

# Recombinant epoetins do not stimulate tumor growth in erythropoietin receptor–positive breast carcinoma models

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## Abstract

We investigated the significance of erythropoietin receptor (EPOR) expression following treatment with recombinant human erythropoietin (rHuEPO; epoetin  $\alpha$ ) and the effect of recombinant epoetins (epoetin  $\alpha$ , epoetin  $\beta$ , and darbepoetin  $\alpha$ ) alone or in combination with anticancer therapy on tumor growth in two well-established preclinical models of breast carcinoma (MDA-MB-231 and MCF-7 cell lines). Expression and localization of EPOR under hypoxic and normoxic conditions in MDA-MB-231 and MCF-7 cells were evaluated by immunoblotting, flow cytometry, and immunohistochemistry. EPOR binding was evaluated using [<sup>125</sup>I]rHuEPO. Proliferation, migration, and signaling in MDA-MB-231 and MCF-7 cells following treatment with rHuEPO were evaluated. Tumor growth was assessed following administration of recombinant epoetins alone and in combination with paclitaxel (anticancer therapy) in orthotopically implanted MDA-MB-231 and MCF-7 breast carcinoma xenograft models in athymic mice. EPOR expression was detected in both tumor cell lines. EPOR localization was found to be exclusively cytosolic and no specific [<sup>125</sup>I]rHuEPO binding was observed. There was no stimulated migration, proliferation, or activation of mitogen-activated protein kinase and AKT following rHuEPO treatment. In mice, treatment with recombinant epoetins alone and in combination with paclitaxel resulted in equivalent tumor burdens compared

with vehicle-treated controls. Results from our study suggest that although EPOR expression was observed in two well-established breast carcinoma cell lines, it was localized to a cytosolic distribution and did not transduce a signaling cascade in tumors that leads to tumor growth. The addition of recombinant epoetins to paclitaxel did not affect the outcome of paclitaxel therapy in breast carcinoma xenograft models. These results show that recombinant epoetins do not evoke a physiologic response on EPOR-bearing tumor cells as assessed by numerous variables, including growth, migration, and cytotoxic challenge in preclinical *in vivo* tumor models. [Mol Cancer Ther 2006;5(2):347–55]

## Introduction

Erythropoietin (EPO) is an essential glycoprotein hormone that regulates erythrocyte production in hematopoietic tissues by stimulating growth, preventing apoptosis, and inducing the differentiation of RBC precursors (1–3). Clinically, recombinant human EPO (rHuEPO; epoetin  $\alpha$ ) has been shown to increase hemoglobin levels and reduce use of transfusion in patients with anemia due to chronic kidney disease, cancer chemotherapy, and HIV therapy (4–6). Treatment with epoetin  $\alpha$  (7, 8) and other recombinant epoetins, such as epoetin  $\beta$  (9, 10) and darbepoetin  $\alpha$  (11, 12), is beneficial in correcting chemotherapy-associated anemia in cancer patients, and guidelines issued by the National Comprehensive Cancer Network and jointly by the American Society of Clinical Oncology and the American Society of Hematology recommend recombinant epoetins as treatment options for patients with cancer and chemotherapy-related anemia (13, 14). However, data from two recent phase III clinical trials indicated lower survival rates relative to placebo in patients with head and neck cancer receiving epoetin  $\beta$  and radiotherapy (15) or with metastatic breast cancer receiving epoetin  $\alpha$  and chemotherapy (16) for patients receiving treatment beyond the correction of anemia (i.e., to hemoglobin levels >12 g/dL).

Recent studies suggest that the function of EPO and its cognate receptor, the EPO receptor (EPOR), are not strictly limited to erythroid lineages (17, 18). EPOR expression has been identified in nonhematopoietic cells and tissues, including endothelial, neuronal, and ovarian (19–21). In addition, several reports have shown EPOR expression on tumor cells (22–26). Expression of EPOR has also been reported in breast cancer cell lines (27–29). These observations, coupled with the function of EPO as a mediator of cell growth and survival, have raised concern that treatment of anemic cancer patients with recombinant epoetins may enhance the proliferation and/or survival of

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the cancer cells, thus promoting tumor growth. Several researchers have examined the question of EPO/EPOR involvement in tumorigenesis with conflicting reports (30). For example, several studies have shown that administration of epoetin  $\alpha$  does not affect cancer cell proliferation (23, 31, 32). In contrast, other investigators have reported that epoetin  $\alpha$  treatment can stimulate proliferation of EPOR-positive cancer cell lines as well as patient samples (33, 34).

Although the potential role of epoetin  $\alpha$  and other recombinant epoetins in tumor proliferation has been suggested following two recent clinical trials (15, 16), it has yet to be mechanistically evaluated following systemic administration along with anticancer therapy in well-established preclinical tumor models. Our objectives were to determine the preclinical activity of epoetin  $\alpha$  and to seek evidence of increased tumor proliferation following treatment with epoetin  $\alpha$ , epoetin  $\beta$ , and darbepoetin  $\alpha$ , each alone or in combination with paclitaxel.

## Materials and Methods

### Cells

MDA-MB-231 and MCF-7 human breast carcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA). Both strains of cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G, 100  $\mu$ g/mL streptomycin sulfate, 0.25  $\mu$ g/mL amphotericin B, 2 mmol/L glutamine, and 25  $\mu$ g/mL gentamicin. MCF-7 cell culture medium was additionally supplemented with 10 mmol/L HEPES and 0.075% sodium bicarbonate. All above listed reagents were obtained from Life Technologies (Grand Island, NJ). For assay purposes, the cells were used from passage 4 to 10 and were never >90% confluent before harvesting. Hypoxic treatment of cells was done in an enclosed chamber (Sanyo Scientific, Bensenville, IL) flushed with premixed gas mixture (1% O<sub>2</sub>, 5% CO<sub>2</sub>, and 94% N<sub>2</sub>) for the times indicated. Normoxic conditions occur at 21% O<sub>2</sub>, 5% CO<sub>2</sub>.

### Flow Cytometry

Flow cytometry analysis was done on cells harvested and stained with the EPOR antibody or an isotype IgG2b control antibody (R&D Systems, Minneapolis, MN) and a rat anti-mouse-phycoerythrin secondary antibody. Intracellular staining was done using the Cytotfix/Cytoperm Plus kit (BD Biosciences, San Jose, CA). Analysis was done using a FACScan with Cell Quest software (Becton Dickinson, San Jose, CA).

### Immunohistochemistry

Cells were grown on two-chamber slides (Nunc) washed in TBS (Sigma, St. Louis, MO) containing 0.2% Tween 20 (TTBS; Sigma), fixed with 4% paraformaldehyde (Electron Microscopy Services, Fort Washington, PA) in PBS for 20 minutes, and permeabilized with 0.25% Triton X-100 (Sigma) in PBS for 10 minutes. Cells were washed with TTBS, blocked with 10% bovine serum albumin (BSA; Sigma) in TBS for 30 minutes, and incubated with primary

EPOR antibody or isotype (IgG2b) antibody 1:50 in TBS 10% BSA for 60 minutes. Cells were washed with TTBS, incubated for 60 minutes with Alexa 488/goat anti-mouse antibody (Molecular Probes, Inc., Eugene, OR) 1:200 in TBS containing 10% BSA, and washed with TTBS. Cells were fixed again as above, then mounted and analyzed using a confocal laser microscope.

### Western Blotting

For EPOR, mitogen-activated protein kinase (MAPK), and AKT analysis, samples were prepared from equal cell number of plated MDA-MB-231 and MCF-7 cells subjected to either normoxic (21% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) conditions for 24 hours. Lysates from UT7 (erythroleukemia cell line) cells were loaded as a positive control for EPOR expression. Briefly, cells were grown under either hypoxic or normoxic conditions in normal growth medium for 24 hours, followed by a 5-hour serum starvation, then treated with study drug for 15 minutes. Cells were washed twice with PBS and lysed directly in sample buffer, boiled for 5 minutes, and placed on ice before loading. Treatment using 10 ng/mL of epidermal growth factor (R&D systems) was used as a positive control for MAPK and AKT phosphorylation. Samples were run on a 10% polyacrylamide gel and transferred to nitrocellulose. Nonspecific binding on membranes was blocked with 5% nonfat dry milk in TBS containing 0.5% Tween 20 (TBST) at room temperature for 60 minutes. The membranes were incubated overnight with specific antibodies (at a 1:1,000 dilution) at 4°C made in TBST containing 0.5% nonfat dry milk. The next day, membranes were washed with TBST and incubated with horseradish peroxidase-conjugated appropriate secondary antibodies (Cell Signaling Technologies, Beverly, MA) diluted in TBST/0.5% nonfat dry milk for 60 minutes. The membranes were then subjected to an enhanced chemiluminescence reaction using the Amersham ECL kit (Amersham Biosciences, Piscataway, NJ). The following antibodies were used in this study: (a) a monoclonal anti-phospho-MAPK (p44/42); (b) a pan polyclonal extracellular signal-regulated kinase 1/2 (p44/42) antibody and anti-phospho-Akt/PKB antibodies (Cell Signaling Technologies); and (c) a monoclonal antihuman EPOR antibody (R&D Systems), which recognizes the NH<sub>2</sub>-terminal (extracellular) region. As a control for equal loading, a glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (Chemicon, Temecula, CA) was used to probe the same gel.

### EPOR Binding Studies

The EPOR binding studies were done essentially as described (35). Briefly, human recombinant (3-[<sup>125</sup>I]iodotyrosyl) EPO ([<sup>125</sup>I]rHuEPO, Perkin-Elmer, Shelton, CT) was used. Cells were plated on six-well plates and allowed to incubate for 3 to 5 days (subconfluency). Cells were then washed with binding buffer (PBS with calcium and magnesium with 1 mg/mL human serum albumin and 0.02% sodium azide). Each well then received 1 mL binding buffer with varying amounts of [<sup>125</sup>I]rHuEPO (0.1, 0.25, 0.5, 1, 2.5, 5, and 10 nmol/L). Samples were incubated at room temperature for 3 hours. Binding was terminated by the

addition of 2 mL washes (done four times) of ice-cold wash buffer (PBS with calcium and magnesium with 1% fetal bovine serum). Cells were then lysed with 1 mL lysis buffer [20 mmol/L HEPES (pH 7.4) with 1% Triton X-100, 10% (v/v) glycerol, and 0.1 mg/mL BSA] and incubated at room temperature for 20 minutes. Lysates were then counted on a  $\gamma$  counter. Nonspecific binding of [ $^{125}$ I]-rHuEPO was determined by adding a 300-fold excess of unlabeled rHuEPO (epoetin  $\alpha$ ) to the binding assay. The resultant radioactivity was subtracted from the total binding to give the specific binding. Assays were done in duplicate with at least five different concentrations of [ $^{125}$ I]rHuEPO. Analysis was done using Graph Pad Prism 4.0.

#### Migration Assays

The migration assays were carried out using the BD FluoroBlok System (BD Biosciences, Bedford, MA). This system consists of a 24-multiwell insert plate with an 8.0  $\mu$ m pore size, BD FluoroBlok membrane designed to facilitate fluorescent reading of only the cells that migrate to the underside of the membrane. Before beginning the assay, MDA-MB-231 cells were "starved" in RPMI 1640 basal medium (Life Technologies-Invitrogen, Carlsbad, CA), containing 0.1% BSA, delipidized (Sigma), for >5 hours. MCF-7 cells were not "starved," as it seemed to inhibit their migratory response. Each cell line was harvested and resuspended at a concentration of  $2 \times 10^5$ /mL in basal medium. For each assay plate, 500  $\mu$ L cells were added to the inserts (100,000 cells per insert) followed by adding 750  $\mu$ L epidermal growth factor or epoetin  $\alpha$  in basal medium to the bottom of the wells. Subsequently, the plate was incubated for 24 hours at 37°C, 5% CO<sub>2</sub>. A 50  $\mu$ g vial of Calcein AM (Molecular Probes)

was solubilized in DMSO, then added to HBSS (Life Technologies), for a final concentration of 4  $\mu$ g/mL. Following the 4-hour incubation, the inserts were placed into a new 24-well plate (Becton Dickinson, Bedford, MA), with each well containing 500  $\mu$ L Calcein AM solution, and the plate was incubated for 90 minutes at 37°C, 5% CO<sub>2</sub>. Plates were read using a Safire (Tecan, Research Triangle Park, NC) at excitation/emission wavelengths of 485/530 nm and a gain of 55. Only those labeled cells that migrated through the pores of the FluoroBlok membrane were detected.

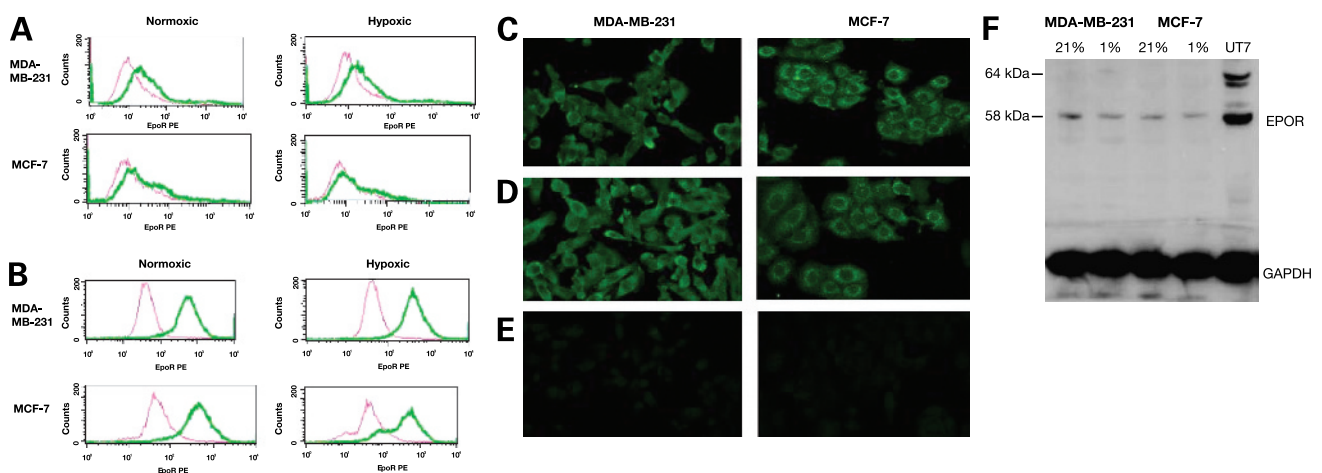
Determination of statistical significance was done using the Student's *t* test. Test values were considered significantly different from control values at  $P \leq 0.05$ , and highly significant at  $P \leq 0.01$ .

#### Proliferation Assays

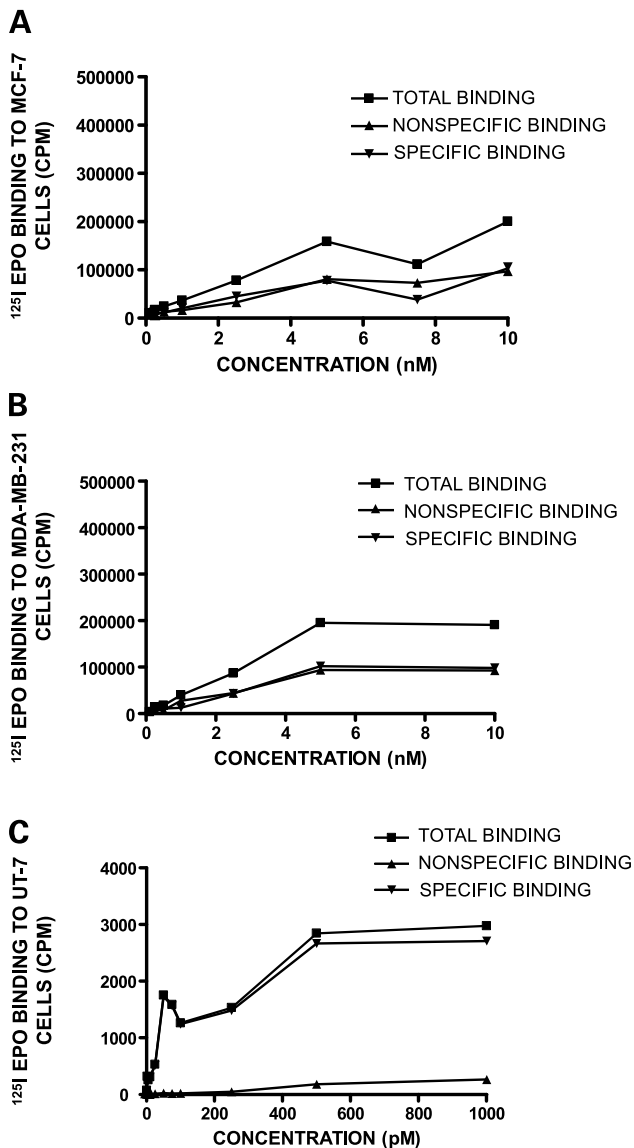
The proliferation assays were carried out using the Cell Titer Blue Cell Viability Assay (Promega, Madison, WI) according to the instructions of the manufacturer.

#### Human Tumor Xenograft Studies

The effects of recombinant epoetins (epoetin  $\alpha$ , darbepoetin  $\alpha$ , and epoetin  $\beta$ ) on paclitaxel therapy in xenograft models were assessed using methods described elsewhere (36). Briefly, female *nu/nu* mice were implanted orthotopically in the mammary fat pad with MCF-7 or MDA-MB-231 cells ( $10^7$  or  $5 \times 10^6$ , respectively) from culture. For the MCF-7 model, mice also received estrogen supplementation. After tumors reached 75 to 144 mm<sup>3</sup>, groups ( $n = 10$ ) were pair matched (day 1). Mice were given saline (control), paclitaxel, or recombinant epoetins starting on day 1. Doses of epoetins were chosen to increase hemoglobin by 1 to 2 g/dL/wk. Group 1 mice received saline administered s.c. on an every-other-day schedule



**Figure 1.** EPOR expression in MDA-MB-231 and MCF-7 breast cancer cell lines under normoxic and hypoxic conditions. Fluorescence-activated cell sorting analysis. *Pink lines*, IgG2b isotype control. *Green lines*, antihuman EPOR antibody. Cells were incubated for 24 h under normoxic conditions or in a hypoxic incubator at 1% O<sub>2</sub> to induce a hypoxic environment. Surface (**A**) and intracellular (**B**) staining is shown. Immunohistochemistry analysis. Cells were grown on slides under normoxic (**C** and **E**) or hypoxic (**D**) conditions for 24 h. Cells were stained with anti-EPOR antibody or an IgG2b isotype control. **F**, Western blot analysis. EPOR expression under hypoxic and normoxic conditions was observed using an anti-EPOR antibody. UT-7 (erythroid leukemia cell line) lysate was run as an EPOR-positive control. The same blot was probed with an anti-glyceraldehyde-3-phosphate dehydrogenase antibody for loading control.



**Figure 2.**  $[^{125}\text{I}]\text{rHuEPO}$  binding to MDA-MB-231 and MCF-7 cells. MCF-7 (A) and MDA-MB-231 (B) tumor cells were exposed to varying amounts of  $[^{125}\text{I}]\text{rHuEPO}$ . Concentration dependence of  $[^{125}\text{I}]\text{rHuEPO}$  binding to the tumor cells was recorded after 3 h. UT-7 cells (C) were used as a positive control. Results are a representative graph of the median binding. Experiments were done at least two independent times with at least five different concentration points.

until end point. Group 2 mice received 10 mg/kg paclitaxel administered i.v. every other day for 5 days. Group 3 mice received 0.0025 mg/kg epoetin  $\alpha$  in vehicle administered s.c. every other day to end point. Group 4 mice received 0.0075 mg/kg darbepoetin  $\alpha$  in vehicle administered s.c. once weekly until end point. Group 5 mice received 0.0025 mg/kg epoetin  $\beta$  in vehicle administered s.c. every other day to end point. Groups 6 to 8 received erythropoietic agents in combination with paclitaxel at the same doses described above.

Individual tumors were measured twice weekly by caliper. Response to treatment was assessed on the basis of tumor growth delay with end points of 1,000 and 1,500 mm<sup>3</sup> for MCF-7 and MDA-MB-231 cells, respectively. Complete and partial ( $\geq 50\%$ ) tumor regressions (CR and PR) and survival (Kaplan-Meier plots were constructed to show the percentage of animals remaining in the study as a function of time) were also assessed. A CR was defined as a tumor volume  $< 13.5$  mm<sup>3</sup> for three consecutive measurements. A PR was defined as a tumor volume  $\leq 50\%$  of its day 1 volume for three consecutive measurements and  $\geq 13.5$  mm<sup>3</sup> for at least one of these three measurements. Long-term tumor-free survivors were mice classified as CRs at the end of the study. At end point, blood samples were collected from all mice for hematology measurements. The log-rank test was used to analyze the significance of the difference in tumor growth delay between two treatment groups.

#### Therapeutic Agents

Johnson & Johnson Pharmaceutical Research & Development provided the test compound epoetin  $\alpha$  (Eprex); darbepoetin  $\alpha$  (Aranesp; Amgen, Thousand Oaks, CA) and epoetin  $\beta$  (NeoRecormon; F. Hoffman-La Roche, Basel, Switzerland) were purchased commercially.

These agents were stored at 4°C. Epoetin  $\alpha$  was provided as a 0.333 mg/mL solution (40,000 units/mL, equivalent to 333  $\mu\text{g}/\text{mL}$ ) and was diluted 1:10 with vehicle to make a 0.0333 mg/mL working stock. Dosing solutions of epoetin  $\alpha$  (0.0005 mg/mL) were prepared fresh each day of dosing in vehicle. Darbepoetin  $\alpha$  was provided as a 0.2 mg/mL stock solution (100  $\mu\text{g}$  per 0.5 mL syringe). Dosing solutions of darbepoetin  $\alpha$  (0.0015 mg/mL) were prepared fresh each day of dosing in vehicle. Epoetin  $\beta$  was provided as a 0.0277 mg/mL stock solution (1,000 units equivalent to 8.3  $\mu\text{g}$  per 0.3 mL syringe). Dosing solutions of epoetin  $\beta$  (0.0005 mg/mL) were prepared fresh each day of dosing in vehicle.

Paclitaxel (Mayne Group Ltd., Melbourne, Australia) dosing solutions were prepared fresh on each day of dosing by diluting a 10 $\times$  stock solution (prepared in 50% ethanol and 50% Cremophor EL) with 5% dextrose in water.

## Results

### Expression Patterns of EPOR in MDA-MB-231 and MCF-7 Cells under Normoxic or Hypoxic Conditions

We observed minimal EPOR on the surface of both the MDA-MB-231 and MCF-7 breast cancer cell lines (Fig. 1). However, appreciable levels of intracellular EPOR were observed in both cell types (Fig. 1). These levels were comparable under normoxic and hypoxic conditions for 24 hours (Fig. 1) and 72 hours (data not shown). Immunohistochemical analysis of these cells revealed intracellular localization of EPOR in most cells (Fig. 1). After 24 hours of hypoxia, there was no difference in the number of positively stained cells, localization of EPOR expression, or in the amount of staining per cell

compared with cells stained under normoxic conditions (Fig. 1). Thus, in these two breast cancer cell lines derived from solid human tumors, hypoxia did not affect the expression of EPOR protein. Using the same specific EPOR monoclonal antibody in the flow cytometry and immunohistochemistry analysis, we detected EPOR expression in the two tumor cell lines by Western analysis (Fig. 1F). EPOR expression levels remain unchanged upon exposure to hypoxic conditions for 24 hours (Fig. 1), corroborating the flow cytometry and immunohistochemical observations.

To further show that the single ~58 kDa band is the EPOR, we immunoprecipitated EPOR from UT-7 (EPOR-positive) cells using the R&D Systems monoclonal antibody followed by immunoblotting with the polyclonal C-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Using this immunoprecipitation/immunoblotting procedure, we detected a single ~58 kDa reactive band (data not shown).

#### Binding of rHuEPO (Epoetin $\alpha$ ) to MDA-MB-231 and MCF-7 Tumor Cells

To confirm the data obtained by flow cytometry and immunohistochemistry and determine whether EPOR expression was functional at the cell surface, the two tumor cell lines were exposed to varying amounts of [ $^{125}$ I]rHuEPO with or without a 300-fold excess of unlabeled rHuEPO (epoetin  $\alpha$ ). The concentration dependence of binding of [ $^{125}$ I]rHuEPO to MCF-7 and MDA-MB-231 cells is shown in Fig. 2A and B, respectively. The receptor binding experiments using [ $^{125}$ I]rHuEPO revealed no significant measurable EPO-specific binding activity present on the surface of either of the tumor cell lines. This is consistent with our observations that EPOR was predominately cytosolic and not present as an active surface receptor (Fig. 1). In contrast to the breast tumor cells, receptor binding using UT-7 cells, a transformed erythroleukemic cell line, showed low pmol/L specific binding activity using [ $^{125}$ I]rHuEPO (Fig. 2C).

#### Expression Patterns of Phospho-MAPK and Phospho-AKT under Hypoxic or Normoxic Conditions following rHuEPO Treatment of MDA-MB-231 and MCF-7 Cells

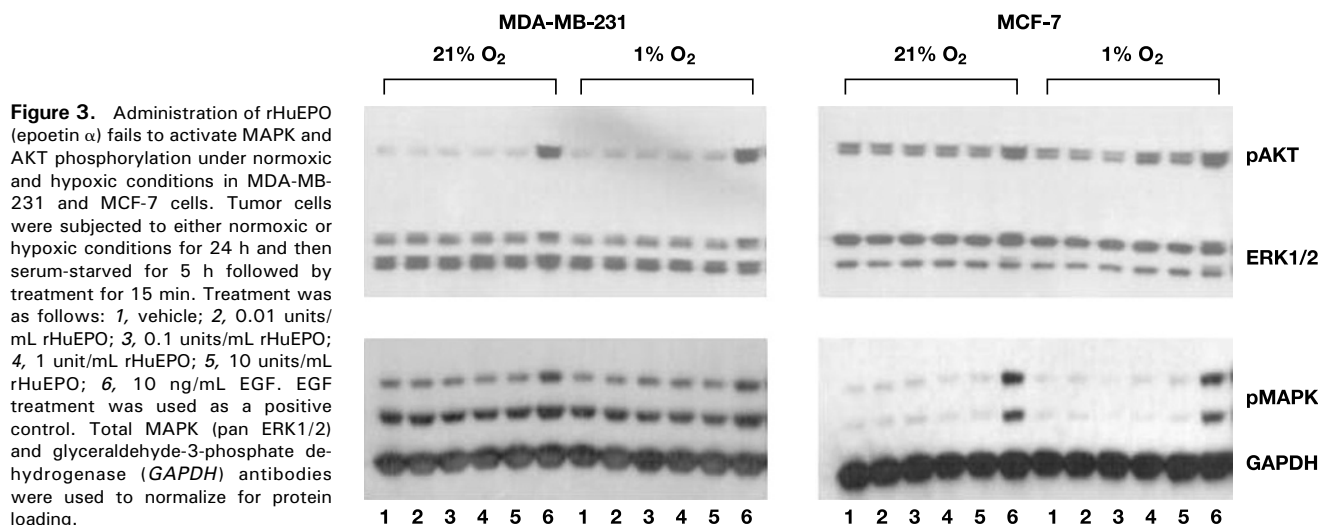
Despite the presence of EPOR expression in the tumor cell lines, epoetin  $\alpha$  treatment failed to induce the phosphorylation state of AKT and extracellular signal-regulated kinase 1/2 under normoxic or hypoxic conditions at physiologic (0.01 units/mL) and pharmacologic (10 units/mL) doses (Fig. 3). Thus, EPOR expression was present, but administering epoetin  $\alpha$  failed to elicit the signaling cascades that are normally activated in hematopoietic cells (37).

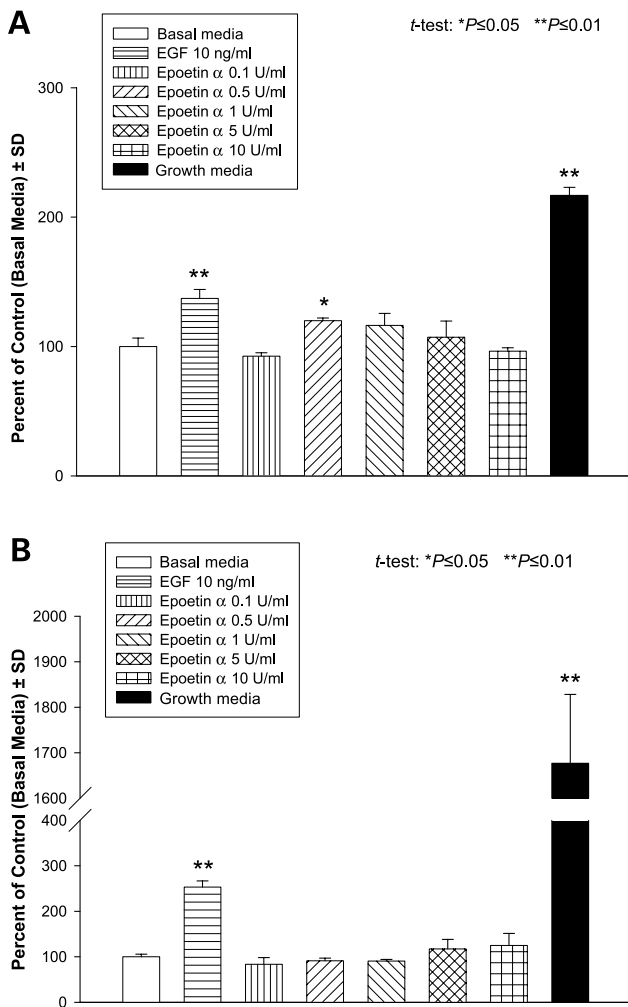
#### Effects of rHuEPO Administration on the Migration of MDA-MB-231 and MCF-7 Cells

To further confirm our observations that EPOR lacks activity on tumor cells, we tested the migratory effects of these breast carcinoma cell lines in the presence of epoetin  $\alpha$ . Figure 4A and B shows the results of migration assays using MCF-7 cells and MDA-MB-231 cells, respectively. Cells that migrated in response to the factors on the underside of the well were labeled with fluorescent dye, and the Fluoroblok coating on the membrane allowed only cells that migrated to be counted using a fluorescent plate reader. MDA-MB-231 cells (Fig. 4B) migrated readily in this system to both growth medium and to epidermal growth factor as a single factor. However, no significant migration was observed when epoetin  $\alpha$  was used as the stimulus in these assays. MCF-7 cells (Fig. 4A) did not migrate as easily in this system, most likely due to their size and tendency to aggregate. However, we still observed significant migration to growth medium and epidermal growth factor and little to no migration in wells where epoetin  $\alpha$  was present.

#### Proliferation of MDA-MB-231 and MCF-7 Cells following Treatment Using rHuEPO

Consistent with the inability of epoetin  $\alpha$  to stimulate tumor cell migration, the proliferation results for MCF-7 cells and MDA-MB-231 cells showed no increase in cell





**Figure 4.** The effects of rHuEPO (epoetin  $\alpha$ ) on MDA-MB-231 and MCF-7 cell migration. Both MCF-7 cells (**A**) and MDA-MB-231 cells (**B**) were plated on the top side of the inserts and serum-free medium containing 10 ng/mL EGF (positive control) or various concentrations of rHuEPO were added to the undersides of the inserts. Growth medium was used as a positive control. Each chemotactic assay was allowed to proceed for 24 h. Data are expressed as percentage of control (wells where no growth factors were added). Columns, mean of triplicate wells; bars, SD. Experiments were done at least two independent times and graphs are from a representative experiment. \*,  $P \leq 0.05$  (t test). \*\*,  $P \leq 0.01$  (t test).

proliferation following treatment with epoetin  $\alpha$  (data not shown). In both tumor cells, epidermal growth factor increased cell proliferation compared with control wells where no growth factor was added (data not shown). In two distinct cellular assays, neither MDA-MB-231 nor MCF-7 cells exhibited epoetin  $\alpha$  responsiveness.

#### Effects on Paclitaxel Therapy following Administration of Recombinant Epoetins in Orthotopically Implanted Breast Carcinoma Xenograft Models in Athymic Mice

Treatment effects of paclitaxel and epoetins either as monotherapy or in combination on the growth of

MDA-MB-231 and MCF-7 tumors on day 18 (an arbitrary day that captures both monotherapy and combination therapy) are summarized in Table 1. The response of MDA-MB-231 xenografts to the combination of paclitaxel with epoetins is shown in Fig. 5 as a growth curve of the mean tumor volume in  $\text{mm}^3$  ( $\pm$ SD). The mean tumor volumes of the groups receiving combination treatment were compared with those of the groups receiving paclitaxel alone. No significant differences in mean tumor volumes were detected by log-rank analysis, consistent with the conclusion that these epoetins do not decrease the efficacy of paclitaxel. The numbers of CRs and PRs were similar among group 2 (paclitaxel alone) and groups 7 (paclitaxel + darbepoetin  $\alpha$ ) and 8 (paclitaxel + epoetin  $\beta$ ). Group 6 (paclitaxel + epoetin  $\alpha$ ) had slightly more CRs and long-term tumor-free survivors (mice classified as CRs at study end) than the other groups (Table 1). Consistent with the MDA-MB-231 tumor regression and regrowth curves (Fig. 5), the effects of recombinant epoetin treatment either alone or in combination with paclitaxel had no effect on tumor growth in the MCF-7 xenografts (data not shown).

In the MDA-MB-231 study, the mean hemoglobin value for group 1 control mice was 12.08 g/dL. All groups that received erythropoietic treatment, whether alone or combination with paclitaxel, had a mean hemoglobin value  $>1.0$  g/dL higher than the mean hemoglobin value of group 1 controls. Similar results were obtained in the MCF-7 xenograft studies (data not shown). These results indicate that the three erythropoietic agents were administered at biologically effective doses. All treatments were acceptably tolerated in the studies. Mean body weight losses were within acceptable limits for all groups and no treatment related deaths were recorded.

Tumor regression and regrowth curves (Fig. 5), mean tumor volumes on individual days (e.g., day 18; Table 1), numbers of CR and PR (Table 1), and overall survival (data not shown) all show that recombinant epoetins do not measurably affect the outcome of paclitaxel therapy in MDA-MB-231 or MCF-7 xenografts.

## Discussion

In the present study, *in vitro* and *in vivo* experimental models were used to characterize the direct effects of epoetin  $\alpha$  in tumor cells that express EPOR. Reports describing EPO and EPOR expression in tumors or cancer cell lines have led to suggestions of a potential role for EPO/EPOR signaling in tumors (38–41). In addition, unfavorable effects of epoetin  $\alpha$  and epoetin  $\beta$  on mortality have been reported in two recent phase III studies in patients with cancer receiving chemotherapy (15, 16). However, our investigations of the biology of EPO/EPOR in two well-established breast carcinoma models (42) revealed that EPOR was predominately cytosolic and not present as an active surface receptor on these cell lines. This was supported by our findings that there was no significant measurable EPO-specific binding activity on the surface of

either cell line, that epoetin  $\alpha$  administration to these cell lines at both physiologic and pharmacologic doses and under normoxic and hypoxic conditions failed to elicit the signaling cascades indicative of activation of the EPOR, and that there was no significant migration or proliferation of these cells in the presence of epoetin  $\alpha$ . Finally, no tumor proliferation effects were detected with any of the recombinant epoetin monotherapies. Paclitaxel treatment was efficacious in these studies, yielding the maximum tumor growth delay achievable, and all mice had either a CR or PR. The addition of recombinant epoetins to treatment with paclitaxel also resulted in the maximum achievable tumor growth delay and regression responses. These results are in direct contrast with those observed with recombinant epoetins on hematopoietic cells. EPO elicits a signaling cascade within seconds leading to tyrosine phosphorylation of cytosolic proteins, including Janus-activated kinase 2, signal transducers and activators of transcription 5, and the EPOR (43). In UT-7 cells in a radiolabeled binding assay, EPO binds to EPOR with an  $IC_{50}$  of 138 pmol/L and stimulates cellular proliferation with an  $EC_{50}$  of 11.5 pmol/L (44). These results show that EPO is a potent cytokine with a well-defined mechanism of action. Delineation of the signaling pathway in hematopoietic cells allows investigation of the EPO/EPOR on non-hematopoietic cells with high resolution.

Recent data have shown that the EPOR is present on nonhematopoietic cells, such as endothelial (35) and neuronal cells (45), but also in tumor cells and tumor cell lines. Acs et al. (27) reported high levels of expression of both EPO and EPOR in the same human breast cancer cells (MDA-MB-231 and MCF-7) used in our study. Additionally, the investigators reported that epoetin  $\alpha$  treatment led to stimulated tyrosine phosphorylation and proliferation of MCF-7 cells. These findings led the investigators to propose that administration of epoetin  $\alpha$  may be detrimental to breast cancer patients who harbor EPOR-positive tumor cells. However, the data that

support their hypothesis are not convincing. It is not unexpected to find EPOR expression (via Western blot) in human breast cancer cells, as EPOR has been found in several nonhematopoietic cells (35, 38–41, 45). Furthermore, the Western analysis used in their study would not discriminate between EPOR expression localized to the cell surface or to cytosol. Additionally, the group has not reported any EPO-specific, cell-surface binding studies. The immunohistochemical analysis in the study revealed extensive cytosolic localization and very little surface staining, similar to our findings. Moreover, the use of the polyclonal (C-20) antibody in the several studies recognizes multiple bands in lysates, so it is inappropriate for use in immunohistochemistry (27, 46). In our study, we used a specific monoclonal antibody that recognizes a single band (~58 kDa) that corresponds to EPOR expression in the erythroleukemia cell line (UT-7). This antibody was also used to determine localization through immunohistochemistry and flow cytometry analysis. Acs et al. (27) also indicated that epoetin  $\alpha$  elicited a strong enhancement of phosphotyrosine levels, a hallmark of EPOR activation in MCF-7 cells. However, the data presented did not show that the EPOR was phosphorylated following epoetin  $\alpha$  treatment, and a suprapharmacologic dose of epoetin  $\alpha$  (250 units/mL) was used. Moreover, the amount of epoetin  $\alpha$ -stimulated proliferation was only 125%, which is nominal compared with what is observed in other cell types, such as UT-7 cells, in which a 500% increase in cell number occurs compared with vehicle control (47). In contrast, there are reports of EPOR-positive hematopoietic and nonhematopoietic malignant cell lines that do not proliferate in response to epoetin  $\alpha$  (22, 31).

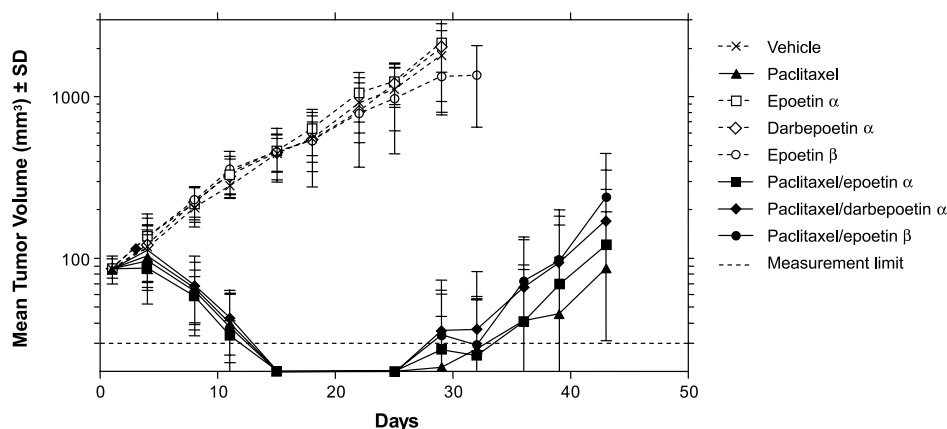
The recent findings of EPO/EPOR expression in human breast cancer as well as in several human breast tumor cell lines (28, 38, 39) have raised important questions about a possible tumor growth-promoting activity associated with administration of epoetin  $\alpha$  or other recombinant epoetins in EPOR-positive tumors. Arcasoy et al. (29)

**Table 1. Treatment response summary for MCF-7 and MDA-MB-231 studies**

Agent	MDA-MB-231				MCF-7			
	MTV $\pm$ SD ( <i>n</i> ) on day 18, mm <sup>3</sup>	No. PRs	No. CRs	No. LTTFS*	MTV $\pm$ SD ( <i>n</i> ) on day 18, mm <sup>3</sup>	No. PRs	No. CRs	No. LTTFS*
Saline	557 $\pm$ 209 (10)	0	1	1	488 $\pm$ 280 (10)	0	0	0
Paclitaxel	6 $\pm$ 7 (10)	4	6	2	0 $\pm$ 0.2 (10)	0	9	5
Epoetin $\alpha$	637 $\pm$ 203 (10)	0	0	0	482 $\pm$ 178 (10)	0	0	0
Darbepoetin $\alpha$	548 $\pm$ 153 (10)	0	0	0	591 $\pm$ 226 (10)	0	0	0
Epoetin $\beta$	541 $\pm$ 263 (10)	0	0	0	458 $\pm$ 195 (10)	0	0	0
Paclitaxel/epoetin $\alpha$	3 $\pm$ 2 (10)	1	9	4	3 $\pm$ 6 (10)	0	6	4
Paclitaxel/darbepoetin $\alpha$	4 $\pm$ 4 (10)	4	6	2	11 $\pm$ 34 (10)	1	5	3
Paclitaxel/epoetin $\beta$	6 $\pm$ 7 (10)	5	3	1	1 $\pm$ 4 (10)	1	6	1

Abbreviations: MTV, mean tumor volume; *n*, number of mice; LTTFS, long-term tumor-free survivors.

\*Mice classified as CRs at the end of the study.



**Figure 5.** The effects of erythropoietic treatment on the outcome of paclitaxel therapy in a MDA-MB-231 xenograft. After MDA-MB-231 tumor development, nude mice ( $n = 10$ ) were treated with vehicle (saline), paclitaxel, erythropoietic agents (epoetin  $\alpha$ , darbepoetin, and epoetin  $\beta$ ), or combinations until an end point as described in the materials and methods section. Points, mean tumor volumes ( $\text{mm}^3$ ) as a function of time; bars, SD.

examined the functional significance of EPO/EPOR expression in breast cancer tissues by implanting rat adenocarcinoma cells into rats and testing the effects of EPO/EPOR antagonist on tumor progression. The results of tumor depth 7 days after implantation showed that treatment with soluble EPOR and an anti-EPO (neutralizing) antibody decreased tumor depth in a dose-dependent manner. These results suggest that EPO/EPOR signaling may be involved in the progression of breast cancer. However, the test compounds were administered only once and the study was of short duration (7 days), suggesting that further investigation is warranted before such a conclusion can be drawn.

Blackwell et al. (48) recently showed that epoetin  $\alpha$ -treated R3230 mammary adenocarcinomas in rats had significantly lower hypoxia measurements compared with vehicle control or rats receiving epoetin  $\alpha$  before tumor implantation. Although there were no differences in tumor volume between epoetin  $\alpha$ -treated tumors and controls in this model, epoetin  $\alpha$ -related improvement in tumor oxygenation was independent of its effects on hemoglobin. In another study, treatment of Lewis lung carcinoma cells in a syngeneic mouse tumor model with either epoetin  $\alpha$  or saline resulted in no statistically significant differences in tumor volume between groups (49). Similarly, using two well-established, preclinical models of breast cancer (orthotopically implanted MDA-MB-231 and MCF-7 cells), no tumor proliferative effects were detected with any of the recombinant epoetin monotherapies. Additionally, the combinations of recombinant epoetins with paclitaxel treatment in these studies did not decrease paclitaxel efficacy against MDA-MB-231 and MCF-7 tumors. However, the administration of these recombinant epoetins in our study led to a statistically significant increase in hemoglobin levels compared with vehicle, confirming biological activity.

Based on our findings presented here, we conclude that expression of the EPOR in these breast carcinoma models does not seem essential for their growth, and that administration of recombinant epoetins does not affect paclitaxel efficacy in our preclinical models of breast

carcinoma. However, until appropriate clinical studies are conducted in anemic and nonanemic patients with cancer, the safety of using recombinant epoetins in the oncology setting cannot be fully appreciated.

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