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Estradiol increases cell growth in human astrocytoma cell lines through ERα activation and its interaction with SRC-1 and SRC-3 coactivators ☆

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ABSTRACT

Estradiol (E2) regulates several cellular functions through the interaction with estrogen receptor subtypes, ERα and ERβ, which present different functional and regulation properties. ER subtypes have been identified in human astrocytomas, the most common and aggressive primary brain tumors. We studied the role of ER subtypes in cell growth of two human astrocytoma cell lines derived from tumors of different evolution grades: U373 and D54 (grades III and IV, respectively). E2 significantly increased the number of cells in both lines and the co-administration with an ER antagonist (ICI 182, 780) significantly blocked E2 effects. ERα was the predominant subtype in both cell lines. E2 and ICI 182, 780 down-regulated ERα expression. The number of U373 and D54 cells significantly increased after PPT (ERα agonist) treatment but not after DPN (ERβ agonist) one. To determine the role of SRC-1 and SRC-3 coactivators in ERα induced cell growth, we silenced them with RNA interference. Coactivator silencing blocked the increase in cell number induced by PPT. The content of proteins involved in proliferation and metastasis was also determined after PPT treatment. Western blot analysis showed that in U373 cells the content of PR isoforms (PR-A and PR-B), EGFR, VEGF and cyclin D1 increased after PPT treatment while in D54 cells only the content of EGFR was increased. Our results demonstrate that E2 induces cell growth of human astrocytoma cell lines through ERα and its interaction with SRC-1 and SRC-3 and also suggest differential roles of ERα on cell growth depending on astrocytoma grade.

1. Introduction

Astrocytomas are the most common primary intracerebral neoplasms in humans, mainly found in adults between 30 and 50 years. They are classified according to their histological characteristics in four groups (I–IV), and the survival of patients is inversely related to the tumor grade [1]. It is well known that the incidence of astrocytomas is higher in men than in women (3:2); however, the role of sex hormones in the development of these neoplasms has been poorly investigated [2–4].

Estradiol (E2) is involved in the regulation of several physiological and pathological processes in the brain and may play a role in the control of brain tumor growth [5,6]. This hormone modulates several functions through the interaction with its intracellular receptors which are ligand-activated transcription factors that regulate the expression of several genes involved in reproduction, development, metabolism and cell proliferation. There are two main intracellular estrogen receptor (ER) subtypes named ERα and ERβ encoded by different genes with distinct functions and regulation [7–9].

ER expression is also differentially regulated by E2 in several cells and tissues. In several cell types ERβ is up-regulated whereas ERα is down-regulated by this steroid [10,11]. ERα down-regulation occurs through a mechanism known as ligand-dependent proteolysis in which E2 induces receptor phosphorylation, a signal for degradation by the ubiquitin–proteasome pathway [12,13].

The transcriptional activity of ER has been linked to the interaction with coactivators which are recruited by the activated steroid receptors to target gene promoters [14–17]. The steroid receptor coactivator family (SRC) consists of three members: SRC-1 (NcoA-1), SRC-2 (GRIP-1/NcoA-2/TIF-2), and SRC-3 (AIB1/ACTR/pCIP/RAC3/TRAM-1) [14,18].

SRCs are expressed in several tissues including brain, where a differential expression and regional distribution have been observed [19]. SRC-1 is involved in rodent neuronal development, brain sexual differentiation [20], and is required for the display of female sexual behavior [21]. SRC-1 enhances ER transcriptional activity and interacts with ligand-bound ER subtypes depending on the rat brain

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region [19,22]. It also participates in tumor progression and survival of several human cancer cell lines [23–25]. SRC-2 has been detected in several rodent brain regions known to mediate a variety of steroid-dependent functions [26–28]. This coactivator interacts with ligand bound ERα, but not ERβ, in rat hippocampus and hypothalamus [29]. In most mice brain structures where SRC-1 is expressed, SRC-2 is present at low levels, and the disruption of SRC-1 gene results in an up-regulation of SRC-2 expression [27]. Besides, SRC-2 together with SRC-1 has been localized in the nucleus of rat astrocytes [30]. SRC-3 is expressed at high concentrations in the rodent hippocampus and it is mainly localized in the cytoplasm of rat astrocytes [19,30,31]. This coactivator is frequently amplified and overexpressed in several cancers such as those of breast, ovary, endometrium, stomach, prostate, pancreas and esophagus [18,32–35]. Our group has detected SRC-1 and SRC-3 expression by RT-PCR and Western blot in U373 and D54 human astrocytoma cell lines [36], but their role in brain tumors is unknown.

ER subtypes have been detected in several human brain tumors such as astrocytomas, meningiomas, chordomas and craniopharyngiomas [2,37–40]. In astrocytomas, an inverse relation between the content of ER and the tumor grade has been reported [2,37,41]. ERα and ERβ are expressed in astrocytes and in astrocytomas but their expression is reduced in biopsies of high-grade human astrocytomas [42] however, the function, regulation, and mechanisms of action of ER subtypes in human astrocytoma cells are not known. Therefore, we studied the role of E2 and its receptors in cell proliferation of U373 and D54 human astrocytoma cell lines (derived from tumors grades III and IV, respectively), the participation of SRC-1 and SRC-3 coactivators in E2 effects, as well as the regulation of ERα expression by E2, and its antagonist ICI 182, 780.

2. Materials and methods

2.1. Cell culture

U373-MG and D54 human astrocytoma cell lines derived from human astrocytoma grades III (ATCC, Manassas, VA) and IV (generously obtained by Dr. Andres Gutiérrez from Dr. Sontheimer laboratory (Bringham, AL)) respectively, were used. Cells were grown in 10 cm dishes and maintained in Dulbecco's modification of Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 1 mM pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids (GIBCO, NY) at 37 °C under a 95% air, 5% CO_2 atmosphere.

2.2. Cell number and treatments

5×10^3 U373 or D54 cells were grown on 48-well plates and maintained as indicated in Cell culture section. 24 h before treatments, medium was changed by DMEM phenol red free medium supplemented with 10% fetal bovine serum without steroid hormones (Hyclone, Utah), 1 mM pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids (GIBCO, NY) at 37 °C under a 95% air, 5% CO_2 atmosphere. The following treatments were applied at day 0: (1) vehicle (0.02% cyclodextrin in sterile water); (2) 17-β estradiol (E2) (10 nM); (3) ICI 182, 780 (2 μM, ER antagonist); (4) E2 + ICI 182, 780; (5) PPT (1 nM, it displays a 410-fold selectivity for ERα compared to ERβ); (6) DPN (1 nM, it displays a 70-fold selectivity for ERβ over ERα). The concentrations used for E2 and ICI 182, 780 have been previously reported by our work group [36,43]. The concentration of PPT and DPN (1 nM) used in the experiments was according to that used by other authors [44–46]. Each experiment was performed in four independent cultures. Cyclodextrin and E2 were purchased from Sigma-Aldrich (St. Louis, MO). ICI 182, 780, PPT and DPN were from Tocris Cookson Inc. (Ellisville, MO). Cells were harvested from incubation every day during 6 consecutive days with PBS 1× + EDTA (1 mM). The number of living cells, evaluated by a blind observer, was measured by trypan blue dye exclusion using an inverted microscope (Olympus CKX41, Center Valley, PA).

2.3. Protein extraction and Western blotting

5×10^5 cells were grown in 10 cm dishes and maintained as it has been described in Cell culture section. 24 h before treatments, medium was changed by DMEM phenol red free medium at 37 °C under a 95% air, 5% CO_2 atmosphere. ER agonists and ICI 182, 780 treatments were applied for 24 h. Each experiment was performed in four independent cultures. After all treatments cells were homogenized in RIPA lysis buffer with protease inhibitors (1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF). Proteins were obtained by centrifugation at 20,000×*g*, at 4 °C for 15 min, and quantified by the method of Bradford (Bio-Rad Laboratories, Hercules, CA). Proteins (70 μg) were separated by electrophoresis on 8% SDS-PAGE at 20 mA. Colored markers (Bio Rad, CA, USA) were included for size determination. Gels were transferred to nitrocellulose membranes for 2 h (Amersham, NJ, USA) (60 mA, at room temperature in semi dry conditions), which were blocked at room temperature with 3% non-fat dry milk and 1% bovine serum albumin for 2 h. Membranes were then incubated with 0.8 μg/ml of antibodies against ERα (Santa Cruz sc-8002, CA, USA), ERβ (Santa Cruz sc-6821), SRC-1 (Millipore 05–522, MA, USA), SRC-3 (Millipore 05–490), EGFR (Santa Cruz sc-003), VEGF (Santa Cruz sc-152), cyclin D1 (Santa Cruz sc-A12) or 3 μg/ml of mouse anti-PR monoclonal antibody (CRM 302C Biocare Medical, CA, USA), which recognizes both PR isoforms (PR-A and PR-B), at 4 °C overnight. Blots were then incubated with 400 μg/ml of secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min.

In order to correct differences in the amount of total protein loaded in each lane, ERα, ERβ, SRC-1, SRC-3, PR isoforms, VEGF, EGFR and cylcin D1 protein content were normalized to that of α-tubulin. Blots were stripped with glycine (0.1 M, pH 2.5, 0.5% SDS) at 4 °C overnight and at room temperature for 30 min, and reprobed with a 1:10,000 dilution mouse anti-α-tubulin monoclonal antibody (Sigma T9026, St. Louis, MO) at room temperature for 2 h. Blots were incubated with a 1:3000 dilution goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Signals were detected by enhanced chemiluminescence (ECL) (Amersham, NJ) with a constant exposure time of 1 min for ERα, ERβ, SRC-1, SRC-3, VEGF, EGFR and cylcin D1, 5 min for PR and 20 s for α-tubulin. The antigen–antibody complex was detected as the area under a peak corresponding to a band density (the area is given in inches, as the default scale is 72 pixels/in.) in a semiquantitative way using HP Scanjet G3110 apparatus (Hewlett-Packard Development Company, USA) and the Scion Image software (Scion Corp., MD, USA). In order to minimize inter-assay variations, all Western blots were carried out in parallel.

2.4. Cell transfection

Four different RNA interferences were purchased from Santa Cruz Biotechnology (CA, USA): one short hairpin RNA (shRNA) targeting coactivator SRC-1, one small interfering RNA (siRNA) targeting coactivator SRC-3 and two control RNAs, shRNA and siRNA, respectively. Both control RNA interferences have a scramble sequence that does not lead to the specific degradation of any known cellular mRNA. U373 and D54 cells were transferred to six-well plates. Cells grown to a confluence of 60% were transfected with 1 μg of RNA interference using FUGENE® HD Transfection Reagent (Roche, Basel, Switzerland) as described by the manufacturer's instruction.

2.5. ER subtypes and coactivators mRNA expression

Total RNA was isolated from U373 and D54 cells with the single-step method based on guanidine isothiocyanate/phenol/chloroform

extraction according to the TRIzol Reagent manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm and its integrity was verified by electrophoresis on 1% denaturing agarose gels in the presence of 2.2 M formaldehyde. The first strand cDNA was synthesized from 5 μg of total RNA by using M-MLV reverse transcriptase and oligo dT_{18} primers according to its protocol (Invitrogen, CA). 10 μl of RT reaction was subjected to PCR in order to simultaneously amplify a gene fragment of ERα, ERβ or SRC-1, SRC-2, SRC-3 and 18S ribosomal RNA, the latter was used as an internal control. The sequences of the specific primers for ERα amplification fragment (from +1760 to +1912) were 5′-[AGC ACC CTG AAG TCT CTG GA]-3′ for the forward primer (F) and 5′-[GAT GTG GGA GAG GAT GAG GA]-3′ for the reverse primer (R), for ERβ amplification region (from +1683 to +1855) were 5′-[AAG AAG ATT CCC GGC TTT GT]-3′ (F) and 5′-[TCT ACG CAT TTC CCC TCA TC]-3′ (R). The sequences of the specific primers for SRC-1 amplification fragment (from +1015 to +1333) were 5′-[CCTCCAGCTATTACGGGTG-TAG]-3′ (F) and 5′-[ATGATGAAAGGTTGCATGTCTG]-3′ (R), for SRC-2 (from +1186 to +1505) were 5′-CTGATGGCACTCTTGTTGCT-3′ (F) and 5′-TTCATTCCCCCAGAACCAC-3′ (R) for SRC-3 amplification region (from +2103 to +2440) were 5′-[GTCATTCCTCCTTGACCAACTC]-3′ (F) and 5′-[ATCCCTGTCCAGCAGGTATCTA]-3′ (R). The 50 μl PCR reaction included: 10 μl of previously synthesized cDNA, 20 mM Tris–HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, and 2.5 units of Taq DNA polymerase. Negative controls without RNA and with non-retrotranscribed RNA were included in all the experiments. After an initial denaturation step at 95 °C for 5 min, PCR reaction was performed for 30 cycles. The cycle profile for ER subtypes and 18S genes amplification was: 30 cycles at 95 °C, 1 min; 60 °C (58 °C for SRCs), 1 min; and 72 °C, 1 min. A final extension cycle was performed at 72 °C for 5 min. All PCR products were always studied and analyzed together throughout the experiments. PCR products were separated on 2% agarose gels and stained with ethidium bromide. The image was captured under a UV transilluminator. The intensity of ERα, ERβ or 18S bands was quantified by densitometry using the Scion Image software (Scion Corp., Maryland). ER subtypes expression level was normalized to that of 18S.

2.6. Statistical analysis

All data were analyzed and plotted utilizing GraphPad Prism version 4.00 for Windows software (GraphPad Software, San Diego, CA). Satistical analysis between comparable groups was performed using ANOVA with Bonferroni's post-test. A value of $p<0.05$ was considered statistically significant as stated in the figure legends.

3. Results

3.1. Effects of E2 and ICI 182, 780 on the growth of U373 and D54 human astrocytoma cell lines

To study the effect of E2 on cell growth of U373 and D54 astrocytoma cell lines, a time-course study over a 6-day period was performed. In U373 cells E2 treatment significantly increased the number of cells on the fourth day of culture and in D54 cells on the

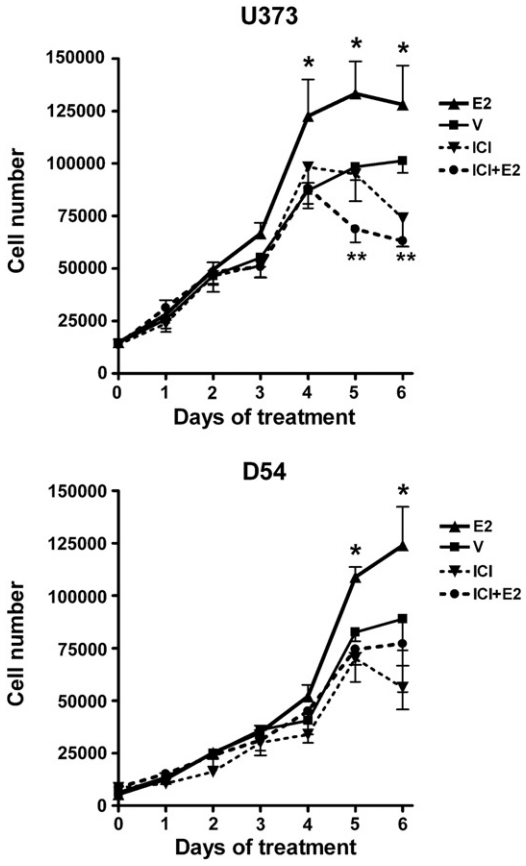

Fig. 1. Effects of E2 and ICI 182, 780 on the cell number of U373 and D54 human astrocytoma cell lines. U373 (*Upper panel*) and D54 (*Lower panel*) cell lines were treated with vehicle (V) (0.02% cyclodextrin), E2 (10 nM), the ER antagonist ICI 182, 780 (2 μM), and E2 plus ICI 182, 780 during a six day time course. Every day cells were harvested and cell number was determined by trypan blue dye exclusion using an inverted microscope. Data are mean ± S.E.M. n = 4; *$P<0.05$ *vs. all groups*, **$P<0.05$ *vs. V*.

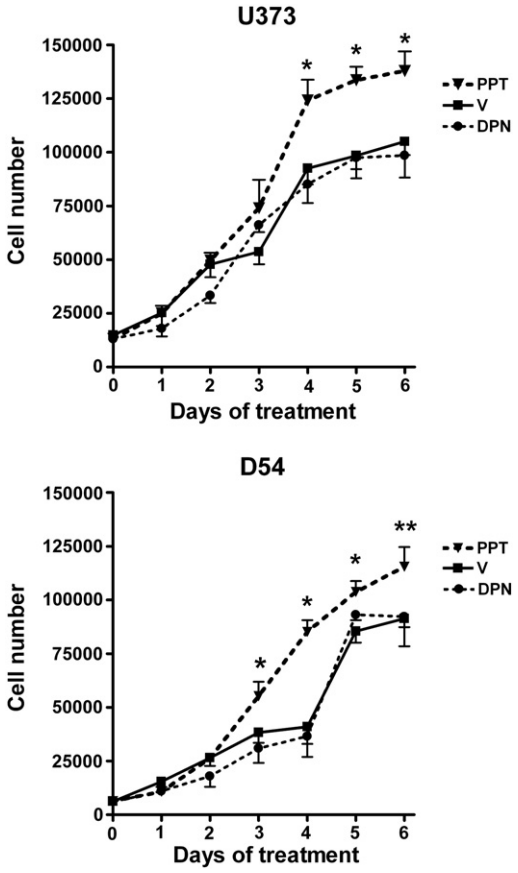

Fig. 2. Effects of ERα agonist PPT and ERβ agonist DPN on cell number of U373 and D54 human astrocytoma cell lines. U373 (*Upper panel*) and D54 (*Lower panel*) cell lines were treated with V (0.02% cyclodextrin), E2 (10 nM), PPT (1 nM) and DPN (1 nM) to determine cell number during a six day time course. Every day cells were harvested and cell number was determined by trypan blue dye exclusion using an inverted microscope. Data are mean ± S.E.M. n = 4. Data were analyzed by ANOVA, for D54 $F = 18.74$, $p<0.001$; for U373 $F = 5.88$, $p<0.001$.*$P<0.05$ *vs. all groups,* **$P<0.05$ *vs. V*.

fifth day. In both cell lines the E2 effect persisted until day 6 (Fig. 1). Treatment with ICI 182, 780 did not significantly change the number of U373 and D54 cells as compared with vehicle. ICI 182–780 co-administered with E2 significantly blocked the effects of this hormone on days 4 and 5, and compared with vehicle in U373 and D54 cells, respectively (Fig. 1). These data suggest that E2 increases cell number through the interaction with ER subtypes.

3.2. Effects of ER stimulation on the growth of U373 and D54 cells

In order to find out which ER subtype is involved in cell growth, we used ER specific agonists: PPT for ERα and DPN for ERβ. A time-course study over a 6-day period with PPT (1 nM) or DPN (1 nM), was performed in U373 and D54 human astrocytoma cell lines. PPT treatment significantly increased the number of cells in a similar manner to that observed with E2. In both cell lines the PPT effect persisted until day 6 (Fig. 2). The treatment with DPN did not significantly change the number of U373 and D54 cells as compared with vehicle (Fig. 2). These results demonstrate that ERα is the subtype implicated in the increase of cell number mediated by E2.

3.3. Expression and regulation of ERα in U373 and D54 cell lines

ER subtype expression was determined by RT-PCR in U373 and D54 cell lines. Fig. 3 shows that ERα content was higher than that of ERβ in both cell lines, however, ERα expression was lower in D54 cells as compared to U373 cells (ERα:ERβ ratio 3:1 in U373 and 1.5:1 in D54) (Fig. 3). We studied the effects of E2 and ICI 182, 780 on the content of ERα in astrocytoma cell lines. We detected a 66 kDa double band corresponding to ERα. E2 treatment down-regulated ERα content as compared with vehicle in U373 cells, and by contrast E2 did not regulate this receptor in D54 cells. ICI 182, 780 down-regulated ERα in both cell lines. E2 plus ICI 182, 780 did not have a synergistic effect over ERα down-regulation in astrocytoma cell lines (Fig. 3). These results suggest that differences in ERα expression and regulation by E2 between both cell lines could be related to the evolution grade of the tumors they derive from. We also detected ERβ as a 55 kDa band (Supplementary Fig. 1), in contrast to the effect observed in ERα, E2 and ICI 182, 780 treatments increased ERβ protein content.

3.4. Role of SRC-1 and SRC-3 coactivators on the growth of astrocytoma cell lines mediated by ERα activation

The transcriptional activity of ERα has been linked to its interaction with general classes of coactivators such as SRC-1, SRC-2, and SRC-3. These coactivators must be coexpressed with steroid receptors in the same cell to mediate the effects of the latter. We found that both cell lines highly expressed SRC-1 and SRC-3. In contrast, the expression of SRC-2 was very low (Supplementary Fig. 2). Therefore, we decided to only evaluate the participation of SRC-1 and SRC-3 in the growth of astrocytoma cell lines dependent on ERα activation. SRC-1 or SRC-3 was silenced with RNA interference in U373 and D54 cells treated with PPT. Coactivator silencing blocked PPT increase in

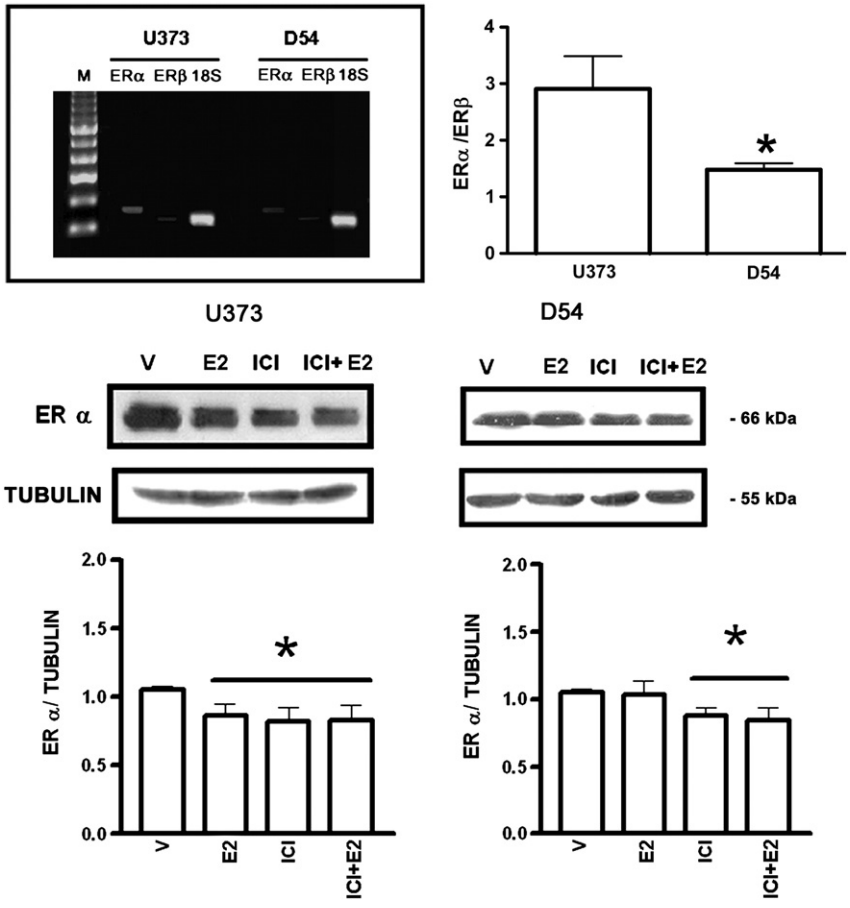

Fig. 3. Expression and regulation of ERα in U373 and D54 cell lines. U373 and D54 cells were lysed and RNA was extracted by a guanidine isothiocyanate/phenol/chloroform assay (*Upper panel*). ERα and ERβ expression was determined by RT-PCR and the 18S ribosomal RNA gene was used as a loading control. Representative image of a 2% agarose gel electrophoresis (*left*), and the statistical analysis (*right*) of the ER-α/ER-β expression ratio for both cell lines are shown. Data are mean ± S.E.M. n = 4; *$P<0.05$ *vs. U373*. Western blot analysis (*Lower panel*) was performed to determine ER-α content in U373 (*left*) and D54 (*right*) cells after treatment with V (0.02% cyclodextrin), E2 (10 nM), ICI 182, 780 (2 μM), and E2 plus ICI 182, 780. Representative blot images for ERα content and control protein tubulin in U373 and D54 are shown. Data are mean ± S.E.M. n = 4; *$P<0.05$ *vs. V*.

astrocytoma cell number during all 6 days of assay (Fig. 4). Silenced coactivator content was determined on day 3 after PPT treatment (Fig. 4). The coactivator silencing was maintained until the sixth day of culture after transfection with RNA interference (Supplemental Fig. 3). In summary, our results suggest that E2 effect on cell growth of U373 and D54 cells depends on ERα activation and its interaction with SRC-1 and SRC-3 coactivators.

3.5. Regulation of PR isoforms, cyclin D1, EGFR and VEGF expression by ERα

Progesterone receptor isoforms (PR-A and PR-B), cyclin D1, VEGF and EGFR [47–49] are involved in metastasis and proliferation of different kinds of tumors such as gliomas. These genes are induced by E2 in cell lines derived from human breast and endometrial tumors [50–52]. Increase in astrocytoma cell number depends on ERα activation; therefore, we studied whether the content of these proteins was regulated by ERα activation in human astrocytoma cell lines. The content of PR isoforms, cyclin D1, EGFR and VEGF was assessed in U373 and D54 cell lines after PPT treatment. Western blot analysis showed that in U373 cells the content of both PR isoforms, cyclin D1, EGFR and VEGF was increased after PPT treatment (Fig. 5) whereas in D54 cells only the content of EGFR was increased after PPT treatment (Fig. 5). Thus, the expression of proteins involved in tumor progression was regulated by ERα depending on the tumor evolution grade from which the cell lines were derived.

4. Discussion

In the present study, we examined the role of E2 and its intracellular receptors (ERα and ERβ) in the regulation of cell growth of two human astrocytoma cell lines: U373 and D54 (derived from tumors of grades III and IV, respectively). The results show that E2 increases cell growth in both cell lines, and the ER antagonist ICI 182, 780 blocked E2 effect. Interestingly, it has been observed that in C6 mouse glioma cells estrogen biosynthesis inhibition by melatonin decreased the growth of these cells [53]. Barone et al. [54] found that E2 decreased tumor growth in nude rats intracerebrally implanted with U87 MG glioblastoma cell line; however, cell proliferation increased at 72 h after 10 nM of 2-hydroxyestradiol treatment in *in vitro* experiments. The invasiveness of glioma cells is mediated in part by their interaction with the surrounding environment that involves astrocytes and extracellular matrix which synthesize many molecules that influence cell proliferation. Differences between *in vitro* and *in vivo* results could be due to the modulator effects of tumor microenvironment on E2 actions.

E2 can exert its effects through genomic or non-genomic mechanisms. Our results suggest that E2 effects on astrocytoma cell growth occur via a classical genomic mechanism through its interaction with ERα, since a specific agonist of this subtype (PPT) increased cell number whereas an ERβ agonist (DPN) did not exert any significant effect.

ERα has been mainly detected in low-grade tumors and in several cases its expression is reduced or lost during tumor development. Nevertheless, it is possible that low levels of ERα have been undetected mostly due to technical procedures such as immunohistochemistry [42]. ERβ expression is known to decline in high-grade tumors and to parallel the loss of differentiation [37]. We have detected ERα and ERβ expression by Western blot in U373 and D54 cell lines in a previous work [36]. In this study the expression of ERα and ERβ diminished in D54 cells compared to U373 cells. The minor ERα expression in D54 cells makes the overall ERα/ERβ ratio to be significantly lower than in U373 cells. These findings correlate with those observed in tumor biospsies where expression of ERs is reduced during tumor development [37,42]. The change in the ratio of ER expression subtypes could be related to a differential regulation of estrogen-dependent genes in cells derived form astrocytomas of the highest evolution grade.

In cell-based studies, ERβ exhibits an inhibitory action on ERα-mediated gene expression and in many cases, opposes the actions of ERα [55]; in breast cancer ERα appears to play a predominant role in cell proliferation, while ERβ is suggested to be antiproliferative [56]. In this work we found that ERα down-regulation via E2 occurred in U373 cells but not in D54 cells while in both cell lines ICI 182, 780 down-regulated ERα. It has been seen that E2 and ICI 182, 780 down-regulate ERα in several cell lines such as MCF-7 breast cancer cells, in which the protein half-life decreased [10,11,57]. Studies in MCF-7 cells have shown that the specific antagonist ICI 182, 780 induces ERα degradation [58]. E2 mediated ERα degradation is dependent on transcription, coactivator recruitment, receptor phosphorylation, ubiquitination and degradation via 26S proteasome, whereas

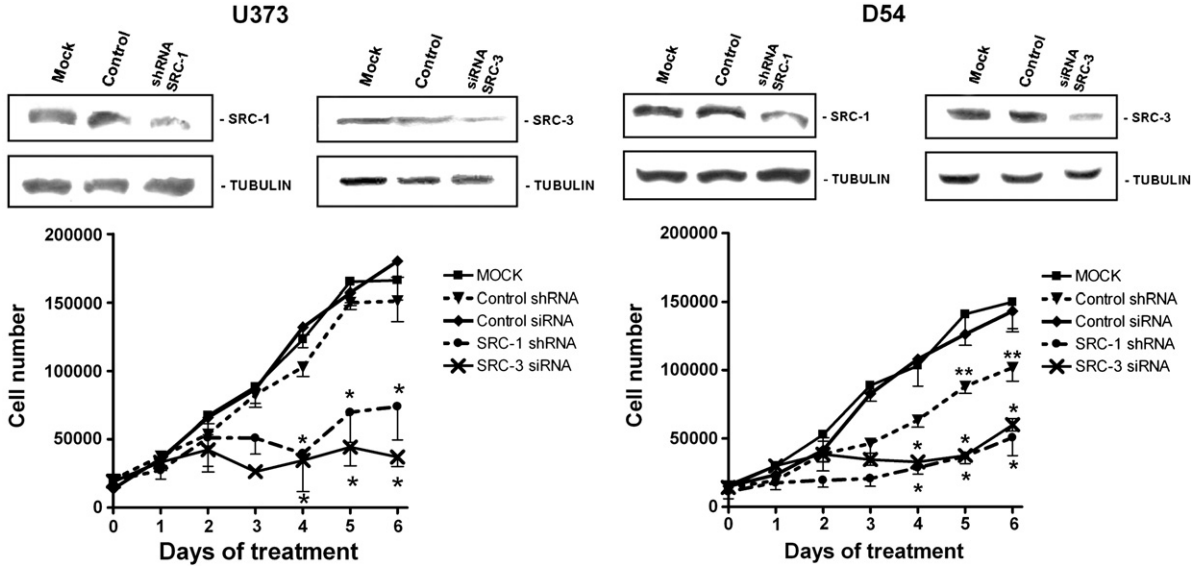

Fig. 4. Effect of SRC-1 and SRC-3 silencing on changes in cell number induced by PPT in U373 and D54 human astrocytoma cell lines. U373 (*Left panel*) and D54 (*Right panel*) cell lines were transfected with SRC-1 shRNA, SRC-3 siRNA and control RNAs interference, and then treated with PPT (1 nM). Every day during six days cells were harvested and cell number was determined as in Fig. 1. Representative Western blots of SRC-1 and SRC-3 from cells lysed at day 3 after PPT treatment are shown in each panel. Mock: vehicle control (cells treated with transfection reagent without RNA interference). Data are mean ± S.E.M. n = 4. *$P<0.05$ *vs. Mock, Control shRNA and Control siRNA.* **$P<0.05$ *vs. Mock.*

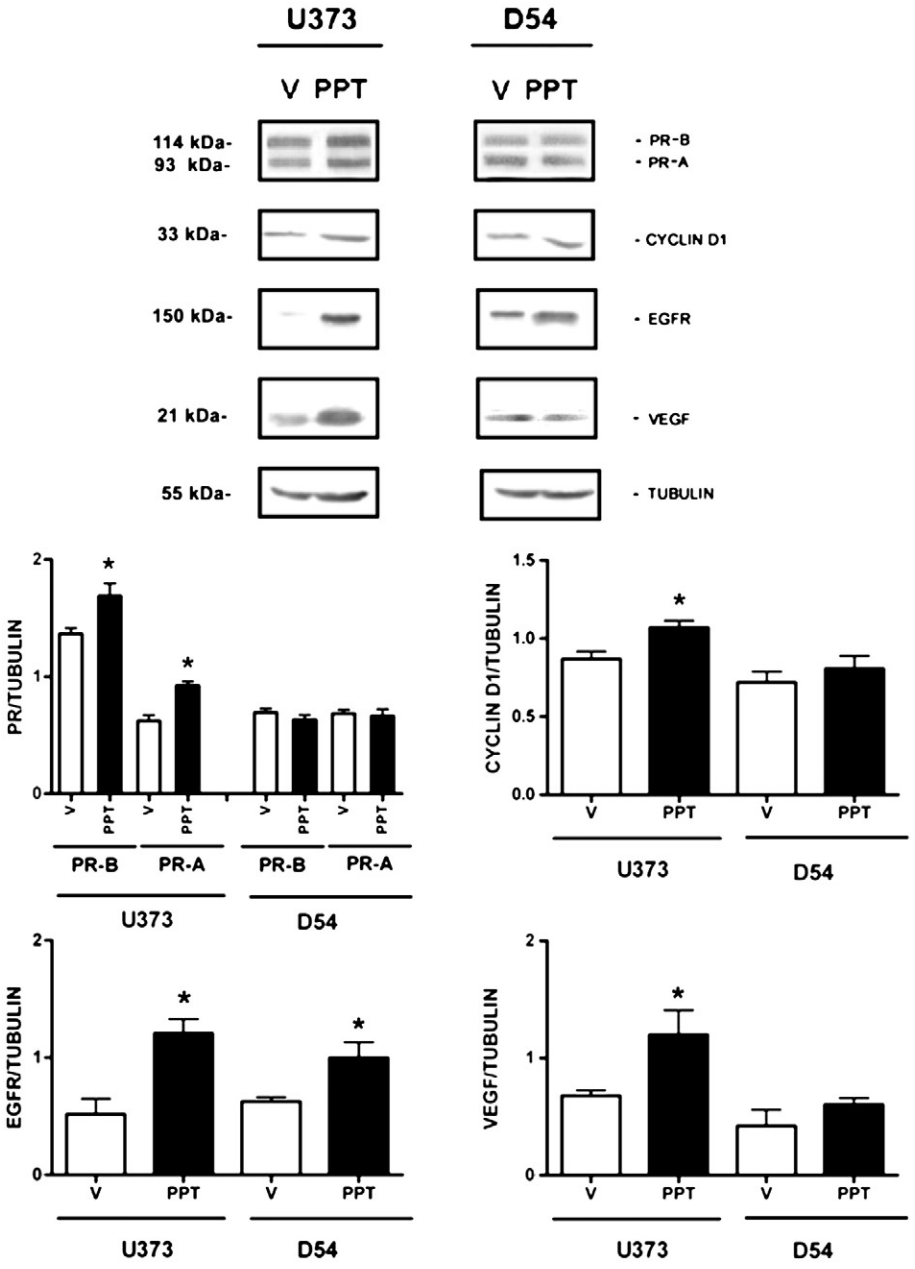

Fig. 5. Effect of ERα agonist PPT on PR, cyclin D1, EGFR and VEGF content in U373 and D54 human astrocytoma cell lines. U373 and D54 cells were lysed after PPT (1 nM) treatment and proteins (70 μg) were separated by electrophoresis on 8% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes and incubated with antibodies for PR, cyclin D1, EGFR and VEGF. Protein antibody complexes were detected by ECL. A representative Western blot is shown (*Upper panel*). Statistical analysis for PR, cyclin D1, EGFR, or VEGF content relative to tubulin in U373 and D54 cell lines is shown (*Lower panel*). Data are mean ± S.E.M. n = 4; $^{*}P<0.05$ *vs. V*.

ICI 182, 780 induces degradation by immobilization of ERα in the nuclear matrix [11]. We observed that the number of U373 cells was lower with E2 + ICI 182, 780 treatment compared to vehicle (Fig. 1). This could be related to a higher ERα degradation induced by the agonist + antagonist treatment as it was observed in Fig. 3.

SRC-1 and SRC-3 interact with ER subtypes in a ligand-dependent manner and enhance transcriptional activity of the ER via histone acetylation and recruitment of additional coactivators [16]. In this study, we determined that depletion of SRC-1 or SRC-3 decreased proliferation of U373 and D54 cells mediated by ERα. The ability of SRC-3 to contribute to cancer cell growth has been relatively well characterized. It has been reported that the reduced proliferation of SRC-3-depleted MCF-7 cells was due to their decreased progression into the S and G_2-M phases of the cell cycle as well as an increase in the number of cells undergoing apoptosis [59]. Besides, SRC-3 stimulates the expression of genes involved in DNA replication, and, hence, cells lacking SRC-3 do not effectively enter into S phase [60]. The ability of SRC-1 to affect cell proliferation is less well studied. An experiment conduced with MCF-7 cells stably overexpressing SRC-1 indicated that this coactivator increased cell growth induced by E2 [24,61].

The control shRNA SRC-1 itself rendered an effect on cell growth compared to our Mock vehicle control (cells treated with transfection reagent without RNA interference) in D54 cells. It is possible that this cell line is more sensitive to the scramble sequence of the control shRNA than the U373 cell line. shRNAs and siRNAs have the potential to cause diverse and nonspecific effects when introduced into cells, in addition to silencing specific target genes. One off-target effect is the sequence-independent activation of the type I IFN pathway [62–64]. This pathway activation may have an effect on astrocytoma cell

growth [65,66]. Although this effect could limit the interpretation of our results, the difference between shRNA SRC-1 and control shRNA is clear, and statistically different to let us state that silencing of SRC-1 reduces cell growth in D54 cells. This effect of transfected control RNAs interference has been reported in other works [67,68].

Growth factors and their receptors, cell cycle control proteins and steroid hormone receptors are associated with the biology of malignant gliomas and other types of cancer. Changes in their expression pattern may result in cell proliferation, metastasis and angiogenesis [4,47,69–71]. Here we show that in U373 cells VEGF, EGFR, cyclin D1 and PR isoforms increased its expression after PPT treatment while in D54 only EGFR augmented its expression. The increase in all these proteins content by PPT suggests that some of the effects of E2 on cell proliferation and metastasis are mediated by changes in the expression of PR isoforms, cyclin D1, VEGF and EGFR through ERα. Differences in protein expression between astrocytoma cell lines could be related to the tumor grade they derive from. A higher evolution grade in tumors implies a more dedifferentiated and malignant phenotype, which may correlate with the loss of gene expression regulation. Other proteins such as PDGF or CXCR4 that have been reported to be necessary for glioma proliferation [72,73] should be further studied in U373 and D54 astrocytoma cell lines.

We conclude that E2 induces cell growth in human astrocytoma cell lines through the interaction with its intracellular receptor ERα, the recruitment of SRC-1 and SRC-3 coactivators, and the regulation of the expression of genes involved in cell cycle, angiogenesis and metastasis. The importance of our study was to identify one pathway through which estrogens can exert their effects on astrocytoma cell lines. Nevertheless, its impact in patients with gliomas, and the study on a glioblastoma *in vivo* model, awaits further work.

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