

Effects of Recombinant Erythropoietin on Breast Cancer–Initiating Cells¹

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Abstract

BACKGROUND: Cancer anemia causes fatigue and correlates with poor treatment outcome. Erythropoietin has been introduced in an attempt to correct these defects. However, five recent clinical trials reported a negative impact of erythropoietin on survival and/or tumor control, indicating that experimental evaluation of a possible direct effect of erythropoietin on cancer cells is required. Cancer recurrence is thought to rely on the proliferation of cancer initiating cells (CICs). In breast cancer, CICs can be identified by phenotypic markers and their fate is controlled by the Notch pathway. **METHODS:** In this study, we investigated the effect of erythropoietin on CICs in breast cancer cell lines. Levels of erythropoietin receptor (EpoR), CD24, CD44, Jagged-1 expression, and activation of Notch-1 were assessed by flow cytometry. Self-renewing capacity of CICs was investigated in sphere formation assays. **RESULTS:** EpoR expression was found on the surface of CICs. Recombinant human Epo (rhEpo) increased the numbers of CICs and self-renewing capacity in a Notch-dependent fashion by induction of Jagged-1. Inhibitors of the Notch pathway and PI3-kinase blocked both effects. **CONCLUSIONS:** Erythropoietin functionally affects CICs directly. Our observation may explain the negative impact of recombinant Epo on local control and survival of cancer patients with EpoR-positive tumors.

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Keywords: CD24^{-low}/CD44⁺ breast cancer cells, breast cancer–initiating cells, rhEpo, Notch, EpoR.

creased death rates in patients treated with ESAs. Whereas some of the patients treated with ESAs experienced thromboembolic events that could be easily related to changes in blood rheology, a negative impact of ESAs on local tumor control most likely resulted from more complex interactions.

We have recently demonstrated that overexpression of the erythropoietin receptor (EpoR) increased the clonogenicity of cancer cells [11] and that local control and overall survival in patients receiving recombinant human erythropoietin (rhEpo) were only negatively affected if the cancers stained positive using an anti–EpoR antibody [12]. A better understanding of the mechanisms underlying these rhEpo-related effects may allow rational selection of patients for treatment and uncover novel targets to improve cancer treatment outcome.

One view of cancer is that it arises from and is maintained by a small number of cancer stem cells (CSCs), which have the ability to self-renew whereas their progenies do not [13]. CSCs can now be identified prospectively in brain tumors [14], breast cancer [15], prostate cancer [16], cancer of the head and neck [6], pancreatic cancer [17], and melanoma [18]. The hypothesis is that CSCs are responsible for the regrowth and metastatic spread of a tumor and the efficacy of any given treatment depends on the killing of this population of cells [13]. In breast cancer, a CD44⁺/CD24^{-low} cell population can be isolated from patient tumor samples or established cell lines that are highly enriched for the putative breast cancer stem cell population exhibiting a 3-log increased tumorigenicity [15].

Furthermore, Dontu et al. [19] demonstrated that mammary development relies on the developmental Notch signaling pathway that regulates the fate of mammary stem cells. On binding of Notch ligands, the Notch receptors undergo intramembranous cleavage by the γ -secretase protease complex.

Introduction

Cancer-related and chemotherapy-induced anemia negatively impacts the quality of life of many cancer patients and is accompanied by a poor prognosis [1–5]. Therefore, anemia is frequently corrected by blood transfusions or application of erythropoiesis-stimulating agents (ESAs) [7]. However, three randomized clinical studies reported a negative impact of ESAs on overall survival and local tumor control in breast [8], head and neck [9], and non–small cell lung cancer patients [10]. More recently, two large clinical trials (DAHANCA 10 and Anemia of Cancer, study by Amgen) were stopped after interim analyses showed in-

Abbreviations: BCIC, breast cancer–initiating cell; CSC, cancer stem cell; Epo, erythropoietin; ESA, erythropoiesis-stimulating agent; GSI, γ -secretase inhibitor; JAK2, Janus kinase 2; NF- κ B, nuclear factor–kappa B; rhEpo, recombinant human erythropoietin

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This releases the intracellular domain of the receptor (Notch-ICD) for translocation into the nucleus, where it switches the function of CBF-1 from a transcriptional repressor into an activator. Function of γ -secretase can be blocked by specific inhibitors, which are already in clinical trials for patients suffering from Alzheimer's disease [20].

The stem cell in breast cancer that is capable of repopulating a tumor from a single cell has not yet been firmly characterized. Therefore, we will join others in calling the population of CD44⁺/CD24^{low} putative stem cells, breast cancer-initiating cells (BCICs). Adopting a technique for the propagation of normal mammary stem cells [21], Ponti et al. [22] recently demonstrated that BCICs can be propagated as mammospheres *in vitro*. BCICs derived from MCF-7 breast cancer cells mimicked the phenotype and tumorigenicity of BCICs derived from primary estrogen receptor-positive breast cancers. This offers an invaluable tool to study the treatment responses of BCICs directly and to compare them to vast literature gained using breast cancer cell lines in the past. Using the same techniques as Ponti et al., we were able to enrich mammospheres to contain up to 40% of CD44⁺/CD24^{low} cells and demonstrate that BCICs are a radio-resistant subpopulation of cancer cells. Remarkably, the number of BCICs increased after sublethal fractionated irradiation *in vitro*. Radiation activated the developmental Notch signaling pathway and inhibition of this pathway prevented the radiation-induced increase in the number of BCICs [23]. The relevance of this cell population was underlined by two other studies demonstrating that BCICs have enhanced invasive properties [24] and that the most early disseminated cells in the bone marrow of breast cancer patients exhibit the CD44⁺/CD24^{low} phenotype [25]. Such cells are also resistant to conventional cancer treatment [23,26,27].

We hypothesized that rhEpo might act on BCICs to increase the size of the BCICs' population and compromise tumor control in breast cancer patients receiving rhEpo treatment. We addressed our hypothesis using our established *in vitro* BCIC system with MCF-7, T47D, and MDA-MB-231 breast cancer cell lines, which account for two thirds of all experimental literature on breast cancer [28].

We found that pharmacological concentrations of rhEpo increased the number of putative BCICs in established breast cancer cell lines. The increase was mediated by the activation of the Notch signaling pathway, could be blocked by inhibiting this pathway, and mimicked by overexpression of a constitutively active Notch-1 receptor. Notch activation occurred through the induction of the Notch receptor ligand Jagged-1 in a phosphoinositide-3 kinase (PI3K)-dependent fashion and could be blocked by a PI3K inhibitor.

Methods

Cell Culture

MCF-7, T47D, and MDA-MB-231 breast cancer cells were purchased from the American Type Culture Corporation (Manassas, VA) and cultured in log-growth phase in min-

imum essential medium (MCF-7 and T47D) (Cellgro, Kansas City, MO) (supplemented with 0.1 mM nonessential amino acids and 1 mM sodium pyruvate) and Dulbecco's modified Eagle's medium (DMEM) (MDA-MB-231) (Cellgro), respectively, after supplementing with 10% heat-inactivated fetal calf serum and 0.01 mg/ml bovine insulin (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere (5% CO₂). Mammosphere cultures were established as described by Ponti et al. [22] under serum-free conditions in phenol red-free DMEM/F12, supplemented with 0.4% bovine serum albumin, 20 ng/ml basic fibroblast growth factor (Sigma), and 10 ng/ml epidermal growth factor (Sigma). Cultures were fed with fresh growth factors every 3 days.

Transfection

All plasmid DNA were prepared using a commercial DNA extraction and isolation kit (Midiprep; Quiagen, Valencia CA). The Notch-ICD plasmid [29] was a gift from Dr. L. Miele (Loyola University Medical Center). The pNICD plasmid was constructed by cloning the intracellular domain of Notch-1 (5309–7655 bp) into the expression vector pcDNA3 (Invitrogen, Carlsbad, CA). The empty pcDNA3 vector was used as a control. MCF-7 cells were transfected with the pNICD plasmid or the pcDNA3 control vector using Lipofectamine2000 (Invitrogen) and OptiMEM (Invitrogen). After 24 hours, the cells were replated and maintained under 1 mg/ml neomycin (Sigma) selection. Individual clones were selected, grown in the presence of 1 mg/ml neomycin, and tested for the expression of intracellular Notch-1 (Notch-1-ICD). Clones overexpressing intracellular Notch-1 were expanded under neomycin selection to generate stable expression of MCF-7-pNICD and MCF-7-pcDNA3 cell lines.

Drug Treatment

RhEpo (1 IU/ml) treatment of monolayer cultures of MCF-7 cells was performed from day 2 to 4 after plating. Cells were harvested on day 5 when flow cytometry was performed to assess the cells' phenotypes. The γ -secretase inhibitor (GSI), GSI XVII (InSolution; Calbiochem, San Diego, CA), was

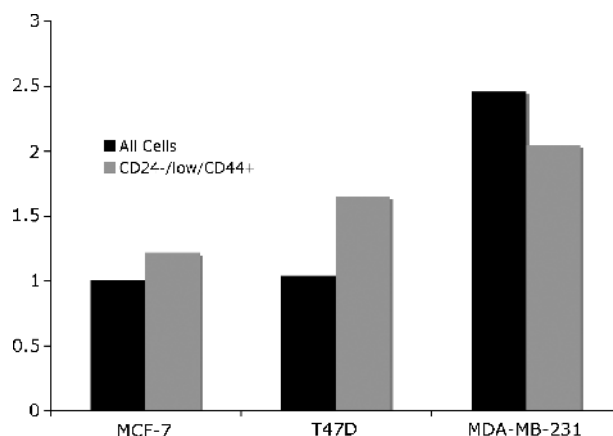


Figure 1. MCF-7, T47D, and MDA-MB-231 monolayer cells were serum-starved for 5 hours, trypsinized, and analyzed for EpoR-expression on the cell surface by flow cytometry. EpoR was expressed on CD44⁺/CD24^{low} cells, the putative stem cell population (gray) as well as on unselected cells (black).

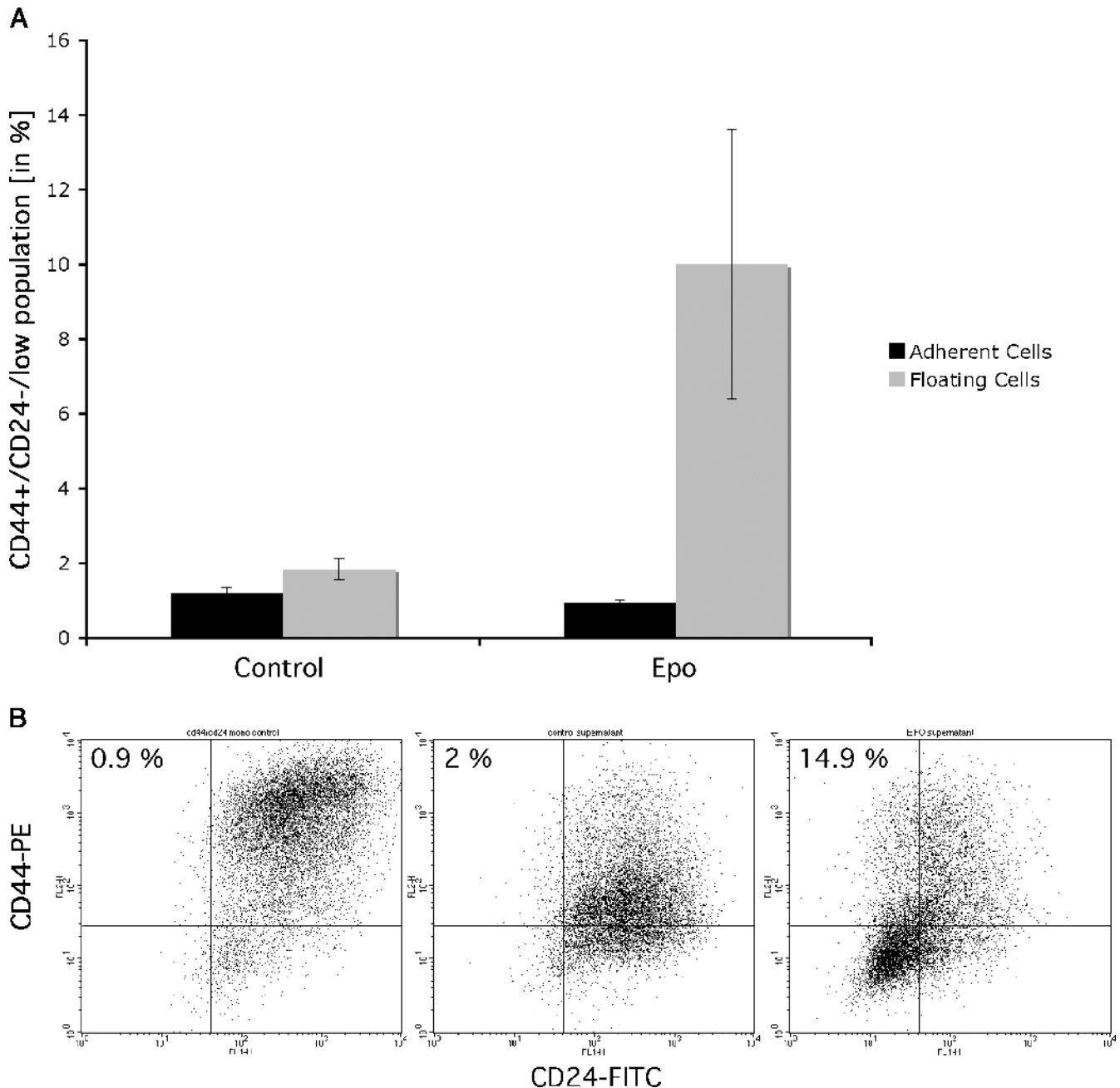


Figure 2. (A) FACS analysis detecting the size of the CD44⁺/CD24^{-/low} cell population in monolayers and supernatant of MCF-7 cells. Treatment with rhEpo (3×1 IU/ml) increased the size of this population 4.8-fold from $2 \pm 0.08\%$ to $9.5 \pm 1.8\%$ ($P < .01$, Student's *t* test). Data from four independent experiments are shown. (B) Representative FACS analysis of MCF-7 monolayer cultures treated with rhEpo (3×1 IU/ml) for 3 consecutive days. About 0.9% of the cell in the adherent cell population was CD44⁺/CD24^{-/low} (left panel). Treatment with rhEpo increased the frequency of CD44⁺/CD24^{-/low} cells in the supernatant from 2% (middle panel) to 14.9% (right panel). (C and D) Primary sphere formation assay of MCF-7 (C) or MDA-MB-231 (D) cells treated with rhEpo (3×1 IU/ml) for 3 consecutive days. RhEpo increased the rate of primary spheres formation (MCF-7: $1 \pm 0.4\%$ for untreated cells, $2.9 \pm 0.6\%$ for Epo-treated cells; MDA-MB-231: 1.2 ± 0.6 for untreated cells, $4.5 \pm 1.1\%$ for Epo-treated cells, $P < .01$, two-sided Student's *t* test; means \pm SEM). Preincubation (30 minutes) with GSI ($5 \mu\text{M}$) treatment prevented the rhEpo-induced increase in primary sphere formation (MCF-7: GSI-treated cells, $1 \pm 0.4\%$; Epo + GSI-treated cells, $1.5 \pm 0.4\%$; MDA-MB-231: GSI-treated cells, $1.5 \pm 0.7\%$; Epo + GSI-treated cells, $1.1 \pm 0.8\%$).

added at a final concentration of $5 \mu\text{M}$ (0.1% final concentration of DMSO). LY294002 (Calbiochem) was dissolved in DMSO and added at a final concentration of $10 \mu\text{M}$. Control cells received 0.1% DMSO only. Cells were serum-starved for 5 hours before the start of the experiment to prevent EpoR internalization through binding of fetal calf serum-derived erythropoietin (Epo). Serum starvation in long-term experiments in which cells were treated with rhEpo for 3

consecutive days was not tolerated by the cells and was therefore omitted.

Flow Cytometry

For analysis of CD24 and CD44 expression, cells were labeled using a mouse anti-human CD24-fluorescein isothiocyanate (BD Pharmingen, San Jose, CA) and a mouse anti-human CD44-phycoerythrin (PE) (BD Pharmingen). In

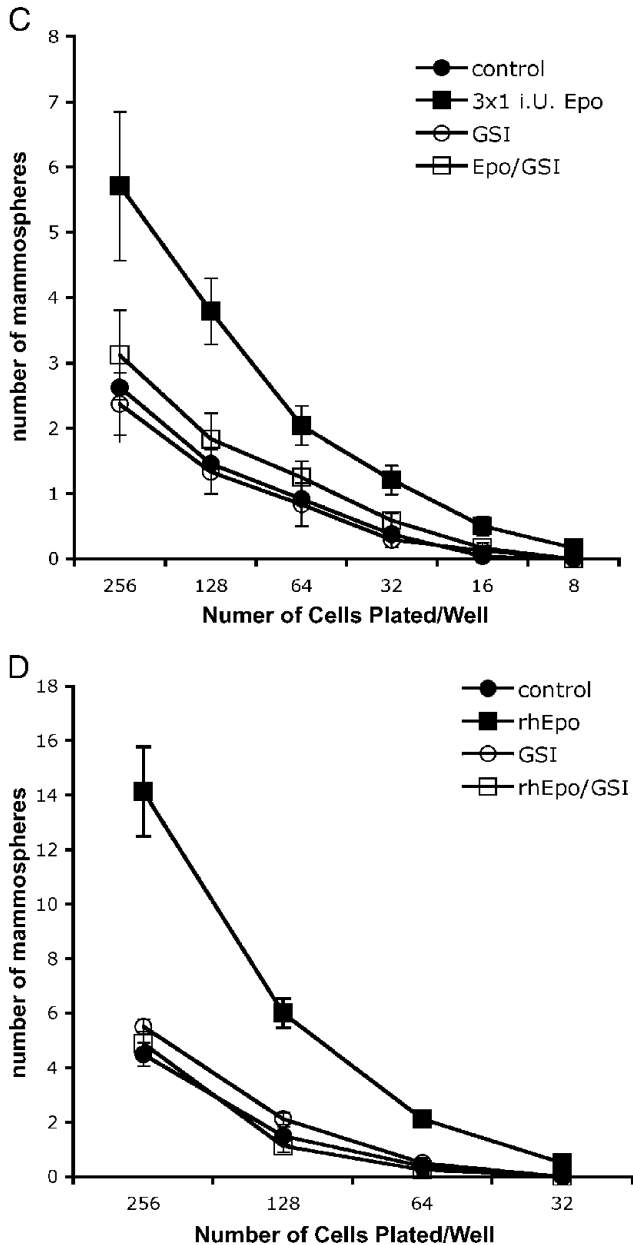


Figure 2. (continued)

experiments investigating EpoR expression, cells were serum-starved for 5 hours. For detecting EpoR expression, cells were labeled with a monoclonal fluorescein isothiocyanate-conjugated mouse anti-human EpoR antibody (FAB307F; R&D Systems, Minneapolis, MN), a PE/Cy5-conjugated mouse anti-human CD44 antibody (BioLegend, San Diego, CA), and a PE-conjugated mouse anti-CD24 antibody (Beckman Coulter, Fullerton, CA) using standard protocols. For analysis of Jagged-1 expression and Notch activation, cells were harvested, fixed in 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.5% saponin, and incubated with a rabbit anti-human Jagged-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit anti-human cleaved Notch-1 antibody (Cell Signaling Inc., Danvers, MA) and fluorochrome-conjugated secondary antibodies using standard protocols. Flow cytometry was performed on a

FACScalibur flow cytometer using the CellQuest software package (BD Biosciences, San Jose, CA).

Primary Sphere Formation Assay

The frequency of cells in the nonadherent population of monolayer cultures that were capable of initiating sphere formation was assessed by harvesting, washing, and resuspending cells in phenol red-free DMEM/F12 medium (supplemented with 0.4% bovine serum albumin, 20 ng/ml basic fibroblast growth factor, and 10 ng/ml epidermal growth factor), and passing them through a 40- μ m sieve. Cells were then counted, diluted, and plated at clonal density into 96-well plates. Mammospheres were counted on day 5.

Results

rhEpo Increases the Size of the Putative BCIC Population

The expression of EpoR on breast cancer cells has been described by others [30], but not its expression on CD44⁺/CD24^{low} cells. MCF-7, T47D, and MDA-MB-231 CD44⁺/CD24^{low} cells were found to express levels of EpoR comparable to levels of EpoR on unselected cells (Figure 1).

To test if rhEpo modulates the size of the CD44⁺/CD24^{low} population, MCF-7 monolayer cultures were treated with 1 IU/ml rhEpo for 3 consecutive days (days 2–4 of culture). About 24 hours later, monolayer cells and cells floating in the supernatant were analyzed for CD24 and CD44 expression. Cells were stained with antibodies against CD24 and CD44 and the size of the CD44⁺/CD24^{low} population of cells was analyzed by flow cytometry. Treatment with rhEpo did not change the number of CD44⁺/CD24^{low} in the adherent cell population but increased the number of CD44⁺/CD24^{low} cells in the supernatant almost 5-fold from 2% (\pm 0.3%) to 9.5% (\pm 1.8%) (means \pm SEM; P < .01, Student's t test) (Figure 2, A and B).

The effect of rhEpo was also demonstrated by its ability to increase in primary sphere formation by cells taken from the supernatant of MCF-7 or MDA-MB-231 monolayer cultures and plated at clonal densities into 96-well plates. Cells derived from cultures treated with 1 IU/ml rhEpo on 3 consecutive days (days 2–4 of culture) showed a significant increase in primary sphere formation (MCF-7: $1 \pm 0.4\%$ for untreated cells, $2.9 \pm 0.6\%$ for Epo-treated cells; MDA-MB-231: 1.2 ± 0.6 for untreated cells, $4.5 \pm 1.1\%$ for Epo-treated cells, P < .01, two-sided Student's t test; means \pm SEM) (Figure 2, C and D).

rhEpo Increases the Number of BCICs in a Notch-Dependent Fashion

We have previously shown that activation of the Notch signaling pathway increases the number of BCICs [23]. To investigate if blocking activation of the Notch-1 pathway would prevent Epo-induced increases in the number of BCICs, monolayer cultures were pretreated with GSI, 30 minutes before each daily treatment with rhEpo. GSI treatment prevented the rhEpo-induced increase in primary sphere formation (MCF-7: GSI-treated cells, $1 \pm 0.4\%$; Epo + GSI-treated

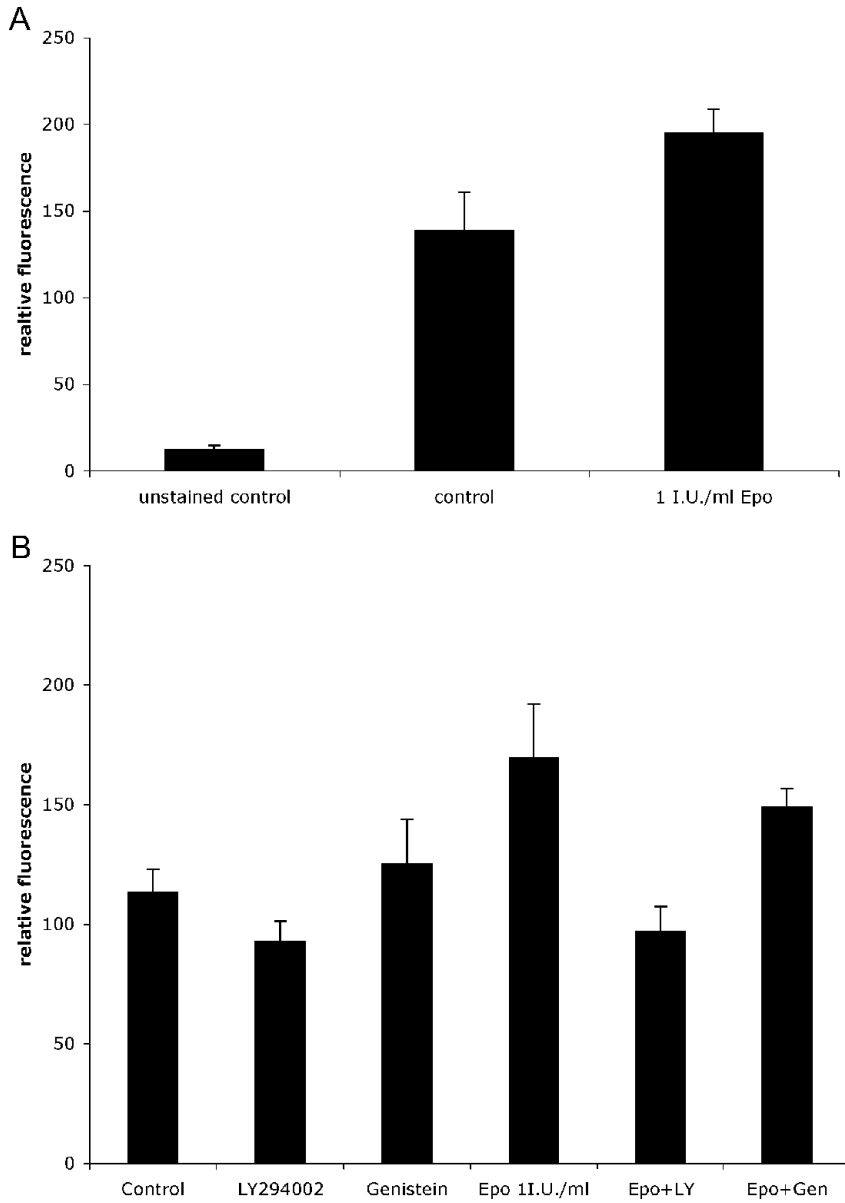


Figure 3. (A) Treatment of MCF-7 monolayer culture with 1 IU/ml rhEpo for 2 hours caused a 1.4 ± 0.16 -fold (mean \pm SEM, $P = .041$, $n = 3$, two-sided paired Student's *t* test) induction of Jagged-1 expression. (B) Treatment of MCF-7 monolayer culture with 1 IU/ml rhEpo for 2 hours caused a 1.5 ± 0.19 -fold (mean \pm SEM; $P = .049$, $n = 3$, two-sided paired Student's *t* test) activation of Notch-1 after 2 hours. Treatment with the PI3K inhibitor LY294002 but not the JAK2 inhibitor genistein prevented rhEpo-induced activation of Notch-1.

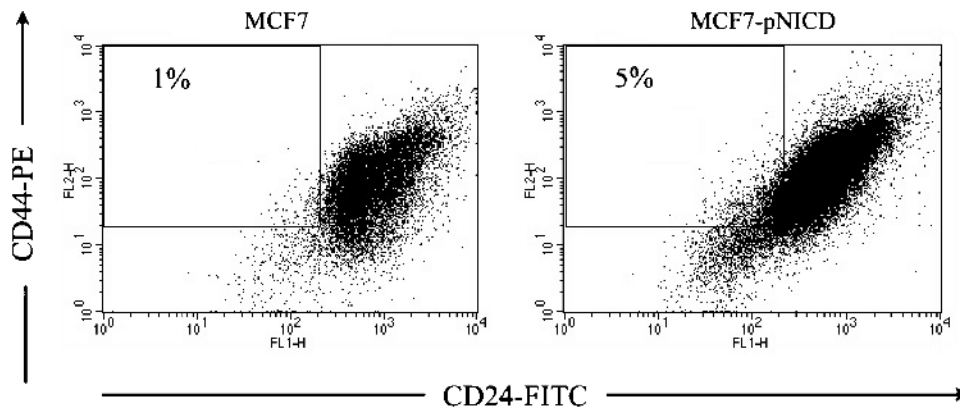


Figure 4. Representative FACS analysis of MCF-7 cells, stable transfected with an expression vector coding for constitutive active Notch, or an empty vector ($n = 2$). Expression of constitutive active Notch increased the population of BCICs.

cells, $1.5 \pm 0.4\%$; MDA-MB-231: GSI-treated cells, $1.5 \pm 0.7\%$; Epo + GSI-treated cells, $1.1 \pm 0.8\%$) (Figure 2, C and D).

To confirm that rhEpo increased the number of BCICs in a Notch-dependent fashion, we treated MCF-7 cells for 2 or 4 hours with 1 IU/ml rhEpo and stained cells with antibodies against Notch-ICD, or Jagged-1, and analyzed expression by flow cytometry. Treatment with rhEpo caused a 1.4 ± 0.16 -fold (mean \pm SEM, $P = .041$, $n = 3$, two-sided paired Student's *t* test) induction of Jagged-1 expression (Figure 3A) and a 1.5 ± 0.19 -fold (mean \pm SEM, $P = .049$, $n = 3$, two-sided paired Student's *t* test) activation of Notch-1 after 2 hours (Figure 3B). To confirm that activated Notch signaling truly increased the number of putative breast cancer stem cells, we stably transfected MCF-7 cells with an expression vector for a constitutively active Notch-ICD. When MCF-7-pNICD cells were stained with antibodies against CD24 and CD44, analyzed by flow cytometry, and compared to mock-transfected cells, the number of CD44⁺/CD24^{low} cells was increased in MCF-7-pNICD cells, consistent with our other data (Figure 4).

rhEpo Activates Notch Signaling through PI3K Pathways

Signaling in response to rhEpo through the Epo receptor is rapid and involves at least three major pathways: Janus kinase 2 (JAK2)-dependent activation of STAT5, activation of the Ras/Raf pathway, and PI3K/Akt-dependent/JAK2-independent activation of nuclear factor- κ B (NF- κ B) [31]. We previously reported activation of NF- κ B in cancer cells after treatment with rhEpo that was dependent on EpoR expression levels [11]. Because NF- κ B is known to induce Jagged-1 expression [32], we repeated our experiment using the PI3K inhibitor LY294002 and the JAK2 inhibitor genistein. As expected, treatment with LY294002 but not genistein prevented rhEpo-induced activation of Notch-1 (Figure 3B). As expected, pretreatment of the cells with the GSI inhibited activation of Notch-1 (Figure 5).

Discussion

The use of ESAs to correct chemotherapy-induced anemia or cancer anemia is currently under debate after five large placebo-controlled clinical trials showed increased cancer-related death rates for patients treated with ESAs [8,10,33] (DAHANCA 10 and Anemia of Cancer, both unpublished). These findings could not be explained by elevated hemoglobin levels, suggesting a direct effect of ESAs on cancer cells. However, experimental evidence for a direct effect of ESAs on cancer cells in the literature is inconclusive. Results from studies investigating direct effects of ESAs and cancers using a broad variety of *in vitro* models, *in vivo* models, and ESAs doses range from chemo- and radiosensitizing effects to chemo- and radioprotective effects, whereas others reported no effect at all [34–42].

EpoR was detected on cells derived from all three breast cancer cell lines confirming previous studies [30,43]. Here we report for the first time that EpoR is expressed on the surface of the BCIC population. It is important to note that the specificity of anti-EpoR antibodies is currently under debate

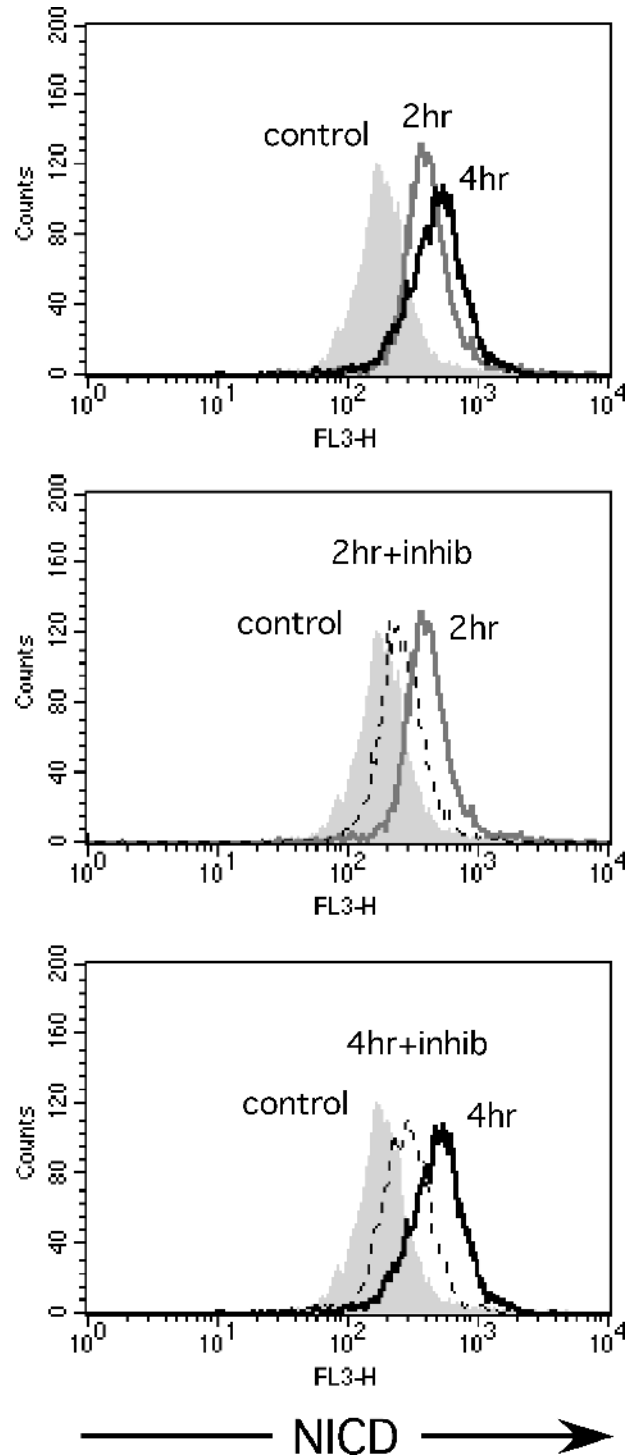


Figure 5. Representative FACS analysis of Notch-1 activation in MCF-7 cells. Treatment with 1 IU/ml rhEpo for 2 or 4 hours (upper panel: gray line and black line; filled histogram, control) caused activation of Notch-1. This activation was inhibited by pretreatment with a GSI for 30 minutes at 2 and 4 hours (middle and lower panels: gray line, rhEpo; dashed line, rhEpo + GSI; filled histogram, control).

[44] but it has been clearly demonstrated that the EpoR signals on rhEpo binding in non-erythroid cells, including cancer cells [45].

Using a pharmacological concentration of rhEpo (1 IU/ml), we found that the size of the CD44⁺/CD24^{low} nonadherent population of BCICs was increased after treatment with rhEpo.

The increase in the number of BCICs observed after rhEpo treatment was significant and the cells were not only viable but, more importantly, exhibited an increased self-renewal capacity as demonstrated by primary *in vitro* sphere formation.

We have previously demonstrated that activation of the Notch signaling pathway is part of the cellular stress response to clinical doses of ionizing radiation [23]. Interestingly, this effect was mediated by increased expression of the Notch receptor ligand Jagged-1 in the non-BCIC population that activated Notch signaling in BCICs. As for radiation [23], we have shown in this study that rhEpo treatment activated Notch signaling in BCICs. It further opens the possibility that EpoR expression may not be essential for BCICs to respond to rhEpo directly, but indirectly as they are the receiving part in the Notch signaling cascade, as long as the surrounding cells in a tumor express the receptor and respond to rhEpo with induction of Notch ligand expression. The role of Notch signaling for the rhEpo-induced increase in BCICs was further underlined by the observations that expression of constitutive active Notch increased the number of BCICs. As in our previous study [23], the number of BCICs was increased in the population of cells floating on top of the adherent cells. Comparable results were initially reported by Ponti et al. [22] and this may reflect the fact that this population usually contains not only dead cells but also cells undergoing mitosis and therefore detach from the adherent population.

We have previously shown that treatment of EpoR-expressing cancer cells activates the transcription factor NF- κ B [11]. This activation is independent of JAK2 [31], mediated through the PI3K pathway [46], and Jagged-1 is a known downstream target gene of NF- κ B [32]. Consistent with these previous findings, inhibition of the PI3K but not JAK2 prevented the increase in BCICs in response to rhEpo. Remarkably, GSI alone did not affect the number of mammospheres formed, which may indicate that GSIs are not toxic for BCICs, rather they only interfere with the switch to symmetric cell division while sphere formation remains intact.

Conclusions

Taken together, our study demonstrates that rhEpo mediates an increase in the number of BCICs in MCF-7 and MDA-MB-231 cultures, which could be blocked by inhibition of Notch signaling. With putative stem cells now identified in other cancers [14,16–18], it will be interesting to investigate if ESAs generally affect the growth of these cells also. Our study provides a possible explanation for the unexpected negative clinical effects of ESAs on local control and survival in cancer patients. Clearly, using established cell lines, our study has limitations and our observations need to be confirmed in primary tumor cell lines derived from patient samples.

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