.5-kb EGFR mRNA transcript was clearly visible in ASPC-1, PANC-1, and T3M4 cells but was only faintly evident in COLO-357 cells (Fig. 1).

Sham (AdLacZ)-infected cells exhibited dose-dependent increases in growth in response to EGF, HB-EGF, or FGF-2 (Fig. 2). The magnitude of these effects was similar for all three growth factors in

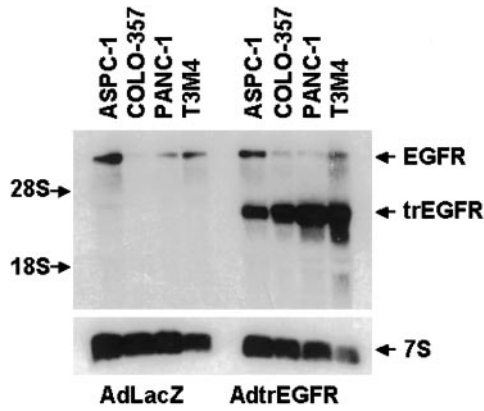


Fig. 1. Northern blot analysis. Total RNA (20  $\mu$ g/sample) was extracted from AdLacZ (control) or AdtrEGFR-infected ASPC-1, COLO-357, PANC-1, and T3M4 cells. An [ $\alpha^{32}$ P]-labeled EGFR cDNA probe (1,000,000 cpm/ml; 1-day exposure) was used for hybridizations. A 7S cDNA probe (50,000 cpm/ml; 6-h exposure) was used to confirm equivalent loading of lanes. The position of 28S and 18S RNase are indicated on the left.

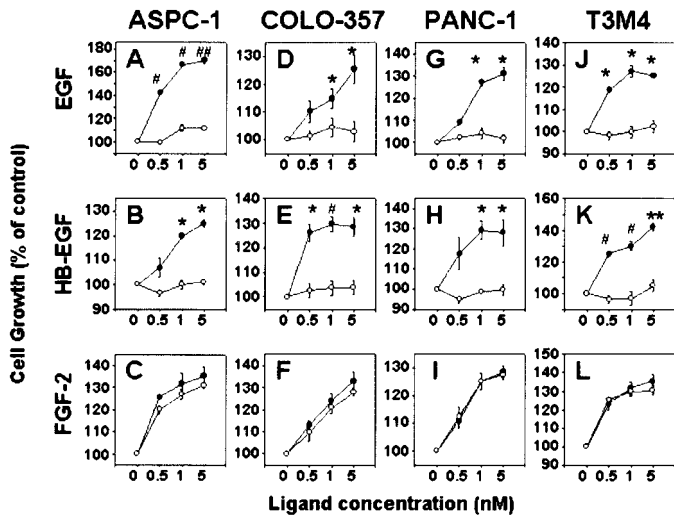
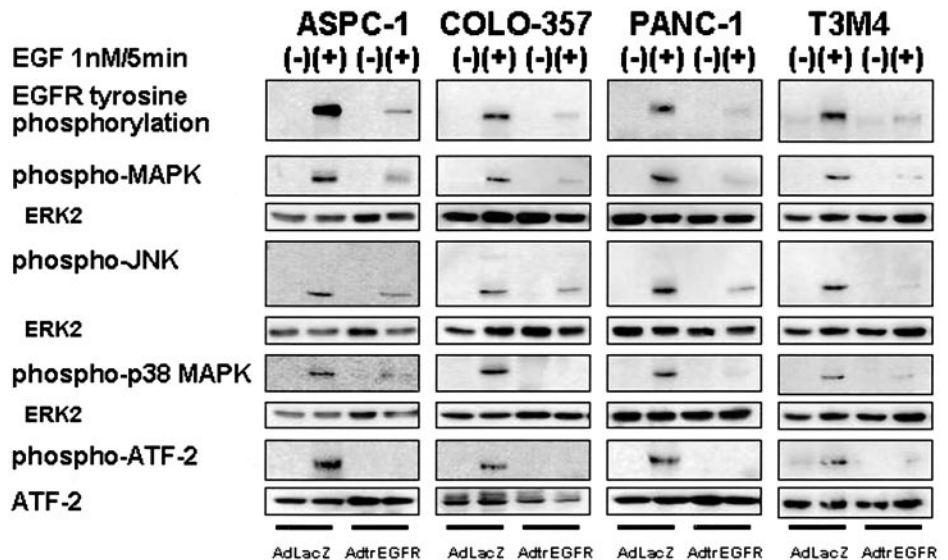


Fig. 2. Effects of AdtrEGFR on cell growth. ASPC-1 (A-C), COLO-357 (D-F), PANC-1 (G-I), and T3M4 (J-L) cells were incubated with AdLacZ (○) or AdtrEGFR (●) before the addition of the indicated concentrations of EGF, HB-EGF, and FGF-2 for 48 h. MTT assays were then performed as described in "Materials and Methods." Data are expressed as percentage change from the respective unstimulated controls and are the means  $\pm$  SE of eight determinations/experiment from three separate experiments. \*,  $P < 0.05$ . #,  $P < 0.005$ , and ##,  $P < 0.001$  when compared with the respective controls.

Fig. 3. Effects of AdtrEGFR on EGFR phosphorylation and signaling. AdLacZ- and AdtrEGFR-infected ASPC-1, COLO-357, PANC-1, and T3M4 cells were incubated for 5 min in the absence (-) or presence (+) of 1 nM EGF. EGFR tyrosine phosphorylation was determined by immunoprecipitating EGFR with anti-EGFR antibody. Immunoprecipitates were then electrophoresed on 7.5% SDS-PAGE and immunoblotted with antiphosphotyrosine antibody (PY20). To perform immunoblotting with phospho-specific antibodies, cell lysates were electrophoresed on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (20  $\mu$ g/lane). The membranes were probed with an antiactive MAPK, JNK, p38 MAPK, and ATF-2 antibodies. Membranes were then stripped and reprobed with anti-ERK-2 or anti-ATF-2 antibodies to confirm equivalent loading of lanes. Visualization was by chemiluminescence.



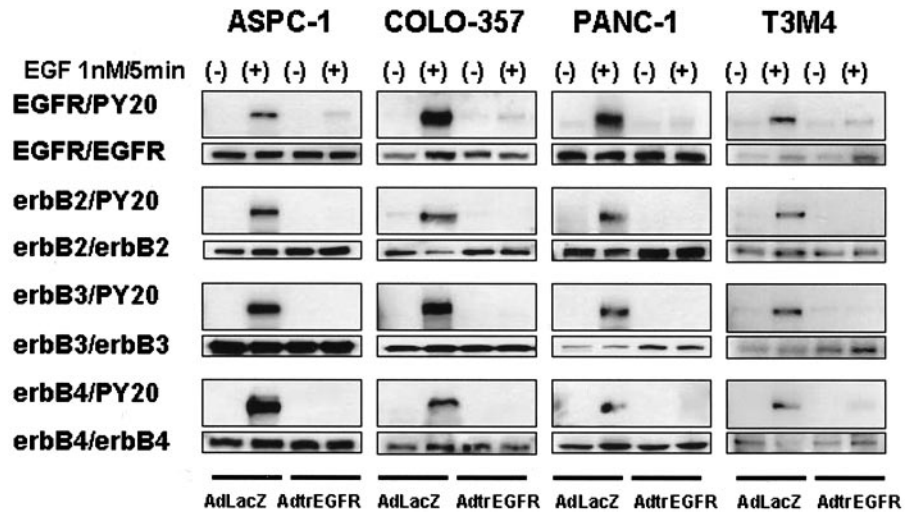
COLO-357, PANC-1, and T3M4 cells. However, in ASPC-1 cells, EGF exhibited a greater stimulatory effect ( $70\% \pm 2.6$ ) than either HB-EGF ( $25\% \pm 1.6$ ) or FGF-2 ( $35\% \pm 4.5$ ). Infection with AdtrEGFR completely attenuated the EGF- and HB-EGF-induced effects on growth in all four cell lines (Fig. 2). In contrast, FGF-2, which activates high affinity FGF receptors, induced growth stimulation in AdtrEGFR-infected cells to the same extent as in noninfected cells (Fig. 2).

**Effects of trEGFR on EGFR Family Tyrosine Phosphorylation and Activation of MAPK Pathways.** In AdLacZ (control)-infected cells, EGF (1 nM) caused a marked increase in tyrosine phosphorylation of endogenous EGFR (Fig. 3). EGF (1 nM) also enhanced the phosphorylation and activation of MAPK, JNK, and p38 MAPK in these cells, as determined by immunoblotting with the respective antiphospho antibodies (Fig. 3). As previously reported by us (17, 18), the effects of EGF on MAPK phosphorylation were always more pronounced on p42 MAPK than on p44 MAPK (Fig. 3). In all four cell lines, AdtrEGFR markedly inhibited (92–98%) the effects of EGF on tyrosine phosphorylation of EGFR. AdtrEGFR also greatly attenuated the ability of EGF to induce the phosphorylation of MAPK (90–94%), p38 MAPK (94–99%), and JNK (70–92%). Furthermore, EGF-induced ATF-2 activation, which is directly downstream of MAPK, p38 MAPK, and JNK (19, 20), was markedly attenuated by AdtrEGFR.

To assess the effects of AdtrEGFR on ligand-induced activation of other members of the EGFR family, cells were incubated with either EGF or HB-EGF, and their effects on tyrosine phosphorylation of EGFR, erbB-2, erbB-3, and erbB-4 were examined. In all four AdLacZ (control)-infected cell lines, EGF (1 nM) and HB-EGF (1 nM) induced tyrosine phosphorylation of the four EGFR family members, whereas infection with AdtrEGFR blocked EGF- and HB-EGF-induced tyrosine phosphorylation of erbB-2, erbB-3, and erbB-4 (Figs. 4 and 5).

**Effects of PD98059, U0126, SB203580, and SP600125 on EGF Responsiveness.** To assess which downstream signaling pathways mediated the mitogenic actions of EGF, the effects of PD98059, a selective MEK1 inhibitor, U0126, a selective MEK1 and MEK2 inhibitor, SB203580, a selective inhibitor of p38 MAPK, and SP600125, a selective inhibitor of JNK (21, 22), on the growth of pancreatic cancer cells were examined next. In COLO-357 cells, PD98059 (50  $\mu$ M) markedly inhibited EGF-induced cell growth (Fig. 6, top panels). In contrast, 20 (T3M4) or 50  $\mu$ M (ASPC-1 and

Fig. 4. Effects of EGF and AdtrEGFR on EGFR family tyrosine phosphorylation. AdLacZ- and AdtrEGFR-infected ASPC-1, COLO-357, PANC-1, and T3M4 cells were incubated in the absence (-) or presence (+) of 1 nM EGF for 5 min. After cell lysis, each EGFR family member was immunoprecipitated with the respective antibody. Immunoprecipitates were then electrophoresed on 7.5% SDS-PAGE, immunoblotted with antiphosphotyrosine antibody (PY20), and visualized by chemiluminescence. Data shown are from a representative of three experiments.



PANC-1) PD98059 were without effect in the other cell lines (Fig. 6, *top panels*). The effects of greater concentrations of PD98059 were not tested in T3M4 cells because of the induction of cell detachment and death in this cell line. When the concentration of PD98059 was increased to 100  $\mu\text{M}$  in the other cell lines, EGF-induced growth stimulation was completely blocked in COLO-357 cells but only partially attenuated in ASPC-1 and PANC-1 cells (data not shown). U0126, a potent and highly specific MEK inhibitor, also completely abrogated EGF-induced mitogenesis in COLO-357 cells (Fig. 6, *bottom panels*). In contrast, even at a concentration of 100  $\mu\text{M}$ , U0126 was without effect in ASPC-1 or PANC-1 cells (Fig. 6, *bottom panels*). U0126 (1  $\mu\text{M}$ ) markedly inhibited the basal growth of T3M4 cells without altering EGF-mediated growth. The effects of higher concentrations of U0126 were not tested in this cell line because of the induction of cell detachment and death.

SB203580 (10  $\mu\text{M}$ ) completely blocked the stimulatory effect of 1 nM EGF in ASPC-1, PANC-1, and T3M4 cells, without altering EGF-mediated growth stimulation in COLO-357 cells (Fig. 7, *top panels*). Increasing the concentration of SB203580 to 20  $\mu\text{M}$  also failed to alter the growth stimulatory action of EGF in COLO-357 cells (data not shown). Although SB203580 (10  $\mu\text{M}$ ) slightly attenuated the growth stimulatory effects of IGF-1 in ASPC-1, COLO-357, and T3M4 cells, this effect was not significant in any of the cells but approached significance ( $P < 0.08$ ) in ASPC-1 cells (Fig. 7, *middle*

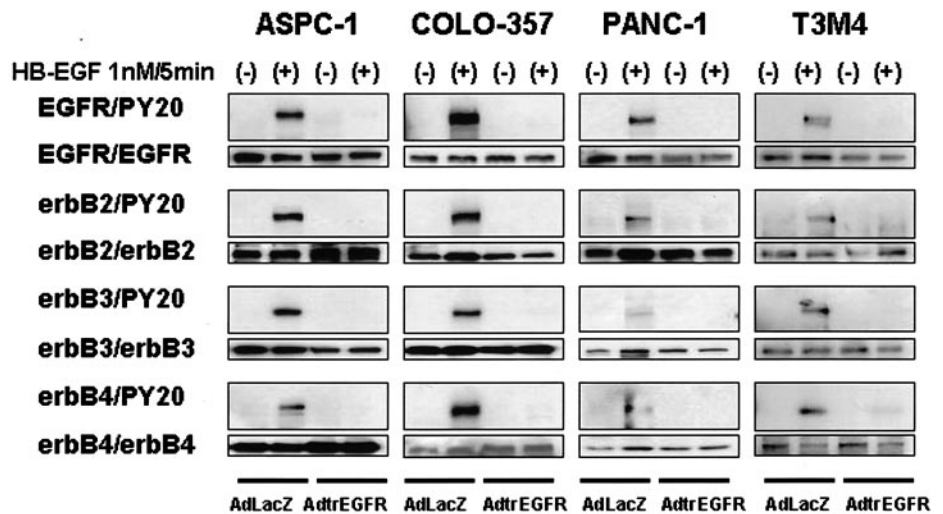
*panels*). In contrast to all of the other inhibitors, SP600125 (10  $\mu\text{M}$ ) did not alter EGF actions on mitogenesis in any of the cell lines (Fig. 7, *bottom panels*). With the exception of PD98059 in T3M4 cells, as noted above, none of the concentrations of PD98059 or SB203580 that were used in this study caused cell detachment or cell death, as determined by trypan blue exclusion.

Immunoblotting with antiactive antibodies revealed that PD98059 (50  $\mu\text{M}$ ) markedly suppressed MAPK activation in COLO-357 (95% inhibition,  $P < 0.05$ ) and PANC-1 cells (99% inhibition,  $P < 0.05$ ) but had no effect in either ASPC-1 (50  $\mu\text{M}$ ) or T3M4 (20  $\mu\text{M}$ ) cells. In contrast, U0126 (1–10  $\mu\text{M}$ ) completely inhibited MAPK activation, SB203580 (10  $\mu\text{M}$ ) completely blocked p38 MAPK activation and SP600125 (1 or 10  $\mu\text{M}$ ) markedly attenuated c-jun phosphorylation in all four cell lines (Fig. 8A). The suppressive efficacy of SB203580 (10  $\mu\text{M}$ ) on the p38 MAPK pathway was confirmed by its ability to block the activation of its downstream substrate MAPKAP kinase 2 as tested in an *in vitro* kinase reaction using Hsp27 as the substrate (Fig. 8B).

## DISCUSSION

EGFR is a glycosylated  $M_r$  170,000 protein consisting of an extracellular ligand binding domain, a transmembrane domain, and an intracellular domain that contains multiple autophosphorylating tyrosine residues (4). Tyrosine phosphorylation stimulates EGFR kinase

Fig. 5. Effects of HB-EGF and AdtrEGFR on EGFR family tyrosine phosphorylation. AdLacZ- and AdtrEGFR-infected ASPC-1, COLO-357, PANC-1, and T3M4 cells were incubated in the absence (-) or presence (+) of 1 nM HB-EGF for 5 min. After cell lysis, each EGFR family member was immunoprecipitated with the respective antibody. Immunoprecipitates were then electrophoresed on 7.5% SDS-PAGE, immunoblotted with antiphosphotyrosine antibody (PY20), and visualized by chemiluminescence. Data shown are from a representative of three experiments.



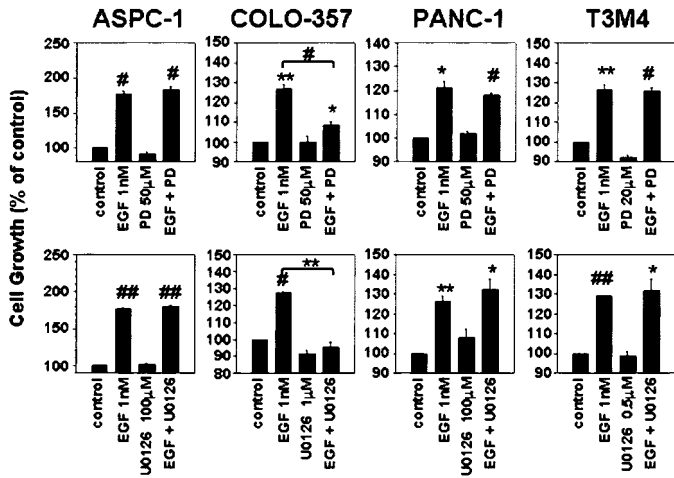


Fig. 6. Effects of PD98059 and U0126 on EGF-mediated cell growth. ASPC-1, COLO-357, PANC-1, and T3M4 cells were incubated for 48 h in the absence or presence of 1 nM EGF, 20 or 50  $\mu$ M PD98059, 1 or 100  $\mu$ M U0126, or the indicated combinations. MTT assays were then performed as described in "Materials and Methods." Data are expressed as percentage change from the respective unstimulated controls and are the means  $\pm$  SE of eight determinations/experiment from three separate experiments. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , #,  $P < 0.005$ , and ##,  $P < 0.001$  when compared with the respective controls or with the indicated additions.

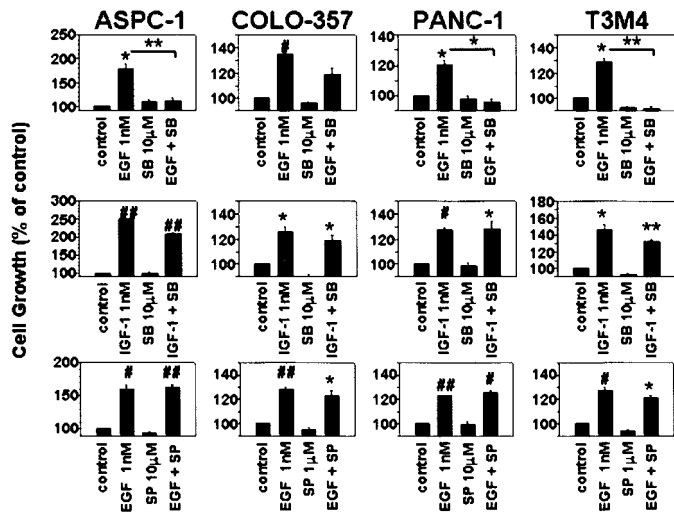


Fig. 7. Effects of SB203580, SP600125, IGF-1, and EGF on cell growth. ASPC-1, COLO-357, PANC-1, and T3M4 cells were incubated for 48 h in the presence or absence of 1 nM IGF-1, 1 nM EGF, 10  $\mu$ M SB203580, 1 or 10  $\mu$ M SP600125, or the indicated combinations. MTT assays were performed as described in "Materials and Methods." Data are expressed as percentage change from the respective unstimulated controls and are the means  $\pm$  SE of eight determinations/experiment from three separate experiments. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , #,  $P < 0.005$ , and ##,  $P < 0.001$  when compared with the respective controls or with the indicated additions.

activity and generates targets for the recruitment of signaling and adapter proteins that contain src-homology-2 and phosphotyrosine binding domains (4). Although erbB-2, erbB-3, and erbB-4 are members of the EGFR family and share considerable amino acid sequence homology with EGFR, they differ from each other in their COOH-terminal tails, a region that is rich in tyrosine residues (4). The lack of homology at these sites allows for the coupling of activated receptor homodimers to different downstream signaling molecules (4, 23). Signaling diversity is enhanced by receptor heterodimerization, which allows for combinatorial interactions and activation of distinct signaling pathways (4, 23, 24).

The cancer cells in PDAC frequently overexpress EGFR, erbB-2, erbB-3, and, less frequently, erbB-4, as well as six ligands that bind

directly to EGFR (2, 7, 8). Therefore, excessive activation of the EGFR family in PDAC may be of fundamental importance in this malignancy. PDACs also harbor a high rate of *K-ras* mutations that may prolong the activation of mitogenic signaling pathways (2). Furthermore, glypican-1, a heparan sulfate proteoglycan that enhances the actions of heparin binding members of the EGF family of ligands by promoting ligand-receptor interactions, and the cell cycle regulator cyclin D1, a modulator of growth factor actions on the cell cycle, are overexpressed in PDAC (25, 26). These findings suggest that multiple components of the pathways that activate the EGFR family are either overexpressed (EGFR family and ligands, glypican-1, and cyclin D1) or mutated (*K-ras*) in PDAC, which may lead to excessive activation of important growth-modulating cascades. In support of this hypothesis, increased expression of EGFR and either EGF or TGF- $\alpha$  (9) and increased expression of erbB-3 (2) are associated with a worse prognosis in PDAC.

To further delineate the biological importance of EGFR in pancreatic cancer cells, we examined the consequences of blocking EGFR-dependent signaling in four pancreatic cancer cell lines. To this end, we used an E3-adenoviral vector (AdtrEGFR) that allows for expression of high levels of a trEGFR without causing nonspecific modulation of endogenous EGFR. PANC-1 and T3M4 cells are poorly differentiated and harbor mutated *K-ras* and *p53* genes (27, 28), whereas ASPC-1 and COLO-357 are moderately well differentiated. ASPC-1 cells also only harbor mutated *K-ras* and *p53* genes, whereas COLO-357 cells only harbor a mutated *K-ras* oncogene (27, 28). All four cell lines express high levels of cell surface EGFR, variable levels of erbB-2 and erbB-3 mRNA, and variable levels of betacellulin, epiregulin, amphiregulin, HB-EGF, and TGF- $\alpha$  (2, 29). These cells are therefore useful for studying EGFR-dependent signaling in a

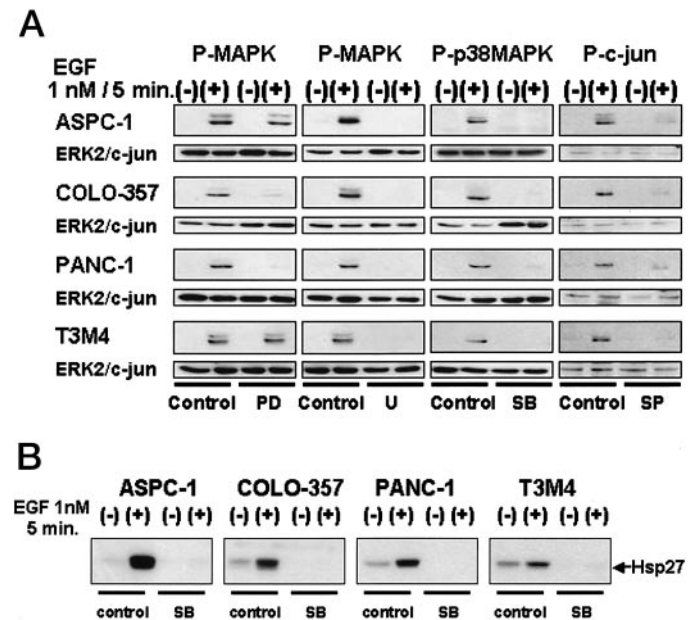


Fig. 8. Effects of inhibitors on EGF signaling. A, immunoblotting with antiactive antibodies. ASPC-1, COLO-357, PANC-1, and T3M4 cells were incubated in the absence or presence of 20  $\mu$ M (T3M4) or 50  $\mu$ M (other three cell lines) PD98059, 1  $\mu$ M (COLO-357, T3M4) or 10  $\mu$ M (ASPC-1 and PANC-1) U0126, 10  $\mu$ M SB203580 and 1  $\mu$ M (COLO-357 and T3M4), or 10  $\mu$ M (ASPC-1 and PANC-1) SP600125 for 2 h before the addition of 1 nM EGF for 5 min. Immunoblotting was performed with the indicated antiactive antibodies and with anti-ERK-2 as a loading control, as described in the legend to Fig. 3. B, MAPKAP kinase 2 activation. ASPC-1, COLO-357, PANC-1, and T3M4 cells were incubated in the absence or presence of 10  $\mu$ M SB203580 for 30 min before the addition of 1 nM EGF for 5 min. Cell lysates were immunoprecipitated with an anti-MAPKAP kinase 2 antibody. MAPKAP kinase activity was assayed using recombinant Hsp27 as a substrate as described in "Materials and Methods." Data shown are from a representative of three experiments.

background that recapitulates the molecular alterations that occur in PDAC.

In this study, EGF induced EGFR tyrosine phosphorylation and activated MAPK, JNK, and p38 MAPK in all four pancreatic cancer cell lines. Infection with AdtrEGFR dramatically attenuated these actions of EGF. In contrast, FGF-2 exerted similar mitogenic effects in control cells and in cells expressing the trEGFR, indicating that the AdtrEGFR-mediated blockade was specific for EGFR. Although EGF has been previously shown to activate MAPK in pancreatic cancer cells, the present findings are the first to demonstrate that EGF activates all three pathways in these cells and that expressing a dominant-negative EGFR can markedly attenuate this activation.

In contrast to EGF, HB-EGF is bifunctional, meaning that it is capable of binding directly to both EGFR and erbB-4. Activation of erbB-4 by bifunctional members of the EGF family of ligands is markedly enhanced when erbB-4 and erbB-2 are coexpressed (30), and pancreatic cancer cell lines express erbB-2 (2). Indeed, in this study, we determined that in addition to stimulating tyrosine phosphorylation of EGFR, HB-EGF enhanced tyrosine phosphorylation of erbB-2, erbB-3, and erbB-4 in all four pancreatic cancer cell lines. Furthermore, AdtrEGFR completely blocked both these actions of HB-EGF and its ability to enhance mitogenesis. These observations suggest that activation of EGFR is necessary for both EGF and HB-EGF-mediated tyrosine phosphorylation of all four members of the EGFR family in pancreatic cancer cells and that mitogenesis by either ligand is dependent on EGFR activation. Alternatively, it is possible that the trEGFR heterodimerizes with other members of this receptor family, thereby directly suppressing signaling through these receptors.

Although it is widely recognized that EGF exerts its mitogenic effects through the MAPK pathway and to activate the JNK and p38 MAPK pathways, it was only recently shown that EGF may also enhance mitogenic signaling via JNK in certain cells (4, 5). To determine which signaling pathways mediate the mitogenic effects of EGF in pancreatic cancer cells, we used the MAPK inhibitors PD98059 and U0126, the p38 MAPK inhibitor SB203580, and JNK inhibitor SP600125. In COLO-357 cells, PD98059 markedly inhibited both EGF-stimulated mitogenesis and MAPK kinase activation, suggesting that in this cell line, EGF enhanced mitogenesis via MAPK. In support of this conclusion, a very low concentration of the highly specific MAPK inhibitor U0126 (1  $\mu\text{M}$ ) completely suppressed the stimulatory effects of EGF on COLO-357 cell growth and MAPK activation. In contrast, neither PD98059 nor U0126 altered EGF-stimulated cell growth in PANC-1, ASPC-1, or T3M4 cells, despite the complete and uniform suppression by U0126 of EGF-mediated MAPK activation in these cells. Furthermore, a low concentration of SB203580 (10  $\mu\text{M}$ ) completely inhibited EGF-stimulated cell growth and dramatically inhibited p38 MAPK and MAPKAP kinase activation in these cells. Although SB203580 also completely blocked this pathway in COLO-357 cells, this effect was not associated with a significant decrease in EGF-mediated mitogenesis. Furthermore, SP600125, a JNK inhibitor, markedly attenuated c-jun phosphorylation but did not alter EGF-mediated growth stimulation in any of the cell lines. In contrast, the same inhibitor suppressed the mitogenic effects of EGF in Chinese hamster ovary cells that express EGFR (data not shown). Together, these observations indicate that EGF enhances mitogenesis via the MAPK pathway in COLO-357 cells but via the p38 MAPK pathway in ASPC-1, PANC-1, and T3M4 cells. Although the exact reasons for this divergence in mitogenic signaling are not readily evident, they may be attributable, in part, to differences in expression of EGFR family members. In addition, differential recycling and degradation of these receptors may lead to differential patterns of heterodimerization and subsequent mitogenic signaling.

There are four p38 MAPK isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) that exhibit 60–70% amino acid sequence homology with each other (31). Although p38 MAPKs have an important role in development and differentiation (31), recent studies suggest that they may also have a role in regulating cell proliferation. Thus, mitogenic signaling by FGF-2 in Swiss 3T3 cells and by granulocyte-stimulating factor in hematopoietic cells is dependent on p38 MAPK (32, 33). In contrast, in NIH 3T3 fibroblasts and CCL39 cells, p38 MAPK inhibits cyclin D1 expression and cell proliferation (34, 35). In this study, SB203580 did not significantly alter the mitogenic actions of IGF-1 in any of the cell lines. This observation underscores the relative specificity of the dependence of the mitogenic actions of EGF on p38 MAPK in ASPC-1, PANC-1, and T3M4 cells. Our findings are thus the first to demonstrate, in any cell type, a mitogenic effect by EGF through the p38 MAPK pathway that is independent of the p42/p44-MAPK pathway. Because SB203580 inhibits p38 $\alpha$  and p38 $\beta$  and does not inhibit the other isoforms (34), our findings indicate that EGF enhances mitogenesis in these cells by activating either p38 MAPK- $\alpha$  or MAPK- $\beta$ .

It is increasingly recognized that EGFR is at the cross-roads of multiple important signaling pathways (4). Thus, mitogenic agonists for serpentine receptors that are G-protein dependent also activate EGFR and MAPK. In some instances, this effect appears to be attributable to activation of proteases that release HB-EGF, which then activate EGFR (36). In other instances, such as in the case of thrombin-induced stimulation of vascular smooth muscle cell proliferation, there is transactivation of EGFR, which then activates p38 MAPK, thereby leading to enhanced mitogenesis (37). EGFR activation also modulates TGF- $\beta$  signaling by inducing Smad2 phosphorylation, thereby interfering with its ability to associate with and be phosphorylated by the type I TGF- $\beta$  receptor and suppressing growth inhibitory signals (38). These observations are relevant to PDAC because there is frequently resistance to TGF- $\beta$ -mediated growth inhibition in this malignancy (39, 40) in conjunction with a high rate of mutation of the *Smad4* gene (3) and overexpression of TGF- $\beta$ s (41). Indeed, in cultured pancreatic cancer cells, EGF suppresses the growth inhibitory actions of TGF- $\beta$ s and exerts additive growth-stimulatory effects with bone morphogenetic protein 2 in those cells that harbor *Smad4* mutations (42). Furthermore, EGFR is expressed at high levels in the endothelial cells within the pancreatic cancer mass (7) and induces the expression of the angiogenic factors vascular endothelial growth factor and interleukin 8 (12, 43). Together, these observations point to the pivotal role of EGFR pathways in PDAC and underscore the therapeutic potential of agents that target EGFR (12, 44, 45). In this context, our findings suggest that abrogation of EGFR-dependent signaling with vectors such as AdtrEGFR may also ultimately have a therapeutic potential in this disorder because AdtrEGFR can block EGF activation of multiple mitogenic pathways as well as the activation of all of the members of the EGFR family.

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