

Recombinant human erythropoietin accelerated the proliferation of non-small cell lung cancer cell lines and reduced the expression of VEGF, HIF-1 α , and PD-L1 under a simulated hypoxic environment in vitro

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Abstract

Background: As erythropoietin (EPO) has been used to treat anemia in cancer patients, negative controversy has continued. Unfortunately, its effects on non-small-cell lung carcinoma (NSCLC) cell lines are uncertain and the phenomenon of inducing immune escape of tumor cells remains to be explored. This study aimed to provide an important basis for the application of exogenous EPO in the treatment of tumor-associated anemia.

Methods: Cells were cultured in 1% O₂, 5% CO₂, and 94% N₂ to simulate a hypoxic environment of the tumor. A549 cell line (lower expression EPOR) and NCI-H838 cell line (higher expression EPOR) were treated with 2 and 8 U/ml recombinant human EPO (rhEPO). CCK-8 method was used to determine the logarithmic growth phase of the cells and to detect cell proliferation. The expression levels of VEGF, HIF-1 α , and PD-L1 were determined by western blot. One-way ANOVA was used for statistical analysis between groups, with $p < 0.05$ indicating a significant difference.

Results: Hypoxia itself could decrease the survival rate of NSCLC cells. Under the hypoxic condition, rhEPO induced tumor cells proliferation, especially in the NCI-H838 cell line, where 2 U/ml rhEPO increased the total number of surviving cells (Hypoxia + rhEPO 2 U/ml vs. Hypoxia, $p < 0.05$). Western blot analysis showed that hypoxia upregulated the expression of VEGF, HIF-1 α , and PD-L1 in NSCLC cell lines (Normoxia vs. Hypoxia, $p < 0.05$), but may not be dependent on the expression levels of EPOR. RhEPO decreased the expression levels of VEGF and HIF-1 α . In the A549 cell line, it depended on the concentration of rhEPO and was particularly obvious in HIF-1 α (Hypoxia vs. Hypoxia + rhEPO 2 U/ml vs. Hypoxia + rhEPO 8 U/ml, $p < 0.05$). A low concentration of rhEPO may not reduce VEGF expression. In the NCI-H838 cell line, the effect of rhEPO on VEGF was more obvious, but it may be independent of rhEPO concentrations. The downregulation of PD-L1 expression by rhEPO was only presented in the A549 cell line and required higher rhEPO concentrations (Hypoxia + rhEPO 8 U/ml vs. Hypoxia + rhEPO 2 U/ml, $p < 0.05$).

Conclusion: The effect of prolonged high concentrations of rhEPO under hypoxic conditions resulted in accelerated cells proliferation of non-small-cell lung cancer and was independent of EPOR expression levels on the cell

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lines surface. Hypoxia resulted in increased expression of VEGF, HIF-1 α , and PD-L1 on the NSCLC cell lines. Under normoxic conditions, rhEPO did not affect the expression of VEGF, HIF-1 α , and PD-L1; but under hypoxic conditions, the application of rhEPO reduced the expression of VEGF, HIF-1 α , and PD-L1, producing an impact on the biological behavior of tumor cells.

KEYWORDS

cell proliferation, hypoxia, hypoxia-inducible factor 1, non-small cell lung cancer, vascular endothelial growth factor

1 | INTRODUCTION

According to the latest statistics, cancers are still the main cause of death in humans. Compared with other cancers, lung cancer has a relatively low survival rate, of which 80% are non-small-cell lung cancers (NSCLC), with a survival rate of only 14%–15%.¹ Patients with NSCLC often suffer from cancer-associated anemia (CRA) due to the cancer itself, the patients' personal constitutions or tumor radiotherapy and chemotherapy, which seriously affects the quality of life and even life and health of patients.² Endogenous erythropoietin (EPO) is produced in the liver during the fetal stage; for adults, the kidney is the main site of production. In 1989, the United States Food and Drug Administration (FDA) approved recombinant human EPO (rhEPO, epoetin) for the treatment of renal anemia, which pioneered the treatment of anemia with exogenous EPO, followed by rhEPO's analogs (darbepoetin alfa, methoxy polyethylene glycol-epoetin beta), collectively known as erythropoiesis-stimulating agents (ESAs).^{3,4} EPO not only has a role in promoting erythropoiesis, but also has the functions of anti-inflammation, cell differentiation regulation, tissue protection, and so on, which are of great significance in a variety of nonhematopoietic organs (including the lung), such as resisting disease progression, maintaining health, and repairing damage.^{5–9} Numerous studies have shown that EPO can also be used for the treatment of CRA in addition to renal anemia, such as breast, ovarian, and NSCLC anemia,^{10–12} increasing hemoglobin (Hb) concentrations to maintain them at baseline levels, reducing patients' blood transfusions, and improving health-related quality of life (HRQL).^{13,14} It is even effective in preventing anemia when combined with chemotherapy.¹⁵ Currently, the application of rhEPO in CRA is second only to renal anemia and preoperative red blood cell mobilization, and it is widely used in the oncology department.¹⁶ It has been confirmed that tumors are in the state of hypoxia, which plays a promoting role in the occurrence and progression of tumors.^{17,18} Under the hypoxic condition, hypoxia-inducible factor-1 α (HIF-1 α) combines with hypoxia-inducible factor-1 β (HIF-1 β) into the nucleus to form

HIF-1. HIF-1 is overexpressed in most cancers, enabling tumor cells to better adapt to the hypoxic environment, which facilitates tumors progression. Some studies have shown that rhEPO enhances the expression of HIF-1,¹⁹ while others have concluded that rhEPO has no effect on the expression of HIF-1.²⁰ There is a higher level of vascular endothelial growth factor (VEGF) expression in NSCLC tissues than in normal lung tissues and other cancer tissues.²¹ RhEPO may also have a certain upregulation effect on VEGF.^{22,23} Programmed death-ligand 1 (PD-L1), also CD274 or B7-H1, with PD-1 in normal tissues can reduce T cells proliferation, but expressed in tumor cells exacerbates tumor growth and promotes immune escape of tumor cells. EPO may enhance the expression of PD-L1 and induce immune escape in tumor cells, but such studies are rare.²⁴ EPO receptor (EPOR) is expressed in various types of tumor cells,²⁵ and the levels of EPOR expression may have a certain impact on the survival time and survival status of cancer patients. A large number of studies have shown that the expression levels of EPOR can regulate the effect of exogenous EPO on the body, and the high expression levels of EPOR can increase the drug resistance of tumor cells, which is also true in NSCLC, and the overexpression of EPOR may reduce the survival probability of patients.^{26–28} In 2007, Wright et al.²⁹ demonstrated that rhEPO reduced survival probability in patients with advanced NSCLC, while in 2018, Frille et al.³⁰ showed that rhEPO did not promote proliferation of NSCLC cells or affect chemotherapy-induced apoptosis, both in normoxic and hypoxic conditions. For the above reasons, this study focuses on the effects of rhEPO with different concentrations (0, 2, and 8 U/ml) on the proliferation of two NSCLC cell lines, A549 (lower EPOR expression) and NCI-H838 (higher EPOR expression), under normoxic and hypoxic conditions. And we also studied the expression of three proteins (VEGF, HIF-1 α , and PD-L1) under different oxygen conditions, different rhEPO concentrations, and different EPOR expression levels. The results of the study can strengthen our cognizance about the application of rhEPO in NSCLC, especially the effects of rhEPO on tumor growth, immune escape of tumor cells and

many other issues under simulated hypoxic condition *in vitro*, providing an important basis for the application of exogenous EPO in the field of CRA treatment.

2 | METHODS

2.1 | Cell culture and growth curve assay

The NSCLC cell lines A549 and NCI-H838 were both purchased from Procell. They were cultured in Ham's F-12K (Kaighn's) medium and RPMI-1640 medium (Roswell Park Memorial Institute Medium; ATCC), both purchased from Gibco (Paisley). The culture medium was supplemented with 10% fetal bovine serum (Excell Bio) and 1% penicillin-streptomycin double-antibody solution (Gibco). Both cell lines were cultured in a cell incubator at 37°C, 5% CO₂ and saturated humidity. Cells in good growth condition were made into 5 × 10⁴ cells/ml single-cell suspension with complete medium and inoculated in the 96-well plate (100 µl/well, 5 duplicated wells, i.e., 5 × 10³ cells/well) every 24 h. After 8 days, the medium was discarded and 100 µl 10% cell counting kit-8 (CCK-8) solution (TransGen Biotech) was added to each well, incubated in a 37% incubator for 1 h, and then the optical density (OD) value at 450 nm was detected by enzyme-labeled instrument (Bio-Rad). The growth curves were drawn to determine the logarithmic growth period of the cell lines for subsequent experiments.

2.2 | Oxygen conditions and experimental groupings

The normoxic condition was 37°C, 95% O₂, and 5% CO₂. The hypoxic condition was 37°C, 1% O₂, 5% CO₂, and 94% N₂. The experiments were divided into six groups: normoxia, hypoxia, normoxia + rhEPO low concentration (2 U/ml), hypoxia + rhEPO low concentration (2 U/ml), normoxia + rhEPO high concentration (8 U/ml), hypoxia + rhEPO high concentration (8 U/ml). RhEPO pretreatment for 6 h and then continued to incubate for 24 and 48 h.

2.3 | Detection of cell proliferation by CCK-8 method

Cells with 90% confluence in a good growth state were digested with trypsin (0.25% Trypsin-EDTA; Gibco) and made into cell suspension, inoculated into a 96-well plate, cultured at 37°C and 5% CO₂ for 24 h. Then the culture medium was discarded, and transfected according to experimental groupings,

and each group was repeated five times. Grouped by points in time, the medium was discarded after 24 and 48 h of intervention, and 100 µl of 10% CCK-8 solution was added to each well. After incubation for 1 h, the OD value at 450 nm was measured with the Microplate Reader.

2.4 | Western blot analysis

According to the experiment groupings, after 48 h intervention of A549 and NCI-H838 cells was completed, collecting 1 × 10⁶ cells to be lysed with 100 µl of RIPA lysate which had been added to protease inhibitors and phosphatase inhibitors, and extracting proteins. The protein concentrations were measured according to the instructions for use of the BCA protein concentration quantification kit (TransGen Biotech). Then, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore). Using 0.22 µm membrane for VEGF and 0.45 µm membrane for HIF-1α, PD-L1, and β-actin, with the transfer time of 90 min for HIF-1α and 60 min for the other proteins. After being closed with TBST buffer (Sangon Biotech) containing 5% skimmed milk powder for 1 h, the primary antibody was diluted with TBST and incubated overnight at 4°C. The secondary antibody diluted with TBST was added and incubated for 1 h at room temperature. The membranes were washed three times with 1 × TBST for 10 min/time. SuperSignal West Pico PLUS chemiluminescence substrate (Thermo Fisher) was used for development exposure, ChemiScope Mini chemiluminescent imaging system (Clinx) was used to detect and photograph, and Image J (1.8.0 version) was used to quantify western blot bands. The ratio between the gray value of the target protein after normalization and the gray value of internal reference β-actin was regarded as the relative expression level of the target protein. Primary antibodies were mouse anti-beta-actin monoclonal antibody (mAb) (1:1000; 100166-MM10; Sino Biological), rabbit anti-VEGF antibody (1:500; bs-1313R; Bioss), rabbit anti-HIF-1α antibody (1:400; bs-0737R; Bioss), and rabbit anti-PD-L1 antibody (1:500; bs-10159R; Bioss); secondary antibodies were goat anti-mouse IgG H&L (HRP) (1:15,000, 1:5000; ab205719; Abcam) and goat anti-rabbit IgG H&L (HRP) (1:5000; ab205718; Abcam).

2.5 | Statistical analysis

All data were expressed as mean ± standard deviation (SD). SPSS 19.0 (IBM SPSS) and GraphPad Prism 9.0 were used for statistical analysis of the data in each group and making diagrams. And one-way analysis of

variance was used for statistics between groups, with $p < 0.05$ indicating a significant difference.

3 | RESULTS

3.1 | Growth curves of NSCLC cells under normoxic condition

The growth curves of A549 and NCI-H838 cell lines were measured under normoxic conditions without rhEPO intervention for 7 consecutive days (Figure 1). The curves were both s-shaped, consistent with the normal cell growth. The logarithmic growth period of the two cell lines was determined, proving that the cell viability was good and could be used for later intervention experiments.

3.2 | Effects of hypoxic condition and rhEPO treatment on the proliferation of NSCLC cell lines

To observe the effects of rhEPO and hypoxic condition on the proliferation of cell lines with different expression levels

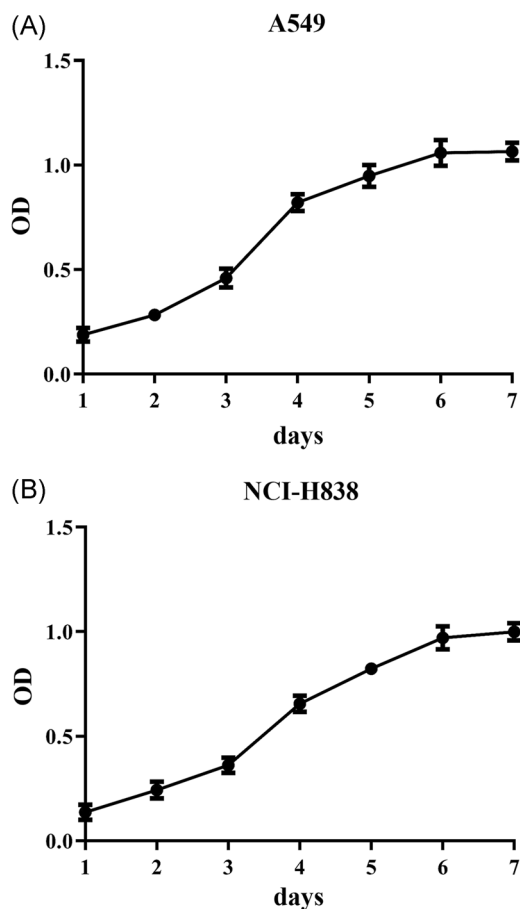


FIGURE 1 (A) Growth curve of the A549 cell line. (B) Growth curve of the NCI-H838 cell line

of EPOR, the CCK-8 method was used to count the two cell lines under six treatment conditions of normoxia, hypoxia, normoxia + rhEPO 2 U/ml, hypoxia + rhEPO 2 U/ml, normoxia + rhEPO 8 U/ml, and hypoxia + rhEPO 8 U/ml, respectively, so as to observe the effects of rhEPO concentrations, oxygen conditions and EPOR expression levels on the proliferation of NSCLC cells, with the normoxia group as the baseline level. According to the cell diagrams of the two cell lines under the microscope ($\times 100$) (Figure 2A), the speed of cells proliferation with high EPOR expression (NCI-H838) was more likely to be affected by rhEPO after 48 h of culture.

In both cell lines, the total number of surviving cells was significantly reduced by hypoxia itself and combined with rhEPO treatment ($p < 0.05$) (Tables 1 and 2 and Figure 2). Under hypoxic conditions, the total number of viable cells in the rhEPO high-concentration group of the A549 cell line increased significantly after 48 h of culture compared to that without rhEPO treatment ($p < 0.05$), while the rhEPO high-concentration group after 24 h of culture and rhEPO low-concentration group had no statistically significant difference from the untreated group (Table 1 and Figure 2B). In the hypoxic condition, the survival rate of the NCI-H838 cell line increased significantly after 48 h of culture in the rhEPO low-concentration group ($p < 0.05$). Compared with the untreated group, the survival rate of cells also significantly increased in the rhEPO high-concentration group after 24 and 48 h ($p < 0.05$), but there was no statistically significant difference from the rhEPO low-concentration group (Table 2 and Figure 2C).

3.3 | Hypoxia upregulated the expression levels of proteins in NSCLC cell lines

We simulated the hypoxic environment within the tumor and cultured the cells in 1% oxygen to explore the effect of hypoxia on EPO-related proteins in A549 and NCI-H838 cell lines. After 48 h of hypoxia culture, the relative levels of VEGF, HIF-1 α , and PD-L1 were significantly increased ($p < 0.05$), indicating that hypoxia induced the expression of the three proteins in NSCLC cell lines, which was not related to the expression levels of EPOR, in other words, the ability of hypoxia to upregulate the proteins may not depend on the expression levels of EPOR (Tables 3 and 4 and Figure 3).

3.4 | Effects of rhEPO on expression of proteins in NSCLC cell lines

To explore whether rhEPO had effects on expression of proteins in NSCLC cell lines, cells were pretreated with different concentrations of rhEPO for 6 h and then the relative expression levels of proteins were measured after 48 h. Western blot analysis showed that the expression levels of VEGF and HIF-1 α in the A549 cell line were

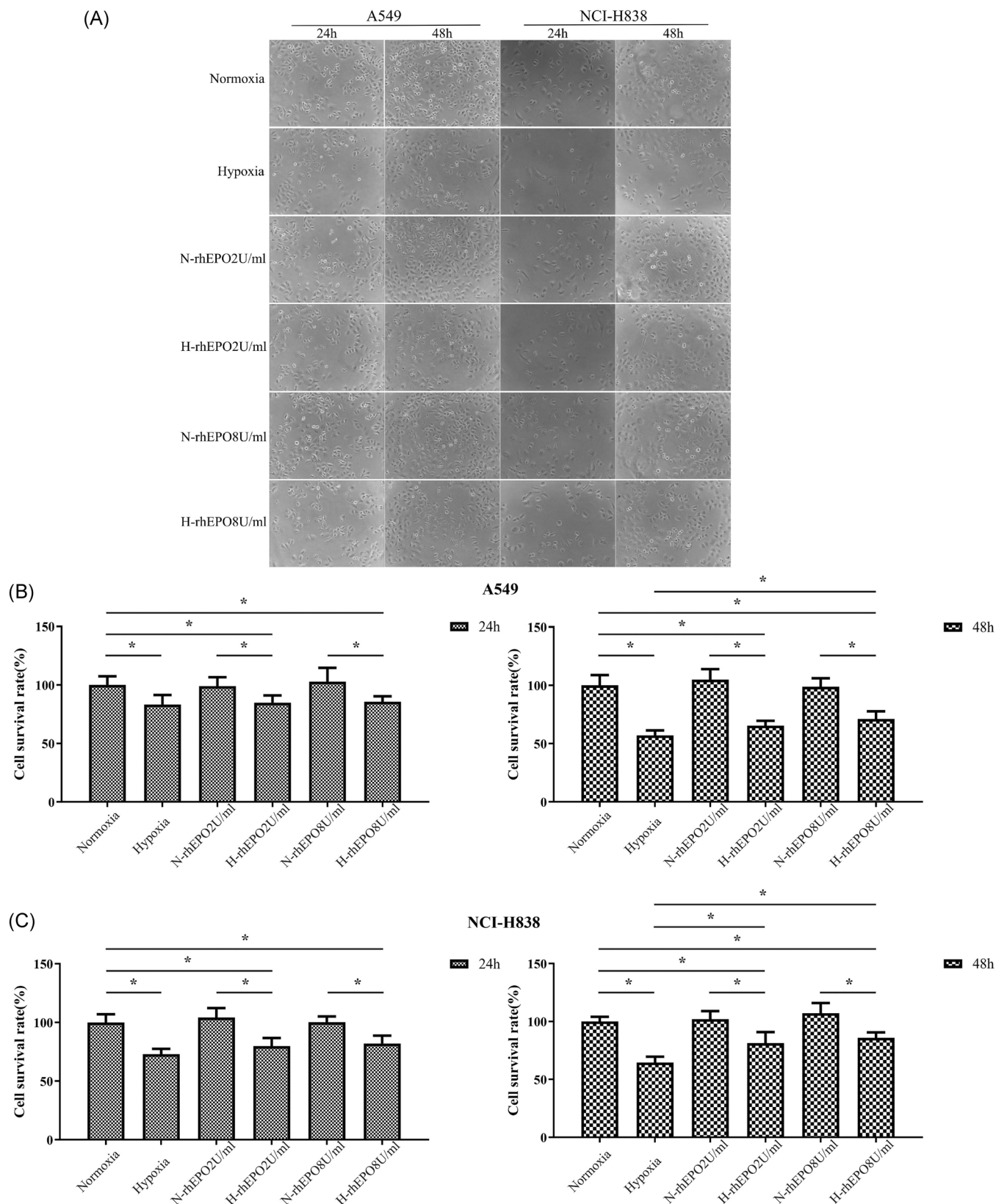


FIGURE 2 Effect of rhEPO on the proliferation of NSCLC cells. The proliferation of the two cell lines was compared after 24 and 48 h intervention with rhEPO (0, 2, and 8 U/ml) under normoxic and hypoxic conditions. (A) Cell diagram under an inverted fluorescence microscope (original magnification $\times 100$). (B) Cell survival rate of the A549 cell line. (C) Cell survival rate of the NCI-H838 cell line. One-way ANOVA was used for statistics ($*p < 0.05$). The results represented the mean \pm SD of five independent experiments. (N: Normoxia; H: Hypoxia). ANOVA, analysis of variance; NSCLC, non-small cell lung cancer; rhEPO, recombinant human erythropoietin

TABLE 1 Effects of different treatments on the proliferation of A549 cells (mean \pm SD, $n = 5$)

Treatments	Cell survival rate (%)	
	24 h	48 h
Normoxia	100.000 \pm 7.459	100.000 \pm 8.802
Hypoxia	83.287 \pm 8.163 ^a	57.101 \pm 4.170 ^a
Normoxia + rhEPO 2 U/ml	99.041 \pm 7.593 ^b	104.880 \pm 9.112 ^b
Hypoxia + rhEPO 2 U/ml	84.766 \pm 6.283 ^{a,c}	65.422 \pm 4.155 ^{a,c}
Normoxia + rhEPO 8 U/ml	102.839 \pm 11.801 ^{b,d}	98.829 \pm 7.256 ^{b,d}
Hypoxia + rhEPO 8 U/ml	85.726 \pm 4.641 ^{a,c,e}	71.205 \pm 6.518 ^{a,b,c,e}

^a $p < 0.05$ versus Normoxia; ^b $p < 0.05$ versus Hypoxia; ^c $p < 0.05$ versus Normoxia + rhEPO 2 U/ml; ^d $p < 0.05$ versus Hypoxia + rhEPO 2 U/ml; ^e $p < 0.05$ versus Normoxia + rhEPO 8 U/ml.

TABLE 2 Effects of different treatments on the proliferation of NCI-H838 cells (mean \pm SD, $n = 5$)

Treatments	Cell survival rate (%)	
	24 h	48 h
Normoxia	100.000 \pm 6.900	100.000 \pm 4.048
Hypoxia	72.940 \pm 4.553 ^a	64.606 \pm 4.956 ^a
Normoxia + rhEPO 2 U/ml	104.149 \pm 7.981 ^b	102.088 \pm 6.799 ^b
Hypoxia + rhEPO 2 U/ml	79.796 \pm 6.851 ^{a,c}	81.287 \pm 9.539 ^{a,b,c}
Normoxia + rhEPO 8 U/ml	100.180 \pm 4.853 ^{b,d}	107.129 \pm 8.827 ^{b,d}
Hypoxia + rhEPO 8 U/ml	81.960 \pm 6.668 ^{a,b,c}	85.909 \pm 4.613 ^{a,b,c,e}

^a $p < 0.05$ versus Normoxia; ^b $p < 0.05$ versus Hypoxia; ^c $p < 0.05$ versus Normoxia + rhEPO 2 U/ml; ^d $p < 0.05$ versus Hypoxia + rhEPO 2 U/ml; ^e $p < 0.05$ versus Normoxia + rhEPO 8 U/ml.

TABLE 3 The expression levels of VEGF, HIF-1 α and PD-L1 in A549 cells (mean \pm SD, $n = 3$)

Treatments	VEGF	HIF-1 α	PD-L1
Normoxia	0.459 \pm 0.038	0.328 \pm 0.050	0.391 \pm 0.014
Hypoxia	0.632 \pm 0.019 ^a	0.579 \pm 0.043 ^a	0.659 \pm 0.035 ^a
Normoxia + rhEPO 2 U/ml	0.467 \pm 0.041 ^b	0.374 \pm 0.021 ^b	0.430 \pm 0.118 ^b
Hypoxia + rhEPO 2 U/ml	0.599 \pm 0.074 ^a	0.510 \pm 0.039 ^{a,b,c}	0.652 \pm 0.020 ^{a,c}
Normoxia + rhEPO 8 U/ml	0.450 \pm 0.090 ^{b,d}	0.311 \pm 0.035 ^{b,d}	0.323 \pm 0.037 ^{b,c,d}
Hypoxia + rhEPO 8 U/ml	0.519 \pm 0.098	0.388 \pm 0.024 ^{b,d,e}	0.523 \pm 0.029 ^{a,b,d,e}

Abbreviations: HIF, hypoxia-inducible factor; PD-L1, programmed death-ligand 1; rhEPO, recombinant human erythropoietin; VEGF, vascular endothelial growth factor.

^a $p < 0.05$ versus Normoxia; ^b $p < 0.05$ versus Hypoxia; ^c $p < 0.05$ versus Normoxia + rhEPO 2 U/ml; ^d $p < 0.05$ versus Hypoxia + rhEPO 2 U/ml; ^e $p < 0.05$ versus Normoxia + rhEPO 8 U/ml.

TABLE 4 The expression levels of VEGF, HIF-1 α , and PD-L1 in NCI-H838 cells (mean \pm SD, $n = 3$)

Treatment	VEGF	HIF-1 α	PD-L1
Normoxia	0.425 \pm 0.080	0.387 \pm 0.031	0.517 \pm 0.063
Hypoxia	0.564 \pm 0.073 ^a	0.558 \pm 0.042 ^a	0.802 \pm 0.058 ^a
Normoxia + rhEPO 2 U/ml	0.432 \pm 0.053	0.404 \pm 0.017 ^b	0.550 \pm 0.024 ^b
Hypoxia + rhEPO 2 U/ml	0.495 \pm 0.034 ^b	0.461 \pm 0.033 ^{a,b,c}	0.753 \pm 0.062 ^{a,c}
Normoxia + rhEPO 8 U/ml	0.452 \pm 0.057	0.410 \pm 0.039 ^b	0.524 \pm 0.091 ^{b,d}
Hypoxia + rhEPO 8 U/ml	0.474 \pm 0.061 ^b	0.449 \pm 0.021 ^{a,b}	0.782 \pm 0.051 ^{a,c,e}

^a $p < 0.05$ versus Normoxia; ^b $p < 0.05$ versus Hypoxia; ^c $p < 0.05$ versus Normoxia + rhEPO 2 U/ml; ^d $p < 0.05$ versus Hypoxia + rhEPO 2 U/ml; ^e $p < 0.05$ versus Normoxia + rhEPO 8 U/ml.

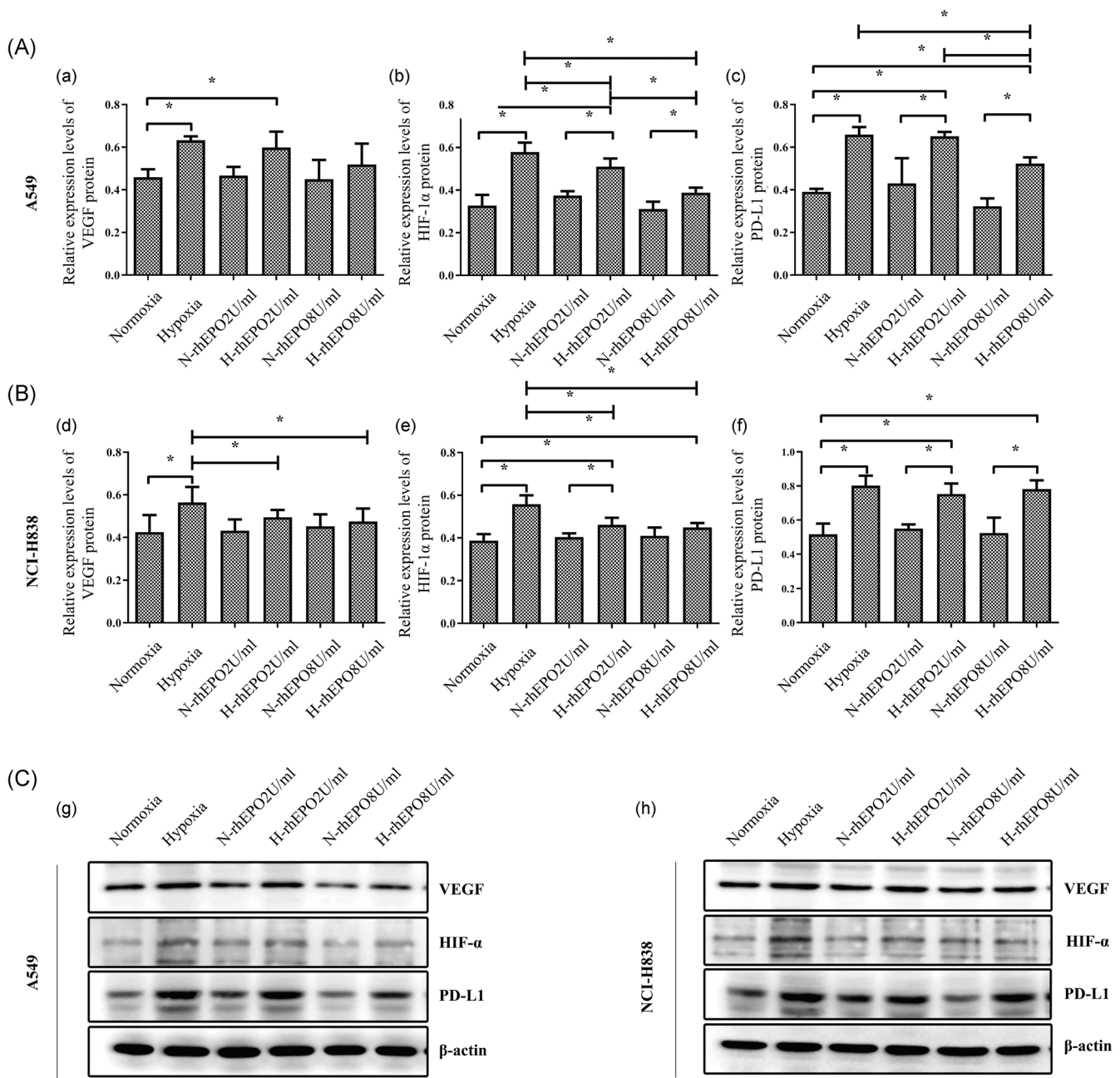


FIGURE 3 Western blot analysis of EPO-related molecules in NSCLC cell lines. The expression of each protein was compared after 48 h intervention with rhEPO (0, 2, and 8 U/ml) under normoxic and hypoxic conditions. (A) The histogram showed the relative expression levels of VEGF, HIF-1 α , and PD-L1 in A549 cells ($*p < 0.05$). (B) The histogram showed the relative expression levels of VEGF, HIF-1 α , and PD-L1 in NCI-H838 cells ($*p < 0.05$). (C) Western blot bands. The immune response was developed by rabbit anti-VEGF antibody, rabbit anti-HIF-1 α antibody, rabbit anti-PD-L1 antibody, and mouse anti- β -actin monoclonal antibody. β -actin was used as a loading control. The results represented the mean \pm SD of three independent experiments. It showed a representative print. (N: Normoxia; H: Hypoxia). EPO, erythropoietin; HIF, hypoxia-inducible factor-1 α ; NSCLC, non-small-cell lung carcinoma; VEGF, vascular endothelial growth factor

also significantly higher in hypoxia and hypoxia + rhEPO 2 U/ml compared to the normoxia group ($p < 0.05$), but the hypoxia + rhEPO 8 U/ml group did not have the advantage of promoting the expression of VEGF and HIF-1 α compared to the normoxia group. Under the condition of hypoxia, the expression level of HIF-1 α was significantly different among 0, 2, and 8 U/ml rhEPO ($p < 0.05$), and the expression level

of HIF-1 α decreased with the increase of rhEPO concentrations, but not in VEGF (Table 3 and Figure 3a, b, g); the expression level of PD-L1 at 8 U/ml rhEPO was significantly decreased compared with that at 0 and 2 U/ml rhEPO, respectively ($p < 0.05$), but there was no statistical difference between rhEPO 0 and 2 U/ml groups, demonstrating that the expression of PD-L1 was reduced only at

higher concentrations of rhEPO, with no effect at lower concentrations (Table 3 and Figure 3c, g).

The expression level of VEGF in the NCI-H838 cell line was not significantly increased under hypoxia and rhEPO intervention compared to the normoxia group, but there was a significant increase in the expression level of HIF-1 α under hypoxia and rhEPO intervention compared to the normoxia group ($p < 0.05$). Under the condition of hypoxia, the expression levels of VEGF and HIF-1 α were significantly decreased at lower and higher concentrations of rhEPO compared to those without rhEPO treatment ($p < 0.05$), but there was no significant difference between 2 U/ml rhEPO and 8 U/ml rhEPO (Table 4 and Figure 3d, e, h). RhEPO did not affect the PD-L1 expression in the NCI-H838 cell line (Table 4 and Figure 3f, h).

The results showed that rhEPO could reduce the expression levels of VEGF and HIF-1 α in NSCLC cell lines under the condition of hypoxia. In the A549 cell line, the effect on HIF-1 α expression became more obvious with higher rhEPO concentrations, and lower concentrations of rhEPO in VEGF expression may have no effect. In the NCI-H838 cell line, rhEPO reduced the expression level of VEGF more significantly, but it may not be related to the concentrations of rhEPO. RhEPO had no effect on the PD-L1 expression of EPOR high expression cell lines, and the effect on EPOR low expression cell lines only occurred when the concentrations of rhEPO were higher.

4 | DISCUSSION

Hypoxia is a common feature of most solid tumors.³¹ In addition, tumor cells show strong adaptability to the hypoxic environment. In the hypoxic microenvironment of the tumor, tumor cells can not only survive but also continue to proliferate. It had been reported that hypoxia can promote tumor cells apoptosis, showing resistance to chemotherapeutic drugs, and had positive effects on cancer progression and metastasis, reducing the survival rate of cancer patients.^{18,32,33} Recombinant human EPO has been used in the treatment of CRA for many years.³⁴

The aim of this study was to explore the correlation between EPO and the growth of NSCLC cell lines under a hypoxic environment, including cell proliferation and the expression of tumor growth, immune escape-related proteins. In our experiments, hypoxia itself reduced the number of survived cells, in other words, compared with normoxic environments, the proliferation ability of tumor cells in a hypoxic environment was reduced, but cells can continue to proliferate.

The safety of exogenous EPO in the treatment of CRA has been controversial. At present, there are four viewpoints: EPO does not protect cells from chemotherapy-induced apoptosis, and has no effect on the proliferation of tumor cells and the survival or prognosis, as seen in cancers such as small cell lung cancer, head and neck

cancer, and lymphoma³⁵⁻³⁹; EPO does not stimulate the proliferation of tumor cells, but protects cells from apoptosis, decreases the sensitivity to chemotherapeutic drugs, and affects immune escape of tumor cells, which has been demonstrated in prostate cancer, cervical cancer and other cancers^{22,40-42}; rhEPO not only induces the proliferation of cells in the tumor microenvironment, but also protects cells from chemotherapy-induced apoptosis, increases resistance to chemotherapeutic drugs, and enhances the risk of tumor progression and death, which has been reported in renal cell carcinoma, hepatocellular carcinoma, breast cancer, and head and neck squamous cell carcinoma⁴³⁻⁴⁶; when combined with antineoplastic drugs, rhEPO has a synergistic inhibitory effect, promoting cells apoptosis, resisting cells proliferation, and inhibiting the progression of tumors, as seen in studies of breast and colorectal carcinoma.^{47,48}

VEGF, HIF-1 α , and programmed death molecule ligand 1 (PD-L1) are the proteins associated with regulating angiogenesis, tumor cells proliferation, metabolism, metastasis, and immune escape. In our study, the expression of HIF-1 α demonstrated the successful establishment of the hypoxia model. Hypoxia can upregulate protein expression levels and increase the progression of tumors and immune escape of tumor cells, but may not be dependent on EPOR expression levels.

Positive EPOR compared with negative EPOR may increase the disadvantage of rhEPO for the treatment of head and neck squamous cell carcinoma.⁴⁹ In our study, rhEPO was also found to promote the proliferation of tumor cells, especially in cell lines with high EPOR expression. However, to a certain extent, rhEPO had an inhibitory effect on the expression of proteins which were related to tumor cells' growth and immune escape. In cell lines with low EPOR expression, this inhibition was correlated with the concentrations of rhEPO, and the inhibition became more serious with the increase of the concentrations. In the cell strains with high EPOR expression, concentrations may not affect the inhibitory effect, and rhEPO did not interfere with the immune escape of tumor cells.

Due to the limitations of this study, we did not explore whether the antagonistic effect of rhEPO on the expression of proteins attributed to the fact that rhEPO itself can downregulate proteins or interfere with the ability of hypoxia to induce proteins. Some studies have shown that HIF-1 can activate the expression of VEGF and the expression of PD-L1 may be dependent on autocrine VEGF.⁵⁰⁻⁵² Therefore, we could not confirm whether the ability of hypoxia to up-regulate the protein levels was due to the independent effect of hypoxia or the indirect promoting effect of protein-protein interaction. At the same time, we also had a limitation of the study. Due to the existence of EPOR, we did not explore the direct effects of EPO on the cell survival and expression of three proteins (VEGF, HIF-1 α , and PD-L1).

Future experiments need to focus on the mechanisms by which hypoxia induces VEGF, PD-L1, and rhEPO reduces the expression levels of proteins. Meanwhile, we are applying for the related topic of EPOR gene knock-down and overexpression in cell lines. It will be a good comparison before and after to knock down EPOR in NCI-H838 cell line by small interfering RNA or transfer overexpressed EPOR gene into the A549 cell line, which will be more convincing. And the direct effect of EPO can be verified by knocking out the EPOR gene.

In addition, we can combine EPO with chemotherapeutic drugs in tumor models to explore how to make the drugs play their proper roles and to better snuff out the tumor cells.

In conclusion, in the simulated tumor hypoxic microenvironment, hypoxia can increase tumor growth and immune escape of tumor cells, and rhEPO can promote the proliferation of tumor cells, but reduce the expressions of VEGF, HIF-1 α , and PD-L1, and to some extent, it is affected by the expression levels of EPOR and rhEPO concentrations.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

REFERENCES

- Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. *CA Cancer J Clin*. 2021;71:7-33. doi:10.3322/caac.21654
- Gainor JF, Curigliano G, Kim DW, et al. Pralsetinib for RET fusion-positive non-small-cell lung cancer (ARROW): a multicohort, open-label, phase 1/2 study. *Lancet Oncol*. 2021;22:959-969. doi:10.1016/S1470-2045(21)00247-3
- Schoener B, Borger J. *Erythropoietin*. StatPearls Publishing; 2021.
- Del Vecchio L, Minutolo R. ESA, Iron therapy and new drugs: are there new perspectives in the treatment of anaemia? *J Clin Med*. 2021;10:839. doi:10.3390/jcm10040839
- Silva I, Alípio C, Pinto R, Mateus V. Potential anti-inflammatory effect of erythropoietin in non-clinical studies in vivo: a systematic review. *Biomed Pharmacother*. 2021;139:111558. doi:10.1016/j.biopha.2021.111558
- Suresh S, de Castro LF, Dey S, Robey PG, Noguchi CT. Erythropoietin modulates bone marrow stromal cell differentiation. *Bone Res*. 2019;7:21. doi:10.1038/s41413-019-0060-0
- Shin YK, Cho SR. Exploring erythropoietin and G-CSF combination therapy in chronic stroke patients. *Int J Mol Sci*. 2016;17:463. doi:10.3390/ijms17040463
- Suresh S, Rajvanshi PK, Noguchi CT. The many facets of erythropoietin physiologic and metabolic response. *Front Physiol*. 2020;10:1534. doi:10.3389/fphys.2019.01534
- Haine L, Yegen CH, Marchant D, Richalet JP, Boncoeur E, Voituron N. Cytoprotective effects of erythropoietin: what about the lung? *Biomed Pharmacother*. 2021;139:111547. doi:10.1016/j.biopha.2021.111547
- Chang J, Couture F, Young S, McWatters KL, Lau CY. Weekly epoetin alfa maintains hemoglobin, improves quality of life, and reduces transfusion in breast cancer patients receiving chemotherapy. *J Clin Oncol*. 2005;23:2597-2605. doi:10.1200/JCO.2004.12.027
- Wilkinson PM, Antonopoulos M, Lahousen M, Lind M, Kosmidis P. EPO-INT-45 Study Group. Epoetin alfa in platinum-treated ovarian cancer patients: results of a multinational, multicentre, randomised trial. *Br J Cancer*. 2006;94:947-954. doi:10.1038/sj.bjc.6603004
- Biesma B, van de Werf PR, Melissant CF, Brok RG. Anaemia management with epoetin alfa in lung cancer patients in The Netherlands. *Lung Cancer*. 2007;58:104-111. doi:10.1016/j.lungcan.2007.05.007
- Witzig TE, Silberstein PT, Loprinzi CL, et al. Phase III, randomized, double-blind study of epoetin alfa compared with placebo in anemic patients receiving chemotherapy. *J Clin Oncol*. 2005;23:2606-2617. doi:10.1200/JCO.2004.10.020
- Kimel M, Leidy NK, Mannix S, Dixon J. Does epoetin alfa improve health-related quality of life in chronically ill patients with anemia? Summary of trials of cancer, HIV/AIDS, and chronic kidney disease. *Value Health*. 2008;11:57-75. doi:10.1111/j.1524-4733.2007.00215.x
- Schouwink JH, Codrington H, Sleeboom HP, Kerkhofs LG, Wormhoudt LW. Prevention of anaemia by early intervention with once weekly epoetin alfa during chemotherapy. *Eur J Cancer*. 2008;44:819-829. doi:10.1016/j.ejca.2008.02.017
- Guan XZ, Wang LL, Pan X, et al. Clinical indications of recombinant human erythropoietin in a single center: a 10-year retrospective study. *Front Pharmacol*. 2020;11:1110. doi:10.3389/fphar.2020.01110
- Strzyz P. Cancer biology: hypoxia as an off switch for gene expression. *Nat Rev Mol Cell Biol*. 2016;17:610. doi:10.1038/nrm.2016.119
- Jing X, Yang F, Shao C, et al. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Mol Cancer*. 2019;18:157. doi:10.1186/s12943-019-1089-9
- Li D, Xie X, Yang Z, Wang C, Wei Z, Kang P. Enhanced bone defect repairing effects in glucocorticoid-induced osteonecrosis of the femoral head using a porous nano-lithium-hydroxyapatite/gelatin microsphere/erythropoietin composite scaffold. *Biomater Sci*. 2018;6:519-537. doi:10.1039/c7bm00975e
- Ceelen W, Boterberg T, Smeets P, et al. Recombinant human erythropoietin alpha modulates the effects of radiotherapy on colorectal cancer microvessels. *Br J Cancer*. 2007;96:692-700. doi:10.1038/sj.bjc.6603568
- Gu A, Lu J, Wang W, Shi C, Han B, Yao M. Role of miR-497 in VEGF-A-mediated cancer cell growth and invasion in nonsmall cell lung cancer. *Int J Biochem Cell Biol*. 2016;70:118-125. doi:10.1016/j.biocel.2015.10.013
- Annese T, Tamma R, Ruggieri S, Ribatti D. Erythropoietin in tumor angiogenesis. *Exp Cell Res*. 2019;374:266-273. doi:10.1016/j.yexcr.2018.12.013
- Yu Y, Ma L, Zhang H, et al. EPO could be regulated by HIF-1 and promote osteogenesis and accelerate bone repair. *Artif Cells Nanomed Biotechnol*. 2020;48:206-217. doi:10.1080/21691401.2019.1699827
- Zhang J, Luo X, Huang C, et al. Erythropoietin prevents LPS-induced preterm birth and increases offspring survival. *Am J Reprod Immunol*. 2020;84:e13283. doi:10.1111/aji.13283
- Debeljak N, Solár P, Sytkowski AJ. Erythropoietin and cancer: the un-intended consequences of anemia correction. *Front Immunol*. 2014;5:563. doi:10.3389/fimmu.2014.00563
- Hardee ME, Cao Y, Fu P, et al. Erythropoietin blockade inhibits the induction of tumor angiogenesis and progression. *PLOS One*. 2007;2:e549. doi:10.1371/journal.pone.0000549
- Ilkovičová L, Trošt N, Szentpéteriová E, Solár P, Komel R, Debeljak N. Overexpression of the erythropoietin receptor in RAMA 37 breast cancer cells alters cell growth and sensitivity to tamoxifen. *Int J Oncol*. 2017;51:737-746. doi:10.3892/ijo.2017.4061
- He L, Wu S, Hao Q, et al. Local blockage of self-sustainable erythropoietin signaling suppresses tumor progression in non-small cell lung cancer. *Oncotarget*. 2017;8:82352-82365. doi:10.18632/oncotarget.19354

29. Wright JR, Ung YC, Julian JA, et al. Randomized, double-blind, placebo-controlled trial of erythropoietin in non-small-cell lung cancer with disease-related anemia. *J Clin Oncol.* 2007;25:1027-1032. doi:10.1200/JCO.2006.07.1514
30. Frille A, Leithner K, Olschewski A, Olschewski H, Wohlkönig C, Hrzenjak A. No erythropoietin-induced growth is observed in non-small cell lung cancer cells. *Int J Oncol.* 2018;52:518-526. doi:10.3892/ijo.2017.4225
31. Bertout JA, Patel SA, Simon MC. The impact of O₂ availability on human cancer. *Nat Rev Cancer.* 2008;8:967-975. doi:10.1038/nrc2540
32. Harris AL. Hypoxia—a key regulatory factor in tumor growth. *Nat Rev Cancer.* 2002;2:38-47. doi:10.1038/nrc704
33. Ma L, Craig AJ, Heinrich S. Hypoxia is a key regulator in liver cancer progression. *J Hepatol.* 2021;75:736-737. doi:10.1016/j.jhep.2021.05.032
34. Spivak JL. The anaemia of cancer: death by a thousand cuts. *Nat Rev Cancer.* 2005;5:543-555. doi:10.1038/nrc1648
35. Grote T, Yeilding AL, Castillo R, et al. Efficacy and safety analysis of epoetin alfa in patients with small-cell lung cancer: a randomized, double-blind, placebo-controlled trial. *J Clin Oncol.* 2005;23:9377-9386. doi:10.1200/JCO.2005.01.8507
36. Hoskin PJ, Robinson M, Slevin N, Morgan D, Harrington K, Gaffney C. Effect of epoetin alfa on survival and cancer treatment-related anemia and fatigue in patients receiving radical radiotherapy with curative intent for head and neck cancer. *J Clin Oncol.* 2009;27:5751-5756. doi:10.1200/JCO.2009.22.3693
37. Engert A, Josting A, Haverkamp H, et al. Epoetin alfa in patients with advanced-stage Hodgkin's lymphoma: results of the randomized placebo-controlled GHSG HD15EPO trial. *J Clin Oncol.* 2010;28:2239-2245. doi:10.1200/JCO.2009.25.1835
38. Park LC, Song YJ, Kim DJ, et al. The effects of erythropoiesis-stimulating agents on the management of chemotherapy-induced anemia and tumor growth in diffuse large B-cell lymphoma patients. *Int J Cancer.* 2019;145:2459-2467. doi:10.1002/ijc.32328
39. Zhou B, Damrauer JS, Bailey ST, et al. Erythropoietin promotes breast tumorigenesis through tumor-initiating cell self-renewal. *J Clin Invest.* 2014;124:553-563. doi:10.1172/JCI69804
40. Shiozawa Y, McGee S, Pienta MJ, et al. Erythropoietin supports the survival of prostate cancer, but not growth and bone metastasis. *J Cell Biochem.* 2013;114:2471-2478. doi:10.1002/jcb.24592
41. Vázquez-Mellado MJ, Cortés-Ballinas LG, Blanco-Flores IC, Aguilar C, Vázquez-Gómez G, Rocha-Zavaleta L. Erythropoietin promotes expression of survivin via STAT3 activation and reduces sensitivity to cisplatin in cervical cancer cells. *Oncol Rep.* 2019;41:1333-1341. doi:10.3892/or.2018.6890
42. Pham TD, Ma W, Miller D, Kazakova L, Benchimol S. Erythropoietin inhibits chemotherapy-induced cell death and promotes a senescence-like state in leukemia cells. *Cell Death Dis.* 2019;10:22. doi:10.1038/s41419-018-1274-6
43. Miyake M, Goodison S, Lawton A, Zhang G, Gomes-Giacoia E, Rosser CJ. Erythropoietin is a JAK2 and ERK1/2 effector that can promote renal tumor cell proliferation under hypoxic conditions. *J Hematol Oncol.* 2013;6:65. doi:10.1186/1756-8722-6-65
44. Miao S, Wang SM, Cheng X, et al. Erythropoietin promoted the proliferation of hepatocellular carcinoma through hypoxia induced translocation of its specific receptor. *Cancer Cell Int.* 2017;17:119. doi:10.1186/s12935-017-0494-7
45. Leyland-Jones B, Bondarenko I, Nemsadze G, et al. A randomized, openlabel, multicenter, phase III study of epoetin alfa versus best standard of care in anemic patients with metastatic breast cancer receiving standard chemotherapy. *J Clin Oncol.* 2016;34:1197-1207. doi:10.1200/JCO.2015.63.5649
46. Overgaard J, Hoff CM, Hansen HS, et al. DAHANCA 10 - Effect of darbepoetin alfa and radiotherapy in the treatment of squamous cell carcinoma of the head and neck. A multicenter, open-label, randomized, phase 3 trial by the Danish head and neck cancer group. *Radiother Oncol.* 2018;127:12-19. doi:10.1016/j.radonc.2018.02.018
47. Rozkiewicz D, Hermanowicz JM, Tankiewicz-Kwedlo A, et al. The intensification of anticancer activity of LFM-A13 by erythropoietin as a possible option for inhibition of breast cancer. *J Enzyme Inhib Med Chem.* 2020;35:1697-1710. doi:10.1080/14756366.2020.1818738
48. Tankiewicz-Kwedlo A, Hermanowicz JM, Domaniewski T, et al. Simultaneous use of erythropoietin and LFM-A13 as a new therapeutic approach for colorectal cancer. *Br J Pharmacol.* 2018;175:743-762. doi:10.1111/bph.14099
49. Topkan E, Yildirim BA. Epoetin receptor status may alter the outcomes in head and neck cancers treated with radiotherapy and darbepoetin- α . *Radiother Oncol.* 2019;130:190. doi:10.1016/j.radonc.2018.07.010
50. Zhang D, Lv FL, Wang GH. Effects of HIF-1 α on diabetic retinopathy angiogenesis and VEGF expression. *Eur Rev Med Pharmacol Sci.* 2018;22:5071-5076. doi:10.26355/eurrev_201808_15699
51. Li G, Ko CN, Li D, et al. A small molecule HIF-1 α stabilizer that accelerates diabetic wound healing. *Nat Commun.* 2021;12:3363. doi:10.1038/s41467-021-23448-7
52. Lai YS, Wahyuningtyas R, Aui SP, Chang KT. Autocrine VEGF signalling on M2 macrophages regulates PD-L1 expression for immunomodulation of T cells. *J Cell Mol Med.* 2019;23:1257-1267. doi:10.1111/jcmm.14027

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