Pharmacological Inhibition of Ras-Transformed Epithelial Cell Growth Is Linked to Down-regulation of Epidermal Growth Factor–Related Peptides

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Background & Aims: Posttranslational farnesylation is required for Ras activation. Farnesyl transferase inhibitors (FTIs) selectively block protein farnesylation and reduce the growth of many Ras-transformed cells in vitro and in vivo. Activated Ras transforms rat intestinal epithelial (RIE-1) cells by a mechanism distinct from NIH 3T3 fibroblasts in that an epidermal growth factor receptor (EGFR) autocrine loop contributes significantly to the Ras-transformed RIE-1 phenotype. **Methods:** The ability of FTIs to block growth of Ras-transformed RIE-1 cells was evaluated, and these results were correlated with decreased EGFR ligand production. **Results:** FTI L744,832 caused a selective, dose-dependent, reversible blockade in proliferation of H-Ras-transformed RIE-1 cells, whereas control cell lines, K-Ras-transformed cells, and activated raf-transfected RIE cells were unaffected. The growth-inhibitory effects of L744,832 correlated with loss of farnesylated H-Ras protein and a marked reduction in transforming growth factor (TGF)- α and amphiregulin expression. Inhibition of proliferation of H-Ras RIE-1 cells by L744,832 was overcome by exogenous TGF- α , and enhanced growth inhibition was achieved by EGFR blockade in combination with L744,832. **Conclusions:** These data suggest that one mechanism by which FTIs inhibit growth of H-Ras-transformed epithelial cells is by reducing Ras-induced EGFR ligand production.

Many cellular proteins undergo posttranslational modification by farnesyl transferase (FTase)¹ or geranylgeranyl-protein transferases (GGPTase I and II).²⁻⁴ Although a variety of proteins such as K-Ras, H-Ras, N-Ras, Rho B, and nuclear lamins A and B are farnesylated, a greater number of proteins undergo geranylgeranylation.⁵ Farnesylation of Ras by FTase is required for translocation of the protein to cellular membranes and its subsequent activation.^{6,7} FTase adds a 15-carbon isoprenyl group through a thioether linkage to the C-terminal CAAX box of Ras.¹

Because Ras mutations occur in many human tumors,

including adenocarcinomas of the pancreas and colon,⁸⁻¹⁰ specific FTase inhibitors (FTIs) have been developed as anticancer drugs to target tumors in which Ras proteins have undergone mutational activation.¹¹⁻¹⁵ Pharmacological inhibition of FTase has been shown to inhibit Ras in both H- and K-Ras-transformed cells, although in most cell types, K-Ras is far less sensitive.^{11,12,15-19} Resistance of K-Ras to FTIs may occur because of its compensatory geranylgeranylation by GGPTase I.²⁰ Unexpectedly, FTIs have no apparent growth-inhibitory effect on normal cells,^{11,12} and inhibition of cells by FTI does not always correlate with Ras mutational status.¹⁹ These observations indicate that further study is required to fully understand and delineate the effects of FTIs in tumor biology.

We previously found that mutant Ras causes transformation of intestinal and mammary epithelial cells via pathways and mechanisms distinct from those described for fibroblasts.^{21,22} For example, activated Ras transformed the rat intestinal epithelial cell line RIE-1 and the mammary epithelial line MCF-10A, whereas activated Raf did not, leading to the conclusion that pathways independent of the conventional Raf/MAPKK (mitogen-activated protein kinase kinase)/MAPK pathway contribute to Ras transformation of epithelial cells.^{21,23} One such pathway is Ras-stimulated increased production and secretion of epidermal growth factor receptor (EGFR) ligands.^{21,22} Blockade of EGFR signaling reverts the

Abbreviations used in this study: AR, amphiregulin; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGFR, EGF receptor; FTase, farnesyltransferase; FTI, FTase inhibitor; GGPTase, geranylgeranyl-protein transferase; HB-EGF, heparin-binding EGF-like growth factor; MAPK, mitogen-activated protein kinase; RIA, radioimmunoassay; SDNS-PBS, phosphate-buffered saline containing 0.02% saponin and 5% normal donkey serum; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TGF, transforming growth factor.

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transformed appearance of Ras-transformed RIE-1 cells and prevents morphological transformation of parental RIE-1 cells caused by Ras-conditioned medium.²² These data indicate that Ras transformation of RIE-1 cells is mediated, at least in part, by EGFR ligand production acting via autocrine pathways.

In this study, we determined that the FTI designated L744,832 significantly inhibits the growth of H-Ras-transformed RIE-1 cells by blocking production of EGFR ligands induced by Ras transformation. The proliferation of control cells, activated Raf-transfected cells, and K-Ras4B-transformed RIE-1 cells is unaffected by L744,832 treatment even at concentrations as high as 50 $\mu\text{mol/L}$. L744,832 causes a selective, dose-dependent, reversible reduction in transforming growth factor (TGF)- α and amphiregulin (AR) expression and cell growth only in H-Ras-transformed RIE-1 cells. FTI growth inhibition is preceded by a loss of farnesylated Ras protein and subsequent reduction in Ras-mediated EGFR ligand production. Growth inhibition induced by FTI in H-Ras RIE cells is overcome by addition of exogenous TGF- α . Blockade of EGFR signaling by a specific EGFR tyrosine kinase inhibitor (PD153035) reduces the growth of H-Ras and K-Ras4B-transformed RIE-1 cells and enhances the growth-inhibitory effects of L744,832, further supporting reduction in EGFR ligand production as an important feature of growth inhibition by FTIs.

Materials and Methods

Materials

L744,832 and PD153035 were obtained from Merck Research Laboratories (West Point, PA)²⁴ and Parke-Davis Pharmaceutical Research (Ann Arbor, MI),²⁵ respectively. L744,832 and PD153035 were dissolved in dimethyl sulfoxide (DMSO) at a 1000 $\times$ concentration and stored at -20°C . Radionucleotides were purchased from New England Nuclear (Boston, MA). All other reagents were analytical grade and purchased from standard suppliers.

Cell Culture

RIE-1 cells, obtained from Dr. Kenneth Brown (Cambridge, England), are a diploid, nontransformed, EGF-responsive cell line derived from rat small intestine.^{26,27} RIE-1 cells stably transfected with control vector construct Neo4F and with constructs encoding activated Raf, H-, and K-Ras4B have been described previously.^{21,22} Stably transfected cells were maintained in growth medium containing 500 $\mu\text{g/mL}$ of G418 (Sigma Chemical Co., St. Louis, MO).

[³H]Thymidine Incorporation

DNA replication was assayed using 10,000 cells/well seeded in 24-well plates. Twenty-four hours after plating,

cultures were treated with the indicated concentrations of drug. After a 22-hour incubation, cells were pulsed with 1 $\mu\text{Ci/well}$ of [³H]thymidine (Amersham, Arlington Heights, IL) for 2 additional hours. Medium was removed, and each well was washed 3 times for 5 minutes with 1 mL/well of cold 10% trichloroacetic acid. Plates were dried for 30 minutes at room temperature, and 300 $\mu\text{L/well}$ of 0.2N NaOH and 40 $\mu\text{g/mL}$ salmon sperm DNA were added. After a 30-minute incubation at room temperature, each sample was transferred into scintillation vials containing 5 mL of aqueous scintillation cocktail and counted for 1 minute. For time course experiments, cells were fixed by addition of 300 μL of 1 mol/L ascorbic acid into the medium of each well. All wells were processed at the completion of the experiment as described above. Results were normalized to cell number that was determined in replicate wells for each condition. Unless otherwise stated, all results are representative of at least 3 separate experiments.

TGF- α Radioimmunoassay

The radioimmunoassay (RIA) for TGF- α was performed in conditioned medium as described previously.²⁸ Unless otherwise stated, all results presented are representative of at least 3 separate experiments.

Isolation of Poly(A) RNA and Northern Blot Analysis

Cells were grown to near confluence, washed twice with isotonic buffer, and then switched to serum-free Dulbecco's modified essential medium for 72 hours. Total cellular RNA was extracted by the method of Schwab et al.²⁹ Oligo(dT)-selected RNA was separated by electrophoresis in 1.2% agarose/formaldehyde gels, and Northern blot analysis was performed.^{30,31} Hybridizations, with species-specific probes labeled by RNA polymerase-directed reverse transcription (EGF, TGF- α , AR, and 1B15) or random primer extension (heparin-binding EGF-like growth factor [HB-EGF]), were performed in hybridization ovens as described previously.^{32,33} 1B15 is a constitutively expressed sequence used to assure equivalent loading and transfer of messenger RNA (mRNA).³⁴ Band intensities were quantified by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA). Unless otherwise stated, all results are representative of at least 3 separate experiments.

Ras Protein Processing

Cells were plated into 12-well plates at 10,000 cells/well. Cells were treated with 10 nmol/L, 100 nmol/L, or 1 $\mu\text{mol/L}$ L744,832 for the indicated times and harvested in 100 μL sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The method for analyzing Ras processing has been described in detail.³⁵ Briefly, samples were separated by 10% SDS-PAGE and transferred to Immobilon (Millipore, Bedford, MA) by Western blotting. Ras proteins were detected by the H-Ras-specific antibody 146-3E4 (Quality Biotech, Camden, NJ) using a peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence (Amersham, Arlington Heights, IL) detection and quantitation.

with a Bio-Rad phosphorimager (Hercules, CA). Unless otherwise stated, all results presented are representative of at least 3 separate experiments.

Ras Protein Immunofluorescence

Cells were plated onto autoclaved coverslips at 10,000 cells/coverslip. Cells were treated for 24 hours with 100 nmol/L, 1 μ mol/L, or 10 μ mol/L L744,832 and fixed in 2% paraformaldehyde for 20 minutes at room temperature. The method for Ras immunofluorescence was adapted from previous methods.³⁵ Briefly, coverslips were washed 3 times with phosphate-buffered saline (PBS) and permeabilized and blocked in PBS containing 0.02% saponin and 5% normal donkey serum (SNDS-PBS) for 30 minutes at room temperature. The coverslips were incubated with the primary pan-Ras antibody Y13259 (Oncogene Science, Manhasset, NY) at 30 μ g/mL in SNDS-PBS for 1 hour at room temperature. Coverslips were washed 3 times for 10 minutes with PBS containing 0.02% saponin. The coverslips were incubated with secondary antibody fluorescein isothiocyanate-conjugated donkey anti-rat immunoglobulin G (Jackson Laboratories, West Grove, PA) at 10 μ g/mL in SNDS-PBS for 30 minutes at room temperature, and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) was added at 5 μ g/mL to label nuclei during the last 5 minutes of the incubation. Coverslips were washed 3 times for 10 minutes with PBS containing 0.02% saponin and mounted for immunofluorescence and visualization.

Results

FTI Selectively Blocks H-Ras RIE-1 Cellular Proliferation

Initial studies were designed to test the effect of FTI L744,832 on growth of parental RIE-1 cells and RIE-1 cells stably transfected with activated raf and ras complementary DNA constructs. L744,832 (from 100 nmol/L to 50 μ mol/L) selectively inhibited proliferation of activated H-Ras-transfected RIE-1 cells as measured

by a dose-dependent reduction in [³H]thymidine incorporation (Figure 1), number of cells (data not shown), and S-phase cell population by flow cytometry (data not shown). No indication of apoptotic cell death was apparent in flow cytometric analysis of FTI-treated H-Ras RIE-1 cells. Proliferation of parental RIE-1 cells, vector-transfected cells, and activated raf-transfected RIE-1 cells was unaffected at FTI concentrations as high as 10 μ mol/L (Figure 1). Further assays showed that concentrations as high as 50 μ mol/L FTI had no significant effect on proliferation of these cells (data not shown). These data are consistent with the reported absence of toxicity in FTI-treated animals, as well as FTI resistance in normal cells and cells transfected with oncogenes downstream of Ras.^{11,12,15-18} Proliferation of K-Ras4B-transformed RIE-1 cells was not affected by FTI up to 50 μ mol/L, but reduction of proliferation at doses higher than 50 μ mol/L was observed because of cytotoxicity (data not shown).

FTI Blocks Ras-Induced TGF- α and AR Growth Factor Expression

Inasmuch as Ras transformation of RIE-1 cells is associated with induction of EGFR ligands and signaling through the EGFR contributes significantly to the Ras-transformed phenotype,^{21,22} we examined the effects of FTI on EGFR ligand production in Ras-transformed RIE-1 cells. As shown in Figure 2, treatment of H-Ras RIE-1 cells for 24 hours with L744,832 caused a dose-dependent decrease in TGF- α protein levels in the culture medium (Figure 2A); this decrease correlated with a parallel reduction in DNA synthesis (Figure 1). Under these experimental conditions, L744,832 had no effect on TGF- α protein levels in the culture medium of FTI-resistant K-Ras4B RIE-1 cells (Figure 2B), nor on mitogenesis (Figure 1). As shown in Figure 3, a single

Figure 1. Effect of L744,832 on DNA replication in control and oncogene-transfected RIE-1 cells. Cells were plated at 10,000 cells/well in 24-well cluster plates. Near-confluent cells were treated for 24 hours with the indicated concentrations of FTI or vehicle (DMSO, 1 μ L/mL). During the last 2 hours of the incubation, cells were pulsed with [³H]thymidine (1 μ Ci/mL) and processed as described in Materials and Methods. Number of cells was determined in replicate wells to normalize data.

Figure 2. L744,832 reduces TGF- α protein levels in (A) H-Ras RIE-1 cells but not in (B) K-Ras4B RIE-1 cells. Under experimental conditions described in Figure 1, medium was removed, and TGF- α protein concentrations were determined by RIA as described in Materials and Methods.

administration of L744,832 also blocked accumulation of TGF- α in the medium of H-Ras RIE-1 cells. Steady-state mRNA expression of EGFR ligands was examined by Northern blot analysis. Dose-dependent decreases in transcripts for TGF- α and AR mRNA were observed after a 24-hour exposure to FTI; for example, at 10 μ mol/L L744,832, TGF- α and AR signals were decreased by approximately 80% and 95%, respectively (Figure 4A). No reduction in TGF- α and AR mRNA expression was observed in FTI-resistant K-Ras4B-transformed RIE-1 cells (Figure 4B).

FTI Growth Inhibition Is Temporally Associated With Decreased TGF- α and AR Expression

We examined the temporal relationship between down-regulation of EGFR ligands and decreased proliferation in FTI-treated H-Ras RIE-1 cells. Administration of a single dose of L744,832 resulted in a rapid decrease in both TGF- α and AR mRNA. This was observed as early as 3 hours, was maximal at 24 hours, and recovered

between 72 and 96 hours (Figure 5). L744,832-mediated reduction of TGF- α and AR mRNA was not affected by cycloheximide treatment (data not shown). The reduction in TGF- α and AR mRNA expression preceded the decrease in cellular proliferation that began to decrease significantly at 12 hours with maximal inhibition at 24 hours and recovery only starting at 96 and 120 hours after FTI treatment (Figure 5).

FTI Rapidly Inhibits H-Ras Farnesylation and Membrane Localization

The rapidity with which L744,832 reduced EGFR ligand mRNA expression was unexpected because previous studies have described a Ras half-life of 20–24 hours in fibroblasts.³⁶ However, the half-life of Ras is dramatically reduced in FTI-treated H-Ras-transformed RIE-1 cells compared with FTI-treated NIH 3T3 fibroblasts (A. Cox, C. Der, and R. Coffey, unpublished observations, June 1998). We determined the effect of a single dose of L744,832 (1 μ mol/L) on H-Ras farnesylation over time (Figure 6A). There was a 50% decrease in farnesylated Ras after 3 hours of FTI treatment. This decrease correlates with a similar reduction in TGF- α and AR mRNA expression 3 hours after L744,832 administration (Figure 5). By 24 hours, most of the Ras protein was unfarnesylated and remained in that form at 48 hours. Most Ras protein returned to its farnesylated form by 72 hours (data not shown). At 24 hours, we compared a dose-range effect of L744,832 on Ras processing in H-Ras and K-Ras4B RIE-1 cells (Figure 6B). There was a dose-dependent decrease in the amount of farnesylated Ras by FTI in H-Ras RIE-1 cells but not in K-Ras4B RIE-1 cells. The farnesylation of K-Ras4B was not affected by L744,832 in concentrations up to 50 μ mol/L (data not shown), a finding that is consistent with its lack of an effect on cellular proliferation at this dose. Western blotting using a pan-Ras antibody showed equal intensity of the p21 Ras signal in H- and K-Ras4B-

Figure 3. L744,832 prevents the accumulation of TGF- α protein in the conditioned medium of H-Ras RIE-1 cells. Cells were plated at 10,000 cells/well in 24-well cluster plates. Near-confluent cells were placed in serum-free medium for 24 hours. FTI (10 μ mol/L; ■) or vehicle (DMSO, 1 μ L/mL; ○) was added to each well; conditioned medium was collected at the indicated times after FTI addition; and TGF- α protein concentrations were determined by RIA. Number of cells was determined in replicate wells to normalize data.

transformed cells, whereas the signal in parental RIE-1 cells was barely detectable (data not shown). The immunolocalization of Ras in response to FTI treatment was also examined (Figure 6C). Treatment with L744,832 (10 $\mu\text{mol/L}$) resulted in loss of H-Ras membrane localization, but not K-Ras4B localization, again supporting a differential effect of FTI on cellular proliferation and Ras protein farnesylation in H- and K-Ras4B-transformed cells.

Addition of TGF- α Overrides FTI Growth Inhibition

TGF- α was administered to L744,832-treated H-Ras RIE-1 cells to further address the importance of decreased EGFR ligand expression in FTI-mediated growth inhibition. Addition of TGF- α (20 ng/mL) simultaneous with or 24 hours after L744,832 fully reversed growth inhibition (Figure 7) and partially

Figure 4. L744,832 decreases TGF- α and AR mRNA expression in (A) H-Ras RIE-1 cells but not in (B) K-Ras4B RIE-1 cells. Cells were plated at 1×10^6 cells/flask in T75 flasks. When cells reached 70%–80% confluency, the cultures were treated with the indicated concentration of FTI or vehicle (DMSO, 1 $\mu\text{L/mL}$) for 24 hours. The cells were then lysed and processed for Northern blot analysis as described in Materials and Methods. IB15 is a constitutively expressed gene that is used to assure equivalent loading and transfer of RNA.

Figure 5. Temporal effect of 10 $\mu\text{mol/L}$ L744,832 on EGFR ligand expression and mitogenesis in H-Ras RIE-1 cells. Experimental conditions and analysis of Northern blots and DNA replication were similar to those described in Figures 4 and 1, respectively. During the time course for DNA replication, 300 μL of 1 mol/L ascorbic acid was added into the medium of each well after a 2-hour pulse of [^3H]thymidine for each time point, and all wells were processed together at the completion of the assay. The effects on DNA replication are expressed as percent of control. $\square$, TGF- α ; $\circ$, AR; $\bullet$, [^3H]thymidine.

restored growth in soft agar (data not shown). Furthermore, coadministration of a TGF- α -neutralizing antibody blocked the ability of TGF- α to restore proliferation in FTI-treated H-Ras RIE-1 cells (Figure 7). These results suggest that down-regulation of EGFR ligand expression in H-Ras-transformed RIE-1 cells by L744,832 plays a central role in the growth inhibition achieved by this compound. However, this may not be the drug's sole mechanism of action because L744,832 treatment of H-Ras-transformed RIE-1 cells for 24 hours resulted in a reduction in MAPK activity, leaving open the possibility that other Ras effectors are also involved. Interestingly, pretreatment of parental RIE-1 cells with FTI had no effect on unstimulated proliferation (Figure 1). L744,832 only partially inhibits (20%–30%) TGF- α -stimulated proliferation and fails to block TGF- α -stimulated induction of EGF-related peptides in parental RIE-1 cells (Barnard et al.³⁷ and data not shown). The relative inability of FTI to block these events in normal RIE-1 cells suggests that they are mediated by K-Ras signaling or by a Ras-independent pathway.

Enhanced Growth Inhibition of Ras-Transformed Cells by Ras and EGFR Blockade

K-Ras-transformed fibroblasts^{38,39} and epithelial cells (Figure 1) are more resistant to FTI growth inhibi-

Figure 6. Treatment with L744,832 causes rapid accumulation of unfarnesylated Ras and loss of cell surface association in H-Ras RIE-1 cells. (A) H-Ras RIE-1 cells were plated at 10^4 cells/well in 12-well cluster dishes. When 70% confluent, cells were treated with L744,832 (1 μ mol/L) or vehicle (DMSO, 1 mg/mL) for the indicated times. Cells were then collected into SDS-PAGE sample buffer for Western blotting as described in Materials and Methods. Data are presented as percentage processed (that is, farnesylated) Ras normalized to untreated controls. (B) Under similar conditions, H-Ras and K-Ras4B RIE-1 cells were treated with increasing concentrations of L744,832 for 24 hours. Fully farnesylated (P) Ras protein migrates faster than the unfarnesylated (U) form. This experiment is representative of 2 separate experiments performed with 3 different concentrations of inhibitor. (C) H- and K-Ras4B RIE-1 cells were plated on coverslips in 12-well cluster dishes. When 70% confluent, cells were treated with FTI (10 μ mol/L) or vehicle (DMSO, 1 mg/mL) for 24 hours, and immunofluorescence for Ras protein was performed as described in Materials and Methods. Ras protein is labeled *green* and nuclei are labeled *blue*.

Figure 7. Exogenous TGF- α overrides FTI growth inhibition of H-Ras RIE-1 cells. Cells were plated at 10,000 cells/well in 24-well cluster plates. Near-confluent cells were treated for 24 hours with the indicated concentrations of FTI or vehicle (DMSO, 1 $\mu\text{L}/\text{mL}$). Cells were then treated for an additional 24 hours with TGF- α (10 ng/mL), TGF- α -neutralizing antibody (S574, 3 $\mu\text{g}/\text{mL}$), or a nonspecific antibody of the same type and species (NS, 3 $\mu\text{g}/\text{mL}$). During the last 2 hours of the final incubation, cells were pulsed with $[^3\text{H}]$ thymidine and then processed as described in Materials and Methods. Number of cells was determined in replicate wells to normalize data.

tion than H-Ras-transformed cells. Because K-Ras mutations occur more commonly in human gastrointestinal neoplasia, we considered whether blockade of EGFR signaling by use of a specific EGFR tyrosine kinase inhibitor (PD153035) might enhance the growth-inhibitory effect of FTI in K-Ras4B-transformed RIE-1 cells.²² Treatment of both H-Ras- and K-Ras4B-transformed RIE-1 cells with PD153035 alone caused a dose-dependent decrease in DNA synthesis (Figure 8A). This occurred without a change in MAPK activation, indicating that EGFR-mediated growth occurs, at least in part, by a pathway independent of the canonical Ras/Raf/MAPKK/MAPK cascade. When H- and K-Ras4B-transformed RIE-1 cells were treated simultaneously with L744,832 and PD153035, there was enhanced growth inhibition of both lines (Figure 8B).

Discussion

The development of FTIs has provided an important tool for studying Ras function and signal transduction. Although FTIs are effective inhibitors of H-Ras transformation in vitro and in vivo,¹¹⁻¹⁸ the lack of an effect in K-Ras-transformed cells and the apparent lack of effects on normal cell signaling suggest that further study of mechanisms underlying FTI drug action is needed. We previously found that activated Ras is capable of transforming RIE-1 cells, whereas activated Raf is not. This contrasts with observations in 3T3 fibroblasts in which both Ras and Raf are transforming.²¹

These observations suggest that Ras transformation in epithelial cells involves additional Ras effector pathways. One such potential pathway is induction of EGF-related peptides; e.g., TGF- α , AR, and HB-EGF. In prior studies, we found that these growth factors are secreted into the conditioned medium of H- and K-Ras4B-transformed RIE-1 cells and are capable of causing morphological transformation and soft agar growth of parental RIE-1 cells.^{21,22} EGFR blockade partially reverts the transformed morphology of Ras RIE-1 cells and prevents the morphological transformation of parental RIE-1 cells caused by Ras-conditioned medium.²²

Using transfected RIE-1 cells, we explored the effect of the FTI L744,832 on Ras-induced EGFR ligand production. We show now that K-Ras4B-transformed intestinal epithelial cells, like K-Ras-transformed fibroblasts^{38,39} are more resistant to growth inhibition by L744,832 than H-Ras-transformed cells. This resistance may be due to a 100-fold greater binding affinity between K-Ras and FTase than H-Ras, or that K-Ras may be a substrate for

Figure 8. Effects of a specific EGFR tyrosine kinase inhibitor PD153035 (TKI) alone and in combination with L744,832 (FTI) on DNA synthesis in Ras-transformed RIE-1 cells. Cells were plated at 10,000 cells/well in 24-well cluster plates. (A) H- and K-Ras4B RIE-1 cells were treated for 24 hours with the indicated concentrations of TKI or vehicle (DMSO, 1 $\mu\text{L}/\text{mL}$). (B) H- and K-Ras4B RIE-1 cells were treated with 10 μM FTI and 1 μM TKI alone and in combination or with vehicle (DMSO, 2 $\mu\text{L}/\text{mL}$), when they had reached 70%–80% confluency. During the last 2 hours of the incubation, cells were pulsed with $[^3\text{H}]$ thymidine and then processed as described in Materials and Methods. Number of cells was determined in replicate wells to normalize data.

compensatory geranylgeranylation in the absence of farnesylation.^{38–42} In fact, an inhibitor of GGPT I disrupts oncogenic K-Ras processing and signaling.³⁷ Parental RIE-1 cells, as well as control transfectants and activated Raf-transfected RIE-1 cells, are unaffected by L744,832 treatment, consistent with previous reports showing that normal cells and cells transfected with oncogenes downstream of Ras signaling are resistant to the effects of FTIs.^{11–18}

The specificity of FTIs for transformed cells is puzzling because Ras function is important in normal mitogenic signaling and is dependent on farnesylation. It is feasible that normal Ras signaling occurs by geranylgeranylated K-Ras in the presence of FTase inhibition.^{20,38–42} Alternatively, normal mitogenic signaling may be mediated through pathways distinct from Ras in some cell types.¹⁹ We observed that L744,832 has no effect on unstimulated parental RIE-1 cellular proliferation and only partially inhibits TGF- α -stimulated proliferation. Interestingly, FTIs also block the growth of a variety of tumor cells without Ras mutations. It is possible that this occurs because activated receptor tyrosine kinases constitutively activate Ras that is then inhibited by FTIs,¹⁹ or that FTI inhibits TGF- α and AR expression, as shown in the present study. In support of this, we recently reported that FTI L744,832 decreases levels of TGF- α in mammary cystic fluid and inhibits the growth of mammary tumors in MMTV-TGF α transgenic mice.⁴³

L744,832 appears to selectively block anchorage-dependent proliferation of H-Ras-transformed RIE-1 cells by inhibiting Ras-mediated EGFR ligand production. Mammalian EGFR ligands include TGF- α , AR, EGF, HB-EGF, betacellulin, and epiregulin.⁴⁴ Of these EGFR ligands, TGF- α , AR, and HB-EGF are induced upon Ras-transformation of RIE-1 cells.²² L744,832 causes inhibition of TGF- α and AR production, which correlates with a parallel loss of functional farnesylated H-Ras protein and its plasma membrane association. In addition, inhibition of proliferation of H-Ras RIE-1 cells by FTI is overcome by addition of TGF- α . FTI-induced loss of farnesylated oncogenic H-Ras protein also lowers MAPK activation; therefore, it is likely that decreases in MAPK activity also contribute to FTI growth inhibition in these cells. However, the ability of the specific EGFR TKI to decrease cellular proliferation in the absence of reduced MAPK activity and the enhanced growth inhibition observed with blockade of both Ras and EGFR signaling emphasize the importance of EGFR signaling to proliferation of Ras-transformed RIE-1 cells. These data suggest that one mechanism of FTI action on Ras-transformed epithelial cells is to block EGF-like autocrine growth factor pathways induced by Ras transfor-

mation. The ability of EGFR blockade to enhance FTI growth inhibition of K-Ras-transformed cells has clinical implications. It is likely that other agents will need to be administered with FTIs to achieve antitumor efficacy because their effects appear cytostatic.⁴⁵

Selected tumor cell lines with activating Ras mutations have been shown to be relatively resistant to effects of L744,832.¹⁹ In these resistant cell lines, FTI did not inhibit MAPK activation in response to growth factor stimulation as it did in sensitive lines.¹⁹ Other effects of FTI, such as those on transformed morphology, do not correlate well with the time course of inhibition of Ras farnesylation.¹⁶ However, some FTI effects on morphology may also be mediated, at least partly, by the loss of secreted factors that contribute to transformed morphology (A. Cox, C. Der, R. Coffey, unpublished results). Collectively, these data indicate that FTI effects on H-Ras transformation may involve multiple complex mechanisms, perhaps involving Ras, Ras-related proteins, and Ras-independent mitogenic signaling pathways.

We have shown that an important mechanism by which FTIs specifically block H-Ras RIE-1 cellular proliferation is by reducing EGFR ligand expression. This observation raises the question as to how essential EGFR ligands are to the Ras-transformed phenotype of epithelial cells in general. This may depend on the cell type under study, the context or stage of carcinogenesis, and the functional redundancy of the EGFR ligand family. In contrast to our finding in RIE-1 cells, TGF- α alone was not able to transform IEC-18 cells, although its depletion in Ras-transformed IEC-18 cells resulted in decreased growth.⁴⁶ Likewise, a role for TGF- α in tumor promotion in the context of Ras transformation has been proposed for skin and mammary carcinoma.^{47,48} A broader issue is the role of EGFR signaling in Ras transformation of epithelial cells. In EGFR null keratinocytes, introduction of oncogenic Ras results in benign tumor formation in nude mouse grafts.⁴⁹ However, growth of *v-ras^{Ha}* papillomas was impaired, suggesting that EGFR signaling may function in tumor promotion rather than initiation.⁴⁹ Current studies will address this in the colon by introduction of oncogenic Ras to a recently established EGFR null mouse colonocyte cell line (R. Whitehead, D. Threadgill, and R. Coffey, personal communication).

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Whipple of Whipple's disease

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George Hoyt Whipple (1878-1976) was born in Ashland, New Hampshire, the son and grandson of country doctors. A boyhood love of the outdoors lasted throughout his life. As an undergraduate at Yale, he distinguished himself as a keen athlete. To support his medical studies at Johns Hopkins he taught at a military academy and in summers worked on lake steamboats in New Hampshire. His M.D. degree in hand, he became a lecturer in pathology, then associate professor at Johns Hopkins. In 1909 he described in meticulous detail the fatal course in a young physician of a previously unrecognized "lipodystrophy," now known as Whipple's disease. In 1914 he returned from a sojourn in Panama gravely ill with malaria; he was admitted to the Presbyterian Hospital in New York where he was cared for by a young intern named Allen Whipple who later became a famous surgeon (no relation, but the two became lifelong friends). On recovery he was lured to the University of California as professor of research medicine, where he furthered his growing reputation. In 1921 he accepted a call to become the founding professor of pathology and dean at the University of Rochester's newly established medical school. Much of his work there centered on the pathophysiology of anemia. With G. R. Minot and W. P. Murphy of Harvard he shared the 1934 Nobel Prize for discoveries leading to the treatment of primary pernicious anemia with liver extract. In tribute to his administration at Rochester, his students composed a doggerel:

"With wisdom born in New Hampshire hills
And high disdain for useless frills,
He sat him down on a three-legged stool,
And man by man there grew a school."

—Contributed by WILLIAM S. HAUBRICH, M.D.
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