EPO-independent functional EPO receptor in breast cancer enhances estrogen receptor activity and promotes cell proliferation

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Abstract
The main function of Erythropoietin (EPO) and its receptor (EPOR) is the stimulation of erythropoiesis. Recombinant human EPO (rhEPO) is therefore used to treat anemia in cancer patients. However, clinical trials have indicated that rhEPO treatment might promote tumor progression and has a negative effect on patient survival. In addition, EPOR expression has been detected in several cancer forms. Using a newly produced anti-EPOR antibody that reliably detects the full-length isoform of the EPOR we show that breast cancer tissue and cells express the EPOR protein. rhEPO stimulation of cultured EPOR expressing breast cancer cells did not result in increased proliferation, overt activation of EPOR (receptor phosphorylation) or a consistent activation of canonical EPOR signaling pathway mediators such as JAK2, STAT3, STAT5, or AKT. However, EPOR knockdown experiments suggested functional EPO receptors in estrogen receptor positive (ER a+) breast cancer cells, as reduced EPOR expression resulted in decreased proliferation. This effect on proliferation was not seen in ER a− cells. EPOR knockdown decreased ER a+ activity further supports a mechanism by which EPOR affects proliferation via ER a-mediated mechanisms. We show that EPOR protein is expressed in breast cancer cells, where it appears to promote proliferation by an EPO-independent mechanism in ER a expressing breast cancer cells.© 2014 The Authors. Published by Elsevier Inc.

1. Introduction
The main and most well described function of erythropoietin (EPO) is the stimulation of erythropoiesis [1,2] by binding to the EPO receptor (EPOR), promoting survival, proliferation and differentiation of erythroidic progenitor cells [3] through the JAK2/STAT, MAPK and/or the PI3 K pathway [4]. Although EPO and EPOR were originally believed to have an exclusive role in erythropoiesis, they are also expressed in many non-hematopoietic cells, for instance in the brain and in the cardiovascular system as well as in tumors of various origins [5,6], but their functions in these tissues are not completely known. In brain, EPO exerts tissue-protective effects on neurons in a paracrine fashion [7] and in the cardiovascular system it protects myocytes against hypoxic injuries [8]. EPOR is expressed in breast cancer cells [9–11] and in breast tumor endothelial cells [12].

The EPOR function in tumor cells remains controversial since contradicting results have been reported [13,14]. Recombinant human EPO (rhEPO) and other erythropoiesis-stimulating agents are used in the treatment of cancer-related anemia although clinical trials have shown an impaired prognosis in patients treated with rhEPO [15]. These reports have led to an increased awareness of yet undefined roles of EPO in tumor growth and progression by interacting with EPOR expressed by tumor and stromal cells. We previously showed a correlation between EPOR expression and tamoxifen response and survival in a clinical breast cancer material [10]. As EPOR expression and function in tumor cells have been questioned and concerns have been raised about the specificity of available antibodies [10,16], we produced a full-length EPOR specific antibody to study the expression and function of EPOR in breast cancer.

2. Material and methods
2.1. Cell culture
The EPO-dependent erythroleukemic UT-7 cells (DSMZ, Braunschweig, Germany) and breast cancer cell lines (ATCC, Manassas,
VA, USA) were cultured under standard conditions as recommended by the suppliers. All cell lines were regularly screened for mycoplasma and replaced by low-passage cells on a trimonthly basis.

2.2. Ethics statement

Breast cancer tissues were obtained from patients at Skåne University Hospital, Sweden. Ethical permission was obtained from the Lund University Regional Ethics Board, Ref. No. 594/2005. The ethics committee waived the need for patient consent and expression data were analyzed anonymously.

2.3. Anti-EPOR antibody production and Western blotting

An antiserum towards the C-terminal of human EPOR (hFL-EPOR) was generated in rabbits using standard procedures. The EPOR peptide with an added cystein residue, C-SLIPAAEPLPPS, conjugated to keyhole limpet hemocyanin was used as immunogen. The antibodies were affinity purified using a column of immobilized peptide antigen as described [17]. Immunoblotting was performed according to standard procedures. Antibodies are listed in Supplemental Table S1.

2.4. PCR, RNA interference and cell proliferation assay

Real-time quantitative PCR (RT-qPCR) was performed in triplicates and normalized to three housekeeping genes, SDHA, UBC, and YWHAZ [18]. Primers are listed in Table S2. For EPOR knockdown, four different siRNAs were tested (Table S2). Transfection was performed using Lipofectamin 2000 (Invitrogen) according to the manufacturers’ instructions. Cells were re-seeded in 96-well plates, 24 h after EPOR knockdown, and proliferation was measured using WST-1 reagent (Roche).

2.5. Immunofluorescence

Cells transfected with siRNA against EPOR were re-seeded on coverslips (30,000 cells/ml) and cultured for 48 h under standard conditions. After fixation and permeabilization the cells were analyzed for Ki67 immunoreactivity and TUNEL activity by confocal microscopy as described [19].

2.6. ERE luciferase assay, EPOR plasmid and EPOR over-expression

Cells were seeded at a density of 1 × 10⁵ cells/well in 12-well plates and transfected with siRNA against EPOR or control siRNA. After 24 h the medium was changed to Phenol Red-free medium supplemented with 10% charcoal stripped serum. After 24 h, cells were transfected with 1.45 μg pG2Luc luciferase reporter plasmid, pERE-luc (ERx responsive element (ERE), kind gift from Dr. Michael S. Denison, UCSD) and 0.05 mg renilla plasmid pRL-SV40 (internal control) using Lipofectamin 2000 (Invitrogen), and cultured with either 10 nM estrogen (17β-estradiol), 1 μM tamoxifen, or both. Luciferase activity was measured after 24 h using the Dual-Luciferase Reporter Assay System (DLR) (Promega) and normalized to internal control.

The Human EpoR cDNA, kindly provided by S. Constantinescu, was subcloned into the BamHI-EcoRI sites of pcDNA 3.1. An HA tag was introduced via PCR after the signal peptide, immediately following amino acid residues PPNL. The cDNA was confirmed by sequencing. For EPOR over-expression experiments, cells were transfected with either control siRNA or EPOR siRNA (siEPOR#3) together with 0.5 μg control (pc) or EPOR (pEPOR) plasmids and 0.5 μg pERE-luc and 0.05 μg renilla plasmids.

2.7. Statistical analysis

The error bars represent the SEM from three or more experiments. Statistical analysis were calculated using Student’s t-test and statistical significance was defined as *p < 0.05; **p < 0.01.

3. Results

3.1. EPOR protein expression in breast cancer cells

We generated a rabbit anti-EPOR serum directed towards the C-terminus and thus, full-length EPOR. The affinity-purified antibody (hFL-EPOR) detected a single protein of the anticipated size (approximately 66 kDa) in the breast cancer cell line CAMA-1 and EPO-dependent erythroleukemic UT7 cells, which served as a positive control (Figs. 1A and S1A). To further establish that the detected 66 kDa protein was EPOR, we transiently targeted EPOR expression using siRNA. RT-qPCR and immunoblot analyses clearly demonstrated a decrease in EPOR mRNA and the 66 kDa protein amount (Fig. 1A and B). A comparison with the C-20 anti-EPOR antibody from Santa Cruz, one of the most commonly used commercial anti-EPOR antibodies, showed that both antibodies detect the EPOR protein but the hFL-EPOR antibody appeared to be more specific by generating cleaner blots (Fig. S1A).

Using the hFL-EPOR antibody we detected EPOR expression in five tested breast cancer cell lines by immunoblotting (Fig. 1C). The EPOR expression was confirmed at the mRNA level (Fig. S1B). EPOR levels did not correlate to ERx protein expression (Fig. 1C). EPOR protein expression at varying levels was also detected in three of four tested primary tumor specimens (Fig. S1C).

3.2. Recombinant human EPO does not promote cell growth or consistently activate canonical EPOR downstream signaling pathways in breast cancer cells

Stimulation with rhEPO had no effect on growth or viability as exemplified by CAMA-1, MDA-MB-231 and T47D cells grown under standard conditions with serum (Fig. 1D). When the experiment was repeated under serum-free and Phenol Red-free conditions, rhEPO did not promote cell growth or increased cell survival (Fig. S2). To investigate the stimulatory capacity of rhEPO, we treated cells for 10 min with 10 U/ml rhEPO after overnight serum starvation. As a positive control UT7 cells were used, where rhEPO treatment induced phosphorylation of EPOR and the canonical EPOR downstream signaling proteins JAK2, STAT3, STAT5, AKT, and ERK1/2 (Fig. 1E). Interestingly, in the breast cancer cells, we could not detect phosphorylation of EPOR after rhEPO stimulation using an anti-phospho-Tyr456 EPOR antibody. We also immunoprecipitated EPOR from rhEPO-treated ERx breast cancer cells, but were unable to detect phosphorylated EPOR. Of the established signaling mediators downstream of EPOR, only ERK1/2 was phosphorylated in ERx cells treated for 10 min with rhEPO (Fig. S3A). Interestingly, the initial high level of AKT phosphorylation (pAKT) decreased slightly in the rhEPO treated cells, but with a slower kinetics than the increase in ERK1/2 phosphorylation (Fig. S3A). In rhEPO stimulated MDA-MB-468 cells, pSTAT3 and pERK1/2 levels increased (Fig. 1E). The possibility that rhEPO further activates EPOR in breast cancer cells by slower kinetics or at higher rhEPO concentrations than needed to activate EPOR in UT7 cells was tested, but uniformly with negative results (representative data shown in Fig. S3B).

3.3. EPOR knockdown leads to impaired cell proliferation in ERx positive breast cancer cells

We next knocked down EPOR using siRNA and evaluated the effects on cell viability and proliferation. Several EPOR siRNAs were
tested as we initially found that knockdown efficiency at the protein level was not as high in CAMA-1 as in UT7 cells (Fig. 1A and B). Notably, EPOR expression in UT7 cells was more rapidly knocked-down than in CAMA-1 cells, where the effects of siEPOR on EPOR protein levels were seen first after 48 and 72 h (Figs. S4A, S4B and S5). The most efficient siRNAs (siEPOR1, siEPOR3 and siEPOR4) were chosen for further analyses (Figs. S4 and S5).

We found a robust decrease in cell proliferation in ERα+ cell lines upon EPOR knockdown using different siRNAs (Figs. 2A and S5). ERα+ cell lines with high EPOR basal levels (CAMA-1 and T47D) were more affected than cells with a low basal EPOR expression (MCF7, Figs. 1C and 2A). A putative relation to ERα status was evaluated by knocking down EPOR in two ERα negative breast cancer cell lines. Interestingly, EPOR knockdown did not have any effect on cell numbers irrespective of whether the basal EPOR level was high (MDA-MB-231) or low (MDA-MB-468) (Figs. 1C and 2B and S5). The EPOR knockdown effect on cell number in ERα+ cells was primarily due to decreased proliferation and not increased cell death. While the number of TUNEL positive cells was very low in both control and siEPOR treated CAMA-1 cells (less than 1%, Fig. 3A), Ki67 expression profoundly decreased after EPOR knockdown (Fig. 3B). In contrast, when the EPO dependent UT7 cells were treated with siEPOR most cells died within a 24 h culture period (Fig. 3A), a result further indicating that the EPOR was indeed targeted by the used siRNA. Taken together, the results imply that EPOR has a function in the growth control of the investigated ERα+ cells despite that we were unable to detect a growth stimulatory effect of rhEPO in these cells. To exclude the possibility that the EPOR dependent growth of breast cancer cells was driven by endogenously produced EPO, we knocked down EPO expression, which did not affect the growth of ERα+ breast cancer cells (Fig. 2C). Overall, EPO mRNA expression in the tested breast cancer cell lines was low, at the limit of detectability by RT-qPCR, a notion in agreement with our previous observation that EPO protein is not produced at detectable levels in the analyzed breast cancer cell lines[10].

3.4. EPOR down-regulation affects ERα activity

Since EPOR knockdown affected proliferation in ERα+ cells, and in light of our previous results showing that EPOR expression correlates with tamoxifen response, we investigated effects of EPOR knockdown on ERα activity using an ERE luciferase assay. As expected, the strong induction of ERα activity upon estrogen stimulation was impaired by tamoxifen treatment in CAMA-1 and T47D cells (Fig. 4A) and tamoxifen stimulation alone had a slight positive effect on ERE luciferase activation (Fig. S6A and B). Over-all, EPO mRNA expression in the tested breast cancer cell lines was low, at the limit of detectability by RT-qPCR, a notion in agreement with our previous observation that EPO protein is not produced at detectable levels in the analyzed breast cancer cell lines[10].
In the presence of endogenous EPOR, estrogen stimulation induced a 27.9 (±8.4)-fold increase in ER\textsubscript{a} activity in CAMA-1 cells whereas in the EPOR knockdown cells the corresponding increase was 11.8 (±2.2)-fold (\(p = 0.003\)). Furthermore, down-regulation of EPOR resulted in a more efficient tamoxifen-induced inhibition of the ER\textsubscript{a} activity; a 2.9 (±0.9)-fold decrease in ER\textsubscript{a} activity in control cells compared to a 3.6 (±0.5)-fold decrease in EPOR knockdown cells (Fig. 4A, left panel). This result is in concordance with our previous observation showing an impaired tamoxifen response in patients with ER\textsubscript{a} positive tumors with high EPOR expression [10]. EPOR knockdown in T47D cells also resulted in a decreased estrogen-induced ER\textsubscript{a} activity (33.7 (±14) vs. 14.5 (±4)-fold induction) while EPOR knockdown did not enhance the effect of tamoxifen on ER\textsubscript{a} activity (8.7 (±3.8) vs. 8.1 (±2.9)) (Fig. 4A).

Subsequently, we tested the effect of overexpression of human wt EPOR on ER\textsubscript{a} activity. Overexpression of wt EPOR significantly increased ER\textsubscript{a} activity during estrogen stimulation. Furthermore, wt EPOR could rescue the siEPOR-inhibited ER\textsubscript{a} activity (Fig. 4B).

As our data indicated that EPOR is constitutively active via an EPO-independent process we searched additional support for this conclusion. We analyzed the effects of EPOR knockdown on phosphorylated AKT (pAKT) and in all three ER\textsubscript{a}+ cell lines pAKT levels were substantially reduced upon EPOR knockdown in keeping with the reduced growth rate of siEPOR-treated cells, while total AKT protein expression was not affected (Fig. 4C).

4. Discussion

We here show that cultured breast cancer cells express EPOR but do not respond to rhEPO stimulation by changes in proliferation or survival. Furthermore, using EPO-dependent UT7 erythroleukemic cells as a positive control, we could not detect rhEPO-induced EPOR phosphorylation or a consistently increased phosphorylation of several mediators of canonical EPOR signal transduction pathways. Our previous observations that EPOR expression levels, analyzed in a large breast cancer material, hold prognostic information and that tamoxifen sensitivity in ER\textsubscript{a}+ tumors could be related to low EPOR expression [10], inferred that EPOR in breast cancer cells mediates not yet defined biological responses. Here, we report that knocking down EPOR in ER\textsubscript{a}+ breast cancer cells resulted in growth inhibition but not cell death. EPOR expression was associated with estrogen-induced ER\textsubscript{a} activity as activation levels decreased in EPOR knocked-down ER\textsubscript{a}+ cells. In these cells EPOR appears to be constitutively active, leading to high AKT activity as demonstrated by decreased pAKT levels after EPOR knockdown. Thus, our data suggest that EPOR has a role in proliferation control of ER\textsubscript{a}+ breast cancer cells while cell survival seems to be unaffected by reduction of EPOR expression.

Stimulation with rhEPO did not result in increased proliferation or survival in the five tested breast cancer cell lines, which is in keeping with recent published data showing that EPOR expressing breast cancer cells do not respond by growth to rhEPO stimulation [20], although contradictory findings have been reported [9]. Published data do however demonstrate that rhEPO stimulation of breast cancer cells results in changes in cell signaling mediators such as AKT, ERK1/2 and STAT3s [14,21,22] and here we present data showing that rhEPO stimulation can increase both ERK1/2 and STAT3 phosphorylation in some of the tested cell lines. Also, knocking down EPOR in ER\textsubscript{a}+ breast cancer cell lines reduces the

![Fig. 2. EPOR knockdown in ER\textsubscript{a} positive breast cancer cells decreases proliferation. (A, B) EPOR knockdown (siEPOR1) effect on cell number in ER\textsubscript{a} positive (CAMA-1, MCF7, T47D) (A) and ER\textsubscript{a} negative (MDA-MB-231, MDA-MB-468) (B) cells (control siRNA (C)) (\(\big|p < 0.05\); \(\big|p < 0.01\)). Knockdown efficiency was determined by immunoblotting. (C) Knockdown effects of endogenous EPO by siEPO1 and siEPO2 versus siControl on proliferation. Knockdown efficiency at the mRNA level is shown in right hand panel.](https://example.com/figure2)

and S6 A and B). In the presence of endogenous EPOR, estrogen stimulation induced a 27.9 (±8.4)-fold increase in ER\textsubscript{a} activity in CAMA-1 cells whereas in the EPOR knockdown cells the corresponding increase was 11.8 (±2.2)-fold (\(p = 0.003\)). Furthermore, down-regulation of EPOR resulted in a more efficient tamoxifen-induced inhibition of the ER\textsubscript{a} activity; a 2.9 (±0.9)-fold decrease in ER\textsubscript{a} activity in control cells compared to a 3.6 (±0.5)-fold decrease in EPOR knockdown cells (Fig. 4A, left panel). This result is in concordance with our previous observation showing an impaired tamoxifen response in patients with ER\textsubscript{a} positive tumors with high EPOR expression [10]. EPOR knockdown in T47D cells also resulted in a decreased estrogen-induced ER\textsubscript{a} activity (33.7 (±14) vs. 14.5 (±4)-fold induction) while EPOR knockdown did not enhance the effect of tamoxifen on ER\textsubscript{a} activity (8.7 (±3.8) vs. 8.1 (±2.9)) (Fig. 4A).

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levels of pAKT and clearly indicates that EPOR transmits signals that change the phosphorylation, and presumably activation status of AKT. The growth-inhibiting effect of knocking down the EPOR receptor was restricted to the ERα+ breast cancer cells and did not require prior activation of EPOR by rhEPO. Thus, the growth promoting activity of EPOR seems to be constitutive in the tested ERα+ cells. The fact that the two studied ERα-negative breast cancer cell lines also express EPOR suggests that this receptor has additional functions in these and presumably also in ERα+ cells. Although our study focuses on the growth-promoting effect of
EPOR in ERα- cells, the presented data do not exclude that the rEPO/EPO signal transduction complex induces other biological responses like cell migration and cell survival gene expression [21]. The rEPO-stimulated increase in ERK and STAT3 phosphorylation in a cell line-dependent manner, seen here and emphasized by more extensively published data on rEPO activated signal transduction mediators [14,21,22], do suggest a broader role of rEPO/EPO in breast cancer and that EPOR signaling is not restricted to proliferation control in ERα- cells.

EPOR expression in clinical breast cancer specimens correlates to outcome [10] and rEPO treatment of ERα-negative xenograft tumors does not affect primary tumor growth but promotes metastasis [23], indicating that EPOR mediates specific, but not yet fully defined activities also in vivo. In ovarian cancer cells, EPOR is constitutively active and signals through classical pathways determined by the activation of the signal transduction mediators JAK, STAT, AKT and ERK. Interestingly, in these cells, EPOR knockdown resulted in reduced proliferation and invasiveness in an EPO-independent way [24,25]. The difference in EPOR turnover revealed to us by the EPOR knockdown experiments in breast cancer versus hematopoietic cells might suggest a different EPOR biology in these two cell types. This dissimilarity in turnover rates could be explained by a difference in activation mechanisms. While rEPO-induced receptor activation in UT7 cells preferentially triggers classical internalization and processing via the lysosomal system, EPOR in breast cancer cells might circumvent these pathways. The explanation could be that the EPORs in breast cancer cells are not exposed at the cell surface and thus have another subcellular localization than in UT7 cells [20]. This scenario would also explain why rEPO robustly triggers EPOR signaling in UT7 but not in the breast cancer cells analyzed.

An interesting observation presented here is the suggested link between EPOR expression and ERα activity. While EPOR knockdown diminished the proliferation of ERα+ breast cancer cells, this effect was not seen in ERα-negative cells. We also observed that EPOR knockdown significantly decreased ERα activity in ERα+ breast cancer cells. This observation provides an explanation of the impact of EPOR on proliferation since the ERα-pathway is a potent regulator of proliferation in ERα+ breast cancer cells [26]. The detailed molecular explanation as to how EPOR can modify ERα activity remains to be established. Our data however indicate that the PI3 K-AKT pathway is constitutively activated by EPOR in the investigated cells, an observation that should be further explored.

Data presented here also support our previously published observations on EPOR expression in relation to tamoxifen response in patients with ERα+ tumors in a clinical trial. In that study, patients with tumors having high EPOR expression were less responsive to tamoxifen treatment compared to those with low EPOR expression [10]. These observations are supported by the EPOR knockdown effects in CAMA-1 cells, which resulted in a more efficient tamoxifen-induced inhibition of ERα activity. The observed putative link between low EPOR expression and improved efficiency of tamoxifen has potential relevance in the clinical setting. One can envision a situation where blocking EPOR in breast cancer cells could increase the treatment efficiency of tamoxifen, perhaps by counteracting tamoxifen resistance. The molecular explanations for the interactions between EPOR and ERα activity might also unravel mechanisms of tamoxifen resistance, questions that need to be addressed in order to find out how EPOR should be targeted to modulate the potent ERα signaling pathways in breast cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.165.

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