

Erythropoietin receptor induces a paclitaxel resistance phenotype in mammary adenocarcinoma cells

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Abstract. While erythropoietin (EPO) regulates erythropoiesis, the erythropoietin receptor (EPOR) has been identified in many non-hematopoietic cells, including cancer. Our previous study demonstrated that overexpression of EPOR altered the cell growth and the sensitivity of RAMA 37 breast cancer cells to tamoxifen. Indeed, results of the present study uncovered the role of EPOR in the resistance of EPOR-overexpressing RAMA 37-28 cells to paclitaxel chemotherapy. In this regard, EPOR silencing in the presence of paclitaxel therapy decreased RAMA 37-28 cell proliferation, confirming its role in the sensitivity or resistance of RAMA 37-28 cells to paclitaxel. Notably, compared to parental RAMA 37 cells, RAMA 37-28 cells also showed a lower rate of apoptosis induced by paclitaxel, as monitored by caspase 3/7 activation and Annexin V by IncuCyte ZOOM system. Moreover, enhanced activation of signaling pathways mediated by pERK1/2 in RAMA 37-28 cells as detected by western blot analysis was demonstrated to be essential for paclitaxel resistance.

Introduction

The resistance of cancer cells appears to be a major issue in current oncology treatment, as it hinders successful treatment and worsens the prognosis of oncology patients. The mechanisms involved in cancer cell resistance also reveal a broad network of interactions at the molecular level and confirm the great complexity of the human organism. As such, further investigation that may utilize the acquired knowledge to predict and successfully treat different diseases, including cancer, is necessary. The goal of scientists should include the

blockage of tumor resistance and improvement of therapeutic options, consequently improving patient prognosis. Several clinical studies have shown a poor prognosis of cancer patients in the case of recombinant erythropoietin (EPO) administration (1,2). This growth hormone has a wide application mostly in the amelioration of anemia that accompanies chemotherapy. Its action is mediated through the EPO receptor (EPOR), and stimulation of EPOR in the cell leads to the activation of different signaling pathways followed by the activation of transcription factors participating in the regulation of many cellular processes. These effects are mitogenic, antiapoptotic, and anti-inflammatory, among other cell protective effects (3). Although it has long been assumed that EPO only acts on blood-forming cells, its action has also been demonstrated in the non-hematopoietic environment, including nerve, retinal, myocardial, and other cells, where the presence of EPOR has been detected (4). Furthermore, EPOR expression has also been identified in several types of cancer cell lines (5,6). In light of the presence of EPOR, activation of EPO/EPOR signaling pathways, and a broad scale of potential effects in the tumor environment, the adverse outcomes of the abovementioned clinical trials can be explained. In contrast, functional EPORs have not been concisely shown to exist in human tumor cell lines (7). Supportive EPO therapy is currently used in cancer patients treated with paclitaxel, which belongs to the group of unique mitotic inhibitors, taxanes (8). Their usage shows great promise in anticancer therapy due to their antiproliferative and antiangiogenic actions observed together with the antimetastatic activity on cancer cells. Although taxanes are assumed to have high potential in the treatment of various types of cancer, they have not been shown to be effective in every case (9). While Larsson *et al* (10) demonstrated a correlation between EPOR and both the estrogen receptor (ER) and progesterone receptor (PR) in breast cancer cells, Volgger *et al* (11) found a positive correlation between the EPOR/ER/PR status and higher local cancer recurrence. In this regard, EPOR silencing was found to reduce the proliferation of both EPOR- and ER α -positive breast cancer cells but not ER α -negative cells (12). Based on our previous study (13) utilizing EPOR-overexpressing and ER α -negative mammary adenocarcinoma RAMA 37-28 cells, we investigated the role of EPOR in the sensitivity and/or resistance of these cells to

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chemotherapeutic agent paclitaxel. The role of EPO in the proliferation and the apoptosis of RAMA 37-28 cells under both control and paclitaxel conditions was also investigated.

Materials and methods

Cell lines and cell culture. Rat mammary adenocarcinoma cell line RAMA 37 and its clone RAMA 37-28 stably transfected with human EPOR were cultivated in RPMI-1640 medium (Gibco/Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco/Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Invitrogen/Thermo Fisher Scientific, Inc.). Cells were maintained at 37°C in a constant atmosphere of 5% CO₂, 21% O₂, 74% N₂ and 95% humidity.

Therapeutic agents. Epoetin α (EPO) (Binocrit; Sandoz) and paclitaxel (Ebewe Pharma) were purchased commercially. Both agents were stored at 4°C. EPO was provided as a 40,000 IU/ml solution and was diluted to make a final concentration of 10 IU/ml. Paclitaxel was provided as a 7 mM solution and was diluted at a ratio 1:69 to construct a 0.1 mM working solution before the preparation of a final concentration of 200 nM. Both EPO and paclitaxel solutions were prepared fresh before use.

Proliferation assays. The proliferation assays were carried out using a clonogenic assay and Incucyte ZOOM system (Essen BioScience).

Clonogenic assay. Both the RAMA 37 and its clone RAMA 37-28 cell lines were seeded on 6-well plates overnight. EPO (10 IU) and/or paclitaxel (200 nM) was then added to the relevant groups of wells. Cells that survived a 72-h incubation period with paclitaxel alone or with the addition of EPO were seeded on other 6-well plates in the amount of 1,000 cells/well and then incubated for 10 days. A similar procedure was adopted in the case of control groups. After 10 days, the medium was aspirated and colonies were stained by methylene blue (500 µl/well) at 1% concentration. The number of colonies was determined using Clono Counter software (14). The results were analyzed statistically using GraphPad Prism 5.01 software (GraphPad Software, Inc.).

Incucyte ZOOM system. The Incucyte ZOOM system allowed the monitoring of the proliferation of both control and treated/experimental groups in a defined environment of a standard incubator. Simplistically this system presents a microscope gantry placed in a humidified incubator, and a networked external controller hard-drive made it possible to gather images and process experimental data. Experimental groups included controls (without the addition of any therapeutic agents), cells with EPO alone or in combination with paclitaxel, and cells with paclitaxel alone. All experimental groups were seeded in a 96-well plate in hexaplicates at a concentration of 3,000 cells/well. Cell proliferation was monitored for 120 h using the Incucyte ZOOM system placed in an incubator. The experiment was initiated by seeding 100 µl of cell suspension/well. Cells were left to adhere and 24 h after seeding, another 100 µl of culture medium with or without

therapeutic agents was added. Experiments were replicated three times. Images of the wells of the 96-well plate were collected every 2 h by IncuCyte ZOOM 4x objective (Nikon Plan Apo Lambda 4x/0.20; cat. no. 4466) IncuCyte ZOOM integrated software (Essen Bioscience) was used to analyze the results.

EPOR silencing. Cells seeded on 96-well plates were treated with 2 µM of on-targeting siRNA (ON-TARGETplus SMARTpool Human EPOR siRNA; Dharmacon). Experimental group of RAMA 37-28 cells treated with non-targeting siRNA (ON-TARGETplus Non-targeting Control siRNA; Dharmacon) at the concentration of 2 µM served as the negative control. Untreated RAMA 37-28 cells served as the controls. Three independent experiments, including samples in triplicates (96-well format), were conducted. Briefly, 100 µl of cell suspension/well with cells at the concentration of 3,000/well was used. Cells were diluted in antibiotic-free complete medium (with the supplement of 10% FBS) and incubated at 37°C overnight. siRNA stock solution (100 µM) was prepared in 1X siRNA buffer and the concentration of siRNA was verified using UV spectrophotometry at 260 nm. Resuspended siRNA was stored at -20°C, and each diluted siRNA at the concentration of 2 µM was prepared freshly before use. The appropriate volume of 2 µM siRNA and the appropriate volume of DharmaFECT transfection reagent (Dharmacon) in the 2 separate tubes were diluted with serum-free and antibiotic-free medium according to the manufacturer's instructions. The contents of each tube were mixed gently by pipetting up and down and subsequently incubated for 5 min at room temperature (RT). They were then merged into 1 tube and further incubated for 20 min at RT. Afterward, a sufficient amount of antibiotic-free complete medium was added to the mix for the desired volume of the transfection medium. Culture medium from the wells of the 96-well plates was removed and replaced by 100 µl of the appropriate transfection medium. After 48 h of cell incubation, the transfection medium was replaced with complete medium with or without the addition of therapeutic agents paclitaxel and/or EPO (200 µl/well). Incucyte monitoring of the cells was stopped after 144 h from cell seeding. Incucyte ZOOM integrated software (Essen Bioscience) was used to analyze the results.

Both transfection procedures, as well as the treatment of cells with or without therapeutic agents, were conducted before western blot analyses using a 6-well plate format. In this case, RAMA 37 and RAMA 37-28 cells were stimulated with paclitaxel for 2 different periods (15 min or 24 h) and lysed afterward to perform western blot analysis.

Western blot analysis. Western blot analysis was carried out according to the generally accepted protocol. Specifically, cells were lysed using lysis buffer in the presence of protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc). Protein samples were separated by 12% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad). The following primary antibodies: anti-p-ERK1/2 (cat. no. 9101S), anti-p-AKT (cat. no. 9271S), anti-STAT5 (cat. no. 9363S), anti-ERK1/2 (cat. no. 9102S), anti-AKT (cat. no. 9272S), anti-BAX (cat. no. 2772S), anti-BCL-XL (cat. no. 2762S), anti-caspase 3 (cat. no. 14220S), anti-β-actin

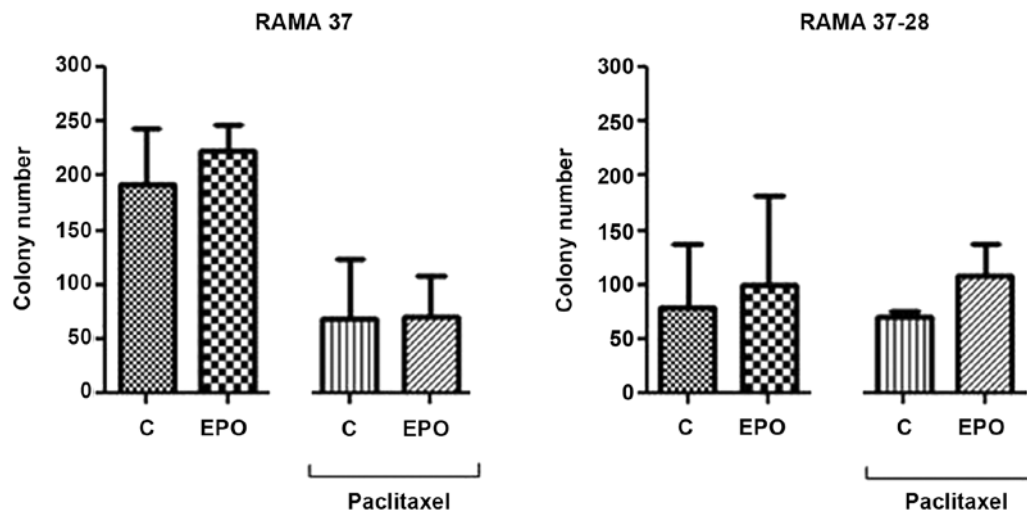


Figure 1. Clonogenic assay of RAMA 37 and RAMA 37-28 cells in the presence of paclitaxel or paclitaxel + EPO. Data are means \pm standard deviations of three independent experiments. A statistically significant difference was not observed; $P > 0.05$. C, control; EPO, erythropoietin.

(cat. no. 3700S) (all from Cell Signaling Technology, Inc.; 1:1,000 dilution), anti-p-STAT5 (cat. no. 50095; Temecula; 1:1,000 dilution), anti-EPOR (A82; Amgen; 1:2,000 dilution), and HRP-conjugated secondary antibodies (Pierce Chemical; 1:2,000 dilution), were used for detection. β -actin antibodies were used as controls for equal protein loading. The visualization was performed using the ECL Western blotting substrate (Thermo Fisher Scientific, Inc.) and Biomax imaging film (Kodak) or ChemiDoc XRS+ Imaging system (Bio-Rad). The films were scanned with the GS-800 Calibrated Densitometer, and the quantification was performed using Image J software version 1.52 (NIH; National Institutes of Health, Bethesda, MD, USA). The results are shown as the mean densities from 3 independent experiments.

Apoptosis assays

Annexin V and caspase 3/7 activity. Cells seeded on a 96-well plate (3,000 cells/well in 100 μ l/well) in triplicates were allowed to adhere overnight. Annexin V reagent (Incucyte Annexin V Green Reagent for Apoptosis; Essen Bioscience, final dilution of 1:200) or Caspase 3/7 reagent (Incucyte Caspase 3/7 Green Apoptosis Reagent; Essen Bioscience, final dilution of 1:1,000) were added together with paclitaxel and/or EPO 72 h after cell seeding and EPOR silencing (experimental groups with siRNA and nt siRNA against EPOR). The rate of activation of Annexin V and both caspase 3/7 in cells was monitored with the Incucyte ZOOM system every 1 h after treatment of the cells. Plates were pre-warmed prior to data acquisition to avoid condensation and expansion of the plate, which would hinder autofocus. The maxima of excitation and emission were 490/515 nm and 500/530 nm for Annexin V and caspase 3/7, respectively. Images of wells of the 96-well plate were collected by Nikon 20x objective. Incucyte ZOOM integrated software (Essen Bioscience) was used to minimize background fluorescence and quantify fluorescent objects.

Statistical analysis. The data were statistically analyzed using ANOVA followed by Tukey's multiple comparison tests in

ORIGIN analysis software (OriginLab Co., Northampton, MA, USA). The results were considered significant at the probability level $P < 0.05$ and $P < 0.01$.

Results

The effects of different concentrations of paclitaxel on the response of rat mammary adenocarcinoma RAMA 37 and RAMA 37-28 cells were monitored by MTT assay (data not shown). From MTT cell survival plots, we determined the concentration of the paclitaxel drug (200 nM), which inhibited the cell survival of both cell lines by 50% (IC_{50}). This concentration was used in other analyses.

Clonogenic assay. Clonogenic assay was first used to evaluate the proliferation of the EPOR-overexpressing RAMA 37-28 cells compared to the parental RAMA 37 cells in control and paclitaxel conditions (Fig. 1). In this regard, the effect of single EPO or its combination with paclitaxel was also studied. Although we observed the overall slower proliferation of RAMA 37-28 cells compared to RAMA 37 cells, the colony number was decreased only slightly after paclitaxel treatment in the EPOR-overexpressing RAMA 37-28 cells as opposed to the parental RAMA 37 cells. In addition, EPO in combination with paclitaxel stimulated (protected) RAMA 37-28 cell proliferation.

Incucyte ZOOM system. The effects of EPO, paclitaxel, and their combination on *in vitro* proliferation of the EPOR-overexpressing RAMA 37-28 cells was also monitored by the Incucyte ZOOM system (Figs. 2B and 3B). We determined the stimulation of RAMA 37-28 cells either after single EPO or after the combination of EPO and paclitaxel. Our results, as demonstrated in Fig. 2A and B, showed that a more pronounced stimulation and/or protection was observed in the RAMA 37-28 cells with stably expressed human EPOR, although low stimulation was also observed in the parental RAMA 37 cells. We chose a specific siRNA against human EPOR to confirm the role of EPOR in the potential resistance of RAMA 37-28 cells to paclitaxel. Indeed, the decrease in RAMA 37-28 cell proliferation, as a result of

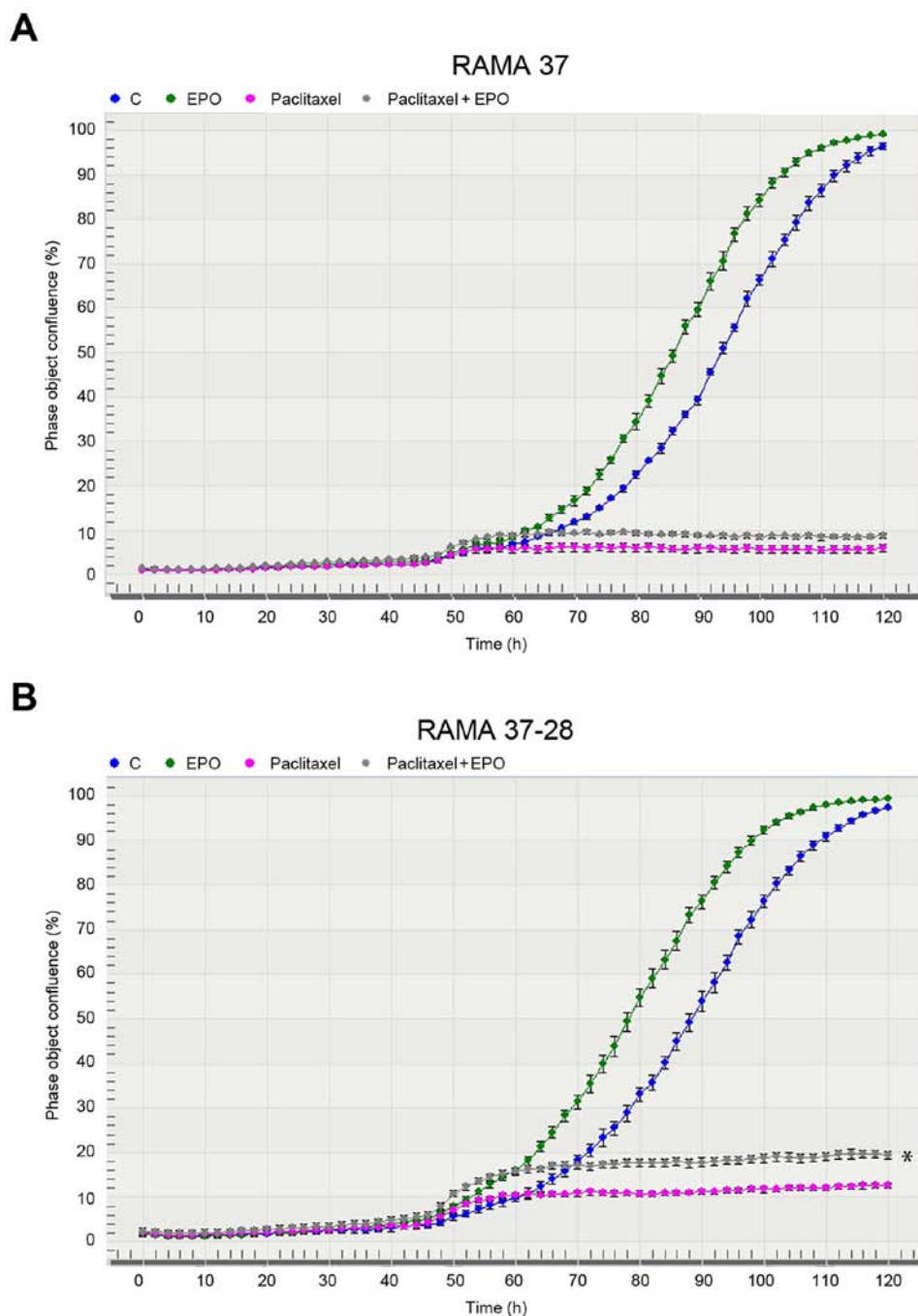


Figure 2. Effect of paclitaxel, EPO and the combination of paclitaxel + EPO on the proliferation of (A) RAMA 37 and (B) RAMA 37-28 cells. The proliferation was monitored using Incucyte ZOOM system. Representative image of three independent experiments is shown. Statistically significant difference of $P < 0.05$ vs. paclitaxel is indicated. C, control; EPO, erythropoietin.

EPOR silencing in the group treated with siRNA and paclitaxel, confirmed the role of EPOR in paclitaxel resistance (Fig. 3A). The RAMA 37-28 cells in the negative control group treated with nt siRNA did not exhibit a difference in cell proliferation in any way, and it showed the same trend in proliferation as the group treated only with paclitaxel. Moreover, an EPO stimulating (protecting) effect, when combined with paclitaxel as opposed to paclitaxel alone, was also successfully minimized using siRNA (Fig. 3B). The specificity and the efficiency of the applied siRNA were confirmed by western blot analysis using a specific anti-EPOR antibody A82. In contrast, the same technique also demonstrated the unspecificity of the nt siRNA.

Analysis of apoptosis. The rate of apoptosis induced by paclitaxel and represented by caspase 3/7 (Fig. 4A) and Annexin V (Fig. 4B) activation was negatively correlated with the EPOR expression in the RAMA cells. Indeed, RAMA 37-28 cells showed a lower rate of apoptosis compared to RAMA 37 cells after paclitaxel treatment. In this regard, the addition of EPO to paclitaxel-treated cells weakened the effect of paclitaxel in terms of decreased activation of Annexin V reagent. On the contrary, such a weakening effect of EPO was not observed in the case of caspase 3/7 activation. Similarly, EPOR silencing did not significantly affect the activation of both reagents (data not shown).

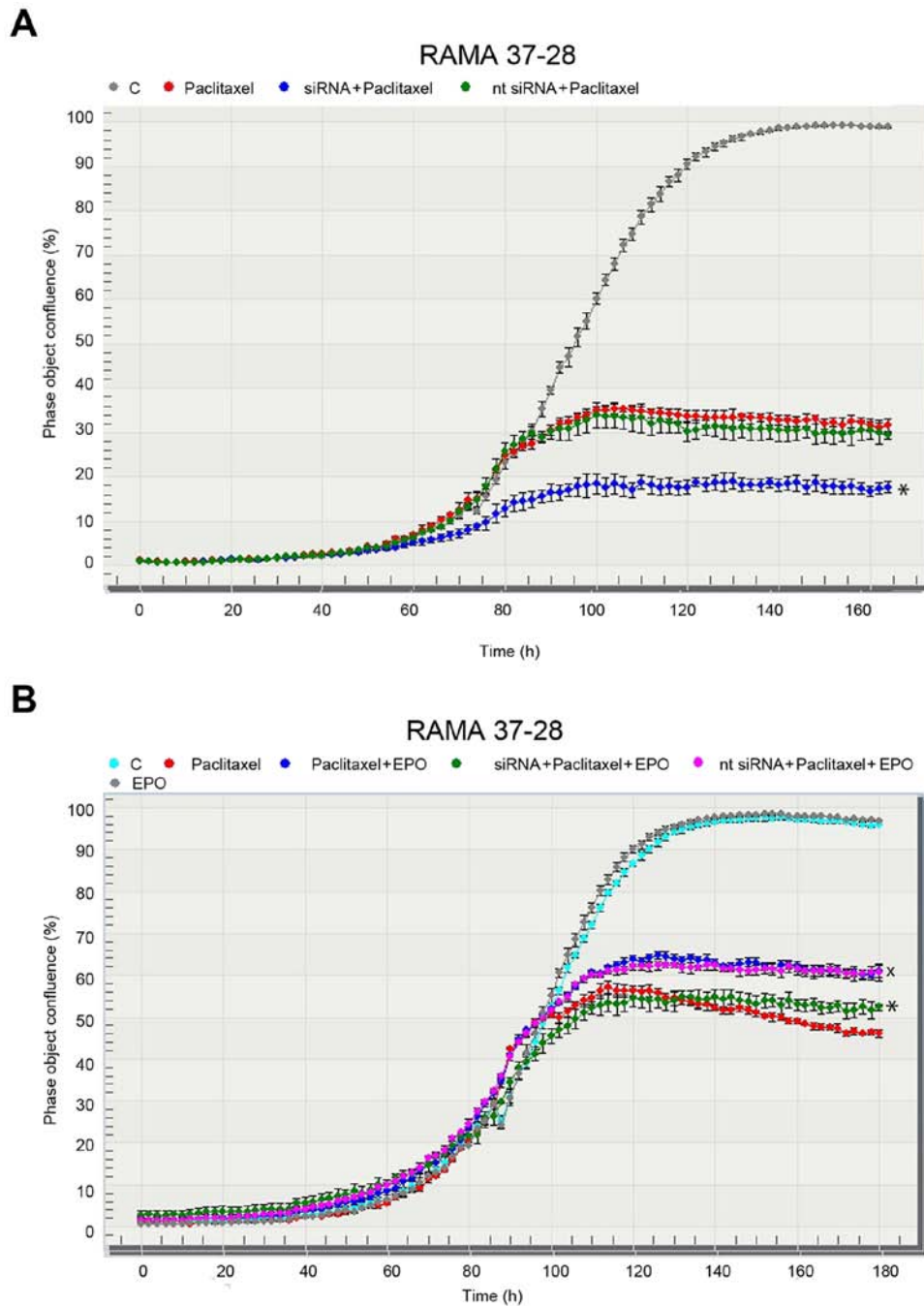


Figure 3. The effect of EPOR silencing on the proliferation of RAMA 37-28 cells in the presence of (A) paclitaxel or in the presence of (B) paclitaxel + EPO. Cell proliferation was monitored using the Incucyte ZOOM system. Representative images of three experiments are shown. Statistically significant difference in A is indicated by * $P < 0.05$ vs. paclitaxel and nt siRNA + paclitaxel. Statistically significant differences in B are indicated by * $P < 0.05$ vs. paclitaxel + EPO and nt siRNA + paclitaxel + EPO and * $P < 0.05$ vs. paclitaxel. C, control; EPO, erythropoietin.

Activation of key proteins. Subsequently, we focused on the key proteins activated in response to paclitaxel and/or EPO (Fig. 5 and Table I). Indeed, protein levels of phosphorylated ERK1/2 (pERK1/2) were higher in RAMA 37-28 cells compared to RAMA 37 cells even under control conditions. In this regard, the rate of pERK1/2 was higher in RAMA 37-28 and RAMA 37-28 + nt siRNA and lower in the group with siRNA. The addition of EPO had no further effect on pERK1/2 levels. A very similar situation occurred 15 min after paclitaxel administration when pERK1/2 was higher in RAMA 37-28 compared to RAMA 37 cells in both

paclitaxel as well as paclitaxel + EPO conditions. Interestingly, a higher rate of pERK1/2 was still observed in RAMA 37-28 and RAMA 37-28 + nt siRNA cells compared to RAMA 37 and RAMA 37-28 cells with EPOR siRNA in the presence of paclitaxel at 24 h. On the contrary, RAMA 37 cells showed a higher pERK1/2 level compared to RAMA 37-28 cells 24 h after the administration of the combination of paclitaxel and EPO (Fig. 5 and Table I).

No phosphorylation of AKT (pAKT) was observed in the control and EPO conditions in both RAMA 37-28 as well as RAMA 37 cells. On the other hand, paclitaxel activated

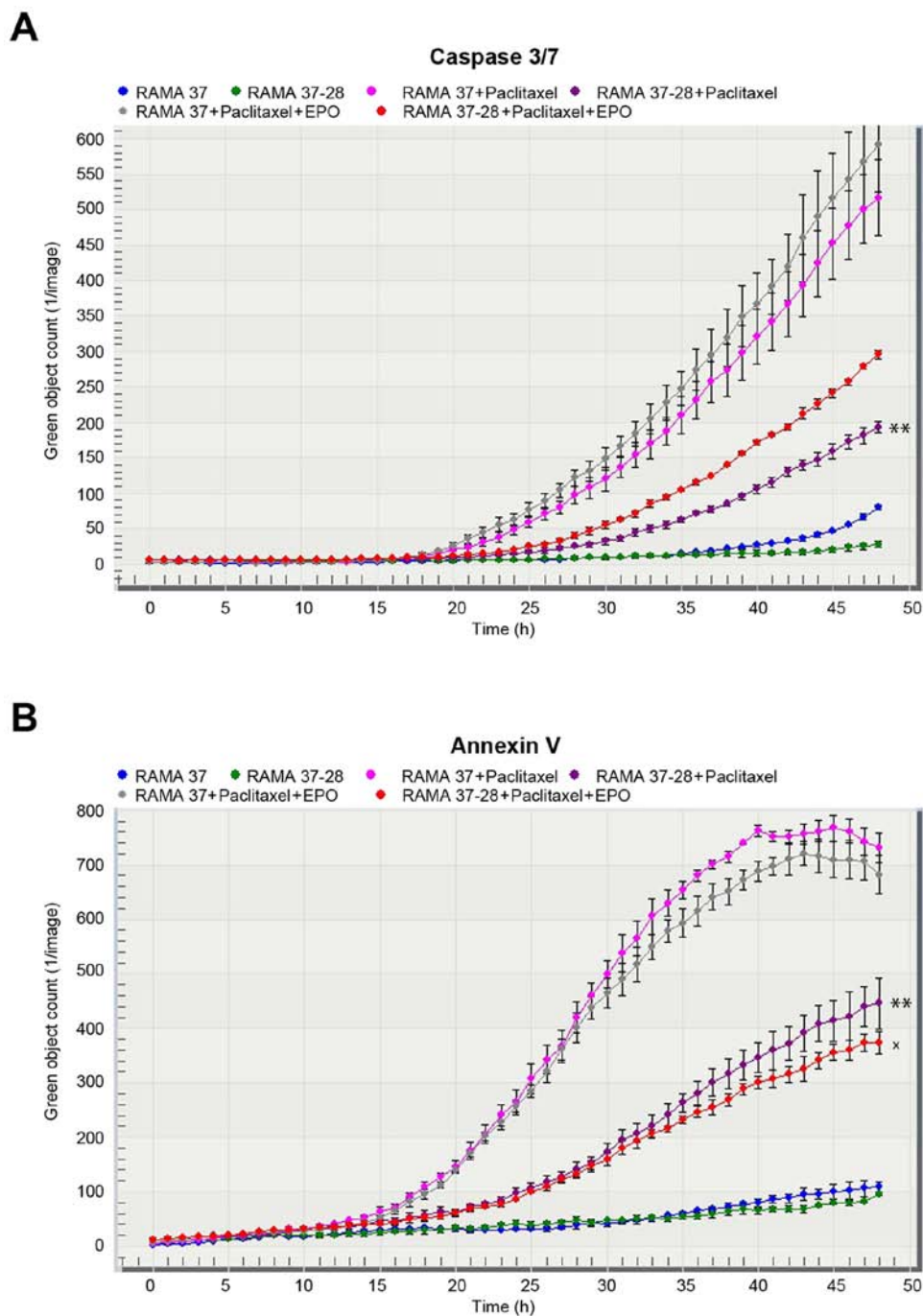


Figure 4. The rate of (A) caspase 3/7 and (B) Annexin V activation in RAMA 37 and RAMA 37-28 cells with addition of paclitaxel or paclitaxel + EPO. Incucyte ZOOM integrated analysis software was used for quantification of fluorescent objects. Representative image of three independent experiments is shown. Statistically significant difference in A is indicated by ** $P < 0.01$ vs. RAMA 37-28. Statistically significant differences in B are indicated by ** $P < 0.01$ vs. RAMA 37-28 and $xP < 0.05$ vs. RAMA 37-28 + paclitaxel. EPO, erythropoietin.

AKT signal transduction only at 24 h, not at the 15-min time point. Notably, paclitaxel did not induce significant pAKT changes in EPOR-overexpressing cells and/or experimental groups: RAMA 37-28 and RAMA 37-28 + nt siRNA compared to RAMA 37 and RAMA 37-28 + siRNA. In addition, a 24-h treatment with paclitaxel + EPO potentiated the pAKT signalization nonspecifically in each experimental group compared to single paclitaxel therapy (Fig. 5 and Table I).

Similar to pERK1/2, the phosphorylation of STAT5 (pSTAT5) in RAMA 37-28 cells occurred without EPO stimula-

tion and disappeared after the silencing of EPOR using siRNA in these cells. No additional pSTAT5 was found in RAMA 37-28 cells either after their incubation with EPO or after paclitaxel treatment, regardless of incubation time. On the contrary, significant pSTAT5 was monitored in RAMA 37-28 cells treated with the combination of paclitaxel and EPO, but only at the 24-h time point. The significance of EPOR in pSTAT5 of RAMA 37-28 cells is evident from their comparison with RAMA 37 cells and also from the silencing of EPOR using specific siRNA. Indeed, RAMA 37 cells did not manifest STAT5 signalization under any tested conditions (Fig. 5 and Table I).

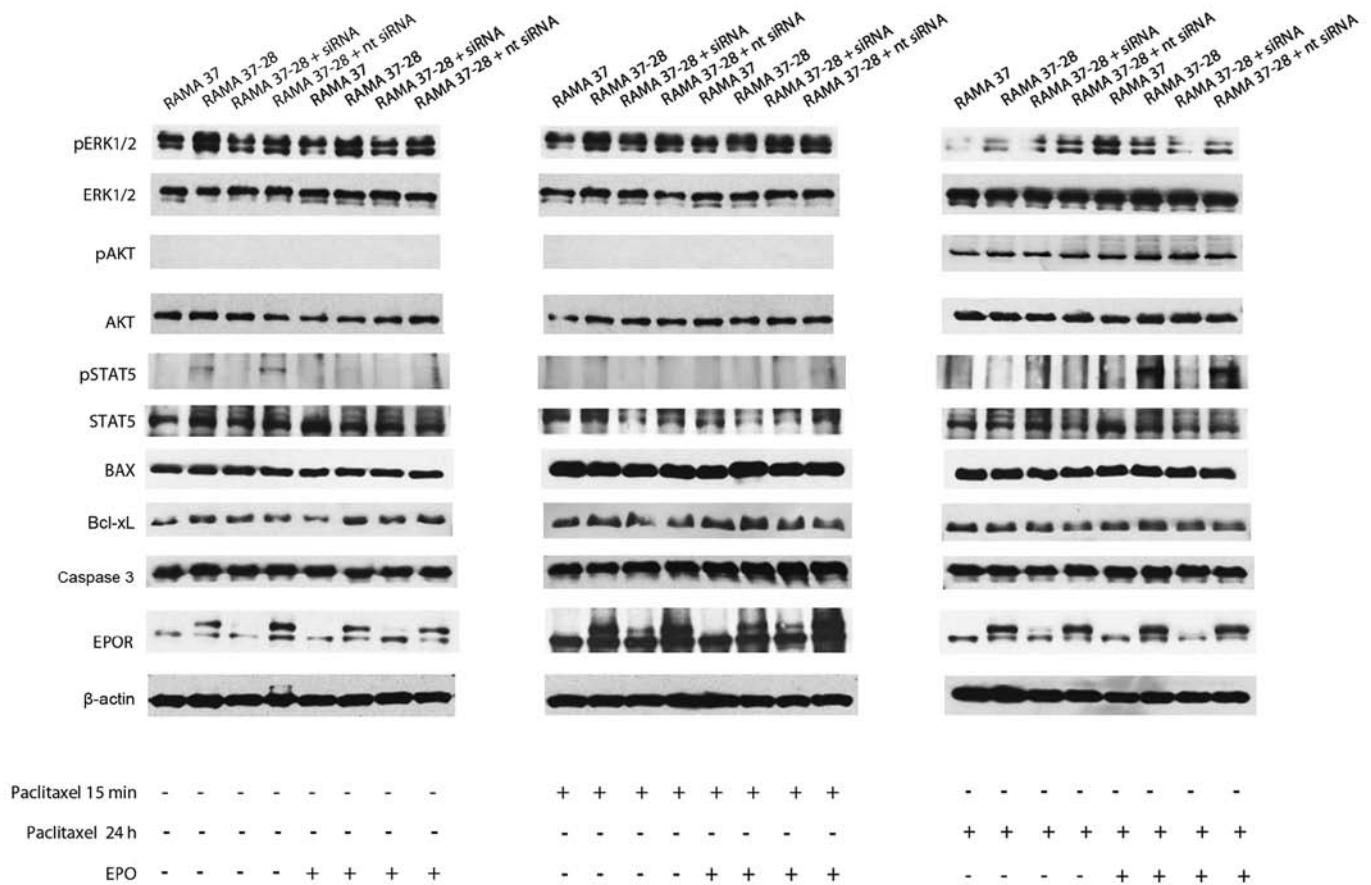


Figure 5. Effects of paclitaxel and/or EPO treatment on the levels of phospho-ERK1/2 (pERK1/2), phospho-AKT (pAKT), phospho-STAT5 (pSTAT5), Bax, Bcl-xL and Caspase 3/7 in RAMA 37 and RAMA 37-28 cells. The groups of RAMA 37-28 cells with EPOR silencing (siRNA) and RAMA 37-28 cells with non-targeting siRNA (nt siRNA) are shown. The cells were grown in standard growth media for 24 h followed by silencing of relevant experimental groups and 48 h later treated with paclitaxel at the concentration of 200 nM for 15 min or 24 h. Cells in the relevant experimental groups were treated with 10 IU of EPO. EPOR silencing was confirmed by the detection of EPOR and equal loading was shown by detection of β -actin. Representative image of three independent experiments is shown. EPO, erythropoietin; EPOR, erythropoietin receptor.

The roles of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-xL were also considered. In this regard, RAMA 37-28 (including experimental groups with siRNA and nt siRNA) and RAMA 37 cells did not show any difference in the Bax protein level under all conditions tested. Neither paclitaxel (in any time of treatment) nor EPO addition altered the level of Bax protein (Fig. 5 and Table I). On the other hand, the level of Bcl-xL was higher in the control RAMA 37-28 cells when compared to the level in the RAMA 37 cells, and silencing of EPOR did not lower the level of the Bcl-xL protein. Moreover, the level of Bcl-xL in the RAMA 37-28 cells was even more pronounced under EPO treatment without paclitaxel treatment. On the contrary, although the Bcl-xL level remained elevated in the RAMA 37-28 cells after paclitaxel and paclitaxel + EPO treatments at 15 min, it decreased under control conditions in each group at 24 h. In the case of caspase 3, no significant differences were observed in the level of this protein between experimental groups (Fig. 5 and Table I).

With the aim to show stable overexpression of EPOR in RAMA 37-28 cells and to confirm the results of EPOR silencing using siRNA, we decided to demonstrate the EPOR level in all experimental groups. In this regard, both control groups (without paclitaxel) and groups with paclitaxel (15 min or

24 h) showed an equal EPOR level in each experimental group. Furthermore, EPO did not have any effect on the level of EPOR in our experimental group (Fig. 5 and Table I).

Discussion

Both the adverse outcomes of various clinical trials (1,2) of cancer patients undergoing recombinant EPO support therapy as well as the confirmation of the presence of EPOR on the cancer cell surface are attracting great research interest. The evidence of the effects of recombinant EPO in regards to tumor progression in cancer patients, however, is unclear in several clinical trials. For example, in the case of Breast Cancer-Anemia and the Value of Erythropoietin (BRAVE) study there was no significant difference in overall survival (15). Furthermore, recent clinical studies failed to confirm the negative effect of recombinant EPO (16-18). To answer the question of whether the presence of EPOR can somehow affect the proliferation and apoptosis of cancer cells, we chose breast cancer cells and monitored their response to paclitaxel chemotherapy. Although paclitaxel is one of the most promising anti-cancer agents in clinical use, the development of paclitaxel resistance in cancer cells decreases the effectiveness of this drug. Poor response of oncological

Table I. Effect of paclitaxel and/or EPO treatment on RAMA 37 and RAMA 37-28 cell signaling: Effect of EPOR siRNA.

A, Effect of EPO treatment		Ratio according to control				Ratio according to EPO treatment			
Protein target	RAMA 37	RAMA 37-28	RAMA 37-28 + siRNA	RAMA 37-28 + nt siRNA		RAMA 37	RAMA 37-28	RAMA 37-28 + siRNA	RAMA 37-28 + nt siRNA
pERK1/2	1.00±0.12	1.93±0.22^a	0.93±0.08	1.46±0.14		0.75±0.09	1.21±0.24	0.82±0.11	1.35±0.18
pAKT	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00
pSTAT5	1.00±0.08	1.28±0.13	1.01±0.20	1.36±0.21		1.17±0.35	1.23±0.22	0.84±0.18	1.18±0.27
Bax	1.00±0.16	1.05±0.08	1.25±0.26	1.22±0.32		1.10±0.31	1.10±0.27	1.19±0.23	1.20±0.19
Bcl-xL	1.00±0.06	1.72±0.09^a	1.22±0.23	1.42±0.08^a		0.26±0.02	2.03±0.31^b	1.37±0.17	2.21±0.27^b
Caspase 3	1.00±0.11	1.12±0.28	1.08±0.17	1.10±0.10		1.02±0.33	0.86±0.09	0.98±0.21	0.98±0.15
EPOR	1.00±0.10	4.36±0.22^c	1.08±0.12	10.98±0.63^c		1.17±0.29	5.91±0.49^c	1.43±0.37	5.42±0.51^c

B, Effect of paclitaxel (15 min) or paclitaxel (15 min) combined with EPO		Ratio according to paclitaxel treatment (15 min)				Ratio according to paclitaxel treatment (15 min) + EPO			
Protein target	RAMA 37	RAMA 37-28	RAMA 37-28 + siRNA	RAMA 37-28 + nt siRNA		RAMA 37	RAMA 37-28	RAMA 37-28 + siRNA	RAMA 37-28 + nt siRNA
pERK1/2	1.00±0.15	1.68±0.26^a	1.40±0.46	2.02±0.21^a		1.15±0.08	1.78±0.18^a	1.39±0.32	1.73±0.23^a
pAKT	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00
pSTAT5	1.00±0.13	0.83±0.27	0.72±0.08	0.71±0.18		0.85±0.26	1.39±0.09	1.17±0.33	1.67±0.16^a
Bax	1.00±0.30	0.86±0.18	0.83±0.17	0.91±0.31		0.77±0.22	1.12±0.25	0.87±0.23	0.90±0.09
Bcl-xL	1.00±0.08	1.44±0.09^a	0.80±0.16	0.89±0.16		1.36±0.24	1.86±0.11^a	0.97±0.14	0.52±0.03
Caspase 3	1.00±0.11	0.82±0.05	1.11±0.21	1.14±0.12		1.31±0.23	1.36±0.41	1.41±0.40	1.42±0.25
EPOR	1.00±0.09	1.98±0.24^b	1.30±0.32	2.37±0.17^b		1.15±0.20	2.09±0.19^b	1.44±0.18	2.65±0.30^b

Table I. Continued.

C, Effect of paclitaxel (24 h) or paclitaxel (24 h) combined with EPO

Protein target	Ratio according to paclitaxel treatment (24 h)						Ratio according to paclitaxel treatment (24 h) + EPO					
	RAMA 37	RAMA 37-28	RAMA 37-28 + siRNA	RAMA 37-28 + nt siRNA	RAMA 37-28 + siRNA	RAMA 37-28 + nt siRNA	RAMA 37	RAMA 37-28	RAMA 37-28 + siRNA	RAMA 37-28 + nt siRNA	RAMA 37-28 + siRNA	RAMA 37-28 + nt siRNA
pERK1/2	1.00±0.15	3.73±0.47^b	1.73±0.58	1.73±0.58	8.00±0.82^c	10.73±0.93^c	5.93±0.72^b	5.93±0.72^b	2.89±0.63^a	4.81±0.77^b	4.81±0.77^b	4.81±0.77^b
pAKT	1.00±0.07	1.47±0.23	1.20±0.36	1.20±0.36	1.55±0.24^a	1.74±0.59	1.92±0.22^a	1.92±0.22^a	1.87±0.31^a	1.77±0.24^a	1.77±0.24^a	1.77±0.24^a
pSTAT5	1.00±0.11	0.32±0.03	0.71±0.06	0.71±0.06	0.76±0.08	0.65±0.07	1.81±0.25^a	1.81±0.25^a	1.46±0.47	2.47±0.31^b	2.47±0.31^b	2.47±0.31^b
Bax	1.00±0.09	0.93±0.10	0.81±0.13	0.81±0.13	0.89±0.20	0.92±0.11	0.98±0.23	0.91±0.19	0.91±0.19	0.92±0.21	0.92±0.21	0.92±0.21
Bcl-xL	1.00±0.08	0.66±0.07	0.52±0.06	0.52±0.06	0.28±0.02	0.57±0.03	0.78±0.08	0.43±0.06	0.43±0.06	0.32±0.03	0.32±0.03	0.32±0.03
Caspase 3	1.00±0.10	0.84±0.09	0.76±0.08	0.76±0.08	0.90±0.11	0.93±0.09	0.96±0.12	1.00±0.20	1.00±0.20	0.85±0.11	0.85±0.11	0.85±0.11
EPOR	1.00±0.07	2.34±0.22^b	0.57±0.19	0.57±0.19	2.22±0.19^b	0.56±0.08	2.78±0.32^b	2.78±0.32^b	0.15±0.02	2.32±0.28^b	2.32±0.28^b	2.32±0.28^b

Ratios ± standard deviation from quantitative densitometric analysis of pAKT, pERK1/2 and pSTAT5 normalized to AKT, ERK1/2 and STAT5, respectively. Bax, Bcl-xL, caspase 3 and EPOR were normalized to β-actin. Ratios in RAMA 37 cells under control and paclitaxel conditions (15 min and 24 h) were arbitrarily set to 1. ^aP<0.05 vs. RAMA 37 without EPO (three independent experiments; one-way ANOVA tests). ^bP<0.01 vs. RAMA 37 without EPO (three independent experiments; one-way ANOVA tests). ^cP<0.001 vs. RAMA 37 without EPO (three independent experiments; one-way ANOVA tests). Values in bold print indicate significant results. EPO, erythropoietin.

patients to paclitaxel raises the need to identify new markers for paclitaxel susceptibility in cancer cells.

Whereas some studies have demonstrated the presence of EPOR in cancer cells (19), other studies have failed to demonstrate high expression of EPOR in cancer cells and EPO-mediated cell stimulation (20,21). The discrepancy in data in terms of the level of EPOR in cancer cells appears to reflect differences in the specificity of used anti-EPOR antibodies or methodological approaches. The problem of unspecificity of anti-EPOR antibodies comes from the possible detection of proteins with improper molecular weight or non-EPOR molecules with the same molecular weight as EPOR (22,23).

The probability of the stimulation of cancer cell growth by EPOR was tested at different levels of EPOR expression in rat mammary adenocarcinoma cells RAMA. Indeed, since RAMA 37 and RAMA 37-28 cells differ in the level of EPOR expression but not in the expression of estrogen receptor α , β and G-protein coupled ER, they represent a suitable model for evaluating the effects of EPO/EPOR on cell physiology (13).

The present study confirmed our previous results (13) and demonstrated a difference in the proliferation of EPOR-overexpressing RAMA 37-28 cells compared to low EPOR-expressing RAMA 37 cells. Even in the absence of EPO, RAMA 37-28 cells proliferated slower compared to RAMA 37 cells. Because the slower proliferation of RAMA 37-28 cells would seem to imply greater resistance to paclitaxel, siRNA was used against EPOR to confirm the role of EPOR in the response of RAMA 37-28 cells to paclitaxel. Indeed, a decrease in the proliferation and increase in the rate of apoptosis after EPOR silencing was observed in the study of Cao *et al* (24) in the case of glioma stem cells. Paragh *et al* (25) showed the presence of phosphorylated EPOR signaling components in A2780 human ovarian adenocarcinoma cells, even when the cells were not exposed to exogenous EPO. In this regard, EPOR knockdown in breast cancer cell lines reduces pAKT levels, which suggests its involvement in transmission of signals, including phosphorylation and activation of AKT (12). Moreover, Ueda *et al* (26) demonstrated the role of JAK2 point mutation in EPOR activation and myeloproliferative neoplasms. Indeed, the EPO-independent EPOR activation needs to be elucidated in more detail. We confirmed the proposed cell stimulation by single EPO and/or by its combination with paclitaxel using IncuCyte monitoring, with pronounced proliferation observed in EPOR-overexpressing RAMA 37-28 cells. A slightly weaker but nevertheless stimulating effect of EPO in control and paclitaxel conditions was found also in RAMA 37 cells where EPOR had an almost undetectable level. This finding could be explained by the existence of receptors other than EPOR, e.g., β common receptor and/or ephrin type-B receptor 4 (4) through which EPO may affect cancer cells. On the contrary, this study clearly demonstrated a decrease in RAMA 37-28 cell proliferation after EPOR silencing in both paclitaxel as well as paclitaxel + EPO groups compared to nt siRNA.

Our findings are in line with an *in vivo* study by Todaro *et al* (27), which showed an increased progression of metastases in the case of combined therapy of paclitaxel + EPO compared to single paclitaxel in human breast cancer stem-like cell (BCSC)-derived orthotopic/metastatic

xenografts. Furthermore, our results are consistent with other *in vivo* studies of mouse models, where the silencing of EPOR expression using short hairpin RNAs prevented the progression of melanoma (28) or prostate cancer (29). Moreover, EPO protected BCSCs from chemotherapy and enhanced metastatic tumor progression through early activation of AKT and ERK signalization and later through an increase in Bcl-xL protein level. Similarly, early after stimulation of RAMA 37-28 cells by EPO, Shi *et al* (30) observed a significant activation of PI3K/AKT, RAS/ERK, and JAK2/STAT5 pathways, while in RAMA 37 cells, they observed no activation of the JAK2/STAT5 pathway and only minor activation of PI3K/AKT and RAS/ERK pathways. On the contrary, Swift *et al* (7) showed the inability of EPO to induce intracellular signaling in NCI-H661 cells.

In our *in vitro* model, EPOR-overexpressing RAMA 37-28 cells, as compared to parental RAMA 37 cells, revealed higher phosphorylation of ERK1/2 in control conditions and higher AKT signal transduction later after paclitaxel addition. Indeed, AKT/ERK signal transductions were shown to be active in paclitaxel-resistant gastric cancer cell lines, and it seems that AKT/ERK activation might have led to the development of paclitaxel and/or multidrug resistance of cancer cells (31). Furthermore, the silencing of EPOR gene expression confirmed the role of the ERK1/2 pathway in our EPOR-induced paclitaxel protection. On the other hand, increased Bcl-xL level in RAMA 37-28 cells did not change after EPOR silencing, and its level was even more pronounced under EPO treatment, regardless of the presence or absence of paclitaxel. Whereas in BCSC model Bcl-xL level was induced by EPO (27), in hepatocellular carcinoma cells SNU-398 (32) was caused by paclitaxel treatment, and in our RAMA 37-28 cells Bcl-xL increase resulted from the simple EPOR overexpression. Consistent with these findings, we also observed a decreased rate of caspase 3/7 activation and Annexin V in cells with EPOR overexpression compared to the rate of apoptosis induced by paclitaxel in RAMA 37-28 vs. RAMA 37 cells. On the contrary, the antiapoptotic effect of EPO in RAMA 37-28 cells was only demonstrated by Annexin V, not by caspase 3/7 activity. The lower sensitivity of IncuCyte monitoring of caspase 3/7 activity compared to enzymatic ELISA or flow cytometric analysis could explain this discrepancy. Moreover, neither Annexin V nor caspase 3/7 demonstrated any significant antiapoptotic effect of EPO in RAMA 37 cells at the endpoints of the monitoring.

Solar *et al* (33) were the first to demonstrate *in vitro* development of a paclitaxel resistance phenotype in human ovarian carcinoma A2780 cells as a result of EPO treatment. Indeed, the results of our present study confirmed the stimulation of EPO in mammary adenocarcinoma cells and the association between EPOR and increased resistance of these cells to paclitaxel. The hypothesized direct effect of EPO on cancer cells highlights the importance of further studies on the EPO/EPOR interactions in cancer cells and the possible modulation of their sensitivity not only to paclitaxel, but also to other chemotherapeutics.

In conclusion, the higher paclitaxel resistance and lower apoptosis rate of EPOR-overexpressing rat mammary adenocarcinoma RAMA 37-28 cells indicate a strong association between EPO/EPOR and tumor progression. The silencing

of EPOR expression under the presence of paclitaxel therapy led to a decrease in RAMA 37-28 cell proliferation and thus confirmed the role of EPOR in the sensitivity of these cells to this therapy. Interestingly, compared to RAMA 37 cells, RAMA 37-28 cells also showed a lower rate of apoptosis induced by paclitaxel and monitored by caspase 3/7 activation and Annexin V. Moreover, enhanced activation of signaling pathways mediated by pERK1/2 in RAMA 37-28 cells was demonstrated to be essential for paclitaxel resistance.

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Availability of data and materials

The datasets used during the present study which are not provided in the manuscript are available from the corresponding author upon reasonable request.

Authors' contributions

Conception and design of the study was carried out by EZ and PS. Acquisition of the data was conducted by EZ, LI, PK and BF. Analysis and interpretation of data was performed by EZ, PS and LI. Writing, review and revision of the manuscript was carried out by EZ, PS, LI, BF and PK. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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