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Original article

Effect of fetal bovine serum on erythropoietin receptor expression and viability of breast cancer cells



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ABSTRACT

Erythropoietin receptors (EPORs) are present not only in erythrocyte precursors but also in non-hematopoietic cells including cancer cells. In this study, we determined the effect of fetal bovine serum (FBS) in culture medium on the EPOR expression and viability of the estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. Using flow cytometry, we showed that the inclusion of 10% FBS in the medium increased the EPOR expressions and viabilities of MDA-MB-231 and MCF-7 cells. The MDA-MB-231 showed greater EPOR expression than MCF-7 cells, suggesting that the presence of ERs on cells is associated with poor expression of EPOR. Culture medium containing 10% FBS also caused increased number of breast cancer cells entering the synthesis phase of the cell cycle. The study also showed that rHuEPO treatment did not affect viability of breast cancer cells. In conclusion, it was shown that the inclusion of FBS in culture medium increased expression of EPOR in breast cancer cells and rHuEPO treatment had no effect on the proliferation of these cancer cells.

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1. Introduction

Erythropoietin (EPO), a heavily glycosylated glycoprotein, plays an essential role as a hematopoietic growth factor in erythro-

Abbreviations: EPOR, erythropoietin receptors; ER, estrogen receptor; FBS, fetal bovine serum; rHuEPO, recombinant human erythropoietin; CFU-e, colony forming units-erythroid; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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poiesis. Recombinant human erythropoietin (rHuEPO) for the treatment of anemia of chronic renal failure first became commercially available in 1986 (Winearls et al., 1986; Eschbach et al., 1989). Since then, the clinical application of rHuEPO broadened significantly and now the drug is also used to treat cancer-associated anemias (Aapro et al., 2019).

The expression of EPO receptors (EPORs) are not restricted to erythrocyte precursors. In fact, various non-hematopoietic organs and cells, including breast cancer cells, also express EPORs (Li et al., 2014; Dulmovits et al., 2017; Chan et al., 2017). EPOR expression in cancer cells was associated with enhanced proliferation and survival of cancer cells, stimulation of angiogenesis in the tumor tissue, and tumor promotion (Kimáková et al., 2017), which suggested that rHuEPO treatment may be contraindicated in cancer patients. This was particularly evident by the results from preclinical and clinical trials, where rHuEPO treatments produced some adverse effects that raised concerns on the safety of rHuEPO for the treatment of anemia of malignancies (Henke et al., 2006;

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Hedley et al., 2011; Tonia et al., 2012; Arantes et al., 2018). However, these concerns remain questionable, since there are many unequivocal evidences that suggested EPO treatment is beneficial to cancer patients and do not produce tumor-promoting effects. In fact, the EPORs are useful targets for EPO-conjugated drugs and drug carrier systems for the treatment of cancers (Beh et al., 2017).

The effect of rHuEPO on cells is dependent on EPO/EPOR signaling, which in erythroid progenitors was shown to result in proliferation (Jelkmann, 2004). Thus, the optimum effect of rHuEPO is expected to occur in cells with high EPOR expressions.

Currently, it is not certain how culture medium affects expression of EPOR in breast cancer cells. It is also not known how the presence of serum in the medium influence expression of EPOR in breast cancer cells. One of the reasons for the dearth of information on the behavior of EPOR-positive cancer cells in culture is the lack of validated techniques for the identification of EPOR, primarily because of the unavailability of specific and sensitive anti-EPOR antibodies for detection of receptors and the poor expression of EPOR in some cells (Elliott et al., 2014; Zaha, 2014; Abd-Elkareem, 2017). EPOR expression is usually detected via immunohistochemistry, Western blotting, binding assays with labeled-receptor ligand, or flow cytometry using monoclonal antibodies (Sinclair et al., 2010; Elliott and Sinclair, 2012; Debeljak et al., 2014).

It has been suggested that the best method of determining EPOR expression is with the use of serum-starved cell cultures (Jia et al., 2009). In this study, we determined the effect of including fetal bovine serum (FBS) in growth medium on the expression of EPOR on the MCF-7 and MDA-MB-231 breast cancer cells. We also determined the effect of rHuEPO on the proliferation and survival of rHuEPO-treated MCF-7 and MDA-MB-231 cell lines.

2. Materials and methods

2.1. Human breast cancer cell lines

MCF-7 and MDA-MB-231 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cell lines were cultured in RPMI-1640 medium (RPMI; GibcoBRL) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 units/mL penicillin and 100 µg/mL streptomycin (GibcoBRL) in a humidified atmosphere of 5% CO₂, and 95% air at 37 °C. 24 h prior to analysis, the cells were grown in RPMI-1640 medium either with or without FBS.

2.1.1. Cell treatment

10 mL of MCF-7 and MDA-MB-231 breast cancer cell lines with the cell viability of greater than 95% were seeded in a T-25 cell culture flask (Nunc, Denmark) at the concentration of 1×10^5 cells/mL. After 24 h, the RPMI-1640 growth medium was discarded and replaced with 10 mL fresh medium, free of serum or containing 10% FBS. Cells in FBS only, served as the serum control. The cells were incubated for a further 24 h before subjecting to flow cytometry and real-time PCR analyses.

2.2. Detection of human erythropoietin receptor via flow cytometry

Treated MCF-7 and MDA-MB-231 cells were detached with 0.05% trypsin and resuspended in 10 mL of RPMI-1640 medium, free of serum or containing 10% of FBS. Then, the cells were pelleted via centrifugation at $300 \times g$ and 4 °C for 10 min before resuspending in 5 mL isotonic phosphate buffered saline (PBS). The cells were then counted using a haemocytometer and cell concentration adjusted to 4×10^6 cells/mL with PBS before incubating for 6 h on a rocker platform. The cells were stained with carboxyfluorescence-

conjugated mouse monoclonal anti-human erythropoietin receptor antibody (R & D Systems) following the manufacturer recommendation and as described by LaMontagne et al., (2006). Approximately 2×10^5 cells were stained with 1 µg of the monoclonal antibody for 45 min on ice. Finally, the cells were resuspend in 1 mL PBS, excited with argon laser at wavelength 488 nm and analyzed via flow cytometry (Coulter Epics, Altra Flow Cytometer, Beckman Counter).

2.3. Detection and quantification of EPOR expression via real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA from control and treatment groups were isolated using the Master Pure™ RNA Purification Kit (Epicentre®, Madison, Wisconsin) and first strand cDNAs were synthesized using Maxime RT PreMix Kit (iNtRON BIOTECHNOLOGY, Korea) based on the recommended protocol. 100 ng of cDNAs generated were then used for PCR amplification in a 12.5 µL PCR reaction. Power SYBR® Green PCR Master Mix containing AmpliTaq Gold® polymerase (Applied Biosystems). The amplification profile was as follows: AmpliTaq Gold® polymerase activation, 95 °C for 10 min, 50 cycles of PCR at 95 °C for 15 s, and 60 °C for 1 min, final extension at 72 °C for 10 min. The PCR products were visualized in 2% agarose gel stained with ethidium bromide. Hypoxanthine phosphoribosyltransferase (HPRT) was used as the housekeeping internal control gene. The forward and reverse primers are listed in Table 1.

2.4. Cell viability assay

Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. 100 µL of suspension containing 1×10^4 of either MCF-7 or MDA-MB-231 cells in serum-free growth medium were seeded in each well of a 96-wells plate and incubated for 24 h in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. The cells were treated for 72 h with 0, 5, 10 or 20% FBS and 10 IU/mL rHuEPO (Eprex, Cilag). Nontreated cells with normal viability and growth served as the positive controls. After 72 h of treatment, 20 µL of 5 mg/mL MTT stock solution (Sigma) were added to each well and the plate incubated in dark for an additional 3 h at 37 °C. The supernatant was then removed and 100 µL dimethylsulphoxide (DMSO, Ajax Chemicals) added to each well. The plate was then further incubated for 1 h at 37 °C. The absorbance was determined in a microplate spectrophotometer (µQuant Universal Microplate Spectrophotometer, BIO-TEK Instruments, Inc.) at 570 nm wavelength. All measurements were performed in quadruplicates. The results were expressed as the percentage cell viability of positive control.

3. Results

3.1. EPOR expression in breast cancer cells

Extracellular EPOR was expressed in both MCF-7 and MDA-MB-231 cell lines (Fig. 1). Regardless of the FBS concentration, more

Table 1
Primers for RT-PCR and qRT-PCR.

Gene	Accession number	Sequence
EPOR	NM_000121	Forward: CCT GAC GCT CTC CCT CAT CC Reverse: GCC TTC AAA CTC GCT CTC TGG
HPRT	NM_000194	Forward: TTA TCA GAC TGA AGA GCT ACT Reverse: TTA CCA GTG TCA ATT ATA TCT TCA ACA ATC

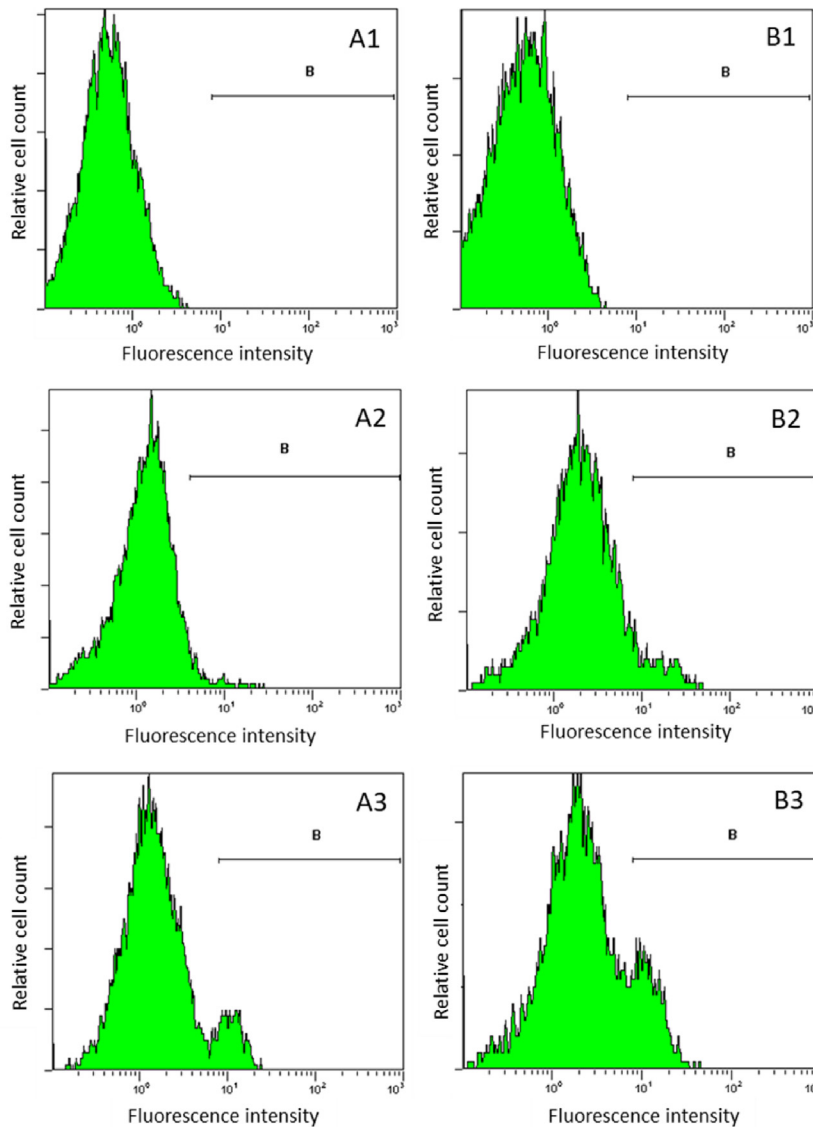


Fig. 1. EPOR expression in MCF-7 and MDA-MB-231 cells determined by flow cytometry. (A1) MCF-7 cells (serum control); (A2) MCF-7 (serum-free medium); (A3) MCF-7 cells (serum-free medium); (B1) MDA-MB-231 cells (serum control); (B2) MDA-MB-231 cells (serum-free medium); (B3) MDA-MB-231 (10% FBS medium). Greater number of MDA-MB-231 cells expressed extracellular EPOR than MCF-7 cells. The EPOR expression level in MDA-MB-231 was enhanced when grown in 10% FBS-containing medium. However, the medium FBS content did not affect expression of EPOR in MCF-7 cells. Cells within gate B are EPOR-positive.

MDA-MB-231 cells expressed EPOR than MCF-7 cells (Table 2). It was in the presence instead of absence of FBS that the percentage of MDA-MB-231 cells expressing EPOR increased markedly. However, the percentage of MCF-7 cells expressing EPOR did vary significantly between those incubated in serum-free and FBS-containing culture medium. It is interesting to note that the expression level of EPOR protein in the breast cancer cells (Fig. 2)

Table 2
EPOR expression in cultured breast cancer cell lines.

Cell Line	Culture Condition	EPOR Expression (% cells)
MCF-7	Serum-free	10.76 ± 0.03
	10% serum	10.82 ± 1.14
MDA-MB-231	Serum-free	16.78 ± 0.11
	10% serum	29.45 ± 3.74

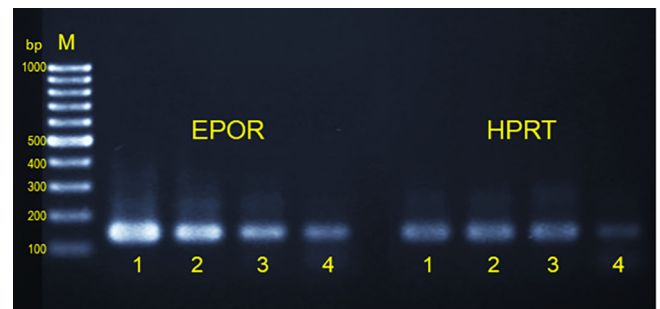


Fig. 2. EPOR expression in MCF-7 and MDA-MB-231 cells cultured in the presence and absence of FBS. M: 100 bp DNA ladder (GeneRuler™, Fermentas), 1: MCF-7 cell (10% FBS medium), 2: MCF-7 cell (serum-free medium), 3: MDA-MB-231 cell (10% FBS medium), 4: MDA-MB-231 cell (serum-free medium). HPRT = Hypoxanthine phosphoribosyltransferase.

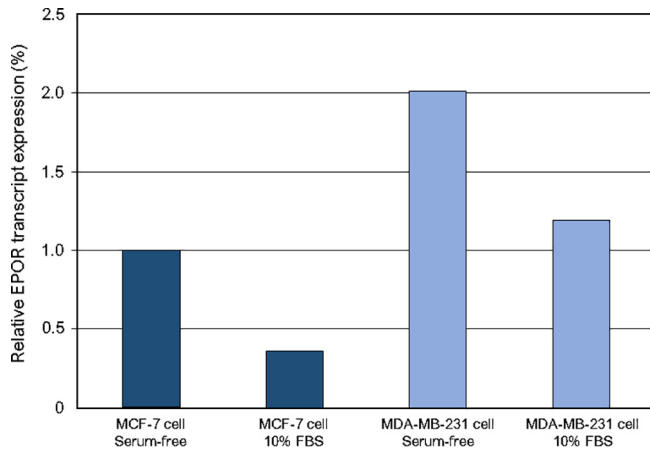


Fig. 3. EPOR transcript expression in MCF-7 and MDA-MB-231 cells cultured in the absence and presence (10%) of FBS in medium at 24 h normalized with HPRT transcript.

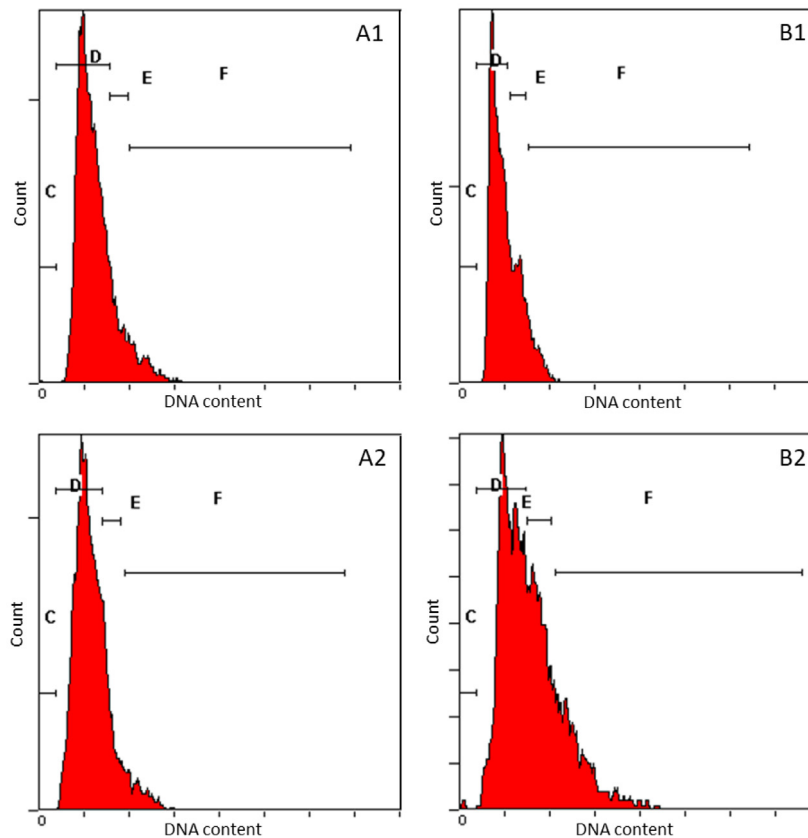
did not correlate with the expression level of EPOR transcript (Fig. 3).

3.2. Effect of serum on breast cancer cell cycle

Absence of serum in culture after 24 h incubation did not affect the survival of MCF-7 or MDA-MB-231 cells. Based on the cell cycle analysis, there were higher numbers of MDA-MB-231 and MCF-7 cells in the synthesis phase in the presence than absence of serum in the culture medium (Fig. 4).

3.3. Effect of serum concentration on viability of breast cancer cells treated with rHuEPO

Supplementing the culture medium with FBS marked increased the number of viable MCF-7 and MDA-MB-231 cells. There seemed to be no difference in viability between cells incubated in medium with various FBS concentrations. The viability of breast cancer cells treated with rHuEPO did not differ significantly from those non-treated (Fig. 5).



Cell cycle phase	Cell population (%)			
	MCF-7 cell		MDA-MB-231 cell	
	Serum-free	10% FBS	Serum-free	10% FBS
Apoptosis (C)	0.23 ± 0.19	0.11 ± 0.032	0.19 ± 0.11	0.63 ± 0.11
G ₀ /G ₁ phase (D)	68.06 ± 16.63	70.97 ± 6.99	42.65 ± 22.58	45.77 ± 2.96
Synthesis phase (E)	16.14 ± 6.08	18.09 ± 5.05	17.72 ± 3.23	23.73 ± 0.61
G ₂ /M phase (F)	14.28 ± 8.88	11.11 ± 7.65	35.83 ± 25.37	25.99 ± 2.89

Fig. 4. Effect of fetal bovine serum (FBS) on the cell cycle stage of MCF-7 and MDA-MB-231 cells. (A1) MCF-7 (10% FBS medium); (A2) MCF-7 (serum-free medium); (B1) MDA-MB-231 (10% FBS medium); (B2) MDA-MB-231 (serum-free medium). In medium with 10% FBS, MDA-MB-231 cells showed marked greater number of cells in the synthesis phase.

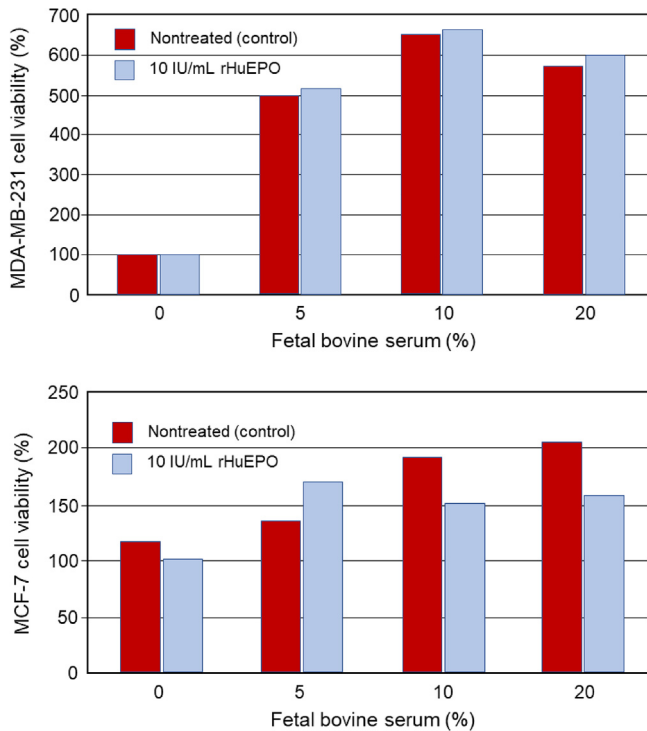


Fig. 5. Effect of fetal bovine serum concentration on viability of breast cancer cells treated with 10 IU/mL rHuEPO. Fetal bovine serum increased viability of both the MCF-7 and MDA-MB-231 cells, with the later showing more marked responses. rHuEPO treatment did not significantly affect the viability of breast cancer cell lines.

4. Discussion

The method of determining of EPOR expression in cancer cells has been a debatable issue, primarily because of the questionable specificity of EPOR antibody and the uncertainty of the effect of culture serum on EPOR expression. The specificity and applicability of the anti-EPOR antibody, particularly the commercial C-20 that is widely used in various preclinical and clinical breast cancer studies is still contentious (Elliott et al., 2010; Sinclair et al., 2010; Elliott and Sinclair, 2012, Patterson et al., 2015). The C-20 antibody also seemed to cross-react with heat shock protein 70 (HSP70) present in various carcinomas including breast carcinoma cells (Elliott et al., 2006; Brown et al., 2007). Like EPOR, HSP70 is up-regulated by hypoxia (Kim et al., 2006; Xia et al., 2009). However, at this juncture, the role of HSP70 protein in cancer development and progression is yet to be fully elucidated (Murphy, 2013, Kasioumi et al., 2019). In our study, we used carboxyfluorescein-conjugated mouse monoclonal anti-human EPOR antibody (Elliott et al., 2014), designed to specifically and quantitatively determine the level of cellular EPOR expression via flow cytometry. The same antibody was used by LaMontagne et al., (2006) and Maxwell et al., (2015) in their studies. We showed that there is significantly more estrogen receptor (ER)-negative MDA-MB-23 expressing EPOR than the ER-positive MCF-7 cells (Gewirtz et al., 2006; Berger et al., 2013). This observation is similar to that reported by others (Albiges et al., 2014; Xu et al., 2014) in breast biopsy specimens stained immunohistochemically using the C-20 antibody. It is most likely that the expression of EPOR in breast cancer cells is inversely related to the level of ER expression. It was suggested these receptors cross-talk during the activation of cellular signaling pathways and to avoid drug-response inhibition (Trošt et al., 2013).

The presence of serum in the culture medium up-regulated the expression level of EPOR expression in both the MCF-7 and MDA-MB-231 cell lines. Since our study also showed that the number

of viable breast cancers increased markedly in the presence of serum in the culture medium, it is possible that the increase in EPOR expression is not serum-dependent but instead it is due to the effect of increased number of viable cells expressing EPOR.

The expression of EPOR may depend on the stage of the cancer cell cycle. In culture medium containing 10% FBS, the MDA-MB-231 cells showed high EPOR expression. The number of cells in the synthesis and to a lesser extent the G₀/G₁ phase also increased. Thus, the inclusion of FBS in culture medium did not only increase level of EPOR expression in breast cancer cells but also drove these cells to enter the synthesis phase. There may be a positive relationship between EPOR expression and the number of breast cancer cells in the synthesis cell cycle phase. This phenomenon is more evident in the MDA-MB-231 than the MCF-7 cells.

The effect of serum and rHuEPO treatment on the viability of MCF-7 and MDA-MB-231 cells in culture was determined via the MTT assay. The viability of MCF-7 and MDA-MB-231 cells grown in culture containing 10% FBS increased significantly. rHuEPO, commonly used for the treatment of various types of anemia including anemia of malignancies, was believed to enhance survival and proliferation of cancer cells through the EPO-EPOR axis (Chan et al., 2017). However, we showed otherwise, that is, although the presence of FBS in culture increased viability of MDA-MB-231 and MCF-7 cells, concurrent rHuEPO treatment did not exacerbate the effect. There is no significant difference in viability between nontreated and rHuEPO-treated breast cancer cells. We postulate in this case that serum in medium is the main factor responsible for the increased survivability and proliferation of the MDA-MB-231 and MCF-7 cells in culture.

At this juncture, it is not clear why the expression of EPOR protein did not correlate with level of EPOR transcript expression in breast cancer cells cultured in serum-containing medium. Our study did not address the effect of FBS on EPOR protein transcription. However, serum albumin as a universal carrier molecule, facilitates transmembrane transportation of its load through interactions with cell surfaces. Thus, it is possible that during these interactions, albumin could have increased EPOR expression by modifying the tertiary structure of EPOR protein and increasing its expression. However, this proposed mechanism warrants further studies.

In conclusion, the study showed no clear evidence that rHuEPO treatment increases proliferation in rHuEPO-treated breast cancer cells, although the level of EPOR expression in these cells was high. It is possible that rHuEPO, irrespective of EPOR expression, has no effect on breast cancer cell proliferation.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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