Diploma thesis

The Role of Erythropoietin and its Receptor in Growth and Survival of non-small cell Lung Cancer cells

submitted by

Armin Frille

Date of birth: July 17, 1983

to attain the academic degree

Doctor medicinae universae

(Dr. med. univ.)

at the

Medical University of Graz

conducted at the

Division of Pulmonology,

Department of Internal Medicine

under supervision of

Dr. Christoph Wohlkönig

and

Prof. Dr. Horst Olschewski

Graz, April 13, 2012 Armin Frille

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Acknowledgements

First and foremost, I would like to thank Dr. Christoph Wohlkönig for the generous offer to conduct my diploma thesis under his supervision in the Lung Cell Laboratory at the Division of Pulmonology. His tireless assistance, patience, and collegiality were exceptional and opened me an outstanding opportunity to develop new skills and techniques. As a consequence, it was a great honor for me to present first results during the Annual Meeting of the Austrian Society of Pneumology in 2010.

I would like to thank as well Prof. Dr. Horst Olschewski for his wise support and leads for new directions throughout the establishment of this work.

In addition, I am very grateful for the generosity of Prof. Dr. Norio Komatsu from the Department of Hematology at the Juntendo University in Tokyo, Japan, for providing us his control cell line he established in his lab.

The pleasant atmosphere in the lab facilitated the continuous and time-consuming work since every co-worker did his great part in contributing to this thesis. In this regard, special thanks deserve Dr. Katharina Leithner and Prof. Dr. Andelko Hrzenjak for their enormous commitment and helpful discussions we were engaged in throughout this work. Sincere thanks are given as well to Dr. Zoltán Bálint for the confocal imaging and Elisabeth Pöllitzer for her excellent technical assistance.

I am also deeply indebted to Dr. Stefan Scheidl for putting me in touch with Dr. Christoph Wohlkönig in the first place and hence made this work possible and valuable for me.

Finally, I would like to thank my beloved family for providing me with continual love, encouragement and support both mentally and financially, especially throughout the diploma thesis and my medical studies.

Zusammenfassung

Einleitung: Lungenkrebspatienten zeigen die höchste Inzidenz von Anämie unter allen Patienten mit soliden Tumoren. Rekombinantes humanes Erythropoetin (rHuEpo) und seine Analoga haben sich in der Behandlung chemotherapie-induzierter Anämie als äußerst wirkungsgvoll erwiesen, die Bluttransfusionsrate zu senken und den Hämoglobinspiegel zu heben. In den letzten Jahren gaben die Ergebnisse klinischer und experimenteller Studien Anlass zu Besorgnis über die Sicherheit ihrer Anwendung. Denn die Verabreichung von Epo und der Nachweis seines Rezeptors (EpoR) auf Tumorzellen wurde verdächtigt, das Tumorwachstum zu beschleunigen und die Therapie ungünstig zu beeinflussen. Das ausgeschriebene Ziel dieser Studie war es, Lungenkarzinomzellen vom nicht-kleinzelligen Typ (NSCLC) hinsichtlich des EpoR, seiner Funktionstüchtigkeit und des Einflusses von Epo auf das Tumorwachstum und Apoptose zu untersuchen.

Materialien und Methoden: Mithilfe von quantitativer RT-PCT, Western-Blot und Immunzytochemie, wurden drei NSCLC-Zelllinien (A427, A549, NCI-H358) auf EpoR und seine Funktionstüchtigkeit hin untersucht. Für die Proliferationsversuche wurden die Zellen unter normoxischen (21% O_2) und unter hypoxischen (1% O_2) Bedingungen kultiviert, initial mit 100 U/ml Epo behandelt und ihre Zellzahl wurde während der drei folgenden Tage bestimmt. Um den Einfluss von Epo auf Apoptose zu analysieren, wurden die Zellen unter normoxischen Bedingungen ebenfalls einmalig mit 100 U/ml Epo, dann zwei Tage später mit dem zytotoxischen Chemotherapeutikum Cisplatin behandelt und anschließend der Anteil an Zellen mit aktivierter Caspase 3 mittels Durchflusszytometrie ermittelt.

Ergebnisse: Wir konnten zeigen, dass alle drei NSCLC-Zelllinien den EpoR sowohl auf mRNA-, als auch auf Proteinebene expremierten, wobei die Rezeptortranskripte in NCI-H358 Zellen durch Hypoxie 2,3-fach hochreguliert wurden ($P = 0,011$). Unabhängig davon, waren die Ergebnisse der Rezeptoraktivierung nicht eindeutig und Epo konnte in diesen Zellen weder das Tumorwachstum beschleunigen, noch die Cisplatin-induzierte Apoptose verhindern.

Schlussfolgerung: Trotz des EpoR-Nachweises in den NSCLC-Zellen, führte die Gabe von Epo im Rahmen dieser Studie weder zu einem Vorteil im Tumorwachstum noch zu einer Chemotherapieresistenz. Dies unterstützt die sichere Anwendbarkeit von Epo bei anämischen Tumorpatienten.

Abstract

Introduction: Patients with lung cancer have the highest incidence of anemia among patients with solid tumors. The use of recombinant human erythropoietin (rHuEpo) and its derivatives have consistently been shown to reduce the need of blood transfusions and increase the hemoglobin level in lung cancer patients with chemotherapy-induced anemia. Over the past decade, there has been continually growing concern from clinical and preclinical studies that Epo and the presence of its receptor (EpoR) on tumor cells is responsible for adverse implications on the patient's outcome. The question has been raised what equivocal role do they play in the treatment of chemotherapy-induced anemia in lung cancer patients in terms of promoting tumor growth and inhibiting cell death in favor of tumor progression and reduced survival of the patient. The aim of this study was to investigate the presence and the functionality of EpoR and the implications of Epo upon growth and survival in lung cancer cells.

Materials and Methods: By using quantitative RT-PCR, Western Blot, and immunocytochemical staining, three non-small-cell lung cancer (NSCLC) cell lines (A427, A549, NCI-H358) were analyzed for the presence and the functionality of EpoR. After single administration of 100 U/mL Epo, proliferation was assessed via cell counting during three consecutive days under ambient and hypoxic conditions (1% O_2). To investigate Epo's effects on apoptosis, cells were also treated once with 100 U/mL Epo under ambient conditions, after two days the cytotoxic chemotherapeutic cisplatin was added, and cells with activated caspase-3 were measured via flow cytometry.

Results: Here, we demonstrate that all three NSCLC cell lines express EpoR on mRNA and protein level to a different degree, while it was significantly upregulated (2.3-fold) by hypoxia in NCI-H358 cells ($P = 0.011$). Nevertheless, while Epo-induced activation and signaling of the receptor remained ambiguous in part, Epo did not promote tumor growth or increased protection from apoptosis in these lung cancer cells.

Conclusion: Epo and the expression of its receptor in these NSCLC cells does not seem to play a pivotal role in tumor growth and survival, supporting the safety profile of Epo within this experimental model.

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Symbols and Abbreviations

Symbols and abbreviations

α	Alpha
β	Beta
γ	Gamma
δ	Delta
ω	Omega
°C	Degree Celsius
∅	Diameter
<	Less than
=	Equals
>	Greater than
ACTB	Beta-actin, β-Actin
ANOVA	Analysis of variance
APS	Ammonium persulfate
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-x_L	B-cell lymphoma-extra large
BSA	Bovine serum albumin
Cat. No.	Catalog number
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CO_2	Carbon dioxide
CT	Computed tomography
DAPI	4',6-diamidino-2-phenylindole
dH2O	Distilled water
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
EpoR	Erythropoietin-receptor
ERK	Extracellular-signal-regulated kinase
ESA	Erythropoietin-stimulating agents
FCS	Fetal calf serum
h	Hour/s
HRP	Horseradish peroxidase

IgG	Immunoglobin G
IMDM	Iscove's Modified Dulbecco's Medium
kDa	Kilodalton
KG	Kilogram
mA	Milliampere
min	Minute/s
mL	Milliliter
mRNA	Messenger ribonucleic acid
MW	Molecular weight
N_2	Nitrogen in its molecular form
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	Non-small-cell lung cancer
O_2	Oxygen in its molecular form
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
pO_2	Oxygen partial pressure
RBC/s	Red blood cell/s
rHuEpo	Recombinant human Erythropoietin
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute 1640 medium
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS-T	Tris-buffered saline and Tween 20
TEMED	N,N,N',N'-tetramethyl-ethane-1,2-diamine
U	Unit
V	Volt

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1 Introduction

1.1 Lung cancer and its anemic consequences

1.1.1 Lung cancer

As a malignancy that originates in the airway or pulmonary parenchyma, lung cancer, or bronchiogenic carcinoma, is the leading cause of cancer deaths in males and second in females worldwide in both developing and developed regions (1). The high mortality of this disease is in part due to the late diagnosis of the majority of lung cancers after regional or distant spread of the malignancy (2), at a point where only palliative treatment options are available (3). Early detection is often hampered due to the lack of biomarkers for early diagnosis of the disease and the fact that even though over 90% of the patients have become symptomatic when diagnosed, yet they present with rather unspecific symptoms, comprising dry cough, weight loss, shortness of breath, diffuse chest pain, or hemoptyses (3). On the contrary, recent findings from the National Lung Screening Trial, which was conducted by the National Cancer Institute in the USA, appeared to be quite encouraging since it revealed that participants who received low-dose helical CT scans had a 20% lower risk of dying from lung cancer than participants who received standard chest X-rays (4).

Histopathologically, the two major forms of lung cancer are small-cell lung cancer (SCLC), which accounts for around 15%, and non-small-cell lung cancer (NSCLC), with around 85% of all diagnosed lung cancers (2). NSCLC is comprised of three main histological subtypes: adenocarcinomas (45%), squamous-cell carcinomas (30%), and large cell carcinomas (10%) (2, 3). Pulmonary adenocarcinomas arise from small bronchi, bronchioles or alveolar epithelial cells, which is why they are typically peripherally located, whereas squamous-cell and small-cell carcinomas are centrally located since they arise from the major bronchi as depicted in Fig. 1 (2, 5). Alongside several other inhalative factors, smoking, and second-hand smoke display the predominant cause in the development of lung cancer (3). While squamous-cell and small-cell carcinoma pathogenesis is strongly linked to smoking, adenocarcinoma is more prevalent in never-smoker patients (5). In this respect, at the molecular level, two major signaling pathways are believed to

mediate lung adenocarcinoma development: an epidermal growth factor receptor (EGFR)-dependent pathway in never-smokers and a Kirsten rat sarcoma oncogene (KRAS)-dependent signaling pathway in smokers (5, 6).

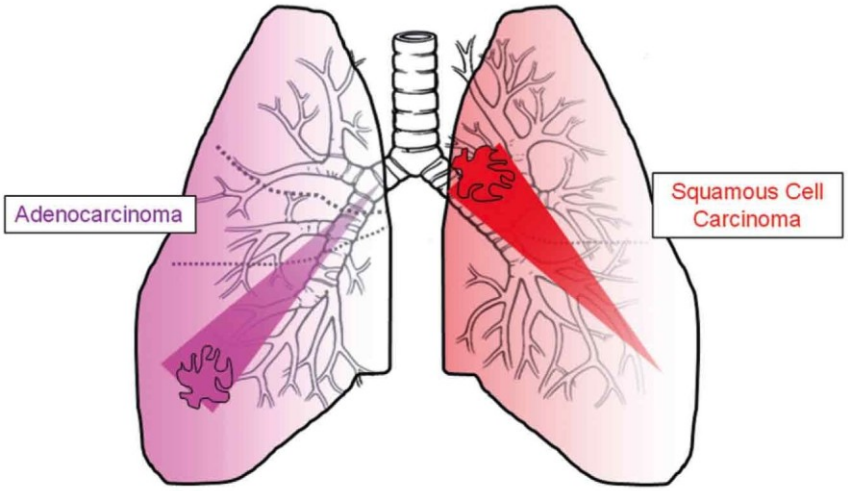

Fig. 1. Overview of the most prevalent localization of two subtypes of human NSCLC. Adapted from Kadara et al. (5).

The aforementioned delay of diagnosis is concurrent with the fact that regional or distant spread of the tumor is already present when diagnosed. As a feature of malignancy, lung cancer cells are able to break out of the local cell agglomeration into vessels and spread throughout the body with a tendency to affect bones, liver, brain, adrenal glands, and bone marrow, which in turn leads to organ failure with its specific symptoms (3). Although the therapeutical regimens differ throughout the lung cancer entities and with tumor progression in terms of staging and grading, the myelosuppressive chemotherapy is a common cornerstone of them all (3). In this regard, the alkylating antineoplastic platinum-based chemotherapy, comprising most importantly cisplatin but also carboplatin, has emerged to one of the standard therapies of most combination regimens studied in lung cancer (3, 7, 8).

Patients suffering from advanced lung cancer that has already infiltrated the bones and being treated with cytotoxic chemotherapeutics are very likely to report severe fatigue that adversely affects their daily life. Altogether, cancer patients are very likely to suffer from cancer-related fatigue. According to the National Comprehensive Cancer Network (NCCN), cancer-related fatigue is defined as “a persistent symptom, a subjective feeling of physical, emotional or cognitive tiredness or exhaustion related to cancer or its treatment that is not proportional to

the recently performed activity, and which can interfere with the usual patient's functional capacity" (9). The severity of fatigue, and the fact not being relieved by rest, are key characteristics that distinguish it from the fatigue of everyday life (10). Anemia is a major etiologic factor involved in the development of cancer-related fatigue (11) apart from other causing factors reviewed in Fig. 2.

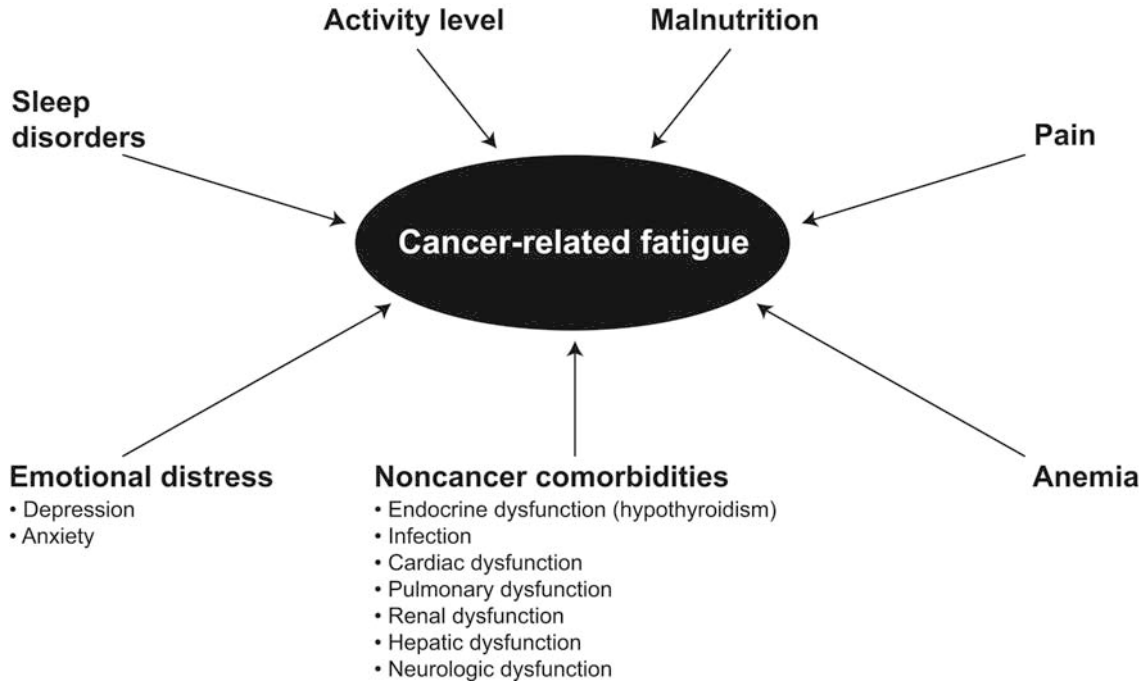

Fig. 2. Schematic overview of etiologic factors of cancer-related anemia. Adapted from Carroll et al. (12).

1.1.2 Anemia due to lung cancer

Patients suffering from lung cancer have the highest incidence of anemia among patients with solid tumors and approximately 50 to 70% of them experience anemia during the course of their disease (11).

In healthy human beings, the oxygen-carrying red blood cells (RBC) are produced in the bone marrow and are maintained by the stimulation of Epo that increases in function of the oxygen pressure in the kidneys and in minor amounts in the liver and in the brain (13).

Anemia is defined as a reduction of hemoglobin concentration, red-cell count, or packed cell volume (hematocrit) to below normal levels (10). The World Health Organization (WHO) and the National Cancer Institute (NCI) classified anemia, where grade 0 signifies hemoglobin values within normal limits: 12 to 16 g/dL for women and 14 to 18 g/dL for men (Tab. 1).

Severity	WHO	NCI
Grade 0 (WNL)	≥ 11.0 g/dL	WNL
Grade 1 (mild)	9.5 - 10.9 g/dL	> 10.0 g/dL to WNL
Grade 2 (moderate)	8.0 - 9.4 g/dL	8.0 - 10.0 g/dL
Grade 3 (serious/severe)	6.5 - 7.9 g/dL	6.5 - 7.9 g/dL
Grade 4 (life-threatening)	< 6.5 g/dL	< 6.5 g/dL

Tab. 1. Grading systems of anemia. WHO, World Health Organization; NCI, National Cancer Institute; WNL, within normal limits. Adapted from Wilson et al. (10)

The nature of anemia associated with cancer and cancer treatment is versatile where cancer is one important cause of anemia (10). The degree of anemia will often fluctuate notably during a cycle of chemotherapy in the sense that hemoglobin level will fall to a nadir 2 to 4 weeks after chemotherapy is given depending on the nature of the chemotherapy and the number of courses administered (10).

The cornerstones of the causing factors leading to cancer-related anemia are given in Fig. 3. In general, direct effects of the neoplasm, such as bleeding, altered iron absorption, and bone marrow replacement due to metastasis of solid tumors (myelophthisis) play an important role (10). Lung cancer itself is able to cause a relative inadequacy of Epo production and impaired erythroid bone marrow response to Epo stimulation which in turn leads to anemia (14). On the other hand, the neoplasm can produce factors, such as cytokines, that impair the RBC production and thus blunt the bone marrow response on naturally secreted Epo, which is why this circumstance may be designated as anemia of inflammation. Interleukin-1 and -6 were identified to be produced by cancer cells and to cause this blunting, while the plasma levels of Epo were slightly above normal (15). In addition to cytokines, other factors may be involved that influence the blood composition in terms of coagulation leading to thrombosis (Trousseau sign) in the course of a paraneoplastic syndrome (16). The third group is comprised of the effects of cancer treatment, such as ionizing radiation and systemic antineoplastic therapy that are potentially able to cause anemia (10). More precisely, the myelosuppressive mechanisms of chemotherapeutics commonly used in lung cancer therapy, such as alkylating platinum-based agents (e.g. cisplatin) and topoisomerase-2 inhibitors (e.g. etoposide) (3), mainly include stem cell death, suppression of renal Epo synthesis due to nephrotoxicity (e.g. cisplatin) (17), oxidant damage to mature hematopoietic cells (18), induction of immune-mediated hematopoietic cell destruction (e.g. cisplatin) (18), and acute bone marrow stromal

damage (19). Furthermore, other potential causes of cancer-related anemia include hemolysis, frequent blood sampling for testing, and reduced appetite due to the tumor itself or chemotherapy causing nutritional deficiencies (iron, vitamin B_{12}, folic acid) (10).

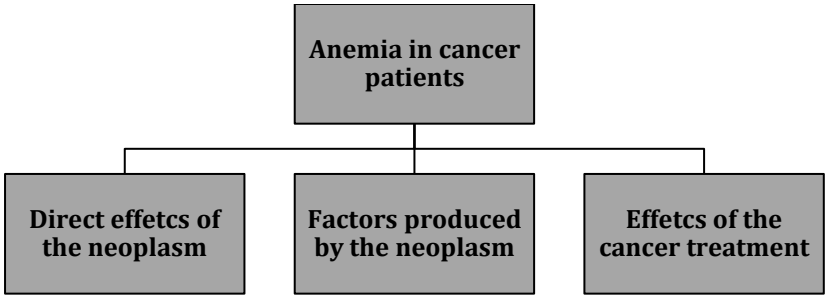

Fig. 3. Overview of different etiologies in cancer-related anemia. Examples are explained in the text.

Given the fact that all organs of the body require oxygen, it is foreseeable that anemia produces diverse symptoms. Fatigue, dizziness, vertigo, headache, pallor, shortness of breath, angina pectoris, heart failure, palpitations, depression, and loss of libido among others are the most common and impairing symptoms of anemia leading up to a significant decrease in the health-related quality of life (20) (Tab. 2).

Part of body affected	Compensatory mechanism	Dysfunction
Brain		Fatigue, tiredness, headaches, dizziness, difficulty thinking/concentrating, depressed mood.
Eyes		Retinal damage.
Heart	Rapid pulse, palpitations	Angina pectoris.
Lungs	Rapid breathing, breathlessness	In severe cases, worsened breathlessness from pulmonary oedema secondary to heart failure.
Kidneys		Water retention.
Gut	Loss of appetite	Indigestion, irregular bowel movements, failure to absorb nutrients from food.
Muscles/legs		Fatigue, reduced exercise capacity, swelling secondary to water retention (due in turn to kidney and heart failure).
Skin	Pallor, feeling cold	Brittle and broken nails.
Reproductive organs		Increased menstrual bleeding, loss of periods, impotence, decreased libido.

Tab. 2. Overview of important symptoms of anemia and possible compensatory mechanisms. Adapted from Wilson et al. (10).

Aside from the fact that the severity of these symptoms depends indeed on the absolute hemoglobin level, the impact on the patient's quality of life is influenced as well by the rapidity of onset, the compensatory mechanisms, and the comorbidities (10, 11).

Of paramount importance is the finding from a systematic, quantitative review that anemia was identified as an independent prognostic factor for survival in patients suffering from lung cancer and is associated with shorter survival times (21). This analysis was later confirmed and complemented by the findings that anemia in early stage NSCLC patients may be a sign of a more aggressive tumor that is at higher risk of failure independent of the treatment modality (22).

To adequately assess anemia, the focus should be on symptoms and severity of anemia as well as laboratory findings comprising storage iron, vitamin levels (vitamin B_{12}, folic acid), and impact on activities of daily life (11). Further, comprehensive questionnaires on health-related quality of life are applied, such as the Linear Analogue Self-Assessment (LASA), the Functional Assessment of Cancer Therapy- Anemia (FACT-An) scales, and the FACT-Fatigue (11, 23). FACT-An and FACT-Fatugue allow a more sophisticated evaluation of anemia, fatigue, and quality of life in cancer patients by differentiating them in terms of the hemoglobin level and performance status.

Taken together, anemia is a major cause of cancer-related fatigue that largely decreases quality of life, causes vast impairment in the patient's daily life, and is associated with a more aggressive tumor and shorter survival in patients with lung cancer.

1.2 Therapeutic options for cancer-related anemia

Given the impact of anemia in cancer patients and the resultant fatigue, its correction would contribute to an amelioration of the symptoms, well-being, and thus the health-related quality of life that would in turn positively affect the patient's compliance with anti-cancer therapy.

In addition to the positive effects in quality of life, hypoxia in the tumoral tissue may be diminished by hemoglobin elevation in anemic cancer patients. In this respect, tumor hypoxia results from an imbalance between cellular oxygen consumption and the oxygen supply to the cells (24). The three principle mechanisms leading to

tumor hypoxia are related to perfusion, diffusion, and anemia (25), while the latter can be corrected through elevated hemoglobin levels. Resistance to radio- and chemotherapy regimens, subsequent tumor progression and poor prognosis has been linked to hypoxic tumors due to anemia, and could be counteracted by increased tumor oxygenation (11, 25).

Thorough investigation of the cause of anemia is essential since it will determine the treatment. According to the 2010 updated clinical practice guidelines issued by the American Society of Hematology in cooperation with the American Society of Clinical Oncology, investigation is comprised of an appropriate history along with a full drug exposure history, physical examination, and diagnostic tests that includes principally peripheral blood-smear, assessment of reticulocyte count, occult blood loss and renal insufficiency (26).

Undirected treatment will not ameliorate the patient's status, if anemia is not the underlying cause. On the other hand, if anemia is not a causative factor for the patient's fatigue, its correction will not improve the patient's well-being either.

1.2.1 Correction of metabolic deficiencies

As delineated above, the etiology of anemia in cancer patients is versatile. Aside from chemotherapy or an underlying hematopoietic malignancy as a cause of anemia, metabolic deficiencies in iron storage, vitamin B_{12}, or folic acid can be supplemented (10). Other causes, such as bleeding predispositions due to coagulation or clotting effects can be treated through restoring of the missing clotting factors (10).

1.2.2 Red blood cell transfusion

Red blood cell transfusion has proved to be a therapeutical option for patients with severe or symptomatic anemia that immediately but often only transiently improves anemia-related symptoms (10, 11). As a rule of thumb, it is estimated that one unit of red blood cells will raise the hemoglobin level of most adults within an hour by 1 g/dL (27). One unit of packed RBC consists of a mixture of young to old RBCs, while approximately 50% of the transfused RBCs will disappear after 50 days, which implies that after 90 days only 0.1 g/dL hemoglobin increase will be left (10).

The clinical impact of RBC transfusion in the treatment of anemia in patients with lung cancer is clearly conveyed by the findings of a large-scale audit of patients with a variety of cancers receiving chemotherapy (28). Approximately 43% of all lung cancer patients required at least 1 RBC transfusion while approximately 22% required more than one transfusion, which exhibited in both cases the highest percentage of all cancers under study. Furthermore, around 25% of the patients receiving blood transfusions required an inpatient admission and overnight stay. Other authors reported as well high blood transfusion requirements in lung cancer (29). Possible reasons for suchlike high transfusion rates are explained by the fact that the potent myelosuppressive chemotherapeutics cisplatin and etoposide are often associated with anemia in lung cancer treatment that required blood transfusions (29).

Apart from the advantage of immediate onset of effect, transfusion of RBCs entails certain risks. In general, possible hazards of blood transfusion are incorrect blood component transfused, acute and delayed transfusion reactions (adverse reactions before or after 24 h, respectively), transfusion-related acute lung injury (TRALI), transfusion-associated graft-versus-host disease, post-transfusion purpura, transfusion-transmitted infections, near-miss events, and adverse reactions associated with autologous transfusion (30).

Altogether, RBC transfusion in anemic lung cancer patients provides a prompt correction of red blood cells and hemoglobin levels that potentially relieves anemia-related fatigue. Nevertheless, several serious hazards are linked to this procedure, whose likelihood is statistically increased in diseases that are associated with high transfusion rates due to anemia, such as in lung cancer.

1.2.3 Recombinant human erythropoietin

Recombinant human Epo provides an alternative to RBC transfusions (31).

Ever since the first synthesis of a recombinant protein therapeutic of Epo, and the successful clinical trial by Eschbach and colleagues in 1987 (32), new possibilities have opened up for an effective treatment of anemia. In further consequence, several synthetic forms of Epo were launched, and named Epoetin (-α, -β, -δ, -ω), and Darbepoetin (-α) subsumed under the general term of erythropoiesis-stimulating agents (ESA) (31). While the amino acid sequence of all Epoetins is

the same, they differ in the carbohydrate structure and, thus, isoforms, whereas Darbepoetin-α differs in five amino acids from Epoetin and has two additional N-glycans (33). A novel Epoetin-β is developed complemented with a polyethylene glycol (PEG) polymer and designated as 'continuous erythropoiesis receptor activator' (CERA) because it provides the highest plasma half-life of all ESAs (Fig. 4) (33).

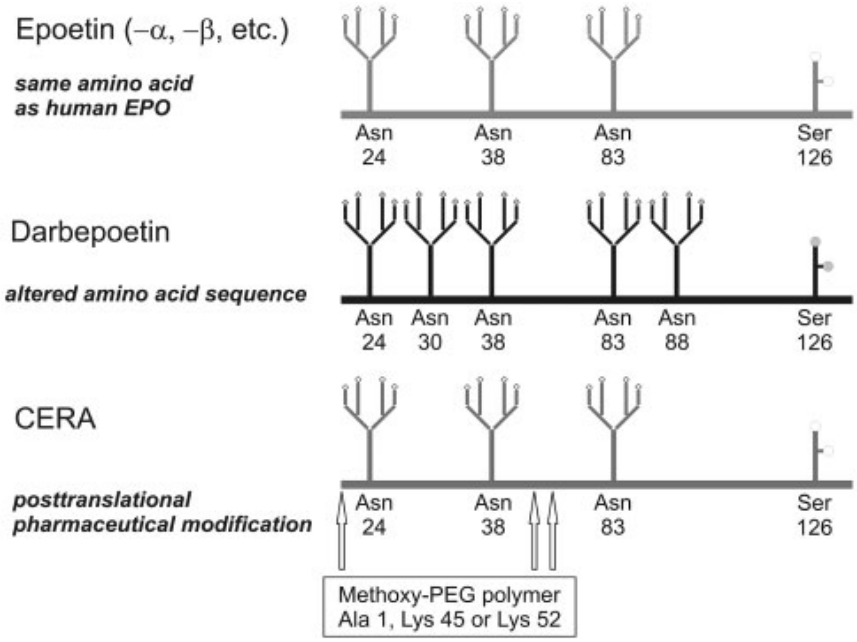

Fig. 4. Schematic overview of the structure of Epoetin, Darbepoetin and CERA (modified Epoetin-β). Adapted from Jelkmann et al. (33).

1.3 Clinical impact on cancer patients receiving ESA

1.3.1 Evidence on potential benefits

Two randomized controlled clinical trials independently provided evidence that both Epoetin alfa and Darbepoetin alfa were able to improve quality of life in lung cancer patients based on LASA or FACT-Fatigue assessment (34, 35). Further, other authors attributed ESA a potential role in alleviating cancer-related fatigue and improving quality of life (11).

Despite these trials, as reviewed in the 2010 clinical practice guideline update for the use of ESA, the only benefit of ESA treatment that has been consistently demonstrated in randomized, placebo-controlled, double-blinded trials and in most recent meta-analyses is to decrease transfusion rates including its risks (26).

1.3.2 Evidence on potential harms

First concerns about the safety profile of ESA use in cancer treatment were raised in 2003 when two large randomized controlled trials issued an increase in tumor progression and mortality in comparison to controls in head and neck (Henke et al. (36)) as well as breast cancer patients (Leyland-Jones et al. ((37))). Including another study, that had to be closed prematurely due to increased thromboembolic and cardiovascular events (38), these studies prompted an Oncologic Drugs Advisory Committee hearing at the Food and Drug Administration (FDA) where the safety properties of ESAs were discussed (31). The following trials confirmed the worrisome risks of ESA use in cancer patients concerning a decrease in patient's overall survival (39), and even decreased progression free survival (40). In this regard, Wright et al. (41) found in an unplanned interim safety analysis in patients with unresectable, advanced NSCLC within the EPO-CAN-20 trial, that the overall survival was decreased in these lung cancer patients that were treated with Epoetin alfa. While these findings led to a premature termination of the study, a significant improvement in quality of life in the ESA-treated arm, earlier defined as the actual end-point of the study, was not shown by then.

In a review, Juneja et al. (42) criticized the design of the studies conducted by Henke, Leyland-Jones, and Wright et al. for aiming too high target hemoglobin levels (> 12 g/dL) than those recommended by current ESA labeling (26). Thus the hemoglobin targets in these studies represented off-label dosing regimens of ESAs in not anemic patients at baseline. There is evidence that an increase of hemoglobin > 14 g/dL would increase viscosity and resistance flow within the chaotic microvasculature of the tumor which in turn would lead to a reduction in oxygen supply (43), which is suggestive of an increase in resistance to chemo- and radiotherapy.

Taken together, serious concerns on the safety profile of ESA use has been published but further clinical investigations with subsequent large-scale meta-analyses are needed to evaluate the influence of tumor progression in cancer patients.

1.3.3 Recommendations of ESA in cancer patients:

Multiple studies und subsequent meta-analyses have provided robust evidence that ESA treatment is potently able to increase hemoglobin levels and reduces the likelihood of RBC transfusions in cancer patients which is comprehensively reviewed by Rizzo et al. in the 2010 clinical practice guideline update on the use of Epoetin and Darbepoetin in adult patients with cancer jointly on behalf of the two American Societies of Hematology and Clinical Oncology (26). These beneficial and salutary effects of ESA determine its indication, likewise. As per 2010 recommendations, ESAs are a therapeutic option that may be considered in patients with chemotherapy-associated anemia and a hemoglobin concentration that has decreased to less than 10 g/dL to decrease blood transfusions (26).

Rizzo et al. concluded in this large-scale meta-analysis that ESAs in patients with chemotherapy-induced anemia only lead to small, statistically significant increases in quality of life, which again was put into perspective since the difference in quality of life in these studies did not meet the psychometric definition of a clinically meaningful change (26). Furthermore, the Update Committee recommended that the goal of ESA use should be to avoid transfusions, without specific consideration of improvement in quality of life in conjunction with clinical judgment of individual risks, benefits, treatment goals, and discussions with patients (26).

It is worth noting that the Food and Drug Administration (FDA) (26) in the USA and the European Medicines Agency (EMEA) (44) decided unanimously in 2008 and 2010 not to approve of ESA use for patients receiving chemotherapy for curative but for palliative intent. The FDA emphasized as well that no study has evaluated by then outcomes of ESA therapy by subgroups defined chemotherapy intent, which is why the goal of the treatment would require clinical judgment in many cases (26).

1.4 Biology of Epo and its receptor in normal and tumor cells

The molecular regulation of the homeostasis of red blood cells that are produced in the bone marrow and send out into the circulation has been extensively investigated and is reviewed most notably by Jelkmann (13, 31), Tsiftsoglou (44), and Ingley (45).

This chapter will focus on the fundamental role of the interaction of Epo and its receptor in hematopoietic cells and their implications on non-hematopoietic and tumor cells.

1.4.1 Expression of EpoR in hematopoietic cells

Principally, the expression of human Epo gene is regulated by hypoxia (13). The most important consequence of hypoxia for a balanced erythropoiesis is its detection in the peritubular fibroblasts in the renal cortex that results in increased stabilization of the transcriptional activator hypoxia inducible factors (HIF)-1α and 2α, which in turn initiates transcription of several hypoxia inducible genes, of which one is the Epo gene. For erythropoiesis, systemic Epo protein exerts its influence on the erythrocytic bone marrow progenitors via its receptor. For the early progenitor cells, this results in increased proliferation, and inhibition of apoptosis, and followed by induction of differentiation in progenitor cells that become committed to maturation through irreversible growth arrest and biosynthesis of heme, globins, and hemeproteins (44) (Fig. 5).

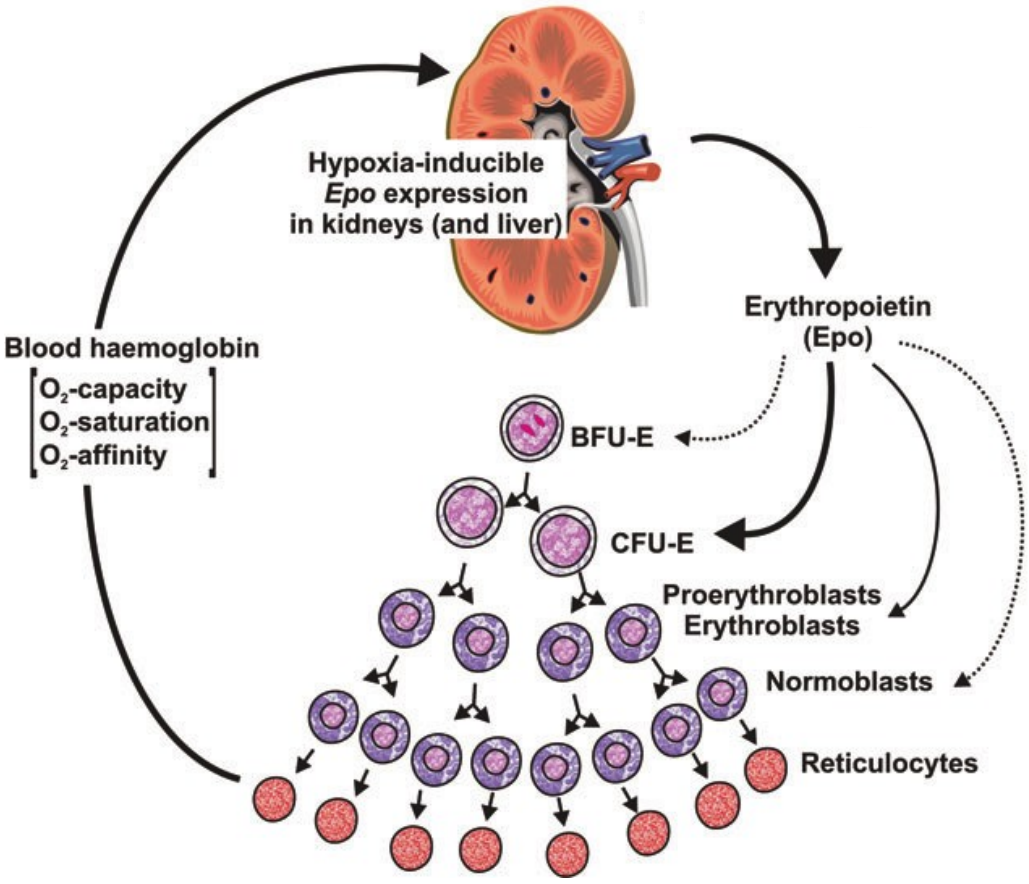

Fig. 5. Schematic overview of the feedback regulation of erythropoiesis. As a survival, proliferation and differentiation factor for the erythrocytic progenitors, Epo particularly stimulates the colony-forming units-erythroid (CFU-Es). **BFU-E**, burst-forming unit-erythroid. Adapted from Jelkmann et al. (13).

Hence, EpoR exhibits the function of a speaking tube that transmits extracellular information into the progenitor cells leading to survival, enhanced cell division, and differentiation.

As a type-1, single-transmembrane cytokine receptor, EpoR is comprised of an extracellular region receiving the Epo protein, followed by a transmembrane and an intracellular region.

There is evidence that EpoR gene undergoes alternative splicing leading to different EpoR variants (46). The full-length form (F-EpoR) is complete and fully functional and weighs approximately 59 kDa, while the truncated form (T-EpoR) lack parts of the cytoplasmic carboxy-terminal portion, yet still sufficient to induce proliferation (31). A soluble third variant (S-EpoR) contains only the extracellular portion suggesting an antagonizing role of Epo in the sense of a decoy receptor (Fig. 6).

Epo binds to two EpoR molecules working as a homodimer, which results in conformational changes of the receptor and subsequent binding of two Janus protein tyrosine kinase 2 molecules (JAK2) to the membrane proximal part of the cytoplasmic region. These changes lead to autophosphorylation and activation of JAK2, which in turn phosphorylates 8 tyrosine residues in the distal part of the cytoplasmic region of the receptor (47). Then, four key signaling pathways are initiated (Fig. 6):

1) *STAT5 pathway (signal transducer and activator of transcription 5):*

JAK2 activates STAT5 via phosphorylation, which then homodimerizes and is translocated into the nucleus in order to enhance the transcription of the anti-apoptotic protein Bcl-x_L (31). Bcl-x_L in turn prevents the activation of effector caspase-3 via inhibition of cytochrome c release from the mitochondria resulting in a missing initiation of apoptosis (48).

2) *PI3K pathway (phosphatidylinositol 3-kinases):*

JAK2 phosphorylates several proteins in an activating manner that leads to the activation of PI3K and a subsequent cascade of phosphorylating activation. Phosphatidylinositol 4,5-bisphosphate (PIP_2) becomes phosphorylated and activates phosphoinositide-dependent kinase-1 (PDK1), which again activates AKT (protein kinase B). As a serine threonine kinase, AKT is able to phosphorylate and now inactivates the pro-apoptotic proteins Bad and the initiator caspase-9, leading to an inhibited initiation of apoptosis (31).

3) MAP kinase pathway (mitogen-activated protein kinase):

Phosphorylated EpoR is able to activate several pathways that lead into the MAP kinase pathway (49). Here, via several adaptor proteins, EpoR activates the monomeric G-protein Ras, which in turn triggers a phosphorylation cascade beginning with Raf (MAP kinase kinase kinase), then MEK 1/2 (MAP kinase kinase), and finally ERK 1/2 (MAP kinase) which is subsequently translocated to the nucleus and induces transcription of cell cycle promoting proteins, such as cyclin D1.

4) PKC pathway (protein kinase C):

Activated EpoR enables phospholipase C-γ to cleave membrane-bound PIP_2 into diacylglycerol (DAG) and inositol trisphosphate (IP_3). After IP_3 opens calcium channels in the endoplasmic reticulum and increases intracellular calcium concentration, protein kinase C is activated by the calcium and is translocated to the membrane-bound DAG, where it phosphorylates several proteins important for cell cycle progression and survival (31).

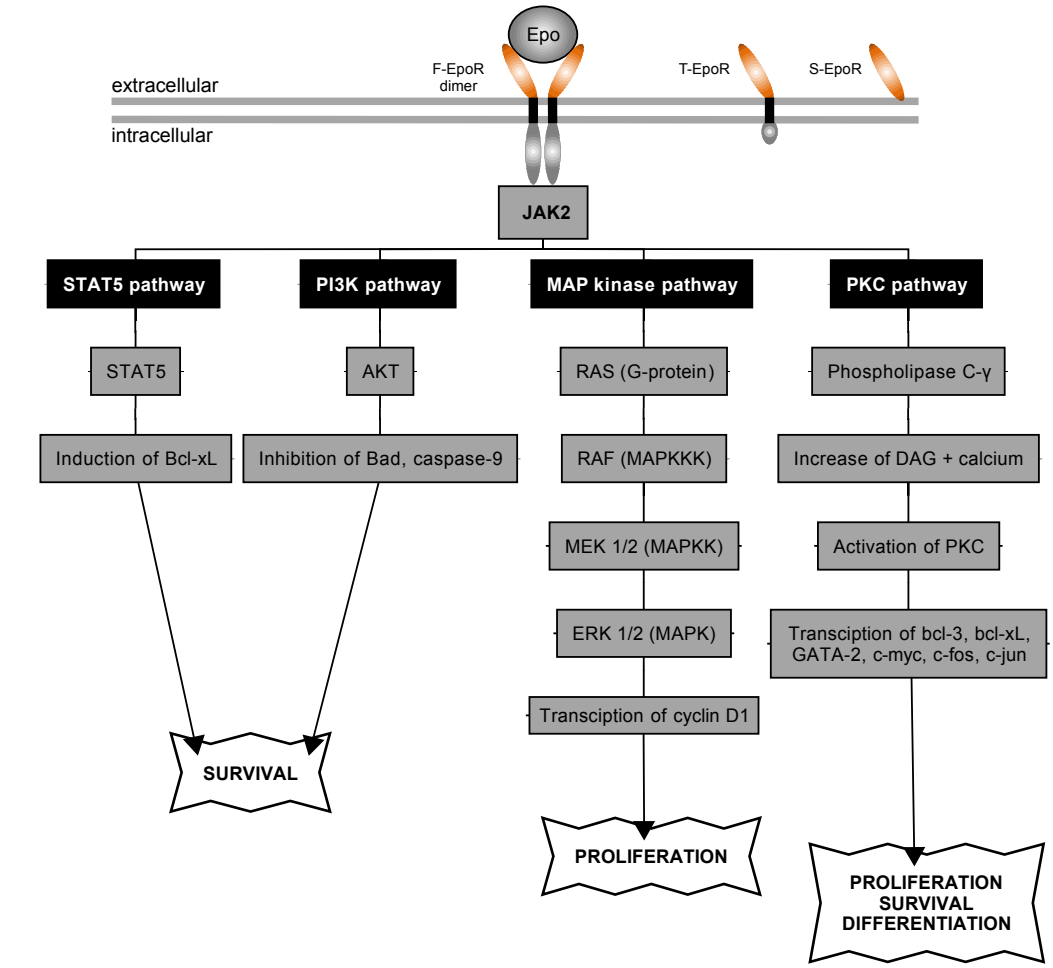

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Fig. 6. Schematic overview of Epo signaling via activated EpoR and its splice variants. F-EpoR represents the functional, full-length, cell surface receptor as a homodimer with complete cytoplasmic portion, while the truncated EpoR (T-EpoR) possesses a short cytoplasmic portion, but sufficient to induce Epo-dependent proliferation. The soluble EpoR (S-EpoR) exclusively consists of an extracellular portion. The activated pathways sustain survival via induction of anti-apoptotic proteins (Bcl-x_L) and inhibition of pro-apoptotic proteins (Bad, caspase-9) as well as proliferation, and differentiation in hematopoietic progenitor cells. **JAK2**, Janus kinase-2; **STAT5**, signal transducer and activator of transcription 5**; Bcl-x_L,** B-cell lymphoma-extra large; **PI3K**, phosphatidylinositol 3-kinases; **AKT**, protein kinase B, **Bad**, BCL2-associated agonist of cell death; **MAP kinase**, mitogen-activated protein kinase; **ERK**, extracellular signal-regulated kinases; **PKC**, protein kinase C; **DAG**, diacylglycerol, **c-myc**, myelocytomatosis cellular oncogene; **c-fos**, FBJ murine osteosarcoma viral oncogene homolog; **c-jun**, jun proto-oncogene; **K**, kinase.

It is worth noting that in these quite complex cascades of activation and inhibition of molecule activity, the addition of phosphate groups is able to change the molecule's activity in both directions, depending on the site of phosphorylation within the molecule (31). Further, there is a vast interplay between these cascades described, which is why they cannot be regarded separately without respect to one another.

Eventually, three major feedback mechanisms in the Epo-EpoR system have been found to avoid hyperstimulation that become effective soon after receptor activation (47):

1) Phosphatases dephosphorylate JAK2 and tyrosine residues in activated EpoR;
2) Inhibition of signaling through suppressors of cytokine signaling (SOCS);
3) Ubiquitination of EpoR resulting in proteasome- and subsequent lysosome-mediated protein degradation. This step leads to receptor downregulation.

To sum up the effects of Epo, the activation of EpoR and its several pathways lead to cell survival, proliferation, and differentiation.

1.4.1.1 Principles of apoptosis with focus on Epo

In the following chapter, to put it simply, only the apoptotic mechanisms important to understand the thinking behind the experiments are depicted at the expense of a comprehensive explanation of its tight regulation.

Apoptosis leads to controlled cell death in the sense of “death of one cell for the benefit of the whole organism”. Consequences are degradation of the genome and termination of the cell cycle machinery. Among several mechanisms to trigger apoptosis, the caspase-induced intrinsic and the extrinsic pathways are two of the most well-investigated (48).

The intrinsic pathway is initiated mostly due to irreparable DNA damage sensed by P53 (48). As a consequence, P53 upregulates several pro-apoptotic proteins, such as Bax and Bak which will gather to form channels in the outer membrane of the mitochondria to release cytochrome c (48). Cytosolic cytochrome c molecules bind to Apaf-1, the apoptotic peptidase-activating factor 1, which then forms a complex with pro-caspase-9, which becomes activated. The activated initiator caspase-9 is able to activate effector caspase-3 (-6, -7), which in turn executes the apoptotic program in an irreversible manner. The release of cytochrome c can be blocked by the anti-apoptotic proteins Bcl-2, or Bcl-x_L (48). In this sense, Epo is able to upregulate Bcl-x_L in order to prevent the initiation of apoptosis.

The extrinsic pathway on the other hand is likely to be initiated by immune cells, such as T lymphocytes, or humoral factors that harm the cell, like members of the tumor necrosis factor family as well as simple deprivation of growth factors or extended hypoxia. Binding of these triggers to the specific death receptor leads to its activation by recruiting Fas –associated death domain (FADD) and pro-caspase-8 which forms a death inducing signaling complex (DISC) (48). As a

result, activated initiator caspase-8 is able to activate effector caspase-3 (-6, -7), which leads to definite initiation of apoptosis. A plain overview of the principles of apoptosis and its consequences is given in Fig. 7.

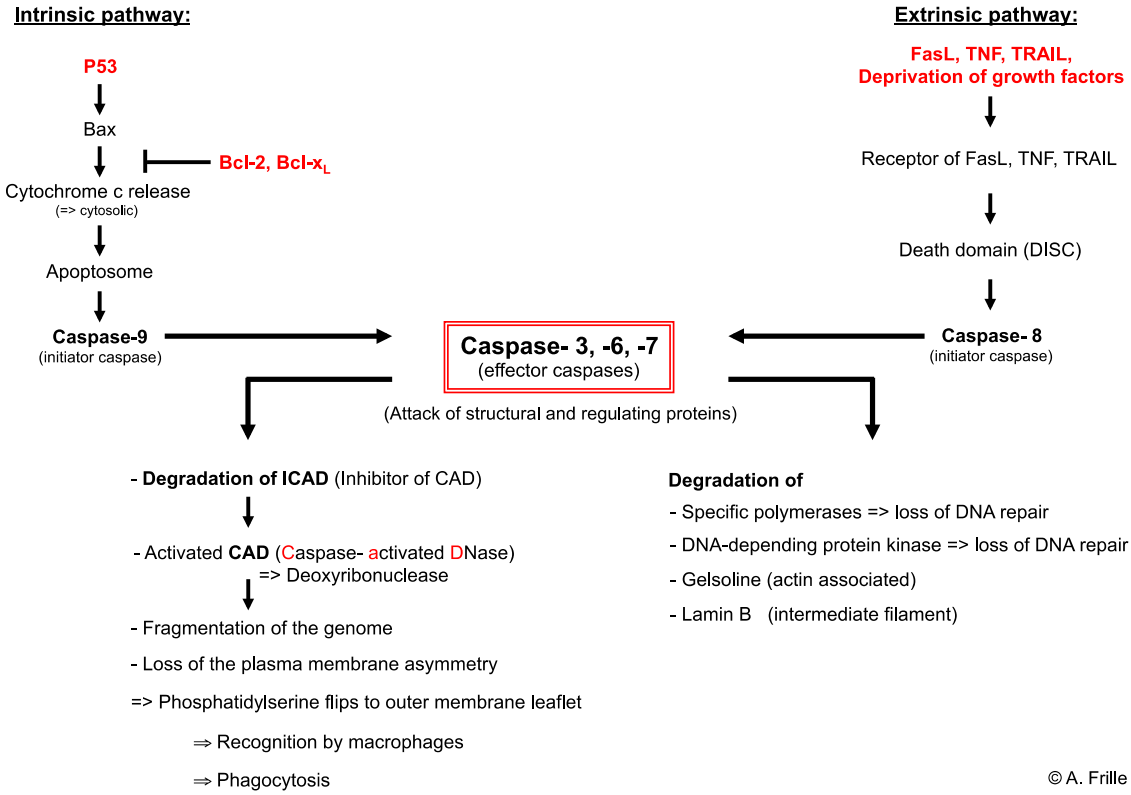


Fig. 7. Schematic overview of apoptosis in human cells. Activation of effector caspase 3 induces irreversible initiation of apoptosis. Epo induces Bcl-x_L to prevent cytochrome c release from the mitochondria into the cytosol. **Bax**, Bcl-2-associated X protein; **Bcl-2**, B-cell lymphoma 2; **Bcl-x_L**, B-cell lymphoma-extra large; **FasL**, Fas ligand, CD95 ligand; **TNF**, tumor necrosis factor; **TRAIL**, TNF-related apoptosis-inducing ligand; **DISC**, death-inducing signaling complex.

1.4.2 Expression of EpoR in non-hematopoietic, non-malignant cells

In addition to the hematopoietic cells, functional EpoR was identified in non-hematopoietic, non-malignant cells suggesting that Epo may play a broader role as a pleiotropic cytokine throughout the entire organism (Tab. 3).

EpoR expression	Function
Astrocytes	Decreased apoptotic cell death
Cardiomyocytes	Mitogenic
Endothelial cells	Mitogenic, endothelin-1 synthesis and release, angiogenic response (proliferation and migration)
Megakaryocytes	Maturation
Mesangial cells	Increased proliferation in vitro
Myeloid cells	Multilineage increase in vitro, immunomodulation
Neurons	Trophic effect, increased monoamine concentration, decreased apoptotic cell death
Renal cells	Mitogenesis
Prostate epithelial cells	Mitogenesis
Vascular smooth muscle cells	Contraction

Tab. 3. EpoR expression and potential functions in normal non-hematopoietic, non-malignant cells. Adapted from Weiss et al. (50).

In a physiological sense, Epo has proved to be implicated in the regulation of cyclic uterine angiogenesis (51) and in the wound healing cascade (52). Additionally, exogenous Epo was shown to be associated with the induction of cellular proliferation in the kidney (53), muscle cells (54), and the intestine (55), in which the EpoR was identified. Other studies demonstrated pro-angiogenic effects of Epo in endothelial cells (56) that were further described as similar to these of the vascular endothelial growth factor (VEGF) (57). Furthermore, some studies could prove that administered rHuEpo improves neuronal survival, by decreasing apoptosis and inflammation (58) as well as in clinical trials, in which Epo showed improvements in clinical recovery and outcomes in acute ischemic stroke patients (59, 60).

At a molecular level, the anti-apoptotic, tissue-protective properties of Epo could be associated in cardiac and neuronal cells with a heterodimerization of EpoR and the common β receptor (cβR), which is a signaltransducing subunit shared by the granulocyte-macrophage colony stimulating factor, and the interleukin-3 and -5 receptors (61) (Fig. 8).

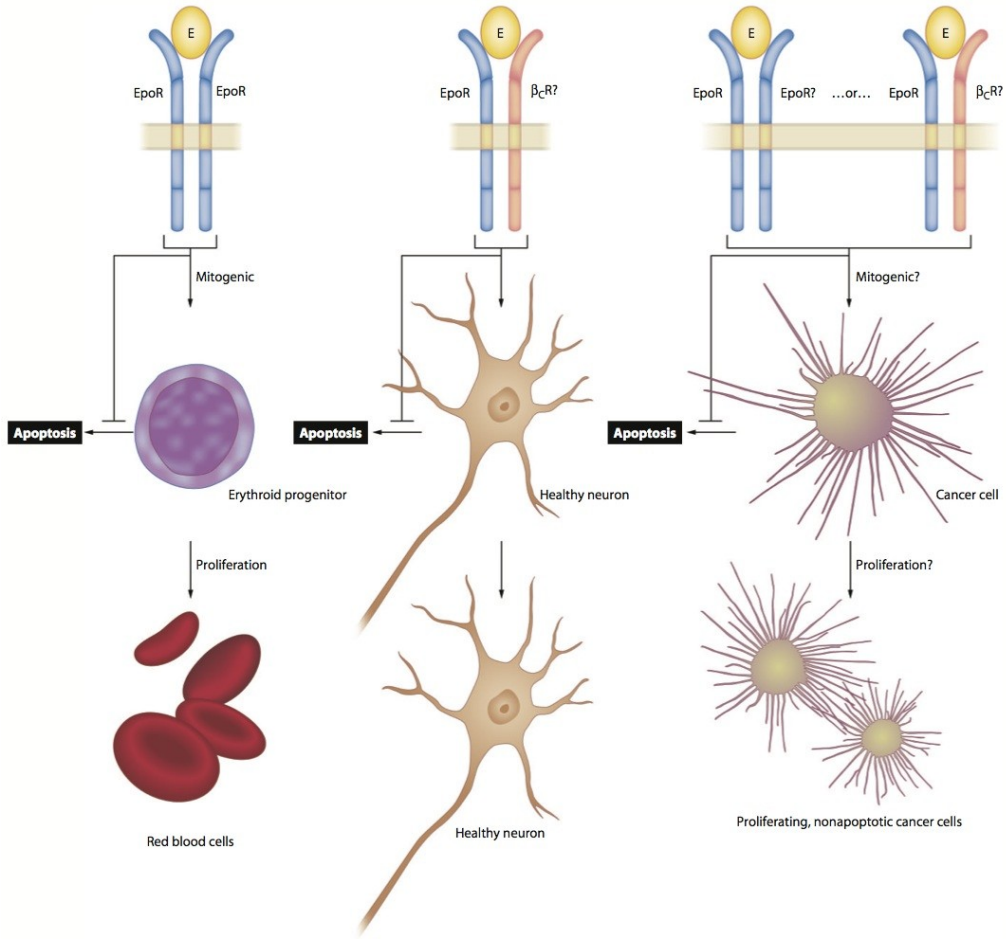


Fig. 8. Overview of Epo's different mode of action in erythropoiesis (**left**), tissue protection (**center**), and the potentially effect on cancer cells (**right**). The Epo molecule binds to the EpoR-EpoR homodimer on hematopoietic (**left**) and cancer cells (**right**) or to the tissue-protective EpoR-βcR heterodimer on healthy, non-hematopoietic (**center**) and cancer cells (**right**). In addition to the anti-apoptotic effects, Epo has a mitogenic effect on hematopoietic progenitors and possibly on cancer cells. E, Epo, βcR, common β receptor. Adapted from Sytkowski et al. (62).

1.4.3 Expression of EpoR in tumor cells

At least since the alarming results of the clinical trials showing adverse effects of Epo treatment in cancer patients with chemotherapy-induced anemia, the attention has been drawn as well to the detection of Epo and EpoR in tumor cell cultures and ex-vivo tissues. The critical question has been raised whether tumor cells express the EpoR and whether they are able to take advantage likewise of the pro-survival, and growth promoting effects of Epo. For several cancer cell lines and ex-vivo specimens, EpoR was identified on mRNA and protein level, as comprehensively reviewed by Sinclair (46), and Österborg (63). In some of them, Epo induced a significant growth advantage (64, 65), reduced initiation of apoptosis (66, 67), and increases angiogenesis (68, 69) in cancer cells under in-vitro conditions.

Moreover, several cancer lines expressed the Epo protein in addition to EpoR suggesting a certain autocrine-paracrine loop of Epo as a strategy to survive and to proliferate (66, 70).

Notably, there is evidence, that NSCLC cells from ex-vivo tissues express both EpoR and Epo while a NSCLC cell line demonstrated a functional EpoR that activated three key Epo-signaling pathways (71, 72).

1.5 From the bedside back to the bench – the aim of the study

What are the possible mechanisms at cellular and molecular level that lead to the adverse clinical findings of Epo in lung cancer patients?

To answer this question, we conducted an in-vitro study that investigated the presence and the functionality of EpoR and the implications of Epo upon growth and survival in three adenocarcinoma cell lines (A427, A549, NCI-H358). With the hypoxia-inducible factor Epo in mind, we mimicked the hypoxic microenvironment of a tumor by performing large parts of the experiments under hypoxia. As a proof of principle for the experimental design, we utilized an EpoR-positive, Epo-depending cell line to reevaluate our results.

With the help of this study, we hope to further our understanding of the biology of Epo and its receptor in lung cancer and to play a part in contributing to the benefit of these patients in the treatment of chemotherapy-induced anemia.

2 Materials and methods

To probe the influence of Epo on selected adenocarcinoma cell lines, we performed techniques that comprise mainly cell culture, RNA isolation and qRT-PCR, Western blot analyses on unstimulated and Epo-stimulated cells as well as inverted phase contrast microscopy, immunocytochemistry using epifluorescent and confocal microscopes. Parts of the proliferation studies were conducted under hypoxia.

2.1 Cell culture

2.1.1 NSCLC cell lines

The human NSCLC cell lines A427 and A549 were purchased from Cell Lines Service (Eppelheim, Germany), and were cultured in DMEM-F12 culture medium (Gibco®, Paisley, UK). The NSCLC cell line NCI-H358 was purchased from American Type Culture Collection (ATCC®, Manassas, VA), and was cultured in RPMI-1640 culture medium (Roswell Park Memorial Institute medium, ATCC®).

2.1.2 UT-7/Epo

The Epo-dependent human megakaryoblastic leukemia cell line UT-7/Epo was a generous gift from Prof. Dr. Norio Komatsu (Department of Hematology, Juntendo University School of Medicine, Tokyo), and was cultured in IMDM (Gibco®). It served as a positive control for EpoR (73). According to the protocol established by Erickson-Miller et al. (74) for a sufficient maintenance of cell metabolism and cell growth, the UT-7/Epo cell line was supplemented with 0.4 U/mL of the rHuEpo Epoetin alfa (Erypo® 10,000 U/mL, Janssen-Cilag Pharma G.m.b.H., Vienna, Austria) every three to four days in the course of medium change.

2.1.3 Maintaining cells in culture

The culture media were supplemented with 10% FCS (Biowest®, Nuaillé, France), 2 mM L-glutamine (Gibco®), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco®).

All four cell lines were cultured in a humidified incubator providing 21% O_2 and 5% CO_2 at 37 °C referred to as normoxia, normoxic, or ambient condition.

Cells were checked microscopically regularly to ensure they are healthy and growing as expected.

2.2 Hypoxic treatment

All four cell lines were as well cultured in a humidified incubator at 37 °C in an atmosphere containing 1% O_2 and 5% CO_2 through a N_2 and a CO_2 gas mixture (Air Liquide, Paris, France) in the automated Xvivo system G300CL (BioSpherix®, Lacona, NY) that served as a hypoxic processing chamber. For the purpose of acclimatization, cells were preincubated for three days for most of the experiments.

2.3 Proliferation and viability

To assess the influence of Epo on proliferation in these NSCLC cells, electronic pulse area analysis (CASY®, Innovatis, Reutlingen, Germany) as a method of cell counting was conducted for all four cell lines with and without Epo both under normoxia and hypoxia for up to 72 h. Therefore, both NSCLC cell lines (A427, A549, NCI-H358) and the control cell line UT-7/Epo were equally plated in duplicates (2×10^5 cells/dish) in 35 mm Petri dishes. For the time period of 24 h, the cell media were totally deprived of FCS. Then, the media were refreshed for each Petri dish and media containing heat-inactivated 10% FCS (57 °C for 30 min, as described by Belenkov et al. (75)) as well as 100 U/mL Epo were added, respectively. After 3 h for settlement of the cells, the dishes were equally distributed to the hypoxic or normoxic incubator. The UT-7/Epo cells were administered 0.4 U/mL, 100 U/mL or no Epo. The Epo dose is considered a suprapharmacological dose as it exceeds 1 U/mL (46). At 24, 48, and 72 h, cells were harvested by trypsinization including cells from the supernatant and counted via CASY®. Based on the principle of this method, viability of the cells was determined at the same time (Fig. 9, 10).

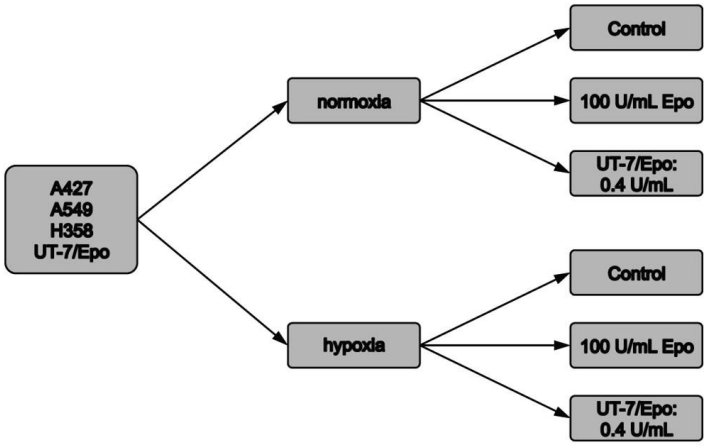


Fig. 9. Schematic overview of the assay design to analyze the proliferative effects of Epo in three selected NSCLC cell lines. UT-7/Epo served as an Epo-dependent control cell line.

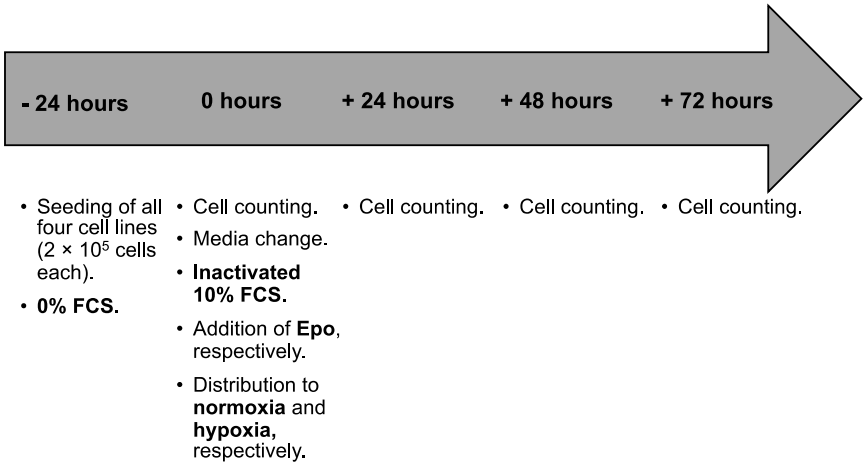


Fig. 10. Schematic overview of cell culture and Epo treatment specifications within the time course of the proliferation assay for three selected NSCLC and the Epo-dependent control cell line.

2.4 Apoptosis

In order to determine apoptosis by active caspase-3 detection in an ambient atmosphere (21% O_2), NSCLC cell lines plus the control cell line UT-7/Epo were preincubated for 48 h in the respective starvation media supplemented with 1% FCS, either with 100 U/mL, or without Epo. An additional group of UT-7/Epo cells received a basal stimulation of 0.4 U/mL Epo. Thereafter, cells were splitted and 5 × 10^5 cells per culture flask were seeded out. After 3 h for settlement, the cells of each condition, both oxygen and Epo, were treated either with or without 8 µM cisplatin for the following 48 h (Fig. 11).

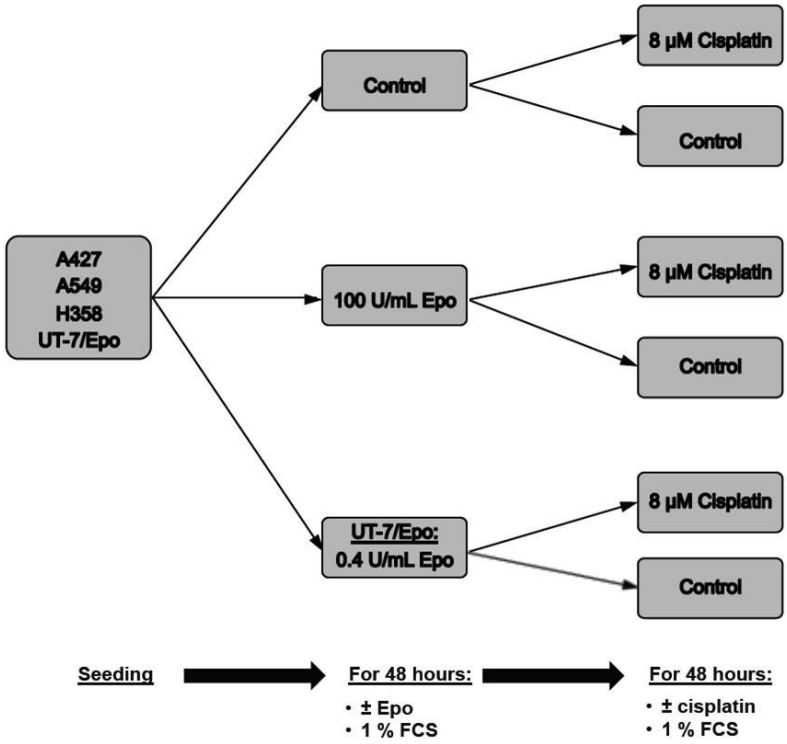


Fig. 11. Schematic overview of the assay design to analyze the effect of Epo after cisplatin-induced apoptosis in three selected NSCLC and the Epo-dependent control cell line.

Then, cells were brought into suspension by trypsinization, collected, and stained with the Caspase-3 Intracellular Activity Assay Kit I (PhiPhiLux® G1D2, Merck, Darmstadt, Germany). For this purpose, approximately 5 × 10^5 cells were centrifuged, resuspended with 25 µL PhiPhiLux-substrate and 25 µL medium according to the cell line. Subsequently, all samples were subjected to 1 hour of incubation at 37 °C in 5% CO_2, washed once with 1 mL ice-cold PBS and resuspended in 400 µL Flow cytometry buffer (PhiPhiLux®). To ensure a sufficiently long-lasting detection of activated caspase-3 signaling, all samples were stored in crushed ice prior to analysis. Finally, cells were analyzed for caspase-3 activity by flow cytometry (FACS Calibur®, BD Biosciences, San Jose, USA).

2.5 RNA isolation and qRT-PCR

2.5.1 RNA isolation

Cells were harvested as described above, centrifuged, supernatant removed by suction, and the pellet was re-suspended in Buffer RLT (guanidine thiocyanate buffer) (QIAGEN®, Hilden, Germany, Cat. No. 79216) for cells lysis before RNA isolation and stored at -20 °C.

Total RNA was extracted by using the RNeasy Mini Kit® (Qiagen®, Hilden, Germany) that included DNA digestion with the help of RNase-Free DNase Set® (Qiagen®) according to the manufacturer's protocol. Complementary DNA was synthesized applying the Revert- AidTM H Minus First Strand cDNA Synthesis Kit® (Fermentas®, St. Leon-Rot, Germany).

2.5.2 Quantitative RT-PCR

Quantitative real-time PCR was conducted with cDNA from 3 (A549, NCI-H358) and 4 (A427) independent cell harvests of the three NSCLC cell lines and carried out in triplicates using an Assay-on-Demand TaqMan® Gene Expression Assay (Applied Biosystems®, Carlsbad, CA, USA) for Epo (Hs00171267_m1), EpoR (Hs00959427_m1), and ACTB (Hs99999903_m1). Amplification and detection of cDNA was performed by the AB 7900 Detection system (Applied Biosystems, Carlsbad, CA, USA).

PCR reaction mix (10 µL):

- 5.0 µL TaqMan® Gene Expression mastermix.
- 0.5 µL Assay-on-Demand TaqMan® Gene Expression Assay, forward and reverse primer of Epo, EpoR, and ACTB.
- 0.5 µL cDNA of each cell line sample.
- 4.0 µL dH2O.

The results are normalized to the endogenous control, the reference gene beta-actin, and displayed as a relative expression of the target gene to normoxic samples, designated as fold change. Calculation was performed using the comparative CT method: Fold change = $2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT_{target} - CT_{reference\ gene\ (ACTB)}$, and $\Delta(\Delta CT) = \Delta CT_{treated} - \Delta CT_{control}$.

2.6 Western blot analyses

2.6.1 Harvesting cells without prior Epo stimulation

NSCLC cell lines that were cultured in their media both in normoxia and hypoxia were initially harvested with ice-cold RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma–Aldrich®, St. Louis, MO, USA) supplemented with protease inhibitor cocktail (Complete mini, Roche) and kept meanwhile on crushed ice.

For thorough lysis, all cell samples were vortexed for 3 to 4 cycles of 1 min each. Alternatively, samples were subjected to Compact Ultrasonic Processor® (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) according to the manufacture's protocol. Briefly, each cell lysate was sonicated for about 3 seconds without provoking foam at an amplitude of 80%.

Subsequently, all cell lysates were centrifuged for 10 min at maximum speed (13,000 rpm), and each supernatant was recovered and stored in a 1.5 mL Eppendorf tube on crushed ice for the time being.

2.6.2 Time course experiments

For the purpose of probing the effect of Epo on lung cancer cells in terms of phosphorylation of proteins involved in the signal transduction of EpoR, three NSCLC cell lines (A427, A549, NCI-H358) and the Epo-dependent human megakaryoblastic leukemia cell line UT-7/Epo were treated with 100 U/mL rHuEpo in a time course (0; 1; 5; 15; 30; 60 min) (Fig. 12).

In detail, 2×10^5 cells/dish of each cell line had been preincubated for 48 h in 35 mm Petri dishes with 1 mL medium (10% FCS) under normoxia and hypoxia. For some experiments, media were changed after 24 h in favor of a lower FCS concentration (0.1 %). On the third day, cells were treated with rHuEpo doses in a corresponding manner and were harvested according to the time points. Therefore, media were aspirated, 150 µL cell lysis buffer (RIPA buffer supplemented with proteinase inhibitor cocktail) were added, cells were gently scraped off the bottom of the Petri dishes with a cell scraper, collected and respectively distributed to the labeled 1.5 mL-Eppendorf tubes. Sample tubes were constantly kept on crushed ice and subsequently stored at -20 °C. For some experiments, cells were washed with PBS once before Epo was added.

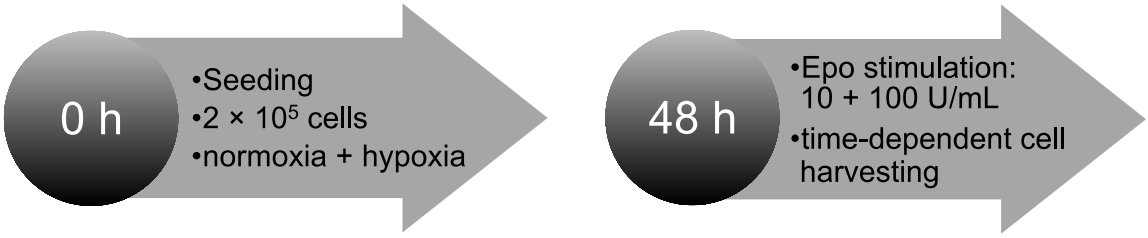


Fig. 12. Schematic overview of cell culture specifications within the time course experiment in order to analyze EpoR functionality and Epo-specific signal transduction pathways.

2.6.3 Protein quantification

For the measurement of protein concentration a BCA Protein Assay Kit (Merck, Darmstadt, Germany, Cat No: 71285-3) (76, 77) was used according to the manufacture's protocol. On a 96-well microtiter plate, a BSA standard curve with a correlation coefficient $R^2 \geq 0.99$ was established (1000 µg/mL; 500 µg/mL; 250 µg/mL; 125 µg/mL; 62.5 µg/mL; 31.25 µg/mL; 15.625 µg/mL). The samples were diluted 1:4 in RIPA buffer and 200 µL BCA working solution was added consisting of 1/50 4% cupric sulfate and 49/50 BCA solution. The optical density was measured at 562 nm using the Spectramax Plus 384 photometer. According to the Lambert–Beer law, the data were analyzed with the Softmax Pro 4.6 software.

2.6.4 Electrotransfer

According to the cell lysate's protein concentration, 10 to 20 µg of total protein was distributed to Eppendorf tubes and equally supplemented by same amount of Laemmli buffer (95% Laemmli-standard [SDS, bromphenol, glycerol], 5% beta-mercaptoethanol) to reduce, linearize, and denaturate the proteins (76, 78). After boiling for 10 min, lysates were separated via SDS-PAGE according to their size in 6 to 12% SDS gels in running buffer at 120 V, and transferred to nitrocellulose membranes (Bio-Rad®, Hercules, CA, USA) in transfer buffer at 400 mA for 90 min in the cooling room at 4 °C. For the purpose of stable buffer temperatures, the ice block was renewed after 45 min. Ponceau S (Acid Red 112) served as an optical control of successful protein transfer to the nitrocellulose membranes.

2.6.5 Immunoreactive staining

To ensure a sufficient specifity of antibody binding by blocking non-specific sites, nitrocellulose membranes were incubated in 5% milk TBS-T for 1 hour at room temperature on a nutating lab shaker. Then they were probed with the appropriate primary antibodies diluted in 1% milk or 5% BSA, and incubated either overnight attached in plastic bags to the spinning wheel in the cooling room at 4 °C, or for 1 h in a plastic box at room temperature. Followed by a washing step (3 × 15 min in TBS-T), membranes were incubated in specific horseradish peroxidase- (HRP-) conjugated secondary antibodies diluted in 1 or 5% milk TBS-T respectively for 1 hour at room temperature once more followed by a washing step. Proteins were detected after incubating the membranes for 5 min in SuperSignal West Pico

Chemiluminescent Substrate® (Thermo Scientific®, Rockford, IL, USA) in a darkened surrounding and developing them on either Amersham Hyperfilm ECL (GE Healthcare Europe GmbH, Vienna, Austria) in function of a satisfactory signal to noise ratio. In case of weak signal, SuperSignal West Femto Chemiluminescent Substrate® was applied.

New antibodies, whose protocol had not yet been established in the workgroup, were analyzed in terms of different polyacrylamide gels and dilution of antibodies, and blocking solution via titration assays.

Further details on the primary and secondary antibodies being used are summarized in Tab. 4.

Primary Antibodies	Company	Cat. No.	Origin	MW	Dilution			Clonality
					Primary	/	Secondary	
Erythropoietin Receptor (M20)	Santa Cruz	Sc 697	rabbit	59 kDa	1:500 (1% milk)	/	1:3000 (1% milk)	polyclonal
Phospho-Epo Receptor	Santa Cruz	Sc 20236-R	rabbit	64-78 kDa	1:200 (1% milk)	/	1:3000 (1% milk)	polyclonal
Akt	Cell Signaling	#9272	rabbit	60 kDa	1:1000 (5% BSA)	/	1:5000 (5% milk)	polyclonal
Phospho-Akt	Cell Signaling	#9271S	rabbit	60 kDa	1:1000 (5% BSA)	/	1:5000 (5% milk)	polyclonal
p44/42 MAPK (ERK1/2)	Cell Signaling	#9102	rabbit	42/44 kDa	1:1000 (5% BSA)	/	1:5000 (5% milk)	polyclonal
Phospho-p44/42 MAPK (ERK1/2)	Cell Signaling	#9101S	rabbit	42/44 kDa	1:1000 (5% BSA)	/	1:5000 (5% milk)	polyclonal
Beta-Actin (C4)	Sanra Cruz	Sc 47778	mouse	43 kDa	1:500 (1% milk)	/	1:2000 (1% milk)	monoclonal
Alpha -Tubulin	Cell Signaling	#2144	rabbit	52 kDa	1:1000 (5% BSA)	/	1:2000 (1% milk)	polyclonal
Secondary Antibodies								
Goat anti-rabbit IgG-HRP	Sanra Cruz	sc-2004	goat					
Goat anti-mouse IgG-HRP	Sanra Cruz	sc-2005	goat					
Alexa Fluor® 555	Invitrogen	A21428	goat					

Tab. 4. Overview on primary and secondary antibody specifications used for Western blots and immunocyto-chemical staining.

2.6.6 Stripping of membranes

As appropriate, Western blot membranes were used several times as long as signal was satisfactory in order to check for phosphorylated and total amount of protein as well as loading control. For the purpose of removing primary and secondary antibody from Western blots, several stripping protocols were applied as suitable. Independently what protocol was followed, after each stripping, membranes were blocked in 5% skim milk blocking solution.

Restore plus -stripping protocol:

Each Membrane was subjected 8 min (5 to 15 min) to a sufficient volume of Restore Plus Western Blot Stripping Buffer (Thermo Fisher Scientific Inc., Rockford, USA). Before and afterwards membranes were sufficiently washed in TBS-T.

NaOH -stripping protocol:

Membranes were subjected 8 min to a sufficient volume of 0.1 M NaOH solution. Before and afterwards membranes were sufficiently washed in dH_2O.

SDS –stripping protocol:

Stripping buffer solution A: 15.15 g Tris + 1 g SDS; pH 6.8; 250 mL dH_2O

Stripping buffer solution B: 20% SDS in H_2O.

Buffer preparation: 12.5 mL solution A; 5 mL solution B; 350 µL beta-mercaptoethanol; filled up to 50 mL with dH_2O

The membranes were incubated for 40 min at 60 °C. After incubation the membranes were thoroughly washed 3 × 20 min in TBS-T.

2.6.7 Digitalization and analysis of immunoblots

The results of immunoreactive staining on the autoradiographs were analyzed by imaging densitometry using the ChemiDoc XRS Imaging System (Bio-Rad) in conjunction with the software package for imaging Quantity One V 4.5.0 (Bio-Rad). The images were digitalized, archived, and the autoradiographic values (intensity/mm^2) were determined.

2.7 Immunocytochemical staining

The presence and intracellular localization of EpoR in three adenocarcinoma cell lines (A427, A549, NCI-H358) was examined applying the principle of indirect immunofluorescence with both epifluorescent and confocal laser scanning microscopy, where a fluorochrome labeled secondary antibody was used to visualize the primary antibody binding on EpoR.

Therefore, 1.5 × 10^5 cells/chamber were seeded out in 2-well chamber slides (Nunc, Langenselbold, Germany) and cultured in complete culture medium for 48 h under normoxia and hypoxia. Adapted from the immunofluorescence protocol of Mechtcheriakova et al. (79, 80), after settlement of the cells and decantation of medium, they were fixed in 4% formaldehyde containing 2% sucrose for 10 min, and permeabilized with PBS containing 0.5% Triton X-100 (Sigma–Aldrich, St. Louis, MO) for 5 min. Antibodies were diluted in PBS containing 1% BSA solution for 1 h at room temperature on a nutating lab shaker, whereof the primary antibody (EpoR: sc-697) was diluted both 1:50 and 1:200, and the secondary antibody (Alexa Fluor® 555: A21428) was diluted 1:200. The secondary antibody was protected from light. Controls included omission of the primary antibody for A427, or using an isotype IgG as primary antibody for A549 and NCI-H358. To visualize cell nuclei, cells were counterstained with a fluorescent mounting medium

containing DAPI (Vector Laboratories, Burlingame, USA), a fluorescent minor groove-binding probe for DNA, cover slides were sealed with nail polish, and slides were stored at 4 °C while being light protected until the analysis. To increase antibody specificity and avoid intermixture of substances, chamber slides were thoroughly washed 2 to 3× with PBS after each step.

For epifluorescent microscopy, cells were analyzed using an Olympus BX51 microscope and an Olympus DP71 camera.

For confocal laser scanning microscopy, a Zeiss Axiovert 200M microscope (Oberkochem, Germany) and LSM 510 release version 4.0 software were applied. Images were taken using a ×63 oil immersion objective with a 1.4 numeric aperture. The optical slice thickness was below 1.4 µm.

2.8 Statistical analyses

Quantitative RT-PCR, Western blot analyses, and proliferation assays were reproduced at least three times. In particular, statistical analyses of qRT-PCR data were performed using the relative expression software tool REST 2008 version 2.0.7 (Corbett Research Pty Ltd and Michael W. Pfaffl, Technical University Munich). The other data were compiled and analyzed using the software package GraphPad Prism version 5.03 (La Jolla, CA) or SPSS (IBM). Group differences were calculated using Student's t-test, one sample t-test, and two-way ANOVA with post hoc analysis (Bonferroni correction). Two-sided P-values smaller than 0.05 were considered significant. Results are expressed as the mean ± standard deviation (SD).

3 Results

3.1 Expression of Epo and its receptor in NSCLC cell lines

3.1.1 EpoR and Epo on mRNA level

All three NSCLC cell lines were examined for the expression of EpoR.

Analysis of the mRNA complement of the NSCLC cell lines by qRT-PCR revealed that all cell lines were producing EpoR mRNA to a different degree (Fig. 13A). In particular, A427 cells expressed the highest level of EpoR mRNA of all three cell lines, regarding the fold change normalized to the reference gene beta actin

(overall difference $P < 0.0001$, Two-way ANOVA): A427 cells expressed EpoR 146-fold higher than in A549 cells, and 71-fold higher than in NCI-H358 cells in normoxia. Hypoxic A427 cells expressed the receptor 191-fold higher than in A549 and 34-fold higher than in NCI-H358 cells (Tab. 5).

Moreover, EpoR mRNA was upregulated 2.3-fold by hypoxia ($P = 0.011$, standard error range 1.313 – 3.591) in NCI-H358 cells while the A427 and A549 cells did not provide evidence of being influenced by the surrounding oxygen concentration within this experimental setting.

Regarding the presence of Epo mRNA, as depicted in Fig. 13B, these cells demonstrated a rather weak transcription level of Epo in comparison to the reference gene showing no statistically significant difference to hypoxic culture conditions, which is why an actual Epo protein synthesis remains uncertain in these cell lines.

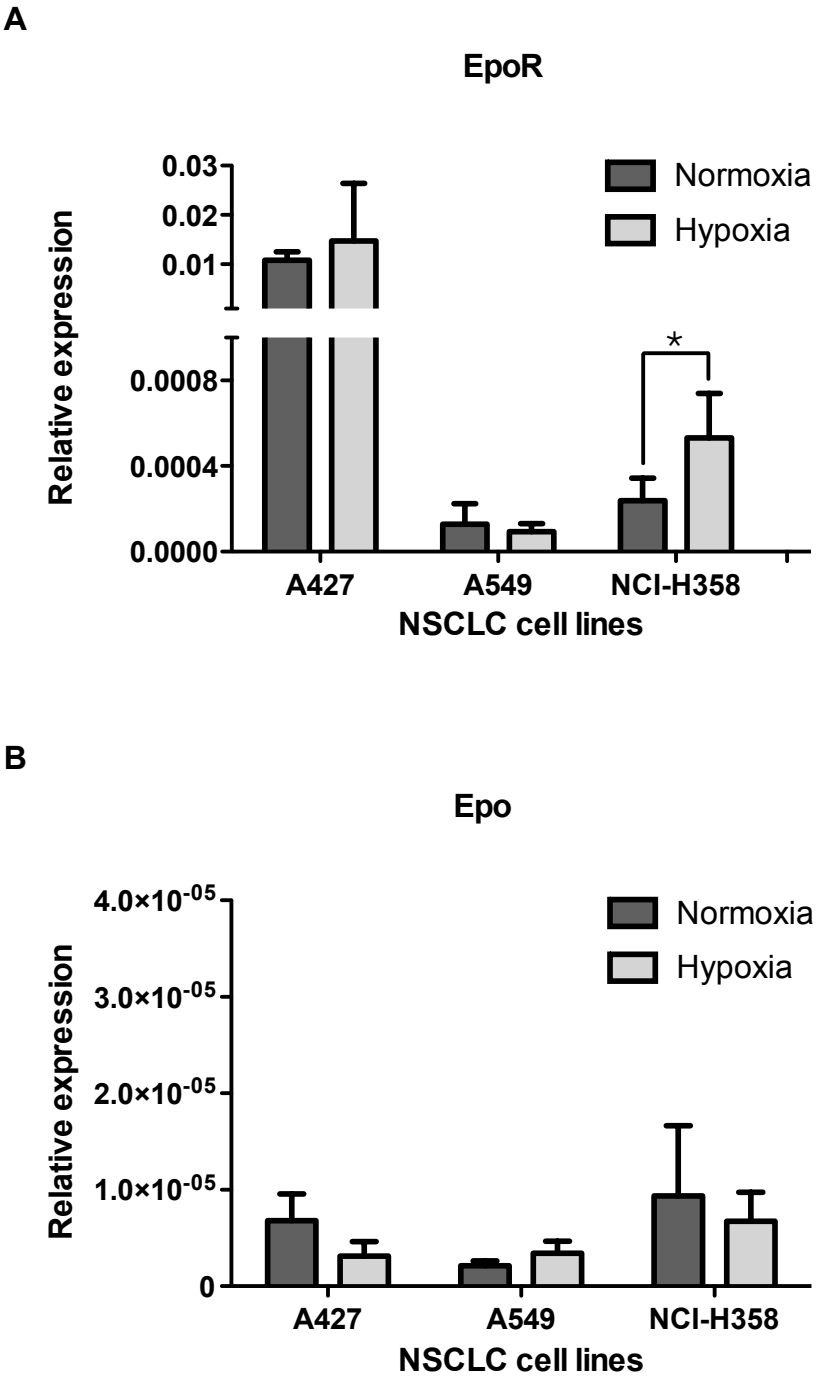


Fig. 13. Relative expression and regulation of EpoR (**A**) and Epo (**B**) mRNA in three NSCLC cell lines (A427, A549, NCI-H358) in normoxic and hypoxic atmosphere, normalized to reference gene beta actin. (**A**) EpoR was significantly upregulated (2.3-fold) by hypoxia in NCI-H358 cells (*P<0.05, REST® v2.0.7). Data represent mean ± SD of at least three independent experiments.

	Fold change of EpoR mRNA (normalized to reference gene)	SD range	Fold change of A427
Normoxia			
A427	1.58×10^{-2}	$6.25 \times 10^{-3} – 4.02 \times 10^{-2}$	1
A549	1.09×10^{-4}	$5.81 \times 10^{-5} – 2.03 \times 10^{-4}$	145.77
NCI-H358	2.23×10^{-4}	$1.07 \times 10^{-4} – 4.64 \times 10^{-4}$	71.13
Hypoxia			
A427	1.69×10^{-2}	$2.48 \times 10^{-3} – 1.15 \times 10^{-1}$	1
A549	8.85×10^{-5}	$4.89 \times 10^{-5} – 1.60 \times 10^{-4}$	191.1
NCI-H358	5.03×10^{-4}	$3.57 \times 10^{-4} – 7.08 \times 10^{-4}$	33.63

Tab. 5. Fold change of EpoR mRNA throughout the three NSCLC cell lines (A427, A549, NCI-H358) under normoxia and hypoxia, plus the fold change of EpoR mRNA in A427 cells in comparison to the other cell lines. A427 cells expressed the highest level of EpoR mRNA of all three cell lines, regarding the fold change normalized to beta actin ($P < 0.0001$, Two-way ANOVA). SD, standard deviation.

3.1.2 EpoR on protein level

EpoR mRNA is translated into protein and present in all three NSCLC cell lines, as indicated by Western blot analysis (Fig. 14). The mature, full-length form of EpoR is visualized at 59 kDa. Optical density measurements of EpoR bands comparing normoxic to hypoxic cells lysates did not yield a statistically significant difference: A427: $P = 0.25$; A549: $P = 0.79$; NCI-H358: $P = 0.25$.

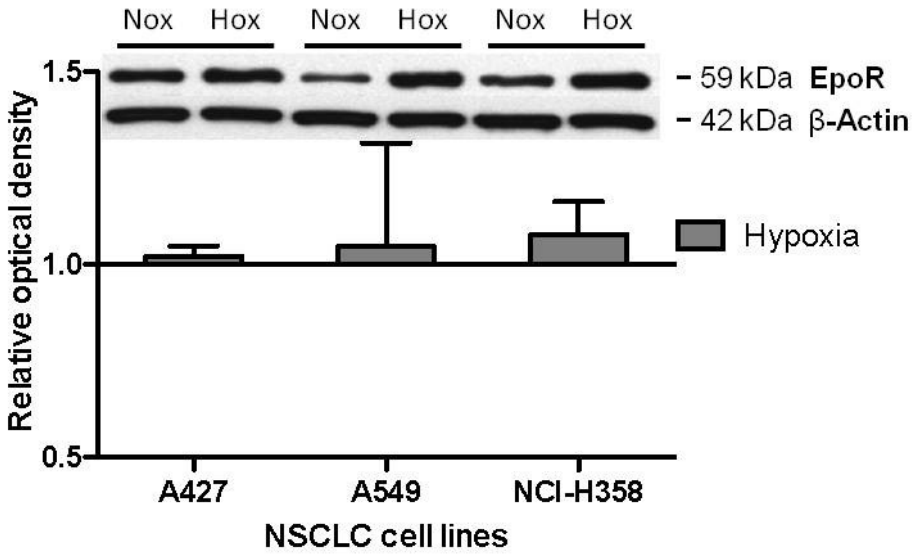
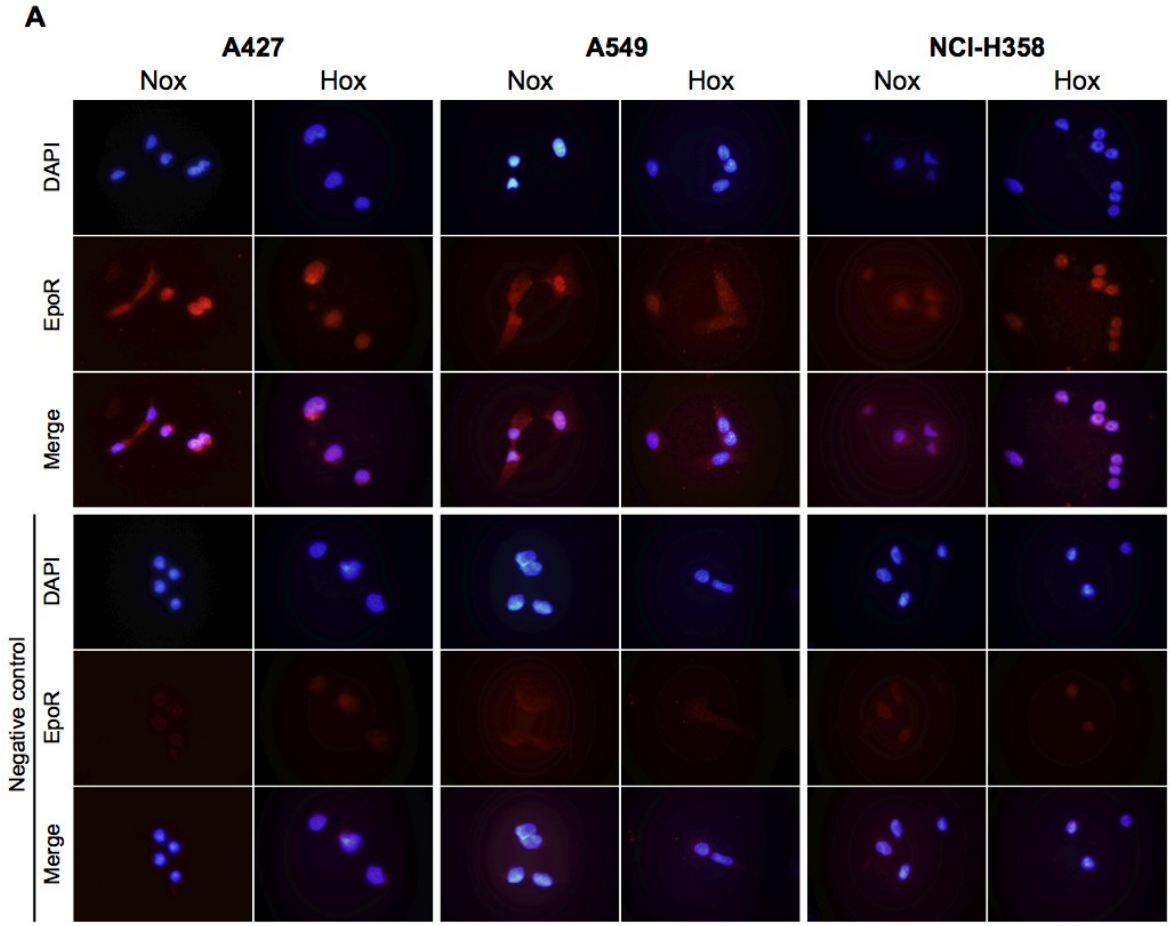


Fig. 14. Expression of EpoR protein in NSCLC cell lines (A427, A549, NCI-H358). Cells were incubated for 3 days under hypoxia prior to cell harvesting and protein isolation. Wells in the gel were evenly loaded with 20 µg protein. Relative optical density of the blots was obtained by normalizing optical densities from hypoxic to normoxic bands. Data represent mean ± SD of three independent experiments. **Nox**, normoxia; **Hox**, hypoxia.

In addition to qRT-PCR and Western blot analysis, EpoR protein was visualized in all three lung cancer cell lines via immunocytochemical staining techniques. All cells expressed the receptor protein to a certain degree both under normoxic and

hypoxic conditions (Fig. 15A). A distinct difference between the two oxygen concentrations could not be appreciated to such an extent as for NCI-H358 cells on mRNA level. But in accordance with the EpoR mRNA data of A427, these cells appeared to have the strongest expression of EpoR protein in comparison to A549 and NCI-H358 cells, as displayed in Fig. 15B. Moreover, in epifluorescent microscopy, a dense intra- and perinuclear agglomeration of EpoR protein staining was recognizable. For further investigation, confocal laser scanning microscopy was applied to provide further information on where the receptor is located intracellularly. By this means indeed, cells showed mainly cytoplasmatic and perinuclear staining of EpoR protein that were organized in small clusters (Fig. 15B).



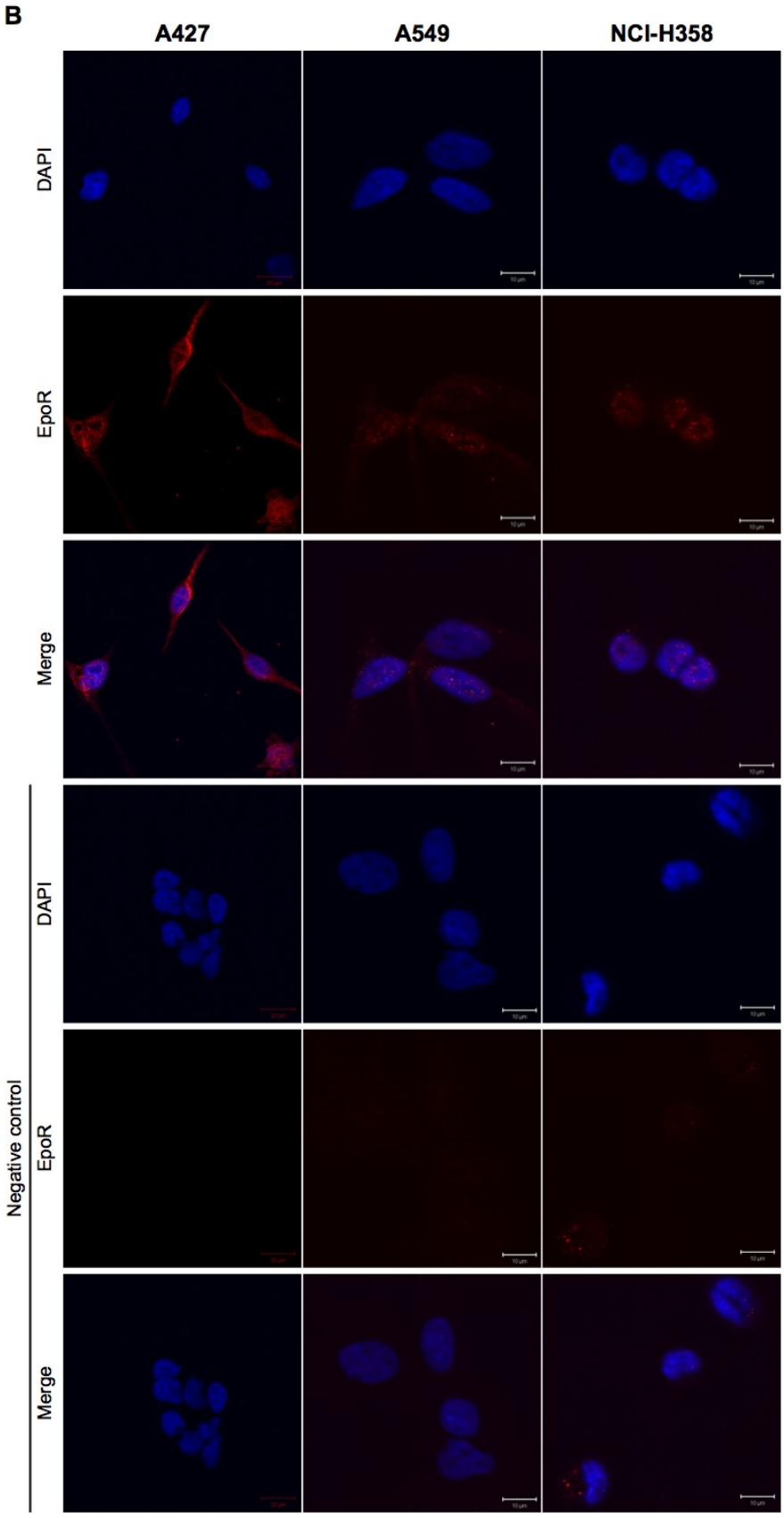


Fig. 15. Immunocytochemical staining of EpoR in three NSCLC cell lines (A427, A549, NCI-H358). (**A**) Epifluorescent microscopy from cells preincubated under either normoxia or hypoxia. (**B**) Confocal laser scanning microscopy from cells preincubated in hypoxia. As a negative control, staining was performed in the absence of the primary antibody for A427, or with an isotype IgG as primary antibody for A549, and NCI-H358. **DAPI**, nucleus staining; **Nox**, normoxia; **Hox**, hypoxia.

3.2 Activity and functionality of EpoR

To assess whether the receptor was functional in all three NSCLC cell lines depending on the different states of receptor maturation and turnover, cells were treated with 100 U/mL rHuEpo and cell lysates were subsequently probed for activation of EpoR for two key Epo-signaling pathways: MAP kinase cascade (analyzed by phospho-ERK1/2), and PI3K cascade (analyzed by phospho-Akt).
Phosphorylation of these proteins can be seen in the cell lines under study given in Fig. 16.
Regarding EpoR, A427 cells showed phosphorylation at each time point throughout the time course with the strongest signal after 60 min under normoxia, and after 30 min under hypoxia. Nevertheless, attention should be paid to the fact that EpoR was as well phosphorylated without any Epo stimulation, suggesting that it must had probably been stimulated before, e.g. by culture medium that contained 10% serum. This finding differed to the signal pattern shown by UT-7/Epo cells under normoxia and hypoxia. Here, EpoR was not phosphorylated in untreated cells but climaxed the strongest signal after 1 min of stimulation that continuously declined in intensity while the total amount of EpoR failed to be present in normoxic, or showed a slight signal in hypoxic untreated cells (Fig. 16A).
Considering the two key Epo-signaling pathways, Western blot analysis revealed phosphorylation of ERK1/2 and Akt proteins in all three lung cancer cell lines but in a rather inconsistent manner. In detail, the proper immunoreactive bands of proteins were distinct and clear in terms of antibody specificity. Epo-untreated A427 cells showed phosphorylation of the key-proteins ERK1/2 and Akt (Fig. 16B). Normoxic NCI-H358 cells might show a time-depending increase in intensity for ERK1/2 to some extent if any in comparison to the total amount of protein. As the internal control, proteins of a same blot were evenly loaded, as indicated by consistent α-tubulin bands.

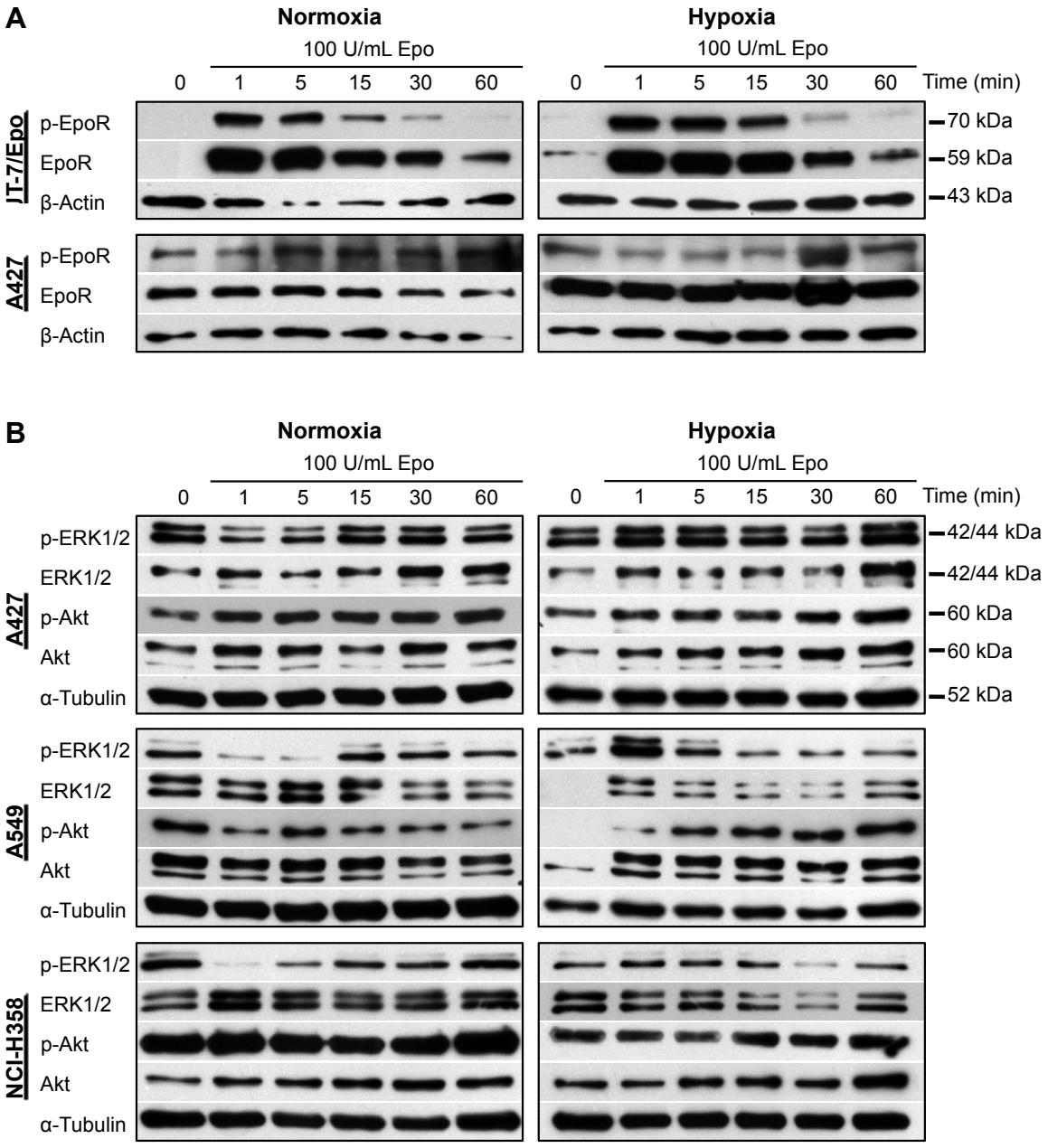


Fig. 16. Comparison of (**A**) UT-7/Epo and A427 signaling for activated EpoR and (**B**) A427, A549, and NCI-H358 signaling for activated downstream proteins over 1 h in response to 100 U/ml rHuEpo. Activated pathways are shown by phosphorylated EpoR, Akt and ERK1/2. The total amount of protein as well as housekeeping proteins are shown as loading controls. Elapsed time displayed in minutes; **p-** , phosphorylated.

3.3 Proliferation in Epo-treated NSCLC cell lines

To investigate whether Epo contributed to increased tumor growth of selected NSCLC cell lines, cell counting assays based on the principle of electronic pulse area analysis (CASY®), were performed with cells treated with 100 U/mL rHuEpo (Fig. 17).

A clear Epo- and time-dependent overall increase in growth ($P < 0.0001$, Two-way ANOVA) was obvious in UT-7/Epo cells, becoming in particular statistically

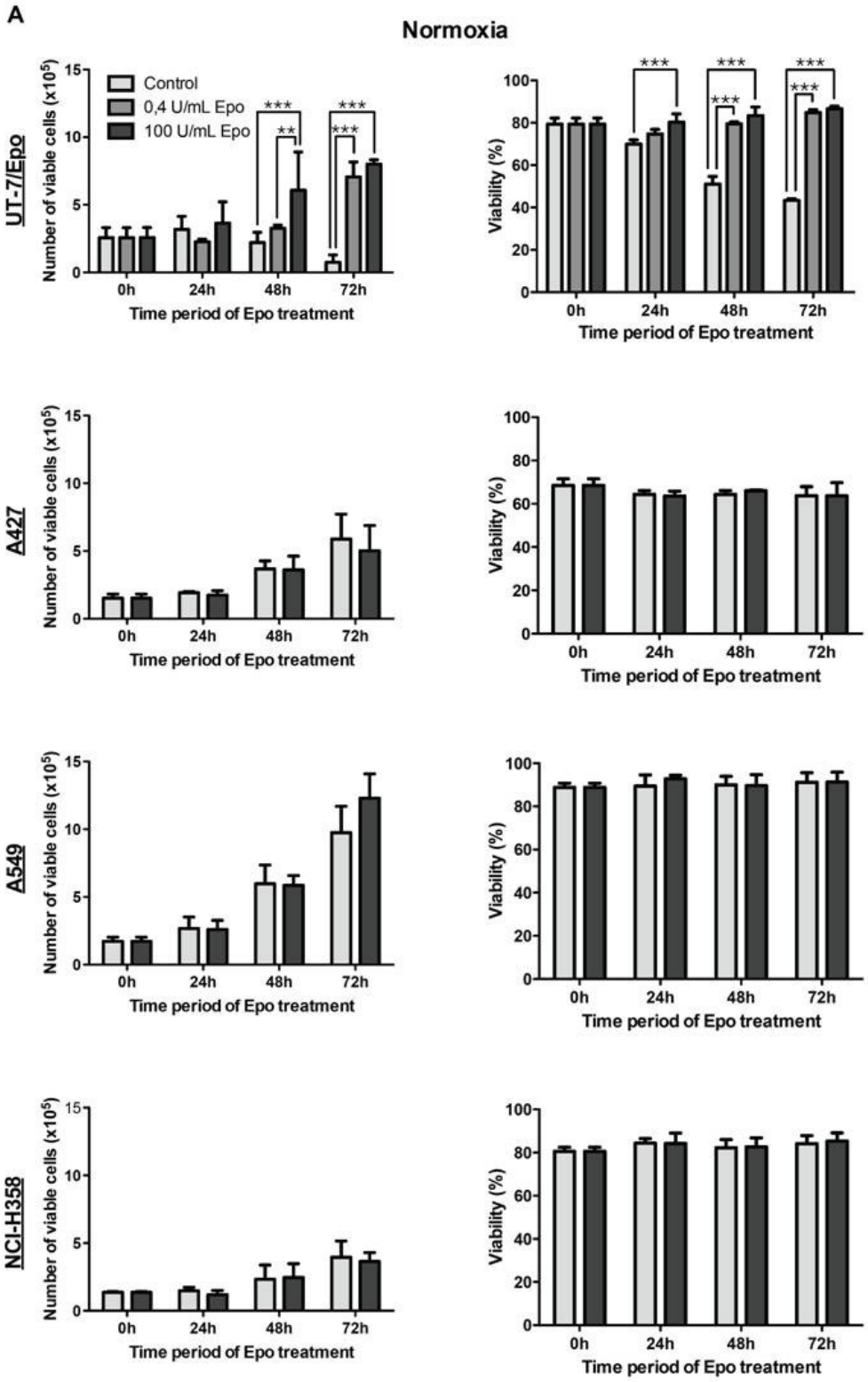
significant after 72 h at 0.4 U/mL ($P < 0.001$) and even after 48 and 72 h at 100 U/mL Epo (each $P < 0.001$) regarding the absolute number of viable cells under normoxia. These differences were fairly the same under hypoxia as depicted in Fig. 17B. The viability panel on the right side in Fig. 17 illustrates the clear Epo dependency of this cell line, showing a continuous decrease in percentage of viable, Epo-naïve cells.

For the NSCLC cell lines under study, even at 100 U/mL Epo, considered as a suprapharmacological dose (46), no statistically significant growth advantage with Epo could be appreciated, neither under normoxia (Fig. 17A) nor under hypoxia (Fig. 17B).

In this regard, the altered oxygen concentration of the atmosphere under hypoxia (1% O_2) did clearly affect the growth rate of all cell lines (Fig. 18). In detail, Epo-naïve A427, A549, and NCI-H358 cells showed a distinct overall impairment in cell growth rate under hypoxia ($P = 0.0008$; $P < 0.0001$; $P = 0.0195$, respectively, Two-way ANOVA), while when treated with 100 U/mL Epo, NCI-H358 cell growth showed no difference to normoxia ($P = 0.1874$). In contrast to this finding, hypoxia had the same growth rate decreasing effect on Epo-treated A427, and A549 cells as in naïve cells ($P = 0.0015$; $P < 0.0001$, respectively).

Interestingly, UT-7/Epo cell treated with 100 U/mL Epo showed a lower proliferation rate in hypoxia compared to normoxia, whereas none or 0.4 U/mL Epo could not lead to a statistically significant difference in growth $P = 0.9596$; $P = 0.2879$, respectively) (Fig. 19) Moreover, given the fact that UT-7/Epo is clearly Epo-dependent, control cells treated with 100 U/mL Epo under normoxia responded to that dose faster than cells treated with 0.4 U/mL, seen in a clear difference in viable cells after 48 h. This difference between dose and response for 0.4 and 100 U/mL Epo is only reversed once again after 72 h under normoxia. Between viable, hypoxic cells treated with 0.4 or 100 U/mL, there was a clear difference in growth after 72 h in favor of 0.4 U/mL ($P < 0.05$) (Fig. 19B).

Taken together, all three NSCLC cell lines did not increase their proliferation after stimulation of 100 U/mL Epo. Hypoxia did significantly diminish the proliferation of all NSCLC cell lines. UT-7/Epo cells seem to immediately respond on high Epo doses while this effect abolishes with time since cells treated with a lower dose will finally catch up in growth. The effect of hypoxia on the proliferative effect on these Epo doses appears to be different to normoxia.


A
Normoxia
UT-7/Epo
A427
A549
NCI-H358
Control
0,4 U/mL Epo
100 U/mL Epo
Number of viable cells (x10^5)
Viability (%)
Time period of Epo treatment
0h
24h
48h
72h
***
**

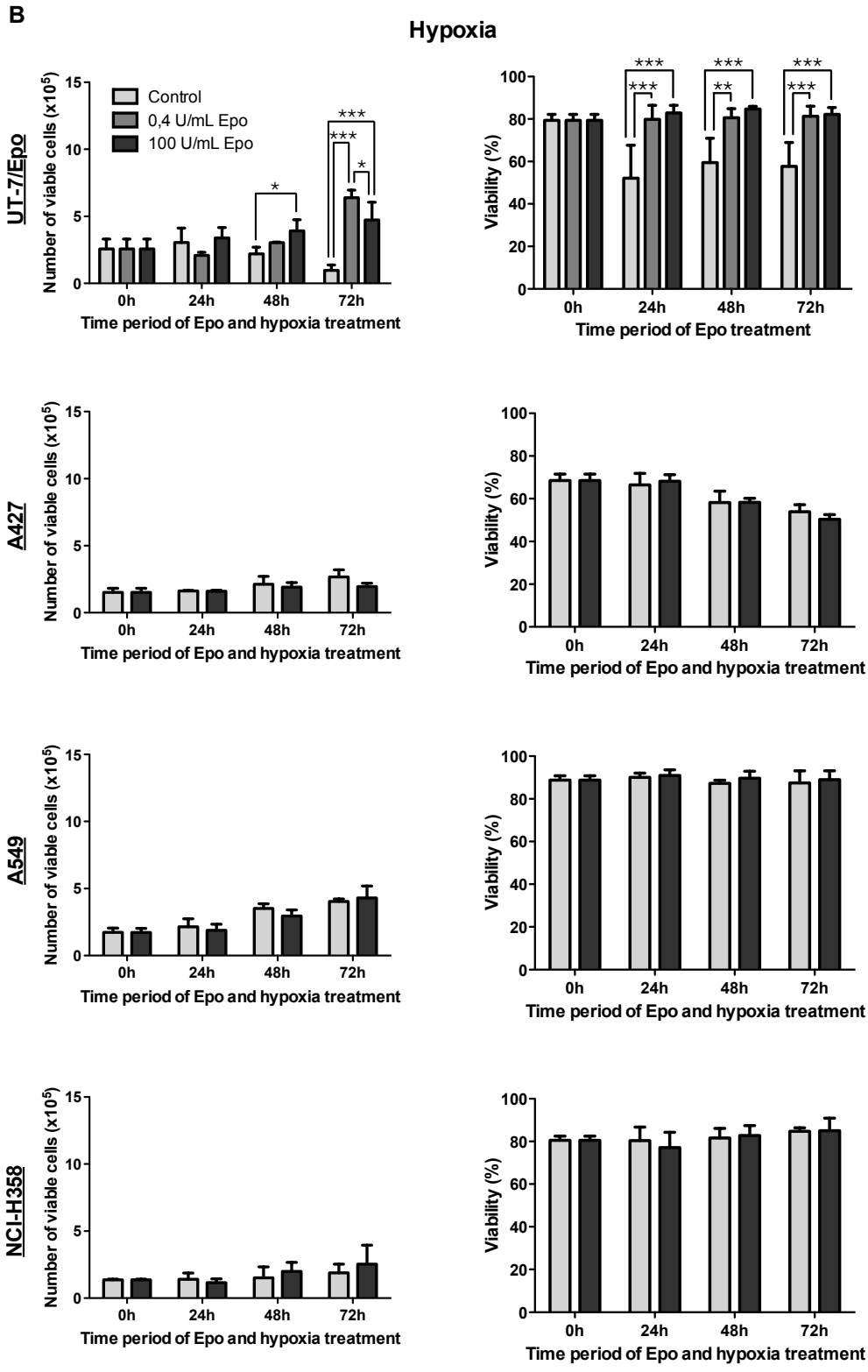


Fig. 17. Proliferation assays of three NSCLC cell lines (A427, A549, NCI-H358) treated with 100 U/mL rHuEpo under normoxic (**A**) and hypoxic (**B**) conditions in the course of three consecutive days. UT-7/Epo cells served as an Epo-depending positive control for the experimental setup. Viability of cells was determined by electronic pulse area analysis (CASY⏜). Statistics were performed using a Two-way ANOVA with Bonferroni correction (*P < 0.05; **P < 0.01; ***P < 0.001). Data represent mean ± SD. The legend in the first diagram applies to the others as well.

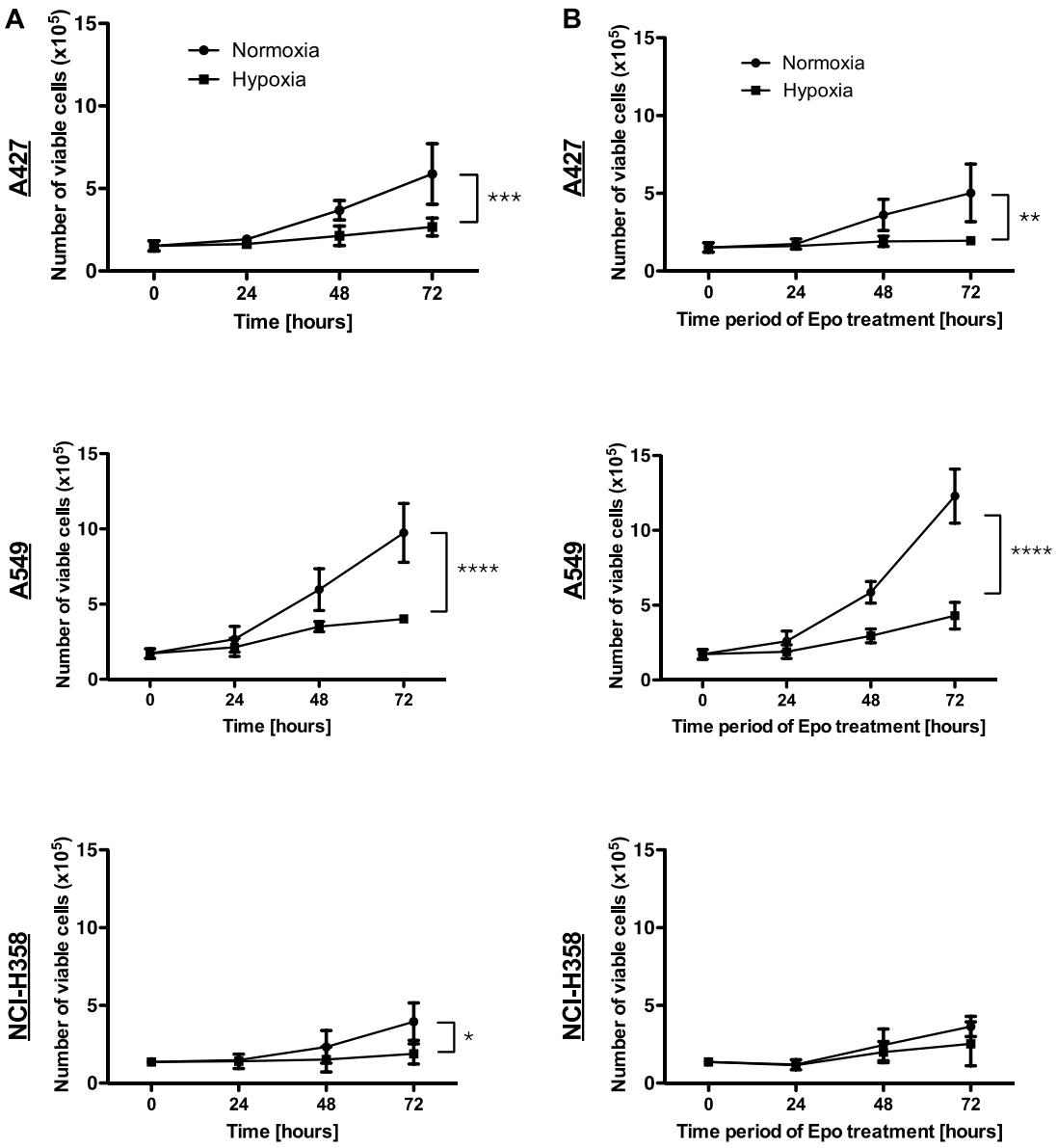


Fig. 18. Effect of altered oxygen concentration on proliferation in three NSCLC cell lines (A427, A549, NCI-H358), without (**A**), and with (**B**) 100 U/mL rHuEpo. These data resulted from the very same proliferation assay, with particular respect to the influence of hypoxia on proliferation. Statistics were performed using a Two-way ANOVA with Bonferroni correction (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001), where the asterisks designate the overall difference, spanning 72 hours of culturing. Data represent mean ± SD. The legend in the first row in (**A**) and (**B**) applies to the other diagrams as well.

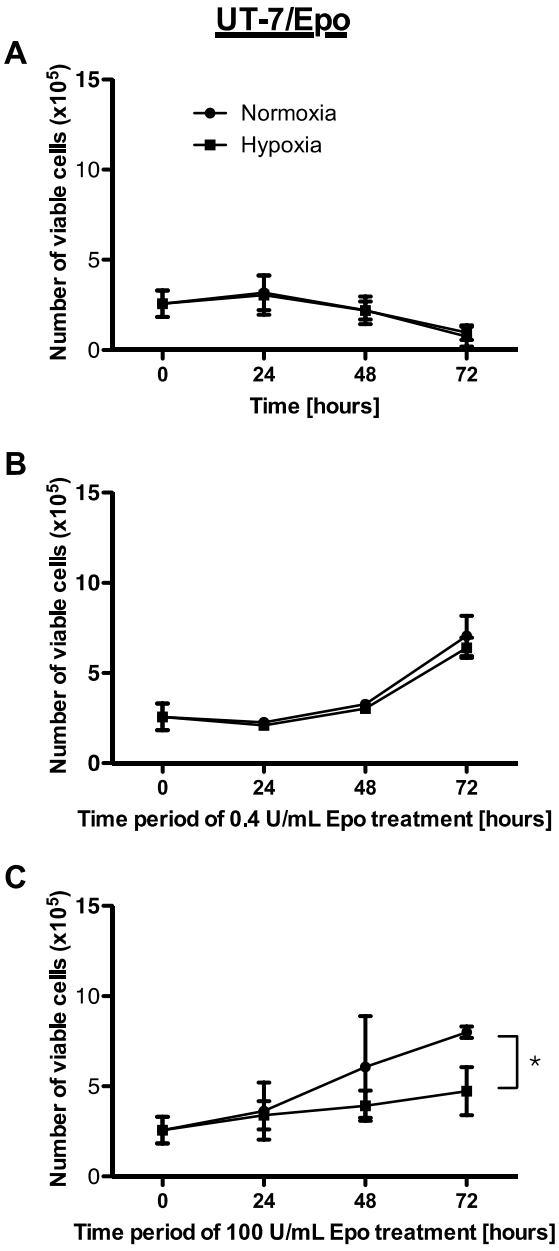


Fig. 19. Effect of altered oxygen concentration on the proliferation in Epo-depending control cell line UT-7/Epo without (**A**), with 0.4 U/mL (**B**), and with 100 U/mL rHuEpo (C). These data resulted from the very same proliferation assay, with particular respect to the influence of hypoxia on proliferation. Statistics were performed using a Two-way ANOVA with Bonferroni correction (*P < 0.05), where the asterisk designates the overall difference, spanning 72 hours of culturing. Data represent mean ± SD. The legend in (**A**) applies to the other diagrams as well.

3.4 Apoptosis in Epo-treated NSCLC cell lines

To study whether Epo was able to protect from cisplatin-induced apoptosis in selected NSCLC cell lines in ambient atmosphere (21% O_2), activated effector caspase-3 as in indicator for irreversible initiation of apoptosis, was detected via flow cytometry within two independent experiments, while viability was simultaneously determined by CASY® cell counting (Fig. 20).

The overall effect of cisplatin (±rHuEpo) significantly led to increased apoptosis in UT-7/Epo ($P = 0.0173$), A427 ($P = 0.0476$), and A549 ($P = 0.0005$). This was not seen in NCI-H358 cells ($P = 0.1227$). However, a clear decrease in the overall viability, as assessed with CASY®, was found in all cell lines (UT-7/Epo: 0.0099, A427: $P = 0.0359$; A549: $P < 0.0001$; NCI-H358: $P = 0.0043$).

In all three NSCLC cell lines, 100 U/mL Epo did not prevent from the cisplatin-induced activation of caspase-3 in a statistically significant manner.

Again, UT-7/Epo proved to be Epo-dependent since the percentage of activated caspase-3 cells clearly decreased with Epo treatment (0.4 U/mL, 100 U/mL: $P < 0.05$, respectively). In addition, both 0.4 and 100 U/mL Epo abolished the apoptotic effect of cisplatin in UT-7/Epo cells ($P < 0.01$, respectively), whereas in the NSCLC cell lines, Epo was not able to reduce the cisplatin-induced apoptosis, suggesting different dependencies of Epo and different affinities to EpoR throughout these cell lines.

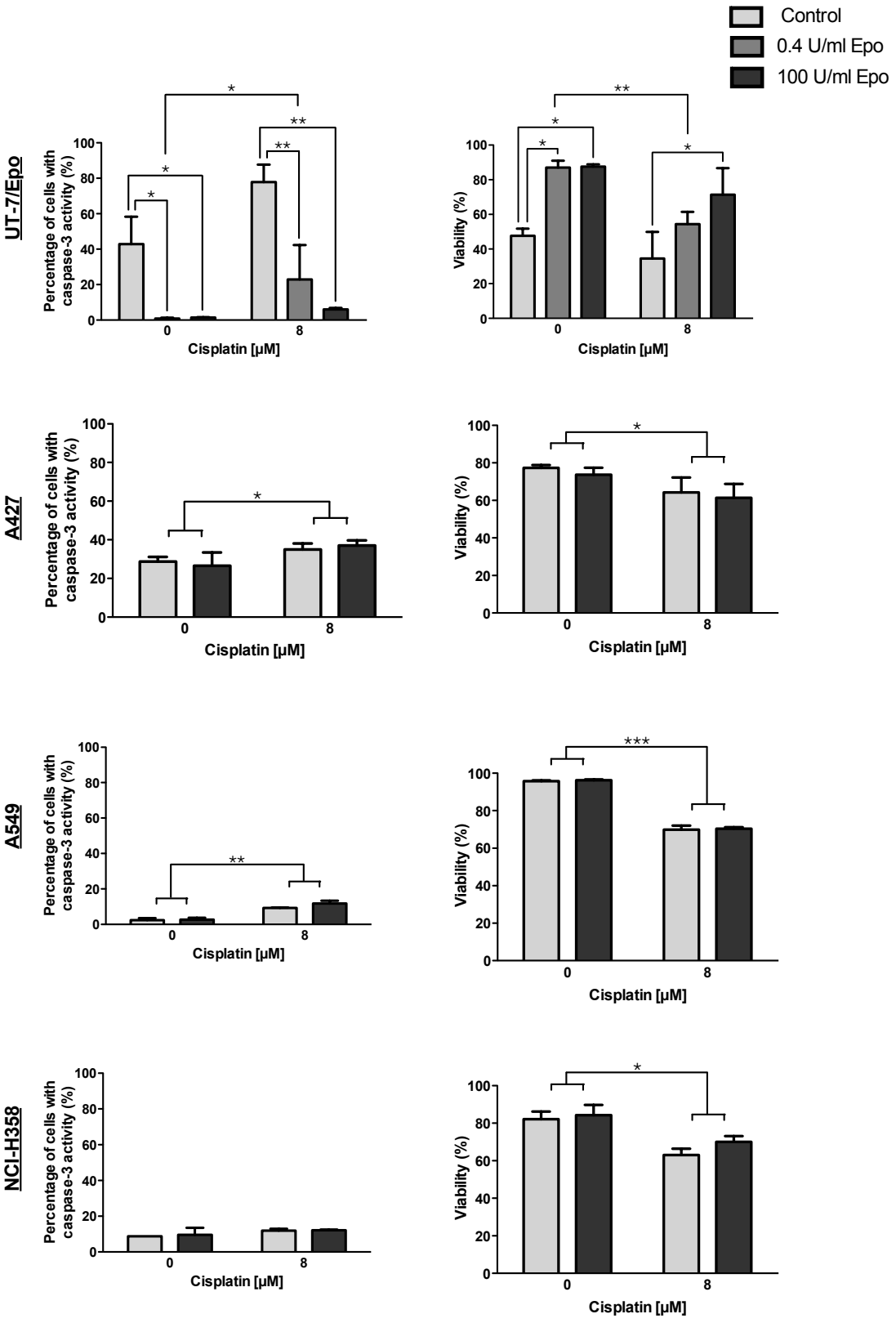


Fig. 20. Apoptosis induction by 8 µM cisplatin and detection of activated caspase-3 in three NSCLC cell lines (A427, A549, NCI-H358) treated with 100 U/mL rHuEpo under normoxic conditions. Number of viable cells was determined by electronic pulse area analysis (CASY⏜). Statistics were performed for two independent experiments using a Two-way ANOVA with Bonferroni correction (*P < 0.05; **P < 0.01; ***P < 0.001). UT-7/Epo cells served as an Epo-depending positive control for the experimental setup. Data represent mean ± SD.

4 Discussion

4.1 The expression of EpoR in NSCLC cell lines

In the first part of our study, we demonstrated that the selected NSCLC cell lines A427, A549, and NCI-H358 expressed EpoR on mRNA and protein level both under normoxic and hypoxic conditions to a different degree making them potentially able to respond to an appropriate Epo stimulation. These mRNA data have to be considered with due care since they result indeed from three, in case of A427 four, independent experiments, and yet have considerable dispersions. To sum up the facts mentioned, while A427 cells showed the highest amount of mRNA transcripts, which is reflected by the fluorescence microscopy data, NCI-H358 cells clearly increased EpoR mRNA when cultured under hypoxia. Western blot analysis supported the presence of EpoR in these cells but not its regulation by hypoxia. When confocal laser scanning microscopy was performed, cells showed a mainly cytoplasmatic and perinuclear fluorescent signal, raising the question, whether these receptors are located on the cell surface and thus can be reached and stimulated by its ligand. Neumann et al. (81) demonstrated that only 60% of the newly synthesized EpoR was processed to the glycosylated receptor protein in Golgi apparatus with a subsequent short half-time of 45-60 min. Westphal et al. (82) concluded from this, that it would be possible that either not only mature receptor but also premature protein in vesicles of endoplasmic reticulum or Golgi apparatus were stained or the fluorescent signal derived from lysosomes where the receptor became degraded. On the other hand, the anti-EpoR antibody applied in this study (M20, Santa Cruz) detected tyrosines of the carboxy-terminal cytoplasmic domain of EpoR, which represents the mature, full-length form, whose molecular weight was verified by its complete size of 59 kDa band with immunoblotting.

In this respect, Elliott, Busse, Brown and their colleagues (83, 84) addressed serious concerns about the specificity of anti-EpoR antibodies used in recent studies for its detection on cancer cells, in which EpoR was allegedly verified by Western blot analysis and immunocytochemistry. The majority of commercially available antibodies were considered unspecific since they recognize additional proteins in a range of 3 to 20 possibly due to cross-reactions. As a matter of fact,

the M20 antibody that we applied rendered one of the most suitable for Western blot analysis while immunocytochemistry results were still considered questionable (83).

In accordance with Neumann et al., the EpoR properties were described as quite dynamic since it is considered as a prime example of receptor that encounters an extreme range of ligand concentrations and is therefore characteristic for this kind of receptor in hematopoietic cells (85). With the help of mathematical core models, Becker et al. (85) provided data on the predominant role of the EpoR turnover that comprises transport of newly synthesized receptor to the plasma membrane and removal of the same by endocytosis both in a ligand-independent manner. According to the authors, these findings were suggestive of large intracellular receptor pools enabling EpoR bearing cells to cope with a wide range of Epo concentrations. This finding is of quite interest since we demonstrated that EpoR in the NSCLC cell lines were mainly localized in intracellular small clusters, which now may be considered as receptor pools with dynamic distribution to the plasma membrane. As a consequence, the cells' dynamics of receptor trafficking from inner receptor pools to the cell surface may determine the ability to convert extracellular signals into receptor activation, and thus to propagate the Epo signaling in these lung cancer cells.

Concerning the EpoR expression, Yasuda et al. (86) found out that A549 cells exhibited a strong EpoR mRNA that showed a significant increase in anoxia, while Epo mRNA reached a rather weak amount of transcripts that we showed likewise for hypoxia (1% pO_2). Since in our selection of NSCLC cell lines Epo synthesis and secretion seemed low to uncertain, an often hypothesized Epo-EpoR autocrine-paracrine loop in cancer cells (66, 87) may play a rather subordinated role.

Furthermore, Westphal and colleagues (82) demonstrated that EpoR was present in all lymphocytes isolated from leukemia patients, in lymphocytes of healthy persons as well as in many samples from human skin tumors except for healthy skin tissue. While they suggested a relationship between EpoR expression and increased malignancy, Selzer et al. interpreted EpoR presence as a progression marker for human melanoma cells (22).

In this respect, an analysis of 158 tumor samples from resected stage I NSCLC by Saintigny et al. (88) revealed, that Epo, EpoR, and Epo-EpoR coexpression respectively detected on these samples were associated with a statistically significant poorer 5-year disease-specific survival. Additionally, in multivariate analysis for disease specific survival, they found that high Epo and EpoR coexpression was an independent prognostic factor for disease-specific survival (hazard ratio: 2.212; 95% CI 1.012 – 4.848; P = .0046) being suggestive of a potential paracrine-autocrine role of Epo in NSCLC aggressiveness.

4.2 The influence of Epo on NSCLC cell lines

The second part of the experiments is directed to the functionality of EpoR and the effects of Epo in terms of promoting proliferation and inhibiting apoptosis on NSCLC cell lines.

For selected cancer cell lines, some studies provide certain evidence that EpoR is capable of transmitting the Epo-driven signal into the cell first and foremost via cascades of activating protein phosphorylations (65, 72, 86, 89, 90). Our time course experiments showed a rather inconsistent phosphorylation pattern. While Epo-treated A427 cells were positive for phosphorylated EpoR in a time-dependent manner, the fact that even untreated cells showed a low but certain signal of phosphorylated EpoR makes this circumstance debatable. Even though Epo mRNA synthesis was questionably weak in all cell lines examined, basal receptor stimulation secondary to the 10% FCS containing culture medium in conjunction with a theoretically possible autocrine/paracrine loop of Epo cannot totally be ruled out which is why the question of receptor affinity remains.

Two of the four key Epo-signaling pathways were under study in these cell lines for its activation revealing that even though the immunoreactive bands of ERK1/2 and Akt were distinct and clear in terms of antibody specificity, they were rather continuously phosphorylated and thus activated. These pathways are very likely to be cross-activated by serum, various growth factors, cytokines, and certain stresses, and yet are tightly regulated due to intensive cross-talks between signaling pathways, as extensively investigated by Lewis, Ahn and their colleagues (49, 91). This may explain why Epo-untreated cells were found to have phosphorylated ERK1/2 and Akt proteins since the culture medium contained 10%

serum. Additional experiments investigating the phosphorylation status should consider medium with less serum in order to lower unspecific stimulation.

Proliferation studies were conducted under normoxia (21% O_2) and hypoxia (1% O_2). As tumor cells have cut themselves off the usual cell metabolism of its original tissue and proliferate independently, rapid tumor growth and ineffective angiogenesis creates an oxygen gradient with a low point of O_2 concentration in the center of the tumor (43). For primary NSCLC, the intratumoral atmosphere was determined intraoperatively revealing a median of 13.5 mmHg O_2, equaling 1.77% pO_2 (92).
For our experiments, to mimic low oxygen levels, as they exist in lung tumors, we cultured the selected NSCLC cell lines for all hypoxic experiments for at least 48 hours at a pO_2 of 1%, equaling around 7.6 mmHg O_2.
Here, A427, and A549 cells exhibited a statistically significant decrease of cell growth under hypoxia, which had already been demonstrated by Wohlkönig et al. for A549 cells (93) and others. Notably, Epo did not alter this hypoxia-induced decrease in cell growth. By contrast, NCI-H358 did not demonstrate the hypoxia-induced decline after administration of 100 U/mL Epo, raising the question whether this was indeed an effect caused by Epo or simply a matter of dispersion of measured values.

As we checked for the proliferative responsiveness to Epo in cell culture via cell counting, even a suprapharmacological dose of 100 U/mL Epo could not stimulate proliferation of the tumor cells within the experimental model suggesting a rather Epo-independent cell cycle. Nevertheless, the study design was confirmed by the fact that the control cell line UT-7/Epo responded to it in terms of a clear Epo dependency in proliferation, which however did not turn out to be dose-dependent. In fact, we precultured the cells for 24 hours in serum-free medium for the purpose of starvation and used heated and thus Epo-inactivated serum for the following 3 days in order to make the cells susceptible to only our administration of Epo, as previously described by Belenkov et al. (75).
Confirming our results, Westphal et al. (82) found various tumor cell lines expressing EpoR, which all lacked in receptor functionality measured by tyrosine

kinase activity while Epo had no influence on the proliferation rate of these tumor cell lines.

Aside from the fact that EpoR activation experiments did not reveal distinct results to answer this question, an unquestionable presence of time- and dose-dependent Epo-induced signaling in lung cancer cells does not inevitably correlate with a growth advantage as Dunlop et al. demonstrated in 2006 (72).

Likewise, in 8 other studies (94-101), no biological response to Epo could be attested to 68 tumor cell lines in terms of proliferation or clonogenic growth assays as critically reviewed by Sinclair and colleagues (46).

By contrast, 4 studies (64, 65, 102, 103) were able to provide evidence that tumor cells can respond to Epo with increased proliferation. In detail, Sinclair et al. questioned the biological relevance in two of these studies (102, 103) for the reasons of lacking establishment of dose-response relations in these cell lines and that the proliferative response in breast cancer cells (102) was not reproducible by 4 other studies (94, 95, 97, 98). Only 2 of the 4 positive studies for proliferation demonstrated a dose-depending proliferation in response to Epo in renal and prostate cancer cell lines ranging from 0.5 to 100 U/mL Epo in both serum-free and serum-containing media (64, 65). Nevertheless, Sinclair et al. criticize the absence of vehicle controls, which should be given instead of Epo, as well as the suprapharmacological doses of Epo in these studies that would limit their usefulness.

In this respect, the averaged basal plasma concentration of Epo in healthy individuals regardless of sex or age-specific differences ranges from 0.005 to 0.04 U/mL, that equals around 10^{-11} mol/L (13, 104, 105), while it can peak to 0.15 to 8 U/mL after subcutaneous (150 U/KG) or intravenous (300 U/KG) administration of clinically relevant doses (Epoetin alfa) (105, 106). On the other hand, Hammerling et al. demonstrated the effect of Epo on an erythroid cell line, in which already pharmacologically relevant doses between only 0.01 and 0.4 U/mL led to an increase of 650% in proliferation (107).

Along with the principle of promoting proliferation, Epo's well-understood role in hematopoietic progenitor cells as survival factor is based in large part on its inhibition of pro-apoptotic proteins mediated mainly through Bcl-x_L (44, 67, 69, 108, 109). We demonstrate that Epo could not protect from cisplatin-induced

apoptosis in these lung cancer cells within our experimental setting. In our approach, Epo effects were determined by measuring the percentage of activated effector caspase-3 after cisplatin-induced apoptosis in these lung cancer cells. Although the concentration of cisplatin applied (8 µM) derived from a cisplatin titration assay that was performed on A427 cells and then extrapolated on the other NSCLC cell lines, this concentration indeed resulted in a statistically significant decrease of overall viability in all cell lines examined but was not able to trigger clear caspase-3 activation in NCI-H358 cells. These data were collected from two independent experiments and should therefore be considered with due care since more data will be needed to become more conclusive. Nevertheless, the experimental model turned out to be working since the control cell line UT-7/Epo exhibited clear abolition of cisplatin-induced apoptosis after administration of Epo in both culturing (0.4 U/mL) and study concentration (100 U/mL).

In line with our results, Liu et al. (96) demonstrated in 2004 that administration of 10 U/mL Epo could not increase Bcl-2 expression in all 7 malignant cell lines under study even though the MAP kinase pathway was shown to be activated resulting in an unchanged high cytotoxicity of cisplatin compared with treatment using cisplatin alone. Further, Epo did not lead to an apparent increase in viability or proliferation.

Conversely, Hardee, Pajonk, and Acs (60, 110, 111) could independently show that exogenous Epo stimulates proliferation and inhibits apoptosis in cancer cells.

In one study published by Belenkov et al. (75), a glioblastoma and a cervical carcinoma cell line became more resistant to ionizing radiation and cisplatin after treatment with 30 U/mL Epo. Selective inhibition of JAK2 kinases could prove that the resistance was Epo-induced.

Astonishingly, one study carried out by Carvalho et al. (112) even reported that Epo inhibited NF-κB-induced anti-apoptotic gene transcription in a renal cancer and in a myelomonocytic leukemia cell line that enhanced chemotherapy-induced cell death suggesting a chemosensitizing effect by Epo.

Taken together, the results from recent proliferation and apoptosis studies on the protective effects of Epo on tumor cells are by now rather contradictory.

4.3 Clinical impact of the results

The use of Epo and its derivatives have consistently been shown to reduce transfusions and increase the hemoglobin level in lung cancer patients with chemotherapy-related anemia (11, 26, 34).

Over the past decade, there has been continually growing concern from both in-vitro and in-vivo studies that Epo has the ability to affect non-hematopoietic malignant cells as it does for the hematopoietic progenitor cells (46, 47). Hereby, the question has been raised what equivocal role does Epo play in lung cancer in terms of promoting cell growth, inhibiting cell death, favoring tumor progression, and in turn reduces survival of the patient, even though it was supposed to relieve anemia.

This in-vitro study summarizes that aside from the fact that these lung cancer cells exhibit EpoR, and its functionality remained ambiguous in part, Epo did not lead to a growth advantage or increased protection from apoptosis in these tumor cells within this experimental model.

In conclusion, the expression of EpoR in these tumor cells does not seem to play a pivotal role for the growth of these cells, which is why this study within its nature-given limits supports the safety profile of Epo.

Still, caution is advised because these results derive from isolated, artificially cultured cancer cells. This model excludes any molecular interplay between tissues of different origin that surrounds the tumor in-vivo. It has been described that functional EpoR is expressed on non-hematopoietic, non-malignant cells, such as endothelial cells (113) and vascular smooth muscle cells (114), thus making this interplay possible in terms of angiogenesis. For instance, Hardee et al. (68) were able to demonstrate Epo-induced enhancement of tumor growth and induction of neovascularization in rodent mammary carcinoma cells inoculated in mice. These effects were reversed after injection of soluble EpoR or anti-Epo antibodies suggesting that Epo is an important angiogenic factor that regulates the induction of tumor cell-induced neovascularization and tumor growth.

To further our understanding on the effects of Epo in lung cancer, additional studies are to be conducted on the potential role of an endogenous Epo-EpoR axis in modulating therapeutic resistance, and on how the balance between pro- and

anti-apoptotic components might be altered by exogenous Epo. And if relevant, whether there is a cell- or tissue-specific manner, in which the Epo-EpoR axis might be targeted clinically for therapeutic intervention.

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Appendix

SDS-PAGE protocols:

SDS-Gels: (∅1.5 mm)

Resolving gel (2X)				**Stacking gel (2X)**	
	6%	8%	12%		
30% Acrylamide	4 mL	5.2 mL	8 mL	**30% Acrylamide**	1.66 mL
dH_2O	10.4 mL	9.2 mL	6.4 mL	dH_2O	6.8 mL
1.5 M Tris pH 8.8	5.2 mL	5.2 mL	5.2 mL	**1.5 M Tris pH 6.8**	1.26 mL
10% SDS	200 µL	200 µL	200 µL	**10% SDS**	100 µL
10% APS	200 µL	200 µL	200 µL	**10% APS**	100 µL
TEMED	16 µL	12 µL	8 µL	**TEMED**	10 µL

Solutions:

Acrylamide (500 mL)	– Acrylamide stock solution: 149.6 g acrylamide; 4 g Bis-AA; fill to 500 mL dH_2O in dark bottle.
Running buffer (1000 mL**)**	– 10x stock solution: 30 g Tris; 144 g glycine; 10 g SDS; dissolved in 1 L dH_2O. – 100 mL 10x running buffer stock solution; 900 mL dH_2O.
Transfer buffer (2250 mL)	– 10 x stock solution: 56 g Tris Base®; 286 g glycine; dissolved to 2 L dH_2O. – 225 mL 10x transfer buffer stock solution; 450 mL methanol; 1575 mL dH_2O.
TBS-T (1000 mL)	– 10x stock solution: 31.5 g Tris-HCL; 80 g NaCl; dissolved in 1 L dH_2O; pH 7.4-7.6. – 100 mL 10x TBS stock solution; 900 mL dH_2O, 1 mL Tween20 (0,1 %).
Blocking solutions (100 mL)	– 10 mL 10x TBS; 90 mL dH_2O; 100 µL Tween20 (0,1 %); • Skim milk powder: 1 g (1 %); 5 g (5 %); or • BSA: 5 g (5%).