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Review Article

# The role of erythropoietin and erythropoietin receptor expression in breast cancer

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**Summary**

Erythropoietin (EPO) plays a number of important functions in the body. Contrary to original beliefs, its activity is not limited to exerting effects on cells along the erythropoietic pathway. Newly published results continue to provide information on novel functions of the protein in other types of tissues, as well as on the important roles played by EPO in pathological processes. With no doubt, EPO has a significant impact on the biology of breast cancer cells by affecting cells' proliferation, apoptosis, resistance to chemotherapy, as well as expression of various types of receptors. EPO exerts its direct action on breast cancer stem-like cells by activation of specific signaling pathways responsible for protection of the tumor from chemotherapy and accelerating disease progression. EPO could inhibit chemotherapeutic drug-induced apoptosis and cytotoxicity. Its correlation with tissue hypoxia may play a significant role in the therapeutic resistance of hypoxic tumors. In recent years, the role of endogenous EPO in regulation of carcinogenesis was also noted. Exogenous EPO, in the form of rhEPO, had been introduced with best intention to treat patients with cancer-related anemia in the course of breast cancer. While it decreases the transfusion requirements and improves the quality of life of cancer patients, randomized trials have demonstrated that rhEPO administration is associated with shorter progression-free and overall survival. Observations allow also to say that EPO antagonizes treatment with the anti-HER2 antibody trastuzumab by activating EpoR/JAK2 downstream effectors, effectively bypassing HER2 signaling. Although increasing amount of information is available regarding the role of EPO and EpoR in breast cancer, elucidation of the activity and involvement of these proteins in complex processes occurring within the cancer cells requires extensive research. Every set of results being published answers some of the questions while instead raise new ones.

*Key words:* Breast neoplasms; Erythropoietin; Hypoxia.

## Erythropoietin (EPO) – structure and function

EPO is a glycoprotein hormone that controls erythropoiesis, i.e. production of red blood cells. The molecule is built of 165 amino acids accounting for the molecular mass of 30.4 kDa; it is encoded by a gene located within chromosome 7. In normal conditions, serum EPO levels are 15-30 IU/L and are subject to diurnal fluctuations reaching peak values during the night. Hypererythropoietinemia is typical for individuals dwelling at high altitudes, while reduced levels of the hormone may be observed in many renal diseases, cancers, or chronic diseases as well as in premature infants [1]. EPO plays a role of a cytokine for erythrocyte precursors within the bone marrow. It is the main regulator of the proliferation and differentiation of erythroblasts. It is produced by interstitial fibroblasts in the kidneys in close association with peritubular capillaries and proximal convoluted tubules. It is also produced in the perisinusoidal cells is the liver. While liver production predominates in the fetal and perinatal periods, renal production is predominant during adulthood (accounting for more than 80% of total production). It was observed that retinoic acid and thyroid hormones increase EPO synthesis in hypoxic conditions. Due to the inability to store the hormone within the synthesizing cells, the quantity of secreted EPO depends only on the rate of its production [2]. Besides playing a role in erythropoiesis, EPO has a number of other systemic functions both in physiological and pathological conditions. It is involved in neuronal response to damage and wound healing process, stimulates angiogenesis, and induces proliferation of smooth muscle fibers. Vasoconstrictive properties of EPO were also reported, facilitating regulation of arterial pressure and regulation of microcirculation. EPO is also involved in the enhancement of gastrointestinal iron absorption by means of suppression of hepcidin gene. An increasing number of results presents the role of EPO as a hormone with tissue-protective effects in various types of tissues, e.g. in ischemic conditions and numerous degenerative disorders. In the brain, where EpoR expression was observed within the neuronal membrane and hypoxia-stimulated EPO synthesis was observed in astrocytes, EPO was shown to protect neurons from ischemic damage. Expression of EPO and EPO receptors in the uter-

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ine wall was shown to impact regulation of angiogenesis during the menstrual cycle [3].

The production of EPO is stimulated by tissue hypoxia that directly stimulates interstitial cells within the renal cortex. This leads to an increase in the number of erythrocytes, improves oxygenation of tissues, and inhibits further secretion and synthesis of EPO in a negative feedback mechanism. EPO is the only hematopoietic growth factor whose expression is regulated by tissue hypoxia [4]. Cytokines released in large quantities by cancer cells reduce the production of EPO, thus becoming one of the main causes of anemia of chronic diseases (ACD). This type of anemia often accompanies proliferative disorders. Endogenous EPO levels in cancer patients was shown to be particularly low in relation to the degree of ischemia [5].

## EPO receptor – structure and function

EPO exerts its effects on cells via a transmembrane receptor (EpoR). The gene encoding human EpoR is located in chromosome 19 and consists of eight exons separated by seven introns. Different splice variants have been reported in murine cell lines and bone marrow as well as in tumor cells [3]. The EpoR protein has molecular mass of 66 kDa and consists of 508 amino acid moieties. The number of EpoRs expressed at the cell surface of normal or transformed erythroid cell is low, approximately up to one thousand receptors per cell. The EpoRs are mainly expressed at the colony-forming unit erythroid (CFU-E) stage. Later, their expression decreases with erythroid maturation, leading to complete absence in reticulocytes and mature red blood cells. Although the highest quantities of EpoRs are found within the cellular membranes of the erythroblast cell line, they were also found to be present in the cells of the brain, myocardium, kidneys, skeletal muscles, liver, lungs, retina, adrenal glands, parathyroid glands, pancreas, placenta, and endothelium. These receptors are also found on the surface of macrocyte precursors [6]. EpoR expression was observed on the surface of malignant tumor cells, for example breast cancer, prostate cancer, squamous cell cancer or multiple myeloma cells as well as in the metastatic foci of these cancers [7]. After binding the EPO molecule, EpoR undergoes homodimerization and is activated by JAK2 cytoplasmic tyrosine kinases. By itself, EpoR molecule lacks the kinase activity. Specific phosphorylated tyrosines within the intracellular domain of the EpoR serve as docking sites for intracellular proteins containing src homology 2 (SH2) domains including STAT5, which is a potent signal transducer and transcription activator. After binding, the proteins may be phosphorylated and activated. Activated STAT5 molecule is transferred to the nucleus to induce transcription of various genes. A direct relationship was also observed between PI3K and EpoR, leading to activation of EPO-dependent intracellular pathways; an alternative PI3K activation pathway proceeding via the adaptor protein IRS2 was also described. Also the RAS/MAPK pathway signaling is activated by EPO binding to the surface receptors and leads to increased proliferation within the erythroid precursor cells. EPO signaling has also been shown to upregulate c-myc mRNA expression via a PKC-dependent pathway. It seems very probable that intensification of EpoR expression on the surface of cancer cells is inversely proportional to cancer differentiation. It is also known that EpoR expression is not observed in all tumors of particular type. Similarly as in the case of erythroblast call lines, EPO exerts its cellular effects via the JAK tyrosine kinase pathway as well as the NF-κB/STAT pathway. Stimulation of these pathways leads to inhibition of apoptosis in cancer-transformed cells. This leads to increased resistance of cancer cells to radio- and chemotherapy and enhances the cancer cells' ability to proliferate, migrate, infiltrate adjacent tissues, and produce metastases [8].

## The role of EPO in breast cancer

Acs *et al.* studied the expression of EpoR in breast cancer cells to demonstrate poor or moderate expression of EPO and EpoR in non-malignant cancer cells [9]. In this study, the expression of EpoR in breast cancer as assessed by immunohistochemical assays was significantly higher compared to non-malignant lesions in both smokers and non-smokers. Expression of EPO in breast cancer was higher compared to non-malignant lesions in non-smokers only. As observed by the research team, EPO expression was most pronounced in cancer cells adjacent to necrotic areas as well as in cells on the surface of lesions infiltrating the surrounding tissues [9]. Another finding was that EpoR expression was markedly higher in lesions characterized by a high degree of histological differentiation with areas of necrosis present within the lesion, as well as by invasion of lymphatic vessels, lymphatic node metastases or loss of hormonal receptor expression. All these findings suggest that the overexpression of EpoR plays a significant role in carcinogenesis in breast cancer since the induction of EPO signaling pathway is a mechanism in which local ischemia leads to neoplastic growth [9].

Anemia is common in cancer patients; however, the incidence estimates are markedly different in different cancer types. It also depends on patient's age, clinical stage of the disease, and treatment applied. It was reported that between 30% and 50% of cancer patients develop anemia at some point during the course of the disease [10]. According to a generally accepted rule, anemia is much more common in malignant tumors that in benign tumors. The highest rates of anemia are observed in patients with hematological cancers. Anemia was shown to be a strong negative prognostic factor for survival in different forms of cancer. Pathogenesis of cancer-related anemia (CRA) is complex and multifactorial and can be due to both the disease itself

and tumor treatment. Factors contributing to CRA include tumor-associated bleeding, hypersplenism with hemophagocytosis, hemolysis, nutritional deficiencies, marrow damage from metastases or myelodysplasia, impaired EPO production, treatment toxicity (chemotherapy and radiotherapy), complex interactions between tumor cells, and immune system, etc. In such conditions EPO synthesis increases in response to hypoxia; however, this mechanism is triggered only upon a marked drop in blood hemoglobin levels. A traditional method to treat CRA was administration of erythrocyte concentrate. However, results of studies conducted in recent years shed doubt on the presumed benefits of this treatment. For example, it showed that blood transfusion may contribute to reduced immunity and accelerated progression of cancer [11]. Currently, a better and more common solution is administration of recombinant human erythropoietin (rhEPO), present in two variants of epoietin-α and epoietin-β. The difference between both proteins consists mainly in different glycosylation patterns, with amino acid sequence being identical to that of endogenous EPO. A similar drug used in CRA is darbepoetin-α. This protein has two additional oligosaccharide chains resulting from substitution of five amino acids in the protein backbone. These modifications were introduced in order to prolong the protein's half-life. In cancer-related anemia, rhEPO was introduced to the management regimens in order to increase the hemoglobin levels, improve the patients' quality of life (by elimination of nagging symptoms of anemia: tachycardia, weakness, skin paleness, retrosternal pain, loss of consciousness), and reduce demand for blood transfusions in patients. Initially, the treatment was considered to be very safe and markedly improving the condition of patients. Numerous reports were published, even suggesting the increase in the survival rates of patients treated with rhEPO. In 2003, first reports on possible detrimental effects of rhEPO treatment in cancer patients were published. The Breast Cancer Erythropoietin Trial (BEST), evaluating rhEPO treatment to prevent anemia in patients with metaplastic breast cancer receiving chemotherapy, was terminated early due to impaired survival in the rhEPO treated group compared to controls [12]. The reason for the differences in survival could not be determined from subsequent analysis after the study. At the same time, another randomized study assessing the efficacy of rhEPO in the treatment of patients with head and neck cancer subjected to radiation therapy revealed impaired locoregional progression free survival in rhEPO treated patients [13]. Of interest is the fact that the results of meta-analyses have changed so dramatically over just a few years. After initial enthusiastic reception, the results of rhEPO treatment turned out to be less effective than previously believed. American Society of Clinical Oncology/ American Society of Hematology guidelines from 2010, one large meta-analysis using individual patient data from 53 randomized controlled trials reported that treatment with erythropoiesis-stimulating agents (ESA) increased study mortality and worsened the overall survival. Further analyses trying to identify subgroups with increased or decreased risk of ESA-induced survival impairment were unsuccessful [14]. These conclusions forced the development of a novel clinical management protocol where rhEPO may only constitute an alternative to erythrocyte concentration transfer which remains the method of first choice in the treatment of CRA. If rhEPO is to be used, it should be used at the lowest doses possible. The mechanisms by which ESAs impact the progression of cancer and patient survival have not been thoroughly studied. Currently, the role of EPO and EpoR in tumor tissues is extensively studied. An increasing amount of data is also available on the role of expression of these proteins in breast cancer.

Larsson *et al.* described the expression of EPO and EpoR in breast cancer cell lines and tissue cultures with particular focus on patients treated with tamoxifen, i.e. an anti-estrogen drug [15]. Tamoxifen exerts its action by competitively binding of estrogen receptors within the cancer cells leading to inhibition of synthesis and release of growth factors, as well as by stimulating the synthesis of progesterone receptors. The main goal of this study was to elucidate and assess the expression of EpoR as the prognostic marker and predictor in breast cancer patients. Expression of both proteins was observed in all tested samples. The expression of EPO and EpoR was also observed in healthy breast tissues. The level of mRNA expression increased slightly in response to hypoxia in all tested samples (1% $O_2$). EPO mRNA was detected in normoxic $ER^+$ and $ER^-$ breast cancer cells. Tamoxifen treatment significantly increased recurrence-free survival (RFS) in patients with $ER^+/PR^+$ tumors with low EpoR expression, but had no effect on RFS in patients with high EpoR expression. In the untreated cohort, RFS was significantly improved for patients with $ER^+$ tumors with high EpoR expression. The finding that EpoR gives prognostic information in $ER^+$ but not in ER- breast cancers has to be further elucidated; however, it can support the hypothesis that EpoR has a specific role associated with estrogen receptor [15].

Observations of negative effects of rhEPO in patients with cancer-related anemia that have been made repeatedly for several years raise the question regarding the clinical implications of the expression of EpoR in tumors [15]. In the aforementioned study, Larsson *et al.* demonstrated the expression of EPO and EpoR; however, as expected, the level of expression differed significantly between individual types of cancer. Regarding the prognosis, high EpoR expression was strongly associated with improved survival in untreated patients with hormone receptor-positive tumors. This association was significant in multivariate analysis and seems to be an independent prognostic factor [15]. The question regarding molecular background of processes responsible for differences in patients' prognoses

remains unanswered. From the clinical standpoint, it is also important to determine whether the adverse effects of rhEPO are correlated with the expression of EpoR in cancer cells since rhEPO still remains an important element of cancer-related anemia treatment protocols. EpoR protein has been proposed to be non-functional in tumor cells due to a non-cell surface location, and therefore, presumably, not available for activation by rhEPO [16].

Recently, clinical trials involving both anemic and non-anemic cancer patients have raised questions concerning the safety of erythropoiesis stimulating agents with respect to survival and promotion of thromboembolic events [17]. Volgger *et al.* retrospectively examined EpoR expression in breast cancer patients and analyzed their association with tumor biology and the course of the disease in breast cancer patients not receiving rhEPO [18]. Unexpectedly, there was no significant difference in the mean expression of EpoR mRNA between malignant and benign lesions. EpoR expression did not depend on menopausal status, age, tumor stage or differentiation. Subsequently, EpoR expression and its correlation with the course of the disease was analyzed. No interdependence was observed between EpoR expression rate and overall survival. However, there was a significant association between EpoR expression and decreased localized disease free survival. Higher EpoR levels were associated with a significantly increased likelihood of locoregional recurrence of cancer and were also significantly correlated with the expression of estrogen and progesterone receptors. This is surprising since a positive estrogen and progesterone receptor status is known to be an indicator for a better prognosis as such patients can be treated with hormone therapy [18]. An increased EpoR mRNA expression in breast cancer biopsies was not associated with an increased mortality; thus, the presence of EpoRs on cancer cells may rather be indicative of a specific, likewise more aggressive, breast cancer phenotype than being a negative predictor per se [18].

Detection of EpoR in cancer cell lines favors supposition that rhEPO has some impact on cancer biology, acting as a growth factor that promotes their progression. However, the studies showed that the level of EpoR expression in cancer cells was significantly lower than in the positive control sample consisting of EPO-responsive cell types and tissues. No differences were also observed in the expression of the protein between cancer tissues and non-cancer tissues. In addition, the EpoR gene itself was only rarely amplified in tumors [19]. These results show that amplification and overexpression of the EpoR gene are not characteristic for cancer.

Studies on the expression of EpoR in normal and cancer tissues were initially conducted using immunohistochemistry or Western blot assays involving anti-EpoR antibodies. The results of the analyses were positive; however, after several years it turned out that the antibodies were not specific [20-22], which raised doubts to the relevance of results. This fact additionally complicates the analysis of available literature on the expression and activity of EPO and EpoR in breast cancers. EPO-stimulating agents (ESAs) were used for more than 20 years as the basis for the treatment of patients with cancer-related anemia. They allowed for increasing the red blood cell counts and limiting the use of blood transfers. In 2002, ESAs were used in nearly 45% of all cancer patients [23]. After clinical trials reporting increased adverse events and/or reduced survival in ESA-treated patients, concerns have been raised regarding the potential role of ESA in promoting tumor progression, possibly through tumor cell stimulation. However, evidence is lacking on the ability of EPO to directly affect cancer stem-like cells which are thought to be responsible for tumor progression and relapse [24].

Todaro *et al.* found that EpoR was expressed in breast cancer stem-like cells (BCSCs) isolated from patient tumors [24]. Of all the studied types of breast cancer, the highest EpoR expression was observed in basal-like cancer. This trial also showed that BCSCs respond to EPO treatment with increased cell proliferation and self-renewal rate. Importantly, EPO stimulation increased BCSCs' resistance to chemotherapeutic agents and activated cellular pathways responsible for survival and drug resistance. Specifically, the Akt and ERK pathways were activated in BCSCs at early time points following EPO treatment, whereas Bcl-xL levels increased at later times. In vivo, EPO administration counteracted the effects of chemotherapeutic agents on BCSCs-derived orthotopic tumor xenografts and promoted metastatic progression both in the presence and in the absence of chemotherapy treatment. [24].

All the aforementioned data demonstrate that EPO exerts its direct action on breast cancer stem-like cells by activation of specific signaling pathways responsible for protection of the tumor from chemotherapy and accelerating disease progression. According to the results of most recent studies, population of cancer stem cells appears to be responsible for the progression, recurrence, and metastases of cancer. In the light of these findings, there is a need to develop a treatment that would specifically target this call population. As is well known for several years now, rhEPO has negative effect on the survival rates of breast cancer patients. Todaro *et al.* were the first to demonstrate the effect of EPO and EpoR on breast cancer stem cells [24]. The results confirmed that EPO was co-involved in the development of chemoresistance and acceleration of tumor growth rate [24].

Already several years earlier, Phillips *et al.* investigated the effect of EPO on cancer stem cells in breast cancer cell lines [25]. They found that pharmacological concentrations of rhEPO increased the number of putative breast cancer initiating cells (BCICs) in established breast cancer cell lines. This increase was mediated by the activation of the Notch signaling pathway. Primarily, the Notch pathway is

important for cell-cell communication which involves gene regulation mechanisms that control multiple cell differentiation processes. Notch activation occurred via the induction of the Notch receptor ligand Jagged-1 in a phophoinositide-3 kinase (PI3K)-dependent fashion. The increase in the number of BCICs observed after rhEPO treatment was significant and the cells were not only viable but, what is more important, exhibited an increased self-renewal capacity as demonstrated by primary in vitro sphere formation [25]. Phillips *et al.* had previously proven that activation of the Notch signaling pathway is a part of the cellular stress response to clinical doses of ionizing radiation [26]. 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, Phillips *et al.* demonstrated that rhEPO treatment activated Notch signaling pathway in BCICs [25].

Studies conducted in recent years facilitated precise examination of the function of EPO and EpoR in various types of cells, including demonstration of their high activity in cancer tissues. Already more than a decade ago, Acs *et al.* described autocrine regulation of apoptosis by these proteins in the course of breast cancer [9]. The researchers incubated cells of the MCF-7 breast cancer cell line in gradually decreasing $O_2$ levels to observe that EPO mRNA expression levels were strictly correlated with the increasingly hypoxic conditions in the culture. Similarly, exacerbating acute hypoxia stimulated the transcription of EpoR mRNA. Verification by Western blot confirmed changes in the EPO and EpoR expression profiles, similar to the changes in the levels of both mRNA transcripts. The study also confirmed that autocrine EPO signaling induced by moderate levels of hypoxia inhibits hypoxia-induced apoptosis and promotes survival in MCF-7 human breast cancer cells. The anti-apoptotic effect of EPO was correlated with upregulation of Bcl-2 and Bcl-$X_L$, and thus its mechanisms appear to be similar to those described in hematopoietic cells. Bcl-2 and Bcl-$X_L$ prevent apoptosis by inhibiting the release of cytochrome c and apoptosis inducing factor (AIF) from mitochondria [27].

The mechanism described above, commonly referred to as "escape from apoptosis" plays one of the key roles in the development and progression of cancer. Avoidance of apoptosis is also one of the most important features determining the malignant character of the lesion.

Acs *et al.* had previously shown that EPO could inhibit chemotherapeutic drug-induced apoptosis and cytotoxicity [8]. The results of their next study suggested that the increased EPO signaling induced by tumor hypoxia could play a significant role in the therapeutic resistance of hypoxic tumors [27].

Hypoxia is a common phenomenon accompanying solid malignancies such as breast cancer. Insufficient distribution of oxygen, most commonly affecting the central parts of the rapidly growing lesions leads to rearrangement of gene expression in hypoxic tumor tissues. As a consequence, synthesis of proteins protecting the cells from adverse conditions is increased. This is achieved for example by blocking the cells' capability to undergo apoptosis. This phenomenon is beneficial for most types of cells but very risky from the standpoint of neoplastic growth. Characteristic proteins that are overexpressed in hypoxic conditions include EPO, EpoR, and HIF-1α. However, another marker of hypoxia may consist in elevated cell levels of signal transducer and activator of transcription 3 (STAT3). This transcription factor is activated through phosphorylation of tyrosine 705. Despite the fact that STAT3 is overexpressed in tumors and has been referred to as an proto-oncogene, its accumulation can be detected in various non-neoplastic conditions of increased cell turnover and their enhanced biosynthesis of various proteins [29, 30]. Linkages in the expression of proteins suggests functional dependences among STAT, HIF-1α, EPO. and EpoR in cell to cell signaling in breast cancer [31]. Breast cancer also involves upregulation of transcriptional agents like STAT3, STAT3 activator - EpoR (erythropoietin receptor), and a HIF-1 downstream protein – EPO [32]. STAT3 contributes to increased EPO expression which is also HIF-1α dependent. Tyrosine phosphorylation of STAT3 is triggered by EPO. In addition, EPO/EpoR signaling was reported to mediate cell survival by targeting Bcl-xL (B-cell leukemia/lymphoma extra-long protein) and counteract Bax-dependent (BCL-2-associated X protein) apoptosis [33, 34].

Overexpression of HIF-1α is indicative of poor prognosis and markedly shorter survival in breast cancer patients [35]. HIF-1 upregulates transcription of angiogenic genes like EPO and vascular endothelial growth factor (VEGF) which induce sprouting of new vessels and consequently increase the risk of metastasis by boosting the contact surface between tumor cells and vasculature. HIF-1 is responsible mainly for cellular adaptation to hypoxic conditions; therefore, genes triggered by this factor are responsible mainly for the improvement in oxygen supply (by increasing angiogenesis, broadening the lumen of existing vessels, increased erythropoiesis or increased iron consumption), adaptation of cells to anaerobic metabolism conditions as well as for other changes facilitating cell survival in the conditions of insufficient oxygen availability and modifying the main metabolic pattern. HIF-1 induces transcription of cytoprotective proteins in malignant cells in hypoxic conditions. HIF-1α predicts poor prognosis in breast cancer [36, 37]. HIF-1 and STAT3 actions were shown to be indirectly related to each other. This relationship was highlighted mainly by interference of STAT3 transcription with small-molecule inhibitor and resultant downregulation of HIF-1 and VEGF that delayed tumor growth and angiogenesis [38].

EPO and EpoR are induced by hypoxia in breast cancer and could contribute to increased survival rate of tumor cells via counteraction to hypoxic injury [39]. EPO coun-

teracts the outflow of cytochrome c from mitochondrion by upregulation of Bcl-xL. EPO prevents apaf-1 complex-dependent activation of caspase 9 and 3 by inhibition of binding cytochrome c to apaf-1 and cyt-c in the cytoplasm [32]. The aforementioned correlations were described for ductal carcinoma by Wincewicz *et al.* that detected STAT3 in 50% of all cancers, HIF-1α in 72% of all cancers, EPO in 89% of all cancers, and EpoR in 72% of all cancers [32]. There were significant relationships between the expressions of STAT3 and HIF-1α. STAT3 was significantly correlated with the expressions of EPO and EpoR in cancers of all patients. HIF-1α was correlated with EPO and EpoR in most of analyzed groups. The data are suggestive of a strict correlation between the levels of all these proteins in the course of breast cancer. All these proteins are overexpressed in hypoxic conditions and all have an important effect on the biology of the tumor. The occurrence of HIF-1α was significantly increased in chemotherapy-spared tumors compared to chemotherapy-treated cancers because chemotherapy could destructively affect cancer cells via inhibition of protein expression [32]. Correlations between STAT3 and EPO suggest that they act in support to the survival of breast cancer cells in human tumors in the same fashion as in cell lines [40, 41].

Some revolutionary insight on the role of EPO and EpoR in breast cancer was provided by the last year' results from Reinbothe *et al.* [42]. According to these results, rhEPO stimulation of cultured EpoR-expressing breast cancer cells did not result in increased proliferation, overt activation of EpoR (receptor phosphorylation), or 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 ($ER\alpha^+$) breast cancer cell lines, as reduced EpoR expression resulted in decreased proliferation, but not in cell death. This effect on proliferation was not seen in estrogen receptor-negative ($ER\alpha^-$) cells. As suggested by these observations, reduction in EpoR expression led to reduced ERα-dependent proliferative activity in breast cancer [42]. EpoR expression seems to play a role in proliferation control of $ER\alpha^+$ breast cancers while survival seems to be unaffected by reduction of EpoR expression.

Studies by Reinbothe *et al.* demonstrated that in EpoR-expressing breast cancers, stimulation of proliferation involves the receptor whereas an EPO-independent mechanism is at work in $ER\alpha^+$ breast cancers. The molecular mechanisms that influenced interactions between EpoR and ERα, as well as their impact on the biology of cancer cells require further studies [42]. As of yet, it is unknown how these act together in regulation of proliferation of breast cancer cells. Questions need to be addressed in order to find out how EpoR should be targeted to modulate the potent ERα signaling pathways. Answers to this query may reveal new potent therapeutic methods in breast cancer. Conclusions from the study conducted by Reinbothe *et al.* [42], suggesting that rhEPO has no effect on the increased proliferative activity or survival in the five tested breast cancer cell lines, are in line with the results published eight years earlier by LaMontagne *et al.* [43], although contradictory findings were reported in study by Acs *et al.* [40]. Discrepancies are also observed in published data with regards to rhEPO stimulation of breast cancer cells resulting in changes in cell signaling mediators such as AKT, ERK1/2, and STATs [44-47].

Despite the fact that EPO was initially proposed to be involved only in regulation of erythropoiesis, it resulted to be an important link between multiple signaling pathways in normal as well as in cancerous non-hematopoietic tissues. Furthermore, functional autocrine/paracrine EPO-EpoR systems were described in recent years in human cancer cells originating from breast cancer, endometrial cancer, malignant melanoma, and prostate cancer. These data suggest that EPO/EpoR may impact the tumor growth, disease progression and formation of metastases [39, 48-52]. Autocrine signaling is a form of intercellular communication in which a cell releases a chemical substance that binds receptors located at the same cell, leading to functional changes within that cell. Paracrine communication is a form of information transfer in which the target cell is located near the signal-releasing cell. Liang *et al.* [45] described the autocrine/paracrine activity of EPO produced by breast cancer cells in both normoxic and hypoxic conditions. They found that the level of EPO produced by these cells was higher in hypoxia than in normoxia. This observation is consistent with the knowledge that EPO is a product of hypoxia-inducible gene expression. Unfortunately, the study did not allow to determine whether the effects of EPO are due to autocrine or paracrine signaling. However, it was proposed with much certainty that both types of intercellular communication had impact on the effects observed in cell line cultures. As also shown by the results presented in the publication, EPO/EpoR autocrine/paracrine signaling may mediate and influence the invasion potential of breast cancer cells [45]. This in turn suggests that autocrine/paracrine EPO signaling may be one of the effector mechanisms in which the HIF-1 factor impacts the invasiveness of breast cancer [53]. Targeting of HIF-1, being actively investigated as a potential strategy for cancer therapy, could inhibit the effects of autocrine/paracrine EPO signaling on cell migration and invasiveness. Inhibition of EPO autocrine/paracrine signaling pathways in cancer cells could be one of the mechanisms explaining the anticancer effects of several anti-HIF-1 agents reported in the literature [54, 55]. Silencing of EPO or EpoR by RNA interference led to marked inhibition of cell signaling and cell migration and invasion. Furthermore, the present authors found that autocrine/paracrine production of EPO also played a role in stimulating tumor sphere growth of breast cancer cells [53].

As shown by previous studies, stimulation of breast cancer cells by rhEPO treatment increased the proportion of tumor-initiating cells [56]. Liang *et al.* [45] demonstrated that also the autocrine/paracrine effects of EPO may induce the proportion of these cells. This is a very adverse phenomenon since tumor-initiating cells play an important role in carcinogenesis through conferring resistance of cancer cells to anticancer treatment and promoting cancer cell self-renewal. Findings by Liang *et al.* suggest that autocrine/paracrine EPO/EpoR loop may be one of the mechanisms by which breast cancer cells maintain stemness [53].

In recent years, Zhou *et al.* confirmed the hypothesis that exogenous EPO has no significant effect on cell proliferation and does not protect cells from chemotherapy-induced apoptosis in vitro [56]. Opposite is observed in vivo, where EPO evidently promotes the progression of breast cancer. In light of these results, researchers hypothesized that EPO's tumor promoting effects are seen in vivo but not with in vitro assays, since they affect a limited fraction of cells, such as breast tumor initiating cells. Thus, the effects might only be seen upon longer periods of EPO administration, such as those achieved in vivo [57]. Moreover, it was observed that the treatment of breast tumor-initiating cells (TICs) with EPO activated JAK/STAT signaling as well as promoted their self-renewal. The protumorigenic role of endogenous EPO in breast tumorigenesis was also confirmed. It was also demonstrated that breast cancer cells as well as tumor-associated endothelial cells are capable of synthesizing EPO and releasing it into the tumor microenvironment. Endogenous EPO expression was hypoxia-inducible in breast cancer cell lines, but not in human mammary epithelial cells. Overexpression of EPO in breast cancer cells is negatively correlated with progression-free survival [57].

A recent study by Marrott *et al.* [58] revealed that the JAK2/STAT3 pathway was crucial for the growth of basal-like, stem-like breast cancer cells. Interleukin 6 (IL-6) was proposed as the main mediator within the JAK/STAT pathways on normoxemic conditions [58]. The study by Zhou *et al.* extended these observations via a functional analysis of the effects of JAK/STAT signaling in a population of breast tumor initiating cells (the $CD44^{+}CD24^{-}EpCAM^{+}$ fraction) [56] and identified EPO as a hypoxia-induced activator of JAK/STAT signaling pathway and cancer cell stemness.

Studies conducted in recent years suggest that EPO plays yet another, clinically important role. Observations allow to state that EPO antagonizes treatment with the anti-HER2 antibody trastuzumab by activating EpoR/JAK2 downstream effectors, effectively bypassing HER2 signaling [60]. Furthermore, the effect of antagonism should be observed only in patients treated with anti-HER2 antibodies. The fact that adverse effects of rhEPO were observed in numerous different subtypes of breast cancer, as well as in patients with cancer-related anemia not treated with trastuzumab, strongly suggests the presence of other, non-HER2-associated pathways for EPO promotion of tumor progression.

These observations are indicative of the important role of both endogenous and exogenous EPO in the natural history of breast cancer. Results obtained by Liang *et al.* show that rather than EPO/EpoR inhibition, inhibition of JAK2 combined with chemotherapy results to be a promising therapeutic strategy in the treatment of breast cancer patients [45].

## Conclusion

EPO plays a number of important functions in the body. Contrary to original beliefs, its activity is not limited to exerting effects on cells along the erythropoietic pathway. Newly published results continue to provide information on novel functions of the protein in other types of tissues, as well as on the important roles played by EPO in pathological processes. With no doubt, EPO has a significant impact on the biology of breast cancer cells by affecting cells' proliferation, apoptosis, resistance to chemotherapy, as well as expression of various types of receptors. In recent years, the role of endogenous EPO in regulation of carcinogenesis was also noted. Exogenous EPO, in the form of rhEPO, had been introduced with the best intention to treat patients with cancer-related anemia in the course of breast cancer. While it decreases the transfusion requirements and improves the quality of life of cancer patients, randomized trials have demonstrated that rhEPO administration is associated with shorter progression-free and overall survival.

Although increasing amount of information is available regarding the role of EPO and EpoR in breast cancer, elucidation of the activity and involvement of these proteins in complex processes occurring within the cancer cells requires extensive research. Every set of results being published answer some of the questions while raising new ones instead.

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