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The effects of erythropoietin signaling on telomerase regulation in non-erythroid malignant and non-malignant cells

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ABSTRACT

Treatment with erythropoietin (EPO) in several cancers is associated with decreased survival due to cancer progression. Due to the major importance of telomerase in cancer biology we hypothesized that some of these effects may be mediated through EPO effect on telomerase. For this aim we explored the possible effects of EPO on telomerase regulation, cell migration and chemosensitivity in non-erythroid malignant and non-malignant cells.

Cell proliferation, telomerase activity (TA) and cell migration increased in response to EPO. EPO had no effect on cancer cells sensitivity to cisplatinum and on the cell cycle status. The inhibition of telomerase modestly repressed the proliferative effect of EPO. Telomere shortening caused by long term inhibition of the enzyme abolished the effect of EPO, suggesting that EPO effects on cancer cells are related to telomere dynamics. TA was correlated with the levels of Epo-R. The increase in TA was mediated post-translationally through the Lyn-Src and not the canonical JAK2 pathway.

1. Introduction

Erythropoietin (EPO) is a growth factor which regulates erythropoiesis and is widely used for the treatment of anemia in cancer patients. However, in several clinical studies reported recently that EPO treatment was associated with decreased survival of cancer patients due to increased cancer progression and aggressiveness [1]. This effect was attributed to a "tissue protective effect" on non-hematopoietic tissues, possibly mediated through a novel heteroreceptor [1].

The importance of telomerase in the biology and prognosis of many types of cancers is well established. Telomerase is a unique reverse transcriptase, expressed almost exclusively in >90% of cancer cells. It prevents telomeric loss in each DNA replication [2] and therefore confers endless replicative potential to cancer cells. Due to its essentiality and specificity to the malignant cell compounds targeting telomerase are in advanced phases of clinical trials [3].

In the light of the major importance of telomerase in cancer biology and due to the detrimental effect of erythropoietin in cancer we sought to study the possible crosstalk between EPO and telomerase activation in non-erythroid malignant and benign cells, assuming that some of the effects of EPO may be mediated through its effect on telomerase.

The activation of telomerase by EPO was recently reported in primary erythroid cell lines and UT7 (erythroleukemia cells) through transcriptional mechanism induced by the canonical JAK2/STAT5/c-Myc pathway [4]. Likewise, EPO activated telomerase in erythroleukemic JAS-REN-A cells which was mediated by both transcriptional and posttranslational mechanisms [5]. The EPO-R signaling pathway activation is inherent to these types of cells and need not necessary activated in non-erythroid malignant cells. To our best knowledge telomerase response to EPO in malignant cells has not been studied yet.

The experimental system consisted of the following cell lines: malignant cells: SK-N-MC (Ewing sarcoma) cells, expressing receptor for erythropoietin (EPO-R) but not dependent on EPO. Two non-erythroid benign cells containing different levels of EPO-R: WT-EPO

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(Ba/F3 cells expressing the wild type EPO-R), NPVY (WT-EPO containing NPVY insertion into the EPO-R, increasing its surface expression). In addition we studied also Ba/F3 cells that express receptor for IL-3. Ba/F3 cells were included in the study since IL-3 receptors have a common signaling pathway with EPO-R. Ba/F3 cells are dependent on IL-3 while WT-EPO and NPVY are dependent on EPO.

2. Materials and methods

2.1. Cell lines

SK-N-MC cells were kindly provided by Dr. G. Lavie (Sheba Medical Center, Ramat-Gan, Israel). Cells were grown in RPMI 1640, 10% heat-inactivated FCS and 1% penicillin/streptomycin (Biological Industries, Beit Haemek, Israel.) Ba/F3, WT-EPO and NPVY were kindly provided by Prof. D. Neumann (Tel Aviv University, Ramat Aviv, Israel). Ba/F3 cells (IL-3 dependent) were grown as SK-N-MC cells with 1 ng/mL recombinant IL-3 (Peprotech, Rehovot, Israel). WT-EPO cells, derivatives of Ba/F3 cells (EPO dependent) containing the wild type EPO receptor (EPO-R) were grown in a similar medium with 0.5 U/ml EPO (Hoffman-La Roche, Basel, Switzerland) instead of IL-3. NPVY cells, other derivatives of Ba/F3 cells, expressing the EPO-R with an insertion of an NPVY sequence, were grown as WT-EPO cells.

2.2. Cell proliferation

Cells proliferation was determined by counting the number of viable cells with the Trypan Blue exclusion assay.

SK-N-MC sensitivity to cytotoxic drugs was detected by the Sulphorodamine B (SRB) assay. 1×10^4 cells/ml were seeded in 24-well plates with the relevant treatments (0.5 U/ml EPO, 0.3 μg/ml cisplatinum). Proliferation was determined with the SRB reagent as described previously [7]. All other cells proliferation was measured by detecting viable cells using the WST-1 assay according to the manufacturer's instructions (Roche, Germany) and as described previously [7].

2.3. Cell cycle

Cells treated with 5 or 10 U/ml EPO were cultured for 3 days, washed with PBS and nuclei were prepared for flow cytometric analysis using a detergent-trypsin method followed by staining with propidium iodide [7]. DNA content was analyzed by FACSCALIBUR (Becton Dickinson, San Jose, CA, USA), using ModFitLT cell cycle analysis software (Verity Software House Inc., Topsham, ME, USA).

2.4. Migration assays

Migration of cells was evaluated by the Boyden chamber assay as described previously [7].

20,000 cells/ml were treated with 10 U/ml EPO and seeded in triplicates on polycarbonate filters with 8 μm pores positioned in 24-well transwell chambers (Costar, Cambridge, Mass, USA). The chambers were coated with 5 μg/filter fibronectin. Culture medium was added and the cells were allowed to migrate for 18 h at 37 °C, the filters were then fixed with methanol and stained with Giemsa stain. Cells that migrated to the filter's bottom side were photographed and counted under a microscope. 5–10 images were screened for each sample.

2.5. Telomerase activity

5×10^4 cells/ml were incubated with EPO or IL-3 in duplicates as above. Measurement of TA was performed by the PCR-based TRAP assay, using the $TRAP_{EZE}$ telomerase detection kit (Intergene, NY, USA), according to the manufacturer's instructions and as previously described [7]. Quantifications were performed using the Quantity-one software for Bio-Rad's Image analysis systems (Bio-Rad Laboratories, Israel). TA was calculated according to the following formula: $TPG=[(X-B)/(C-B)]:[(r-B)/Cr*100]$, where TPG = total product generated, X = sample signal, C = 36 bp PCR control, r = TSR8 quantification control, B = global gel background.

2.6. mTERT expression

Real time PCR reaction was used to detect the expression of the mTERT gene. Total RNA isolation was done by the EZ-RNA2 RNA isolation kit (Biological Industries, Israel) according to the manufacturer's instructions. 1 μg RNA was then reverse transcribed with the cDNA reverse transcription kit (Applied Biosystems, CA. USA). PCR reactions were carried out with the readymix PCR master mix Taqman based kit (Thermo Scientific, Worcester, MA, USA), mTERT and HPRT-1 (as an internal control) primers labeled with FAM (Applied Biosystems, CA, USA), run and analyzed on the prism 7700 sequence detection system (Applied Biosystems, CA, USA).

2.7. Inhibition of telomerase

Telomerase inhibitor, GRN163L, was kindly provided by Dr. S. Gryaznov, Geron Corporation (Menlo Park, CA, USA). WT-EPO and SK-N-MC cells were exposed twice a week to 1 μM GRN163L for two periods: a short one – 3 days and a much longer one – 26 months to allow sufficient shortening of telomeres. These cells were treated with EPO as described later in the text.

2.8. Western Blotting

WT-EPO and Ba/F3 cells were deprived from growth factors (EPO and IL-3, respectively) for 16 h and re-exposed to these growth factors to induce Akt and PKCα phosphorylation. Cells were harvested, washed by PBS and lysed using CHAPS lysis buffer ($TRAP_{EZE}$ kit). Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories) and subjected to PAGE. The expression of proteins was detected by monoclonal antibodies (Anti pPKCα and PKCα antibodies: Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti pAKT and AKT: Cell Signaling, Beverly, MA, USA) in 1:1000 dilution followed by fluorescence-labeled secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA). Visualization and quantification of the proteins' levels were performed by using the Odyssey Infrared Imaging System (LI-COR Biosciences NE, USA).

2.9. Immunoprecipitation

WT-EPO and Ba/F3 cells were deprived from serum and growth factors (EPO and IL-3, respectively) for 16 h and then re-exposed to them. The expression of the phosphorylated form of mTERT (p-mTERT) was measured by immunoprecipitation followed by Western blotting. p-mTERT and total mTERT (t-mTERT) were immunoprecipitated by anti-telomerase antibodies (20 μg/ml) and protein A/G-Sepharose beads (from Santa Cruz Biotechnology, CA, USA) and were detected by anti-phospho-serine antibody or anti t-mTERT (Santa Cruz Biotechnology, CA, USA).

2.10. Exposure to inhibitors of AKT, JAK2 and Lyn

Cells were deprived of either EPO or IL-3 for 16 h, re-stimulated with them and grown for additional 24 h and grown with EPO with the following inhibitors: 10 mM AG490, JAK2 inhibitor (Sigma,

Rehovot, Israel); 25 μM AKT inhibitor-X, AKT inhibitor; 50 μM PP2, 30 μM PP1, both Lyn inhibitors (Merck, Darmstadt, Germany).

2.11. Statistical analyses

Statistical analyses included Student's *T*-test, two or one ways ANOVA. The results are expressed as mean ± standard deviation or standard error. *P* values <0.05 were considered as statistically significant and is shown in the figures by ∗.

3. Results

3.1. EPO increases cell proliferation and does not affect the cell cycle

The proliferative effect of EPO on SK-N-MC cells was measured after 3 days of cell's exposure to 10 U/ml EPO. EPO significantly enhanced cells proliferation by ∼40% [($P < 0.018$, one sample Student *T* test) (Fig 1A)].

Treatment of the malignant SK-N-MC cells with the cytotoxic agent cisplatinum resulted as expected in significant decrease of cell proliferation. Addition of EPO attenuated the cisplatinum mediated damage (Fig 1A).

SK-N-MC cells were exposed to 5 or 10 U/ml EPO for three days and the cell cycle status was determined by FACS. Both EPO concentrations did not significantly affect the cell cycle status (Fig 1B).

Ba/F3 and WT-EPO cells were grown in the absence of EPO or IL-3 respectively for 16 h. Then these growth factors were added to the growth medium and cells were grown for additional 24 h. Viable cells were counted by using the Trypan Blue exclusion assay at baseline and 24 h after their exposure to the growth factors. As shown in Fig 1C, an addition of EPO and IL-3 resulted in a significant increase in cells proliferation ($P < 0.001$).

In contrast to the SK-N-MC cells in the non-malignant cells EPO treatment resulted in an increase in S phase (Fig 1D).

3.2. EPO and IL-3 increase telomerase activity

SK-N-MC cells were exposed to EPO for 6, 24 and 32 h and TA was measured by the TRAP assay. TA increased 6 h post EPO treatment, reached a peak level after 24 h (X5) and started to decline 32 h after exposure to EPO (Fig 2A and B) ($P < 0.05$).

This increase was specific to telomerase, as DNA polymerase α activity did not change after EPO administration (not shown).

TA was also measured in Ba/F3 and WT-EPO cells which were exposed to EPO or IL-3 as described above. As shown in Fig 2C and D, the addition of EPO to the growth medium of WT-EPO cells increased this activity 3.15-fold. The addition of IL-3 increased telomerase activity in Ba/F3 cells 1.65-fold ($P < 0.05$).

The effect of EPO was also determined in Ba/F3 cells overexpressing the EPO-R on the cells basal membrane, due to a NPVY insertion to the EPO-R ORF ("NPVY cells"). These cells were exposed to EPO as described above and TA is shown in Fig 2D. Exposure to EPO for 24 h increased TA 4.25-fold compared to those of baseline, implying that the extent of the TA in Ba/F3 cells in response to EPO correlates with the level of expression of EPO-R (315%, 425% in WT-EPO and NPVY cells respectively).

3.3. The Effect of EPO on cancer cell migration

Cell migration assay is an accepted surrogate marker for metastatic potential of cancer cells. Exposure of 24 h to EPO resulted in a marked enhancement (70%) in the migration of SK-N-MC through fibronectin coated membranes (Fig 3A and B; $P < 0.05$).

3.4. The role of telomerase in EPO-dependent cancer cell proliferation

To decipher a possible role of telomerase activation in the proliferative ability of SK-N-MC cells, TA was inhibited using a template antagonist of telomerase, GRN163L. The inhibition of telomerase was performed in two time scales: inhibition for a short time (three days) and a very long term inhibition of the enzyme's activity (26 months), which resulted in about 50% shortening of their telomeres. A short term inhibition of telomerase attenuated the proliferative effect induced by EPO on the cells (Fig 3D). However in cancer cells with shortened telomeres caused by long term telomerase inhibition EPO completely failed to induce cell proliferation (Fig 3D). In contrast, inhibition of telomerase in WT-EPO cells for 3 days did not affect their proliferation induced by EPO (Fig 3C). These results may imply that long term telomerase inhibition which results in telomeres attrition may eliminate the proliferative effect of EPO specifically in cancer cells.

3.5. The regulatory pathways mediating the EPO-dependent upregulation of telomerase

To decipher the mechanism by which EPO exerts its effect on TA experiments were performed with WT-EPO Ba/F3 cells since the effect of EPO was similar in all three cell lines.

TA is regulated mainly at transcriptional and posttranslational levels.

3.6. Transcriptional level

The level of the mTERT gene expression was measured in Ba/F3 and WT-EPO cells after IL-3 or EPO exposure. 16 h deprivation of growth factors did not decrease the expression of the mTERT gene. Fig. 4 In fact, mTERT expression even increased after growth factor withdrawal. Similarly, the reintroduction of the growth factors to the medium did not result in an increase of the mTERT expression (Fig. 4A). In addition alternative splicing variations in response to EPO or IL-3 could not be detected (not shown). Therefore we conclude that the response of telomerase activity to EPO or IL-3 is not transcriptionally mediated.

3.7. Post translational level

The phosphorylation of telomerase was measured by immunoprecipitation. Ba/F3 and WT-EPO cells were grown without serum and growth factors and then stimulated with EPO or IL-3. The phosphorylation status of telomerase markedly increased in WT-EPO cells after 30′ (∼200%) and returned to basal levels after 1 h (Fig. 4B and C), albeit not statistically significant. In Ba/F3 cells no changes in telomerase phosphorylation by IL-3 were detected.

AKT is the main kinase responsible for posttranslational regulation of TA. The phosphorylation of AKT markedly increased ($P < 0.05$) after EPO stimulation in WT-EPO cells, and to a much lesser extent in Ba/F3 cells after IL-3 stimulation. As shown in Fig. 4D and E, this stimulation was maximal after 30′. These results point to a posttranslational modification of telomerase by AKT in response to EPO stimulation.

3.8. The role of downstream EPO pathways in telomerase regulation

To further characterize EPO effect on TA, we studied the roles of JAK2, Lyn and AKT by using their respective inhibitors. The inhibition of JAK2, which is considered the chief mediator of signals stemming from EPO-R and IL-3 activation, was done by exposing the cells to 10 μM AG490 concomitantly with EPO or IL-3. TA was measured in this setting as the end product. The inhibition of JAK2 did not change TA induced by EPO or IL-3 in WT-EPO

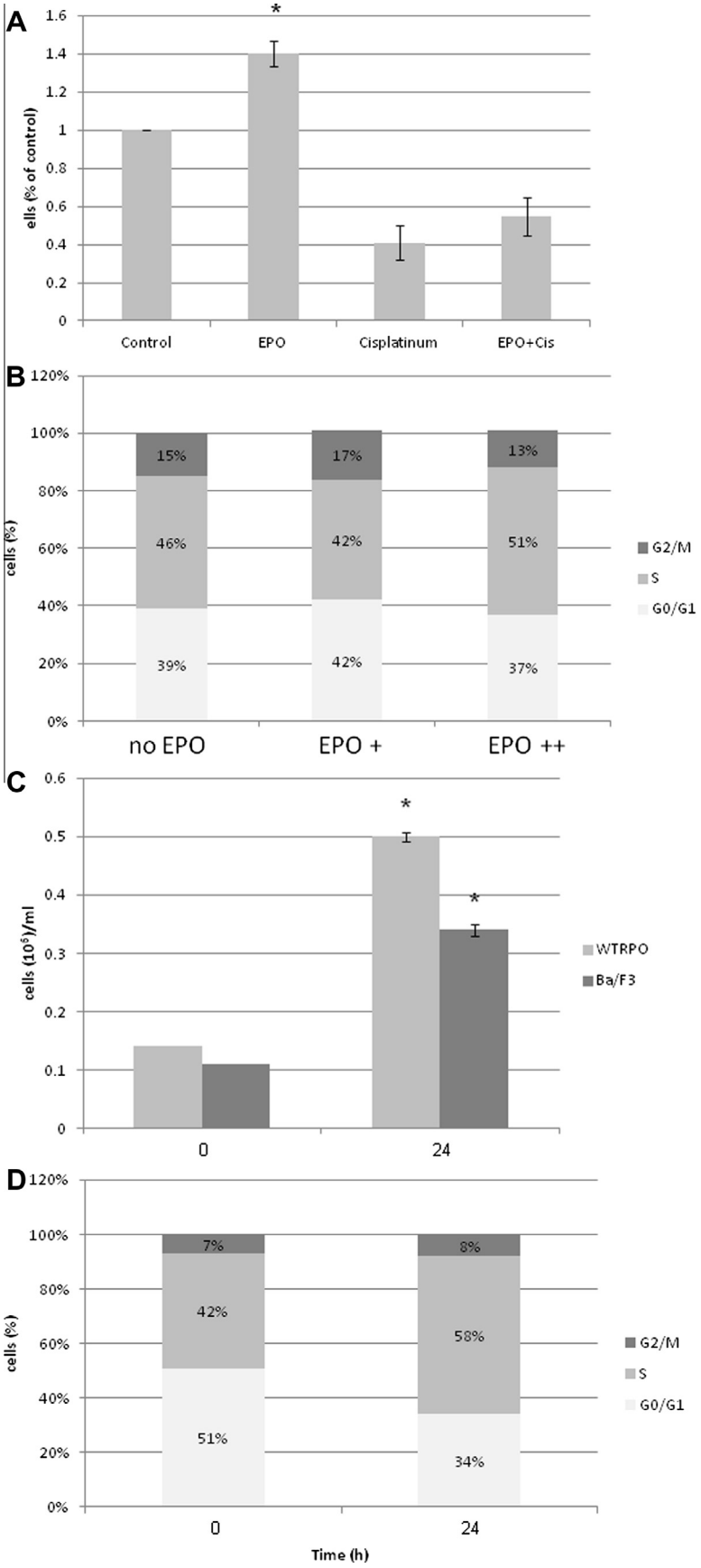

Fig. 1. The effect of EPO on cells proliferation. (A) SK-N-MC cells proliferation was assessed by the SRB assay after exposure to erythropoietin and cisplatinum. (B) Cell cycle analysis performed by FACS on SK-N-MC cells. (C) Ba/F3 and WT-EPO cells were exposed to IL-3 or EPO respectively after starvation and their proliferation was assessed by the trypan exclusion assay. o: 16 h of growth factors (EPO/IL-3) starvation; 24: 24 h growth in the presence of growth factors. (D) WT-EPO cell cycle analysis performed by FACS.

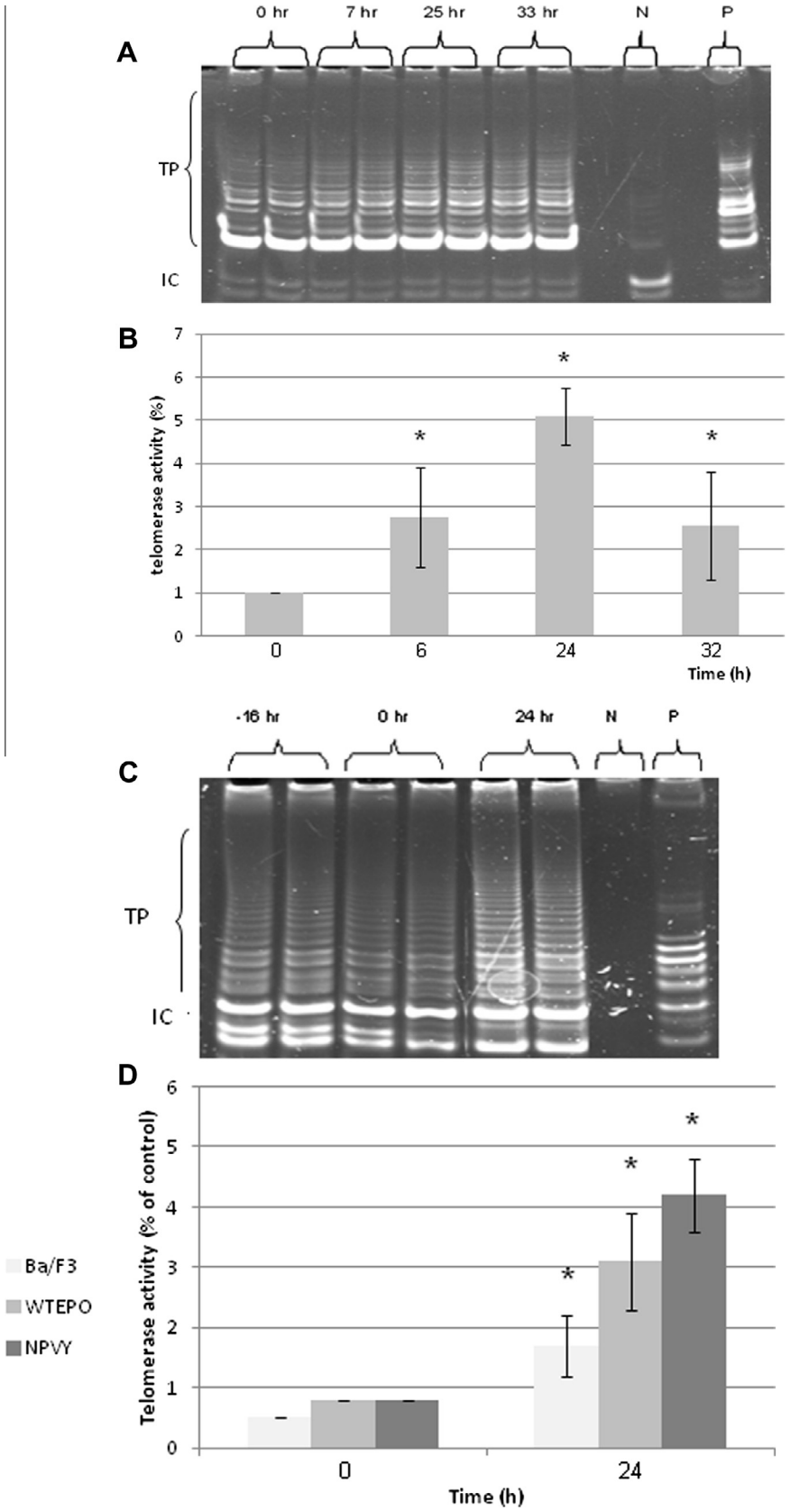

Fig. 2. The effect of EPO on telomerase activity in SK-N-MC, Ba/F3, EPO, and NPVY cells. Cells were exposed to EPO or IL3 as described earlier and TA was examined by the TRAP assay. (A) An example of the TRAP assay used to measure TA. (B) Quantitation of TA. Values are relatively to the non-treated cells ("0"). (C) An example of the TRAP assay in WT-EPO cells. (D) Quantitation of TA.

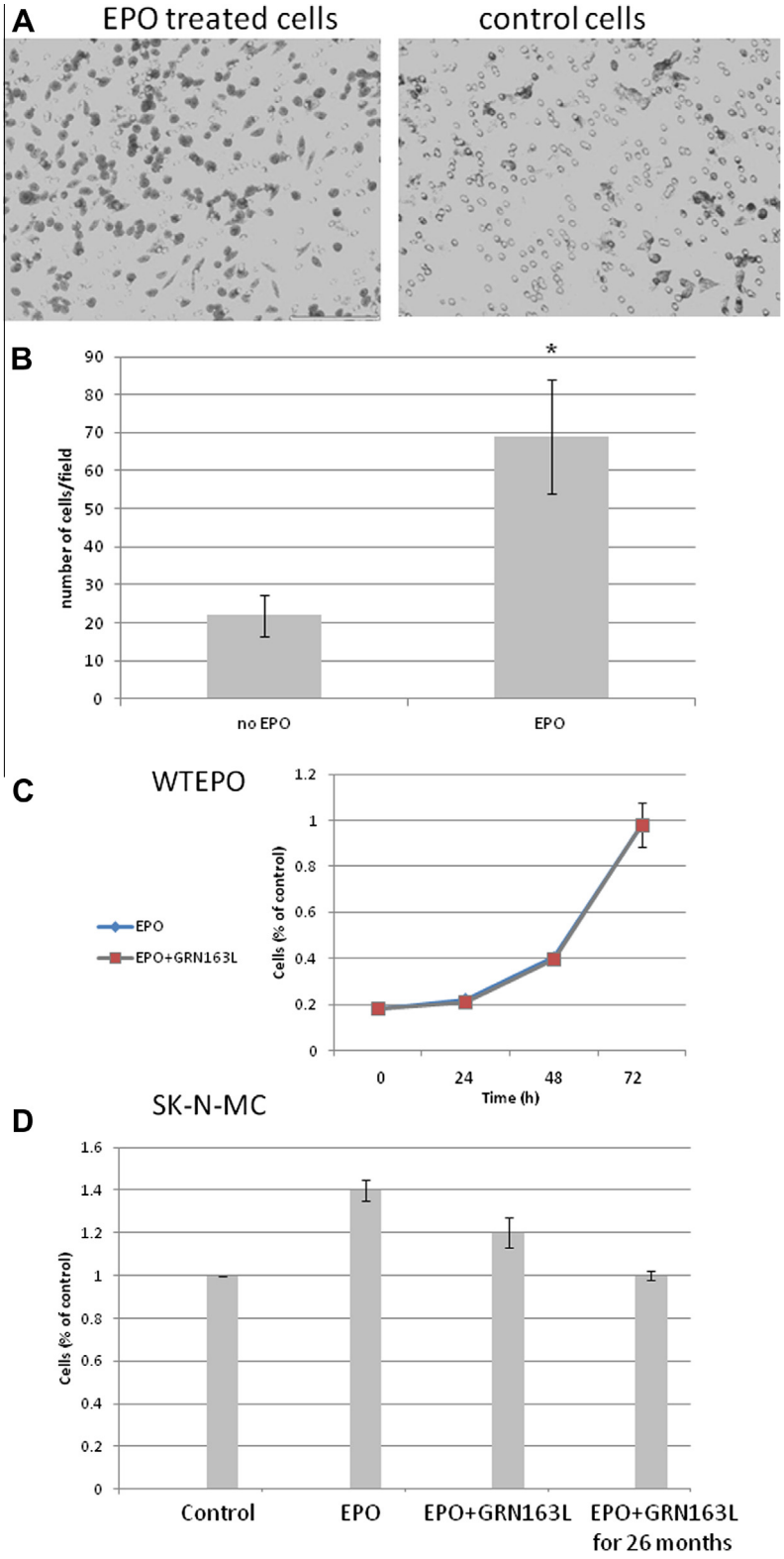

Fig. 3. The effect of EPO on cell migration and the consequence of telomerase inhibition on cell proliferation triggered by EPO. SK-N-MC and WT-EPO cells were grown in the presence of EPO and their migration was followed by the Boyden chamber assay (A, B). Cells were treated with telomerase inhibitor GRN163L for three days or for 26 months and exposed to EPO as before. Their proliferation was assessed by the WST-1 and SRB assays, respectively (C, D). (A) A representative picture of migrated cells in response to EPO. (B) A Quantitation of the migration assays. (C) WT-EPO proliferation with EPO, with or without inhibition of telomerase. Proliferation was assessed 24, 48 and 72 h after EPO administration. (D) SK-N-MC cells proliferation three days after EPO administration and telomerase inhibition. Telomerase was inhibited by GRN163L for three days (middle-right bar) or 26 months (right bar) prior to EPO exposure.

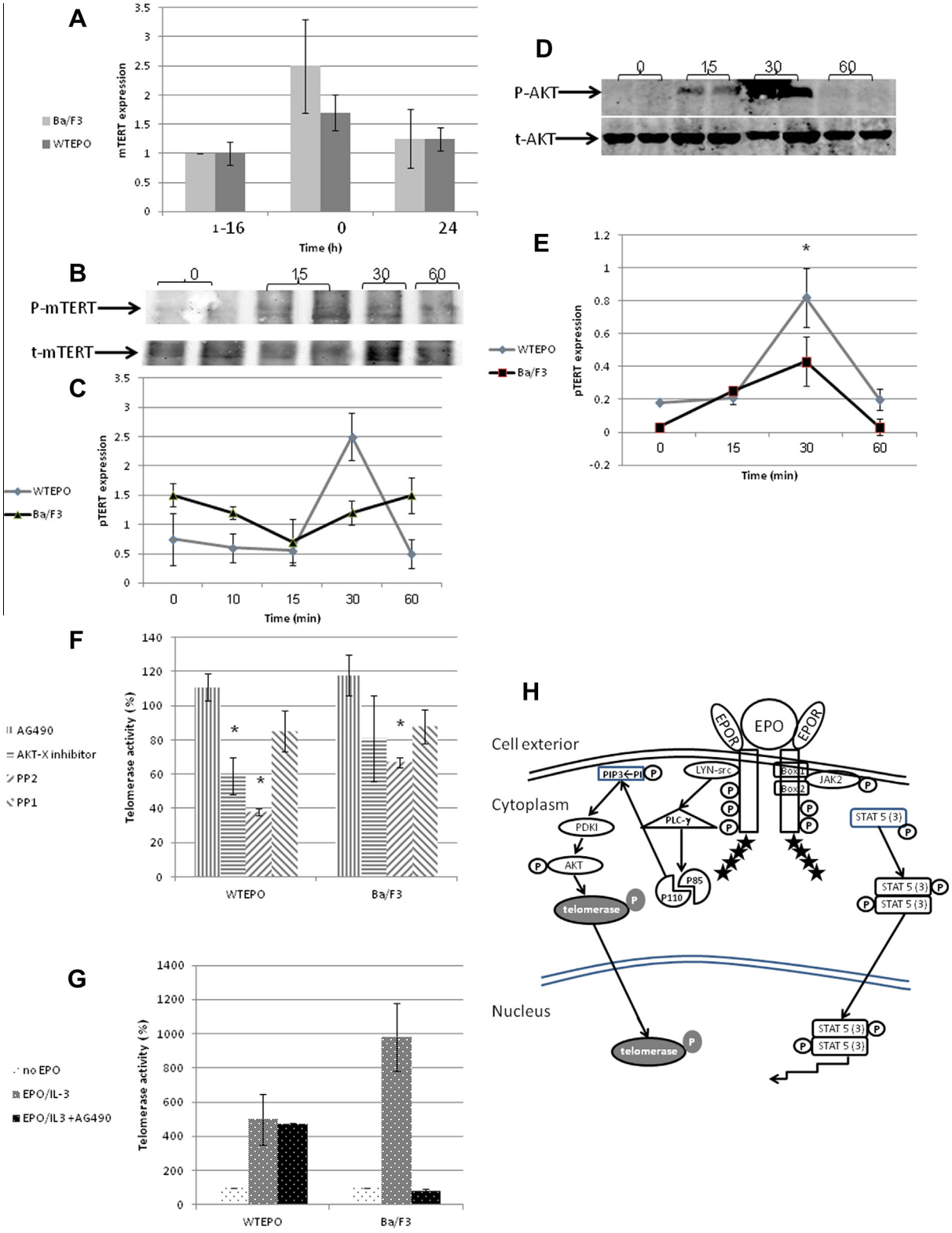

Fig. 4. The effect of EPO on hTERT transcription and post translation status, Ba/F3 and WT-EPO cells were assessed of phosphor-telomerase, after cells starvation for growth factors or serum for two hours. A schematic model is shown on panel h. (A) mRNA levels of mTERT as assessed by real time PCR. "16" is the baseline prior to 16 h growth factors removal. (B) Phospho-telomerase (p-mTERT) was compared to total telomerase (t-mTERT) and detected by immune-precipitation followed by Western blot (a representative example). (C) A summary of three independent experiments. (D) The level of phosphorylated AKT (p-AKT) versus total AKT (t-AKT) was followed during 60 min after the addition of IL-3 or EPO as detected by Western blot (a representative example). (E) A summary of three independent experiments. (F) The effect of various inhibitors on telomerase activity in Ba/F3 and WT EPO cells. Ba/F3 and WT-EPO cells were grown for 24 h in the presence of the following inhibitors: JAK2 inhibitor, AG490, (10 μM), AKT inhibitor, AKT X (μM25 (and two inhibitors of Src kinase Lyn, PP1 (50 μM) and PP2 (30 μM). Telomerase activity was determined by the TRAP assay. (G) The effect of JAK 2 inhibitor on the phosphorylation level of AKT in response to EPO or IL-3 stimulations. Ba/F3 and WT-EPO cells were grown in the presence of JAK2 inhibitor, AG490 and the levels of the phosphorylated form of JAK2 was measured and compared to that of total JAK2 30 min after the addition of EPO or IL-3. (H) The mechanism by which EPO stimulates telomerase activity (modified with permission from Ref. [13]).

and Ba/F3 cells, as shown in Fig. 4F. Therefore, it seemed that the effect of EPO or IL-3 on telomerase activation is not mediated by JAK2.

We then used two different inhibitors of Lyn, a Src kinase that serves as an alternative mediator of EPO-R signaling. 30 μM PP1 or 50 μM PP2 were added to the growth media as described above and TA was measured. The inhibition of Lyn with PP2 reduced TA in 64% and 35% in WT-EPO and Ba/F3 cells respectively ($P < 0.00001$ for WT-EPO and $P < 0.004$ for Baf3 cells) compared to its activity without the inhibitor. PP1 inhibited TA by 26% in WT-EPO cells but had no effect on its activity in Ba/F3 cells (Fig. 4F). Taken together, these results imply that the upregulation of telomerase by EPO is mediated by Lyn and not by JAK2.

AKT kinase is phosphorylated by PI3K kinase operating downstream to the EPO-R –Lyn axis and has been shown by others and by us to phosphorylate telomerase. Its inhibition resulted in a 42% reduction of TA ($P < 0.0325$) induced by EPO in WT-EPO cells (Fig. 4F). A smaller reduction in TA was obtained in Ba/F3 cells stimulated with IL-3. These results define AKT as an important player in the EPO-R-Lyn-telomerase axis activated by EPO.

3.9. The role of JAK2 in the phosphorylation of AKT

To study the role of JAK2 in AKT phosphorylation both cell lines were grown after growth factors starvation with JAK2 inhibitor, AG490 and the status of AKT phosphorylation was measured. As shown in Fig. 4G, JAK2 inhibition did not affect the phosphorylation of AKT after EPO stimulation in WT-EPO cells. In contrast, JAK2 inhibition markedly reduced AKT phosphorylation to baseline levels in Ba/F3 cells followed IL-3 stimulus. These results emphasize the differences in the mechanisms operated by IL-3 or EPO in the two cell lines affecting telomerase activation.

The inhibition of Lyn by PP2 or PP1 reduced the levels of phosphorylated AKT to base line levels in both cell lines (not shown), suggesting that Lyn phosphorylates AKT, highlighting the involvement of AKT in the effect of EPO on telomerase upregulation.

4. Discussion

Our results describe for the first time the promoting effect of EPO on TA and its regulation in malignant and non-malignant non-erythroid cells. Together with its effect on the proliferation of the cells EPO specifically increased the activity of telomerase. TA increased in SK-N-MC cells and in WT-EPO cells. This effect was correlated with the degree of EPO-R expression. The increase in TA was specific, as DNA polymerase α was not affected by EPO treatment and increased proliferation. The mild increase in S phase of the cell cycle after EPO stimulation also points to specificity in the elevation of TA, as many studies linked induction of TA to the S phase of the cell cycle [6].

These results have also potential clinical implications. Addition of EPO to cancer cells attenuated the toxicity of cisplatinum suggesting that it may be involved in resistance to chemotherapy. Interestingly, we have shown that telomere dynamics increased cancer cells sensitivity to cisplatinum [7]. In addition, the increased the migration of cancer cells induced by EPO implicate its potential role in the metastatic process. Considering the report of TA relatedness to metastatic potential of cancer cells [8], these findings may be interconnected. Thus, telomerase inhibition might have a protective role from EPO's effects. Our results strengthen this avenue suggesting that telomere shortening, more than telomerase inhibition, may attenuate EPO induced proliferation of cancer cells. This is also in line with our previous report [7] showing that telomere shortening rather than telomerase inhibition is important in antitumor activities.

To decipher the molecular pathway underlying the effect of EPO on TA, we used three cell lines with differential expression of the EPO-R: Ba/F3, which has no EPO-R but expresses the IL-3-R, WT-EPO, which expresses the EPO-R and NPVY, in which NPVY residues were inserted into the ORF of EPO-R, promoting its maturation and membranal localization. IL-3 and EPO are close cytokine family members [9]. The major homology in the extracellular domain of EPO-R, IL-2,3,4,6,7, prolactin, growth hormone and G-MCSF receptors, unify them in a novel family of receptors. EPO-R, IL-2 and IL-3-R share homology at the intracellular domain as well [10]. Additionally, they induce similar signal transduction pathway (activation of JAK2 promotes tyrosine phosphorylation of the cognate receptors and a similar set of SH-2-containing signaling molecules including Shc, SHP-2, and Stat5 [11]). Likewise, the C-terminal of EPO-R and IL-3-R are required for Ras signaling pathway activation [12]. In our study the downstream effects of EPO or IL-3 were quite similar, but differed in the extent of TA in response to EPO and in the involvement of AKT in the IL-3 mediated pathway. Although we deciphered the mechanism of EPO action on TA in WT-EPO cells, one cannot exclude that a somewhat different mechanism promotes EPO's effects on cancer cells. Another difference may be attributed to the sources of cell lines: whereas WT-EPO cells are from mouse, SK-N-MC are human cells.

Our study shows that the upregulation in TA is not mediated by the major signaling pathway of EPO-R, involving mainly JAK2. Rather, it is mediated by the Lyn-Src pathway (Fig. 4H). The binding of EPO to the EPO-R stimulates the activation of Lyn-Src, which transduces the signal to PLC-y. This protein activates the subunits of PI3K, p85 and p110, which are recruited to the cell membrane, undergo auto-phosphorylation and subsequently phosphorylate PDK1 which phosphorylates AKT. AKT than phosphorylates telomerase which enters the cell nucleus and probably binds the telomere substrate (Fig. 4H).

Since the shortening of telomeres in SK-N-MC cells totally abolished the effect of EPO on cell proliferation, we concluded that telomerase and intact telomeres may be the mediators of the proliferative effects induced by EPO. In the view of these findings, we propose that the safety of EPO administration in cancer patients requires a better understanding of EPO effect on cancer cells in general and on telomerase activity in particular. Therefore, the effect of EPO on this enzyme may have translational applications for clinical use.

A similar increase in TA was shown in breast cancer cell line MCF10 cells. An increase of two folds was detected after EPO administration in these cells, and in MCF10 over expressing EPO-R, this increase was similar (L. Feldman, personal communications). On the contrary results from our laboratory show that EPO does not activate telomerase in cells lacking telomerase expression such as human mononuclear cells (not shown).

If the results of this study are validated over a wider spectrum of cancer cells, they may point to employing telomerase inhibition strategy in cancer patients, especially if EPO administration is considered.

References

[1] A.J. Sytkowski, Does erythropoietin have a dark side? Epo signaling and cancer cells, Sci. STKE 395 (2007) pe38.
[2] F. Lavelle, J.F. Riou, A. Laoui, P. Mailliet, Telomerase: a therapeutic target for the third millennium?, Crit Rev. Oncol. Hematol. 34 (2000) 111–126.
[3] E. Skordalakes, Telomerase structure paves the way for new cancer therapies, Future Oncol. 5 (2009) 163–167.
[4] N. Prade-Houdellier, E. Frébet, C. Demur, et al., Human telomerase is regulated by erythropoietin and transforming growth factor-beta in human erythroid progenitor cells, Leukemia 21 (2007) 2304–2310.
[5] M. Akiyama, T. Kawano, Y. Mikami-Terao, et al., Erythropoietin activates telomerase through transcriptional and posttranscriptional regulation in human erythroleukemic JAS-REN-A cells, Leuk. Res. 35 (2011) 416–418.

[6] S. Marcand, V. Brevet, C. Mann, et al., Cell cycle restriction of telomere elongation, Curr. Biol. 10 (2000) 487–490.
[7] O. Uziel, E. Beery, V. Dronichev, et al., Telomere shortening sensitizes cancer cells to selected cytotoxic agents: in vitro and in vivo studies and putative mechanisms, PLoS ONE 5 (2010) e9132.
[8] S. Bougel, B. Lhermitte, G. Gallagher, et al., Methylation of the hTERT promoter: a novel cancer biomarker for leptomeningeal metastasis detection in cerebrospinal fluids, Clin. Cancer Res. 19 (2013) 2216–2223.
[9] N. Sumaya, H. Hamadmad, K. Matthew, Erythropoietin receptor signal transduction requires protein geranylgeranylation, J. Pharmacol. Exp. Ther. 316 (2006) 403–409.
[10] A.D. D'Andrea, L.I. Zon, Erythropoietin receptor. Subunit structure and activation, J. Clin. Invest. 86 (1990) 681–687.
[11] J.N. Ihle, Cytokine receptor signaling, Nature 19 (1995) 591–594.
[12] H. Chin, T. Saito, A. Arai, et al., Erythropoietin and IL-3 induce tyrosine phosphorylation of CrkL and its association with Shc, SHP-2, and Cbl in hematopoietic cells, Biochem. Biophys. Res. Commun. 239 (1997) 412–417.
[13] J. Szenajch, G. Wcislo, J.Y. Jeong, C. Szczylik, L. Feldman, The role of erythropoietin and its receptor in growth, survival and therapeutic response of human tumor cells from clinic to bench – a critical review, Biochim. Biophys. Acta 2010 (1806) 82–95.