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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 (ERα$^+$) breast cancer cells, as reduced EPOR expression resulted in decreased proliferation. This effect on proliferation was not seen in ERα negative cells. EPOR knockdown decreased ERα activity further supports a mechanism by which EPOR affects proliferation via ERα-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 ERα expressing breast cancer cells.

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 erythrocytic 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,

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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 tri-monthly 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 cyctein 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 manufactures' 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 1×10^5 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 pGL2 luciferase reporter plasmid, pERE-luc (ERα responsive element (ERE), kind gift from Dr. Michael S. Denison, UCD) and 0.05 mg renilla plasmid pRL-SV40 (internal control) using Lipofectamin 2000 (Invitrogen), and cultured with either 10 nM estrogen (17b-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 ERα 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 ERα$^+$ breast cancer cells, but were unable to detect phosphorylated EPOR. Of the established signaling mediators downstream of EPOR, only ERK1/2 was phosphorylated in ERα$^+$ cells treated for 10 min with rhEPO (Fig. S3A). Interestingly, the initially 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 ERα positive breast cancer cells

We next knocked down EPOR using siRNA and evaluated the effects on cell viability and proliferation. Several EPOR siRNAs were