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1 **TSPO over-expression increases motility, transmigration**
2 **and proliferation properties of C6 rat glioma cells**

3

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20 **Key words:** Peripheral-type benzodiazepine receptor; Glioma; Tumor invasiveness;
21 Proliferation; Ligand-binding; over-expression

22

1 SUMMARY

2 Gliomas are one of the most malignant cancers. The molecular bases regulating the
3 onset of such tumors are still poorly understood. The translocator protein (TSPO),
4 formerly known as the peripheral-type benzodiazepine receptor, is a mitochondrial
5 permeability transition (MPT)-pore protein robustly expressed in gliomas and
6 involved in the regulation of apoptosis and cell proliferation. TSPO expression
7 levels have been correlated with tumor malignancy. Here we describe the production
8 of C6 rat glioma cells engineered to over-express the TSPO protein with the aim of
9 providing the first direct evidence of a correlation between TSPO expression level
10 and glioma cell aggressiveness. We observed that TSPO potentiates proliferation,
11 motility and transmigration capabilities as well as the ability to overcome contact-
12 induced cell growth inhibition of glioma cells. On the whole, these data demonstrate
13 that TSPO density influences metastatic potential of glioma cells. Since several data
14 suggest that TSPO ligands may act as chemotherapeutic agents, in this paper we also
15 demonstrate that TSPO ligand-induced cell death is dependent on TSPO density.
16 These findings suggest that the use of TSPO ligands as chemotherapeutic agents
17 could be effective on aggressive tumor cells with a high TSPO expression level.

18

1 INTRODUCTION

2 The translocator protein (18 KDa) (TSPO), formerly known as the peripheral-type
3 benzodiazepine receptor (PBR) [1], is an abundant mitochondrial protein expressed
4 in peripheral tissues, as well as in astrocytes and microglial cells within the central
5 nervous system (CNS) [2] and [3]. Increased TSPO expression has been observed in
6 cancer, including brain tumors [4], [5], [6], [7] and [8]. TSPO has been implicated in
7 many physio-pathological conditions including apoptosis, microglial activation
8 related to brain damage, steroidogenesis, and cell proliferation [9]. In mitochondria,
9 TSPO is associated with other proteins, including the voltage-dependent anion
10 channel (VDAC) and the adenine nucleotide carrier (ANT), making up the
11 mitochondrial permeability transition (MPT)-pore [10] whose modulation has been
12 suggested as a critical event in the regulation of processes underlying cellular
13 survival/death [2], [10], [11] and [12]. It has been demonstrated that specific TSPO
14 ligands show pro-apoptotic activity and may act, through the modulation of the
15 MTP-pore opening, as potential drugs useful in cancer therapy [5], [13], [14], [15],
16 [16] and [17]. Among tumors, gliomas represent an elective target for TSPO ligand
17 mediated chemotherapy; this protein being virtually absent in the neurons and over-
18 expressed in glioma with respect to normal glial cells [3], [18], [19], [20], [21] and
19 [22].

20 It is interesting to note that TSPO expression level positively correlates with cell
21 malignancy in several tumors including gliomas [18], [23], [24] [25], [26], [27] and
22 [28]. However, the role of TSPO up-regulation in brain tumor cells is still puzzling.

1 For these reasons over the last years we have been focussing our attention on the
2 study of TSPO and TSPO ligand effects in glioma aggressivness. Up to now no
3 studies have been performed by direct comparison of brain tumor cells engineered to
4 over-express TSPO with wild type cells of the same cell line. Here, we describe the
5 production of glioma cells engineered to over-express the TSPO and examine the
6 relationship between TSPO up-regulation and growth, motility, transmigration and
7 susceptibility to the TSPO ligand PK11195-induced apoptosis of glioma cells.

8

1 MATERIALS AND METHODS

2 CELL CULTURES

3 Rat C6 glioma cells (a kindly gift from Professor Damir Janigro, Cleveland Clinic
4 Foundation, Cleveland, OH) were cultured as described in [15].

5 RNA ISOLATION, REVERSE TRANSCRIPTION-PCR, AND CLONING OF 6 RAT *TSPO*

7 Total RNA was extracted from 5×10^6 cells using the Nucleospin RNA II kit
8 (Machery-Nagel) according to manufacturer's instructions. For RT-PCR, about 1 μ g
9 DNase-digested total RNA was reverse-transcribed using Superscript II reverse
10 transcriptase (Invitrogen) following the recommendations of the manufacturer. Full-
11 length cDNA of TSPO was amplified using TSPO specific forward primer
12 (5'CCCAAGCTTATGTCTCAATCCTGGGTACC3') containing a restriction site
13 for the endonuclease Hind III at the 5' end and a TSPO specific reverse primer
14 (5'GCGAGGATCCAAAGCAGGATGACCTCAACC3') containing a restriction
15 site for the endonuclease BamH I at the 5' end. The condition for PCR were as
16 follows: 2 min at 95°C, 30 cycles of 30 sec at 95°C, 1 min at 55°C, and 30 sec at
17 72°C. A final extension was carried on at 72°C for 7 min. The amplified fragment
18 was Hind III and BamH I digested and then cloned into the vector pFLAG-cmv-2
19 (Sigma) in frame with a short flag epitope in the aminoterminal position. After
20 transformation in competent *E. coli* cells, some clones were isolated and plasmid
21 DNAs were sequenced by automated fluorescent cycle sequencing (ABI). One of
22 these clones (TSPO-pFLAG-cmv-2) was used in the following applications.

23 TRANSFECTION

1 For transfection, 2 μ g of TSPO-pFLAG-cmv-2 or pFLAG-cmv-2 (control) were
2 mixed with 10 μ L Lipofectamine 2000 reagent (Invitrogen) in 0,5 mL of Optimem
3 (Gibco). 90% confluent C6 glioma cells were transfected with the mixture and
4 incubated for 6 hours according to manufacturer's instructions. Then the transfection
5 medium was replaced with complete medium and the cells were used for
6 experiments.

7 **WESTERN BLOT ANALYSIS**

8 Whole cell extract of TSPO-pFLAG-cmv-2 or pFLAG-cmv-2 transfected C6 cells
9 were used for Western blot analysis. 24, 48, 72 and 96 hours after transfection C6
10 cells were detached with 2mM EDTA, collected by centrifugation (1000g for 5 min)
11 and lysed by adding 80 μ L of lysis buffer (1% Triton X100, 10% glycerol, 20mM
12 Tris HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulphonyl
13 fluoride (PMSF), 0.5 μ M aprotinin, 0.5 μ M leupeptin) for 30 min on ice. Then
14 samples were frozen-thawed for three times and centrifuged to collect the
15 supernatants. Protein concentration was measured by using the Micro BCA protein
16 assay kit (Pierce). 25 μ g of proteins were separated on a SDS-PAGE gel and
17 electrotransferred onto a nitrocellulose membrane. After blocking with 5% non-fat
18 dry milk (Biorad) in TPBS (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 4
19 hours at room temperature, the FLAG-TSPO recombinant protein was detected by
20 adding mouse anti-FLAG antibody (Sigma, 1:1000) in 3% non-fat dry milk in TPBS
21 for 1 hours at room temperature. Filters were then washed in TPBS and incubated
22 for 1 hour at room temperature with 1:100000 dilution of POD-conjugated anti-
23 mouse IgG antibody (Biorad). After several washes in TPBS, signal cross-

1 hybridization was revealed by chemoluminescence using the Supersignal West Dura
2 substrate (Pierce). To test that an equal amount of total protein were loaded in each
3 line, after FLAG-TSPO revelation, filters were stripped and re-probed with mouse
4 anti-ERK antibody (Santa cruz, 1:1000) and revealed as described above.

5 **RADIOLIGAND BINDING ASSAYS**

6 Membranes from transfected C6 glioma cells with TSPO-pFLAG-cmv-2 or pFLAG-
7 cmv-2 (collected 24 hours after transfection) were prepared as previously described
8 by Gorman and Veenman [28] and [29] with minor modifications. Briefly, confluent
9 cells were washed with phosphate-buffered saline, harvested by scraping and
10 collected by centrifugation at 1000 x g for 10 min at 4°C. Cell pellet was suspended
11 in 5 mM Tris-HCl buffer, pH 7.4 containing 160 µg/ml benzamidine, 200 µg/ml
12 bacitracin and 20 µg/ml trypsin inhibitor, homogenized using Ultraturrax
13 homogenizer and centrifuged at 48000xg for 15 min at 4°C. Pellet was suspended in
14 50 mM Tris-HCl buffer, pH 7.4 (assay buffer), homogenized and centrifuged at
15 48000 x g for 15 min at 4°C. Protein content was estimated by the Bradford method
16 [30] using the Bio-Rad Protein Assay reagent, according to the manufacturer's
17 protocol, with bovine serum albumin (BSA) as standard.

18 [³H]PK 11195 (specific activity 69.9 Ci/mmol) binding assays were conducted
19 incubating cell membranes (40 µg of protein), with 0.5-26 nM [³H]PK 11195 in a
20 final volume of 500 µl of 50 mM Tris/HCl, pH 7.4 buffer. Non-specific binding was
21 determined in the presence of 1 µM unlabelled PK11195. Binding equilibrium was
22 reached following a 90 min incubation at 0°C. Samples were filtered rapidly under

1 vacuum through GF/C glass fiber filters, washed four times with 3 ml of assay
2 buffer. Finally, filters were placed in vials containing 4 ml of scintillation liquid and
3 bound radioactivity was measured by a scintillation counter (Tri-Carb 2800 TR,
4 Perkin Elmer).

5 Scatchard analyses of saturation binding data, graphic presentation and statistical
6 analyses were performed using the non-linear multipurpose curve-fitting Graph-Pad
7 Prism computer program (Graph Pad Software, version 4.0; San Diego, CA). In
8 detail, for saturation binding experiments one-site binding curve fitting was used.

9 **IMMUNOFLUORESCENCE MICROSCOPY**

10 Approximately 2×10^4 TSPO-pFLAG-cmv-2 or pFLAG-cmv-2 transfected C6 cells
11 were plated onto poly-D-lysine slides and incubated overnight. Cells were then fixed
12 in paraformaldehyde (4%) for 30 min at room temperature, washed in PBS and
13 blocked in blocking solution (0.1% Triton X-100, 0,5% bovine serum albumin in
14 PBS) for 30 minutes. Then, cells were incubated for 1 hour at room temperature in
15 blocking solution containing rabbit anti-FLAG antibody (Sigma, 1:100) and mouse
16 anti-cytochrome c antibody (Santa Cruz, 1:50). After washing in PBST (0.1% Triton
17 in PBS), cells were incubated for 1 hour at room temperature in blocking solution
18 containing FITC-conjugate anti-rabbit secondary antibody, (1:200, Invitrogen) and
19 rhodamine-conjugate anti-mouse secondary antibody (1:200, Santa Cruz). Slides
20 were then mounted and analyzed under the Axioplan fluorescent microscope (Zeiss).

21 **CELL PROLIFERATION ASSAYS**

22 To determine the proliferation rate, the same number (4000 cells for well) of TSPO-
23 pFLAG-cmv-2 or pFLAG-cmv-2 transfected C6 cells, were seeded in 24-well plates,

1 immediately after transfection, and allowed to proliferate for 24, 48, 96 h. Both
2 attached and floating cells were taken for counting with a hemocytometer. We
3 designed this experiment in way to maintain cells in the exponential phase of growth
4 (not confluent) up to about 120 hours. For each cell line the experiment was
5 performed on three independent transfections. The growth curves were confirmed
6 repeating the entire experiment for three times. The number of TSPO-pFLAG-cmv-2
7 or pFLAG-cmv-2 transfected C6 cells were also evaluated after they had been
8 confluent for three days. For this last analysis, 90% confluent C6 glioma cells were
9 transfected with TSPO-pFLAG-cmv-2 or pFLAG-cmv-2 vectors as described above.
10 Three days after transfection the medium covering cells was collected, the adherent
11 cells trypsinized, added to the collected medium and counted with a hemocytometer.
12 For each cell line the experiment was performed on ten independent transfections
13 To calculate the mitotic index of C6 cells overexpressing TSPO, 6-well plates
14 containing 90% confluent C6 glioma cells were transfected with TSPO-pFLAG-
15 cmv-2, pFLAG-cmv-2, or pEGFP-C1 (a vector that drives the expression of the
16 Green Fluorescent Protein in the cytoplasm). Three independent transfection were
17 performed for each vector. Six hours after transfection, cells of each well were
18 detached and split in three 6-well plates (to reach 30% confluence) and allowed to
19 proliferate for 24 and 72 hours.
20 Cells were then detached, collected by centrifugation and resuspended in 40 μ l of
21 glycerol, acetic acid, PBS (1:1:13) solution containing 1x Hoechst. Two 5 μ l
22 aliquots of cell suspension for each sample were spotted on a glass slide and allowed
23 to dry. The number of mitotic figures was counted under the fluorescence

1 microscope Axiovert 25 (Zeiss). Two 10 μ l aliquots for each sample were used to
2 count the number of total cells with a hemocytometer. For each cell line the mitotic
3 index [(mitotic figures/ total cells) x1000] was calculated in 9 replicate wells. Two
4 independent experiments were performed.

5 **MOTILITY ASSAY**

6 Equal amount of C6 cells were plated in 6-well plates (95% confluent), transfected
7 with TSPO-pFLAG-cmv-2, pFLAG-cmv-2 or pEGFP-C1 and a scratch (to remove
8 cells) was done along the diameter of all 6-well plates 6 hours after transfection.
9 Photographs were taken immediately after scratching (T0) in three different points
10 along the scratch. 20, 25, 40 hours after the scratch the same points were
11 photographed at the same magnification to determine the colonization of the scratch.
12 The scratch of each picture was measured by using the Adobe Photoshop ruler. The
13 migration rate (cm/h) was calculated for each treatment considering the mean
14 difference between the scratch width at T0 and at each time of analysis in the three
15 photographed points. For each cell line the experiment was performed on six
16 independent transfections.

17 **TRANSMIGRATION ASSAY**

18 Cell ability to transmigrate was assayed in modified Boyden chambers (Neuro
19 Probe). 10% FBS in C6 growth medium supplemented with 0.1% BSA was placed
20 in the lower chamber as source of chemoattractant. Polyvinylpyrrolidone-free 8 μ m-
21 pore polycarbonate filters were coated with 5 mg/L porcine skin gelatin (Sigma) and
22 placed between upper and lower chambers. TSPO-pFLAG-cmv-2 or pFLAG-cmv-2
23 transfected C6 cells were trypsinized, resuspended in growth medium supplemented

1 with 0.1% BSA and then placed in the upper chamber at a density of 2×10^5 cells for
2 chamber. After 5 hours of incubation at 37°C in a humidified atmosphere, filters
3 were fixed in absolute ethanol for 30s and stained with 2% toluidine blue (Sigma)
4 for 10min at room temperature. Cells incapable of migrating were gently scraped
5 from the upper side of the filter, while migrated cells were counted. Fifteen
6 microscope fields (10X) for each filter were counted.

7 **CELL VIABILITY ANALYSIS BY MTS CONVERSION ASSAY**

8 TSPO-pFLAG-cmv-2 or pFLAG-cmv-2 transfected C6 cells were seeded in 96-well
9 plates at a density of 1×10^4 cells for well. The following day or 96 hours after
10 transfection, culture medium was replaced with fresh medium supplemented with
11 different PK 11195 concentrations (1-100 μM). Each cell line has been also treated
12 with the vehicle ethanol. 24 hours after the treatment the number of living cells was
13 measured by quantitative colorimetric MTS assay (Promega) using a microplate
14 reader (Wallac Victor 2, 1420 Multilabel counter, Perkin-Elmer) as previously
15 described by [15].

16 Each treatment was performed in duplicate and three independent experiments were
17 performed.

18 Graphic presentation and data analysis were performed using the non-linear
19 multipurpose curve-fitting Graph-Pad Prism computer program (Graph Pad
20 Software, version 4.0; San Diego, CA).

21

1 RESULTS

2 TSPO OVER-EXPRESSION IN C6 RAT GLIOMA CELLS

3 TSPO cDNA was isolated by RT-PCR using C6 total RNA as template, and cloned
4 in pFLAG-cmv-2 vector in frame with the nucleotide sequence encoding the eight
5 aminoacid FLAG peptide (at the N-terminus of TSPO). The cDNA sequence
6 isolated from C6 cell was identical to that deposited in DDBJ/EMBL/GenBank
7 under accession number NM_012515. To assess the effectiveness of our expression
8 system, the expression of the FLAG-tagged TSPO was initially monitored by
9 immunoblotting on whole extract of TSPO-pFLAG-cmv-2 or pFLAG-cmv-2 cells
10 obtained 24, 48, 72 and 96 hours after transfection. Anti-FLAG antibody revealed
11 the presence of a cross-reactive band of the expected molecular weight (20 kDa) in
12 TSPO-pFLAG-cmv-2 transfected samples. Interestingly, no SDS and beta-
13 mercaptoethanol resistant polymers were found with anti FLAG antibodies. The
14 recombinant protein was expressed at high level in samples collected 24 hours after
15 transfection and its expression was significantly reduced in cell extracts collected 48
16 and 72 hours after transfection. Recombinant protein was no longer detectable in the
17 extracts collected 96 hours after transfection (Fig 1 A). No cross-reactive bands were
18 visible in whole extract obtained from pFLAG-cmv-2 transfected cells (control
19 cells).

20 In order to verify if the introduction of the FLAG epitope at the N-terminus of TSPO
21 could modify the binding affinity for the specific and selective TSPO ligand,
22 PK11195, homogenate membranes of TSPO-pFLAG-cmv-2 transfected cells,
23 collected 24 hours after transfection, were used for saturation studies of specific

1 [³H]PK11195 binding. Scatchard analysis of [³H]PK11195 binding saturation data
2 revealed a single population of binding sites with a dissociation constant (Kd)
3 value of 5.0 +/- 0.52 nM . C6 glioma cells transfected with TSPO-pFLAG-cmv-2
4 showed higher levels (about 2 folds) of [³H]PK specific binding sites than control
5 cells (Fig 1 B).

6 We also analyzed the sub-cellular localization of TSPO-pFLAG recombinant protein
7 by immunofluorescence microscopy. Anti-FLAG antibody revealed a cytoplasmic
8 localization of TSPO-FLAG protein that appeared accumulated in well-defined sub-
9 cellular structures (Fig 2A). Co-localization experiments performed by using anti-
10 cytochrome c antibody as a mitochondrial marker (Fig 2B) revealed an exclusive
11 mitochondrial localization for TSPO-FLAG recombinant protein (Fig 2C).

13 **TSPO OVER-EXPRESSION INCREASES THE PROLIFERATION** 14 **CAPABILITIES OF C6 GLIOMA CELLS**

15 With the aim of analyzing the relationship between TSPO expression level and cell
16 growth (Fig. 3), we assessed TSPO-pFLAG-cmv-2, pFLAG-cmv-2 or pEGFP-C1
17 transfected cell mitotic index. As shown in Fig. 3C, TSPO-pFLAG-cmv-2
18 transfected cells showed a higher mitotic index then control cells one day after
19 transfection. This difference became less pronounced 3 days after transfection when,
20 according to western blot analysis, the expression of TSPO recombinant protein is
21 strongly reduced (data not shown). Further support for TSPO positive influence on
22 cell proliferation, derived from the analysis of TSPO-pFLAG-cmv-2, pFLAG-cmv-
23 2 or pEGFP-C1 transfected cell proliferation curves. As reported in Fig 3A, TSPO-

1 pFLAG-cmv-2 transfected C6 cells have a higher proliferation rate than control
2 cells. Significant differences appeared 72 hours after seeding (* $p < 0.05$). Although
3 this data could appear in disagreement with the early effect of TSPO up-regulation
4 on mitosis, it has to be taken in mind that we designed our experiments in
5 way to maintain cells in the exponential phase of growth – up to about 120 hours.
6 Thus, the difference in cell number (also if small) that is produced by TSPO
7 overexpression during the first days is amplified in the following days as long as one
8 of the cell line reach the confluence and reduces cell growth rate.

9 We also evaluated the ability of TSPO-pFLAG-cmv-2 or pFLAG-cmv-2 transfected
10 C6 cells to grow after having reached the confluence to test TSPO influence on
11 cell ability to overcome contact-induced cell growth inhibition. As reported in Fig
12 3B the number of TSPO-pFLAG-cmv-2 transfected C6 cells was 25% higher
13 ($p < 1 \times 10^{-5}$) than that of control cells.

14

15 **TSPO OVER-EXPRESSION INCREASE MIGRATION RATE OF C6** 16 **GLIOMA CELLS**

17 With the aim of analyzing the relationship between TSPO expression level and cell
18 migration capabilities, we assessed the rate at which controls (pFLAG-cmv-2 and
19 pEGFP-C1) or TSPO-pFLAG-cmv-2 transfected cells recovered a scratch done on a
20 layer of confluent cells. Colonization of the scratch was monitored 20, 25 and 40
21 hours after the scratching. As reported in Fig 4, TSPO-pFLAG-cmv-2 transfected C6
22 cells reduced the clear area (Fig 4 A, B) and migrated into the scratch at a
23 significantly higher rate than that of controls cells ($p < 0.05$) (Fig 4 C).

1 **TSPO OVER-EXPRESSION INCREASES TRANSMIGRATION** 2 **CAPABILITIES OF C6 GLIOMA CELLS**

3 One of the most important characteristics of aggressive tumoral cells is the ability to
4 penetrate anatomic barriers and diffuse in cell matrix. Therefore, we assessed C6
5 tumor cell transmigration across artificial barriers *in vitro* to evaluate tumor cell
6 invasion properties of TSPO-pFLAG-cmv-2 transfected cells and control cells. We
7 separated populations of cells able to invade a gelatin layer and counted the number
8 of cells that reached the lower side of a polycarbonate filter. As reported in Fig 5, the
9 number of cells that more actively invade the gelatin layer was significantly higher
10 in TSPO-pFLAG-cmv-2 transfected C6 than control cells ($p < 0.05$).

11 **TSPO OVER-EXPRESSION SENSITIZES C6 GLIOMA CELLS TO TSPO** 12 **SPECIFIC LIGAND TREATMENT**

13 To assess whether the TSPO over-expression affect C6 cell sensitivity to the pro-
14 apoptotic activity of its specific ligand PK 11195, we evaluated cell viability of
15 control and TSPO-pFLAG-cmv-2 transfected cells following 24 hours exposure to
16 micromolar increasing PK 11195 concentrations. The 24 hour treatment was
17 performed starting from the 24th hour or from the 96th hour after transfection. As
18 shown in Fig 6, PK 11195 caused a concentration-dependent inhibition of C6 cell
19 viability in both TSPO-pFLAG-cmv-2 transfected and control cells treated starting
20 from the 24th hour after transfection. Interestingly, we observed that TSPO-pFLAG-
21 cmv-2 transfected C6 cells showed a higher sensitivity to the antiproliferative
22 activity of PK 11195 in comparison with control cells. These differences were
23 observed for each PK 11195 concentration. Accordingly the strong reduction in

1 recombinant TSPO expression that occurs 96 hours after transfection, no more
2 differences in sensitivity to PK11195 was observable when the treatment was
3 initiated 96 hours after transfection.

4

ACCEPTED MANUSCRIPT

1 DISCUSSION

2 In recent years, attention has been focused on TSPO role in the regulation of cell
3 proliferation, as several literature data report that TSPO is strongly up-regulated in
4 tumoral tissues or tumor cell lines with respect to untransformed control tissues [4],
5 [6], [7] and [8]. Much research has been focused on TSPO as a promising protein
6 target for chemotherapy, suggesting its ligands as potential therapeutic drugs [13],
7 [15], [16], [31], [32] and [33]. However, only indirect data are available to elucidate
8 the meaning of TSPO up-regulation on tumor cells. TSPO is highly expressed in
9 glioma cells and to understand the role of its up-regulation in glioma aggressiveness
10 we produced, for the first time, genetically modified glioma cells over-expressing
11 TSPO protein and we evaluated the influence of TSPO density on the typical
12 features that give an invasive/aggressive phenotype (i.e. proliferation, motility and
13 transmigration capabilities) to a tumor cell. Among glioma cell lines we selected C6
14 cells because we previously characterized in this cell line TSPO expression and sub-
15 cellular localization and established cell response to TSPO ligand treatment [15] and
16 [16].

17 By western blot analysis, TSPO recombinant protein expression was detectable 24
18 hours after transfection and some of it remained up to 72 hours, a sufficiently long
19 period to carry out functional studies. Radioligand binding assays demonstrated that
20 the FLAG epitope addition to the amino terminus of TSPO does not change the
21 binding affinity for this ligand. Indeed, the [³H]PK11195 binding affinity for FLAG-
22 tagged TSPO resulted comparable to Kd value previously reported for native rat C6
23 glioma cells [29]. Moreover, radioligand binding experiments showed an increase of

1 the [³H]PK11195 specific binding sites in cells expressing TSPO recombinant
2 protein with respect to control cells, confirming western blot results. By
3 immunofluorescence assays we also demonstrated that TSPO recombinant protein
4 accumulated at mitochondrion level accordingly with the main distribution of TSPO
5 in these cells, as previously demonstrated by [16].

6 These results demonstrate the success in producing C6 cells over-expressing a
7 recombinant TSPO protein with peculiarities similar to those of the endogenous
8 TSPO protein and pushed us to undertake functional experiments to test the
9 proliferation, motility and transmigration properties of C6 cells over-expressing the
10 TSPO protein. We found that TSPO over-expression increased C6 cell proliferation
11 rate and the mitotic index as well as the cell ability to overcome the contact-
12 dependent inhibition of cell growth that is linked to tumor invasion capabilities.
13 Moreover we found that TSPO over-expression improved the motility rate and
14 transmigration capability of C6 cells, demonstrating that the increase of TSPO
15 expression level may contribute to the acquisition of an invasive/aggressive
16 phenotype in gliomas. This is the first direct evidence that TSPO density influences
17 glioma cell aggressiveness and is consistent with previous reports that correlate
18 TSPO expression level and tumor malignancy grade [18], [23], [24] and [28].

19 Our data agree with recent findings, obtained in breast cancer cells, demonstrating
20 that a siRNA mediated reduction of TSPO endogenous level and TSPO over-
21 expression induce a decrease and an increase in cell proliferation, respectively [34].
22 However, all these data are in contrast with a previous report describing that TSPO
23 knockdown by stable antisense transfection induces an increase of *in vitro*

1 tumorigenicity and a reduction of apoptosis in C6 cells [35]. These authors have
2 suggested that increased cell proliferation in TSPO knockdown cells was due to a
3 loss of TSPO-related apoptosis. According to these interpretations, we should have
4 found an increase in apoptotic cell death and a reduced proliferation rate in TSPO
5 over-expressing cells. Contrarily, in our experimental condition, we did not find an
6 increase in the number of died cells and, in addition, TSPO transfected cells
7 proliferated at higher rate than controls. Although apparently contrasting, these
8 findings could be explained by the idea that TSPO could be found, inside the outer
9 mitochondrial membrane, as a monomer, heteropolymer or SDS and beta-
10 mercaptoethanol resistant homopolymer. In the heteropolymers, TSPO is bound to
11 the mitochondrial permeability transition pore proteins [36] and [9] and the
12 heteropolymerization of TSPO in the pore complex is limited by the density of
13 VDAC [37]. The silencing of TSPO expression affects the monomeric,
14 homopolymeric and the heteropolymeric forms. On the contrary, the over-expression
15 of the FLAG-TSPO in the C6 cell mitochondrial membranes preferentially acts by
16 increasing the monomeric TSPO. Indeed, the amount of the heteromeric TSPO is
17 limited by VDAC density and unlikely the number of hetheropolymers could
18 increase without a parallel increase in VDAC expression. Moreover, our western
19 blot analysis exclude that the majority FLAG-TSPO protein is expressed as a SDS
20 and beta-mercaptoethanol resistant homopolymer. This explains why the silencing
21 and up-regulation have similar effects on the cell growth. Indeed, the reduction of
22 TSPO inside the pore makes the cells more resistant to endogenous apoptotic
23 stimuli, as suggested by [35] while the increase of the monomeric form promote

1 cholesterol transport [36] and, as a consequence, the biogenesis of the inner
2 mitochondrial membrane that positively correlate with cell proliferation, motility
3 and invasiveness.

4 Finally, it is well known that micromolar PK 11195 concentrations induce apoptosis
5 in C6 glioma cells [18]. In the present study we tested the PK 11195 effects on cell
6 viability of C6 glioma cells expressing different TSPO levels. We found that TSPO
7 over-expression causes an increased susceptibility of C6 glioma cells to the
8 antiproliferative activity of PK 11195, suggesting that this ligand may be useful also
9 for the therapeutic treatment of gliomas with aggressive phenotype and high TSPO
10 density.

11

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1 **FIGURE LEGENDS**

2

3 **FIGURE 1:** TSPO-FLAG recombinant protein expression. (A) Western blot
4 analysis of TSPO-FLAG recombinant protein in TSPO-pFLAG-cmv-2 and pFLAG-
5 cmv-2 transfected cells. The molecular weight axis indicated on the right, anti ERK
6 has been used as loading control. (B) Saturation curves and Scatchard plots (inset) of
7 specific [³H]PK 11195 binding to membranes of C6 glioma cells transfected with
8 TSPO-pFLAG-cmv-2 (▼) or with pFLAG-cmv-2 (■). Membranes were incubated
9 with increasing concentrations of [³H]PK 11195 ranging from 0.5 to 26 nM. Non
10 specific binding was measured in the presence of 1 μM PK 11195. Data are from a
11 single experiment carried out in triplicate. For C6 glioma cells transfected with
12 TSPO-pFLAG-cmv-2 or with pFLAG-cmv-2 the K_d values were, 6.0 and 5.0 nM
13 and mean B_{max} values were, 10.98 and 5.72 pmol/mg of protein, respectively.

14 **FIGURE 2:** Assessment of the subcellular distribution of TSPO-FLAG recombinant
15 protein in TSPO-pFLAG-cmv-2 transfected C6 cell by fluorescent microscopy. (A)
16 Distribution of TSPO-FLAG recombinant protein as visualized by using anti-FLAG
17 antibody. (B) Subcellular distribution of mitochondria as revealed by using anti-
18 cytochrome c antibody. (C) Merged panels A and B show that in TSPO-pFLAG-
19 cmv-2 transfected cell (arrow), TSPO-FLAG recombinant protein is expressed in the
20 mitochondria.

21 **FIGURE 3:** Assessment of TSPO transfected cell proliferation capabilities. (A) Cell
22 proliferation curves of pFLAG-cmv-2 and TSPO-pFLAG-cmv-2 transfected cells.
23 Each value is expressed as mean ± SD of three independent experiments done in
24 triplicate. (* p < 0.05, calculated by student *t* test). (B) number of TSPO-pFLAG-

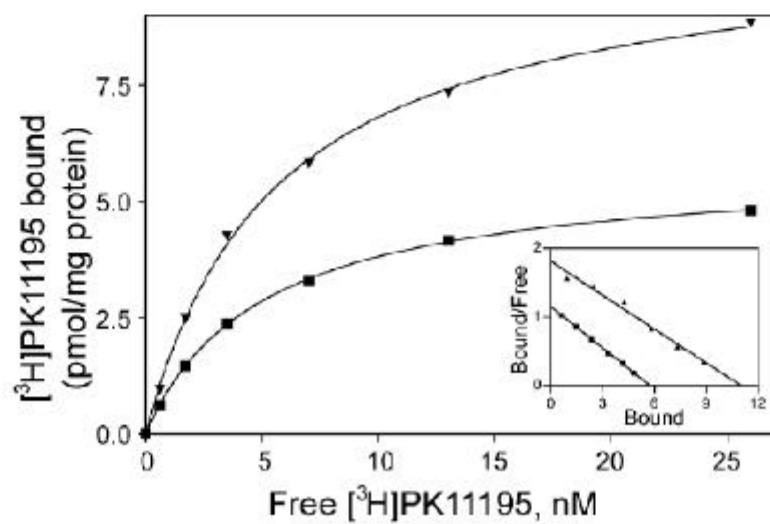
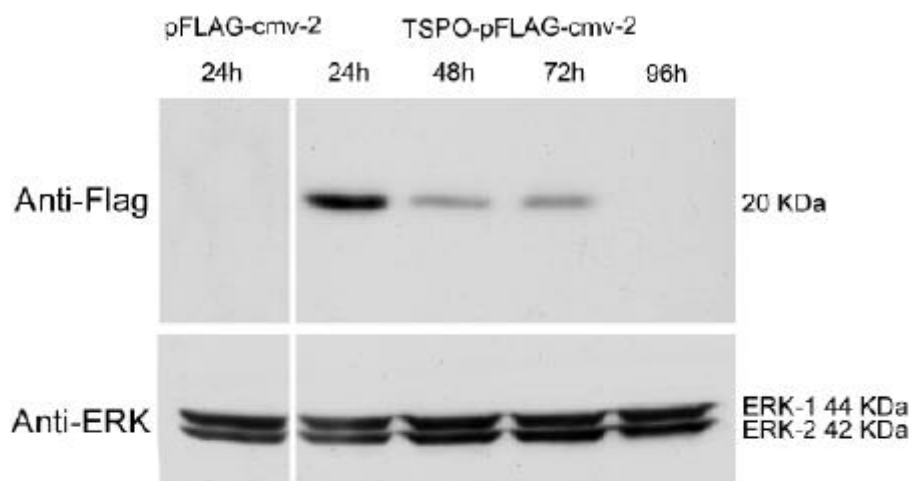
1 cmv-2 or pFLAG-cmv-2 transfected C6 cells after they had been confluent for three
2 days. Each value is expressed as mean \pm SD of the cell number counted in ten
3 independent transfections (* $p < 1 \times 10^{-5}$, calculated by student *t* test). (C) Mitotic
4 index of pFLAG-cmv-2, pEGFP-C1 and TSPO-pFLAG-cmv-2 transfected cells 24
5 hours after transfection. (* $p < 0.001$, calculated by student *t* test between TSPO-
6 pFLAG-cmv-2 and pFLAG-cmv-2)

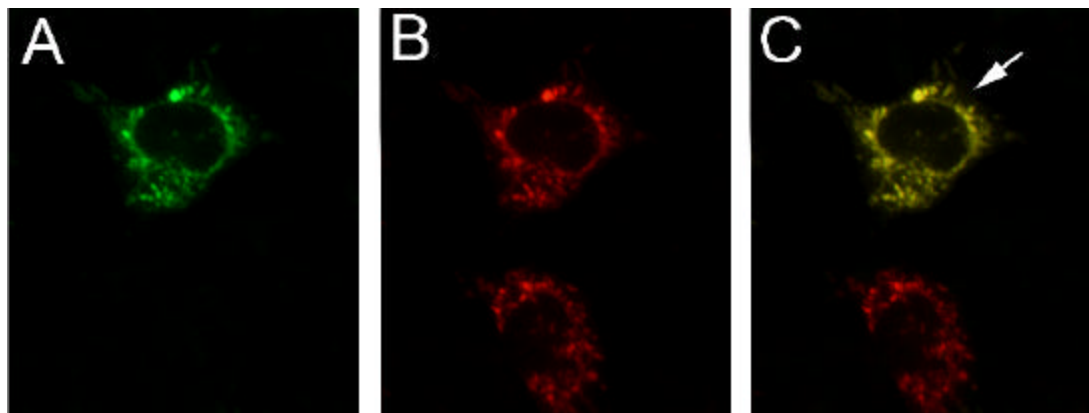
7 **FIGURE 4:** Effect of TSPO over-expression on cell motility. (A) A representative
8 example of TSPO-pFLAG-cmv-2 transfected C6 cells recovering the scratch
9 surface. (B) Reduction of the scratch area 20, 25 and 40 hours after scratching in
10 pFLAG-cmv-2, pEGFP-C1 or TSPO-pFLAG-cmv-2 transfected cells. Mean values
11 \pm SD were obtained normalizing the scratch area at each time of analysis versus the
12 scratch area measured immediately after scratching (* $p < 0.05$, ** $p < 0.01$, calculated
13 by student *t* test between TSPO-pFLAG-cmv-2 and pFLAG-cmv-2). (C) Migration
14 rate (cm/h) of TSPO-pFLAG-cmv-2, pEGFP-C1 and pFLAG-cmv-2 transfected
15 cells. Each bar shows the rate as mean \pm SD calculated in three point of six
16 independent transfections at 20, 25 and 40 hours (* $p < 0.05$, calculated by student *t*
17 test between TSPO-pFLAG-cmv-2 and pFLAG-cmv-2).

18 **FIGURE 5:** Effect of TSPO over expression on transmigration of C6 cells through
19 an artificial barrier. (A) Cells were allowed to migrate through gelatin-coated filters
20 in response to chemoattraction for 5 hours. (B) Number of cells passed through the
21 filter has been counted in 15 microscope fields (10 X). Each bar shows the mean
22 number \pm S.D of cells counted in three independent experiments (* $p < 0.05$,
23 calculated by student *t* test).

1 **FIGURE 6:** TSPO-pFLAG-cmv-2 and pFLAG-cmv-2 transfected C6 cell viability
2 inhibition by PK 11195. Cells were treated with increasing concentrations (ranging
3 from 1 to 100 μ M) of PK 11195 for 24 hours. After incubation, cell viability was
4 measured by MTS conversion assay. The results are expressed as a percentage of
5 viable cells observed after treatment with PK 11195 vs vehicle treated cells (100%)
6 and shown as mean \pm SD. The data are obtained from three independent experiments
7 done in duplicate.

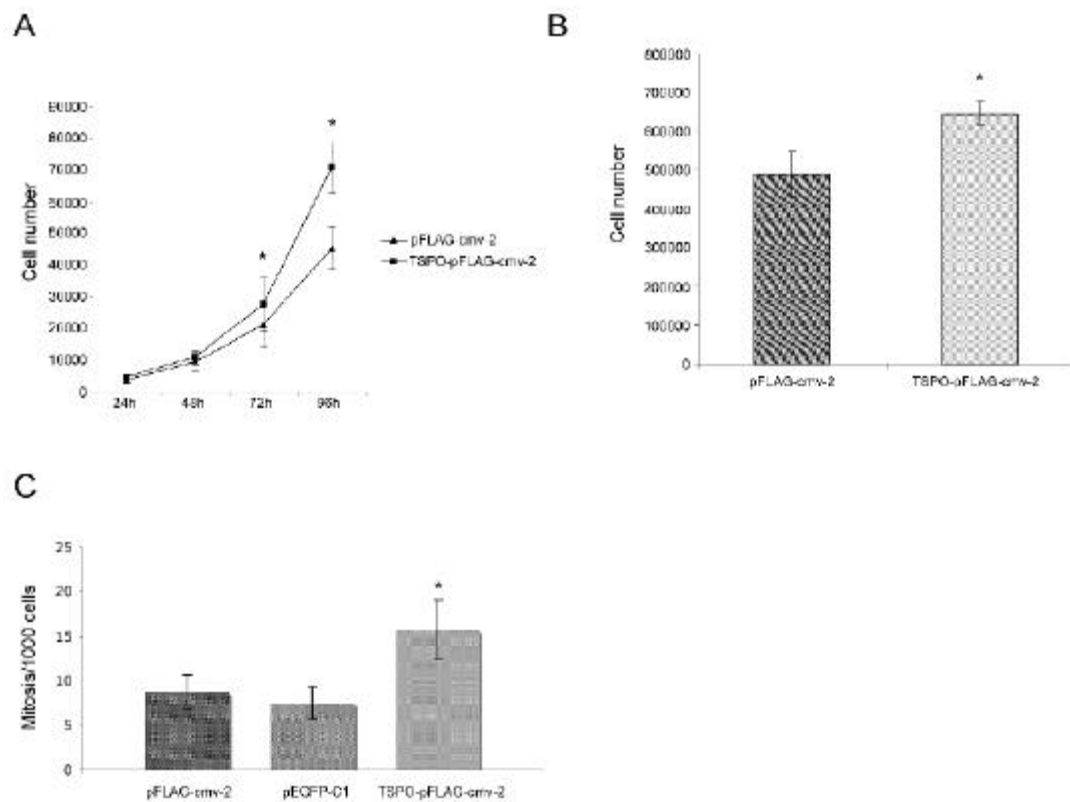
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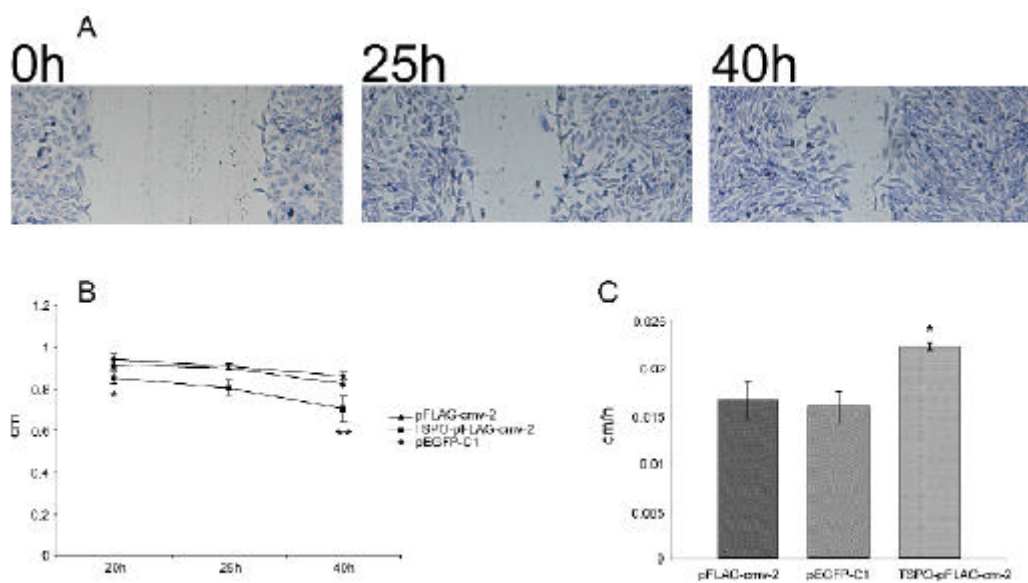
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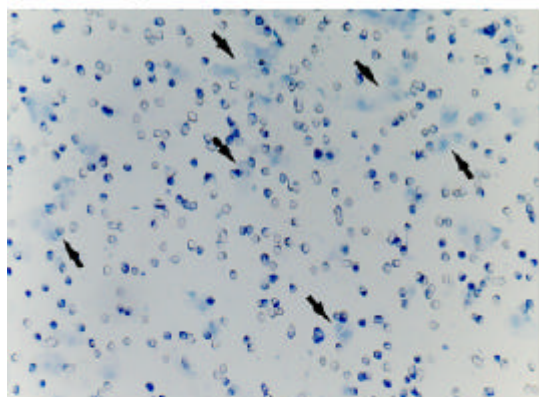


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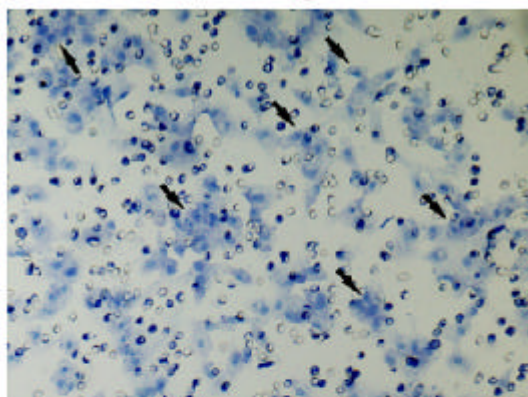
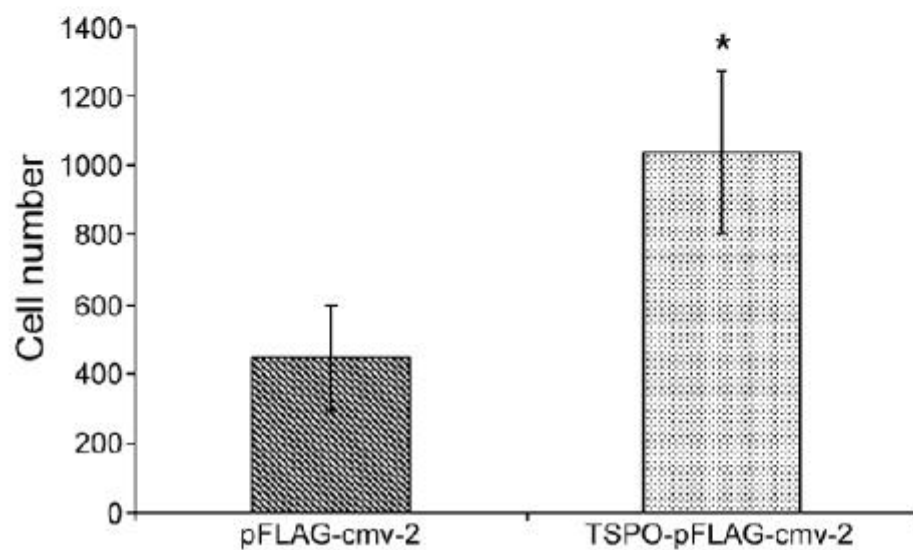
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pFlag-cmv-2

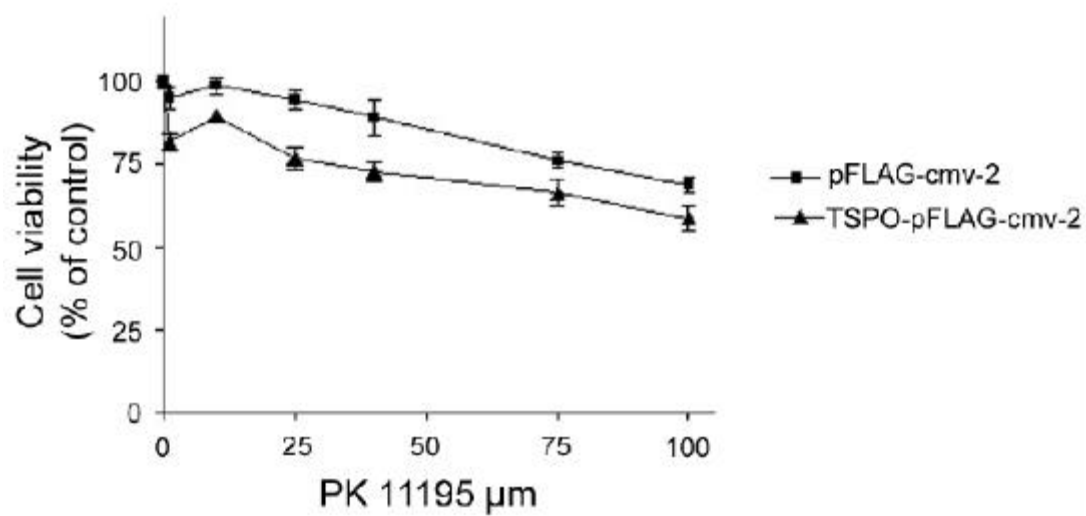


TSPO-pFlag-cmv-2

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