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

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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., 1994).

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; Ali-mandi 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.
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, and 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 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−/−−2 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-
sulted in strong oncogenic transformation compared with ErbB-2 alone (Fig. 2B and Fig. S2A, 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<sup>−/−</sup> cells expressing ErbB-2 and -3 combinations (Fig. 2A). Interestingly, the restoration of FAK in FAK<sup>−/−</sup>-2/3 and -2 rescued the ability of ErbB to induce anchorage-independent growth on soft agar but did not in control FAK<sup>−/−</sup> cells or FAK<sup>+/+</sup>-2/3 cells overexpressing the kinase-deficient receptor ErbB-3 (Fig. 2B and Fig. S2A).

**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<sup>+/+</sup>-2 and -2/3 cells exhibited an increase in invasion upon HRG stimulation, with cells overexpressing ErbB-2/3 being the most chemotactic (Fig. 2C and Fig. S2B). FAK<sup>−/−</sup> cells expressing control retroviral particles or any of the ErbB-2 and -3 combinations were weakly invasive, whereas FAK<sup>+/+</sup>-2 and -2/3 in which wild-type FAK was reconstituted (FAK<sup>+/+</sup>-2–FAK and FAK<sup>+/+</sup>-2/3–FAK, respectively) exhibited an increase in invasion upon HRG stimulation. This rescue was not observed in control FAK<sup>−/−</sup> and FAK<sup>+/+</sup>-3 stably expressing wild-type FAK (Fig. 2D and Fig. S2B).

**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<sup>+/+</sup> nor FAK<sup>−/−</sup> cells that were transduced with control retroviral particles or ErbB-3 receptor formed tumors after >35 d (Fig. 3A). The overexpression of ErbB-2 and -2/3 in FAK<sup>+/+</sup>-2/3 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<sup>3</sup> in <14 d for FAK<sup>+/+</sup>-2/3 vs. >18 d for FAK<sup>−/−</sup>-2/3 cells). In contrast, in FAK<sup>−/−</sup>-2/3 cells, tumor sizes were <0.5 cm<sup>3</sup> for both ErbB-2 and -2/3 (the maximal size was ~0.5 cm<sup>3</sup> 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 ≥ 8 mice per condition for each experiment.

To further demonstrate that differences in tumor growth between FAK<sup>−/−</sup>-ErbB and FAK<sup>+/+</sup>-ErbB is directly related to FAK, we examined the impact of FAK reconstitution on tumorigenesis of FAK<sup>−/−</sup>-2/3 cells, focusing on ErbB-2/3 as the most aggressive in FAK<sup>+/+</sup>-2/3. As shown in Fig. 3B, the expression of wild-type FAK in FAK<sup>−/−</sup>-2/3 clearly restored tumor formation. In contrast, the restoration of FAK in control FAK<sup>−/−</sup>-2/3 did not induce tumor formation. In FAK<sup>−/−</sup>-2/3–FAK, we noted a clear delay for the tumors to reach exponential tumor growth phase compared with FAK<sup>+/+</sup>-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<sup>+/+</sup> and FAK<sup>−/−</sup> control cells that were transduced with

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**Figure 2. FAK is essential for ErbB-induced oncogenic transformation and chemotaxis.**

(A) Western blot analysis to show FAK expression status in FAK<sup>−/−</sup> 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<sup>+/+</sup>-ErbB-2/3 cells are included as a control. (B) Oncogenic property of FAK<sup>−/−</sup>- and FAK<sup>+/+</sup>-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. S2A, available at http://www.jcb.org/cgi/content/full/jcb.200504124/DC1). Each bar on the graph represents the mean ± SD. (C and D) ErbB-induced cell invasion in FAK<sup>+/+</sup> (C), FAK<sup>/−</sup> and FAK<sup>−/−</sup>-expressing ErbB-2/3 cells in which FAK was restored (D). FAK<sup>+/+</sup>, FAK<sup>−/−</sup>, and FAK<sup>−/−</sup>-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.
empty retroviral particles or FAK\textsuperscript{+/+} and FAK\textsuperscript{-/-} 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\textsuperscript{-/-} cells compared with cells overexpressing ErbB-2 receptor alone or FAK\textsuperscript{+/+} 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\textsuperscript{-/-} cells compared with FAK\textsuperscript{+/+} cells is directly related to FAK, we examined the impact of FAK on lung metastasis formation in FAK\textsuperscript{-/-} cells in which wild-type FAK was restored. As shown in Fig. 3 D, the restoration of FAK in FAK\textsuperscript{-/-} cells partially rescued the deficiency in invasion (P < 0.05 when comparing FAK\textsuperscript{-/-} with FAK\textsuperscript{-/-}–FAK). This partial rescue may be contributed by lower levels of FAK expression in FAK\textsuperscript{+/+} cells compared with endogenous FAK in FAK\textsuperscript{-/-} 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\textsuperscript{-/-} 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-
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+/−/− cells compared with control FAK+/+ and FAK−/−/− cells. Interestingly, the reconstitution of FAK in FAK−/−/− cells restored ERK phosphorylation to approximately the same levels seen in FAK+/−/− 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+/−/− 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+/−/− cells and FAK-reconstituted FAK−/−/− cells. In contrast, only PP2 or Src dominant mutants prevented ErbB-2/3–induced chemotaxis (Fig. 4 E).
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 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−/−/− 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+/−/− 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−/−/− cells 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.

Figure 5. ErbB-2 colocalizes with FAK at focal adhesions in motile cells. (A) ErbB-2 and FAK colocalize in motile FAK+/−/− cells. Confluent cells were scratch wounded and allowed to heal for the indicated time points before fixation. Cells were then immunostained 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−/−/− 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+/−/− 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−/−/− cells 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.

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We then examined the subcellular localization of ErbB-2 and FAK by confocal microscopy. FAK−/−/− 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−/−/− 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−/−/− 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).
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 throughout the cytoplasm (E). Bar, 30 \(\mu\)m.

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.
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 coinmunolabeled 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 coexpression in FAK-proficient cells, but not in FAK-deficient cells, induced an-
chorage-independent growth on soft agar as well as tumor formation in vivo. Consistently, the restoration of FAK can rescue the inability of FAK<sup>-/-</sup>/3– as well as FAK<sup>-/-</sup>/2– overexpressing cells to induce growth on soft agar and tumor formation in vivo. In contrast, this rescue was not observed in FAK<sup>-/-</sup> 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)anthracene, 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). 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<sup>−/−</sup>-2/3–FAK cells may be explained by low levels of exogenous FAK in FAK<sup>−/−</sup>-2/3 compared with FAK<sup>+/+</sup> 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<sup>−/−</sup> 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 increase 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 αvβ3 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 (Holmggaard 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-MB-231 cells were obtained from the American Type Culture Collection. The metastatic variants MDA-MB-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 385) and polyclonal anti-ErbB-2 (Ab-1; Oncogene); polyclonal anti-ErbB-3 (clone C-17), polyclonal anti-FAK (A-17; C-20), and polyclonal anti-phospho-FAK (Y925) (Santa Cruz Biotechnology, Inc.); antiphospho-FAK Y397 and antiphospho-FAK Y861 (Biosource International); antiphosphotyrosine antibody (4G10) and anti–ERK-2 (clone B389; Upstate Biotechnology); antiphospho-FAK Y397 and antiphospho-FAK Y861 (Biosource International); antifluorescein isothiocyanate (FITC); and antiphospho–FAK-Y925 (clone B389; Upstate Biotechnology); antiphospho-FAK Y397 and antiphospho-FAK Y861 (Biosource International); antiphosphotyrosine antibody (4G10) and anti–ERK-2 (clone B389; Upstate Biotechnology); antiphospho-FAK Y397 and antiphospho-FAK Y861 (Biosource International); antiphosphotyrosine antibody (4G10) and anti–ERK-2 (clone B389; Upstate Biotechnology). Human cancer cells were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS and penicillin/streptomycin.

Stable expression of ErbB receptors in FAK−/− cells

ErbB receptors were expressed in a polyclonal cell population as described previously (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 (Axiohot; 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 Northern 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.).

Generation of cells expressing stable FAK siRNA

A specific 19 nt sequence spanning positions 466–484 of FAK human gene (GenBank/EMBL/DBJ accession no. L10616) was cloned into vectors 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 (Novagen). 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 chemoattractants 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 (Axiohot; 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 Northern 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-MB-231-M2). Tumor volumes were measured every second or third day as described previously (Alooui-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 immunoprecipitate 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.

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