

## Erythropoietin Enhances Migration of Human Neuroblastoma Cells: In vitro Studies and Potential Therapeutic Implications

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**Abstract:** The erythropoietin receptor (EpoR) is expressed by cells from the erythroid lineage; however, evidence has accumulated that it is also expressed by some other non-hematopoietic tissues including several solid tumor cells and proposed candidates for cancer stem cells. This is an important concern, because recombinant erythropoietin (EPO) is frequently employed in cancer patients as a drug to ameliorate anemia related to chemo/radiotherapy. In our studies, we employed three human neuroblastoma (NB) cell lines and found in all of them the expression of EpoR and EPO mRNA. The functionality of EpoR in RMS cell lines was evaluated by chemotaxis, adhesion, and direct cell proliferation assays. We noticed that all three human NB cell lines responded to EPO stimulation by enhanced chemotaxis and cell adhesion. However, at the same time we did not observe any significant effect of EPO on proliferation. Based on this EPO supplementation in NB patients employed because of radio/chemotherapy induced anemias may have an unwanted side effect on tumor metastasis.

**Keywords:** Neuroblastoma, Erythropoietin (EPO), Erythropoietin receptor (EpoR), Metastasis.

### INTRODUCTION

Neuroblastoma (NB) is a type of pediatric sarcoma that most frequently starts from one of the adrenal glands, but can also develop in several other locations including abdomen, chest, neck and spinal cord [1]. It was first described by Rudolf Virchow as "abdominal glioma". NB develops from certain types of very primitive cells in the embryo and it is the third most common childhood cancer after leukemia and brain cancer [2] and it represents 7% of the total cases in group of age 0–14 [3]. Moreover, when taking into consideration the first year of life, it is the most common cancer [3, 4].

It belongs to a family of so-called "small round blue tumor cells" that comprise its histological mimics, such as rhabdomyosarcoma, nephroblastoma (Wilms tumor) and Ewing's sarcoma [5]. The great majority of NB cases are sporadic and non-familial, however 1–2% of cases have been linked to specific gene mutations in the ALK gene [6] and germline mutations in the PHOX2B [7, 8]. NB may be also a part of neurofibromatosis type 1 as a result of the loss

of imprinting at Igf2-H19 locus part of Beckwith-Wiedemann Syndrome [9, 10]. Children with neuroblastoma are most commonly treated with surgery, chemotherapy and/or radiation therapy and those with high-risk disease may receive high-dose chemotherapy followed by autologous hematopoietic stem cell transplantation [11]. These latter patients have also the greatest risk of treatment-related complications - including severe hearing loss, infertility, cardiac toxicity, and secondary cancer.

Erythropoietin (EPO) is synthesized mainly by interstitial cells in the renal cortex with additional amounts being produced in the liver, and the vascular pericytes as well as in the brain tissue [12–15]. However, EPO was for a long time considered a "classical hematopoietic" cytokine that could stimulate erythropoiesis, and the erythropoietin receptor (EpoR) has been found to be expressed by cells from the erythroid lineage [16], recently additional evidence has accumulated that EpoR is also found in a number of other tissues including heart [17], skeletal muscles [18], kidney [19] and neural tissues [20]. EpoR is also expressed by several solid tumors [21–30] including rhabdomyosarcoma [31], Ewing sarcoma [32], pediatric brain tumors [33] (medulloblastoma, astrocytoma, and ependymoma), Wilms tumor [34], hepatoblastoma [35], and it has been detected in NB cells [36, 37].

EPO has been also reported to stimulate angiogenesis in NB [37, 38]; however, it does not enhance the proliferation of tumor cells [39, 40], it inhibits apoptosis in response to

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TNF- $\alpha$  [41, 42] and as recently reported in response to some cytostatic drugs such as etoposide and vincristine [31, 43]. On other hand EPO is available for use as a therapeutic agent produced by recombinant DNA technology in treating chemotherapy induced anemia in patients with cancer including those suffering from NB.

What is also important EpoR has been proposed to be expressed by some putative cancer stem cells by promoting their stemness in EPO-dependent autocrine/paracrine manner [44] and preventing breast cancer stem cell candidates from chemotherapy induced apoptosis [45]. Since one of the challenging problems of radio/chemotherapy is recurrence and metastasis of cancer stem cells that survive classical treatment protocols, therefore focused in this study on a role of EPO in promoting motility of cancer cells with the prospective of targeting cancer stem cells.

In the present study, we report expression of EPO and EpoR mRNA in all three tested human NB cell lines. Importantly, EpoR was functional in all our cell lines tested in response to stimulation by erythropoietin (EPO) increased their chemotaxis and adhesion. The presence of functional EpoR in NB and its effect on cell migration cells provides evidence that EPO supplementation may have an unwanted side effect of facilitating tumor motility and metastasis in NB patients.

## MATERIAL AND METHODS

### Cell Lines

We used three human NB cell lines purchased from ATCC®: CRL22271 (SK-N-BE.2), CRL2266 (SH-SY5Y) and HTB11(SK-N-SH). CRL2266 and CRL2271 cells were cultured in 1:1 mixture of ATCC-formulated Eagle's Minimum Essential Medium (EMEM) and F12 Medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 10  $\mu$ g/ml streptomycin. HTB11 cells were cultured in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 10  $\mu$ g/ml streptomycin. All cells were placed in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C and the medium was changed every 72 hours.

### Conventional RT-PCR

Total RNA from various cells was isolated using the RNeasy Mini kit (Qiagen Inc.), including treatment with DNase I (Qiagen Inc.). The mRNA (200 ng) was reverse-transcribed with Taqman Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's instructions. The resulting cDNA fragments were amplified (1 cycle of 8 min at 95°C, 2 cycles of 2 min at 95°C, 1 min at 60°C, 1 min at 72°C, and subsequently 35 cycles of 30 s at 95°C, 1 min at 60°C, 1 min at 72°C, and 1 cycle of 10 min at 72°C). All primers were designed using the NCBI/Primer-Blast program, as at least one primer included an exon–intron boundary. ( *$\beta$ -actin*: F-GGA TGC AGA AGG AGA TCA CTG, R-CGA TCC ACA CGG AGT ACT TG; hEpoR: F-CTC CCT TTG TCT CCT GCT CG,

R-TAG GCA GCG AAC ACC AGA AG; *hEPO*: F-TCA TCT GTG ACA GCC GAG TC, R-GCC ACT GAC GGC TTT ATC CA).

### Cell Proliferation

Cells were grown in 24-well culture plates at an initial density of  $7.5 \times 10^3$  cells/well. After 24 h, the medium was changed to new medium with 0.5% BSA and supplemented with or without EPO (0.5 and 20 iU/ml). Medium with 0.5% BSA was used as a negative control. The cell number was calculated at 24h (1 day) and 120 h (5 days) after the change of medium. At the indicated time points, cells were harvested from the culture plates by trypsinization.

### Chemotaxis Assay

Chemotaxis assays were performed in a modified Boyden's chamber with 8- $\mu$ m-pore polycarbonate membrane inserts (Costar Transwell; Corning Costar, Lowell, MA, USA) as described previously [46]. In brief, cells detached with 0.25% trypsin were seeded into the upper chamber of an insert at a density of  $4.5 \times 10^4$  in 120  $\mu$ l. The lower chamber was filled with pre-warmed culture medium containing 0.5% BSA and EPO (0.5, 5 and 20 iU/ml). Medium supplemented with 0.5% BSA was used as a negative control. After 24 hours, the inserts were removed from the Transwell supports. The cells that had not migrated were scraped off with cotton wool from the upper membrane, and the cells that had transmigrated to the lower side of the membrane were fixed and stained with HEMA 3 (protocol, Fisher Scientific, Pittsburgh, PA) and counted on the lower side of the membrane using an inverted microscope.

### Adhesion Assay to Fibronectin

Cells were made quiescent for 3 hours with 0.5% BSA in EMEM or EMEM/F12 (1:1) before incubation with EPO (0.5, 5 and 20 iU/ml). Subsequently, cell suspensions ( $2 \times 10^3$ /100  $\mu$ L) were added directly to 96-well plates covered with fibronectin and incubated for 5 min at 37°C. The wells were coated with fibronectin (10  $\mu$ g/ml) overnight at 4°C and blocked with 0.5% BSA for 1 hour before the experiment. Following incubation, the plates were vigorously washed three times to remove non-adherent cells, and the adherent cells were counted using an inverted microscope.

### Phosphorylation of Intracellular Pathway Proteins

The HTB11 neuroblastoma cell line were incubated overnight in EMEM medium containing low levels of BSA (0.5%) to render the cells quiescent. After the cells were stimulated with EPO (0.5 or 20 IU/ml), or the medium level with 10% FBS as a positive control at 37°C for 5 min, the cells were lysed for 10 min on ice in RIPA lysis buffer containing protease and phosphatase inhibitors (Santa Cruz Biotechnology). The extracted proteins were separated on a 4–12% SDS-PAGE gel and transferred to a PVDF membrane. Phosphorylation of the serine/threonine

kinase AKT (yielding phospho-AKT<sup>473</sup>) and p44/42 mitogen-activated kinase (yielding phospho-MAPK<sup>44/42</sup>) was detected by phosphospecific p44/42 MAPK mouse and AKT rabbit polyclonal antibodies (Cell Signaling Technology, Danvers, MA, USA) with HRP-conjugated goat anti-mouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology). Equal loading in the lanes was evaluated by stripping the blots and reprobing with anti-p42/44 MAPK monoclonal antibody (Cell Signaling Technology) and anti-AKT polyclonal antibody (Cell Signaling Technology). The membranes were developed with an enhanced chemiluminescence (ECL) reagent (Amersham Life Science, Arlington Heights, IL, USA), dried, and subsequently exposed to film (Hyperfilm; Amersham Life Science).

### Statistical Analysis

All results were presented as mean  $\pm$  SD. Statistical analysis of the data was done using Student's t test for unpaired samples, with  $p \leq 0.05$  considered significant.

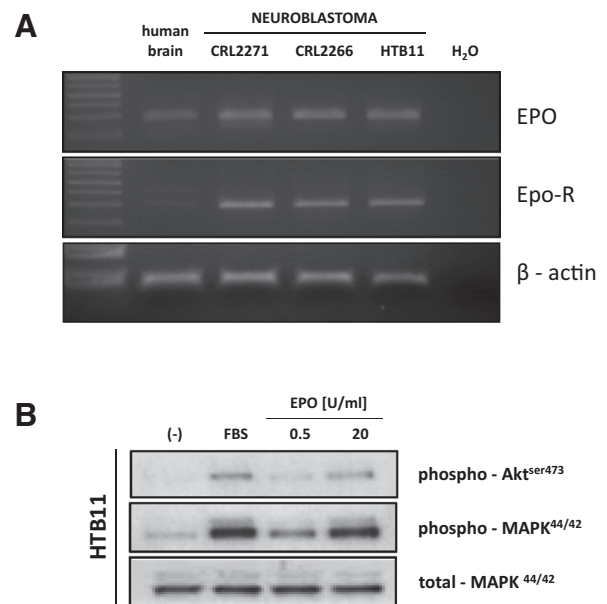
## RESULTS

### Human NB Cell Lines Express mRNA for EpoR and EPO and EpoR Is Functional on NB Cells

First, we performed RT-PCR studies to evaluate mRNA expression in human NB cell lines. Figure 1A shows that we were able to detect EpoR mRNA by RT-PCR in all cell lines employed in this study. Furthermore, our RT-PCR analysis revealed expression of endogenous EPO in all studied NB cell lines. To address if EpoR is functional on NB cells we stimulated these cells by EPO and noticed dose-dependent increase in phosphorylation of AKT and MAPKp42/44 (Figure 1B).

### EpoR Induces Motility and Adhesion of Cells in Human NB Cell Lines

Next, to evaluate whether EpoR may induce cell migration and the adhesion of human NB cell lines, we performed chemotaxis and cell adhesion studies. Thus, by employing Transwell chemotaxis assays (Figure 2) we used as the chemoattractant EPO at doses of 0.5, 5 and 20 IU/ml. We observed that EPO induced motility of all NB cell lines



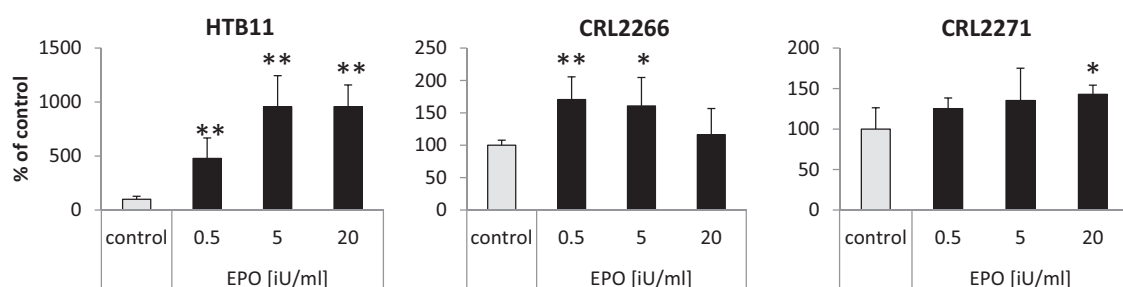
**Figure 1. Panel A - mRNAs for EpoR and EpoR are expressed in human neuroblastoma (NB) cell lines.** Expression of EpoR and EPO at the mRNA level by NB cells was assessed by RT-PCR. Representative results are shown. Panel B - EPO activates phosphorylation of phospho-Akt<sup>ser473</sup> and phospho-MAPK<sup>44/42</sup> signaling in HTB11 cells. Cells were non-stimulated (negative control) or stimulated for 5 min with FBS (positive control) or stimulated for 5min with EPO (0.5 or 20 IU/ml). Representative results are shown.

evaluated in our studies. The highest responsiveness was observed for HTB11 cells.

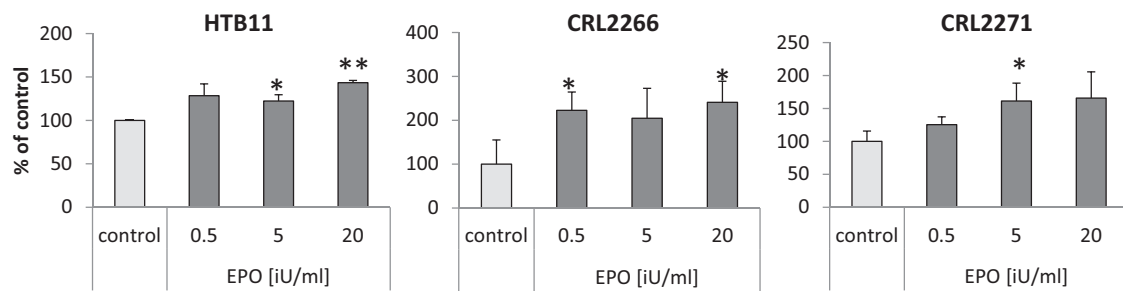
Another important feature of metastasizing cancer cells is their adhesion at the site of metastasis. Therefore, as next we studied the effect of EPO on adhesion of NB cell lines to fibronectin. Depending on the dose employed, we found that EPO may induce adhesion of NB cells to fibronectin (Figure 3), and this effect was particularly visible for CRL2266 cells.

### EPO Does Not Induce Significant Proliferation of NB Cells

EPO is a known growth factor for erythroid cells [16] as well as for some tumor cell lines [21–31]. Therefore, based on receptor expression studies (Figure 1), we exposed NB



**Figure 2. Erythropoietin (EPO) enhances migration of human NB cell lines.** Effect of EPO (0.5, 5 or 20 IU/ml) on the chemoattraction of NB cells across Transwell membranes covered with gelatin. Combined data from three independent experiments are shown. \* $p \leq 0.05$ , \*\* $p \leq 0.001$ .



**Figure 3. Erythropoietin (EPO) enhances adhesion of human NB cell lines.** Effect of EPO (0.5, 5 or 20 IU/ml) on adhesion of RMS cells to fibronectin. Combined data from three independent experiments are shown. \* $p \leq 0.05$ , \*\* $p \leq 0.001$ .

cells to 0.5 and 20 IU/ml EPO and evaluated its effect on the proliferation of these cells. We found that EPO only slightly stimulated the proliferation of CRL2266 cells, however this increase was not significant.

## DISCUSSION

The salient observation of our work is that human NB cell lines express mRNA for EPO and EpoR and that this receptor is functional and induces pro-migratory behavior of NB cells. Therefore EPO is a previously unrecognized pro-metastatic factor for this tumor, which indicates a potential risk in treating NB patients with human recombinant EPO because of cancer-related anemia.

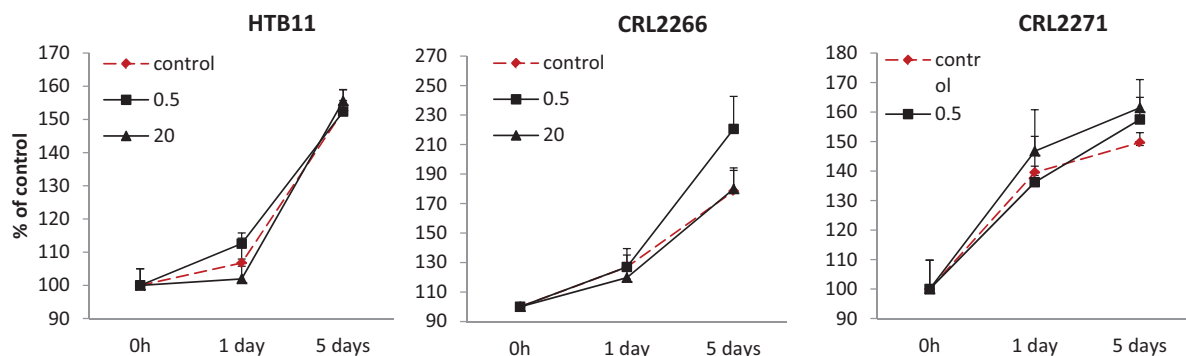
It is well known that systemic anemia related to radio/chemotherapy or tumor-induced cachexia is an independent prognostic factor for poor survival in cancer patients [47–49]. Therefore, it has been proposed that recombinant human EPO be used in clinical settings to treat this complication [48–50]. Unfortunately, such treatment has often resulted in rapid tumor progression and reduced patient survival [47–49]. To support this, EpoR has been identified as a negative prognostic factor in gastric, renal, breast, and head and neck cancer and in melanoma [21–28].

The EpoR has been already reported to be expressed on human NB cells [36–40], however since it did not promote proliferation of these cells [39, 40] supplementation with

EPO has been proposed to be safe. Nevertheless, as recently demonstrated EPO-EpoR axis may exert several pleiotropic effects on NB grow such as promoting angiogenesis [38] and making NB cells more resistant to some cytostatic [43]. Moreover, EPO–EpoR axis has also been reported to be involved in the autocrine progression of NB [39], and our data are in agreement with this observation, as NB cell lines expressed both EPO and EpoR. EpoR has been also reported to be upregulated on NB cells in response to hypoxia [53].

The recurrence of tumor growth after a successful initial treatment and the fatal tendency of cancerous cells to migrate and to spread to different vital organs are major problems affecting the survival of cancer patients. The metastasis of NB cells, however, is directed by several other factors, including chemokines (e.g., stromal-derived factor 1 alpha) and growth factors (e.g., hepatocyte growth factor). Herein, for the first time, we identified EPO as cytokine that promotes migration and adhesion of human NB cells that may direct their spread to distant locations.

We recently demonstrated the presence of functional EpoR in human and murine germline-derived cell lines, including teratocarcinomas and ovarian cancer cells [54]. Interestingly, 150-years ago, Rudolf Virchow [55] and Julius Conheim [56] proposed the so-called "embryonic rest hypothesis of cancer development", in which malignancies may develop from dormant embryonic or germ cells residing in adult tissues. Small round blue cell



**Figure 4. Effect of EPO on proliferation of human NB cells.** Effect of EPO (0.5 and 20 IU/ml) on proliferation of the HTB11, CRL2266 and CRL2271 cell lines. Data from three independent experiments are combined.  $P < 0.05$ .

tumors, including NB, are candidates for such malignancies. However, this tempting 150 years old hypothesis requires further experimental support to identify cells that give origin to those tumors. Potential candidate cells in which small round blue cell tumors develop are very small embryonic like stem cells (VSELs) [57]. Thus, it would be important to explore potential possibility that mutated or transformed VSELs could give rise to NB stem cells. In fact we already reported that VSELs express EpoR [31]. We also found functional EpoR to be highly expressed by human rhabdomyosarcoma cells that is another tumor type belonging to the family of small round blue cell tumors [31]. This indicates a possibility that several pediatric sarcomas may originate in mutated VSELs [58]. Moreover, since one of the challenging problems of radio/chemotherapy is recurrence and metastasis of cancer cells that survive classical treatment protocols, treatment with EPO could endow putative cancer stem cells with enhanced migratory potential to sites of future metastases [59].

In conclusion, since recombinant human EPO is frequently employed to ameliorate chemotherapy-related anemia in cancer patients, our in vitro data has important clinical implications. The presence of functional EpoR in NB cells indicates that EPO supplementation because of therapy and tumor cachexia induced anemia may have the unwanted side effect of tumor progression and should perhaps be avoided. In addition based on the observation that EPO and EpoR is expressed in NB cells we cannot exclude the contribution of autocrine EPO-EpoR interactions in the pathogenesis of NB, and this phenomenon is currently being investigated in our laboratory.

## CONFLICTS OF INTEREST

None.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

## AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript.

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