FAK signaling is critical for ErbB-2/ErbB-3 receptor cooperation for oncogenic transformation and invasion

Naciba Benlimame,[1,2] Qiang He,[1,2] Su Jie,[1,2] Dingzhang Xiao,[1,2] Ying Jie Xu,[1,2] Martin Loignon,[1,2] David D. Schlaepfer,[3] and Moulay A. Alaoui-Jamali[1,2]

[1]Department of Medicine and [2]Department of Oncology, Lady Davis Institute of the Sir Mortimer B. Davis Jewish General Hospital, McGill University, Montreal, Quebec H3T 1E2, Canada
[3]The Scripps Research Institute, La Jolla, CA 92037

The overexpression of members of the ErbB tyrosine kinase receptor family has been associated with cancer progression. We demonstrate that focal adhesion kinase (FAK) is essential for oncogenic transformation and cell invasion that is induced by ErbB-2 and -3 receptor signaling. ErbB-2/3 overexpression in FAK-deficient cells fails to promote cell transformation and rescue chemotaxis deficiency. Restoration of FAK rescues both oncogenic transformation and invasion that is induced by ErbB-2/3 in vitro and in vivo. In contrast, the inhibition of FAK in FAK-proficient invasive cancer cells prevented cell invasion and metastasis formation. The activation of ErbB-2/3 regulates FAK phosphorylation at Tyr-397, -861, and -925. ErbB-induced oncogenic transformation correlates with the ability of FAK to restore ErbB-2/3–induced mitogen-activated protein kinase (MAPK) activation; the inhibition of MAPK prevented oncogenic transformation. In contrast, the inhibition of Src but not MAPK prevented ErbB–FAK-induced chemotaxis. In migratory cells, activated ErbB-2/3 receptors colocalize with activated FAK at cell protrusions. This colocalization requires intact FAK. In summary, distinct FAK signaling has an essential function in ErbB-induced oncogenesis and invasiveness.

Introduction

The ErbB family of tyrosine kinase receptors includes EGF receptor (ErbB-1), ErbB-2, -3, and -4. The overexpression of specific members of the ErbB tyrosine kinase receptor family, particularly ErbB-2 (Her-2), has been associated with poor prognosis and invasiveness in human cancer (Slamon et al., 1987) as well as ErbB-2/Neu transgenic mice (Guy et al., 1992).

ErbB receptor ligands are divided into three categories: (1) those that bind EGF receptor alone, such as EGF; (2) those that bind to ErbB-3 or -4, which are represented by heregulins (HRGs); and (3) those that bind to ErbB-4 or EGF receptor, such as betacellulin. Ligand binding to the receptor induces receptor autophosphorylation, homodimerization, and heterodimerization, with ErbB-2 being the preferred partner for heterodimerization (Pinkas-Kramarski et al., 1996). The biological activity of ErbB receptors is attributed primarily to cooperative signaling via ErbB heterodimers, whereas homodimers are weakly active or are devoid of kinase activity (e.g., ErbB-3; Guy et al., 1994). Interestingly, the cooverexpression of multiple ErbB receptors within the same tissue and cell is common in invasive cancers from humans (Lemoine et al., 1992; Alimandi et al., 1995; Naidu et al., 1998; Xia et al., 1999) and transgenic mice (Siegel et al., 1999).

The mechanisms by which ErbB overexpression contributes to tumor cell invasion are not fully understood. One important early event that has been implicated as a potential molecular switch for cell migration induced by growth factors is the activation of the nonreceptor FAK. FAK is a major protein of the focal adhesion complex that plays a key role in cell migration and matrix survival signals (Ilic et al., 1995; Frisch et al., 1996; Sieg et al., 1999). FAK is activated by a number of growth factors, including the ErbB ligands EGF (Sieg et al., 2000; Lu et al., 2001) and HRG (Vadlamudi et al., 2002), and follows integrin clustering in response to components of the extracellular cell matrix.

N. Benlimame and Q. He contributed equally to this paper.

Correspondence to Moulay A Alaoui-Jamali: moulay.alaoui-jamali@mcgill.ca

Q. He's present address is Department of Hepatobiliary Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510089, China.

Abbreviations used in this paper: CT, COOH terminus; ERK, extracellular regulated kinase; HRG, heregulin; NT, NH_2 terminus; siRNA, short inhibitory RNA.

The online version of this article contains supplemental material.

The Journal of Cell Biology, Vol. 171, No. 3, November 7, 2005 505–516
http://www.jcb.org/cgi/doi/10.1083/jcb.200504124

In this study, we dissected the function of FAK in oncogenic transformation versus cell invasion that is induced by the cooperation between ErbB-2 and -3 tyrosine kinase receptors in the context of receptor overexpression. These receptors were overexpressed as single and paired combinations using $FAK^{+/+}$ cells, $FAK^{-/-}$ cells, $FAK^{-/-}$ in which FAK was reconstituted, and invasive human breast cancer cells in which FAK was inhibited by short inhibitory RNA (siRNA). We demonstrate that ErbB-induced oncogenic transformation and cell invasion are dependent on FAK. ErbB-2/3–induced oncogenic transformation is FAK–Src–MAPK dependent, whereas ErbB-2/3–induced cell invasion is FAK–Src dependent.

Results

Expression and activation of ErbB receptors in $FAK^{+/+}$ and $FAK^{-/-}$ cells

Both $FAK^{+/+}$ and $FAK^{-/-}$ cells express very low levels of ErbB-1, but -2, -3, and -4 were not detected by Western blot analysis (Fig. 1 A), making this model very appropriate to address the biological impact of ErbB overexpression. We focused on ErbB-2 and -3 overexpression based on preliminary data showing that FAK-proficient cells cooverexpressing the ErbB-2 and -3 combination were the most invasive in vivo and on the Boyden chamber assay compared with cells overexpressing the other ErbB receptor combinations (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200504124/DC1). $FAK^{+/+}$ and $FAK^{-/-}$ cells were stably transduced with a retrovirus that expresses ErbB-2 or -3 receptor and enhanced GFP. Receptor expression in cells that were transduced with control retroviral particles or particles encoding ErbB-2 and/or -3 receptors was confirmed by Western blot assay (Fig. 1 A) and immunofluorescence analysis (Fig. 1 B).

ErbB tyrosine phosphorylation after 10 min of cell stimulation with 20 ng/ml EGF or HRG was measured by receptor immunoprecipitation and Western blot analysis (Fig. 1 C). Constitutive receptor autophosphorylation in the absence of ligand was seen in both $FAK^{-/-}$ and $FAK^{+/+}$ cells overexpressing ErbB-2 receptor alone or in combination with ErbB-3, which is consistent with the property of ErbB-2 to undergo constitutive autophosphorylation in the absence of ligand activation (Pinkas-Kramarski et al., 1996). In contrast, EGF or HRG induced no or a slight increase in ErbB-3 phosphorylation in cells overexpressing ErbB-3 alone (Fig. 1 C). ErbB-3 has an impaired kinase activity (Guy et al., 1994), and the slight increase in phosphorylation upon stimulation with EGF or HRG (Fig. 1 C) is likely attributed to transphosphorylation by endogenous ErbB-1 that is present in these cells (Fig. 1 A). Coexpression of ErbB-2 with -3 in $FAK^{-/-}$ and $FAK^{+/+}$ cells resulted in robust ErbB phosphorylation, particularly after stimulation with the ErbB-3 ligand HRG as a result of receptor heterodimerization and transphosphorylation.

FAK is required for ErbB-induced oncogenic transformation

Parental $FAK^{-/-}$ and $FAK^{+/+}$ cells exhibit no apparent morphological changes that reflect cell transformation, and they

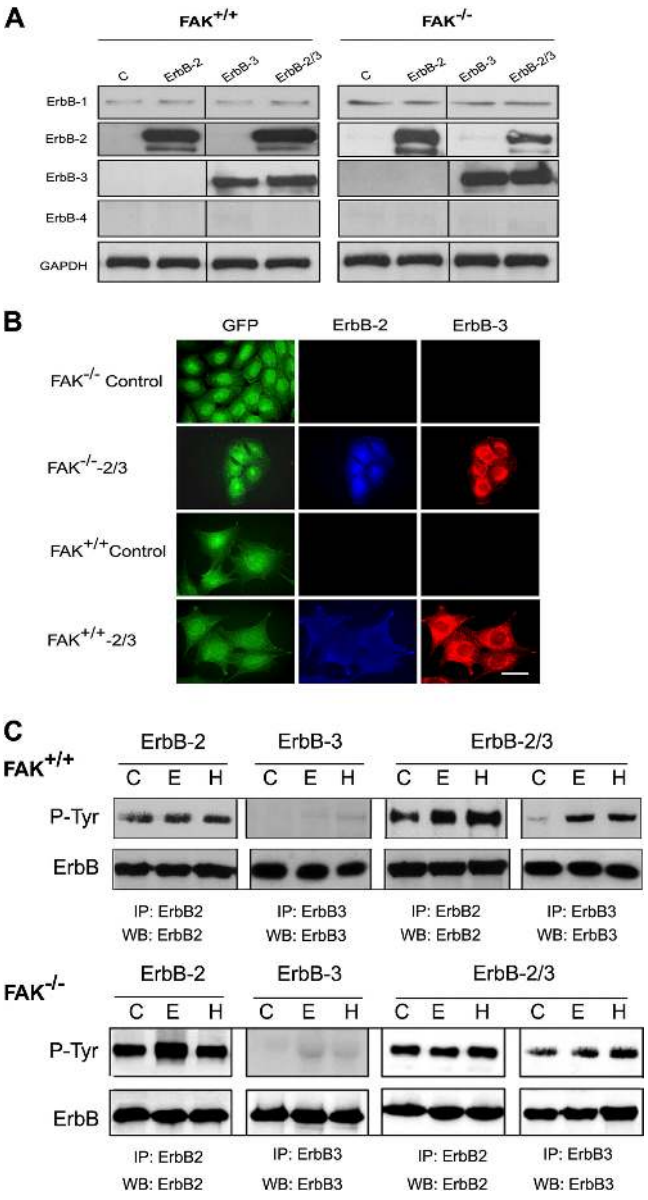

Figure 1. **Overexpression of ErbB-2, -3, and -2/3 in $FAK^{+/+}$ and $FAK^{-/-}$ cells.** (A) Cells expressing control retroviral particles or ErbB receptors were subjected to Western blotting using specific ErbB antibodies as described in Materials and methods. (B) Cells were fixed and double stained for ErbB-2 and -3 using specific antibodies and were examined by immunofluorescence microscopy. Note that $FAK^{-/-}$ and $FAK^{+/+}$ control cells do not express any ErbB-2 or -3 receptors, whereas $FAK^{-/-}$-2/3 and $FAK^{+/+}$-2/3 exhibited strong labeling for both receptors. Bar, 40 μm. (C) Cells were serum starved for 24 h and kept unstimulated (control, C) or were stimulated with 20 ng/ml EGF (E) or HRG (H) for 10 min. Cell lysates were immunoprecipitated with anti-ErbB, and the blots were probed using antiphosphotyrosine antibody and reprobed with the corresponding ErbB-specific antibody.

lack the ability to grow on soft agar and form tumors in immunocompromised mice (see Fig. 3). Neither control $FAK^{+/+}$ nor $FAK^{-/-}$ cells expressing empty retroviral particles that were used to express ErbB receptors formed colonies in soft agar. In contrast, $FAK^{+/+}$-2 and -2/3 cells, but not $FAK^{+/+}$-3 or any $FAK^{-/-}$–ErbB-expressing cells, were able to grow on soft agar and form large foci; the coexpression of ErbB-2 with -3 re-

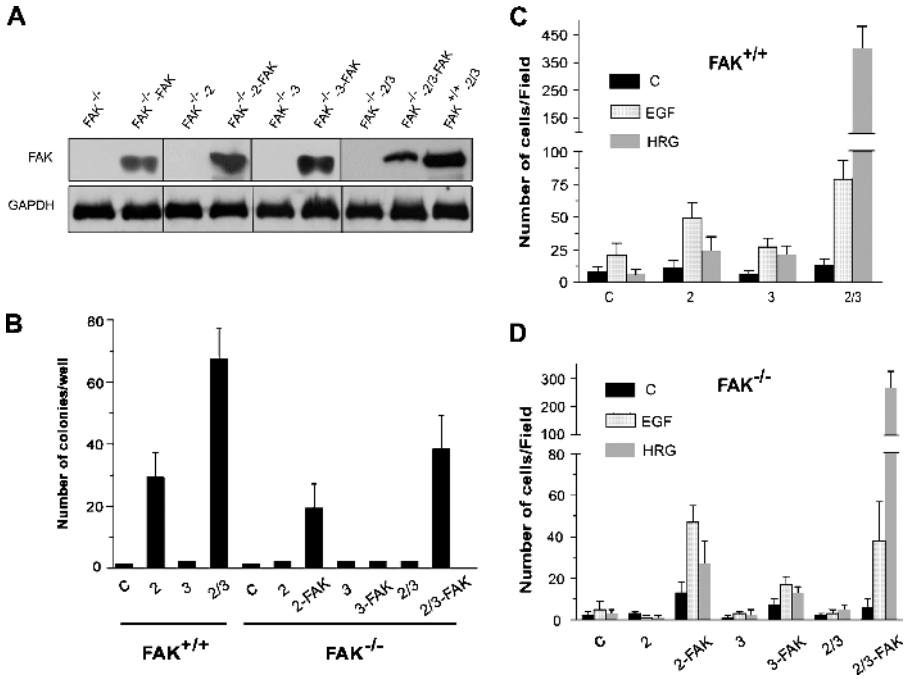

Figure 2. **FAK is essential for ErbB-induced oncogenic transformation and chemotaxis.** (A) Western blot analysis to show FAK expression status in $FAK^{-/-}$ cells expressing various ErbB receptor combinations and their matched cells in which wild-type FAK was reconstituted by stable transfection, as indicated in Materials and methods. $FAK^{+/+}$–ErbB-2/3 cells are included as a control. (B) Oncogenic property of $FAK^{-/-}$- and $FAK^{+/+}$-expressing ErbB-2/3 receptors. Cells were cultured in medium containing soft agarose, and colony formation was determined 4 wk later by counting the number of cell foci of >20 μm in diameter (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200504124/DC1). Each bar on the graph represents the mean number of colonies per well from three independent experiments ± SD. (C and D) ErbB-induced cell invasion in $FAK^{+/+}$ (C), $FAK^{-/-}$, and $FAK^{-/-}$ cells in which FAK was restored (D). $FAK^{+/+}$, $FAK^{-/-}$, and $FAK^{-/-}$-reconstituted cells expressing ErbB receptors were cultured in the upper chamber, whereas EGF or HRG was used as a chemoattractant in the lower chamber. Each bar of the graphs represents the mean ± SD (error bars) of invading cells from three independent experiments. C, control.

sulted in strong oncogenic transformation compared with ErbB-2 alone (Fig. 2 B and Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200504124/DC1).

To prove that FAK is required for ErbB-induced cell transformation, we reconstituted FAK in $FAK^{-/-}$ cells expressing ErbB-2 and -3 combinations (Fig. 2 A). Interestingly, the restoration of FAK in $FAK^{-/-}$-2/3 and -2 rescued the ability of ErbB to induce anchorage-independent growth on soft agar but did not in control $FAK^{-/-}$ cells or $FAK^{-/-}$ cells overexpressing the kinase-deficient receptor ErbB-3 (Fig. 2 B and Fig. S2 A).

FAK is required for ErbB-induced cell chemotaxis

Upon EGF or HRG stimulation, no to very low invasive activity was detected in control cells and in cells overexpressing the kinase-deficient ErbB-3, whereas $FAK^{+/+}$-2 and -2/3 cells exhibited an increase in invasion upon HRG stimulation, with cells overexpressing ErbB-2/3 being the most chemotactic (Fig. 2 C and Fig. S2 B). $FAK^{-/-}$ cells expressing control retroviral particles or any of the ErbB-2 and -3 combinations were weakly invasive, whereas $FAK^{-/-}$-2 and -2/3 in which wild-type FAK was reconstituted ($FAK^{-/-}$-2–FAK and $FAK^{-/-}$-2/3–FAK, respectively) exhibited an increase in invasion upon HRG stimulation. This rescue was not observed in control $FAK^{-/-}$ and $FAK^{-/-}$-3 stably expressing wild-type FAK (Fig. 2 D and Fig. S2 B).

ErbB-induced tumorigenicity and metastasis formation are dependent on FAK

To investigate the impact of FAK on ErbB-induced in vivo tumorigenicity, control and ErbB-transduced (ErbB-2, -3, and -2/3) cells were transplanted subcutaneously into Scid mice. Neither $FAK^{+/+}$ nor $FAK^{-/-}$cells that were transduced with control retroviral particles or ErbB-3 receptor formed tumors after >35 d (Fig. 3 A). The overexpression of ErbB-2 and -2/3 in $FAK^{+/+}$ cells induced aggressive tumor growth within 20 d, with ErbB-2/3 cells being the most aggressive compared with ErbB-2 ($P < 0.05$; tumors reached a size of ~1.25 cm^3 in <14 d for $FAK^{+/+}$-2/3 vs. >18 d for $FAK^{+/+}$-2 cells). In contrast, in $FAK^{-/-}$ cells, tumor sizes were <0.5 cm^3 for both ErbB-2 and -2/3 (the maximal size was ~0.5 cm^3 after 40 d, but most tumors regressed or became necrotic thereafter). This pattern of tumor growth was confirmed in three independent experiments using different cell stocks and with $n \geq 8$ mice per condition for each experiment.

To further demonstrate that differences in tumor growth between $FAK^{-/-}$–ErbB and $FAK^{+/+}$–ErbB is directly related to FAK, we examined the impact of FAK reconstitution on tumorigenesis of $FAK^{-/-}$-2/3 cells, focusing on ErbB-2/3 as the most aggressive in $FAK^{+/+}$. As shown in Fig. 3 B, the expression of wild-type FAK in $FAK^{-/-}$-2/3 clearly restored tumor formation. In contrast, the restoration of FAK in control $FAK^{-/-}$ did not induce tumor formation. In $FAK^{-/-}$-2/3–FAK, we noted a clear delay for the tumors to reach exponential tumor growth phase compared with $FAK^{+/+}$-2/3. This difference cannot be attributed to a loss of ErbB receptor expression in vivo because we confirmed by immunohistochemistry that ErbB receptors were highly expressed in tumor sections collected at the time of killing or in primary cells established from these tumors (unpublished data).

To determine whether the impact of FAK on ErbB-induced tumorigenicity paralleled metastasis formation, we examined the capacity of ErbB-overexpressing cells to form lung metastases after intravenous cell administration, which mimics a late stage of the metastatic process (extravasation). Both $FAK^{+/+}$ and $FAK^{-/-}$ control cells that were transduced with

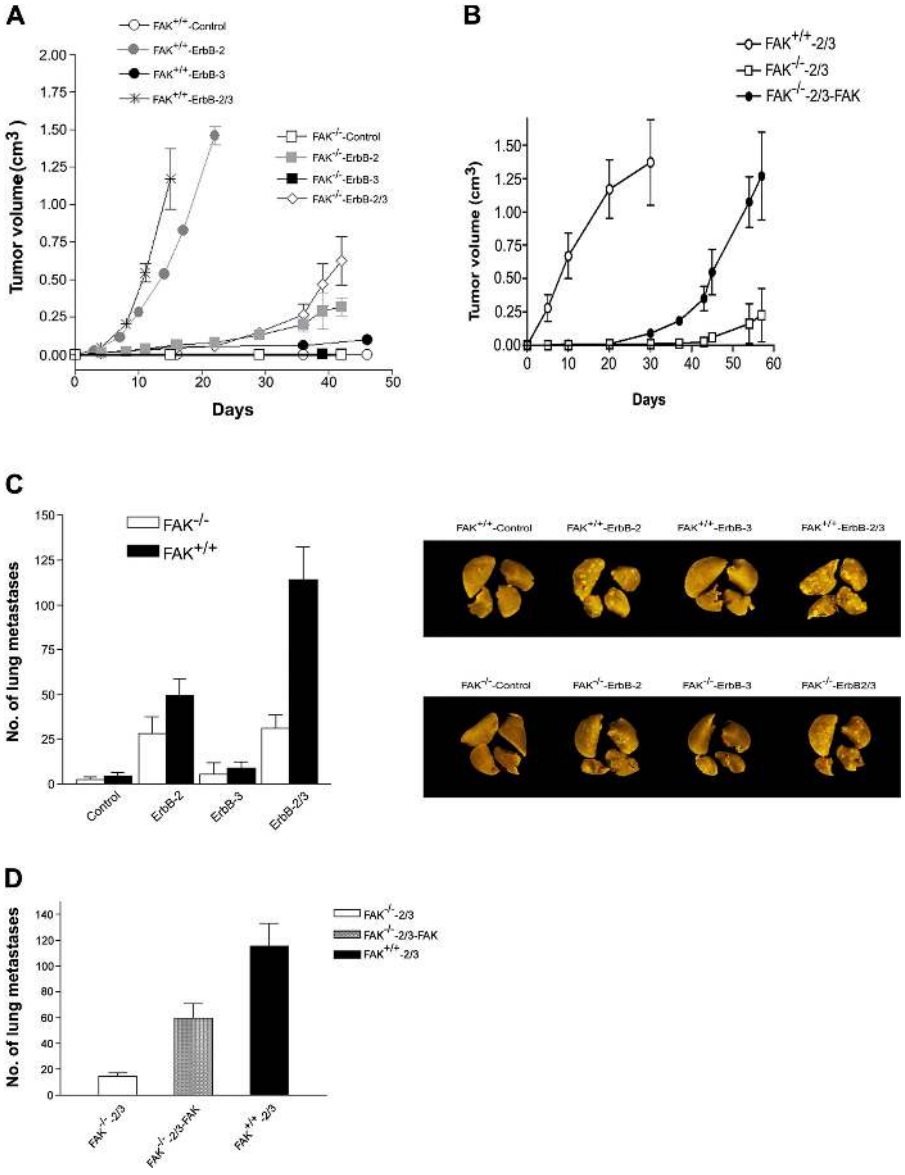

Figure 3. **FAK is required for ErbB-induced tumor progression and tumor invasion in vivo, whereas the restoration of FAK in FAK$^{-/-}$ cells rescued the deficiency in tumor invasion.** (A) Tumor growth kinetics after subcutaneous implantation of cells into the flank of Scid mice. (B) Tumor growth kinetics after subcutaneous implantation of FAK$^{-/-}$-2/3 and FAK$^{-/-}$-2/3–reconstituted cells (FAK$^{-/-}$-2/3–FAK); FAK$^{+/+}$-2/3 cells were used as positive controls. Tumor growth was monitored over time as indicated in Materials and methods. Each point represents the mean of five to eight mice ± SEM. (C) Quantification of lung surface metastases induced by intravenous cell administration. (a) Mean surface lung metastases (n = 8–10) ± SEM. (b) Representative lungs from mice inoculated with FAK$^{-/-}$ and FAK$^{+/+}$ control cells (expressing empty retroviral particles) and cells overexpressing ErbB-2, -3, or -2/3 receptors. (D) Mean lung metastases (n = 8) ± SEM (error bars) induced by FAK$^{-/-}$-2/3 or FAK-reconstituted FAK$^{-/-}$-2/3 (FAK$^{-/-}$-2/3–FAK). FAK$^{+/+}$-2/3 cells were used as positive controls.

empty retroviral particles or FAK$^{+/+}$ and FAK$^{-/-}$ overexpressing ErbB-3 induced no or very few macroscopic lung nodules. The overexpression of ErbB-2 or -2/3 increased the incidence of lung metastases. However, the ErbB-2/3 combination was much more potent in inducing macroscopic lung metastases in FAK$^{+/+}$ cells compared with cells overexpressing ErbB-2 receptor alone or FAK$^{-/-}$ overexpressing ErbB-2 or -2/3 receptors ($P < 0.05$; Fig. 3 C).

To further demonstrate that the reduced number of lung metastases in FAK$^{-/-}$-2/3 compared with FAK$^{+/+}$-2/3 is directly related to FAK, we examined the impact of FAK on lung metastasis formation in FAK$^{-/-}$-2/3 cells in which wild-type FAK was restored. As shown in Fig. 3 D, the restoration of FAK in FAK$^{-/-}$-2/3 cells partially rescued the deficiency in invasion ($P < 0.05$ when comparing FAK$^{-/-}$-2/3 with FAK$^{-/-}$-2/3–FAK). This partial rescue may be contributed by lower levels of FAK expression in FAK$^{-/-}$-2/3 compared with endogenous FAK in FAK$^{+/+}$ cells (Fig. 2 A).

ErbB-induced oncogenic transformation and invasion are mediated via distinct FAK signaling

To dissect the FAK-dependent signaling involved in ErbB-induced oncogenesis and invasion, we examined the impact of ErbB on FAK phosphorylation and its interaction with downstream signaling partners. FAK phosphorylation occurs at several tyrosine sites, including Tyr-397, -861, and -925; these sites are selectively regulated after ErbB activation (Sieg et al., 2000; Lu et al., 2001; Vadlamudi et al., 2002, 2003). In FAK$^{+/+}$ control cells, the stimulation of ErbB by EGF resulted in a small increase in the level of FAK phosphorylation over time (Fig. 4 A, top left), whereas HRG had no effect on basal FAK phosphorylation (not depicted). The stimulation of cells overexpressing ErbB-2 with EGF increased total FAK phosphorylation as well as phosphorylation at Tyr-397, -861, and -925. This increase is likely a result of ErbB-2 transactivation by the low endogenous ErbB-1 present in these cells. FAK ac-

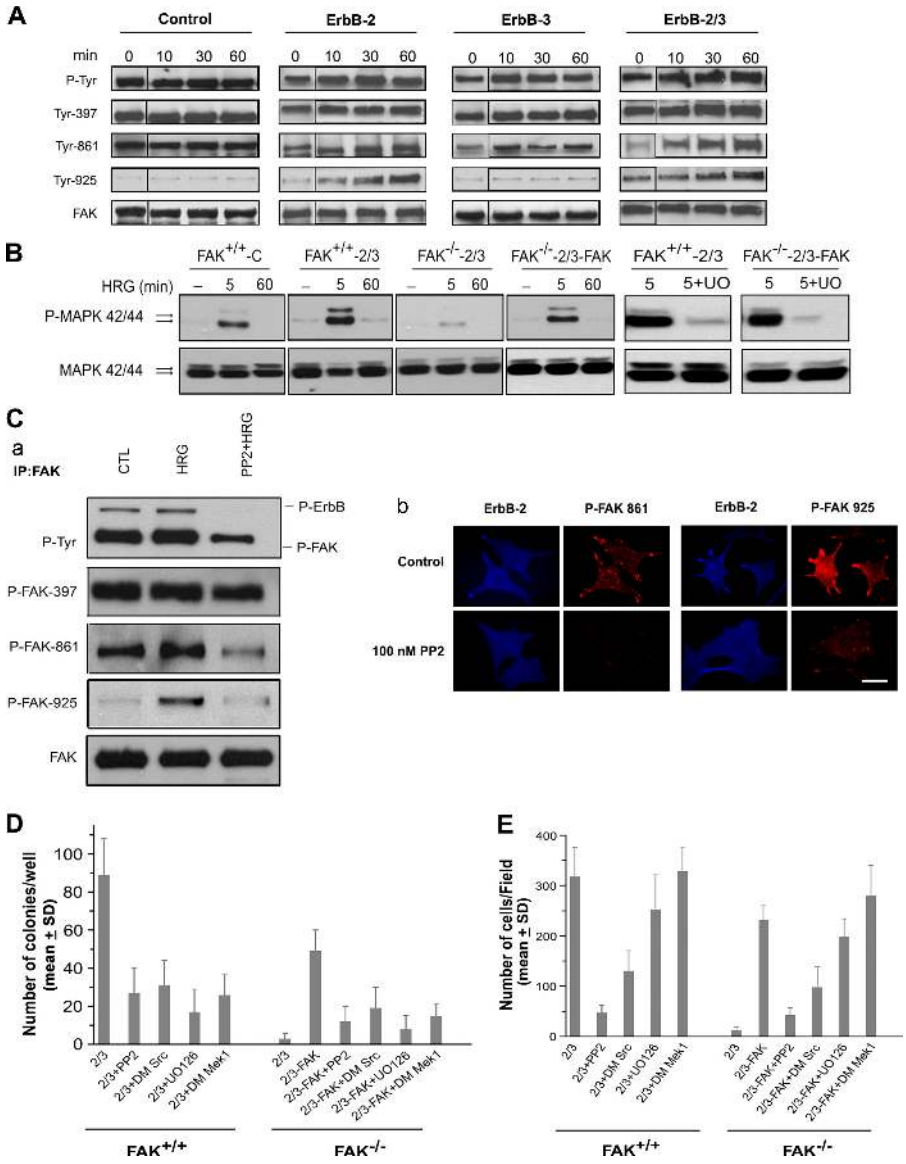

Figure 4. **ErbB-induced oncogenic transformation and invasion are mediated via distinct FAK signaling.** (A) Differential regulation of FAK phosphorylation by ErbB-2 and -3 receptors. Serum-starved cells were stimulated with 20 ng/ml EGF (control cells) or HRG (ErbB-2, -3, and -2/3–overexpressing cells) for the indicated times. Proteins were immunoprecipitated with anti-FAK and probed with antiphosphotyrosine antibody, antiphospho-FAK antibodies specific to different residues, and anti-FAK antibody. (B) Differential regulation of MAPK phosphorylation by ErbB-2 and -3. Cell lysates from HRG-stimulated adherent cells were blotted with P-MAPK and reprobed with MAPK antibody as indicated in Materials and methods. The figure shows an increase in P-MAPK after 5 min of stimulation with HRG in $FAK^{+/+}$-2/3 cells and FAK-reconstituted $FAK^{-/-}$-2/3 cells compared with control cells. Exposure to UO126 strongly inhibited P-MAPK in FAK-proficient cells. (C) Inhibition of Src reduced HRG-induced FAK phosphorylation at Tyr-861 and -925. $FAK^{+/+}$-2/3 cells were serum starved for 24 h and pretreated with PP2 at 100 nM for 60 min followed by treatment with 20 ng/ml HRG for 30 min. (a) Whole cell lysates were immunoprecipitated with anti-FAK and probed with antiphosphotyrosine antibody. Membranes were subsequently reprobed with antiphospho-FAK antibodies specific to different residues and total FAK. (b) $FAK^{+/+}$-2/3 cells were coimmunostained for ErbB-2 and the indicated phospho-FAK in the absence or presence of PP2. Bar, 40 μm. (D) Inhibition of colony formation on agar by Src and MAPK inhibition. Cells were cultured in medium containing soft agarose either in the absence and presence of PP2 or UO126 or were transfected with dominant mutants for MEK1 or Src. Colony formation was determined 4 wk later by counting the number of cell foci. (E) Inhibition of cell invasion by Src but not MAPK inhibition. Cells were cultured in the upper chamber of the Boyden chamber in the absence and presence of PP2 or UO126 or after being transfected with dominant mutants for MEK1 or Src. HRG was used as a chemoattractant in the lower chamber. Each bar of the graphs represents the mean ± SD (error bars) of invading cells from three independent experiments.

tivation was weak in cells overexpressing kinase-deficient ErbB-3 that was stimulated with HRG (Fig. 4 A). In cells overexpressing ErbB-2 and -2/3, a clear increase in total FAK phosphorylation was seen, which was associated with the increased phosphorylation of Tyr-397, -861, and -925; the most pronounced increase was seen on Tyr-861 and -925 phosphorylation sites (Fig. 4 A).

ErbB receptors are coupled to several signaling molecules that are also recruited by FAK. These include the MAPK and Src kinases. We used inhibitors for these kinases to delineate the signaling events that are associated with ErbB–FAK-dependent oncogenic transformation versus cell invasion. As shown in Fig. 4 B, MAPK/extracellular regulated kinase (ERK) phosphorylation is strongly induced after 5 min of stimulation with HRG in $FAK^{+/+}$-2/3 cells compared with control $FAK^{+/+}$ and $FAK^{-/-}$-2/3 cells. Interestingly, the reconstitution of FAK in $FAK^{-/-}$-2/3 cells restored ERK phosphorylation to approximately the same levels seen in $FAK^{+/+}$-2/3 cells. In both FAK-proficient and FAK-reconstituted cells, inhibition of the dual-specificity protein kinase Mek1/2 by UO126 efficiently prevented ErbB-induced MAPK activation (Fig. 4 B, bottom). In $FAK^{+/+}$-2/3 cells, immunoprecipitation assay and immunostaining revealed that the activation of FAK phosphorylation at Tyr-861 and -925 by HRG was inhibited by the Src inhibitor PP2 (Fig. 4 C) but not by UO126 (not depicted).

We next addressed the impact of MAPK and Src inhibition on ErbB-induced cell transformation versus cell invasion. As shown in Fig. 4 D, MAPK inhibition by UO126 or Mek1 dominant mutants prevented ErbB-induced anchorage-independent growth in soft agar in $FAK^{+/+}$-2/3 cells and FAK-reconstituted $FAK^{-/-}$-2/3 cells. In contrast, only PP2 or Src dominant mutants prevented ErbB-2/3–induced chemotaxis (Fig. 4 E).

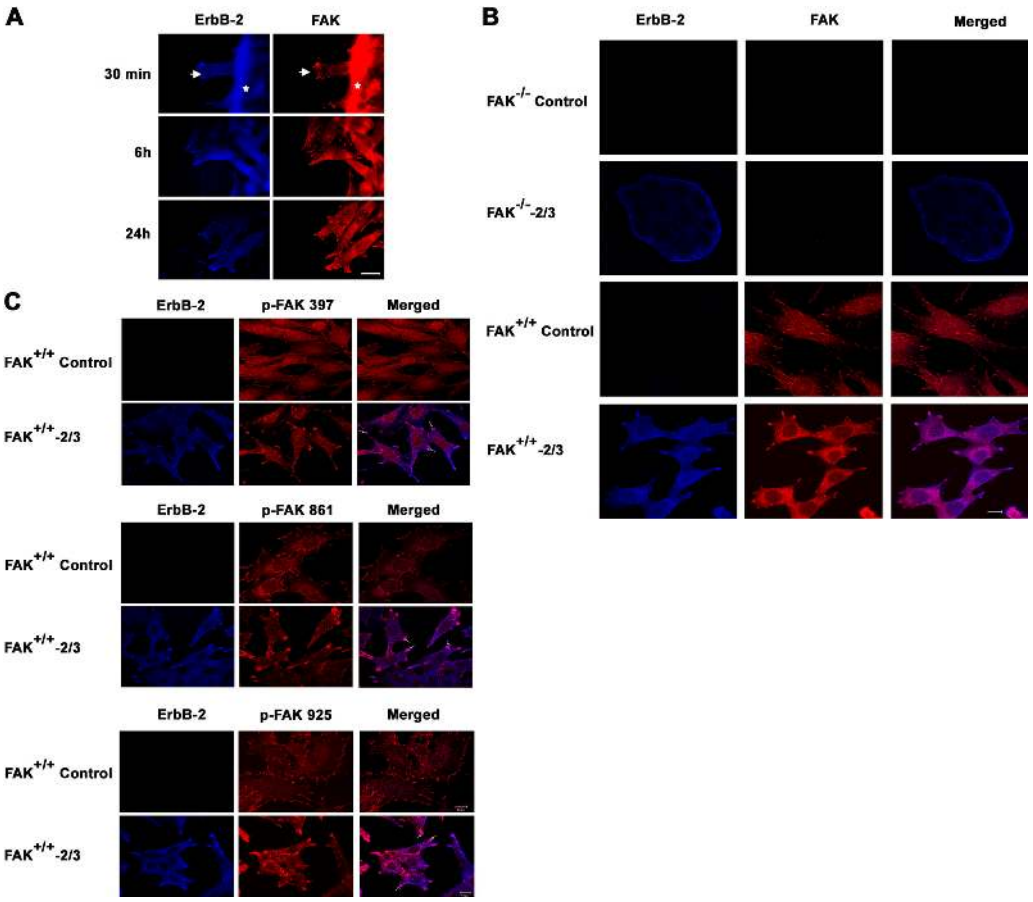

Figure 5. **ErbB-2 colocalizes with FAK at focal adhesions in motile cells.** (A) ErbB-2 and FAK colocalize in motile $FAK^{+/+}$-2/3 cells. Confluent cells were scratch wounded and allowed to heal for the indicated time points before fixation. Cells were then coimmunostained with anti–ErbB-2 and anti-FAK antibodies followed by appropriate secondary antibodies as described in Materials and methods. Note that both ErbB-2 and FAK are recruited into newly formed lamellipodia near the leading edge of the wounded cells during cell migration to the acellular area (30 min). Typical ventral focal contacts that were stained for FAK become detectable 6 h after wound healing and become more pronounced after 24 h. Arrowheads indicate the newly formed protrusions. Stars indicate the folded cell layer at the wounded area. Bar, 30 μm. (B) Colocalization of ErbB-2 and FAK at the cell protrusion. Cells were fixed, permeabilized, and double immunostained with anti–ErbB-2 and anti-FAK antibodies followed by appropriate secondary antibodies conjugated either to aminomethylcoumarin (AMCA) or Texas red to detect ErbB-2 and FAK, respectively. The figure shows that $FAK^{-/-}$ control cells were negatively stained for both ErbB-2 and FAK, whereas $FAK^{-/-}$-2/3 cells were strongly labeled for ErbB-2 receptors, which were homogeneously distributed around the cell periphery. In contrast, $FAK^{+/+}$ control cells exhibit strong labeling of FAK, which was localized to cell extensions and ventral focal contact sites within the cells, whereas $FAK^{+/+}$-2/3 cells exhibit strong labeling of FAK at cell protrusions, which colocalize partially with ErbB-2 receptors as revealed by dual-color merged confocal images. (C) Tyrosine-phosphorylated FAK colocalized with ErbB-2. $FAK^{+/+}$ control cells and $FAK^{+/+}$-2/3 were grown in complete medium, fixed, and immunostained with ErbB-2 in combination with either FAK Tyr-397, -861, or -925 antibodies. Confocal microscopy reveals partial colocalization of phospho-FAK with ErbB-2 receptor at the cell protrusions (arrows). In contrast, control cells that do not express any ErbB exhibit homogeneous distribution of phospho-FAK at the focal contacts. (B and C) Bars, 50 μm.

FAK is required for ErbB-2 localization at the cell protrusion

To further understand the impact of ErbB–FAK interaction on the formation of focal adhesions in migratory cells, we first used the scratch-wound assay to follow ErbB–FAK localization at the cell protrusion. Fig. 5 A reveals that both ErbB-2 and FAK are recruited into newly formed lamellipodia near the leading edge of wounded cells during cell migration to the acellular area (Fig. 5 A, 30 min). Typical ventral focal contacts that were stained for FAK became detectable 6 h after wound healing and become more pronounced after 24 h (Fig. 5 A, 24 h).

We then examined the subcellular localization of ErbB-2 and FAK by confocal microscopy. $FAK^{-/-}$-2/3 cells exhibited strong labeling of ErbB-2 that was homogeneously distributed at the cell membrane. In contrast, FAK labeling was localized to cell extensions at ventral focal contact sites in $FAK^{+/+}$ control cells, whereas $FAK^{+/+}$-2/3 cells exhibited multiple protrusions at the cell periphery, where FAK and ErbB-2 colocalized (Fig. 5 B). This colocalization was also seen using specific antibodies against the FAK phosphosites Tyr-397, -861, and -925 (Fig. 5 C). Because ErbB receptors physically interact with FAK (Sieg et al., 2000; Vartanian et al., 2000), we examined whether ErbB-2 and FAK colocalize to the cell membrane protrusion as separate proteins or as a complex. Immunoprecipitation studies on isolated plasma membranes from nonstimulated and stimulated $FAK^{+/+}$-2/3 cells revealed that ErbB-2 and FAK were present on the cell membrane, at least in part, as a preformed complex (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200504124/DC1).

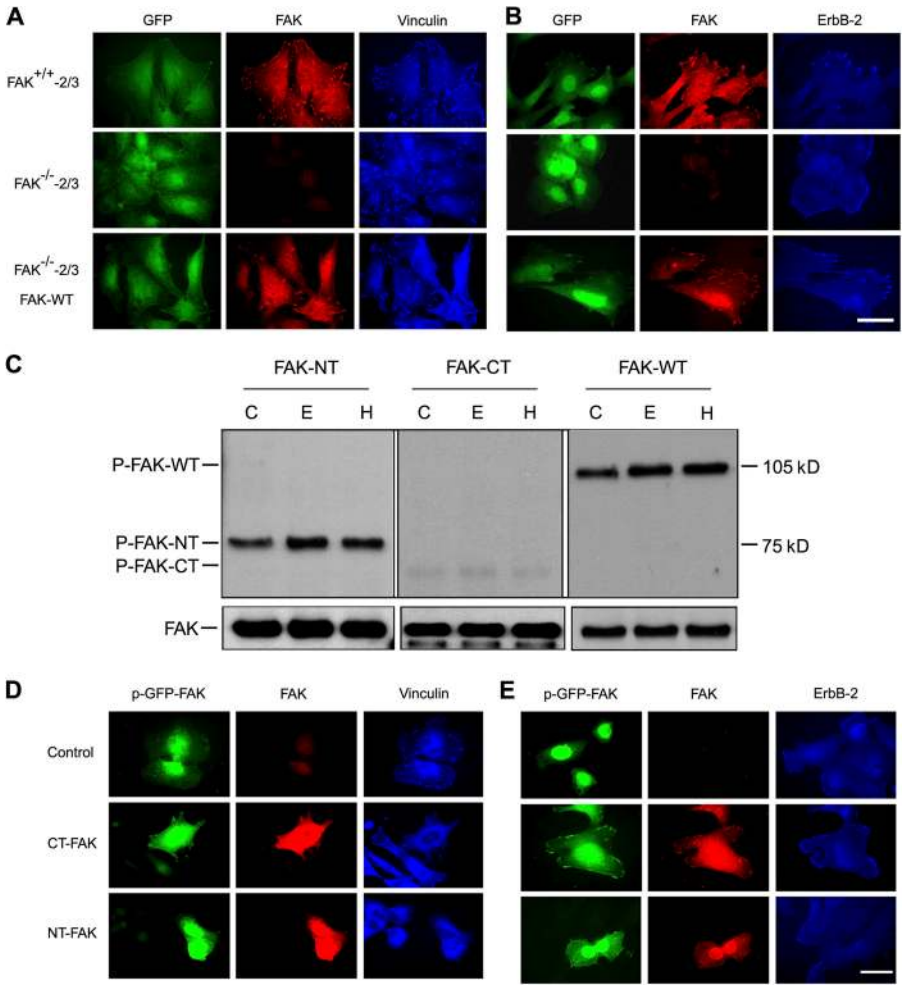

Figure 6. **Restoration of FAK in FAK$^{-/-}$ cells rescued focal adhesions and ErbB-2 relocalization to focal protrusions.** (A) FAK$^{-/-}$ cells cooverexpressing ErbB-2 and -3 receptors were stably transfected with wild-type FAK as described in Materials and methods. Double immunofluorescence labeling of FAK and vinculin, a marker for focal adhesion, demonstrated that FAK$^{-/-}$-2/3 cells in which FAK was restored (right) exhibited many focal adhesions that were labeled for both FAK and vinculin, which is similar to normal FAK$^{+/+}$-2/3 cells. (B) Double immunofluorescence of FAK and ErbB-2 revealed that the restoration of wild-type FAK in FAK$^{-/-}$-2/3 cells induces the relocalization of ErbB-2 receptors from the cell membrane to fingerlike protrusions. This ErbB-2 pattern is similar to FAK$^{+/+}$-2/3 cells in which FAK and ErbB-2 receptors colocalized at cell protrusions. Bar, 30 μm. (C) Expression of NH_2 terminus (NT) and COOH terminus (CT) FAK in FAK$^{-/-}$-2/3. Cells expressing the NT or CT were stimulated without (C) or with 20 ng/ml EGF (E) or 20 ng/ml HRG (H) for 30 min. Equal amounts of protein were immunoprecipitated with anti–FAK-A17, which targets at NT-FAK, or anti-FAK–C-20 which targets at CT-FAK. Blots were probed with antiphosphotyrosine antibody. (D and E) Immunofluorescence analysis. FAK$^{-/-}$-2/3 cells expressing control, CT-FAK–GFP, or NT-FAK–GFP fusion protein (see Materials and methods). After transfection, the cells were incubated in complete medium for 24 h and were fixed and coimmunostained for FAK and vinculin (D) or FAK and ErbB-2 receptor (E). Note that focal adhesions were seen only when cells were transfected with CT-FAK but not with NT-FAK (D). In contrast, cells transfected with CT-FAK exhibited a homogeneous distribution of ErbB-2 receptors at the cell membrane, whereas transfection with NT-FAK exhibited a reduced staining of ErbB-2 throughout the cytoplasm (E). Bar, 30 μm.

Next, we confirmed the colocalization of FAK and ErbB-2 in FAK$^{-/-}$-2/3 cells in which FAK was reconstituted by immunofluorescence. FAK$^{-/-}$-2/3–FAK cells exhibited many focal adhesions that were labeled for both FAK and vinculin, which is similar to FAK$^{+/+}$-2/3 cells (Fig. 6 A). The restoration of FAK in FAK$^{-/-}$-2/3 cells induced the relocalization of ErbB-2 from the straight parts of the cell membrane to fingerlike protrusions in a similar pattern to that seen in FAK$^{+/+}$-2/3 cells (Fig. 6 B).

To further examine the importance of FAK domains for ErbB localization, we compared the localization of ErbB-2 with cell protrusion in FAK$^{-/-}$-2/3 cells that were transiently transfected with wild-type FAK, FAK NH_2 terminus (NT), or FAK COOH terminus (CT); both were fused to GFP. The expression and activation of these truncated forms of FAK after 30 min of stimulation with EGF or HRG was confirmed by immunoprecipitation analysis (Fig. 6 C). CT-FAK showed no clear changes in phosphorylation after stimulation with HRG. Immunofluorescence labeling revealed the presence of focal adhesions that colocalize with vinculin in cells expressing CT-FAK, but the NT-FAK–transfected cells developed less focal adhesions, as detected by vinculin staining (Fig. 6 D). Furthermore, ErbB-2 was homogeneously distributed at the cell membrane in cells transfected with control or CT-FAK–GFP-expressing plasmid (Fig. 6 E). In contrast, NT-FAK–GFP-transfected cells exhibited a reduced staining of ErbB-2, which was localized throughout the cytoplasm.

Together, these results indicate that ErbB-induced cell migration involves interaction with FAK and relocalization and accumulation of ErbB-2 at the cell protrusion.

ErbB-2–induced invasion by FAK colocalizes in human cancer cells

To confirm the relevance of the results in mouse embryonic fibroblasts to human cells, we examined the importance of FAK for cell invasion in a panel of human breast cancer cells, including SKBR3 and T47D cells that overexpress ErbB-2 constitutively, and two metastatic variants of the breast carcinoma cells MDA-231-M2 and MCF7-M4 that were selected in vivo from parental cells overexpressing ErbB-2. Fig. 7 A shows the ErbB-2 status in these cells, which were examined by Western blot analysis, and Fig. 7 B shows the efficiency of siRNA to down-regulate FAK. We next examined the impact of FAK down-regulation on cell invasion by using the Boyden chamber assay on matched control and FAK siRNA cells.

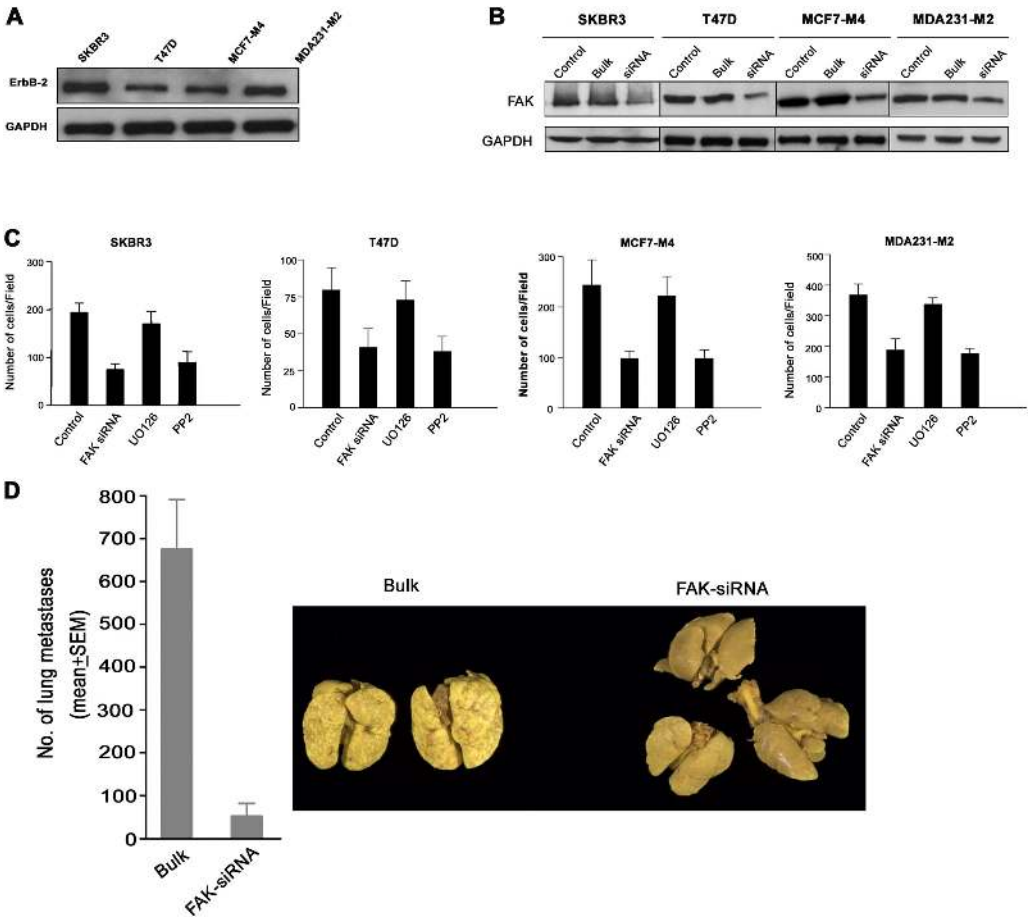

Figure 7. **FAK regulates ErbB-induced cell invasion and metastasis formation that is induced by human breast cancer cells.** (A) ErbB-2 expression status examined by Western blot analysis of cell extracts from cells constitutively overexpressing ErbB-2 (SKBR-3 and T47D) and metastatic cell variants (MCF7-M4 and MDA-231-M2) that were isolated in vivo from MCF7 and MDA-231 cells engineered to overexpress ErbB-2. (B) Western blots on cells stably expressing control (bulk) or FAK siRNA. Note that FAK-specific siRNA induced a marked decrease of FAK expression. GAPDH used as an internal control was unaffected. (C) Inhibition of FAK by siRNA reduced cell invasion to a similar level as observed after Src inhibition but not MAPK inhibition. Cells expressing siRNA or treated with PP2 or UO126 were cultured in the upper chamber, whereas HRG was used as a chemoattractant in the lower chamber. Each bar of the graphs represents the mean ± SD of invading cells from three independent experiments. (D) The metastatic MDA-231-M2 cells were implanted into the mammary fat pad. Animals were sacrificed 50 d later, lungs were fixed in Bouin, and surface lung metastases were counted. Each bar represents the mean of five mice ± SEM (error bars). Image is of representative lungs from these experiments.

As noted in Fig. 7 C, cells expressing siRNA have reduced invasive activity. The exposure of cells to PP2 but not UO126 inhibitors mimics the inhibitory effect of cell invasion that was observed with FAK siRNA. Equally important, an in vivo study using the highly invasive MDA-231-M2 cells indicated that the expression of FAK siRNA is stable in vivo, as shown by immunohistochemistry on tumor tissues taken at the time of killing (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200504124/DC1), and drastically reduced the invasive capacity of these cells when implanted into the mammary fat pad. 50 d after tumor implantation, the number of lung nodules was reduced by ~90% compared with control cells expressing control bulk siRNA (Fig. 7 D).

To confirm the colocalization of ErbB and FAK in SKBR3, T47D, MDA-231-M2, and MCF7-M4, the ErbB-2 receptor was coimmunolabeled with antibodies against ErbB-2 and FAK. Fig. 8 shows that both ErbB-2 and FAK colocalize to cell protrusions in all of these cells. Double labeling of ErbB-2 and FAK indicate that cells with ErbB-2 overexpression exhibit a strong colocalization of FAK with ErbB-2 at the cell lamellipodia. In all cases, confocal microscopy indicates that ErbB and FAK localization is partial.

Discussion

Increasing evidence highlighted a key role for the regulation of cell invasion by cooperation between a number of growth factor receptors, including the ErbB tyrosine kinases, and integrin-dependent pathways. This cooperation has been suggested to result from the amplification of cell signal transduction pathways, including the recruitment of FAK and its network of interactors. In this study, we dissected the importance of FAK for ErbB-induced cell invasion versus oncogenesis in the context of combinatorial receptor overexpression, a situation that is common in invasive cancers.

FAK is indispensable for ErbB-induced oncogenic transformation

Our results provide evidence that ErbB-2/3 cooverexpression in FAK-proficient cells, but not in FAK-deficient cells, induced an-

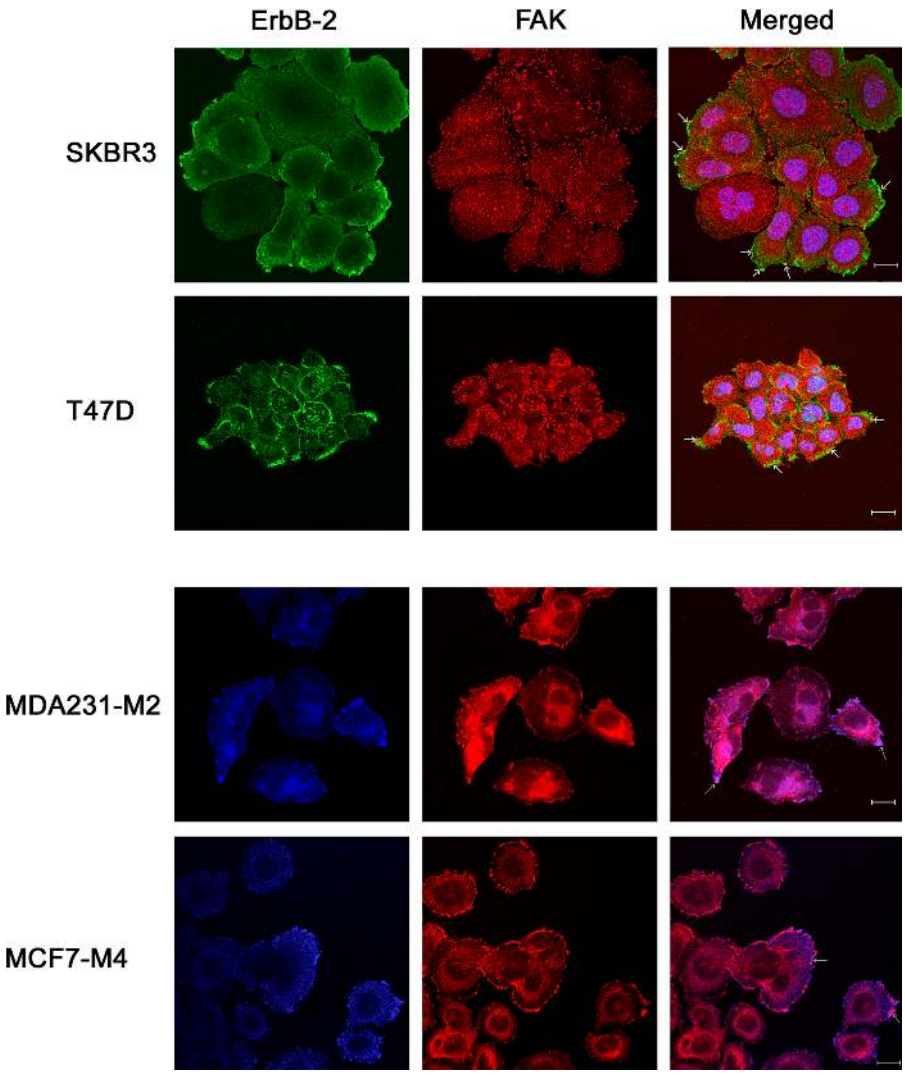

Figure 8. **ErbB-2 colocalizes with FAK at focal adhesions in invasive breast cancer cells.** Cells were fixed, permeabilized, and subjected to confocal microscopy after double immunostaining with anti–ErbB-2 and anti-FAK antibodies followed by appropriate secondary antibodies conjugated either to Cy2 or Texas red (left) or conjugated to AMCA and Texas red (middle) to detect ErbB-2 and FAK, respectively. The panels show a strong labeling of FAK, which was localized to cell extensions and ventral focal contact sites within the cells. For all cell lines, dual-color merged confocal images reveal a partial colocalization of ErbB-2 with FAK (arrows). Bars, 50 μm.

chorage-independent growth on soft agar as well as tumor formation in vivo. Consistently, the restoration of FAK can rescue the inability of $FAK^{-/-}$-2/3– as well as $FAK^{-/-}$-2–overexpressing cells to induce growth on soft agar and tumor formation in vivo. In contrast, this rescue was not observed in $FAK^{-/-}$ cells expressing the kinase-deficient ErbB-3 receptor, which relies on heterodimerization with other members of the ErbB family, particularly with ErbB-2, for transmitting oncogenic signals (Pinkas-Kramarski et al., 1996). An essential role of FAK for oncogenic transformation induced by v-Src, Ras, 12-dimethylbenz(a)nthracene, or TPA has been reported (Renshaw et al., 1999; McLean et al., 2000; Lim et al., 2004). In contrast, FAK was found to be dispensable for v-Src–induced oncogenic transformation in contrast to cell motility (Hauck et al., 2002; Hsia et al., 2003).

Cross talk between ErbB and FAK is multidirectional. The strong tumorigenic and invasive potential of the ErbB-2/3 combination is consistent with the potent mitogenic signals emanating from the ErbB-2/ErbB-3 heterodimer compared with monomeric receptors (Alimandi et al., 1995; Pinkas-Kramarski et al., 1996). For instance, ErbB-2 overexpression can lead to constitutive autophosphorylation and activation of the kinase-deficient ErbB-3, whereas activation of ErbB-3 by HRG can transphosphorylate ErbB-2; this cooperation results in the amplification of cell signaling (Riese et al., 1995; Pinkas-Kramarski et al., 1996).

ErbB-induced cell transformation has been linked to several signaling molecules that were also recruited by FAK, including the Ras-MAPKs and Src. Like ErbB, FAK activation results in the activation of multiple signaling molecules, including Src family kinases and the adaptor protein Grb2. We demonstrate that ErbB activation induces FAK phosphorylation at several sites, including Tyr-397, -861 and -925. These sites have been shown to be the major regulated phosphorylated sites after ErbB activation by EGF or HRG (Sieg et al., 2000; Lu et al., 2001; Vadlamudi et al., 2002, 2003). In our model, ErbB-2/3 activation by HRG increased FAK phosphorylation, which contrasts with other studies in which ErbB receptor activation in cells overexpressing a single receptor was shown to induce FAK dephosphorylation (Lu et al., 2001; Vadlamudi et al., 2002), but is in agreement with others (Brunton et al., 1997; Hauck et al., 2001; Golubovskaya et al., 2002). Nevertheless, changes in FAK phosphorylation status by ErbB does not correlate with the potency of ErbB to induce tumor invasion, because we observed a similar pattern of FAK phosphorylation in cells overexpressing ErbB-1 or -1/3 recep-

tors despite the fact that these cells are less invasive compared with cells overexpressing ErbB-2/3. This would support the idea that FAK contributes to ErbB-induced cell invasion primarily via its downstream pathways. Using inhibitors for MEK1 and Src, we demonstrate that FAK involves two distinct primary signaling molecules: namely, MAPK/ERK for cell transformation and Src for cell invasion. First, oncogenic transformation by ErbB-2/3 in FAK-proficient and FAK-reconstituted cells correlated with a rapid ERK-1/2 activation compared with FAK-deficient cells, supporting the idea that FAK is an important mediator for the potent MAPK activation reported previously for heterodimeric forms containing ErbB-2 (Olayioye et al., 2000). Interestingly, the inhibition of MAPK prevented ErbB–FAK-dependent oncogenic transformation both in ErbB-2/3 FAK-proficient and FAK-reconstituted cells. This also depended on FAK–Src interaction because Src inhibition can prevent ErbB-induced oncogenesis, which is in agreement with previous studies on FAK–Src–MAPK-dependent signaling for v-Src–induced cell transformation or chemotaxis (Schlaepfer et al., 1998; Westhoff et al., 2004). Moreover, a study by Renshaw et al. (1999) reported that unlike nontransformed cells, oncogene-transformed cells induced anchorage independency via MAPK activation, which bypasses a requirement for cell adhesion and growth factor stimuli. Whether such a scenario can account for ErbB–FAK-dependent oncogenic transformation in our models will require further studies.

FAK is essential for ErbB-induced cell chemotaxis, tumorigenesis, and metastasis formation

It has been suggested that the relationship between ErbB receptor overexpression and cancer invasiveness is the result of the amplification of cell signal transduction, which is primarily attributed to interreceptor heterodimerization. FAK-deficient cells have defects in cell migration compared with FAK-proficient cells (Ilic et al., 1995). We demonstrate that the overexpression of ErbB-2/3 receptors failed to induce tumorigenesis, which is in contrast to FAK-proficient cells. However, the restoration of FAK can rescue the capacity of ErbB-2/3 to induce tumor formation. The delay of tumor growth that was induced by $FAK^{-/-}$-2/3–FAK cells may be explained by low levels of exogenous FAK in $FAK^{-/-}$-2/3 compared with $FAK^{+/+}$ cells or by other mechanisms that operate in vivo (e.g., regulation of tumor angiogenesis or stromal–host interactions; Yen et al., 2002; Alaoui-Jamali et al., 2003).

The incidence of lung metastases was drastically reduced in ErbB-2/3 FAK-deficient cells compared with ErbB-2/3 FAK-proficient cells, but the restoration of FAK in $FAK^{-/-}$ cells rescued the invasive potential of ErbB in these cells both in vitro and in vivo. Nevertheless, we noted that metastasis formation in vivo was not completely abolished in nonreconstituted ErbB–FAK-deficient cells compared with FAK-proficient cells. However, lung metastases in this model form after the intravenous injection of cells, which represents a late stage process of metastasis. Alternatively, ErbB-2/3 receptors may be able to override the requirement for FAK in vivo via alternative mechanisms such as a possible compensatory role for Pyk2, which binds to ErbB-1 and is overexpressed in FAK-deficient cells (Ivankovic-Dikic et al., 2000). Interestingly, in human invasive cancer cells expressing ErbB-2/3 receptors, the inhibition of FAK efficiently prevented the formation of lung metastases from distant primary tumors implanted into the mammary fat pad.

Both activated ErbB-2/3 and FAK can associate with c-Src. c-Src utilizes Tyr-397 of FAK to interact with FAK (Schaller et al., 1994), although others have shown that Src itself can induce FAK tyrosine phosphorylation independently of Tyr-397 (McLean et al., 2000). HRG was shown to up-regulate the Tyr-215 of c-Src and to increases Src kinase activity, and increased Src activity is associated with reduced cell–cell adhesion (Hamaguchi et al., 1993) and increased metastatic potential (Irby et al., 1999). Furthermore, cooperation between Src and ErbB in tumorigenesis and invasion has been previously reported (Maa et al., 1995). Interestingly, FAK-related nonkinase expression in v-Src–transformed NIH3T3 cells inhibited FAK phosphorylation at Tyr-861 and formation of lung metastases but did not inhibit the growth of primary tumors (Hauck et al., 2002).

Because the inhibition of Src is found to prevent ErbB–FAK colocalization to focal adhesions, one possibility is that Src may regulate the focal adhesion turnover of podosome-associated Src substrates, as reported previously (Fincham and Frame, 1998; Carragher et al., 2002). Additional mechanisms may imply a regulation of cell cytoskeleton reorganization via FAK signaling because changes in several cytoskeleton proteins were noted in ErbB-transformed cells (Alaoui-Jamali et al., 2003).

ErbB–FAK localizes specifically to cell protrusions in migratory cells

The connection between ErbB and cell invasion can occur at multiple levels, including the regulation of focal adhesions. Focal adhesions are primarily localized to the cell periphery, are highly phosphorylated, contain proteins such as $\alpha v\beta 3$ integrin, vinculin, and paxillin, and function primarily by providing anchors to the extracellular matrix, thus allowing the contractile actomyosin system to pull the cell body and trailing edge forward. As noted in our results, migratory ErbB-2/3 cells display fingerlike protrusions at highly organized plasma membrane structures. Interestingly, ErbB localization with vinculin, which is a marker for focal adhesions, was dependent on activated FAK, as exogenous FAK can restore ErbB localization to focal adhesion sites in FAK-deficient cells. Confocal microscopy combined with immunoprecipitation assay on purified cell membranes confirmed that ErbB and FAK colocalize, in part, as a preformed complex, which is also supported by the previously reported physical interaction between ErbB and FAK (Sieg et al., 2000; Vartanian et al., 2000).

However, bidirectional receptor endocytosis/exocytosis raises a question about the specificity of ErbB localization to focal adhesions versus receptor recycling mechanisms. ErbB-2, unlike ErbB-1, has a long half-life at the plasma membrane that is attributed to its capacity to overcome clathrin-mediated endocytosis and proteolysis and/or increased recycling (Baulida et al., 1996; Waterman et al., 1998). In a panel of human invasive cancers, we confirmed that FAK regulates ErbB-induced cell invasion and metastasis formation when cells were implanted into the mammary fat pad. Of relevance to this study, ErbB-2 is found to be

preferentially associated with membrane protrusions in SKBR3 cells, where it becomes highly resistant to internalization (Hommelgaard et al., 2004). This seems to be independent of ErbB-2 association with (1) lipid rafts or the actin cytoskeleton (Mineo et al., 1999); (2) caveolae, which represents a subset of rafts (Nagy et al., 2002); or (3) the actin cytoskeleton (Feldner and Brandt, 2002). These observations support that ErbB–FAK colocalization at cell protrusions is involved in cell migration. In summary, our data provide evidence that the potent invasive property of ErbB-2/3 receptors is mediated via FAK-dependent mechanisms.

Materials and methods

Reagents and cell lines
Mouse embryonic fibroblast ($FAK^{+/+}$ or $FAK^{-/-}$) cells were provided by D. Ilic (University of California, San Francisco, San Francisco, CA). SKBR-3, T47D, MCF-7, and MDA-231 cells were obtained from the American Type Culture Collection. The metastatic variants MDA-231-M2 and MCF7-M4 were established from metastatic lung nodules induced in vivo by parental cells that were engineered to overexpress ErbB-2 and implanted into the mammary fat pad of Scid mice. The following antibodies were used: monoclonal anti–ErbB-2 (Ab-3; clone 3B5) and polyclonal anti–ErbB-2 (Ab-1; Oncogene Research Products); polyclonal anti–ErbB-3 (clone C-17), polyclonal anti-FAK (A-17; C-20), and antiphospho–FAK-Y925 (Santa Cruz Biotechnology, Inc.); antiphospho–FAK-Y397 and antiphospho–FAK-Y861 (Biosource International); antiphosphotyrosine antibody (4G10) and anti–ERK-2 (clone B3B9; Upstate Biotechnology); antiphospho-MAPK (New England Biolabs, Inc.); monoclonal antivinculin (clone hVIN-1; Sigma-Aldrich); and anti-GAPDH (Cedarlane). EGF was obtained from Sigma-Aldrich, HRG was purchased from Neomarkers, and PP2 and UO126 were obtained from Alexis Biochemicals. The dominant-negative mutants for MEK1 and Src were provided by S. Meloche (Montreal University, Montreal, Canada) and D. Shalloway (Cornell University, Ithaca, NY), respectively.

Cell culture
$FAK^{+/+}$ or $FAK^{-/-}$ cells were cultured in DME (Life Technologies) supplemented with 10% FBS, 1 mM sodium pyruvate, 1% (vol/vol) nonessential amino acids, 100 μM 2-mercaptoethanol, and penicillin/streptomycin. Human cancer cells were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS and penicillin/streptomycin.

Stable overexpression of ErbB receptors in $FAK^{+/+}$ and $FAK^{-/-}$ cells
ErbB receptors were expressed in a polyclonal cell population as described previously (Yen et al., 2002).

Construction of plasmids and transfection
The plasmid encoding mouse FAK was described previously (Sieg et al., 2000). The cDNA encoding FAK residues 231–1,538 and residues 2,281–3,378 were amplified by PCR from a human osteosarcoma cDNA library. Both fragments were subcloned into the BglII–ApaI sites of the pEGFP-N2 expression vector (CLONTECH Laboratories, Inc.). For both stable and transient transfections, cells at 50% confluence were transfected with 2 μg plasmid DNA for 18 h using LipofectAMINE (Invitrogen). For transient expression, cells were collected 24–48 h after incubation in complete medium. For stable expression, cells were transfected for 18 h and were cultured in completed medium containing 500 μg/ml hygromycin for 14 d. Clones with a stable expression of FAK were isolated.

Anchorage-independent cell growth
5,000 cells were suspended in 0.3% SeaPlaque agarose (Mandel Scientific Company) diluted in complete medium and were poured on a 0.7% preformed layer of agarose. Cells were incubated at 37°C and reefed every 3 d with fresh DME. 4 wk later, cell foci that were >20 μm in diameter were counted.

Western blot and immunoprecipitation assays
Western blot and immunoprecipitation assays were described previously (Yen et al., 2002). When indicated, cells were either pretreated with PP2 at 1–10 μM or UO126 at 10 μM or were transiently transfected with dominant mutants for Src and MEK1 using Fugene reagent (Roche Diagnostics) following similar experimental conditions for FAK as described in Construction of plasmids and transfection.

Generation of cells expressing stable FAK siRNA
A specific 19-nt sequence spanning positions 466–484 of FAK human gene (GenBank/EMBL/DDBJ accession no. L10616) was cloned as inverted repeats into pSuper-retro puromycin vector according to the manufacturer's instructions (Oligoengine). Control retroviral vector pRetro-Super puromycin alone or expressing FAK siRNA was transfected into Phoenix cells using Genejuice (Novagene). After 48 h after transfection, the supernatant of Phoenix cells was filtered through a 0.45-μm filter and was used to infect target cell lines twice, 24 h apart, in the presence of 8 μg/ml polybrene. 48 h after infection, polyclonal populations were selected for resistance to 1 μg/ml puromycin for 2 wk to generate stable siRNA-expressing cells and matched (bulk) controls.

Scratch motility assay
Cells grown on coverslips were wounded by cell scraping with a micropipette tip. Cultures were washed and incubated in complete medium. Cells were incubated at 37°C for different periods of time to allow migration toward the gap and were then fixed, permeabilized, and immunostained for both ErbB-2 and FAK.

Invasion assay
Cell invasion experiments were performed with 8-μm porous chambers coated with matrigel (Becton Dickinson) according to the manufacturer's recommendations. 20 ng/ml EGF or HRG were used as chemoatractants in the lower compartment. Cells were allowed to invade through the matrigel membrane for 48 h. The invasive cells underneath were fixed and stained.

Immunofluorescence labeling
$FAK^{-/-}$ and $FAK^{+/+}$ cells overexpressing ErbB receptors were processed for immunofluorescence as previously described (Yen et al., 2002). When indicated, cells were incubated for 24 h in serum-free medium and were pretreated with 100 nM PP2 before adding 20 ng/ml EGF or HRG for 15 min. After labeling, the cells were viewed with a fluorescent microscope (Axiophot; Carl Zeiss MicroImaging, Inc.) equipped with a 63× plan Apochromat objective and selective filters. Images were acquired from a cooled CCD camera (Retiga 1300; Q Imaging) and displayed on a high resolution monitor. Images were analyzed by the Northen Eclipse Image analysis system (Carl Zeiss MicroImaging, Inc.). Confocal analyses were performed with an inverted confocal microscope (McGill University; model LSM 510; Carl Zeiss MicroImaging, Inc.).

In vivo tumorigenic and invasion studies
In vivo studies were approved by the McGill Animal Care Committee (protocol 4101) and were conducted in accordance with institutional and Canadian federal guidelines. For primary tumors, one million cells were injected subcutaneously into the flank of Scid mice ($FAK^{+/+}$ and $FAK^{-/-}$ cells) or into the mammary fat pad (MDA-231-M2). Tumor volumes were measured every second or third day as described previously (Alaoui-Jamali et al., 2003). For tumor invasion, one million cells per 100 μl were injected intravenously ($FAK^{+/+}$ and $FAK^{-/-}$ cells) or into the mammary fat pad (human breast cancer cell line MDA-231-M2). Animals were killed 4–8 wk after cell inoculation. The lungs were fixed in 10% Bouin's fixative, and lung surface metastases were counted.

Online supplemental material
Fig. S1 shows the impact of ErbB receptors that are expressed as single or paired combinations on the metastatic and chemotactic properties of mouse embryonic fibroblast ($FAK^{+/+}$) cells described in this study. Fig. S2 shows representative images of colony formation in agar and cell chemotaxis through the matrigel of the Boyden chamber. Fig. S3 shows that ErbB-2 and FAK coimmunoprecipitate from purified plasma membranes. Fig. S4 shows that FAK siRNA is stable in MDA-231-M2 tumors growing into the mammary fat pad, as revealed by immunohistochemistry analysis. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200504124/DC1.

We thank Jacynthe Laliberté (Department of Pharmacology, McGill University) for her assistance with the confocal microscopy.

This work was supported by the Canadian Breast Cancer Research Alliance, the Cancer Research Society Strategic Grant on Cancer Metastases, and, in part, by the Canadian Institutes for Health Research. M.A. Alaoui-Jamali is a Fonds de Recherches en Sante du Quebec Scholar and a recipient of the Dundi and Lyon Sachs Distinguished Scientist award. D. Schlaepfer is an established investigator of the American Heart Association.

Submitted: 22 April 2005
Accepted: 5 October 2005

References

Alaoui-Jamali, M.A., D.J. Song, N. Benlimame, L. Yen, X. Deng, M. Hernandez-Perez, and T. Wang. 2003. Regulation of multiple tumor microenvironment markers by overexpression of single or paired combinations of ErbB receptors. *Cancer Res.* 63:3764–3774.

Alimandi, M., A. Romano, M.C. Curia, R. Muraro, P. Fedi, S.A. Aaronson, P.P. Di Fiori, and M.H. Kraus. 1995. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene.* 10:1813–1821.

Baulida, J., M.H. Kraus, M. Alimandi, P.P. Di Fiore, and G. Carpenter. 1996. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J. Biol. Chem.* 271:5251–5257.

Brunton, V.G., B.W. Ozanne, C. Paraskeva, and M.C. Frame. 1997. A role for epidermal growth factor receptor, c-Src and focal adhesion kinase in an in vitro model for the progression of colon cancer. *Oncogene.* 14:283–293.

Carragher, N.O., M.A. Westhoff, D. Riley, D.A. Potter, P. Dutt, J.S. Elce, P.A. Greer, and M.C. Frame. 2002. v-Src-induced modulation of the calpain-calpastatin proteolytic system regulates transformation. *Mol. Cell. Biol.* 22:257–269.

Feldner, J.C., and B.H. Brandt. 2002. Cancer cell motility—on the road from c-erbB-2 receptor steered signaling to actin reorganization. *Exp. Cell Res.* 272:93–108.

Fincham, V.J., and M.C. Frame. 1998. The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. *EMBO J.* 17:81–92.

Frisch, S.M., K. Vuori, E. Ruoslahti, and P.Y. Chan-Hui. 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. *J. Cell Biol.* 134:793–799.

Golubovskaya, V., L. Beviglia, L.H. Xu, H.S. Earp III, R. Craven, and W. Cance. 2002. Dual inhibition of focal adhesion kinase and epidermal growth factor pathways cooperatively induces death receptor-mediated apoptosis in human breast cancer cells. *J. Biol. Chem.* 277:38978–38987.

Guy, C.T., M.A. Webster, M. Schaller, T.J. Parsons, R.D. Cardiff, and W.J. Muller. 1992. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA.* 89:10578–10582.

Guy, P.M., J.V. Platko, L.C. Cantley, R.A. Cerione, and K.L. Carraway 3rd. 1994. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA.* 91:8132–8136.

Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, and Y. Nagai. 1993. p60v-src causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO J.* 12:307–314.

Hauck, C.R., D.J. Sieg, D.A. Hsia, J.C. Loftus, W.A. Gaarde, B.P. Monia, and D.D. Schlaepfer. 2001. Inhibition of focal adhesion kinase expression or activity disrupts epidermal growth factor-stimulated signaling promoting the migration of invasive human carcinoma cells. *Cancer Res.* 61:7079–7090.

Hauck, C.R., D.A. Hsia, X.S. Puente, D.A. Cheresh, and D.D. Schlaepfer. 2002. FRNK blocks v-Src-stimulated invasion and experimental metastases without effects on cell motility or growth. *EMBO J.* 21:6289–6302.

Hommelgaard, A.M., M. Lerdrup, and B. Van Deurs. 2004. Association with membrane protrusions makes ErbB2 an internalization-resistant receptor. *Mol. Biol. Cell.* 15:1557–1567.

Hsia, D.A., S.K. Mitra, C.R. Hauck, D.N. Streblow, J.A. Nelson, D. Ilic, S. Huang, E. Li, G.R. Nemerow, J. Leng, et al. 2003. Differential regulation of cell motility and invasion by FAK. *J. Cell Biol.* 160:753–767.

Ilic, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, T. Yamamoto, and S. Aizawa. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature.* 377:539–544.

Irby, R.B., W. Mao, D. Coppola, J. Kang, J.M. Loubeau, W. Trudeau, R. Karl, D.J. Fujita, R. Jove, and T.J. Yeatman. 1999. Activating SRC mutation in a subset of advanced human colon cancers. *Nat. Genet.* 21:187–190.

Ivankovic-Dikic, I., E. Gronroos, A. Blaukat, B.U. Barth, and I. Dikic. 2000. Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. *Nat. Cell Biol.* 2:574–581.

Lemoine, N.R., D.M. Barnes, D.P. Hollywood, C.M. Hughes, P. Smith, E. Dublin, S.A. Prigent, W.J. Gullick, and H.C. Hurst. 1992. Expression of the ERBB3 gene product in breast cancer. *Br. J. Cancer.* 66:1116–1121.

Lim, Y., I. Han, J. Jeon, H. Park, Y.Y. Bahk, and E.S. Oh. 2004. Phosphorylation of focal adhesion kinase at tyrosine 861 is crucial for Ras transformation of fibroblasts. *J. Biol. Chem.* 279:29060–29065.

Lu, Z., G. Jiang, P. Blume-Jensen, and T. Hunter. 2001. Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase. *Mol. Cell. Biol.* 21:4016–4031.

Maa, M.C., T.H. Leu, D.J. McCarley, R.C. Schatzman, and S.J. Parsons. 1995. Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: implications for the etiology of multiple human cancers. *Proc. Natl. Acad. Sci. USA.* 92:6981–6985.

McLean, G.W., V.J. Fincham, and M.C. Frame. 2000. v-Src induces tyrosine phosphorylation of focal adhesion kinase independently of tyrosine 397 and formation of a complex with Src. *J. Biol. Chem.* 275:23333–23339.

Mineo, C., G.N. Gill, and R.G. Anderson. 1999. Regulated migration of epidermal growth factor receptor from caveolae. *J. Biol. Chem.* 274:30636–30643.

Nagy, P., G. Vereb, Z. Sebestyen, G. Horvath, S.J. Lockett, S. Damjanovich, J.W. Park, T.M. Jovin, and J. Szollosi. 2002. Lipid rafts and the local density of ErbB proteins influence the biological role of homo- and heteroassociations of ErbB2. *J. Cell Sci.* 115:4251–4262.

Naidu, R., M. Yadav, S. Nair, and M.K. Kutty. 1998. Expression of c-erbB3 protein in primary breast carcinomas. *Br. J. Cancer.* 78:1385–1390.

Olayioye, M.A., R.M. Neve, H.A. Lane, and N.E. Hynes. 2000. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.* 19:3159–3167.

Pinkas-Kramarski, R., L. Soussan, H. Waterman, G. Levkowitz, I. Alroy, L. Klapper, S. Lavi, R. Seger, B.J. Ratzkin, M. Sela, and Y. Yarden. 1996. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.* 15:2452–2467.

Renshaw, M.W., L.S. Price, and M.A. Schwartz. 1999. Focal adhesion kinase mediates the integrin signaling requirement for growth factor activation of MAP kinase. *J. Cell Biol.* 147:611–618.

Riese, D.J., II, T.M. Van Raaij, G.D. Plowman, G.C. Andrews, and D.F. Stern. 1995. The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol. Cell. Biol.* 15:5770–5776.

Schaller, M.D., J.D. Hildebrand, J.D. Shannon, J.W. Fox, R.R. Vines, and J.T. Parsons. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol. Cell. Biol.* 14:1680–1688.

Schlaepfer, D.D., K.C. Jones, and T. Hunter. 1998. Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol. Cell. Biol.* 18:2571–2585.

Sieg, D.J., C.R Hauck, and D.D. Schlaepfer. 1999. Required role of focal adhesion kinase FAK for integrin-stimulated cell migration. *J. Cell. Sci.* 16:2677–2691.

Sieg, D.J., C.R. Hauck, D. Ilic, C.K. Klingbeil, E. Schaefer, C.H. Damsky, and D.D. Schlaepfer. 2000. FAK integrates growth-factor and integrin signals to promote cell migration. *Nat. Cell Biol.* 2:249–256.

Siegel, P.M., E.D. Ryan, R.D. Cardiff, and W.J. Muller. 1999. Elevated expression of activated forms of Neu/erbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implication for human breast cancer. *EMBO J.* 18:2149–2164.

Slamon, D.J., G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, and W.L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science.* 235:177–182.

Vadlamudi, R.K., L. Adam, D. Nguyen, M. Santos, and R. Kumar. 2002. Differential regulation of components of the focal adhesion complex by heregulin: role of phosphatase SHP-2. *J. Cell. Physiol.* 190:189–199.

Vadlamudi, R.K., A.A. Sahin, L. Adam, R.A. Wang, and R. Kumar. 2003. Heregulin and HER2 signaling selectively activates c-Src phosphorylation at tyrosine 215. *FEBS Lett.* 543:76–80.

Vartanian, T., A. Goodearl, S. Lefebvre, S.K. Park, and G. Fischbach. 2000. Neuregulin induces the rapid association of focal adhesion kinase with the erbB2-erbB3 receptor complex in schwann cells. *Biochem. Biophys. Res. Commun.* 271:414–417.

Waterman, H., I. Sabanai, B. Geiger, and Y. Yarden. 1998. Alternative intracellular routing of ErbB receptors may determine signaling potency. *J. Biol. Chem.* 273:13819–13827.

Westhoff, M.A., B. Serrels, V.J. Fincham, M.C. Frame, and N.O. Carragher. 2004. SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol. Cell Biol.* 18:8113–8133.

Xia, W., Y.K. Lau, H.Z. Zhang, F.Y. Xiao, D.A. Johnston, A.R. Liu, L. Li, R.L. Katz, and M.C. Hung. 1999. Combination of EGFR, HER-2/neu, and HER-3 is a stronger predictor for the outcome oral squamous cell carcinoma than any individual family members. *Clin. Cancer Res.* 5:4164–4174.

Yen, L., N. Benlimame, Z.R. Nie, D. Xiao, T. Wang, A.E. Al Moustafa, H. Esumi, J. Milanini, N.E. Hynes, G. Pages, and M.A. Alaoui-Jamali. 2002. Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers. *Mol. Biol. Cell.* 13:4029–4044.