

Signaling and Regulation

Erythropoietin-Induced Activation of the JAK2/STAT5, PI3K/Akt, and Ras/ERK Pathways Promotes Malignant Cell Behavior in a Modified Breast Cancer Cell LineZhanzhong Shi¹, Vivien M. Hodges¹, Elaine A. Dunlop⁴, Melanie J. Percy³, Alexander P. Maxwell², Mohamed El-Tanani¹, and Terry R.J. Lappin¹**Abstract**

Erythropoietin (Epo), the major regulator of erythropoiesis, and its cognate receptor (EpoR) are also expressed in nonerythroid tissues, including tumors. Clinical studies have highlighted the potential adverse effects of erythropoiesis-stimulating agents when used to treat cancer-related anemia. We assessed the ability of EpoR to enhance tumor growth and invasiveness following Epo stimulation. A benign noninvasive rat mammary cell line, Rama 37, was used as a model system. Cell signaling and malignant cell behavior were compared between parental Rama 37 cells, which express few or no endogenous EpoRs, and a modified cell line stably transfected with human EpoR (Rama 37-28). The incubation of Rama 37-28 cells with pharmacologic levels of Epo led to the rapid and sustained increases in phosphorylation of signal transducers and activators of transcription 5, Akt, and extracellular signal-regulated kinase. The activation of these signaling pathways significantly increased invasion, migration, adhesion, and colony formation. The Epo-induced invasion capacity of Rama 37-28 cells was reduced by the small interfering RNA-mediated knockdown of EpoR mRNA levels and by inhibitors of the phosphoinositide 3-kinase/Akt and Ras/extracellular signal-regulated kinase signaling pathways with adhesion also reduced by Janus-activated kinase 2/signal transducers and activators of transcription 5 inhibition. These data show that Epo induces phenotypic changes in the behavior of breast cancer cell lines and establishes links between individual cell signaling pathways and the potential for cancer spread. *Mol Cancer Res*; 8(4): 615–26. ©2010 AACR.

Introduction

Erythropoietin (Epo) is a glycoprotein hormone, produced by the kidney in response to hypoxia, and acts through its cognate receptor (EpoR) to trigger signaling cascades that result in proliferation, differentiation, and survival of erythroid progenitors (reviewed in refs. 1, 2). Following the detection of expression of Epo and its receptor on multiple cell types, it has become clear that Epo has pleiotropic effects extending well beyond the maintenance of red cell mass (3). In recent years, multiple investigators have documented the presence of EpoR expression in numerous tumor cell lines and carcinomata (4–14) but the specificity of commercially available EpoR antibodies has been questioned (15, 16). Functional studies are needed

to determine whether EpoR activation modifies tumor cell growth.

Recombinant human Epo treatment has transformed the quality of life for millions of anemia sufferers, especially those with chronic kidney disease. More recently, erythropoiesis-stimulating agents (ESA) have been used to treat the cancer-related anemia, but there is emerging evidence that such therapies may be harmful to some groups of cancer patients. In 2003, Henke and colleagues (17) described the outcome of Epo treatment in a randomized trial of 351 anaemic head and neck cancer patients undergoing radiotherapy. Unexpectedly, locoregional progression-free survival was worse in the Epo treatment arm compared with placebo. In the same year, the Breast Cancer Erythropoietin Survival Trial reported that overall survival was poorer in the patients assigned to the Epo treatment arm (18). A Cochrane Review collated data on 9,353 cancer patients in 57 trials in which ESAs were given to prevent or treat anemia (19). There was a significantly higher relative risk for thromboembolic events in ESA-treated patients compared with controls (relative risk, 1.67; confidence interval, 1.35–2.06; ref. 20). A recent Food and Drug Administration alert has highlighted concerns about the potential serious adverse effects of ESA treatment of anemic cancer patients and postulated that this could be due to an increased risk of thrombosis, tumor growth, and/or neovascularisation (21, 22). These regulatory concerns have led to further revision of the clinical guidelines for use of ESAs in

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anaemic cancer patients (23). Recently, two meta-analysis reports that included data from a total of 13,933 cancer patients enrolled in 53 trials using ESAs (24) and 52 trials with 12,009 patients (25) have both concluded that ESAs are associated with an increased risk of death with active treatment and worse overall survival.

EpoR, a 508-amino acid transmembrane protein, is a type I cytokine receptor belonging to a family of proteins that includes the prolactin, growth hormone, and interleukin-3 receptors. After Epo binding, preformed Epo receptor dimers undergo a conformational change that activates three major signal transduction pathways: the Janus-activated kinase-signal transducer and activator of transcription (JAK2-STAT5), phosphatidylinositol-3-kinase-Akt (PI3K-Akt), and Ras-extracellular signal-related kinase (Ras-ERK) cascades (reviewed in ref. 1).

Recent studies using the non-small cell lung carcinoma cell line H838 have confirmed that Epo at pharmacologic levels can activate the PI3K/Akt, JAK2/STAT5, and Ras/ERK signaling cascades in nonerythroid cells (26). Down-regulation of Epo-induced signaling was found to be impaired in these cells (27), suggesting that Epo may provide a growth advantage to tumor cells. To further investigate the ability of Epo to enhance tumor growth and the potential for metastatic spread, we have compared Rama 37, a benign noninvasive rat mammary cell line expressing low levels of endogenous EpoR, to a modified Rama 37 cell line stably transfected with human EpoR to express a higher level of receptor (Rama 37-28). Both cell types, Rama 37 and Rama 37-28 expressing low and high levels of EpoR, respectively, were incubated with pharmacologic levels of Epo (10 U/mL). Here, we present evidence that Epo signaling through the JAK2/STAT5, PI3K/Akt, and Ras/ERK pathways can mediate an increase in malignant cell behavior in the presence of EpoR.

Materials and Methods

Cell Lines and Cell Culture

The rat mammary Rama 37 nonmetastatic benign tumor-derived cell line (28), the derivative stably transformed cell subclones, and MDA-MB-435s human breast epithelial cell line (29, 30) were cultured in DMEM with high glucose (4.5 g/L), 10% (v/v) FCS, 100 μ g/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen) at 37°C in an atmosphere of 5% (v/v) CO₂. Erythroid progenitors were derived from PBSC harvests, frozen pre-2005, and consented for research at that time. Cells (6×10^5 per mL) were plated in triplicate in HSC-CFUlite methylcellulose medium with EPO (Miltenyi Biotec). Blast-forming unit (erythroid) colonies were harvested after 12 d of culture at 37°C.

Stable and Transient Transfections

Human EpoR was amplified from cDNA and cloned into the pcDNA3.1/V5-His-TOPO using the following primers: forward, TTT TTT AAG CTT ATG GAC CAC CTC GGG GCG; reverse, TTT TTT TTG AAT TCC

AGA GCA AGC CAC ATA (Eurofins MWG Operon). For the production of stable transformant cell lines, Rama 37 cells were seeded in six-well plates at 1×10^5 /mL in antibiotic-free medium. Rama 37 cells were transfected with an expression vector pcDNA3.1 for wild-type human EpoR and selected in 1.0 mg/mL geneticin (Invitrogen) as previously described (31). Single-cell clones of transformants, which overexpressed the transfected EpoR as determined by quantitative real-time reverse transcriptase-PCR and Western blot screening, were generated. Two clones, Rama 37-28 and Rama 37-50, with significantly increased EpoR mRNA levels, were selected for further study.

Transient transfection of human EpoR small interfering RNA (siRNA; Ambion) into Rama 37-28 and MDA-MB-435s cell lines was carried out in six-well plates using the siPORT NeoFX transfection agent according to the manufacturer's instructions (Ambion). Briefly, before the transfection, cells were trypsinized and suspended in media without antibiotics at a cell density of 1×10^5 /mL. SiPORT NeoFX (5 μ L) was diluted into Opti-MEM medium (95 μ L; Invitrogen), incubated at room temperature for 10 min, then mixed with an equal volume of appropriately diluted EpoR siRNA solution (final concentration, 10 nmol/L). After incubation at room temperature for 10 min, siRNA transfection complexes (200 μ L) were dispensed into a six-well plate. Cell suspensions (2.3 mL) were overlaid onto the transfection complexes, gently mixed, and incubated for 72 h at 37°C, 5% CO₂.

Quantitative Real-time Reverse Transcriptase-PCR

EpoR mRNA expression was measured in the parental cells and transfected cell lines by Q-PCR. Briefly, total RNA was extracted from cell lines with Trizol reagent (Invitrogen). Following reverse transcription, cDNA was amplified by Taqman probe-based chemistry as previously described (32) using predesigned primer/probe sets for human and rat EpoR (human: Hs 00181092.m1; rat: Rn00566533_m1; Applied Biosystems). For comparison, a primer/probe set for the endogenous control 18S RNA, which recognizes human, mouse, and rat transcripts, was used (Applied Biosystems). Samples that gave an 18S C_T value outside the range of 10 ± 3 were excluded from further analysis. Relative Q-PCR EpoR Δ C_T values were calculated by comparison with 18S levels. A standard curve using linearized EpoR in pCDNA3.1 plasmid DNA was generated to calculate the number of EpoR transcripts per 10-ng total RNA from a real-time PCR C_T value. The equation generated from the standard curve was C_T value = $-3.012 \log(\text{EpoR copy number}) + 34.65$.

Western Blot Analysis

Western blot analysis was done as previously described (26) with minor modifications. For the detection of EpoR expression, cells were harvested by trypsinisation and were lysed in radioimmunoprecipitation assay buffer. Cells for signaling pathway immunoblotting analysis were plated at a density of 1×10^5 /mL and incubated overnight.

Following three washes with PBS, the cells were serum starved for 24 h and then, on the day of the experiment, were treated with Epo (10, 2, or 0.2 U/mL) for 0, 5, 15, 30, 60, and 120 min with additional controls without Epo for 30- and 60-min incubation times. Whole-cell lysates were obtained by harvesting cells into a Laemmli buffer followed by sonication and boiling for 5 min. Samples were separated on 10% polyacrylamide gels; transferred to polyvinylidene difluoride membranes; and probed with antibodies for pSTAT5 (Upstate), STAT5, pAkt, Akt, pERK 1/2, and ERK 1/2 (all from Cell Signaling, New England Biolabs). Detection was done by horseradish peroxidase-conjugated secondary antibodies (DAKO) and enhanced chemiluminescence plus Western Blotting Detection System (Amersham Biosciences). For the signaling pathway inhibition experiments, cells were pretreated with JAK inhibitor 1, U0126, or PI-103 (all from Calbiochem, Merck), at optimized final concentrations of 5, 20, and 0.5 $\mu\text{mol/L}$, respectively, for 1 h before the addition of Epo. Cell lysates prepared by direct addition of Laemmli buffer to cells at 0-, 15-, 30-, and 60-min time points were retained at -80°C .

***In vitro* Tests for Cell Adhesion, Invasion, and Colony Formation**

Assays for cell adhesion, colony formation, and invasion through Matrigel in Boyden chambers were carried out as previously described (33). Cells for adhesion, migration, invasion, and colony formation assays were washed thrice with PBS and then plated in serum-free media for 24 h before treatment with Epo.

Invasion Assay

Invasion was measured using Matrigel-coated multiwell inserts as previously described (31) with minor modifications. Matrigel-coated invasion chambers (6.4-mm diameter; 8- μm pore size; BD Biosciences) were used to assess the invasive capacity of parental Rama 37 and EpoR-transfected Rama 37-28 and Rama 37-50 cell lines. Briefly, 1×10^5 cells were resuspended in serum-free, phenol red-free DMEM media and placed in the upper invasion chambers. The cultures were incubated with or without Epo treatment with or without kinase inhibitors for 18 h at 37°C in a 5% (v/v) CO_2 atmosphere. The upper surfaces of the filters were wiped clean of cells and the filters were then fixed by immersion in 100% (v/v) methanol and stained by 0.5% crystal violet for 25 min. Each membrane was washed in running distilled water and left to air dry. After washing with sodium citrate/ethanol for 30 min, 200 μL of the solution were transferred into a 96-well plate and read at 570 nm in a Tecan plate reader. The percentage of invasion of each cell type was normalized using the percentage of invasion of parental Rama 37 cells without Epo treatment as 100%.

Migration Assay

The migration assay was similar to the invasion assay described above except that the insert membrane was not

coated with Matrigel. The percentage of migration of each cell type was normalized using the percentage of migration of parental Rama 37 cells without Epo treatment as 100%, as outlined above.

Adhesion Assay

Cell adhesion assays were done as previously described (31) with minor modifications. Briefly, cells were plated at 2×10^5 cells per well in a six-well plate coated with Matrigel (BD Biosciences) in conditioned media, treated with or without 10 U/mL Epo, and incubated at 37°C for 18 h in an atmosphere of 5% (v/v) CO_2 . Cells were washed with PBS, fixed with methanol, stained with 0.5% crystal violet, washed with distilled water, and air dried. Sodium citrate/ethanol solution was added and the cells were gently agitated for 30 min. An aliquot (50 μL) from each well was transferred into a 96-well plate and read at 570 nm in a Tecan plate reader. The percentage of adhesion of each cell type was normalized using the percentage of adhesion of parental Rama 37 cells without Epo treatment as 100%.

Colony Formation Assay

Colony formation assays were done on soft agar. Base agar was prepared by mixing an equal volume of 1% agar with $2 \times$ DMEM culture media containing high glucose (4.5 g/L), 10% (v/v) FCS, 100 $\mu\text{g/mL}$ penicillin, and 100 $\mu\text{g/mL}$ streptomycin (Invitrogen). A 1.5-mL aliquot of this mixture was added to a 35-mm Petri dish and allowed to set. Cells (2×10^4) were seeded into 10-mL centrifuge tubes containing 3 mL of $2 \times$ DMEM culture media and 3 mL of 0.7% Agar. The solution was mixed gently and 1.5 mL were added on top of the base agar in triplicate. Plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 10 to 14 d. Plates were stained with 0.5 mL of 0.005% crystal violet for over 1 h and colonies were counted using a dissecting microscope.

Results

Epo Receptor Overexpression

EpoR mRNA expression in Rama 37 and Rama 37-28 cells was quantified by Q-PCR using both human- and rat-specific primer probe sets (Table 1) and immunoblotting (Fig. 1A). In the parental Rama 37 cells, EpoR levels were barely detectable ($C_T > 35$). By comparison, high levels of expression for both EpoR mRNA and protein were observed in Rama 37-28 cells with stable transfection of EpoR. This model system permitted direct comparison of benign noninvasive mammary epithelial cells expressing very low EpoR with cells expressing an increased number of receptors. To investigate the effect of Epo on the cellular properties of parental Rama 37 cells compared with EpoR-transfected Rama 37-28 cells, a series of invasion, adhesion, migration, and colony formation assays were done in the presence or absence of Epo. Epo treatment of parental Rama 37 cells did not affect invasion, adhesion, or migration. However, Rama 37-28 cells stably transfected with EpoR showed significantly increased invasion ($P < 0.001$),

Table 1. EpoR mRNA expression level as determined by Q-PCR and expressed as corrected C_T

Cell line	Human EpoR C_T value	Rat EpoR C_T value
Rama 37	>35	>35
Rama 37-28	19.28 ± 0.43	>35

adhesion ($P < 0.001$), and migration ($P < 0.01$) in response to Epo stimulation (Fig. 1B-D) compared with nontreated controls. The number of Rama 37-28 colonies also increased significantly in the presence of Epo ($P < 0.001$), but there was no change in the colony-forming potential in the parental Rama 37 cells exposed to Epo (Fig. 1E). Invasion capacity was also tested with a second stably transfected clone with a lower level of Epo receptors (Rama 37-50; EpoR C_T , 22.8 ± 0.14) and also a range of Epo concentrations (0, 0.2, 2, and 10 U/mL; see Fig. 1F). There were no significant differences ($P > 0.05$) in invasion between 0 and 0.2 U/mL Epo in any of the three Rama cell lines. At 2 U/mL Epo, the invasion capacity of the Rama 37-28 cells was significantly higher than both Rama 37-50 and Rama 37 parental cells, whereas at 10 U/mL Epo, both the Rama 37-50 and Rama 37-28 had significantly higher invasion capacity than Rama 37 parental cells. Thus, invasion potential seems to be dependent on the combination of EpoR copy number and the concentration of Epo used (Fig. 1F). EpoR mRNA levels in our models are higher than in a range of cancer cell lines but comparable with levels in human erythroid progenitors and a human erythroleukemia cell line, UT7, which have been reported to express ~10,000 receptors per cell (Table 2; ref. 34).

Epo Receptor Knockdown

Epo-induced increases in adhesion, migration, invasion, and colony-forming potential in the Rama 37-28 EpoR-transfected cell line suggest that EpoR overexpression alone can increase malignant potential. In this model, silencing of EpoR expression using RNAi indicated that changes in cell behavior were mediated directly through the Epo stimulation of EpoR. Specific siRNA-mediated knockdown of the EpoR in Rama 37-28 cells (Table 3; Fig. 2A) resulted in a significant reduction in the invasive capacity of the cells (Fig. 2B). To confirm the role of EpoR in malignant cell behavior, the effects of Epo stimulation on a human cell line expressing endogenous EpoR were analyzed. EpoR mRNA and protein levels were assessed in parental MDA-MB-435s cells and, following siRNA-mediated EpoR knockdown, showed a significant reduction in receptor levels (Table 3; Fig. 2A). Parental MDA-MB-435s cells show a significant increase in invasion and adhesion capacity in response to Epo stimulation ($P < 0.05$), which is abrogated following EpoR knockdown ($P < 0.05$; Fig. 2C). The results suggest a role for Epo/EpoR in increasing malignant cell behavior in a human cancer cell line.

Activation of Three Signaling Pathways by Erythropoietin

To assess EpoR signaling activity, Epo activation of the three pathways was investigated. Phosphorylation of STAT5, Akt, and ERK1/2 were monitored over a period of 2 hours after Epo stimulation by immunoblotting. Parental Rama 37 cells (serum starved for 24 hours) showed no activation of the JAK/STAT pathway and only minor, short-lived activation of the PI3K/Akt and Ras/ERK pathways (Fig. 3A). In contrast, Epo stimulation of Rama 37-28 cells resulted in the significant and sustained activation of all three signaling pathways (Fig. 3B). Activation was observed only 5 minutes following stimulation with maximal levels seen at 15 minutes (highlighted). The level of total STAT5, Akt, and ERK1/2 protein remained unchanged. This confirmed that the transfected EpoR in Rama 37-28 cells was functionally active in response to Epo and activation of these pathways mediated a significant shift toward a metastatic cell phenotype. Signaling pathways are still activated in these cells at only 0.2 U/mL Epo (Fig. 3C), although activation levels are decreased and slightly delayed, particularly for the STAT5 pathway.

Identification of Signaling Pathway-Mediating Malignant Cell Behavior

Kinase inhibitors were used to assess the roles of the activated signaling pathways in mediating Rama 37-28 cell invasion. Concentrations of three inhibitors, JAK inhibitor 1 (5 μ mol/L), PI-103 (0.5 μ mol/L), and U0126 (20 μ mol/L), which inhibit JAK, PI3K (p110 α subunit), and mitogen-activated protein kinase kinase, in the JAK2/STAT5, PI3K/Akt, and Ras/ERK pathways, respectively, were optimized to determine the lowest concentration required to abolish Epo-induced signaling as determined by immunoblotting but without significantly affecting the other pathways (Fig. 4A). The traditional JAK-STAT inhibitor AG-490 failed to abrogate levels of phosphoSTAT5 even at a concentration of 100 μ mol/L (data not shown). These inhibitors were then included in the assessment of Rama 37-28 cell invasion. Inhibition of the PI3K/Akt and Ras/ERK pathways abrogates invasion potential even in the presence of Epo (Fig. 4B). Although the JAK2/STAT5 signaling is effectively inhibited with a decrease in levels of phosphorylated STAT5, the decrease in invasion in response to Epo was not significant compared with Rama 37-28 cells without inhibitor. In addition, the presence of Epo still conferred a significant increase in invasion compared with nontreated cells even in the presence of JAK inhibitor 1. However, inhibition of all three pathways led to a significant decrease in adhesion potential (Fig. 4C).

Discussion

The anemia of cancer is associated with poor prognosis, shortened survival, and a reduced quality of life (35). Correction of anemia by ESAs provides an attractive alternative to regular blood transfusions but concerns about the use of ESAs have been reported in clinical trials involving a range

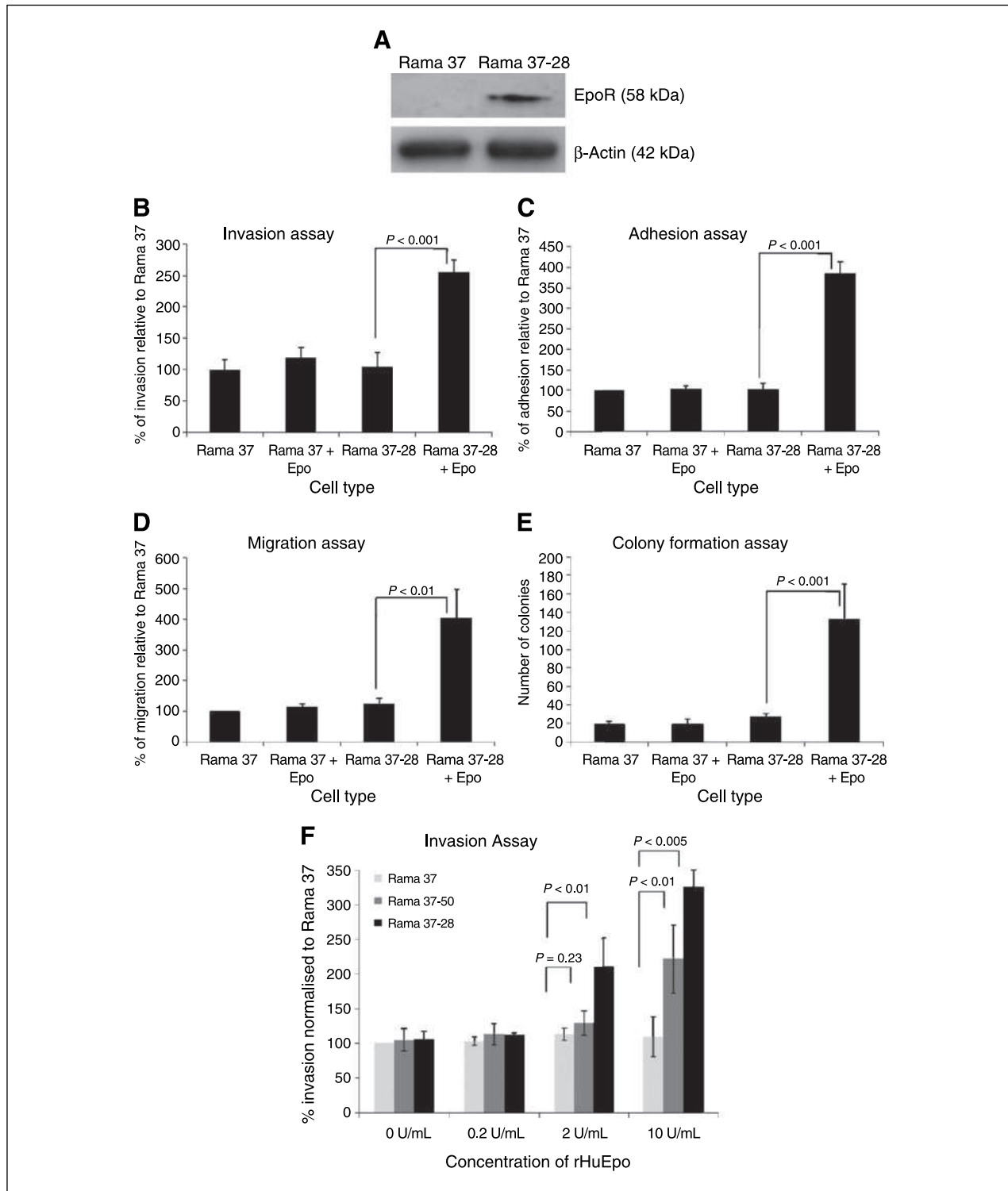


FIGURE 1. EpoR overexpression. A, immunoblot analysis of EpoR protein expression. Cell lysates were diluted and 15 μ g of protein were loaded on a SDS 8% (w/w) polyacrylamide gel. Specific proteins were detected using antibodies to EpoR or β -actin. B, invasion, (C) adhesion, (D) migration, and (E) colony formation capacity were measured in the presence/absence of Epo. Percentage invasion, migration, adhesion, and colony formation were calculated relative to Rama 37 (-Epo) control. Rama 37-28 cell malignant cell behavior was significantly increased in the presence of Epo compared with the Rama 37 parental cells using Matrigel-coated multiwell plates. Columns, mean from three independent experiments; bars, SD. F, invasion potential was also measured in three cell lines with varying levels of EpoR (low, Rama 37; medium, Rama 37-50; and high, Rama 37-28) at four different concentrations of Epo (0, 0.2, 2, and 10 U/mL).

Table 2. Q-PCR analysis of EpoR mRNA expression in a range of cancer cell lines, with conversion to copy number per 10 ng RNA

Cells	Cell type	C _T value	EpoR copy number	% compared with erythroid progenitors
Rama 37	Rat mammary epithelial	>35	N/D	N/D
Rama 37-50	Rat mammary epithelial	22.8 ± 0.14	8,595 ± 1,303	28
Rama 37-28	Rat mammary epithelial	19.28 ± 0.43	133,652 ± 59,987	435
BFU-E	Human erythroid progenitors	21.16 ± 0.34	30,753 ± 8,408	100
MDA-MB-435S	Human mammary epithelial	25.03 ± 0.35	1,608 ± 429	5
MDA-MB-231	Human mammary epithelial	25.39 ± 0.23	1,205 ± 205	4
MCF7	Human mammary epithelial	25.68 ± 0.11	951 ± 75	3
MCF10A	Human mammary epithelial	27.17 ± 0.32	310 ± 77	1
MCF10AT	Human mammary epithelial	26.02 ± 0.30	745 ± 157	2
MCF10A-CA1a	Human mammary epithelial	28.64 ± 0.59	104 ± 46	0.3
C9	Human cervical epithelial	>35	N/D	N/D
H838	Human non-small cell lung carcinoma	26.30 ± 0.28	603 ± 134	2
H157	Human non-small cell lung carcinoma	25.31 ± 0.33	1294 ± 309	4
H23	Human adenocarcinoma	25.82 ± 0.29	870 ± 180	3
UT7	Human erythroleukemia (AML-M6)	21.91 ± 0.83	19,893 ± 10,642	65
K562	Human chronic myeloid leukemia	25.51 ± 0.69	1,210 ± 519	4

Abbreviation: N/D, nondetectable using 10 ng RNA template.

of cancers (reviewed in ref. 36). An inverse relationship between EpoR expression and disease-free and overall survival in breast cancer has been reported (37) and Epo may only have a negative effect in cancers that are positive for EpoR expression using immunohistochemistry (35). However, further studies are needed with more specific antibodies and greater sample numbers to determine the prognostic significance of EpoR expression on tumors. The biological effects of Epo stimulation of tumor cells is still debated with reports of enhanced survival (8, 11), proliferation (4, 6, 38-40), resistance to treatment (38, 41, 42), tumor angiogenesis (43-45), chemotaxis (46), invasion (47-50), and migration (40, 46, 51). Others have reported no discernible effects of Epo (52).

In the present study, we have examined the role of EpoR in mediating malignant cell behavior in a rat mammary epithelial cell line model. The parental Rama 37 cells with low endogenous EpoR expression display a benign phenotype. Generation of a modified cell line with stable expression of EpoR (Rama 37-28) resulted in significant changes in cell signaling in response to Epo stimulation and the creation of a cell line with a malignant phenotype.

The increased EpoR expression in the Rama 37-28 cell line resulted in the activation of all three major signaling pathways downstream of EpoR in response to Epo with significant increases in levels of phospho-STAT5, phospho-Akt, and phospho-ERK1/2 >60 min after Epo stimulation. Parental Rama 37 cells showed only minor activation of two signaling pathways and no significant change in cell behavior in response to Epo, confirming their benign phenotype. In contrast, Epo treatment caused an increase in

adhesion, invasion, migration, and colony formation in Rama 37-28 cells, suggesting that the presence of functional EpoR is capable of mediating transformation to a malignant cell phenotype.

When invasion capacity was compared among Rama 37 parental cells, Rama 37-28, and Rama 37-50 (a clone with an intermediate level of EpoR overexpression), no significant differences were found at 0 and at 0.2 U/mL Epo in any of the three Rama cell lines. At 2 U/mL Epo, Rama 37 parental and Rama 37-50 were similar, but the invasion capacity of Rama 37-28 was significantly higher than Rama 37 parental cells. At 10 U/mL Epo, both Rama 37-50 and Rama 37-28 have significantly higher invasion capacity than Rama 37 parental cells. In this context, it is

Table 3. Q-PCR analysis of EpoR mRNA expression in Rama 37-28 cells, parental MDA-MB-435s cells, and following siRNA-mediated knockdown

Cell line	C _T value	Knockdown efficiency
Rama 37-28 (0 h)	19.9	
Rama 37-28 + siRNA (48 h)	22.0	72.4 ± 8.6%
MDA-MB-435s (0 h)	25.3	
MDA-MB-435s + siRNA (48 h)	27.9	84.1%

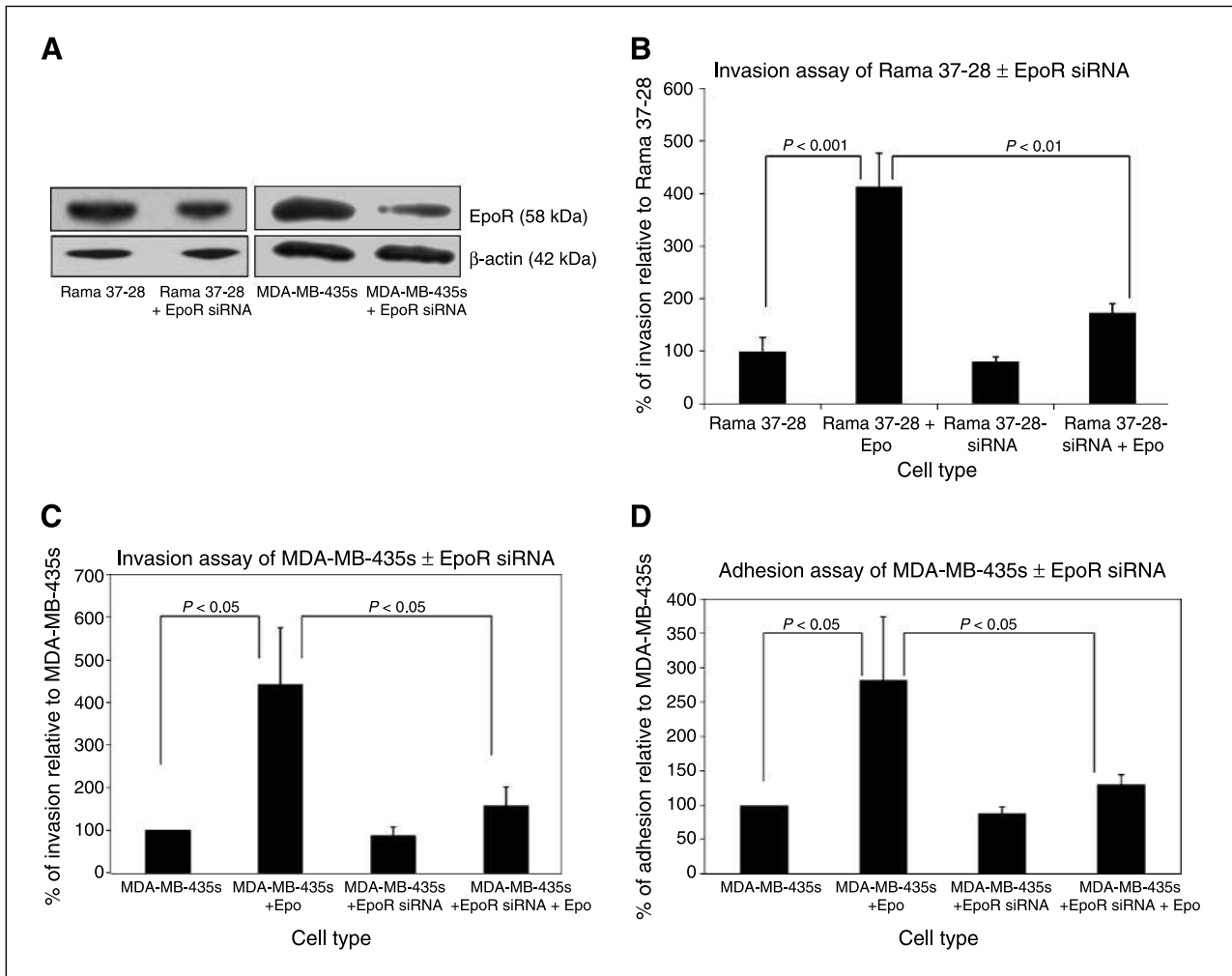


FIGURE 2. RNAi-mediated EpoR knockdown. A, immunoblot analysis of EpoR mRNA expression in Rama 37-28 cells, parental MDA-MB-435s cells, and following siRNA-mediated knockdown. B, abrogated invasion capacity in response to Epo compared with the Rama 37-28 control cells. C, invasion and (D) adhesion capacity following Epo stimulation of endogenous EpoR in MDA-MB-435s and following EpoR knockdown. Columns, mean from three independent experiments; bars, SD.

of interest to note that with patients receiving recombinant human Epo therapy, serum levels of ~4 U/mL Epo have been reported in patients receiving 120 U/kg Epo (53).

In our study, preliminary investigations with the human invasive cell line MDA-MB-435s, which expresses endogenous EpoR, shows a significant increase in invasion and adhesion in response to Epo. This is clearly mediated through EpoR as siRNA-mediated receptor knockdown resulted in significant attenuation of the invasion and adhesion potential in the presence of Epo compared with parental controls. These results confirm in a human model system that Epo stimulation of endogenous EpoR promotes malignant cell behavior.

Multiple signaling pathways are activated by Epo binding to the EpoR including the JAK/STAT, PI3K/Akt, and Ras/ERK cascades in our mammary epithelial model. Activation of all three signaling cascades through other class I cytokine receptors (e.g., prolactin and leptin) in breast cancer has been previously reported (54, 55). Activation of

these intersecting biochemical networks may drive tumorigenesis irrespective of normal biological cues. A range of signaling intermediate inhibitors were used to determine which pathways are involved in mediating Epo-induced malignant cell behavior.

PI3Ks are lipid kinases that are activated by receptor tyrosine kinases and other cell surface receptors to synthesize the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate. Phosphatidylinositol-3,4,5-trisphosphate acts as a docking site at the plasma membrane to recruit and activate proteins containing phospholipid-binding domains such as Akt, a serine-threonine kinase that signals to suppress apoptosis and promote cell growth. The PI3K pathway is frequently activated in cancer due to amplification or gain-of-function mutations of the *PIK3CA* gene, which encodes the p110 α subunit of Class I PIK, loss of function of PTEN, which catalyses the conversion of phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol 4,5-bisphosphate, or

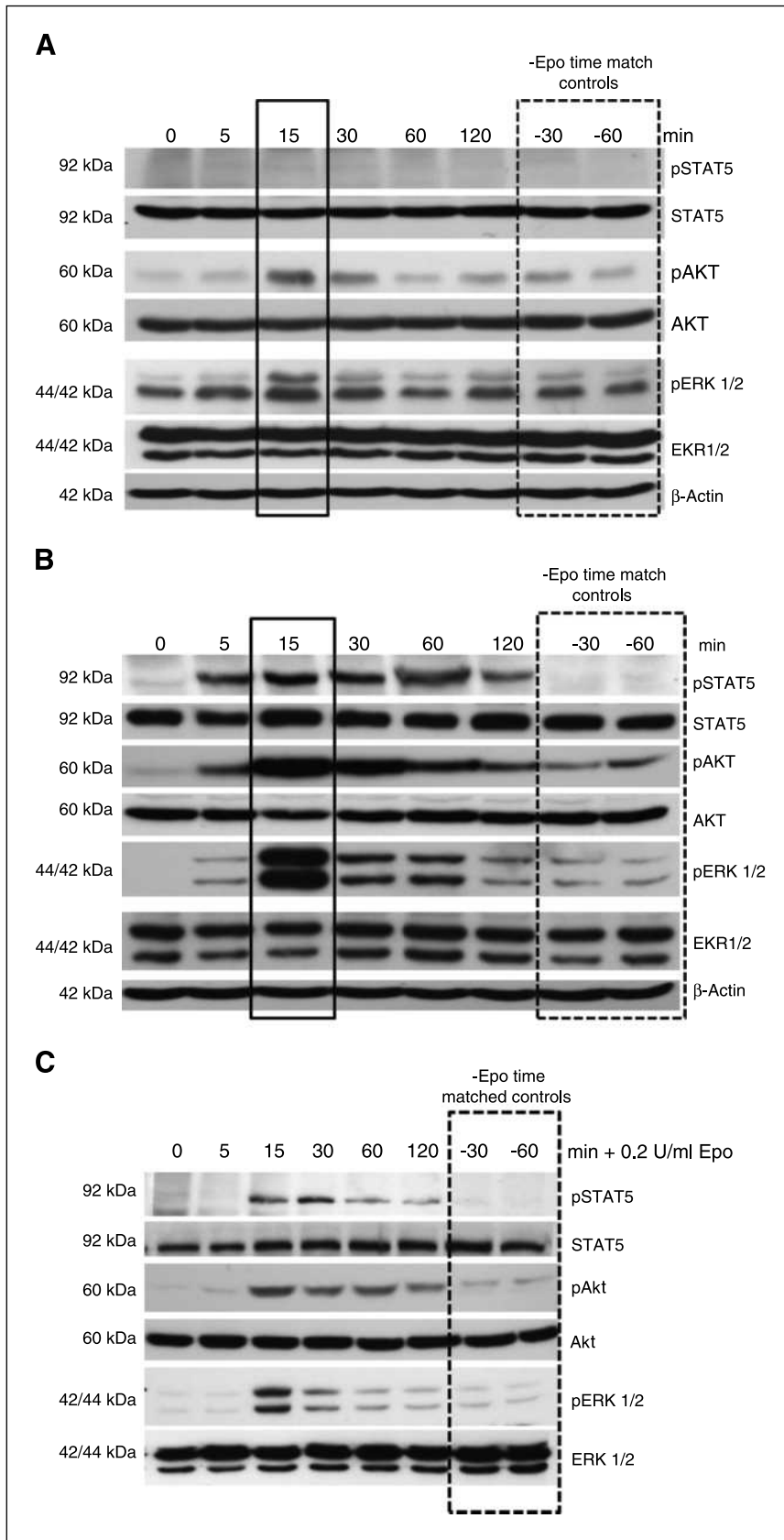
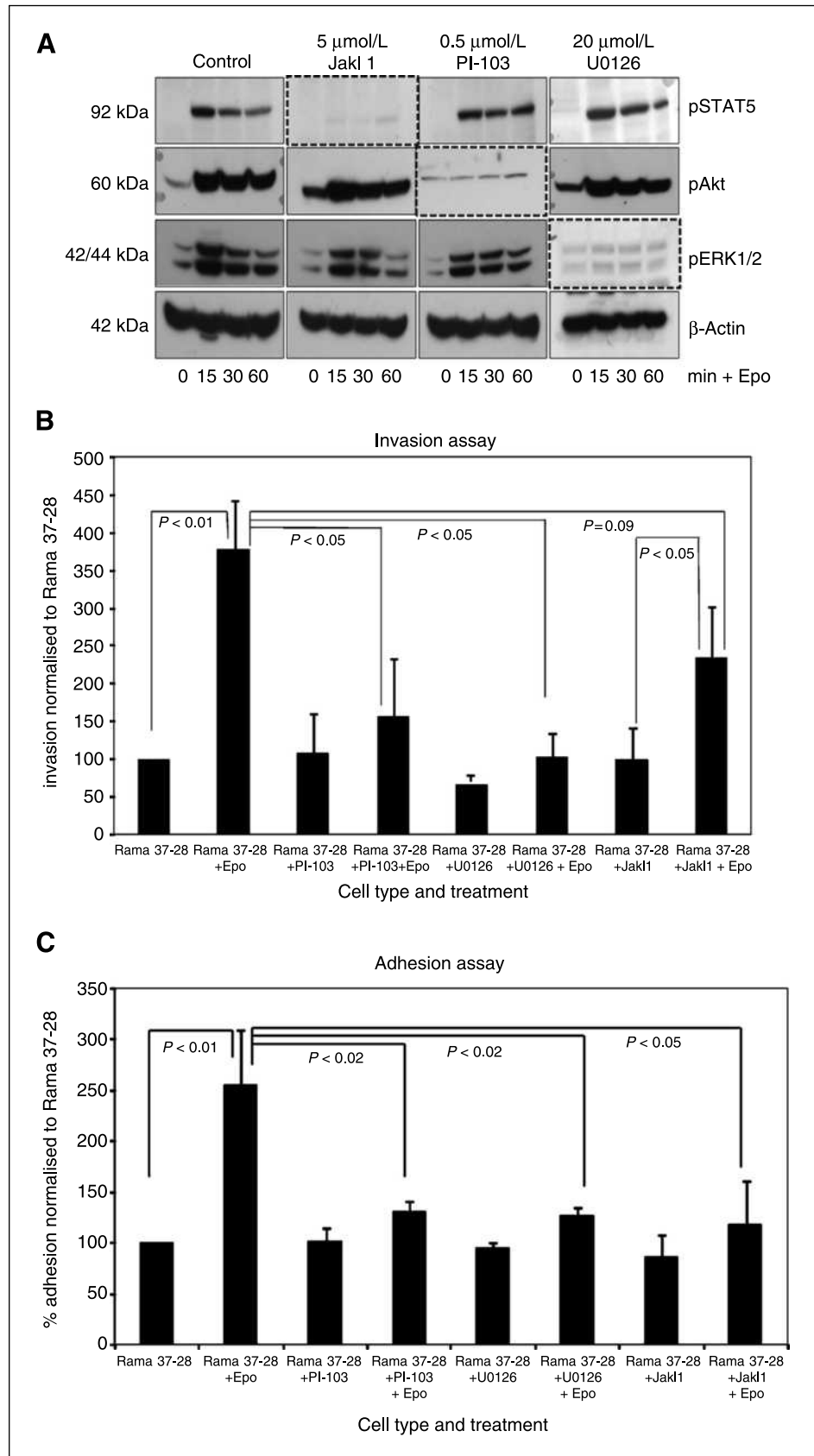


FIGURE 3. Signaling pathway activation in response to Epo stimulation. Epo-induced signaling was analyzed (A) in Rama 37 parental cells at 10 U/mL Epo, (B) in Rama 37-28 cells at 10 U/mL Epo, and (C) in Rama 37-28 cells at 0.2 U/mL Epo. Protein lysates were analyzed on a 10% SDS polyacrylamide gel. Specific proteins were detected using antibodies to phospho-Stat5, Stat5, phospho-Akt, Akt, phospho-ERK1/2, ERK1/2, and β -actin. Bands were quantified using densitometric analysis and normalized to β -actin. A representative blot from triplicate experiments is shown. Solid box, maximum signaling at 15 min. Dashed box, time-matched-Epo controls.

FIGURE 4. The effect of signaling pathway inhibition on cell behavior. A, inhibition of JAK2/STAT5, PI3K/Akt, and Ras/ERK pathways using 5 $\mu\text{mol/L}$ JAK inhibitor 1, 0.5 $\mu\text{mol/L}$ PI-103, and 20 $\mu\text{mol/L}$ U0126 small-molecule inhibitors, respectively. The cells were pretreated with inhibitor for 1 h before the addition of Epo. At these concentrations, only the targeted pathway showed significantly reduced signaling capacity. B and C, cell behavior analyzed in the presence of the small-molecule inhibitors. Percentage (B) invasion and (C) adhesion were calculated relative to Rama 37-28 (–Epo) controls. SDs from triplicate experiments are shown and *t* tests were used to determine statistically significant differences.



aberrant growth factor or integrin receptor signaling (reviewed in ref. 56). Thus, the PI3K pathway has emerged as an attractive target for small-molecule inhibitors with therapeutic potential for disrupting the initiation and progression of cancers. The arylmorpholine agent PI-103 inhibits both PI3K and mammalian target of rapamycin, enabling the inhibition of both the forward signal from PI3K and the negative feedback from mammalian target of rapamycin. PI-103 inhibits invasion in breast and ovarian cancer xenograft models (57) and induces proliferative arrest of glioma cells, mediated through its dual inhibitory action (58). In the Rama 37-28 cell model, a low concentration of PI-103 (0.5 $\mu\text{mol/L}$) effectively abolished the phosphorylation of the PI3K downstream target Akt and resulted in a significant abrogation of invasion and adhesion potential, even in the presence of Epo.

The Ras/ERK pathway couples signals from cell surface receptors to a wide range of cellular processes including proliferation, differentiation, apoptosis, and cell cycle progression (59). Amplification of *ras* genes and activating mutations in this pathway occur frequently in cancer, leading to constitutive signaling activation (reviewed in ref. 60). Ras is a small GTP-binding protein that lies upstream of both the Raf/mitogen-activated protein kinase/ERK and PI3K pathways. It is activated by growth factor/mitogen binding to their reciprocal receptor, which leads to the association of the Src homology and collagen/growth factor receptor binding protein 2/SOS complex and the subsequent conformational activation of Ras. Activation of the Ras-ERK and mitogen-activated protein kinase/c-Jun-NH₂-kinase pathways has been implicated in mediating proliferation and migration of breast cancer cells (40). In our study, exposure of EpoR expressing Rama 37-28 cells to Epo in the presence of the specific mitogen-activated protein kinase kinase inhibitor U0126 resulted in the complete abolition of the Epo-induced induction of ERK1/2 phosphorylation. This in turn eliminated the invasion and adhesion capacity of this cell line despite Epo stimulation.

JAK/STAT inhibitors are currently used to treat hematological malignancies (reviewed in ref. 61). However, the role of inhibitors of this pathway in solid tumors is less well understood. Epo-induced invasion of head and neck squamous cell carcinoma through the JAK/STAT pathway has been reported (46) but our results suggest that this pathway does not play a major role in mediating invasion of mammary epithelial cells. Although a decrease in invasion capacity was shown, Epo stimulation, even in the presence

of a JAK inhibitor, was still capable of eliciting a significant increase in invasion when compared with controls. However, inhibition of JAK/STAT signaling resulted in abrogating the adhesion potential in Rama 37-28 cells, suggesting that this signaling cascade may play different roles in promoting malignant cell behavior.

Growth factors stimulate cells to take up excess nutrients, and to use them for anabolic processes and activation of their signaling pathways can play a major role in mediating changes in cell metabolism (62). An altered metabolic phenotype with a switch to aerobic glycolysis is one of the hallmarks of tumorigenesis first noted by Otto Warburg (63). Akt, one of the most frequently activated protein kinases in human malignancy, which transduces growth factor effects on cell survival, growth, and proliferation, is also a critical mediator of accelerated glycolytic and oxidative metabolism (64). Our present data indicate that stimulation of EpoR by pharmacologic levels of Epo leads to rapid and sustained activation of Akt, underscoring the key role played by this pathway in the transformation from a benign to a tumorigenic cell phenotype.

Breast cancer progression depends not only on primary tumor growth but also on the ability of tumor cells to metastasize to distant sites; thus, the invasion, adhesion, and migration capacities increased by Epo activation of the EpoR in our study represents an additional mechanism for induction of pathways leading to a metastatic phenotype. Clearly, this is only one model system and further experiments *in vivo* are required to fully understand the role of the Epo receptor in malignant progression. Whether Epo is capable of accelerating tumor growth in cancer patients remains an open question.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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