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Research Article

Progesterone Receptor Subcellular Localization and Gene Expression Profile in Human Astrocytoma Cells Are Modified by Progesterone

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Abstract. Intracellular progesterone receptor (PR) has been identified in human astrocytomas, the most common and aggressive primary brain tumors in humans. It has been reported that PR cell distribution affects their transcriptional activity and turnover. In this work we studied by immunofluorescence the effects of estradiol and progesterone on the subcellular localization of PR in a grade III human astrocytoma derived cell line (U373). We observed that total PR was mainly distributed in the cytoplasm without hormonal treatment. Estradiol (10 nM) increased PR presence in the cytoplasm of U373 cells, whereas progesterone (10 nM) and RU486 (PR antagonist, 1 μM) blocked this effect. To investigate the role of PR activity in the regulation of gene expression pattern of U373 cells, we evaluated by microarray analysis the profile of genes regulated by progesterone, RU486, or both steroids. We found different genes regulated by steroid treatments that encode for proteins involved in metabolism, transport, cell cycle, proliferation, metastasis, apoptosis, processing of nucleic acids and proteins, adhesion, pathogenesis, immune response, cytoskeleton, and membrane receptors. We determined that 30 genes were regulated by progesterone, 41 genes by RU486 alone, and 13 genes by the cotreatment of progesterone+RU486, suggesting that there are many genes regulated by intracellular PR or through other signaling pathways modulated by progesterone. All these data suggest that PR distribution and activity should modify astrocytomas growth.

Keywords: progesterone receptor, progesterone regulated genes, human astrocytomas, gliomas, immunofluorescence, microarrays

1. Introduction

Progesterone (P4) participates in the regulation of diverse functions and diseases in the brain by interacting with its intracellular receptors (PR) [1–3]. In humans, two PR isoforms with different functions and regulation have been characterized: PR-A (94 kDa) and PR-B (116 kDa). Both isoforms are encoded by the same gene but are regulated by

distinct promoters and generated from alternative transcription initiation sites [4–6].

PR has been detected in several human brain tumors such as astrocytomas, meningiomas, chordomas, and craniopharyngiomas [7–11]. In astrocytomas, a direct relation between PR expression and tumor grade has been reported [9, 10, 12, 13]. The most frequent and aggressive human brain tumors are astrocytomas, which are glial cell derived tumors (gliomas) with high malignant potential. They arise from astrocytes, glial progenitor cells, or cancer stem cells [14–18]. They originate anywhere in the brain but are mainly located in the cerebral cortex, appearing more frequently in adults between 40 and 60 years old [19]. Astrocytomas are classified according to their histopathological and molecular features into four grades (I-IV), where grade IV, also known as glioblastoma, represents the maximal evolution stage. The survival of patients is inversely related to the degree of tumor progression [19, 20].

PR is expressed in biopsies from human astrocytomas [9, 12, 21] and cell lines U373 and D54 which are derived from human astrocytomas grades III and IV, respectively [19]. The content of PR increased after estradiol treatment in U373 cells [13]. In many cell types, PR expression is upregulated by estradiol at transcriptional level by estrogen-responsive elements located in the PR promoter [22], while P4 induces phosphorylation of PR which marks it to be degraded by the proteasome pathway resulting in PR downregulation [23].

Proliferation of many cancer cells is under P4 control. P4 significantly increased the number of D54 cells from the second day of culture and the number of U373 cells on days 3-5 whereas the PR antagonist, RU486, blocked P4 effects in both astrocytoma cell lines [21]. A transient increase in phase S of cell cycle was seen in U373 astrocytoma cells after P4 treatment, which was correlated with the induction of genes associated with cell cycle progression, such as cyclin D1 [21, 24]. Growth factors and their receptors have been proposed as candidate mediators of P4 effects on cell proliferation. The mRNA and protein expression of vascular endothelial growth factor and epidermal growth factor receptor were increased by P4 in astrocytoma cells, and this increase was blocked by RU486 [24]. However, the effects of PR activation on the profile of gene expression in U373 cells are unknown.

Since the subcellular distribution and the expression of PR are critical for cell function, we studied PR localization by immunofluorescence as well as the gene expression pattern in U373 cells after P4 and RU486 treatments.

2. Materials and Methods

2.1. Cell culture and treatments. Human astrocytoma derived cell line U373 (ATCC, Manassas, VA) grade III was used. For immunofluorescence experiments 5 x 10^3cells were plated in 4-well glass slides and for microarrays and RT-PCR experiments 1×10^6 cells were plated in 10 cm dishes. Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) for U373 cells, supplemented with 10% fetal bovine serum, 1 mM pyruvate, 2 mM glutamine, 0.1 mM nonessential amino acids (GIBCO, NY) for 24 h. Medium was changed by DMEM phenol red free medium supplemented with 10% fetal bovine serum without steroid hormones (HyClone, Utah), at 37 °C under a 95% air, 5% CO_2 atmosphere during 24 h. The following treatments were applied for locating PR by immunofluorescence assays: (1) vehicle (0.02% cyclodextrin in sterile water), 48 h; (2) estradiol (10 nM), 48 h; (3) estradiol, 48 h followed by P4 (10 nM), 24 h; (4) estradiol, 48 h followed by RU 486 (PR antagonist, 1 μM), 24 h; (5) estradiol, 48 h followed by P+RU 486, 24 h. Each experiment was performed in three independent cultures. Cyclodextrin, P4, estradiol, and RU486 were purchased from Sigma-Aldrich (St. Louis, MO, USA). In the case of the gene expression profile determined by microarray assays, cells were treated with vehicle, 10 nM of P4, 1 μM of RU486, or both steroids for 12 h.

2.2. Immunofluorescence. Indirect immunofluorescence was used to characterize total PR subcellular location in U373 cells. After all treatments cells were rinsed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min at room temperature, washed with PBS, and permeabilized with 100% methanol for 6 min at 4°C. After washing again with PBS, nonspecific binding was blocked by applying 5% normal goat serum and 1% BSA for 1 h at room temperature. Cells were incubated with primary antibody (rabbit anti-PR polyclonal antibody SC-539, Santa Cruz Biotechnology, Dallas, TX, USA) (8 μg/ml) at 4°C overnight. Cells were incubated with the secondary anti-rabbit antibody conjugated with the fluorophore FITC (Invitrogen, Carlsbad, CA, USA) (1:1000), for 2 h at room temperature. Nuclei were stained with 1 ng/ml of Hoechst 33258 (Sigma, St. Louis, MO, USA). Negative controls consisted of cells in which the primary antibody was omitted. These experiments did not produce any staining (data not shown). Images were acquired in an Olympus BX43 microscope (Olympus, PA, USA), to detect FITC and Hoechst fluorescence in a sequential manner, by exciting with different wavelengths. To establish coexpression of the used markers, merged images were generated. The examiner was unaware of the treatment condition of cells.

2.3. Microarrays and analysis. TRIzol Reagent (Invitrogen, CLD, CA, USA) was employed to isolate total RNA according to manufacturer's recommendations. RNA quantity and purity were assessed by using the spectrophotometer NanoDrop-2000 (Thermo Scientific, Waltham, MA, USA). RNA samples were tested on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to evaluate RNA integrity. RNA samples with RIN above 9 were used to generate labeled cRNA, which were hybridized to Human

Gene 1.0 ST Array microarrays (Affymetrix, Cleveland, OH, USA). RNA was obtained with these features, and exogenous positive controls included in the GeneChip Eukaryotic Poly-A RNA Control Kit (Affymetrix, Cleveland, OH, USA) were added. Subsequently, we used the WT Expression Kit (Ambion, Life Technologies, Waltham, MA, USA) for the synthesis and amplification of complementary DNA (cDNA) which was fragmented and labeled at its 3 'end with the WT Terminal Labeling Kit (Affymetrix, Cleveland, OH, USA). GeneChip Human Gene 1.0 ST Array (Affymetrix, Cleveland, OH, USA) consisting of 28,000 full-length human genes was used for hybridization of fragmented cDNA that was added to a hybridization mixture and stained with streptavidin/phycoerythrin.

The data were preprocessed and analyzed using the oligo and LIMMA (linear models for microarray data) libraries, both part of the bioconductor project, on the R statistical environment. Raw intensity data were normalized using quantile normalization. Differential expression between different groups was analyzed using empirical Bayes method implemented in the LIMMA package and P values were computed. P value cutoff of < 0.05 and fold change cutoff of >1.5 were used as criteria to identify differences in gene expression.

2.4. RNA isolation and RT-PCR. TRIzol Reagent (Invitrogen, CLD, CA, USA) was employed to isolate total RNA according to manufacturer's recommendations. RNA quantity and purity were assessed by using the spectrophotometer NanoDrop-2000 (Thermo Scientific, MA, USA). RNA samples were tested on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to evaluate RNA integrity. cDNA was synthesized from 3 μg of total RNA by using SuperScript II reverse transcription (Invitrogen CLD, CA, USA) and oligo $(dT)_{12-18}$ primers (Sigma-Aldrich, St. Louis, MO, USA) according to its protocol. 3 μL of RT reaction was subjected to PCR in order to simultaneously amplify different genes fragments. 18S ribosomal RNA was used as an internal control. The sequences of the specific primers (Sigma-Aldrich, St. Louis, MO, USA) for GLIPR2, IL7R SREBF1, IL18, TGFβ2, MAP1B, ANLN, HBG1, STARD4, AOC3, and 18S amplification fragments are indicated in Table 1. The 25 μl PCR reaction included 2 μl of previously synthesized cDNA, 2.5 μl 10X buffer PCR, 1.25 mM $MgCl_2$, 0.25 mM of each dNTP, 15 μM of each primer, and 2.5 units of Taq DNA polymerase. Negative controls without RNA and with nonretrotranscribed RNA were included in all the experiments. After the initial denaturation step at 94 °C for 5 min, PCR reaction was performed for 30 cycles. The cycle profile for each gene and 18S amplification was 30 s at 94 °C, 30 s at the melting temperature of each primer, and 30 s at 72 °C. A final extension cycle was performed at 72 °C for 5 min. The number of performed cycles was within the exponential phase of the amplification process. 25 μl of PCR products was separated on 2% agarose gel and stained with GelRed^{TM} (Biotium, Hayward, CA, USA). The image was captured under a UV transilluminator. The intensity of amplified fragments and 18S bands was quantified by densitometry using the ImageJ software (National Institute of Health, WA). Gene expression levels were normalized to those of 18S.

2.5. Statistical analysis. All images were analyzed and quantified by using ImageJ (Image Processing and Analysis in Java). All data were analyzed and plotted by using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA, USA). All data are presented as arbitrary units of fluorescence intensity/cell (mean $\pm$ S.E.M.). For immunofluorescence and RT-PCR studies, statistical analysis between comparable groups was performed with an ANOVA followed by a Bonferroni's post test. A value of $P< 0.05$ was considered statistically significant as stated in figure legends.

3. Results

3.1. Subcellular localization of PR in U373 cells . First, we determined the subcellular localization of total PR in human astrocytoma cells by immunofluorescence. We observed that PR was mainly located in the cytoplasm of U373 cells independent of hormone treatment (Figures 1 and 2). Estradiol increased PR presence in the cytoplasm of U373 cells that was reduced with P4 and/or RU486 treatments after estradiol PR induction (Figures 1 and 2). Experiments using P4 or RU486 alone or combining both steroids without the previous estradiol treatment were done; nevertheless, P4 and RU486 downregulated PR cytoplasmic content in such a manner that fluorescence quantification was not possible (data not shown).

3.2. Groups of genes regulated by P4, RU486, and P4+RU486 in U373 cells. After microarray analysis, genes were organized into three groups based on the different treatments: P4, RU486, and P4+RU486 (Supplementary Tables). We found that regulated genes are encoded for proteins involved in metabolism, transport, cell cycle, proliferation, metastasis, apoptosis, processing of nucleic acids and proteins, adhesion, pathogenesis, immune response, docking complexes, cytoskeleton, and membrane receptors. We also found genes encoding for siRNAs or for some products with no specific assigned function.

3.3. Validation by RT-PCR of genes regulated by P4, RU486, and P4+RU486. After the review of gene function and its exchange rate ≥ 1.5 relative to vehicle, 10 genes were chosen for microarray validation. The main criterion for choosing these genes was the fact that they have been involved in cancer development, and specifically in astrocytomas growth. In this regard, genes implicated in processes such as immune response, transcription, cytoskeletal function, metabolism,

Table 1: Primers for PCR Analysis.

Gene	Primer sequence	Amplified fragment
GLIPR2	FW 5'- CCTGTGGGTGTATGTGCTTG -3'	157 pb
GLIPR2	RV 5'- CCCCAATCCAAATAATCGTG -3'	
IL7R	FW 5'- CTGAGGCTCCTTTTGACCTG -3'	159 pb*
IL7R	RV 5'- TCACATGCGTCCATTTGTTT -3'	
SREBF1	FW 5'- TGCATTTTCTGACACGCTTC -3'	171 pb*
SREBF1	RV 5'- CCAAGCTGTACAGGCTCTCC -3'	
IL18	FW 5'- GGAATTGTCTCCCAGTGCAT -3'	177 pb*
IL18	RV 5'- ACTGGTTCAGCAGCCATCTT -3'	
TGFβ2	FW 5'- TGCTTTGGCTTTCTGGTTCT -3'	199 pb
TGFβ2	RV 5'- TTTGTTTGTGGTGCAGTGGT -3'	
MAP1B	FW 5'- AATCGAGAAGACCAGCCTGA -3'	245 pb
MAP1B	RV 5'- AATCCGTTGAGCGGTGTAAC -3'	
ANLN	FW 5'- ATGCAGTGTGGTGCACATTT -3'	195 pb
ANLN	RV 5'- AACCCAAACACTTTGGCAAG -3'	
HBG1	FW 5'- GCAAGAAGGTGCTGACTTCC -3'	176 pb*
HBG1	RV 5'- GAATTCTTTGCCGAAATGGA -3'	
STARD4	FW 5'- GGCGAGTTGCTAAGAAAACG -3'	219 pb*
STARD4	RV 5'- TGTAACGCATCACACAGCAA -3'	
AOC3	FW 5'- CAGGGGACACTGAACCTTGT -3'	233 pb
AOC3	RV 5'- CCTTTCCAGCTCAGCTATGG -3'	
18S	FW 5'- CGCGGTTCTATTTTGTTGGT -3'	219 pb
18S	RV 5'-AGTCGGCATCGTTTATGGTC -3'	

FW:FORWARD, RV:REVERSE,*Amplification of exon-exon union

transport, proliferation, adhesion, and pathogenesis were chosen. Pseudogenes were excluded, as well as genes whose products were related to noncoding RNAs (including those whose names begin with "SNOR" and "ncRNA"). The selected genes and their functions are shown in Table 2.

In order to validate the data obtained from microarrays (Figure 3A), gene expression was determined by RT-PCR. In all cases the results are derived from at least three independent experiments. For mRNA expression of GLIPR2 and ANLN we did not detect significant changes after P4 treatment; however, a significant increase with RU486 treatment alone or combined with P4 was observed (Figures 3B and 3H). In contrast, a significant decrease in SREBF1 expression was produced by both treatments (Figure 3D). Regarding IL7R and HBG1 genes, we determined that P4 did not regulate their expression, but P4 together with RU486 significantly decreased it (Figures 3C and 3I). IL18 mRNA expression did not change after any treatment (Figure 3E). TGFβ2 expression increased after all treatments as compared with vehicle (Figure 3F) whereas MAP1B expression decreased with all of them (3G). STARD4 expression was increased by the combined treatment of P4 + RU486 while AOC3 gene expression was increased by P4 and P4 + RU486 (Figures 3J and 3K).

The effects of the different treatments in the expression of genes tested by microarray assay and RT-PCR in U373 cells are summarized in Table 3. We observed that GLIPR2 expression evaluated by microarrays entirely coincides with that performed by RT-PCR. It is shown that, in several genes such as TGFβ2, AOC3, or MAP1B, there was only one coincidence in the change observed by microarrays and RT-PCR. In other cases such as IL18, the lack of effects produced by the treatments with RU486 was observed with both methods. There was only one gene, HBG1, with no correlation observed between the results obtained by microarrays or RT-PCR (Table 3).

4. Discussion

Our study shows the cytoplasmic and nuclear distribution of PR after hormonal treatments and the regulation of the gene expression profile by P4 in U373 human astrocytoma cells. We found that total PR was principally located in the cytoplasm. Estradiol increased the presence of PR in the cytoplasm as compared with vehicle, and the treatments with P4 and RU486 alone or combined diminished it compared to estradiol. According to this result, in a previous work our group had demonstrated by western blot that the content

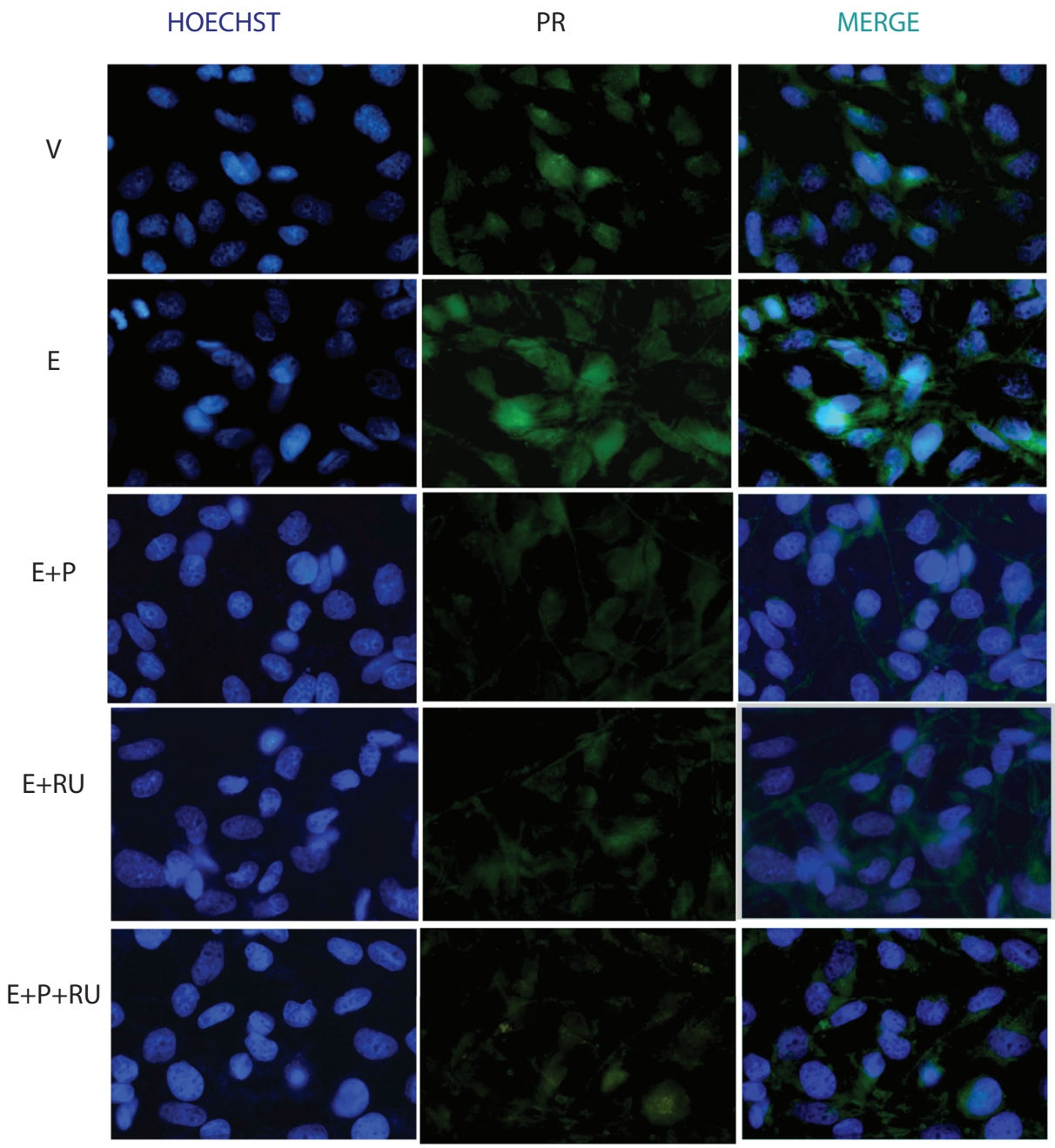

Figure 1: **PR localization in U373 human astrocytoma cells.** PR was stained by indirect immunofluorescence using FITC (green) labeled secondary antibody. Cells were treated with vehicle (V); 48 h with estradiol (E) (10 nM); E followed by progesterone (P) (10 nM) for 24 h (E+P); E followed by RU486 (PR antagonist, 1 μM) for 24 h (E+RU); E followed by P+RU486 for 24 h (E+P+RU). Nuclei were counterstained with HOECHST. A representative assay of five independent experiments is shown.

of both PR isoforms increased after estradiol treatment and diminished with P4 in these cells [13]. In many cell types, PR expression is upregulated by estradiol at transcriptional level by estrogen-responsive elements located in the PR promoter [22], while P4 induces phosphorylation of PR which marks it to be degraded by the proteasome pathway resulting in PR downregulation [23]. RU486 antagonizes progestins action by its binding with PR allowing dimerization and binding with DNAs hormone response elements but avoids transcription [25]. After RU486 binding, PR, phosphorylation can be induced and mark it to degradation by 26S proteasome even without transcriptional activation. Previously, a reduction of PR isoform expression at protein level after RU486 administration has been reported in the

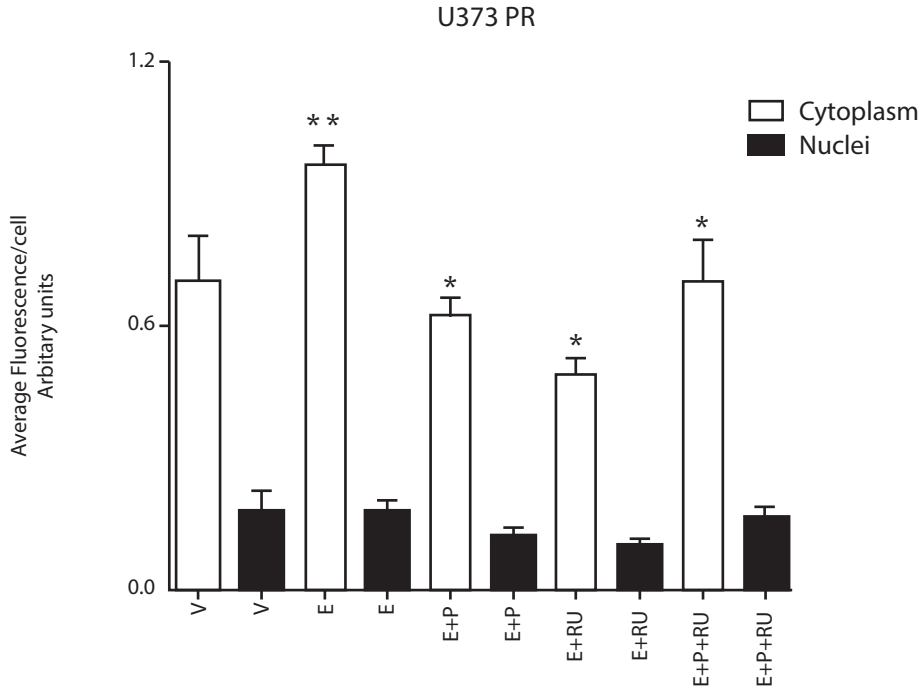

Figure 2: **PR expression in U373 human astrocytoma cells.** Immunofluorescence images were quantified and analyzed as described in Materials and Methods. Vehicle (V); 48 h with estradiol (E) (10 nM); E followed by progesterone (P) (10 nM) for 24 h (E+P); E followed by RU486 (1 μM) for 24 h (E+RU); E followed by P+RU486 for 24 h (E+P+RU). Results are expressed as mean ± SEM; n=5. ***P*<0.05 vs all groups; **P*<0.05 vs the other groups except V in cytoplasm.

Table 2: Selected genes validated by RT-PCR.

Gene	Name	Function
GLIPR2	Glioma pathogenesis- related 2	The product of this gene increases the activation of fibroblasts and induces epithelial mesenchymal transition (Baxter et al., 2007).
IL7R	Interleukin 7 receptor	High levels of this receptor correlate with tumor aggressiveness in breast cancer (Al- Rawi et al., 2004).
SREBF1	Sterol regulatory element binding transcription factor	It has been found to be involved in signaling pathways related to cancer (Shao and Espenshade, 2012).
IL18	Interleukin 18	This cytokine is involved in carcinogenesis and it is secreted by tumor cells, e.g., kidney (Sözen et al., 2004) and prostate cancer (Binay Lebel et al., 2003).
TGFβ2	Transforming growth factor β-2	Disruption of this gene function has been implicated in a variety of cancers (Buck and Knabbe, 2006; Nilsson EE, Skinner MK, 2002; Steiner, 1995)
MAP1B	Microtubule-associated protein 1B	This gene encodes a protein that belongs to the microtubule-associated protein family, which are involved in microtubule assembly. (Riederer, 2007).
ANLN	Anillin, actin binding protein	Alterations in this gene have been linked to oral cancer. Its upregulation is a common feature in the carcinogenic process of lung tissue (Suzuki et al., 2005).
HBG1	Hemoglobin, gamma A	This gene product is part of the fetal hemoglobin. It has been reported that some therapies directed to differentiate malignant cells result in the synthesis of fetal hemoglobin (Patrinos et al, 2005; Kieslich et al, 2003).
STARD4	STAR-related lipid transfer (START) domain containing	The level of expression of this gene has been linked to the endoplasmic reticulum stress (Yamada et al., 2006).
AOC3	Amine oxidase, copper	It has been associated with migration and metastasis in cancers such as colorectal, prostate, and melanoma (Jalkanen and Salmi, 2007; Toiyama, et al, 2009;. Ekblom et al, 1999. Forster-Horváth et al, 2004).

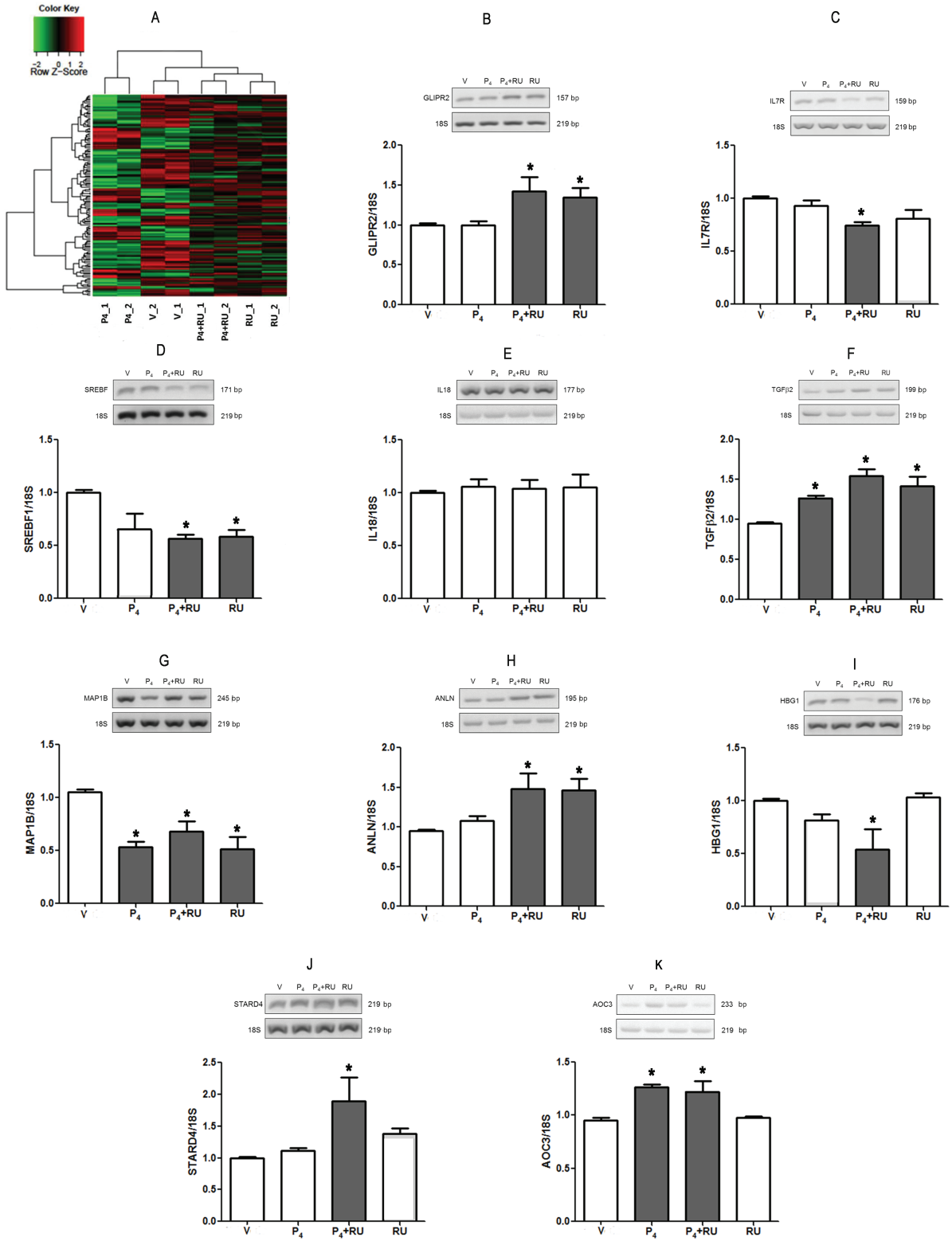

Figure 3: **Validation by RT-PCR of genes regulated by P4, RU486, and P4+RU486.** Total RNA of U373 cells treated during 12 h with vehicle (V) (0.02% cyclodextrin), P4 (10 nM), RU486 (RU 1 μM), or P4 + RU was used for RT-PCR assays. PCR products were separated on 2% agarose gel, stained with gel red, and detected with UV light (upper panels). Densitometric analysis of different mRNAs expression was corrected by using data of 18S mRNA values. A) Heat Map of microarrays, B) GLIPR2, C) IL7R, D) SREBF1, E) IL18, F) TGFβ2, G) MAP1B, H) ANLN, I) HBG1, J) STARD4, and K) AOC3. Results are expressed as mean ± SEM n=3. *P<0.05 vs vehicle (V).

Table 3: Effects of P4 and RU486 on the expression of genes tested by Microarrays and validated by RT-PCR in U373 cells.

Gene	P_4	Microarray P_4+RU	RU	P_4	RT-PCR P_4+RU	RU
GLIPR2	-	Increase	Increase	-	Increase	Increase
TGFb2	-	Increase	-	Increase	Increase	Increase
AOC3	Increase	-	-	Increase	Increase	-
HBG1	Increase	-	Increase	-	Decrease	-
IL7R	-	Decrease	-	-	Decrease	-
IL18	Decrease	-	-	-	-	-
MAP1B	-	Decrease	-	Decrease	Decrease	Decrease
ANLN	-	Decrease	-	-	Increase	Increase
SREBF1	-	-	Decrease	-	Decrease	Decrease
STARD4	-	Decrease	Decrease	-	Increase	-

preoptic area, uterus, ovary, and breast cancer cell lines [26–28].

In nuclei we did not find significant changes in PR content with any treatment. In T47D breast cancer cells PR nuclear translocation occurred at 1h after progestin or RU486 treatment [29, 30]. In order to determine if this hormone or RU486 induces nuclear translocation in U373 cells we need further investigation at shorter times.

The results obtained using microarrays showed that at 12 h P4 regulates, positively or negatively, the expression of various genes, many of which are involved in immunological processes, proliferation, adhesion, and metabolism that may have an important role in the development of tumors. This agrees with the fact that malignant tumors including astrocytomas have a complex process in which the expression of various genes is modified to allow the tumor cells to have oxygen supply and nutrients, escape to the immune system, and have the ability to migrate and invade [31–33]. The genes whose expression was altered by P4 treatment observed in this work are also modified by this hormone in other types of cancer and/or other pathological conditions [34–36]; however, we also determined the effect of these hormones over other genes that have not been reported before.

GLIPR2 encodes a protein still poorly studied and its function is not well characterized [37, 38]; found in many glioma cell lines and in vitro studies, this protein can induce epithelial mesenchymal transition (essential in cellular plasticity during development) and cancer progression [37, 39]. IL7R is a gene whose product has a critical role in the development, differentiation, growth, and activation of lymphoid cells [40] and has been associated with decreased immune response responsible for preventing the development of tumor in high grade gliomas of evolution [41].

AOC3 gene was only modified by P4; its product is a protein which catalyzes the oxidation of amines to aldehydes but also it has been involved in cell migration and extravasation induced by inflammatory processes [42]. It should be noted that this regulation seems to be through a nonclassical mechanism since the effect of P4 was not blocked by RU486. This regulation could occur through membrane PR [43].

Interestingly, it was observed for some genes such as GLIPR2, ANLN, and SREBF1 that RU486 treatment or cotreatment with P4 + RU486 regulates its expression without P4 effects. RU486 is a type II antagonist which promotes PR dimerization and allows binding of the dimers to DNA. It has been shown that RU486-bound PR-A:PR-A dimers are transcriptionally silent, whereas RU486-bound PR-B:PR-B dimers can activate transcription. RU486-bound PR-A:PR-B dimers act to distinctly inhibit transcriptional activation, and it is the activity that is commonly observed in P4 responsive cells [44, 45]. It is important to mention that in U373 cells PR-B content is three times higher than that of PR-A [21, 46] which could lead to an increased formation of PR-B:PR-B dimers and an activation of transcriptional activity upon RU486 treatment.

It should be noted that U373 cells are not being distributed by ATCC anymore, since a sequencing study demonstrated similarities between U-373 and the glioblastoma cell line, U-251 [47]. However, both cell lines have characteristics of aggressive astrocytomas.

Authors' Contribution

Aliesha González-Arenas and Alejandro Cabrera-Wrooman equally contributed to this work.

References

[1] C. Guerra-Araiza, O. Villamar-Cruz, A. González-Arenas, R. Chavira, and I. Camacho-Arroyo, Changes in progestrone receptor isoforms content in the rat brain during the oestrous cycle and after oestradiol and progesterone treatments, *Journal of Neuroendocrinology*, **15**, no. 10, 984–990, (2003).

[2] I. Camacho-Arroyo, A. González-Arenas, and G. González-Morán, Ontogenic variations in the content and distribution of progesterone receptor isoforms in the reproductive tract and

brain of chicks, *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, **146**, no. 4, 644–652, (2007).

[3] R. D. Brinton, R. F. Thompson, M. R. Foy, M. Baudry, J. Wang, C. E. Finch, T. E. Morgan, C. J. Pike, W. J. Mack, F. Z. Stanczyk, and J. Nilsen, Progesterone receptors: Form and function in brain, *Frontiers in Neuroendocrinology*, **29**, no. 2, 313–339, (2008).

[4] W. L. Kraus, M. M. Montano, and B. S. Katzenellenbogen, Cloning of the rat progesterone receptor gene 5'-region and identification of two functionally distinct promoters, *Molecular Endocrinology*, **7**, no. 12, 1603–1616, (1993).

[5] P. H. Giangrande and D. P. Mcdonnell, The A and B isoforms of the human progesterone receptor: Two functionally different transcription factors encoded by a single gene, *Recent Progress in Hormone Research*, **54**, 291–314, (1999).

[6] P. Kastner, A. Krust, B. Turcotte, U. Stropp, L. Tora, H. Gronemeyer, and P. Chambon, Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B, *EMBO Journal*, **9**, no. 5, 1603–1614, (1990).

[7] C. Bozzetti, R. Camisa, R. Nizzoli, L. Manotti, A. Guazzi, N. Naldi, S. Mazza, V. Nizzoli, and G. Cocconi, Estrogen and progesterone receptors in human meningiomas: Biochemical and immunocytochemical evaluation, *Surgical Neurology*, **43**, no. 3, 230–234, (1995).

[8] I. Camacho-Arroyo, G. González-Agüero, A. Gamboa-Domínguez, M. A. Cerbón, and R. Ondarza, Progesterone receptor isoforms expression pattern in human chordomas, *Journal of Neuro-Oncology*, **49**, no. 1, 1–7, (2000).

[9] R. S. Carroll, J. Zhang, K. Dashner, M. Sar, P. M. Black, and C. Raffel, Steroid hormone receptors in astrocytic neoplasms, *Neurosurgery*, **37**, no. 3, 496–504, (1995).

[10] J. Honegger, C. Renner, R. Fahlbusch, and E. F. Adams, Progesterone receptor gene expression in craniopharyngiomas and evidence for biological activity, *Neurosurgery*, **41**, no. 6, 1359–1364, (1997).

[11] A. Omulecka, W. Papierz, A. Nawrocka-Kunecka, and I. Lewy-Trenda, Immunohistochemical expression of progesterone and estrogen receptors in meningiomas, *Folia Neuropathologica*, **44**, no. 2, 111–115, (2006).

[12] G. González-Agüero, R. Ondarza, A. Gamboa-Domínguez, M. A. Cerbón, and I. Camacho-Arroyo, Progesterone receptor isoforms expression pattern in human astrocytomas, *Brain Research Bulletin*, **56**, no. 1, 43–48, (2001).

[13] E. Cabrera-Muñoz, A. González-Arenas, M. Saqui-Salces, J. Camacho, F. Larrea, R. García-Becerra, and I. Camacho-Arroyo, Regulation of progesterone receptor isoforms content in human astrocytoma cell lines, *Journal of Steroid Biochemistry and Molecular Biology*, **113**, no. 1-2, 80–84, (2009).

[14] D. Friedmann-Morvinski, E. A. Bushong, E. Ke, Y. Soda, T. Marumoto, O. Singer, M. H. Ellisman, and I. M. Verma, Dedifferentiation of neurons and astrocytes by oncogenes can induce gliomas in mice, *Science*, **338**, no. 6110, 1080–1084, (2012).

[15] S. Alcantara Llaguno, J. Chen, C. Kwon, E. L. Jackson, Y. Li, D. K. Burns, A. Alvarez-Buylla, and L. F. Parada, Malignant Astrocytomas Originate from Neural Stem/Progenitor Cells in a Somatic Tumor Suppressor Mouse Model, *Cancer Cell*, **15**, no. 1, 45–56, (2009).

[16] L. Cheng, S. Bao, and J. N. Rich, Potential therapeutic implications of cancer stem cells in glioblastoma, *Biochemical Pharmacology*, **80**, no. 5, 654–665, (2010).

[17] D. L. Schonberg, D. Lubelski, T. E. Miller, and J. N. Rich, Brain tumor stem cells: Molecular characteristics and their impact on therapy, *Molecular Aspects of Medicine*, (2013).

[18] D. Cho, S. Lin, W. Yang, H. Lee, D. Hsu, H. Lin, C. Chen, C. Liu, W. Lee, and L. Ho, Targeting cancer stem cells for treatment of glioblastoma multiforme, *Cell Transplantation*, **22**, no. 4, 731–739, (2013).

[19] J. T. Huse, H. S. Phillips, and C. W. Brennan, Molecular subclassification of diffuse gliomas: Seeing order in the chaos, *GLIA*, **59**, no. 8, 1190–1199, (2011).

[20] C. Daumas-Duport, B. Scheithauer, J. O'Fallon, and P. Kelly, Grading of astrocytomas: A simple and reproducible method, *Cancer*, **62**, no. 10, 2152–2165, (1988).

[21] G. González-Agüero, A. A. Gutiérrez, D. González-Espinosa, J. D. Solano, R. Morales, A. González-Arenas, E. Cabrera-Muñoz, and I. Camacho-Arroyo, Progesterone effects on cell growth of U373 and D54 human astrocytoma cell lines, *Endocrine*, **32**, no. 2, 129–135, (2007).

[22] C. Guerra-Araiza and I. Camacho-Arroyo, Progesterone receptor isoforms: Function and regulation, *Revista de Investigacion Clinica*, **52**, no. 6, 686–691, (2000).

[23] C. A. Lange, T. Shen, and K. B. Horwitz, Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome, *Proceedings of the National Academy of Sciences of the United States of America*, **97**, no. 3, 1032–1037, (2000).

[24] O. T. Hernández-Hernández, T. K. González-García, and I. Camacho-Arroyo, Progesterone receptor and SRC-1 participate in the regulation of VEGF, EGFR and Cyclin D1 expression in human astrocytoma cell lines, *Journal of Steroid Biochemistry and Molecular Biology*, **132**, no. 1-2, 127–134, (2012).

[25] F. Cadepond, A. Ulmann, and E.-E. Baulieu, RU486 (mifepristone): Mechanisms of action and clinical uses, *Annual Review of Medicine*, **48**, 129–156, (1997).

[26] G. E. Carrillo-Martínez, P. Gómora-Arrati, A. González-Arenas, G. Roldán-Roldán, O. González-Flores, and I. Camacho-Arroyo, Effects of RU486 in the expression of progesterone receptor isoforms in the hypothalamus and the preoptic area of the rat during postpartum estrus, *Neuroscience Letters*, **504**, no. 2, 127–130, (2011).

[27] X. Fang, S. Wong, and B. F. Mitchell, Effects of RU486 on estrogen, progesterone, oxytocin, and their receptors in the rat uterus during late gestation, *Endocrinology*, **138**, no. 7, 2763–2768, (1997).

[28] L. D. Read, C. E. Snider, J. S. Miller, G. L. Greene, and B. S. Katzenellenbogen, Ligand-modulated regulation of progesterone receptor messenger ribonucleic acid and protein in human breast cancer cell lines, *Molecular Endocrinology*, **2**, no. 3, 263–271, (1988).

[29] L. Tseng and H. H. Zhu, Regulation of progesterone receptor messenger ribonucleic acid by progestin in human endometrial stromal cells, *Biology of Reproduction*, **57**, no. 6, 1360–1366, (1997).

[30] L. K. Pierson-Mullany and C. A. Lange, Phosphorylation of progesterone receptor serine 400 mediates ligand-independent transcriptional activity in response to activation of cyclin-dependent protein kinase 2, *Molecular and Cellular Biology*, **24**, no. 24, 10542–10557, (2004).

[31] R. Weinstain, J. Kanter, B. Friedman, L. G. Ellies, M. E. Baker, and R. Y. Tsien, Fluorescent ligand for human progesterone receptor imaging in live cells, *Bioconjugate Chemistry*, **24**, no. 5, 766–771, (2013).

[32] I. Kareva and P. Hahnfeldt, The emerging "Hallmarks" of metabolic reprogramming and immune evasion: Distinct or linked? *Cancer Research*, **73**, no. 9, 2737–2742, (2013).

[33] M. Nakada, S. Nakada, T. Demuth, N. L. Tran, D. B. Hoelzinger, and M. E. Berens, Molecular targets of glioma invasion, *Cellular and Molecular Life Sciences*, **64**, no. 4, 458–478, (2007).

[34] P. Friedl and K. Wolf, Tumour-cell invasion and migration: Diversity and escape mechanisms, *Nature Reviews Cancer*, **3**, no. 5, 362–374, (2003).

[35] R. H. Paulssen, B. Moe, H. Grønaas, and A. Ørbo, Gene expression in endometrial cancer cells (Ishikawa) after short time high dose exposure to progesterone, *Steroids*, **73**, no. 1, 116–128, (2008).

[36] C. A. Lapp, M. E. Thomas, and J. B. Lewis, Modulation by progesterone of interleukin-6 production by gingival fibroblasts., *Journal of Periodontology*, **66**, no. 4, 279–284, (1995).

[37] J. Fan, Y. Shimizu, J. Chan, A. Wilkinson, A. Ito, P. Tontonoz, E. Dullaghan, L. A. M. Galea, T. Pfeifer, and C. L. Wellington, Hormonal modulators of glial ABCA1 and apoE levels, *The Journal of Lipid Research*, **54**, no. 2, 3139–3150, (2013).

[38] R. M. Baxter, T. P. Crowell, J. A. George, M. E. Getman, and H. Gardner, The plant pathogenesis related protein GLIPR-2 is highly expressed in fibrotic kidney and promotes epithelial to mesenchymal transition in vitro, *Matrix Biology*, **26**, no. 1, 20–29, (2007).

[39] M. R. Groves, A. Kühn, A. Hendricks, S. Radke, R. L. Serrano, J. B. Helms, and I. Sinning, Crystallization of a Golgi-associated PR-1-related protein (GAPR-1) that localizes to lipid-enriched microdomains, *Acta Crystallographica Section D: Biological Crystallography*, **60**, no. 4, 730–732, (2004).

[40] B. D. Craene and G. Berx, Regulatory networks defining EMT during cancer initiation and progression, *Nature Reviews Cancer*, **13**, no. 2, 97–110, (2013).

[41] M. A. A. Al-Rawi, K. Rmali, G. Watkins, R. E. Mansel, and W. G. Jiang, Aberrant expression of interleukin-7 (IL-7) and its signalling complex in human breast cancer, *European Journal of Cancer*, **40**, no. 4, 494–502, (2004).

[42] H. Ardon, B. Verbinnen, W. Maes, T. Beez, S. Van Gool, and S. De Vleeschouwer, Technical advancement in regulatory T cell isolation and characterization using CD127 expression in patients with malignant glioma treated with autologous dendritic cell vaccination, *Journal of Immunological Methods*, **352**, no. 1-2, 169–173, (2010).

[43] S. Jalkanen and M. Salmi, VAP-1 and CD73, endothelial cell surface enzymes in leukocyte extravasation, *Arteriosclerosis, Thrombosis, and Vascular Biology*, **28**, no. 1, 18–26, (2008).

[44] M. Singh, C. Su, and S. Ng, Non-genomic mechanisms of progesterone action in the brain, *Frontiers in Neuroscience*, **7**, p. 159, (2013).

[45] C. A. Sartorius, S. D. Groshong, L. A. Miller, R. L. Powell, L. Tung, G. S. Takimoto, and K. B. Horwitz, New T47D breast cancer cell lines for the independent study of progesterone B- and A-receptors: Only antiprogestin-occupied B-receptors are switched to transcriptional agonists by cAMP, *Cancer Research*, **54**, no. 14, 3868–3877, (1994).

[46] Z. Liu, D. Auboeuf, J. Wong, J. D. Chen, S. Y. Tsai, M. Tsai, and B. W. O'Malley, Coactivator/corepressor ratios modulate PR-mediated transcription by the selective receptor modulator RU486, *Proceedings of the National Academy of Sciences of the United States of America*, **99**, no. 12, 7940–7944, (2002).

[47] V. Hansberg-Pastor, A. González-Arenas, M. A. Peña-Ortiz, E. García-Gómez, M. Rodríguez-Dorantes, and I. Camacho-Arroyo, The role of DNA methylation and histone acetylation in the regulation of progesterone receptor isoforms expression in human astrocytoma cell lines, *Steroids*, **78**, no. 5, 500–507, (2013).

[48] N. Ishii, D. Maier, A. Merlo, M. Tada, Y. Sawamura, A. Diserens, and E. G. Van Meir, Frequent Co-alterations of TP53, p16/CDKN2A, p14(ARF), PTEN tumor suppressor genes in human glioma cell lines, *Brain Pathology*, **9**, no. 3, 469–479, (1999).