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CXCR4 Suppression Attenuates EGFRvIII-Mediated Invasion and Induces p38 MAPK-Dependent Protein Trafficking and Degradation of EGFRvIII in Breast Cancer Cells

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Abstract

Our previous report has shown that the constitutively activated EGFR variant, EGFRvIII, up-regulates the pro-metastatic chemokine receptor CXCR4 in breast cancer cells. Here we evaluated the biological effect and cell signaling effects of silencing CXCR4 expression in EGFRvIII-expressing breast cancer cells. Short hairpin RNA (shRNA)-mediated suppression of CXCR4 expression significantly reduced the invasive potential and proliferation of EGFRvIII-expressing breast cancer cells. These cells exhibited a reduction of EGFRvIII activity and protein expression due to increased protein degradation and altered protein trafficking. In conclusion, suppression of CXCR4 inhibits EGFRvIII-mediated breast cancer cell invasion and proliferation.

Keywords

EGFR; EGFRvIII; CXCR4; Growth; Invasion

1. Introduction

Chemokines have been reported to be associated with breast cancer metastasis. The chemokine and chemokine receptor axis of CXCL12/CXCR4 is believed to home CXCR4-expressing cells to organ sites expressing CXCL12 [1]. The expression of CXCR4 in human breast cancer specimens are also associated with metastatic spread and poor prognosis [2–8]. CXCR4 neutralizing antibodies and other CXCR4 antagonists developed primarily to inhibit HIV-viral entry have shown to inhibit the invasive potential of breast cancer cells *in vitro* as well as reduce breast cancer metastasis in nude mice [1;9–12].

Silencing of CXCR4 expression with RNA-interference (RNAi) has shown to inhibit breast cancer cell migration, invasion, and metastasis *in vitro* and *in vivo*. Initially, an inducible knock-down of CXCR4 using RNAi technology resulted in a significant reduction of breast cancer cell migration [13]. Down-regulation of CXCR4 with short hairpin RNA (shRNA) in breast cancer cells with high metastatic potential (MDA-MB-231) not only lowered lung metastasis, it also reduced breast cancer cell proliferation [14]. Decreased proliferation of

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breast cancer cells by suppressed expression of CXCR4 also resulted in the failure of these cells to grow tumors in SCID mice [15]. Targeting CXCR4 with the combination of two small interfering RNA (siRNA) duplexes also impaired breast cancer cell invasion using matrigel invasion assays and breast cancer metastasis in an animal model [16]. ErbB2-mediated breast cancer metastasis is also dependent on CXCR4 up-regulation as it was shown that shRNA knock-down of CXCR4 in ErbB2-overexpressing breast cancer cells inhibited breast cancer metastasis to the lung [9]. However, the signaling cascades or other molecular events by which this inhibition occurs are not well described.

Our previous studies have found increased expression of the Epidermal Growth Factor Receptor (EGFR) variant, EGFRvIII, in breast cancer metastasis [17]. Expression of this variant has also been detected in circulating breast cancer cells and was found to be correlated with metastatic disease, however, the mechanism by which this tumor specific, constitutively active oncoprotein promotes breast cancer metastasis is not understood [18;19]. Recently, we reported that EGFRvIII up-regulates CXCR4 in breast cancer cells regardless of estrogen and progesterone receptor (ER/PgR) status or levels of endogenous ErbB-receptors [20]. Furthermore, CXCR4 was transcriptionally up-regulated in EGFRvIII-expressing breast cancer cells via up-regulation of HIF-1 α and/or post-translationally through decreased protein degradation and presumably increased receptor recycling and protein trafficking involving p38 MAPK activity and down-regulation of the endosomal sorting molecules β -Arrestin 1/2 and the Nedd4-like E3 ubiquitin ligase AIP4 [20]. More importantly, these cells also had enhanced SDF-1/CXCR4-mediated invasion [20]. Since EGFRvIII-expressing breast cancer cells universally had an up-regulation of CXCR4 expression and metastatic breast cancer cells that require CXCR4 for breast cancer metastasis often express high levels of ErbB-receptors such as EGFR and ErbB2, we were interested in understanding potential changes in the activity or expression of these ErbB-receptors upon suppression of CXCR4 expression.

Here we report that suppression of CXCR4 with shRNA significantly reduces not only the invasive potential of EGFRvIII-expressing breast cancer cells, it also reduces the proliferation of the cells. Cross-talk between CXCR4 and EGFRvIII is bidirectional. Suppression of CXCR4 expression reduces the protein levels of EGFRvIII through enhanced EGFRvIII protein degradation. Inhibition of the proteasome and protein trafficking revealed that both of these pathways are involved in the turnover of EGFRvIII in CXCR4-shRNA cells. Furthermore, inhibition of p38 MAPK, a key molecule which has been shown to be involved in EGFR internalization and down-regulation, reverses EGFRvIII protein suppression by CXCR4 knockdown. However, activation of p38 MAPK under hypoxic conditions increases EGFRvIII protein levels suggesting an essential role of p38 MAPK in EGFRvIII protein trafficking.

2. Materials and Methods

2.1. Cell Culture and Reagents

MDA-MB-361 and BT474 breast carcinoma cell lines and their EGFRvIII-expressing derivatives were maintained in IMEM supplemented with 10% FBS. Since endogenous EGFRvIII expression is lost in cancer cells under *in vitro* conditions [21], stable EGFRvIII-expressing breast cancer cells were generated as previously described [22]. Cycloheximide, MG132, chloroquine, monensin, leupeptin, and SB203850 were purchased from Sigma-Aldrich (St. Louis, MO). PD150606 and PD98059 were purchased from Calbiochem (Gibbstown, NJ).

2.2. CXCR4 shRN

CXCR4 human shRNA constructs were purchased from Origene (Rockville, MD). MDAMB-361 and BT474 cells expressing EGFRvIII were transfected with negative control constructs [empty vector (pRS) and GFP-targeting] and four shRNA constructs targeting CXCR4 using calcium phosphate precipitation as previously described [22]. Transfected cells were selected using puromycin. Stable cell lines were generated using individual clones and pooled clones. Knock-down of CXCR4 was verified using fluorescence-activated cell sorting analysis and quantitative real-time PCR and at least two cell lines transfected with two different CXCR4 shRNA constructs that had the most CXCR4 knock-down were used. Pooled clones transfected with the negative control constructs were used as negative controls.

2.3. Fluorescence-Activated Cell Sorting (FACS) Analysis

Cells ($0.5\text{--}1.0 \times 10^6$) were harvested and then stained for 1 hour with anti-CXCR4 (mab172 or mab173; R&D Systems; Minneapolis, MN) antibodies at 4°C. Stained cells were then washed with cold PBS. A secondary FITC-anti-mouse antibody (KPL; Gaithersburg, MD) was added for 30 minutes, and the CXCR4 levels were quantified by flow cytometry.

2.4. Quantitative Real-Time PCR

RNA was reverse transcribed from random hexamers using SuperScript® III Reverse transcriptase (Invitrogen; Carlsbad, CA). Real-time quantitative PCR was performed using the Real-time PCR system 7900 (Applied Biosystems; Foster City, CA). In brief, the PCR amplification reaction mixtures (25 µL) contained cDNA, RT² PCR Primer Assay (SA Biosciences; Frederick, MD), and RT² Real-Time SYBR Green Master Mix (SA Biosciences) (performed in triplicates). The thermal cycle conditions included maintaining the reactions at 50°C for 2 minutes and at 95°C for 10 minutes, and then alternating for 40 cycles between 95°C for 15 seconds and 60°C for 1 minute. The relative gene expression for each sample was determined using the formula $2^{(-\delta Ct)} = 2^{(Ct(GAPDH) - Ct(target))}$, which reflected the target gene expression normalized to GAPDH levels.

2.5. Invasion Assays

Invasion was measured using 24-well cell culture inserts with membranes with 8 µm pores and a matrigel-coating to mimic the basement membrane (BD Biosciences; San Jose, CA). Breast cancer cells were suspended in serum-free medium with 0.1% BSA and 2.0×10^5 cells were plated in the top part of the insert. The inserts were placed in wells containing 10% FBS in IMEM. After incubation at 37°C for 48 hours, residual cells were wiped off the top of the membranes with cotton swabs, and invaded cells on the underside of the membranes were fixed and stained using the HEMA-3 kit (Fisher Diagnostics; Pittsburgh, PA). Cells were counted in 10 fields from three inserts per experimental condition. Experiments were performed in a minimum of three independent studies.

2.6. Anchorage-Dependent Growth assays

Cancer cells in normal growth media were seeded in triplicates in 24-well plates. Using a cell counter, cancer cells were counted on days 0, 1, 7, and 10. Experiments were performed in a minimum of three independent studies.

2.7. Immunoblot and Immunoprecipitation analysis

Breast cancer cells were plated in culture plates and grown to 50–80% confluence. Unless otherwise specified, cells were lysed after the removal of growth media. Some cultures were pretreated with MG132, chloroquine, monensin, leupeptin, or PD150606, and then cycloheximide for the specified times. Hypoxia experiments were performed in a computer

monitored hypoxia chamber (94% nitrogen, 5% carbon dioxide, and 0.5 to 1% oxygen) for 24 hours. Cells were rinsed, lysed, and equal amounts of protein were then separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis. Antibodies against phospho-EGFR (Thr998, Tyr1148, Tyr845, Tyr1173, Tyr1068), phospho-ErbB2 (Tyr877), phospho-p38 MAPK (Thr180/Tyr182), and p38 MAPK were purchased from Cell Signaling Technology (Danvers, MA); the antibodies for EGFR and ErbB2 were purchased from NeoMarkers (Fremont, CA); the antibody for HIF-1 α was purchased from BD Biosciences; the antibodies for phospho-Cbl (Tyr774) and Cbl were purchased from Upstate (Billerica, MA); and the antibody for GAPDH was purchased from Sigma-Aldrich. Densitometry measurements were performed using Scion Image software (Scion Corporation; Frederick, MD).

Immunoprecipitation experiments were performed using 500–1000 μ g of lysate and 1 μ g of anti-EGFR antibody (Neomarkers). After overnight precipitation at 4°C, protein A-agarose beads (Amersham Biosciences, NJ, USA) were added and left for a 2-hour incubation at 4°C. The immunocomplexes were then separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis using anti-phosphotyrosine (Upstate) and anti-EGFR (Neomarkers) antibodies.

2.8. Statistics

Statistical analysis was performed using ANOVA, followed by the Tukey test using SigmaStat software. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. CXCR4 suppression inhibits the invasion and proliferation of EGFRvIII-expressing breast cancer cells

Our early study showed that expressing EGFRvIII in breast cancer cells results in increased expression of CXCR4 [20], therefore, we determined the consequences of stably suppressing CXCR4 expression in these cells with two different shRNAs targeting CXCR4. Successful reduction of CXCR4 mRNA and protein was achieved in EGFRvIII-expressing MDA-MB-361 (“MDA-MB-361/vIII”) and BT474 (“BT474/vIII”) breast cancer cells, although complete suppression of CXCR4 protein was not achieved by any shRNA constructs (Figure 1A and B). Both of the shRNA constructs specifically targeted CXCR4 as expression of the closely related CXCR7 was not suppressed and CXCR7 did not compensate for loss of CXCR4 as the expression of this chemokine receptor continued to remain relatively low in these CXCR4-shRNA transfectants (data not shown). It is worthwhile to note that transfecting the EGFRvIII-expressing breast cancer cells with the CXCR4-shRNA constructs resulted in very few clones with stable suppression of CXCR4 expression, which implicated that significant suppression of CXCR4 may inhibit the proliferation or survival of breast cancer cells expressing EGFRvIII. Therefore, the EGFRvIII-expressing breast cancer cells that maintained a sufficient level of CXCR4 protein levels were viable. Only clones which maintained sufficient CXCR4 knock-down were used in these studies.

We then evaluated the biological effects of silencing CXCR4 expression in EGFRvIII-expressing breast cancer cells. Using *in vitro* invasion assays, it was determined that CXCR4 suppression substantially reduces the invasive potential of MDA-MB-361/vIII breast cancer cells (Figure 1C). The reduction of invasion was correlated with the level of CXCR4 suppression as clones with the most reduction of CXCR4 expression had the most reduction in invasion (Figure 1C and Supplemental Figure 1). Invasion assays for BT474/vIII breast cancer cells with suppressed CXCR4 levels were unsuccessful as these cells usually have low invasive potential. However, both MDA-MB-361/vIII and BT474/vIII breast cancer

cells with suppressed CXCR4 expression exhibited a significant reduction in proliferation (Figure 1D).

3.2. CXCR4 suppression reduces EGFRvIII phosphorylation and protein expression

Although the biological effects of CXCR4 suppression in breast cancer cells has been previously reported, signaling changes in these cells remain to be established [9;13– 16]. We determined whether there were any changes to the phosphorylation and protein levels of ErbB-receptors, including EGFRvIII, when CXCR4 levels are reduced. CXCR4 suppression in EGFRvIII-expressing breast cancer cells resulted in reduced phosphorylation and expression of EGFRvIII in both MDA-MB-361/vIII and BT474/vIII cells under steady-state conditions (Figure 2A). The degree by which EGFRvIII phosphorylation and protein levels were reduced was correlated to the level of CXCR4 protein reduction (Supplemental Figure 1). This reduction in EGFRvIII protein levels also correlated with reduction in invasive potential (Supplemental Figure 1). Although transactivation and crosstalk between EGFR and ErbB2 with CXCR4 has been reported [23], we found increased phosphorylation and expression of ErbB2 in MDA-MB-361/vIII cells when CXCR4 expression is suppressed (Figure 2B). This may be attributed to a feedback mechanism resulting from decreased activity of CXCR4 or EGFRvIII as these cells express higher levels of CXCR4 and lack high expression of wild-type EGFR. However, in the BT474/vIII breast cancer cells, which express intermediate levels of endogenous wild-type EGFR, CXCR4 suppression led only to a decrease in ErbB2 phosphorylation (Figure 2B), and the phosphorylation and expression of wild-type EGFR remained unchanged despite the significant decreased expression of EGFRvIII (Figure 2C). This observation may result from decreased heterodimerization between EGFRvIII and ErbB2 and the ability of wild-type EGFR to compensate for loss of CXCR4 and EGFRvIII expression.

3.3. CXCR4 suppression increases the protein degradation of EGFRvIII through protein trafficking and proteasomal degradation

Cbl-mediated ubiquitination and proteasomal degradation attenuates activated wild-type EGFR signaling, a process EGFRvIII evades [24]. Since EGFRvIII levels are reduced by suppression of CXCR4, it was determined whether the rate of EGFRvIII degradation was altered. Treating MDA-MB-361/vIII and BT474/vIII breast cancer cells with the protein synthesis inhibitor, cycloheximide, revealed that the rate of EGFRvIII degradation is increased when CXCR4 levels are knocked-down (Figure 3A and Supplemental Figure 2). In addition, the rate of ErbB2 degradation was slightly reduced in MDA-MB-361/vIII breast cancer cells, whereas in the BT474/vIII breast cancer cells the degradation rate of ErbB2 was increased when CXCR4 expression is suppressed. These results suggest that the two different cell lines have different compensatory feedback mechanisms and potentially different ErbB-receptor degradation pathways, which is likely depended upon the genetic background of the cells (Figure 3B). The degradation of wild-type EGFR appears to also be accelerated (Figure 3A and Supplemental Figure 2), although the expression of wild-type EGFR is relatively low in both cell lines and a high wild-type EGFR-expressing cell line would need to be used to assess the impact of CXCR4 loss on its degradation. Also, these alterations were unlikely a result of increased Cbl-mediated degradation as the phosphorylation and/or protein levels of Cbl were not increased in EGFRvIII-expressing breast cancer cells with suppressed CXCR4 expression (Supplemental Figure 3).

Next, these cells were treated with MG132 (proteasome inhibitor), chloroquine (receptor recycling inhibitor), monensin (intracellular protein trafficking inhibitor), and leupeptin and PD150606 (lysosomal protease inhibitors), to determine if these pathways were responsible for EGFRvIII down-regulation (Figure 4A). While it was expected that MG132 treatment would partially inhibit the degradation of EGFRvIII, we also discovered that monensin also

partially prevented the degradation of EGFRvIII in the MDA-MB-361/vIII breast cancer cells with suppressed CXCR4 expression (Figure 4). However, monensin did not have a similar effect in the BT474/vIII breast cancer cells with suppressed CXCR4 expression, suggesting that the two cell lines do actually have different mechanisms which induce degradation of EGFRvIII (Figure 4B). The unglycosylated form of EGFRvIII (lowest protein band) seemed to be more sensitive to monensin, while MG132 did not prevent the degradation of the unglycosylated form of EGFRvIII (Figure 4). This led us to conclude that suppression of CXCR4 may induce signaling cascades which induce increased EGFRvIII protein turnover through the proteasome and protein trafficking.

3.4. Suppression of increased p38 MAPK activity in CXCR4 shRNA cells reverses the reduction of EGFRvIII protein expression

Since the suppression of CXCR4 expression caused a reduction in the phosphorylation and expression of EGFRvIII, alterations in signaling pathways downstream of growth factor receptors were investigated. Surprisingly, very few alterations in signaling pathways were found in CXCR4 shRNA expressing MDA-MB-361/vIII breast cancer cells (data not shown), despite that these cells exhibited reduced invasion and proliferation. Another unexpected result was increased p38 MAPK activity in MDA-MB-361/vIII breast cancer cells with suppressed CXCR4 protein levels (Figure 5A). Enhanced activation of p38 MAPK was also observed in BT474/vIII breast cancer cells when CXCR4 expression is suppressed (Figure 5A), but this cell line has low endogenous p38 MAPK phosphorylation and protein expression. While this is inconsistent with our previous results, this result is in agreement with other results which have shown that activation of p38 MAPK induces EGFR internalization and down-regulation [25;26]. Furthermore, inhibition of p38 MAPK with SB203580 reversed the down-regulation of EGFRvIII when CXCR4 is suppressed in breast cancer cells (Figure 5B). However, the p44/42 MAPK inhibitor PD98059 did not have this effect, suggesting that p38 MAPK activity is involved in the process of EGFRvIII down-regulation (Figure 5C). Although the phosphorylation of EGFRvIII and ErbB2 were significantly down-regulated under hypoxic conditions which increase the phosphorylation of p38 MAPK, the EGFRvIII protein levels were increased (Figure 6). This suggests that p38 MAPK is involved in the protein trafficking of EGFRvIII and possibly other ErbB-receptors as well, although the activation of this pathway may positively or negatively regulate ErbB-receptor protein expression based on the cellular context of the cells and the tumor microenvironment.

4. Discussion

The SDF-1/CXCR4 axis plays a pivotal role in breast cancer metastasis and our previous report found that the naturally occurring EGFR variant, EGFRvIII, up-regulates CXCR4 through multiple mechanisms [20]. Our current study found that suppression of CXCR4 in EGFRvIII-expressing breast cancer cells decreased the invasive potential and the proliferation of EGFRvIII-expressing breast cancer cells. Suppression of CXCR4 expression also increased the degradation of EGFRvIII through the proteasome as well as altered protein trafficking, and the activity of p38 MAPK seemed to play an essential role in protein trafficking and down-regulation of EGFRvIII.

We discovered that complete suppression of CXCR4 protein levels was unattainable in EGFRvIII-expressing breast cancer cells although CXCR4 mRNA levels were significantly suppressed by shRNA constructs targeting CXCR4. Previously, we showed that increased p38 MAPK activity mediated by EGFRvIII induces increased cell surface expression of CXCR4 presumably through altered protein trafficking [20]. Both the ectopic expression of EGFRvIII in breast cancer cells and hypoxic conditions that induce p38 MAPK activity, lead to an up-regulation of CXCR4 [20]. Here we also unexpectedly discovered increased

p38 MAPK activity when CXCR4 expression is suppressed. Increased activation of this pathway may explain why we observed less suppression of CXCR4 protein levels although CXCR4 mRNA levels were highly suppressed in most of our stable cell lines. Forced suppression of CXCR4 expression may induce a stress response by which cells maintain CXCR4 protein levels by activating p38 MAPK. Subsequently, activation of p38 MAPK negatively regulates EGFRvIII expression as inhibition of the p38 MAPK pathway restored EGFRvIII expression in breast cancer cells with suppressed CXCR4 suppression. Interestingly, hypoxic conditions induced p38 MAPK activity and a reduction in EGFRvIII and ErbB2 phosphorylation, but EGFRvIII protein levels were increased. Therefore, the p38 MAPK pathway may regulate the protein trafficking and regulation of ErbB-receptors in order to meet the requirements of cancer cells under stress conditions to facilitate cellular signaling, proliferation, and invasion. Whether activation and up-regulation of CXCR4 under stress conditions such as low oxygen tension directly influences the phosphorylation and protein expression of oncogenes such as ErbB-receptors as well as the activity of p38 MAPK remains to be investigated.

Our results also showed that EGFRvIII down-regulation in breast cancer cells with suppressed CXCR4 expression may be mediated by different pathways. This is also the case for the up-regulation of CXCR4 in EGFRvIII-expressing breast cancer cells as CXCR4 is primarily regulated post-translationally in MDA-MB-361 breast cancer cells expressing EGFRvIII (estrogen-independent cells), while it is regulated transcriptionally and post-translationally in BT474 breast cancer cells expressing EGFRvIII (estrogen-dependent cells) [20]. We are unable to elucidate whether CXCR4 suppression has any impact on transcriptional regulation of EGFRvIII since cancer cell lines do not express endogenous EGFRvIII under cell culture conditions. It is plausible that heterogeneity between breast cancer cells allows for more transcriptional control of protein expression in BT474 breast cancer cells expressing EGFRvIII, while MDA-MB-361 breast cancer cells expressing EGFRvIII regulate the expression of these proteins through posttranslational mechanisms such as protein stability as these cells have higher activity of the p38 MAPK pathway. Activation of p38 MAPK was shown to alter the expression of wild-type EGFR from the cell surface to the cytoplasm [27]. EGFRvIII is also found to be expressed in cytoplasm and whether p38 MAPK activation by the constitutive activity of EGFRvIII alters the localization of EGFRvIII and possibly other ErbB-receptors remains to be determined [28]. Furthermore, MDA-MB-361 breast cancer cells expressing EGFRvIII become estrogen-independent and tamoxifen-resistant, characterized by decreased levels of estrogen receptor (ER) α and loss of the progesterone receptor. However, these molecular changes do not occur in BT474 breast cancer cells expressing EGFRvIII [29]. Activation of p38 MAPK was shown to be involved in tamoxifen resistance of breast cancer cells, and whether a more active p38 MAPK signaling pathway in EGFRvIII-expressing MDA-MB-361 leads to the tamoxifen-resistant phenotype of these cells needs to be investigated, as blockage of this pathway may resensitize these cells to tamoxifen [30]. Since CXCR4 is also an estrogen-regulated gene, the impact of CXCR4 suppression on the tamoxifen-sensitivity of these cells is also warranted.

EGFRvIII has been shown to protect cancer cells from radiation and hypoxic conditions [31]. However, the effect of low oxygen tension on EGFRvIII expression and signaling has never been described. Although we discovered that hypoxic conditions reduce EGFRvIII phosphorylation and induce protein expression, EGFRvIII phosphorylation and protein expression persist which may result in the protective effect on cancer cells under stress conditions. Increased CXCR4 expression in combination with EGFRvIII expression in cancer cells may play an important role in this protective effect, and therefore, suppression of CXCR4 in EGFRvIII-expressing breast cancer cells may reduce the resistance of these cells to radiation and hypoxia. These results continue to implicate that crosstalk and

crossregulation by CXCR4 and ErbB-receptors is a dynamic, yet important interaction during breast cancer progression and metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

FACS analysis and quantitative RT-PCR shows shRNA constructs targeting CXCR4 suppressed mRNA (A) and protein (B) expression of CXCR4 in MDA-MB-361 and BT474 breast cancer cells stably expressing EGFRvIII in comparison to cells transfected with control constructs (EGFRvIII-expressing cells are labeled "vIII"; shRNA negative control constructs are labeled "CTRL"; individual clones from two different CXCR4 shRNA constructs are labeled "shRNA1" and "shRNA2") (* = $p < 0.05$; ** = $p < 0.001$; ANOVA). FACS bar graphs represent the relative expression based on the mean geometric fluorescence of cells in each group/control cells. (C) Invasion of MDA-MB-361/vIII cells was significantly inhibited by stable CXCR4 suppression with shRNA (* = $p < 0.001$; ANOVA). Cells were plated in 0.1% BSA/IMEM in the upper chamber of transwells with membranes with 8 μ m pores, and with serum containing media in the lower chamber. After 48 hours of incubation, the membranes were fixed and stained, and cells counted on the underside of the membranes. (D) Anchorage-dependent growth of MDA-MB-361/vIII and BT474/vIII cells was significantly reduced by stable CXCR4 suppression with shRNA (* = $p < 0.05$; ANOVA).

Fig. 2.

(A) Immunoblot analysis revealed decreased phosphorylation and expression of EGFRvIII in EGFRvIII-expressing breast cancer cells with suppressed CXCR4 expression. (B) Immunoblot analysis revealed altered phosphorylation and expression of ErbB2 in EGFRvIII-expressing breast cancer cells with suppressed CXCR4 suppression. (C) Immunoblot analysis revealed decreased phosphorylation of EGFRvIII at various phosphorylation sites as well as EGFRvIII protein levels, while wild-type EGFR phosphorylation/expression was unchanged with suppressed CXCR4 expression.

Fig. 3. (A) Immunoblot analysis of MDA-MB-361/vIII and BT474/vIII breast cancer cells with suppressed CXCR4 expression treated with the protein synthesis inhibitor cycloheximide (10 μ g/mL) showing that CXCR4 suppression decreases the stability of EGFRvIII. (B) Immunoblot analysis of MDA-MB-361/vIII and BT474/vIII breast cancer cells with suppressed CXCR4 expression treated with the protein synthesis inhibitor cycloheximide (10 μ g/mL) showing that CXCR4 suppression increases the stability of ErbB2 in MDA-MB-361/vIII breast cancer cells, but decreases the stability of ErbB2 in BT474/vIII breast cancer cells.

Fig. 4.

(A) Immunoblot analysis of MDA-MB-361/vIII breast cancer cells with suppressed CXCR4 expression pre-treated with the proteasome inhibitor MG132 (10 μ M), the inhibitor of protein recycling chloroquine (100 μ M), the inhibitor of protein trafficking monensin (25 μ M), the inhibitor of lysosomal proteases leupeptin (10 μ M), and the inhibitor of lysosomal proteases PD150606 (10 μ M), then treated with the protein synthesis inhibitor cycloheximide (10 μ g/mL) for 48 hours showing that CXCR4 suppression decreases the stability EGFRvIII mainly through altered protein trafficking. (B) Immunoblot analysis of MDA-MB-361/vIII and BT474/vIII breast cancer cells with suppressed CXCR4 expression pre-treated with the proteasome inhibitor MG132 (10 μ M) and the inhibitor of protein trafficking monensin (25 μ M), then treated with the protein synthesis inhibitor cycloheximide (10 μ g/mL) for 24 hours showing that CXCR4 suppression decreases the stability of EGFRvIII through proteasomal degradation and altered protein trafficking in MDA-MB-361/vIII breast cancer cells, but only proteasomal degradation in BT474/vIII breast cancer cells.

Fig. 5.

(A) Immunoblot analysis of MDA-MB-361/vIII and BT474/vIII breast cancer cells with suppressed CXCR4 expression showing that CXCR4 suppression increases the phosphorylation of p38 MAPK. (B) Immunoblot analysis of lysates from MDA-MB-361/vIII breast cancer cells with suppressed CXCR4 expression showing that EGFRvIII protein levels can be induced after 24 hours of treatment with the p38 MAPK inhibitor SB203580 (10 μ M), while 24 hours of treatment with the p44/42 MAPK inhibitor PD98059 (2 μ M) does not have any effect on EGFRvIII expression (C).

Fig. 6.

Immunoblot analysis of MDA-MB-361/vIII breast cancer cells under hypoxic conditions for 24 hours showing that stress conditions such as low oxygen tension induce the phosphorylation of p38 MAPK, but suppression of EGFRvIII and ErbB2 phosphorylation and an induction of EGFRvIII protein levels.