Heregulin Regulates Prolactinoma Gene Expression

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Abstract

To investigate the role of p185her2/neu/ErbB3 signaling in pituitary tumor function, we examined these receptors in human prolactinomas. Immunofluorescent p185her2/neu was detected in almost all (seven of eight), and ErbB3 expression in a subset (four of eight) of tumors (seven adenomas and one carcinoma). Quantitative PCR also showed abundant ErbB3 mRNA in tumor specimens derived from a rarely encountered prolactin-cell carcinoma. Activation of p185her2/neu/ErbB3 signaling with heregulin, the ErbB3 ligand, in rat lactosomatotroph (GH4C1) tumor cells specifically induced prolactin (PRL) mRNA expression ~5-fold and PRL secretion ~4-fold, whereas growth hormone expression was unchanged. Heregulin (6 nmol/L) induced tyrosine phosphorylation and ErbB3 and p185her2/neu heterodimerization, with subsequent activation of intracellular ERK and Akt. The Akt signal was specific to ErbB3 activation by heregulin, and was not observed in response to epidermal growth factor activation of epidermal growth factor receptor. Gefitinib, the tyrosine kinase inhibitor, suppressed heregulin-mediated p185her2/neu/ErbB3 signaling to PRL. Heregulin induction of PRL was also abrogated by transflecting cells with short interfering RNA directed against ErbB3. Pharmacologic inhibition of heregulin-induced phosphoinositide-3-kinase/Akt (with LY294002) and ERK (with U0126) signaling, as well as short interfering RNA–mediated mitogen-activated protein kinase-1 down-regulation, showed ERK signaling as the primary transducer of heregulin signaling to PRL. These results show ErbB3 expression in human prolactinomas and a novel ErbB3-mediated mechanism for TKI regulation in experimental lactotroph tumors. Targeted inhibition of up-regulated p185her2/neu/ErbB3 activity could be useful for the treatment of aggressive prolactinomas resistant to conventional therapy.

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Introduction

Prolactinomas arising from pituitary lactotrophs account for ~40% of all pituitary tumors (1, 2). Excess prolactin (PRL) secretion may lead to infertility, sexual dysfunction, and osteoporosis (1). Prolactinoma mass effects may lead to local compressive symptoms including visual field defects and hypopituitarism. Tumorous lactotrophs usually express functional dopamine (D2) receptors, enabling medical therapy with dopamine agonists to suppress PRL synthesis and secretion and tumor growth (1). However, dopamine agonist resistance encountered in a subset of patients with PRL-secreting pituitary adenomas limits therapeutic efficacy (3). Effective treatments are also needed for invasive refractory macroprolactinomas and for those aggressive tumors rarely undergoing malignant transformation resulting in a poor prognosis (4–6).

Mechanisms underlying pituitary tumor aggressive behavior and malignant transformation are largely unclear. Dopamine agonist resistance may be due to decreased D2 receptor or differential isoform expression, and or disrupted autocrine growth factor signaling (1, 3). ErbB receptors are comprised of four subtypes (EGFR, p185her2/neu, ErbB3, and ErbB4; ref. 7), and the kinase-deficient ErbB3 requires dimerization with p185her2/neu to affect signaling (8). Reports of increased ErbB receptor member expression, particularly in aggressive pituitary tumors and pituitary carcinomas (9–13), could reflect tumor progression to a more dedifferentiated state and associated loss of growth control. For such patients, targeted ErbB receptor inhibition could provide an alternative medical control of tumor growth and or hormone secretion, as recently shown for experimental rat prolactinomas (14).

Discovery of aberrant ErbB receptor activation in human cancers has led to selective therapeutic targeting particularly of epidermal growth factor receptor (EGFR) and p185her2/neu (ErbB2) with monoclonal antibodies and small compound tyrosine kinase inhibitors (TKI; ref. 7). However, recent studies examining the mechanisms for TKI resistance have highlighted the importance of kinase-inactive ErbB3 expression, and associated activation of the phosphatidylyl-3-OH kinase (PI3K)/Akt survival pathway (15, 16).

Here, we show ErbB3 receptor expression in a subset of human prolactinomas and report the functional role of p185her2/neu/ErbB3 signaling in experimental rat lacto-somatotroph hormone regulation.

Materials and Methods

Materials. Ham's F10 media, fetal bovine serum, penicillin, streptomycin, and amphoteracin B were purchased from Invitrogen. Epidermal growth factor (EGF) was from Sigma, and heregulin (NRG1-β1/HRG1-β1) was from R&D Systems. Gefitinib (Iressa) was purchased from Biaffin GmbH & Co., U0126 from Promega, and LY294002 from Calbiochem.

Cell cultures. GH4C1 rat lacto-somatotroph cells were purchased from the American Type Culture Collection. After synchronization by serum starvation (medium containing 0.2% bovine serum albumin for ~24 h), cells were plated in 100-mm dishes (~1.5 × 106 cell density), six-well plates (~0.5 × 106 cell density), or 24-well plates (~50,000 cell density) and treatment agents added with fresh serum-depleted medium (with 0.2% bovine serum albumin) and samples collected as indicated.

Pituitary glands obtained from female Wistar-Furth rats (140–160 g body weight) were dissociated to single-cell suspensions by enzymatic degradation of the extracellular adhesion proteins with the Neural Tissue Dissociation Kit (papain) according to the manufacturer's instructions (Miltenyi Biotech GmbH). Animal procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee. Cells
were incubated in complete medium (DMEM containing 10% fetal bovine serum and antibiotics) for 24 h and then in serum-free medium (0.3% bovine serum albumin) for 8 h, followed by treatment with heregulin for the indicated times. At the end of each experiment, supernatants were collected for growth hormone (GH) and PRL assays, and cells were dissolved in TRIZOL reagent (Invitrogen) and stored at –80°C.

Short interfering RNA transfections. GH4C1 cells grown in six-well plates to ~50% confluency were serum-starved overnight and transfected with 100 pmol of Silencer Select Negative Control no. 1 (Scr) short interfering RNA (siRNA). Silencer Select Predesigned rat mitogen-activated protein kinase-1 (MAPK1) siRNA (ID: siR18100), ErbB2 (Neu) siRNA (ID: siR077710), ErbB3 siRNA (ID: siR13557; Applied Biosystems/Ambion) or both ErbB2 (Neu) and ErbB3 siRNA (50 pmol each) for 24 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After an additional 8 h of incubation (serum-free medium), treatments were added and samples collected at the indicated times.

Templates for probes and Northern blot analysis. RNA extraction was performed using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The β-actin probe was a 1.076 kb fragment of the mouse β-actin gene (Ambion). Probes for rat GH and PRL were generated and Northern blot analysis performed as previously described (14).

Immunoprecipitation and Western blotting. After completion of treatments, cells were placed on ice and washed with cold PBS. For whole cell protein extraction, cells were lysed in 150 μL of radioimmunoprecipitation assay buffer (Sigma), and for immunoprecipitation experiments, cells were lysed in 500 μL of modified radioimmunoprecipitation assay buffer [1% Triton X-100, 1% deoxycholate, 0.1% NaDodSO4, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate (pH 7.4), 2 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, and 400 μmol/L sodium orthovanadate], containing complete protease inhibitor cocktail tablets (Roche Molecular Biochemicals) and phosphatase inhibitor cocktail 2 (Sigma). Lysates were centrifuged at 13,000 × g for 20 min at 4°C and protein concentrations determined by Bradford's method (Bio-Rad). Approximately 1 mg of protein was immunoprecipitated with rabbit polyclonal anti-EGFR (1005), anti-ErbB3 (C-17; 2 μg; Santa Cruz Biotechnology) and with monoclonal antibody 7.16.4 (ref. 17; 3 μg; a kind gift from Dr. M.I. Greene, University of Pennsylvania, Philadelphia, PA) which reacts specifically with rat p185 molecules. Preclearing was performed with A/G PLUS-Agarose beads (20 μL; Sigma) overnight at 4°C. Immunoprecipitation with appropriate antibody titers was performed for 1 h prior to the addition of A/G PLUS-Agarose beads (20 μL) overnight at 4°C. Immunoprecipitates were washed once in 10-fold wash volume in washing buffer and resuspended in SDS sample buffer (pH 6.8) as previously described (18).

Western blot analysis was performed according to the guidelines of NuPAGE electrophoresis system protocol (Invitrogen). In brief, whole cell lysates (~50 μg protein per lane) or immunoprecipitation samples were heated for 5 min at 100°C, respectively. Proteins were separated on NuPAGE 4% to 12% Bis-Tris gels and electrotransferred for 1 h to polyvinylidene difluoride (Invitrogen). Membranes were blocked for 1 h in 2% nonfat dry milk (or 5% bovine serum albumin) in TBS-T buffer, and incubated overnight with primary antibody. The following primary antibodies were used: mouse anti-ERK1/2, rabbit anti-ERK1/2 (1:100; Santa Cruz Biotechnology), mouse monoclonal anti-pTyr (P999), rabbit polyclonal anti-EGFR (1005), anti-Neu (C-18), anti-ErbB3 (C-17, I:200; Santa Cruz Biotechnology), rabbit monoclonal anti-pAkt (phosphorylated S473, I:1,000; Abcam), rabbit polyclonal anti-Akt, and anti-pGAPDH (1:1,000; Cell Signaling). After washing with TBS-T, membranes were incubated with peroxidase-conjugated secondary antibody for 1.5 h (~20% nonfat dry milk or 5% bovine serum albumin in TBS-T buffer). Blots were washed and hybridization signals measured by enhanced chemiluminescence detection system (Amersham).

Immunofluorescence. Tumor specimens were fixed in 10% formalin and embedded in paraffin. After deparaffinization of the sections, antigen retrieval was performed using citrate and permeabilization by 0.1% Triton X. Slides were blocked in 10% goat serum in 1% bovine serum albumin-PBS and then incubated with primary antibody overnight at 4°C. The following antibodies were used: rabbit polyclonal anti-Neu (C-18) and anti-ErbB3 (C-17; I:100; Santa Cruz Biotechnology). Following washes, slides were incubated with Alexa Fluor goat anti-rabbit 488 (H+L) secondary antibody (1:500; Invitrogen) for 2 h at room temperature. Nuclei were stained using 1:500 Topro-3 iodide 1 mmol/L solution (1:250 in PBS; Molecular Probes, Inc.) for 2 h at room temperature, and following such, slides were mounted with Prolong Gold antifade reagent (Invitrogen). Confocal microscope images were obtained using a TCS-SP confocal scanner (Leica Microsystems). To detect the contributions of autofluorescence in these paraffin-embedded tissues, a spectral imaging approach was used. The confocal spectrophotometer was set to detect specific FITC fluorescence ranging from 505 to 540 nm. A second channel detecting autofluorescence with wavelengths from 560 to 600 nm was used, and both channels color-coded and merged. Green represents specific fluorescence from FITC and red, autofluorescence. The staining was strong and autofluorescence was very low in comparison to the specific signal. Only erythrocytes showed appreciable autofluorescence and appear dark orange in the images. A Leica PlanApo 20× 0.7 numerical aperture lens was used for overview images and a PlanApo 40× 1.2 numerical aperture lens was used for high-magnification images.

Quantitative real-time PCR. Total RNA was extracted with Trizol Reagent (Invitrogen) according to the instructions of the manufacturer. Total RNA was reverse-transcribed into first-strand cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative PCR reactions were carried out in the iQ5 Multicolor Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) as follows: 95°C for 3 min, 1 cycle; 95°C for 15 s, 8°C for 1 min, 40 cycles. Melting curve analysis was performed to ensure product specificity. Certified RT2 qPCR Primer Assays for human ErbB2 and ErbB3 were purchased from SuperArray. Human housekeeping gene primer set (10 genes) was from Real-time Primers. This set included primers for β-glucoronidase (GUSB), β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transferring receptor (TFRC), phosphoglycerate kinase 1 (PGK1), hypoxanthin phosphoribosyl-transferase (HPRT1), peptidyl-propyl-isomerase A (PPIA), ribosomal protein L13A (RPL13A), TATA box-binding protein (TBP), and β2-microglobulin (B2M).

Hormone assays. RIA for rat GH and PRL were performed using reagents provided by the National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases (Harbor-UCLA Medical Center, Torrance, CA) as described (14).

Statistical analysis. Cell cycle phases were analyzed by ModFit LT software (version 2.0, Becton Dickinson). NIH Image 1.59 software was used for densitometric analysis of specific bands in blots and comparisons evaluated using two-tailed Student’s t test. Results are expressed as mean ± SE of independently performed experiments. Statistical significance was set at P < 0.05.

Results

ErbB family receptor expression and activation in rat pituitary GH4C1 cells. The functional role of pituitary prolactin-norma ErbB3 expression was examined in rat lacto-somatotroph GH4C1 cells. Immunoprecipitation and immunoblotting with receptor-specific antibodies showed the expression of EGFR (175 kDa), p185Neu (185 kDa), and ErbB3 (185 kDa) in GH4C1 cells. The EGFR ligand, EGF (5 nmol/L), induced EGFR and p185Neu tyrosine phosphorylation, but did not result in ErbB3 phosphorylation. In contrast, heregulin (6 nmol/L), the ErbB3 and ErbB4 ligand, induced tyrosine phosphorylation of ErbB3 and p185Neu but not that of EGFR (Fig. 1A). Thus, ErbB1–3 receptors seem to be functional in GH4C1 cells, and p185Neu likely heterodimerizes with both activated EGFR and ErbB3.

Increasing heregulin concentrations selectively induced PRL mRNA expression up to ~5-fold at 48 hours (P < 0.05), with no observed effect on GH mRNA expression (Fig. 1B). Dose-dependent heregulin-mediated induction of PRL secretion was also confirmed in the cell culture medium (Fig. 1C). Heregulin increased PRL
release ~4-fold at 48 hours ($P < 0.05$), with no effect on GH secretion. Heregulin did not induce GH4C1 cell proliferation, as assessed by cell cycle analysis and cell proliferation assays (data not shown). Heregulin did not induce PRL in nontumorous mixed primary rat pituitary cell cultures derived from female Wistar-Furth rats (data not shown).

**Heregulin-mediated p185$^c$-neu/ErbB3 signaling.** In time course experiments (Fig. 2A), treatment of GH4C1 cells with heregulin (6 nmol/L) induced rapid (within 5 min) ErbB3 tyrosine phosphorylation. High levels of receptor phosphorylation were observed for up to 60 min, and gradually declined thereafter. Heregulin also induced marked p185$^c$-neu tyrosine phosphorylation for up to 30 min. Similar to the patterns of immunoprecipitated receptor activation, heregulin induced ERK and Akt phosphorylation in whole cell extracts as detected by Western blot. Although ERK activation occurred both in response to EGF (with EGF; Fig. 2B) and ErbB3 (with heregulin; Fig. 2A), induction of Akt signaling was specific for p185$^c$-neu/ErbB3 signaling. Formation of heregulin-mediated p185$^c$-neu/ErbB3 heterodimers was confirmed by immunoprecipitation (Fig. 2C).

As gefitinib blocks heregulin signaling by inducing the formation of inactive heterodimers between ErbB3 and other ErbB receptor members (19, 20), we examined the effects of TKI on heregulin-induced ErbB receptor activation and downstream signaling. As shown in Fig. 3A, pretreatment with increasing gefitinib concentrations suppressed both receptor activation and signaling. Interestingly, inhibition of heregulin-induced ERK and Akt phosphorylation was observed at the lowest concentration tested (0.1 $\mu$mol/L), whereas suppression of p185$^c$-neu and ErbB3 tyrosine phosphorylation was observed with higher gefitinib doses (0.5 and 5 $\mu$mol/L). Gefitinib also dose-dependently prevented heregulin-induced p185$^c$-neu/ErbB3 heterodimerization (Fig. 3B) and PRL secretion (Fig. 3C).

**Functional role of pituitary p185$^c$-neu/ErbB3 heterodimer signaling.** To further explore the mechanisms for the observed heregulin-mediated effects through p185$^c$-neu and ErbB3 receptor activation, we used siRNA to selectively down-regulate these respective ErbB receptor members. Under these experimental conditions, we observed up to 60% reduction of p185$^c$-neu and ErbB3 expression, which was associated with decreased total...
tyrosine phosphorylation in response to heregulin (Fig. 4A). With down-regulated p185c-neu and or ErbB3 levels, heregulin-induced PRL mRNA (Fig. 4B) and PRL hormone (Fig. 4C) expression were attenuated to almost basal levels, whereas no effects on GH were observed, demonstrating both the specificity of, and requirement for, both p185c-neu and ErbB3 for heregulin-mediated PRL induction.

As heregulin induces ErbB receptor–mediated ERK and Akt signaling (Figs. 2A and 3A) we used a pharmacologic approach to block ERK or Akt activation, to identify pathways, specifically mediating heregulin action on the lactotroph. The PI3K inhibitor LY294002 dose-dependently suppressed heregulin-induced Akt phosphorylation (Fig. 5Aa), confirming the activity of the inhibitor used. However, at the same concentrations, LY294002 had no effect...
on heregulin-induced PRL mRNA (Fig. 5A) and PRL hormone (Fig. 5C) expression. The dose-dependent effects of the MEK inhibitor U0126 on heregulin-induced ERK phosphorylation are shown in Fig. 5D. In contrast to PI3K/Akt signaling blockade, the MEK inhibitor dose-dependently suppressed heregulin-induced PRL mRNA expression (Fig. 5E) and PRL secretion (Fig. 5F). The specific involvement of ERK in mediating heregulin-induced PRL expression was confirmed by siRNA experiments (Fig. 5B). Suppression of MAPK1 by siRNA attenuated heregulin-induced PRL expression was confirmed by siRNA experiments (Fig. 5B). The specific involvement of ERK in mediating heregulin-induced PRL mRNA expression (Fig. 5A) and ERK phosphorylation was confirmed by siRNA experiments (Fig. 5B). The specific involvement of ERK in mediating heregulin-induced PRL expression was confirmed by siRNA experiments (Fig. 5B). The specific involvement of ERK in mediating heregulin-induced PRL expression was confirmed by siRNA experiments (Fig. 5B). The specific involvement of ERK in mediating heregulin-induced PRL expression was confirmed by siRNA experiments (Fig. 5B).

**ErbB2 and ErbB3 expression in human PRL-secreting pituitary adenomas.** Immunofluorescence staining for ErbB2 (p185her2/neu) and ErbB3 was performed in eight human PRL-secreting tumors (Supplementary Table). Human breast carcinoma tissue samples were used as immunoreactive controls, whereas antibody-specific blocking peptides (sc-284-P and sc-285-S) suppressed the fluorescent signal, confirming the specificity of the antibody reactions (Supplementary Figs. S1 and S2).

Two patients (cases 7 and 8) underwent multiple surgeries due to dopamine agonist resistance; case 8 eventually evolved into a carcinoma with loss of PRL immunoreactivity. Positive ErbB2 staining with varying intensities was observed in seven of eight tumor specimens, whereas tumor tissue obtained from four cases was immunoreactive for ErbB3 (Fig. 6A), and also exhibited a Ki-67 index of ≥4% (Supplementary Table).

ErbB2 and ErbB3 mRNA expression were compared by quantitative PCR analysis of two tumor specimens derived from a patient who initially underwent surgery for an invasive but histologically benign prolactinoma (specimen obtained in 1997). Tissue was not available for immunostaining. Progressive resistance to high-dose dopamine agonists necessitated four consecutive trans-sphenoidal pituitary surgeries due to tumor remnant regrowth. Gamma-knife radiotherapy temporarily stabilized the tumor mass and PRL levels, however, subsequent tumor enlargement caused loss of unilateral vision. The surgical tumor specimen obtained in 2008 showed a Ki-67 index of 20%. When we used two housekeeping genes (GAPDH and TFRC) for internal normalization, ErbB2 and ErbB3 mRNA expression levels were observed to be elevated ~5 and 41-fold, respectively, in the 2008 specimen (Fig. 6Ba). When 10 housekeeping genes (most of which also exhibited up-regulation) were evaluated in the assay, ErbB2 and ErbB3 expression levels were shown to be increased ~1 and 11-fold, respectively (Fig. 6Bb).

**Discussion**

Pituitary adenomas account for ~15% of primary intracranial neoplasms and are discovered in up to 25% of unselected autopsy specimens (2). PRL-secreting adenomas account for ~40% of all pituitary tumors (1). Indications for prolactinoma therapy include hyperprolactinemia-associated hypogonadism, infertility, and osteoporosis, as well as central compressive effects (1, 21). Drug intolerance or the development of dopamine agonist resistance may constrain medical therapy of benign prolactinomas (3). Effective drug therapies are also required for recurrent invasive macroprolactinomas or those that undergo rare malignant transformation and are resistant to attempts at surgery and radiation (4–6).

We show here that human prolactinomas express ErbB3 receptors, and that heregulin, an ErbB3 ligand, induces PRL gene expression. Expression of other ErbB receptor family members EGFR (22–27) and p185her2/neu (11, 13, 25) have been reported in the normal anterior pituitary. EGFR (9, 10, 13, 25–27) and p185her2/neu (11, 25, 28, 29) expression in pituitary adenomas have been associated with more invasive tumor phenotypes (9–13), suggesting a role for these receptors in aggressive tumor behavior. Estrogen-mediated rat lactotroph hyperplasia is also associated with the up-regulation of pituitary transforming growth factor-α, an EGFR ligand (30), and lactotroph transforming growth factor-α.
overexpression in transgenic mice results in hyperplasia and pituitary adenoma formation (31). EGFR tyrosine kinase inhibition was shown to inhibit EGF-induced rat lactotroph differentiation and tumor growth (14), indicating a role for EGFR signaling in prolactinoma regulation.

ErbB3 activation and subsequent PI3K/Akt signaling may contribute to TKI resistance in breast and lung cancer (15, 16). p185her2/neu and ErbB3 are functionally incomplete receptors. The extracellular domain of p185her2/neu seems to be devoid of ligand-binding activity, whereas ErbB3 contains a nonfunctional kinase domain devoid of catalytic kinase activity. However, the p185her2/neu/ErbB3 heterodimer is the most active signaling dimer in the ErbB family (8). Ligand induction leads to ErbB3 transphosphorylation (32), the only ErbB receptor which couples directly to PI3K/Akt (33). Although pituitary heregulin (glial growth factor) expression has been reported (34), ErbB3 expression and the role of p185her2/neu/ErbB3 signaling in pituitary tumorigenesis were not heretofore known.

Rat lacto-somatotroph GH4C1 cells are shown here to express functional ErbB receptor members (Fig. 1A), and p185her2/neu tyrosine phosphorylation was observed in response to both EGF and ErbB3 activation, suggesting p185her2/neu as the preferred dimerization partner in these cells. p185her2/neu tyrosine phosphorylation was detected only in response to ligand induction, suggesting the expression of non-mutated proto-oncogenic p185her2/neu, similar to the observed absence of p185her2/neu activating mutations in human pituitary adenomas (28).

Heregulin induction of PRL (Fig. 1B and C), suggests a functional role for p185her2/neu/ErbB3 signaling in PRL but not GH control, consistent with known GH regulation (35). In contrast to EGFR and p185her2/neu (11, 13, 22–27), nontumorous pituitary lactotrophs likely do not express ErbB3, as we did not observe PRL regulation by heregulin in primary rat pituitary cell cultures. ErbB receptor-mediated PRL regulation may be selective for transformed lactotrophs with altered intracellular signaling and loss of dopaminergic control. Indeed, GH3/GH4C1 cells do not express functional D2 dopamine receptors (36) and are resistant to dopamine agonists, similar to drug-resistant aggressive prolactinomas.

Aberrant Akt (37) and MAPK (38) activity in human pituitary tumors, and heregulin-mediated ErbB3 and p185her2/neu tyrosine phosphorylation and receptor heterodimerization we observed, was associated with ERK and Akt activation. EGF, the EGFR ligand, also activated ERK but not PI3K/Akt signaling, as previously shown for GH3 cells (14), demonstrating the requirement of pituitary ErbB3 activation for PI3K/Akt signaling induction. As targeted ErbB3-specific therapies are not currently available, we used pharmacologic inhibition of the receptor with gefitinib, an EGFR TKI, which induces the formation of inactive heterodimers between ErbB3 and other ErbB receptor members, thereby blocking heregulin signaling (19, 20). Indeed, gefitinib treatment suppressed ErbB3 and p185her2/neu tyrosine phosphorylation and downstream signaling. Dose-dependent differences of gefitinib inhibition on receptor (higher doses) versus ERK and Akt activation (lower

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**Figure 5.** ERK signaling mediates heregulin-induced PRL. A, GH4C1 cells were serum-starved overnight and pretreated for 45 min with the PI3K inhibitor LY294002 (a–c) or the MEK inhibitor U0126 (d–f). Cells were treated with HRG (6 nmol/L) for 10 min (a and d) or 48 h (b–c and e–f). Phosphorylated Akt and total Akt (a), as well as phosphorylated ERK and total ERK (d), were detected by Western blot in whole protein extracts. GH and PRL mRNA expression and protein secretion were determined by Northern blot (b and e) and RIA (c and f) as for Fig. 1. Representative of three independently performed experiments (mean ± SE). *, P < 0.05 versus control group; †, P < 0.05 versus HRG only (–) group. B, GH4C1 cells were serum-starved overnight, transfected with negative control (Scr) or rat MAPK1 (100 pmol) siRNA (24 h). After an additional 8 h (serum-free medium), cells were treated with HRG (6 nmol/L) for 10 min (a) or 48 h (b and c). Phosphorylated ERK and GAPDH loading controls were detected by Western blot in whole protein extracts. GH and PRL mRNA expression and protein secretion were determined by Northern blot and RIA as for Fig. 1. Representative of three independently performed experiments (mean ± SE). ***, P < 0.001 versus Scr HRG group.
doses) are likely due to differences in assay sensitivities, as the blots for immunoprecipitated ErbB3 and p185<sup>c-neu</sup> show total tyrosine phosphorylation load, and are not reflective of specific tyrosine residues which signal for ERK and Akt. Although low doses of gefitinib could inhibit transactivated EGFR on ERK signaling by heresulin, the ligand did not seem to induce EGFR transactivation (Fig. 1A).

Individual p185<sup>c-neu</sup> and ErbB3 down-regulation suppressed heresulin effects, indicating the requirement of functional pituitary p185<sup>c-neu</sup>/ErbB3 signaling for heresulin action on PRL. In the presence of heresulin, specific pharmacologic and siRNA-mediated inhibition of ERK, but not Akt signaling, mediated PRL regulation, consistent with known Ras/Raf/MAPK/Ets-1–mediated PRL regulation in GH3/GH4 cells (39, 40). However, downstream involvement of additional Ras/MAPK-independent mechanisms (as has been shown for EGF–mediated PRL regulation; refs. 41, 42) cannot be excluded. In contrast to PRL-releasing peptide and insulin, however, which signal to the proximal rat PRL promoter in a pathway that involves PI3K/Akt (43), p185<sup>c-neu</sup>/ErbB3–mediated PI3K/Akt activation does not seem to induce lactotroph differentiation but could reflect changes leading to a more aggressive cell phenotype.

The unavailability of functional human pituitary cell lines and scarcity of surgically obtained human prolactinoma specimens renders mechanistic analysis of heregulin signaling in human prolactinomas an experimental challenge. We examined p185<sup>her2/neu</sup> and ErbB3 expression in seven benign prolactinomas and one carcinoma (Hardy class 4) by immunofluorescence and showed p185<sup>her2/neu</sup> expression in the majority of tumors (seven of eight). In addition, we now also show ErbB3 expression in a subset of these tumors (four of eight), a novel finding which may indicate altered patterns of tumor differentiation. Tumors which were immunoreactive for ErbB3 expressed relatively high levels of Ki-67, a marker of increased pituitary cell proliferation (44). ErbB3 immunoreactivity was also detected in an aggressive carcinoma specimen. Quantitative PCR analysis of available RNA samples derived from a single individual who initially presented with a benign prolactinoma, which recurred as a malignant prolactinoma with dopamine resistance and failed surgery and radiation, revealed dramatic up-regulation of ErbB3 mRNA expression in the second tumor sample.

In addition to the potential efficacy of temozolomide therapy (45, 46), and the development of new generation PRL receptor antagonists (47), these results highlight the importance of examining ErbB receptor expression in surgical prolactinoma specimens. Benign prolactinomas usually respond effectively to medical therapy with dopamine agonists, and drug resistance may be an early sign of aggressive tumor behavior. In very rare cases of
malignant tumor transformation during the observed course of the disease, and resistance to conventional therapy, information on progressive ErbB receptor status would provide an additional tool for determining therapy options for controlling tumor cell growth and excess PRL secretion.

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

References
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