

Research Article

Modulation of Gene Expression Associated with the Cell Cycle and Tumorigenicity of Glioblastoma Cells by the 18 kDa Translocator Protein (TSPO)

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Abstract

After PK 11195 (25 µM) exposure (24 and 48 hours) and permanent TSPO silencing by siRNA to U118MG glioblastoma cells, microarray analysis of gene expression, followed up by validation with real time polymerase chain reaction (RT-PCR), showed effects on gene expression related to the cell cycle. Other affected genes are related to apoptosis, oxidative stress, immune responses, DNA repair, and membrane functions, including adhesion and transport. In relation to post transcriptional and post translational effects, TSPO ligand and TSPO knockdown affected gene expression for many short nucleolar RNAs. Applying a 2-fold, cut off to micro array analysis revealed that 24 and 48 hours of PK 11195 exposure affected respectively 128 and 85 genes that were also affected by TSPO silencing. Taking a 2.5 fold, cut off, only gene expression of v-FOS was found to be modulated by each of these TSPO manipulations. Analyzing the effects of TSPO knockdown and PK 11195 exposure, RT-PCR indicated that 5 genes related to the cell cycle are under TSPO control. Focusing further on the cell cycle: 1) TSPO silencing increased numbers of cells in the S (to 321%) and G2/M (to 131%) phases, and caused a decrease in the G0/G1 phase (to 69%); 2) 24 hours of PK 11195 exposure caused decreases in the S (to 81%) and G2/M (to 94%) phases, and an increase in the G0/G1 (to 112%) phase; 3) 48 hours of PK 11195 exposure caused decreases in the S (to 70%) and G0/G1 (to 96%) phases, and an increase in the G2/M (to 124%) phase. Thus, TSPO appears to provide for regulation of the cell cycle via specific modulation of gene expression for key gene products in these glioblastoma cells.

Keywords: Glioblastoma; 18 kDa Translocator protein (TSPO); Gene expression; FOS; Cell cycle; siRNA knockdown; PK 11195; Brain tumor

Abbreviations

BRCA1: Breast Cancer 1, Early Onset; CDC2: Cell Division Cycle 2; CDC6: Cell Division Cycle 6; EGR1: Early Growth Response 1; FGIN-1-27: *N*, *N*-Dihexyl-2-(4-fluorophenyl)Indole-3-Acetamide; GBM: Glioblastoma Multiforme; MCM2: Minichromosome Maintenance Complex Component 2; MCM4: Minichromosome Maintenance Complex Component 4; PK 11195: 1-(2-Chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-Isoquinolinecarboxamide; PPIX: Protoporphyrin IX; RANBP3: Ran-Binding Protein 3; Ro5-4864: Benzodiazepine Ro5 4864 (4'-chlorodiazepam); ROS: Reactive Oxygen Species; RT-PCR: Realtime Polymerase Chain Reaction; TSPO: 18 kDa Translocator Protein; v-FOS: v-fos FBJ murine osteosarcoma viral oncogene homolog

Introduction

Glioblastoma (GBM) presents the most aggressive form of malignant glioma, including spread and destruction of tissue throughout the brain [1,2]. Moreover, current treatment regimens only provide marginal impact on patient survival [3]. Survival rate after diagnosis of GBM typically is less than 6 months. GBM presents 80% of the malignant gliomas [4-6]. The challenge is to understand and arrest the cellular processes contributing to the

malignancy of glioblastoma. [7]. Previous studies by us have shown that glioblastoma cells could be transformed to even more malignant strains due to silencing of the 18 kDa translocator protein (TSPO) by genetic manipulation [8-12]. Thus, it is important to understand the role of the TSPO in malignancy [13,14].

TSPO can be found in normal glial cells as well as healthy peripheral tissues such as the heart, lung, kidney, adrenal, and reproductive organs [14,15]. In addition, TSPO expression typically is enhanced in tumorigenic tissues and cells. In the mitochondria, TSPO can be found in association with several proteins that are related to various functions such as cell metabolism, programmed cell death, heme metabolism, ROS generation, ATP production, and steroidogenesis [11,16-20]. The association of the TSPO with the cell nucleus is thought to take part in regulation of gene expression by the TSPO [21,22]. Synthetic TSPO ligands include the benzodiazepine Ro5 4864 (4'-chlorodiazepam), as well as the isoquinoline carboxamide derivative PK 11195, and have been used to study the functions of the TSPO [23,26]. The TSPO has been implicated in various functions, for example, programmed cell death, steroidogenesis, mitochondrial respiration, reactive oxygen species (ROS) generation, regulation of the mitochondrial membrane potential ($\Delta\Psi_m$), inflammation, effects on the immune and phagocytic host-defense response,

microglial activation, ischemia, cell growth and differentiation, cancer cell proliferation, regulation of the cell cycle, and mental and neuropathological disorders [14,17,20,25-29]. Several of these functions have implications for TSPO involvement in tumorigenicity. We have previously described the effect of TSPO knockdown by siRNA as well as treatments with the specific TSPO ligands PK 11195 and Ro5 4864 on programmed cell death of U118MG glioblastoma cells [11,12,18,30,31]. In addition, enhanced TSPO expression in cancer cells correlates with their proliferation [9,32]. Furthermore, TSPO knockdown as well as application of the TSPO ligand PK11195 reduced adhesion and enhanced migratory characteristics of glioma cells in cell culture *in vitro* and increased invasiveness *in vivo* in animal models [2]. Microarray analysis of gene expression suggested that the TSPO may regulate gene expression related to tumorigenicity of U118MG cells [22]. The influence of TSPO knockdown on the cell cycle of glioblastoma cells was for example studied on the rat C6 cell line [9]. In that study the TSPO knockdown resulted in a lower percentage of knockdown clones in the G0/G1 phase and a larger percentage of the same clones in the G2/M phases in comparison to the control. It also was shown that synthetic TSPO ligands, such as FGIN-1-27, PK 11195, and Ro5 4864, dose-dependently inhibited cell proliferation of via an arrest of the G0/G1 phase in various human cancer cells [33-36]. Thusfar, none of these studies proposed a mechanism whereby TSPO may be able to affect the cell cycle, nor do they present an explanation why opposing, apparently contradictory results can be found.

To better understand the involvement of the TSPO in regulation of the cell cycle due to TSPO modulations, we performed microarray analysis of gene expression. Our methodology includes study of the effects of the synthetic TSPO ligand PK 11195 applied for 24 and 48 hours in comparisons with the effects of permanent TSPO knockdown by siRNA introduced by stable transfection. By this approach we consider the likelihood that effects that are common to these different methods indeed are due to effects on the TSPO, while effects unique to either one of these methods do not necessarily relate directly to the TSPO. After screening the general effects on gene expression in our paradigm, we focussed on gene expression associated with the cell cycle, and also on actual changes in the cell cycle phases in these complementary experimental models.

Material and Methods

Materials

Materials required for our cell cultures were obtained from Biological Industries (Beit Ha'emek, Israel), Biochrom (Berlin, Germany), and Lonza (Basel, Switzerland). Oligonucleotides for real time polymerase chain reaction (RT-PCR) were from IBA (BioTAGnology, Göttingen, Germany) and SYBR Green Mastermix from Applied Biosystems (Foster City, CA). Materials for microarray analysis were from Agilent (Life science group, Penzberg, Germany) and Affymetrix (Santa Clara, CA). TRIzol reagent used for RNA extraction of microarray samples was purchased Invitrogen (Darmstadt, Germany). 1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) was obtained from Alexis (Switzerland).

Cell culture

Cells of the human glioblastoma cell line U118MG were cultured

at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity. U118MG wild type (WT) cells were cultured in MEM-EAGLE cell culture medium supplemented with 10% FBS, 2% glutamine, and 0.05% gentamycin (50 mg/ml). We applied stable transfection with siRNA to silence TSPO expression in U118MG cells (TSPO knockdown) and also prepared sham control scrambled siRNA cells (Scr control) as described before [12]. Expression of TSPO was routinely assessed using RT-PCR and Western blot, as described by us in detail previously [11,12]. In the present study, RT-PCR assays showed that gene expression of the TSPO protein was reduced to 20% of the levels detected in Scr control cells (n = 6, p < 0.01, for each assay), similar to what was described in previous studies [11,12,31]. For the assays of the present study (flow cytometry for cell cycle analysis; and RNA extraction for microarray and RT-PCR), TSPO knockdown cells, the Scr control cells, and the WT cells were grown until they reached 90% confluency. Cells were collected by trypsinization and centrifugation, as described previously [9,11,12,22], to study the effects of our treatments on gene expression and the cell cycle.

Exposure to PK 11195

WT U118MG cells (1×10^6) were seeded in 75 cm² cell culture flasks and allowed to acclimatize overnight. Then the cells were exposed to 25 μM of PK 11195 (Alexis, Switzerland). The exposure times to PK 11195 were 24 and 48 hrs. Flow cytometry and RNA extraction were executed immediately after the PK 11195 exposures. Stock solution of PK 11195 with a concentration of 20 mM was prepared in 100% ethanol. For exposures, final concentration of PK 11195 was 25 μM in the culture medium and final concentration of ethanol was 1%. Control cells were exposed to vehicle i.e. 1% ethanol final concentration [30]. The concentration of 25 μM of PK 11195 was previously found in other studies to be very effective and consistent, providing effects that were similar to those of TSPO knockdown by genetic manipulation in those studies [2,11,12,18,22,30,31].

Microarray analysis of gene expression

For microarray analysis of gene expression, 3×10^4 U118MG cells (Scr control and siRNA TSPO knockdown) were seeded and grown in cell culture flasks (75 cm²), until they reached 90% confluency as described above. For these assays, also WT U118MG cells were seeded, cultured, and exposed to PK 11195 for 24 and 48 hrs as described above. Experiments were repeated at least three times, and each experiment was done in triplicates. RT-PCR was performed as described before [2]. Cells were collected by trypsinization and in this way 3 samples of 3×10^5 cells were obtained per experimental group, each sample coming from a separate flask. Cell pellets were prepared by centrifugation (1000 × g, 7 min, 20°C). The RNA isolation was done according to the "TRIzol RNA extraction protocol" (Invitrogen, Darmstadt, Germany). The RNA pellet was dissolved in 100 μl RNase free water and mixed by repetitive pipetting. For checking RNA quality and quantity, 2 μl of each sample was used. Measurements of the 28S:18S ratios to determine the integrity of RNA samples were done using Agilent 2100 bioanalyzer (Agilent Technologies, Genomics, Santa Clara, CA), as described previously [11,22,37,38]. The "Low RNA Input linear Amplification Kit Plus, two color" (Agilent, 5188-5340) and the "RNA Spike-In Kit" (Agilent, 5188-5279) were used for cDNA synthesis and *in vitro* transcription according to the manufacturer's recommendations. Quantity and dye incorporation rates of the amplified cRNAs were determined using

the NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1 (NanoDrop Technologies, Wilmington, DE). cDNA was labeled for microarray hybridization with the help of an *in vitro* transcription (IVT) labeling reaction. The IVT labeling was performed with the help of Affymetrix GeneChip® IVT Labelling Kit (Affymetrix, High Wycombe, UK). Fragmentation and hybridization of labeled amino-allyl-RNA (aRNA) GeneChip® Human Exon 1.0 ST Array (Affymetrix) was performed in the DNA Microarray Facility at the Medical Faculty of the Georg-August-University of Göttingen [37,39-42]. Briefly, Affymetrix Whole Transcript chips were used and hierarchical gene cluster analysis was applied. The raw intensity data were normalized with quantile normalization. Differentially expressed genes were identified by an ANOVA fixed effects model. Adjusted p-values were obtained by the Benjamini-Hochberg method to control the false discovery-rate [43,44]. To further avoid false positives in our interpretations, we considered only 2- fold differences and higher between gene expression of detected genes in Scr and TSPO knockdown cells, and between vehicle control and PK 11195 exposure detected with our microarray analysis. For the present study, microarray assays were applied to U118MG TSPO knockdown cells and their Scr control cells, and to WT U118MG cells exposed for 24 hrs and 48 hrs to PK 11195 and their vehicle controls.

Validation of candidate genes with real time polymerase chain reaction (RT-PCR)

To verify the results of the screening by microarray assay, as another measure to avoid false positives, RT-PCR was applied as described [2,22]. For the present study we applied RT-PCR to genes coding for proteins with relatively well defined functions in the cell cycle that were firstly detected by microarray applied to U118MG TSPO knockdown cells in comparison to Scr control cells (Table 1), applying the criterion of a 2-fold cut off. For these genes we also applied RT-PCR to U118MG cells exposed to PK 11195 for 24 hrs and 48 hrs, if they showed significant differences with the microarray analysis (Table 1). RNA extraction was applied according to the instructions provided with the kit (RNeasy® Mini Kit, Qiagen, Germany). The concentrations of RNAs were determined by NanoDrop™ measurement. Reverse transcription of RNA was performed with iScript cDNA Synthesis Kit (BioRad, Hercules, CA), according to the instructions of the manufacturer. RT-PCR was performed using 10 ng target cDNA and 3 pmol of each primer in question (see list below) in SYBR-GreenMix (SYBR Green, Applied Biosystems, Foster City, CA). The reaction was carried out with a final volume of 10 µl in 384 well optical plates. For each sample, triplicates were performed and water was taken as control. 18S was chosen as an internal standard because its expression is stable irrespective of treatment of cells [45]. The constancy of the cycle threshold (ct) of 18S can be measured and analyzed after 12 cycles for all experiments. The reaction was carried out at the ABI PRISM 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA) with 40 cycles of 15 seconds at 95°C, and one cycle of one minute at 60°C followed by the measurement of the dissociation curve. Data analysis of RT-PCR was performed using SDS 2.4 software (Applied Biosystems, Foster City, CA). For this purpose, the threshold was adjusted to the beginning of exponential fluorescence increase in a logarithmic plot of fluorescence over the amount of cycles. This was done to eliminate the background noise of fluorescence, as described previously [45]. The point of intersection between fluorescence curve and threshold defines the ct-value (cycle

threshold) of the respective gene in the respective sample i.e. the time point at which the chosen threshold was attained defined the level of change in gene expression. The mean of the triple ct-values was normalized to the internal standard creating the Δ ct-value. The ct-value of the internal standard 18S did not differ between all analyses of gene expressions at 12 cycles, indicative of the consistency of the RT-PCR assays for our analyses. The difference between Δ ct-values of TSPO knockdown or PK 11195 treated cells and their respective control cells defines the $\Delta\Delta$ ct-value. Due to the exponential relation of the DNA amount to the respective cycle, the normalized difference of mRNA that was present in the original samples is described by $2^{-\Delta\Delta t}$. This value represents the fold change of expression induced by the treatment [45].

Primer sequences used for realtime-PCR analysis:

BRCA1	Forw	5'-TTCCAGATCCACAAGCCCAAC-3'
	Rev	5'-GGCACTATTCTCTGATGACCCG-3'
CDC2	Forw	5'-TGATCCAGCCAAACGAATTC-3'
	Rev	5'-GCTACATCTTCTTAATCTGATTGTCCAA-3'
CDC6	Forw	5'-CCGTAACCTGTCTCTCTCGTGT-3'
	Rev	5'-TCTTGCCCTTGCTTTGGTGA-3'
Claspin	Forw	5'-GGATCCGCCCCACCATGACAGGCGAGGTGGGT-3'
	Rev	5'-TCTAGACTCGAGGCTCTCCAAATATTTG-3'
CyclinE2	Forw	5'-TGTTGGCCACCTGTATTATCTGG-3'
	Rev	5'-ATCTGGAGAAATCACTTGTCTCTATTTTC-3'
CyclinG2	Forw	5'-GCTGAAAGCTTGCAACTGCCGACTC-3'
	Rev	5'-GGTATCGTTGGCAGCTCAGGAAC-3'
EGR1	Forw	5'-ACCGCAGAGTCTTTTCTGACA-3'
	Rev	5'-TTGGTCATGCTCACTAGGCCAC-3'
KIAA	Forw	5'-AACATGGTGCAGACTAAAGCA-3'
	Rev	5'-CCTCGATGAAACTGATGTCGAA-3'
MCM2	Forw	5'-AGACGAGATAGAGCTGACTG-3'
	Rev	5'-CACCACGTACCTTGTGCTTG-3'
MCM4	Forw	5'-CCTTCAGAGATTTCTTCAGCG-3'
	Rev	5'-TCACCAATAACATTAATCTCCC-3'
v-FOS	Forw	5'-TGGCGTTGTGAAGACCATGA-3'
	Rev	5'-CCCTTCGGATTCTCCTTTCTC-3'
RANBP3	Forw	5'-ACAGCAAGCACTGACTGTGG-3'
	Rev	5'-GGCACTGGCCTGAATTAATA-3'
18S	Forw	5'-AACTTTCGATGGTAGTCGCCG-3'
	Rev	5'-GGATGTGGTAGCCGTTCTCAG-3'

Cell cycle analysis with flow cytometry

For cell cycle analysis, Fluorescence Assisted Cell Sorting (FACS)

analysis of the cell cultures of our study was performed each time by 3 independent experiments each done in triplicate. In brief, cells were harvested by trypsinization. Cells were pelleted by centrifugation, resuspended, and fixed in 500 μ l of Nicoletti buffer [0.1% (w/v) Sodium Citrate, 0.1% (v/v) of TritonX-100 and 50 μ g/ml of propidium iodide (PI)]. The cell suspension was vortexed and incubated for 60 min at 4°C in the dark. Cells were analyzed immediately for PI labeling using the FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). For each measurement, 10,000 cells were counted. Dot plots and histograms were analyzed by Cell-Quest Pro software (BD Biosciences, Heidelberg, Germany).

Statistical analysis

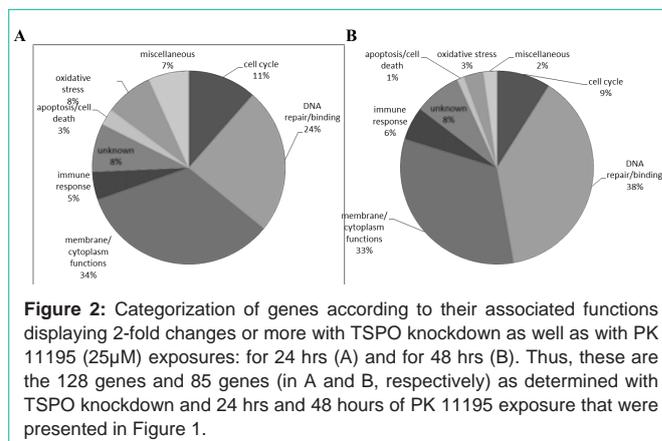
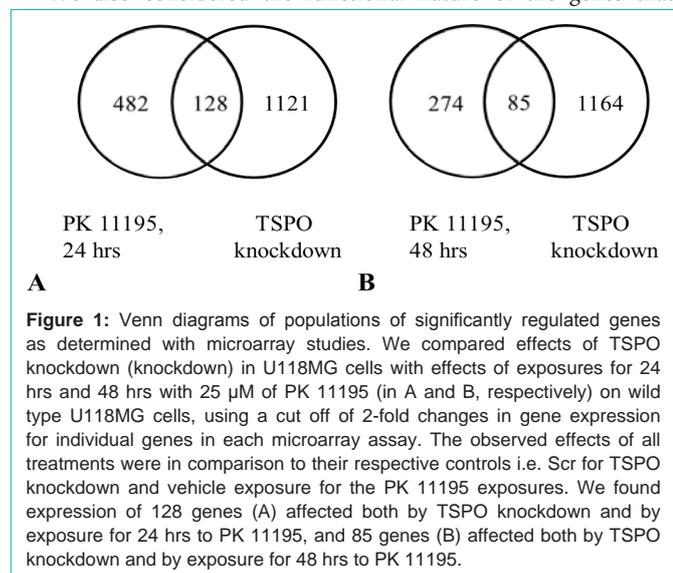
All assays were at least 3 independent experiments, each done in triplicate (total $n \geq 9$). Error bars represent the standard deviation (SD). For statistical analysis paired Student's t-Test was applied as appropriate. Statistical significance is presented in the following manner: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

Changes in gene expression determined with microarray and Venn analysis

Applying a 2.0 fold change cut off for our micro array screening, Venn diagrams present the number of genes that underwent significant changes in expression common for TSPO knockdown cells and WT cells exposed to PK 11195 for 24 hrs and 48 hrs, respectively (Figure 1) (see also the appendices 1, 2, 3). When WT U118MG cells were exposed for 24 and 48 hours to 25 μ M of PK 11195, significant changes in expression detected in this way were respectively 610 and 359 genes (Figure 1A and B). Compared to U118MG Scr control cells, knockdown of TSPO with siRNA resulted in significant changes in gene expression levels for 1249 genes (Figure 1) [22]. Furthermore, the Venn diagrams (Figure 1) demonstrated an overlap of 128 genes having their expression changed by TSPO knockdown as well as 24 hrs of PK 11195 exposure (Figure 1A). With the same approach, WT cells exposed to PK 11195 for 48 hrs and TSPO knockdown cells presented an overlap of 85 genes (Figure 1B).

We also considered the functional nature of the genes that



showed significant 2 fold changes effected by TSPO knockdown as well as by PK 11195 exposure for 24 hours and 48 hours, as compared to their respective controls. These genes were categorized into functional entities that included: membrane and cytoplasm functions (transport, cytoskeleton, and adhesion), effects on DNA, the cell cycle, oxidative stress, immune response, and cell death (Figure 2). The proportions of the overlaps between the groups of genes found with these comparisons were about the same for most of the functional categories (Figure 2). One exception was the effect on expression of genes related to oxidative stress, which constituted 8% of the total population of affected genes found to be affected both in TSPO knockdown cells and cells exposed for 24 hours to 25 μ M of PK 11195. However, such genes formed only 3% of the total overlap of changes in gene expression between TSPO knockdown cells and cells exposed for 48 hours to 25 μ M of PK 11195. Another functional entity showing considerable changes in gene expression both in TSPO knockdown cells and cells exposed for 24 hours to 25 μ M of PK 11195 was DNA repair and binding to DNA, presenting an overlap which constituted 24% of the total population of affected genes in this comparison. This increased to 38% of the total overlap of changes in gene expression between TSPO knockdown cells and cells exposed for 48 hours to 25 μ M of PK 11195. Furthermore, a general observation, our microarray analysis showed that gene expression affected by TSPO knockdown and TSPO ligand exposure corresponded to functions typically attributed to the TSPO [14,22,26].

Subsequently, we focused on the effects of TSPO knockdown and PK 11195 exposure (24 hrs and 48 hrs) on gene expression related to cell cycle, presented by microarray screening (Table 1). Table 1 shows this screened gene expression related to the cell cycle, including the direction of the changes (+ or -) and the magnitudes of the changes. Table 1 also presents the specific functions in the cell cycle related to the genes in question.

Table 1 also presents v-FOS and RANBP3 gene expression, which showed the biggest changes in expression in the present study. With a cut off of a 2.5 change, only v-FOS FBJ murine osteosarcoma viral oncogene homolog (v-FOS) showed a change in gene expression due to TSPO knockdown, as well as both the 24 hrs and 48 hrs exposures to PK 11195 (Table 1). In more detail, v-FOS expression levels were decreased to 0.2 fold in U118MG TSPO knockdown cells, to 0.3 fold after 24 hrs as well as 48 hrs of PK 11195 exposure of WT cells (Appendices 1, 2, 3, and Table 1). With this 2.5 cut off applied, Ran-

Table 1: Direction, magnitude and significance of changes in expression of genes related to the cell cycle (BRCA1, CDC2, CDC6, Claspin, CyclinE2, CyclinG2, EGR1, KIAA, MCM2, MCM4), and also of genes known to regulate gene expression and nuclear transport (FOS, RANBP3), as resulting from TSPO knockdown as well as PK 11195 exposure, as screened with microarray.

Gene	Target in cell cycle or nuclear function	TSPO knockdown Microarray expression level (x-fold)	PK 11195 24 hrs Microarray expression level (x-fold)	PK 11195 48 hrs Microarray expression level (x-fold)
BRCA1	S/G2	(+) 2.1	(+) 2.7	(+) 1.7
CDC2	G2/M	(+) 2.5	(+) 1.8	(+) 1.7
CDC6	G1/S	(+) 2.4	(+) 3.4	(+) 1.8
Claspin	S/G2	(+) 2.3	(+) 3.1	(+) 1.6
CyclinE2	G1	(+) 2.1	(+) 3.5	(+) 1.5
CyclinG2	G2/M	0.4	(-) 0.5	no effect
EGR1	G1	0.4	no effect	(-) 0.7
KIAA	G2/M	0.4	no effect	(+) 2.1
MCM2	S	(+) 2.3	(+) 2.0	no effect
MCM4	G2M	(+) 2.2	(+) 2.6	no effect
v-FOS	gene expression	(-) 0.2	(-) 0.3	(-) 0.3
RANBP3	nuclear transport	(-) 0.1	(-) 0.4	(-) 0.5

binding protein 3 (RANBP3) also showed a change in gene expression with TSPO knockdown and with 24 hrs exposure to PK 11195 (Table 1), but not with 48 hrs exposure to PK 11195. In more detail, changes in expression levels of RANBP3 were 0.1 fold for TSPO knockdown, 0.4 fold for 24 hrs of PK 11195 exposure, and 0.5 fold for 48 hrs of PK 11195 exposure (Appendices 1, 2, 3, and Table 1). It has been reported that the v-FOS gene either encodes or induces an activator of transcription that recognizes specific sequences in promoters [46]. RANBP3 is a cofactor for Crm1-mediated nuclear protein export [47].

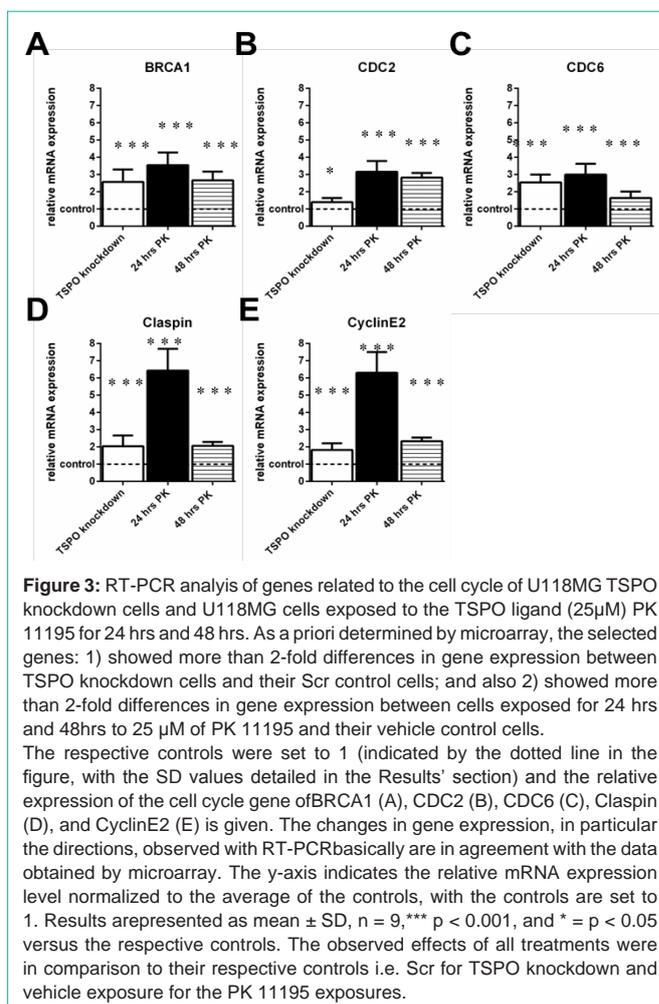
RT-PCR for gene expression related to the cell cycle

To verify the effects of TSPO knockdown and PK 11195 exposure (24 hrs and 48 hrs) on gene expression related to cell cycle (Table 1, appendices 1,2,3), RT-PCR was subsequently applied (Figures. 3-5). In addition, RT-PCR was subsequently applied to the v-FOS and RANBP3 genes (Figure 6), which had displayed more than 2.5-fold changes in expression as observed with microarray analysis of TSPO knockdown cells, as presented above. Figures 3, 4, and 5 show these RT-PCR results:

Figure 3 presents genes that show changes due to TSPO knockdown as well as both 24 hours and 48 hours of PK 11195 exposure, as obtained with RT PCR: Breast cancer 1, early onset (BRCA1), Cell division cycle 2 (CDC 2), Cell division cycle 6 homolog (CDC6), Claspin homolog (Claspin), and CyclinE2 (CyclinE2). In more detail:

BRCA1 was upregulated to 257% ± 73 after TSPO knockdown (Control 100% ± 15), to 355% ± 73 after 24 hrs of PK 11195 exposure (Control 100% ± 22), and to 267% ± 50 after 48 hrs of PK 11195 exposure (Control 100% ± 13) (Figure 3A).

CDC 2 was upregulated to 140% ± 24 after TSPO knockdown (Control 100% ± 28), to 316% ± 62 after 24 hrs of PK 11195 exposure



(Control 100% ± 20), and to 283% ± 27 after 48 hrs of PK 11195 exposure (Control 100% ± 5) (Figure 3B). CDC6 was upregulated to 255% ± 45 after TSPO knockdown (Control 100% ± 14), to 300% ± 63 after 24 hrs of PK 11195 exposure (Control 100% ± 21), and to 164% ± 37 after 48 hrs of PK 11195 exposure (Control 100% ± 28) (Figure 3C).

Claspin was upregulated to 204% ± 63 after TSPO knockdown (Control 100% ± 24), to 643% ± 126 after 24 hrs of PK 11195 exposure (Control 100% ± 19), and to 207% ± 23 after 48 hrs of PK 11195 exposure (Control 100% ± 18) (Figure 3D). CyclinE2 was upregulated to 183% ± 38 after TSPO knockdown (Control 100% ± 28), to 630% ± 120 after 24 hrs of PK 11195 exposure (Control 100% ± 18), and to 244% ± 20 after exposure for 48 hrs of PK 11195 exposure (Control 100% ± 16) (Figure 3E).

Figure 4 presents genes that show changes due to TSPO knockdown as well as 24 hours of PK 11195 exposure, but have no significant changes after 48 hours of PK 11195 exposure. After TSPO knockdown and after exposure for 24 hrs of PK 11195, the gene expression levels for minichromosome maintenance complex component 2 (MCM2), minichromosome maintenance complex component 4 (MCM4), and CyclinG2 were changed more than 2-fold by both these two treatments of U118MG cells, as firstly seen with microarray (Table 1). Applying RT-PCR, Figure 4A shows that gene

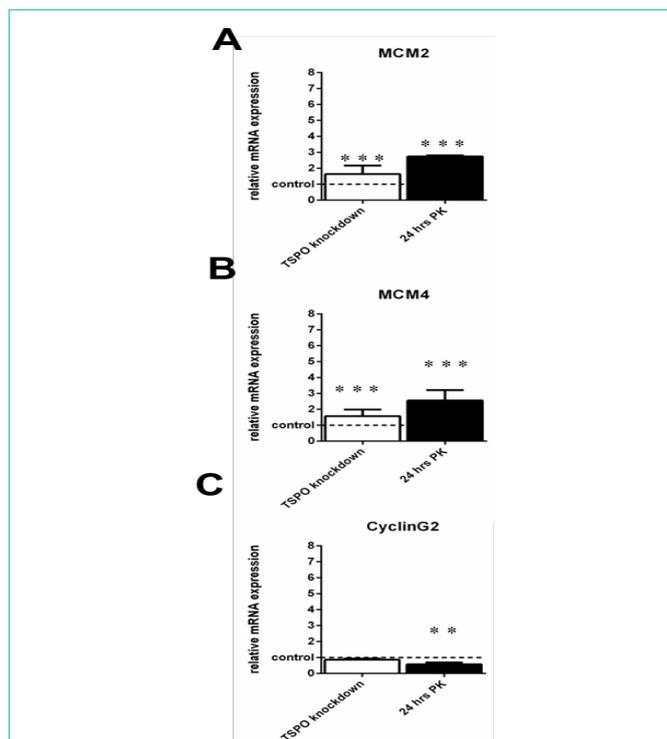


Figure 4: RT-PCR analysis of genes related to the cell cycle of U118MG TSPO knockdown cells and U118MG cells exposed to the TSPO ligand PK 11195 (25 μ M) for 24 hrs. As a priori determined by microarray, the selected genes: 1) showed more than 2-fold differences in gene expression between TSPO knockdown cells and their Scr control cells; and also 2) showed more than 2-fold differences in gene expression between cells exposed for 24 hrs to 25 μ M of PK 11195 and their vehicle control cells, and 3) did not show such effects after 48 hrs of exposure to PK 11195.

The respective controls were set to 1 (indicated by the dotted line in the figure with the SD values detailed in the Results' section) and the relative expression of the cell cycle genes of MCM2 (A), MCM4 (B), and CyclinG2 (C) is given. Changes in gene expression, in particular the direction, as observed with RT-PCR were basically in agreement with the data obtained by microarray. As an exception, in U118MG TSPO knockdown cells, CyclinG2 (C) did not show a significant difference when analyzed with RT-PCR, but nonetheless did so after 24 hrs of PK 11195 exposure. The y-axis indicates the relative mRNA expression level normalized to the average of the controls, where the controls are set to 1. Results are presented as mean \pm SD, $n = 9$, *** $p < 0.001$, ** $p < 0.01$ versus the respective controls. The observed effects of all treatments were in comparison to their respective controls i.e. Scr for TSPO knockdown and vehicle exposure for the PK 11195 exposures.

expression of MCM2 in TSPO knockdown cells was upregulated to $164\% \pm 53$ (Control $100\% \pm 36$) and after 24 hrs of PK 11195 exposure to $274\% \pm 8$ (Control $100\% \pm 13$). The gene expression for MCM4 was upregulated to $157\% \pm 41$ in TSPO knockdown cells and after 24 hrs of PK 11195 exposure to $256\% \pm 65$ (Control $100\% \pm 2$) (Figure 4B). Gene expression of CyclinG2 (CyclinG2) was downregulated to $86\% \pm 7$ after TSPO knockdown (Control $100\% \pm 31$), and to $57\% \pm 13$ after 24 hrs of PK 11195 exposure (Control $100\% \pm 6$) (Figure 4C).

Figure 5 presents genes that show changes due to TSPO knockdown as well as 48 hours of PK 11195 exposure, but have no significant changes after 24 hours of PK 11195 exposure. Figure 5 shows the RT-PCR results for early growth response 1 (EGR1) and KIAA (KIAA 1524) in TSPO knockdown cells and after exposure of PK 11195 for 48 hrs, which a priori showed more than two fold changes in gene expression as measured with microarray (Table 1).

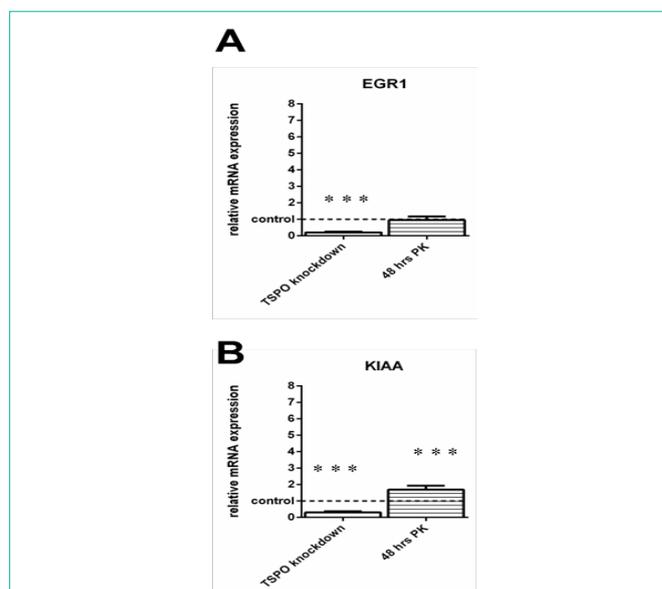


Figure 5: RT-PCR analysis of genes related to the cell cycle of U118MG TSPO knockdown cells and U118MG cells exposed to TSPO ligand PK 11195 (25 μ M) for 48 hrs. As a priori determined by microarray, the selected genes: 1) showed more than 2-fold differences in gene expression between TSPO knockdown cells and their Scr control cells; and also 2) showed more than 2-fold differences in gene expression between cells exposed for 48 hrs to 25 μ M of PK 11195 and their vehicle control cells, and 3) did not show such effects after 24 hrs of exposure to PK 11195.

The respective controls were set to 1 (indicated by the dotted line in the figure with the SD values detailed in the Results' section) and the relative expression of the cell cycle genes of EGR1 (A) and KIAA (B) were given. For TSPO knockdown cells, changes in gene expression, in particular the direction, as observed with RT-PCR for EGR1 (A) and KIAA (B), were basically in agreement with the data obtained by microarray. However, after PK 11195 exposure for 48 hrs U118MG cells did not show a significant difference for EGR1 expression when analyzed with RT-PCR, while KIAA expression appeared increased with RT-PCR (instead of decreased as observed with microarray). The y-axes indicate the relative mRNA expression level normalized to the average of the controls, where the controls are set to 1. Results are presented as mean \pm SD, $n = 9$, *** $p < 0.001$ versus the respective controls. The observed effects of all treatments were in comparison to their respective controls i.e. Scr for TSPO knockdown and vehicle exposure for the PK 11195 exposures.

The gene expression of EGR1 was significantly downregulated in TSPO knockdown cells to $20\% \pm 5$ (Control $100\% \pm 18$), but not so after 48 hrs of PK 11195 exposure (to $96\% \pm 22$) (Control $100\% \pm 33$) (Figure 5A). Furthermore, KIAA was downregulated to $30\% \pm 7$ after TSPO knockdown (Control $100\% \pm 21$) and upregulated to 169% after PK 11195 exposure for 48 hrs (Control $100\% \pm 25$) (Figure 5B).

RT-PCR for gene expression of RANBP3 and v-FOS

Interestingly, as mentioned above, v-FOS and RANBP3 showed the biggest changes in gene expression with TSPO knockdown in the microarray analysis (Table 1). RT-PCR showed that TSPO knockdown led to a decrease in v-FOS expression to $59\% \pm 21$ (Control $100\% \pm 15$) and after 24 hrs and 48 hrs of exposure to PK 11195 to downregulations to $80\% \pm 7$ and $66\% \pm 21$, respectively (Control $100\% \pm 27$) (Figure 6A). Furthermore, TSPO knockdown led also to a decrease in RANBP3 expression to $10\% \pm 2$ (Control $100\% \pm 3$) and decreases in expression to $24\% \pm 24$ and $62\% \pm 22$ (Control $100\% \pm 21$) after PK 11195 exposure for 24 hrs and 48 hrs, respectively, as seen with RT-PCR (Figure 6B).

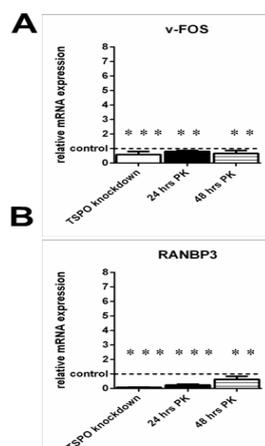


Figure 6: RT-PCR analysis of v-FOS (A) and RANBP3 (B) genes in U118MG TSPO knockdown cells and in U118MG cells exposed to TSPO ligand PK 11195 (25 μ M) for 24 hrs and 48 hrs. As a priori determined by microarray, these selected genes: 1) showed a more than 10-fold difference in gene expression of RANBP3 and a more than 5-fold difference in gene expression of v-FOS between TSPO knockdown cells and their Scr control cells; 2) showed more than 2-fold changes (RANBP3) and 3-fold changes (v-FOS) in gene expression in cells exposed for 24 hrs and 48 hrs to 25 μ M of PK 11195 compared to their vehicle control cells. In fact, after TSPO knockdown, v-FOS and RANBP3 gene expression was affected more than of other genes, as determined with microarray (Table1, Appendices 1, 2, 3).

The respective controls were set to 1 (indicated by the dotted line in the figure with the SD values detailed in the Results' section) and the relative RT-PCR-derived observations for gene expression of v-FOS (A) and RANBP3 (B) are given. For all treatments (TSPO knockdown and PK 11195 exposure), changes in gene expression, in particular the direction, as observed with RT-PCR were basically in agreement with the data obtained by microarray. The y-axis indicates the relative mRNA expression level normalized to the average of the controls, where the controls are set to 1. Results are presented as mean \pm SD, n = 9, *** p < 0.001 and ** p < 0.01 versus the respective controls. The observed effects of all treatments were in comparison to their respective controls i.e. Scr for TSPO knockdown and vehicle exposure for the PK 11195 exposures.

Cell cycle analysis with flow cytometry

Cell cycle analysis / distribution of U118MG TSPO knockdown cells and PK 11195 (24 and 48 hrs) exposed U118MG cells compared to control cells was performed by FACS analysis. TSPO knockdown led to a significant reduction in the proportion of the number of cells in the G0/G1 phase [from 72.66% \pm 0.80 to 49.99% \pm 0.64, ***p < 0.001], an increase in the proportion of the number of cells in the S phase [from 7.08% \pm 0.33 to 22.75% \pm 1.44, **p < 0.01], and an increase in the proportion of the number of cells in the G2/M phase [from 13.53% \pm 0.92 to 17.72% \pm 1.05, *p < 0.05] (Figure 7A).

PK 11195 exposure for 24 hrs led to an increase in the proportion of the number of cells in the G0/G1 phase [from 69.14% \pm 0.57 to 77.60% \pm 0.68 (***p < 0.001)] and decreases in the proportions of the number of cells in the S phase [from 11.02% \pm 0.53 to 8.91% \pm 0.21, **p < 0.01] and the G2/M phase [from 7.62% \pm 0.47 to 7.16% \pm 0.33, *p < 0.05] (Figure 7B).

U118MG exposure to PK 11195 for 48 hrs led to small decreases in the proportions of the number of cells in the G0/G1 phase [from 82.19% \pm 0.86 to 79.00% \pm 1.08, ***p < 0.001] and in the S phase [from 4.28% \pm 0.2 to 3.0% \pm 0.24, **p < 0.01], and an increase in the proportion of the number of cells in the G2/M phase [from 5.86% \pm 0.24 to 7.00% \pm 0.50, ***p < 0.001] (Figure 7C).

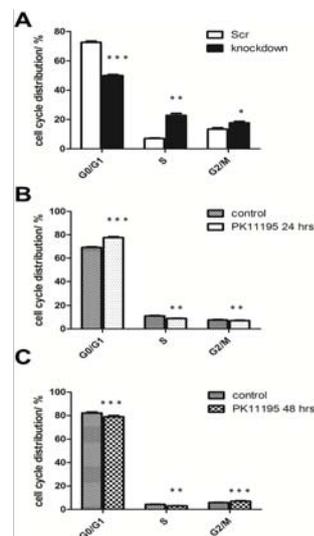


Figure 7: Quantification of cell cycle phases with the aid of PI. The relative proportion of cells in each of the G0/G1, S, and G2/M phases from U118MG Scr control cells and U118MG TSPO knockdown cells are shown in (A), WT U118MG vehicle (24 hrs exposure) control cells and cells exposed for 24 hrs to 25 μ M of PK 11195 are shown in (B) and WT U118MG vehicle (48 hrs exposure) control cells and cells exposed for 48 hrs to 25 μ M of PK 11195 in (C). Results are presented as mean \pm SD, n = 9, *** p < 0.001, ** p < 0.01, and * p < 0.05 versus the respective controls.

Discussion

Several previous studies have suggested that the TSPO is involved in glioblastoma tumorigenicity [26,32,48]. For example knockdown studies of TSPO, as well as application of TSPO ligands, have indicated pro cell death functions of the TSPO that include promotion of collapse of the mitochondrial membrane potential ($\Delta\Psi_m$), Reactive Oxygen Species (ROS) generation at mitochondrial levels, cardiolipin oxidation, leading to programmed cell death [9,12,16-20,30,32,49]. Furthermore, in cell culture, TSPO knockdown enhances anchorage independent cell proliferation (i.e. suspended in agar), reduces cell adhesion, and enhances cell migration of glioma cells [2,8,9]. Finally, *in vivo* studies in whole animal models have shown that TSPO knockdown in cancer cells causes enhanced glioblastoma tumor proliferation in the brain [2] and reduces survival rate of the host animals [2,8,9]. Nonetheless, a complete understanding of TSPO involvement in tumorigenicity is not simple. Part of this enigma is that the TSPO is involved in various and numerous functions [14,22,26]. We consider the possibility that TSPO's involvement in regulation of gene expression may at least partly explain TSPO's association with these functions.

We are driven by the realization that the TSPO presents a central component for processes related to the host defense response [17,26]. Thus, to better understand how TSPO can be involved in various and numerous functions, in particular related to tumorigenesis of glioblastoma, we studied TSPO involvement in gene expression. In particular, we selected 24 hours and 48 hours of exposure to the specific TSPO ligand PK 11195, at a concentration of 25 μ M. This concentration of PK 11195 is known to inhibit TSPO functions, including programmed cell death in U118MG cells induced by various agents [2,12,30,31]. In addition, we applied stable knockdown of TSPO, which presents a permanent inhibition of

Table 2: Correlations between changes in gene expression and effect on the cell cycle following TSPO knockdown by siRNA and application of PK 11195. The genes affected by our exposures to PK 11195 and by TSPO knockdown are presented in the first column. In the second, third, and fourth columns previously reported effect by the individual gene products in question on cell cycle phases is presented. In the fifth column representative references reporting the effects of the gene products in question on the cell cycle are given. The following paired columns (6 and 7; 8 and 9; 10 and 11) show the effects on gene expression and cell cycle phase by respectively, PK 11195 for 24 hours, PK 11195 for 48 hours, and TSPO knockdown by siRNA. Symbols: Black arrows pointing up mean (1) increase in numbers of cells in a particular cell phase (2) increase in gene expression. White arrows pointing up mean (1) decrease in numbers of cell in a particular cell phase (2) decrease in gene expression. Shaded cells (in pairs) show when the effects on the cell cycle phases are in accord with the effects on gene expression following exposure to PK 11195 for 24 hours, to PK 11195 for 48 hours, and due to TSPO knockdown by siRNA as can be predicted from previous reports on the effects on the cell cycle by the gene products in question.

Genes in question	Previous reported effects on cell cycle of the gene products in question				Effects observed in our study regarding gene expression and effects on the cell cycle						
					24 hr PK 11195		48 hr PK 11195		TSPO knockdown		
					Gene expression	Phase	Gene expression	Phase	Gene expression	Phase	
	G0/G1	S	G2/M	Ref.							
CDC6		↑		(63)	↑	S	↑	S	↑	↑	S
CyclinE2		↑		(69)	↑	S	↑	S	↑	↑	S
BRCA1		↓		(67,75)	↑	S	↑	S	↑	↑	S
CDC2			↑	(64)	↑	G2/M	↑	G2/M	↑	↑	G2/M
Claspin			↓	(65,77)	↑	G2/M	↑	G2/M	↑	↑	G2/M

TSPO function, including enhanced tumorigenicity and attenuation of programmed cell death in U118MG and other cell lines, an effect that can also be induced by TSPO ligands [2,8-12,18,19,22,30,31]. We consider that differences in the outcomes regarding gene expression following 24 and 48 hours of exposure to 25 μM of PK 11195, and stable TSPO knockdown may be due to the duration of changes in TSPO function (i.e. 24 hrs, 48 hrs, and permanent). Indeed, we found that 24 hrs of PK 11195 (25 μM) exposure affected more genes than 48 hours of exposure to PK 11195 (25 μM) (610 genes versus 359 genes). TSPO knockdown by stable siRNA transfection affected 1249 genes (when applying a minimum of a 2.0 fold, cut off), which was much more than found with 24 and 48 hours of PK 11195 exposure, applying the same cut off. When considering the overlapping populations of genes affected by the differing TSPO manipulations, also the number of genes affected both by 48 hours of PK 11195 (25 μM) and TSPO knockdown was considerably smaller than the number of genes affected both by 24 hrs of PK 11195 (25 μM) and TSPO knockdown (85 genes vs. 128 genes).

As regulation of the cell cycle is a major component for tumorigenesis we paid extra attention to the group of genes related to this cell function. Previous studies applying TSPO knockdown and various TSPO ligands already suggested TSPO involvement in the cell cycle [9,34-36,50,51]. The present study applied the approach

of determining by microarray and RT-PCR which genes for proteins related to the cell cycle are under the control of the TSPO. With the present study we found that TSPO knockdown by siRNA, as well as exposure to 25 μM of the TSPO ligand PK 11195 for 24 hours and 48 hours, significantly modulates gene expression for several proteins associated with the cell cycle (Table 1). These individual proteins each regulate one or more of the several phases of the cell cycle. Details how these proteins may regulate various aspects of the cell cycle have been studied intensively by several groups [52-57]. As these genes were affected by TSPO knockdown as well as by exposure to PK 11195 we assume that such genes actually may be under the control of the TSPO. Genes that were affected only by TSPO knockdown or only by exposure to PK 11195 were not further scrutinized in the present study. They are however in the appendices 1, 2, and 3 added to this work and a previous work [22].

In table 2 we summarize gene expression related to the cell cycle that is affected by 24 and 48 hours of exposure to PK 11195, and by TSPO knockdown, as indicated by microarray as well as by RT-PCR. The following 5 genes, CDC6, CyclinE2, BRCA1, CDC2, and Claspin indicated in Table 2, fulfill these criteria i.e. they displayed changes after all three TSPO manipulations. We were also interested whether these changes in gene expression actually match changes in the cell cycle. In Table 2 we present correlations between changes in gene expression and effects on the cell cycle following application of PK 11195 and of TSPO knockdown by siRNA. It is known that the proteins coded by CDC6 and CyclinE2 affect the S phase positively [53,58-61,65]. The protein coded by BRCA1 affects the S phase negatively [56,64]. CDC2 and Claspin code for proteins that affect the G2/M phase positively and negatively, respectively [54,55,66,67]. In this way, in TSPO knockdown cells, the upregulation of CDC6, CyclinE2, and CDC2 match well the upregulation of the S and G2/M phase (and consequently the downregulation of the G0/G1 phase in these cells) (Table 2). In cells exposed to PK 11195 for 48 hours, upregulation of the genes BRCA1 and CDC2 match well the downregulation of the S phase and the upregulation of the G2/M phase, respectively (Table 2). In cells exposed to PK 11195 for 24 hours, the upregulation of BRCA1 and Claspin match well the downregulation seen for both the S phase and the G2/M phase, respectively (and consequently the upregulation of the G0/G1 phase in these cells) (Table 2). Thus, while 24 and 48 hours of exposure to PK 11195 as well as TSPO knockdown all upregulate the expression of these 5 genes of CDC6, CyclinE2, BRCA1, CDC2, and Claspin (Figs. 3-5, and Table 1,2) the net functional consequences under these three conditions are not similar (Figure 7, and Table 2). In particular the functional effects of 24 hours of PK 11195 exposure and TSPO knockdown appear to be the exact opposite, while 48 hours of PK 11195 shows partial similarities with both these treatments. We considered the possibility that quantitative differences in changes in gene expression might explain the different functional outcomes, however, no clear cut association in magnitude of gene expression in correlation with different changes in the cell cycle could be discerned (Figures 3-5, and Table 2). As a consequence, we considered the possibility that pretranscriptional and posttranslational effects may play a role.

With this approach, we found that TSPO ligands and TSPO knockdown also affect gene expression of numerous non-coding

Table 3: Gene expression of non protein coding genes (for Small nucleolar RNA).

Small nucleolar RNA gene expression changes in U11MG glioblastoma cells						
24 hrs of PK11195	48 hrs of PK11195	Permanent TSPO knockdown by stable siRNA transfection				
SNORD30	SNORD30	SNORD49B	SNORA74A	SNHG12	SNORD28	RNU105B
SNORA20	SNORA20	SNORD30	SNORA16A	SNORD15A	SNORA3	SNORD116-14
SNORA43	LOC85391	SNORD25	SNORD26	SNORD1B	SNORD29	SNORD13
SNORD22	SNORD49A	SNORD49A	SNORD22	NOL9	NOC3L	SNORD116-15
SNORD28	NUSAP1	SNORD27	SNORD42A	NOLC1	SNORA1	SNORD116-8
NOC3L	SNORD49B	SNORD77	SNORD14C	SNORD42B	SNORD1C	SNORD116-1
SNORA73A	SNORD28	SNORD12C	SNORD43	SNORD83A	SNORD102	SNORA22
	SNORD4A	SNORD80	SNORD75	SNORA75	SNORD6	SNORD116-3
	LOC85389	SNORD78	SNORD47	SNORA4	SNORD45B	SNORD116-3
	SNORA51	SNORD4A	SNORD74	SNORD14E	SNORD45C	SNORD116-5
	SNORA73A	SNORA61	SNORD79	SNORD4B	SNORA62	SNORD116-5
		SNORD44	SNORD82	SNORA27	SNORD76	SNORD116-24
		SNORD31	SNORD20	NOL11	SNORD116-20	
		SNORA74A	SNORA44	NOP2	SNORD109A	

Summary:

-7-Of the 610 genes affected by 24 hours of PK 11195 exposure **7** were for small nucleolar RNAs

-11-Of the 359 genes affected by 48 hours of PK 11195 exposure **11** were for small nucleolar RNAs

-68-Of the 1249 genes affected by stable TSPO knockdown **68** were for small nucleolar RNAs

genes, including small nucleolar RNAs (snRNAs) (Table 3) (see also appendices 1,2, and 3). snoRNAs, function as a ribonucleoprotein particle (RNP) in the nucleolus. Certain snoRNAs have been reported to act as either oncogenes or tumor suppressors *in vitro* [68]. The central functions of snoRNAs have long been believed to modify, mature, and stabilize rRNAs. These post-transcriptional modifications are important for the production of efficient and accurate ribosomes [69]. However, snoRNAs can also partake in modifications of snRNAs that mediate mRNA splicing [70]. Furthermore, snoRNA transcripts serve as the precursors of miRNA-like small RNAs and the regulators of alternative splicing [71,72]. Interestingly, progressively larger numbers of such noncoding genes are affected by 24 hours of PK 11195 exposure, 48 hours of PK 11195 exposure, and TSPO knockdown by siRNA, respectively (Table 3) (see also appendices 1, 2, 3).

The importance of TSPO for regulation of gene expression was already shown by previous studies. Microarray analysis applied in a previous study suggested regulation of cell cycle related genes by TSPO ligands [35]. For example, the TSPO ligand-mediated signaling in colorectal cancer cells involved the upregulation of the cyclin-dependent kinase inhibitors p21WAF1/CIP1 and p27Kip1, cdc16, and the cell cycle inhibitors gadd45 and gadd153, the downregulation of the cyclins D1 and B1, as well as the inactivation of ERK1/2 [35]. Other studies have shown that exogenous TSPO ligands like FGIN-1-27, PK 11195, and Ro5-4864 dose dependently (10 μ M – 100 μ M) inhibited cell proliferation of human breast cancer and colorectal cancer cells measured after 72 hrs, probably via an arrest of cells in the G0/G1 phase as observed after 24 hrs, which is similar to the cell cycle effects we observed after 24 hrs [33,34]. Thus, our results are in accord with previous studies, regarding effects of particular durations of application of TSPO ligands as well as of stable TSPO knockdown.

Very interesting regarding the capability of the TSPO to regulate

gene expression is the strong reduction of the expression of v-FOS, also known as c-FOS [73], that we found after TSPO knockdown and exposure to 25 μ M of PK 11195 for 24 and 48 hours. Also FOSB is affected (FBJ murine osteosarcome viral oncogene homolog B; see appendices). The FOS gene family consists of 4 members: FOS, FOSB, FOSL1, and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of gene expression for cell proliferation, differentiation, and transformation. In some cases, expression of the FOS gene has also been associated with apoptotic cell death [67]. Interestingly, the relative magnitudes of changes in v-FOS gene expression (TSPO knockdown > 48 hours of PK 11195 exposure > 24 hours of PK 11195 exposure) is in accord with our prior expectations regarding relative changes in gene expression. Here it can be also noted that from 24 hours to 48 hours of PK 11195 exposure the proportion of the group of genes related to repair and binding of DNA increases till it becomes the largest group of the total population of genes that are affected both by 48 hours of exposure to PK 11195 (25 μ M) and TSPO knockdown (Figure 2). This gives further indication that regulation of DNA functions such as gene expression is one of the major TSPO functions.

In addition to v-FOS, Ran-binding protein 3 (RANBP3) gene expression was most strongly reduced by TSPO knockdown as well as by exposure to 25 μ M of PK 11195 for 24 and 48 hours. RANBP3 is a ~55-kDa protein that functions as a cofactor for Chromosome Region Maintenance (Crm1) - mediated nuclear export [46,59]. RANBP3 mediates nuclear export of Smad2 and Smad3 in a Ran-dependent manner. Depletion of RANBP3 expression enhances TGF beta-induced antiproliferative and transcriptional responses [60]. Thus, it may be that TSPO is able to regulate transport of molecules in and out of the nucleus, which may contribute to TSPO's capability to modulate gene expression. Previously, it was suggested the transport

of cholesterol by TSPO into the nucleus serves to modulate gene expression [74].

Summarizing, our data indicate firstly that TSPO is involved in regulation of expression of numerous genes related to various functions. One such function appears to be the cell cycle. Some genes and groups of genes show increasing changes in proportion to the duration of TSPO manipulation (i.e. 24 and 48 hrs of PK 11195 exposure, and permanent disabling by stable knockdown by siRNA transfection), in particular: c-FOS, RANBP3, gene expression related to repair and binding to DNA, and gene expression for small nucleolar RNAs. As the effects of TSPO on gene expression are myriad, it can be expected that more studies in this area will lead to important insights regarding tumorigenesis, and also regarding potential anti-cancer mechanisms.

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References

- Bognár L, Klekner A, Szollosi J. Expression pattern of invasion-related molecules in cerebral tumors of different origin Magyar Onkologia. 2009; 53: 253-258.
- Bode J, Veenman L, Caballero B, Lakomek M, Kugler W, Gavish M. The 18 kDa translocator protein influences angiogenesis, as well as aggressiveness, adhesion, migration, and proliferation of glioblastoma cells. Pharmacogenetics and genomics. 2012; 22: 538-550.
- Kaur B, Tan C, Brat DJ, Post DE, Van Meir EG. Genetic and hypoxic regulation of angiogenesis in gliomas. J Neurooncol. 2004; 70: 229-243.
- Kleihues P, Sobin LH. World Health Organization classification of tumors. Cancer. 2000; 88: 2887.
- Kleihues P, Ohgaki H. Phenotype vs genotype in the evolution of astrocytic brain tumors. Toxicol Pathol. 2000; 28: 164-170.
- DeAngelis LM. Brain tumors. N Engl J Med. 2001; 344: 114-123.
- Sabag AD, Bode J, Fink D, Kigel B, Kugler W, Neufeld G. Semaphorin-3D and semaphorin-3E inhibit the development of tumors from glioblastoma cells implanted in the cortex of the brain. PLoS One. 2012; 7: e42912.
- Weisinger G, Kelly-Herschkovitz E, Veenman L, Spanier I, Leschiner S, Gavish M, et al. Peripheral benzodiazepine receptor antisense knockout increases tumorigenicity of MA-10 Leydig cells in vivo and in vitro. Biochemistry. 2004; 43: 12315-12321.
- Levin E, Premkumar A, Veenman L, Kugler W, Leschiner S, Spanier I, et al. The peripheral-type benzodiazepine receptor and tumorigenicity: isoquinoline binding protein (IBP) antisense knockdown in the C6 glioma cell line. Biochemistry. 2005; 44: 9924-9935.
- Shoukrun R, Veenman L, Shandalov Y, Leschiner S, Spanier I, Karry R, et al. The 18-kDa translocator protein, formerly known as the peripheral-type benzodiazepine receptor, confers proapoptotic and antineoplastic effects in a human colorectal cancer cell line. Pharmacogenet Genomics. 2008; 18: 977-988.
- Zeno S, Veenman L, Katz Y, Bode J, Gavish M, Zaaroor M. The 18 kDa mitochondrial translocator protein (TSPO) prevents accumulation of protoporphyrin IX. Involvement of reactive oxygen species (ROS). Curr Mol Med. 2012; 12: 494-501.
- Zeno S, Zaaroor M, Leschiner S, Veenman L, Gavish M. CoCl₂ induces apoptosis via the 18 kDa translocator protein in U118MG human glioblastoma cells. Biochemistry. 2009; 48: 4652-4661.
- Chelli B, Salvetti A, Da Pozzo E, Rechichi M, Spinetti F, Rossi L, et al. PK 11195 differentially affects cell survival in human wild-type and 18 kDa translocator protein-silenced ADF astrocytoma cells. J Cell Biochem. 2008; 105: 712-723.
- Gavish M, Bachman I, Shoukrun R, Katz Y, Veenman L, Weisinger G, et al. Enigma of the peripheral benzodiazepine receptor. Pharmacol Rev. 1999; 51: 629-650.
- Gavish M, Bar-Ami S, Weizman R. The endocrine system and mitochondrial benzodiazepine receptors. Mol Cell Endocrinol. 1992; 88: 1-13.
- McEnery MW. The mitochondrial benzodiazepine receptor: evidence for association with the voltage-dependent anion channel (VDAC). J Bioenerg Biomembr. 1992; 24: 63-69.
- Veenman L, Papadopoulos V, Gavish M. Channel-like functions of the 18-kDa translocator protein (TSPO): regulation of apoptosis and steroidogenesis as part of the host-defense response. Curr Pharm Des. 2007; 13: 2385-2405.
- Veenman L, Shandalov Y, Gavish M. VDAC activation by the 18 kDa translocator protein (TSPO), implications for apoptosis. J Bioenerg Biomembr. 2008; 40: 199-205.
- Veenman L, Alten J, Linnemannstons K, Shandalov Y, Zeno S, Lakomek M, et al. Potential involvement of F0F1-ATP(synth)ase and reactive oxygen species in apoptosis induction by the antineoplastic agent erucylphosphohomocholine in glioblastoma cell lines : a mechanism for induction of apoptosis via the 18 kDa mitochondrial translocator protein. Apoptosis. 2010; 15: 753-768.
- Caballero B, Veenman L, Gavish M. Role of mitochondrial translocator protein (18 kDa) on mitochondrial-related cell death processes. Recent Pat Endocr Metab Immune Drug Discov. 2013; 7: 86-101.
- Mukherjee S, Das SK. Translocator protein (TSPO) in breast cancer. Curr Mol Med. 2012; 12: 443-457.
- Veenman L, Bode J, Gaitner M, Caballero B, Pe'er Y, Zeno S, et al. Effects of 18-kDa translocator protein knockdown on gene expression of glutamate receptors, transporters, and metabolism, and on cell viability affected by glutamate. Pharmacogenet Genomics. 2012; 22: 606-619.
- Benavides J, Quarteronet D, Imbault F, Malgouris C, Uzan A, Renault C, et al. Labelling of "peripheral-type" benzodiazepine binding sites in the rat brain by using [³H] PK 11195, an isoquinoline carboxamide derivative: kinetic studies and autoradiographic localization. J Neurochem. 1983; 4: 1744-1750.
- Benavides J, Malgouris C, Imbault F, Begassat F, Uzan A, Renault C, et al. "Peripheral type" benzodiazepine binding sites in rat adrenals: binding studies with [³H]PK 11195 and autoradiographic localization. Arch Int Pharmacodyn Ther. 1983; 266: 38-49.
- James ML, Selleri S, Kassiou M. Development of ligands for the peripheral benzodiazepine receptor. Curr Med Chem. 2006; 13: 1991-2001.
- Veenman L, Gavish M. The peripheral-type benzodiazepine receptor and the cardiovascular system. Implications for drug development. Pharmacol Ther. 2006; 110: 503-524.
- Papadopoulos V, Baraldi M, Guilarte TR, Knudsen TB, Lacapère JJ, Lindemann P, et al. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci. 2006; 27: 402-409.
- Veenman L, Gavish M. Peripheral-type benzodiazepine receptors: Their implication in brain disease. Drug Development Research volume. 2000; 50: 355-370.
- Ricchelli F, Sileikyte J, Bernardi P. Shedding light on the mitochondrial permeability transition. Biochim Biophys Acta. 2011; 1807: 482-490.
- Kugler W, Veenman L, Shandalov Y, Leschiner S, Spanier I, Lakomek M, et al. Ligands of the mitochondrial 18 kDa translocator protein attenuate apoptosis of human glioblastoma cells exposed to erucylphosphohomocholine. Cell Oncol. 2008; 30: 435-450.

31. Shargorodsky L, Veenman L, Caballero B, Pe'er Y, Leschiner S, Bode J, Gavish M. The nitric oxide donor sodium nitroprusside requires the 18 kDa Translocator Protein to induce cell death. *Apoptosis: an international journal on programmed cell death*. 2012; 17: 647-665.
32. Veenman L, Levin E, Weisinger G, Leschiner S, Spanier I, Snyder SH, et al. Peripheral-type benzodiazepine receptor density and in vitro tumorigenicity of glioma cell lines. *Biochem Pharmacol*. 2004; 68: 689-698.
33. Carmel I, Fares FA, Leschiner S, Scherübl H, Weisinger G, Gavish M. Peripheral-type benzodiazepine receptors in the regulation of proliferation of MCF-7 human breast carcinoma cell line. *Biochem Pharmacol*. 1999; 58: 273-278.
34. K Maaser, M Höpfner, A Jansen, G Weisinger, M Gavish, A P Kozikowski, et al. Specific ligands of the peripheral benzodiazepine receptor induce apoptosis and cell cycle arrest in human colorectal cancer cells. *Br J Cancer*. 2001; 85: 1771-1780.
35. Maaser K, Sutter AP, Krahn A, Höpfner M, Grabowski P, Scherübl H. Cell cycle-related signaling pathways modulated by peripheral benzodiazepine receptor ligands in colorectal cancer cells. *Biochem Biophys Res Commun*. 2004; 324: 878-886.
36. Maaser K, Sutter AP, Scherubl H. Mechanisms of mitochondrial apoptosis induced by peripheral benzodiazepine receptor ligands in human colorectal cancer cells. *Biochem Biophys Res Commun*. 2005; 332: 646-652.
37. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol*. 2006; 7: 3.
38. Kletsas D, Li W, Han Z, Papadopoulos V. Peripheral-type benzodiazepine receptor (PBR) and PBR drug ligands in fibroblast and fibrosarcoma cell proliferation: role of ERK, c-Jun and ligand-activated PBR-independent pathways. *Biochem Pharmacol*. 2004; 67: 1927-1932.
39. Jung K, Grade M, Gaedcke J, Jo P, Opitz L, Becker H, et al. A new sensitivity-preferred strategy to build prediction rules for therapy response of cancer patients using gene expression data. *Comput Methods Programs Biomed*. 2010; 100: 132-139.
40. Opitz L, Salinas-Riester G, Grade M, Jung K, Jo P, Emons G, et al. Impact of RNA degradation on gene expression profiling. *BMC Med Genomics*. 2010; 3: 36.
41. Novota P, Zinöcker S, Norden J, Wang XN, Sviland L, Opitz L, et al. Expression profiling of major histocompatibility and natural killer complex genes reveals candidates for controlling risk of graft versus host disease. *PLoS One*. 2011; 6: e16582
42. Draï D, Benjamini Y, Golani I. Statistical discrimination of natural modes of motion in rat exploratory behavior. *J Neurosci Methods*. 2000; 96: 119-131.
43. Klipper-Aurbach Y, Wasserman M, Braunsiegel-Weintrob N, Borstein D, Peleg S, Assa S, et al. Mathematical formulae for the prediction of the residual beta cell function during the first two years of disease in children and adolescents with insulin-dependent diabetes mellitus. *Med Hypotheses*. 1995; 45: 486-490.
44. Venn J. On the diagrammatic and mechanical representation of propositions and reasonings. *Dublin Philosophical Magazine and Journal of Science*. 1880; 9: 1-18
45. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; 29: e45.
46. Lindsay ME, Holaska JM, Welch K, Paschal BM, Macara IG. Ran-binding protein 3 is a cofactor for Crm1-mediated nuclear protein export. *J Cell Biol*. 2001; 153: 1391-1402.
47. Vlodayvsky E, Soustiel JF. Immunohistochemical expression of peripheral benzodiazepine receptors in human astrocytomas and its correlation with grade of malignancy, proliferation, apoptosis and survival. *J Neurooncol*. 2007; 8: 1-7.
48. Midzak A, Akula N, Lecanu L, Papadopoulos V. Novel androstenediol interacts with the mitochondrial translocator protein and controls steroidogenesis. *J Biol Chem*. 2011; 286: 9875-9887.
49. Garnier M, Boujrad N, Oke BO, Brown AS, Riond J, Ferrara P, et al. Diazepam binding inhibitor is a paracrine/autocrine regulator of Leydig cell proliferation and steroidogenesis: action via peripheral-type benzodiazepine receptor and independent mechanisms. *Endocrinology*. 1993; 132: 444-458.
50. Ikezaki K, Black KL. Stimulation of cell growth and DNA synthesis by peripheral benzodiazepine. *Cancer Lett*. 1990; 49: 115-120.
51. Lee DH, Kang SK, Lee RH, Ryu JM, Park HY, Choi HS, et al. Effects of peripheral benzodiazepine receptor ligands on proliferation and differentiation of human mesenchymal stem cells. *J Cell Physiol*. 2004; 198: 91-99.
52. Borlado LR, Méndez J. CDC6: from DNA replication to cell cycle checkpoints and oncogenesis. *Carcinogenesis*. 2008; 29: 237-243.
53. Lee MG, Nurse P. Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature*. 1987; 327: 31-35.
54. Chini CC, Chen J. Claspin, a regulator of Chk1 in DNA replication stress pathway. *DNA Repair (Amst)*. 2004; 3: 1033-1037.
55. Zimmermann M, Arachchige-Don AS, Donaldson MS, Dallapiazza RF, Cowan CE, Horne MC. Elevated cyclin G2 expression intersects with DNA damage checkpoint signaling and is required for a potent G2/M checkpoint arrest response to doxorubicin. *J Biol Chem*. 2012; 287: 22838-22853.
56. Deng CX. BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Res*. 2006; 34: 1416-1426.
57. Hosokawa M, Takehara A, Matsuda K, Eguchi H, Ohigashi H, Ishikawa O, et al. Oncogenic role of KIAA0101 interacting with proliferating cell nuclear antigen in pancreatic cancer. *Cancer Res*. 2007; 67: 2568-2576.
58. Hwang HC, Clurman BE. Cyclin E in normal and neoplastic cell cycles. *Oncogene*. 2005; 24: 2776-2786.
59. Nemergut ME, Lindsay ME, Brownawell AM, Macara IG. Ran-binding protein 3 links Crm1 to the Ran guanine nucleotide exchange factor. *J Biol Chem*. 2002; 277: 17385-17388.
60. Dai F, Lin X, Chang C, Feng XH. Nuclear export of Smad2 and Smad3 by RanBP3 facilitates termination of TGF-beta signaling. *Dev Cell*. 2009; 16: 345-357.
61. Parra E, Ortega A, Saenz L. Down-regulation of Egr-1 by siRNA inhibits growth of human prostate carcinoma cell line PC-3. *Oncol Rep*. 2009; 22: 1513-1518.
62. Yan H, Merchant AM, Tye BK. Cell cycle-regulated nuclear localization of MCM2 and MCM3, which are required for the initiation of DNA synthesis at chromosomal replication origins in yeast. *Genes Dev*. 1993; 7: 2149-2160.
63. Komamura-Kohno Y, Karasawa-Shimizu K, Saitoh T, Sato M, Hanaoka F, Tanaka S, et al. Site-specific phosphorylation of MCM4 during the cell cycle in mammalian cells. *FEBS J*. 2006; 273: 1224-1239.
64. Mullan PB, Quinn JE, Harkin DP. The role of BRCA1 in transcriptional regulation and cell cycle control. *Oncogene*. 2006; 25: 5854-5863.
65. van den Heuvel S, Harlow E. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science*. 1993; 262: 2050-2054.
66. Freire R, van Vugt MA, Mamely I, Medema RH. Claspin: timing the cell cycle arrest when the genome is damaged. *Cell Cycle*. 2006; 5: 2831-2834.
67. Hughes R, Parry J, Beynon J, Jenkins G. Molecular changes consistent with increased proliferation and invasion are common in rectal cancer. *Clin Transl Oncol*. 2011; 13: 753-759.
68. Nallar SC, Kalvakolanu DV. Regulation of snoRNAs in cancer: close encounters with interferon. *J Interferon Cytokine Res*. 2013; 33: 189-198.
69. Decatur WA, Fournier MJ. rRNA modifications and ribosome function. *Trends Biochem Sci*. 2002; 27: 344-351.
70. Nizami Z, Deryusheva S, Gall JG. The Cajal body and histone locus body. *Cold Spring Harb Perspect Biol*. 2010; 2: a000653.
71. Ender C, Krek A, Friedländer MR, Beitzinger M, Weinmann L, Chen W, et al. A human snoRNA with microRNA-like functions. *Mol Cell*. 2008; 32: 519-528.

72. Kishore S, Khanna A, Zhang Z, Hui J, Balwierz PJ, Stefan M, et al. The snoRNA MBII-52 (SNORD 115) is processed into smaller RNAs and regulates alternative splicing. *Hum Mol Genet.* 2010; 19: 1153-1164.
73. Setoyama C, Frunzio R, Liao G, Mudryj M, de Crombrughe B. Transcriptional activation encoded by the v-fos gene. *Proc Natl Acad Sci U S A.* 1986; 83: 3213-3217.
74. Hardwick M, Fertikh D, Culty M, Li H, Vidic B, Papadopoulos V, et al. Peripheral-type benzodiazepine receptor (PBR) in human breast cancer: correlation of breast cancer cell aggressive phenotype with PBR expression, nuclear localization, and PBR-mediated cell proliferation and nuclear transport of cholesterol. *Cancer Res.* 1999; 59: 831-842.