The 18-kDa Mitochondrial Translocator Protein in Gliomas: From the bench to bedside

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Abstract

The 18kDa mitochondrial Translocator Protein (TSPO) is known to be highly expressed in several types of cancer including gliomas while expression in normal brain is low. TSPO functions in glioma are still incompletely understood. The TSPO can be quantified pre-operatively with molecular imaging making it an ideal candidate for personalized treatment of patient with glioma. Studies have proposed to exploit the TSPO as a transporter of chemotherapics to selectively target tumour cells in the brain. Our studies proved that PET-imaging can contribute to predict progression of patients with glioma and that molecular imaging with TSPO-specific ligands is suitable to stratify patients in view of TSPO-targeted treatment. Finally, we proved that TSPO in gliomas is predominantly expressed by tumour cells.

Key words: Translocator protein, glioma, PET-imaging, pathology

Introduction

Astrocytomas and oligodendrogliomas are the most common primary brain tumours in adults [1]. They are graded according to the World Health Organization classification scheme in grade II (low grade), grade III (anaplastic) and for astrocytomas only, grade IV (glioblastoma). Glioblastoma (GBM) can occur *de novo* or result from the progression of a lower grade lesion. Low-grade astrocytomas (LGA) and oligodendrogliomas (LGO) almost invariably progress to ultimately fatal lesions but the timescale for transformation is highly unpredictable. Most patients die within 10 years from presentation though oligodendrogliomas often show more indolent behaviour than astrocytomas and are more responsive to chemotherapy [2]. Neuroimaging follow-up of patients with low-grade glioma is largely based on post-contrast structural MRI. However, the presence of contrast enhancement does not reliably predict transformation [3]. More sophisticated methodologies such as relative cerebral blood volume (rCBV) from perfusion MRI have higher sensitivity but they are not free of limitations, particularly in oligodendrogliomas [4]. The identification of biomarkers of transformation is therefore relevant to early treatment intervention. Gliomas are genetically, molecularly and metabolically heterogenous [5]. For this reason a

personalized approach is needed to more effectively target neoplastic cells but such an approach requires adequate stratification of patients and in-depth knowledge of the function and distribution of target molecules [6].

The Translocator Protein (TSPO) is a potential candidate for individualised approach to gliomas as its expression is enhanced in astrocytomas but it is low in the normal brain. Experimental studies proposed the TSPO as a direct target of anticancer drug or as a transporter for selective delivery into brain cancer cells [7]. Finally the TSPO can be visualized and quantified with PET-imaging allowing for pre-operative stratification of patients [8].

TSPO in gliomas

Over two decades ago, PET-imaging studies first documented high density of TSPO ligand binding sites in glioma patients and binding sites were shown to correspond to histologically determined areas of the tumour, to be higher than normal brain with no binding in the necrotic regions [8, 9].

Several studies also examined TSPO expression in glioma cell line models [reviewed in 11] but considerably less has been done in human glioma tissue. Using immunohistochemistry and *in situ* hybridization, Miettinen et al. [12] investigated 86 human astrocytomas of different grade and documented increase in TSPO mRNA and protein levels in higher grades but also an overlap between WHO grade II and III lesions. Similar results were obtained by Vlodavsky et al. by looking at 130 gliomas [13]. Both authors suggested a correlation between TSPO expression and outcome. More recently, Takaya et al [14] combined PET-imaging and neuropathology and observed low TSPO expression in two cases of anaplastic astrocytoma (AA) but also downregulation in glioma associated microglia/macrophages (GAMs). Notably, none of previous studies investigated oligodendroglial tumours.

The evidence of high TSPO in GBM led to propose the TSPO as a target molecule for treatment of this lethal tumour. The use of anticancer drug conjugated to TSPO ligands for selective brain delivery was first suggested by Guo et al. [15] using a PK11195-gemcitabine conjugate in a preclinical glioma model. Other studies followed to evaluate the efficacy of new TSPO ligand-anticancer drug conjugates [16,17]. Musacchio et al [18] proposed the use of PEG-PE micelles loaded with paclitaxel and surface-modified by the TSPO ligand CB86 and more recently Denora et al [19] assessed the toxicity of TSPO-ligand ARA-C compound. Finally, PK11195 coated dendrimers have more recently been proposed [20] as they offer the advantage to attach multiple copies of the targeting agent on a single particle, they are biocompatible and soluble, their cost of production is low. In the last decade, the use of nanocarrier such as polymer, emulsion, liposome, nano-crystals and micelles have boosted the progress in the field for drug targeting and imaging [21].

PET imaging of TSPO in gliomas and pathology correlates

PET-scan has increasingly been used as non-invasive quantitative imaging modality to assess the biodistribution and pharmacokinetics of drugs and to determine their efficacy and safety in the treatment of patients with cancer. Such a task can only be achieved by acquiring an in-depth knowledge of the extent of tissue expression and distribution of molecular targets. With this in mind, we designed the two studies to investigate the suitability of PET imaging to stratify gliomas

with low and high TSPO expression [22].

Initially, we assessed the first generation TSPO probe [^{11}C]-(*R*)PK11195 to study human gliomas and documented its suitability to investigate low and high-grade lesions [23]. PK11195 is a pharmacological antagonist of TSPO with no known effects in humans. Despite its limitations and difficulties in modelling, this compound is the best-characterised TSPO radioligand and the most commonly used probe in imaging CNS diseases [8]. Several of its properties make [^{11}C]-(*R*)PK11195 suitable for gliomas. It is lipophilic and therefore readily crosses the blood-brain barrier (BBB) that is usually intact in low grade lesions. It rapidly clears from the normal brain, allows good discrimination between neoplastic and normal tissues, and its binding is not affected by the previously identified substitution Ala147Thr in the fifth transmembrane domain of TSPO [24].

Secondly, we characterised gliomas of different grade using [^{11}C]-(*R*)PK11195 PET-imaging, compared structural MRI and rCBV maps with PET imaging and validated findings with analysis of the tumour tissue [25]. Our results proved that [^{11}C]-(*R*)PK11195 PET-imaging can be used to stratify patients for TSPO targeted treatment and that such treatment would predominantly challenge neoplastic cells. The partial overlap between regions of high rCBV and high [^{11}C]-(*R*)PK11195 uptake suggests that [^{11}C]-(*R*)PK11195 binding is not influenced by the disruption of the BBB, and by changes in blood volume or neoangiogensis.

For the first time, we documented low [^{11}C]-(*R*)PK11195 binding in LGO and considerably increased ligand uptake in anaplastic examples. Though limited to seven cases, this result may suggest that [^{11}C]-(*R*)PK11195 PET can predict anaplastic transformation in oligodendroglial tumours. Pre-operative distinction between low grade and anaplastic lesions is challenging with structural and physiological imaging and correct grading can also be difficult at histology, particularly on small biopsies. A few PET imaging studies have investigated oligodendrogliomas. FDG or ^{11}C-MET or both ligands in combination did not prove to be of any value to predict progression in oligodendrogliomas as tracer uptake was high irrespective of their WHO grade [26, 27].(figure 1)

As a validation cohort, we examined 50 supratentorial astrocytic and oligodendroglial tumours and observed similar features. In both series, we detected no staining in reactive astrocytes, oligodendrocytes, neurons and found low expression in GAMs. In low-grade lesions, endothelial cells demonstrated TSPO express similar to normal brain while TSPO appeared reduced in newly formed vessels seen in GBMs.

The human *TSPO* is a 13-kbp gene mapped to chromosome 22q13.31 and consists of four exons. Exon 1 encodes a 5′ untranslated segment and is separated from exon 2 by an intron of approximately 6 kbp. The 100 bp upstream lack TATA or CAAT boxes but it is GC rich. The transcriptional start site 25 bp upstream from the first exon/ intron junction. *In silico* analysis of the cloned human *TSPO* promoter sequence revealed high GC content in the proximal region of the promoter and that the *Tspo* gene is located within a CpG island extending approximately 470 bp upstream and 615 bp downstream of the transcription initiation site [reviewed in 28].We therefore investigated possibility that *TSPO* gene expression in gliomas can be modulated by epigenetic silencing. Methylation Sensitive PCR analysis and pyrosequencing of TSPO promoter in 50 human glioma tissues and glioma cell lines (U-87MG, GaMG, DBTRG and 42MG-BA) demonstrated methylation in 21% of astrocytomas and over 72% of oligodendrogliomas while no methylation was observed in the four astrocytoma cell lines tested, this finding being consistent with their high protein expression. This result suggested a different regulation of TSPO in astrocytomas and oligodendrogliomas.

We finally ask the question if TSPO can be predictive of outcome as previously suggested [12,13]. We correlated proteins expression and methylation status to the overall survival in patients with astrocytoma but we did not observe any significant correlation (figure 2). Interestingly, we found a positive trend in the univariate analysis when survival was correlated with *TSPO* methylation. The relationship between TSPO methylation status and patient outcome could however be related the co-occurrence with other epigenetic events. A study on the predictive value of TSPO in oligodendroglioma is ongoing in our lab.

In vitro modeling of TSPO in gliomas

The TSPO has been studied in several *in vitro* cancer models including murine and human breast and colon carcinoma, melanoma and glioma suggesting a role in cell proliferation and survival, migration, apoptosis and cell response to stress. However, the literature on the function of TSPO in cancer cells is still conflicting. The majority of reports of the potential involvement of TSPO in apoptotic cell death are based on *in vitro* experiments utilising TSPO ligands and PK11195 in particular [reviewed in 11]. Recently Bode et al [11] examined the role of TSPO in brain tumour formation using the human U118MG glioma cell line in two different in-vivo models and observed an increase in angiogenesis, tumour formation with TSPO knockdown cells and reduction in adhesion in TSPO knockdown in U118MG glioma cells with an increase in migration. They also observed similar effect with PK 11195 at the concentration of 25 mmol/l but not at higher doses.

In our lab, we first analysed TSPO expression in a panel of human glioma cell lines (U-87MG, GaMG, DBTRG-05MG, 42-MG-BA and SNB-19) using Western blotting. Notably, all cell lines expressed monomeric TSPO and no obvious difference in TSPO protein level was found. All the subsequent experiments were conducted using U-87MG cells (purchased at the American Type Culture Collection, Washington DC, USA) as a well-established and widely used *in vitro* model of human glioma. We conducted knock-down experiments after silencing the TSPO gene with small interfering RNAs. The efficiency of transfections was assessed at 72 hours post transfection and was confirmed by Western blotting and by immunofluorescence analysis. Our experiments showed that TSPO gene silencing in U87MG did not affect cell growth, cell cycle, formation of clones or apoptosis and growth in low nutrient conditions (0.1% Fetal Bovine Serum), despite the level of knock-down surpassing that of published reports [29]

TSPO expression in glioma associated microglia and macrophages.

A full understanding of the pathology behind a disease is a prerequisite to the correct application of molecular imaging. This principle is particularly relevant to gliomas given their heterogenous microenvironment that consists of a mixed population of neoplastic and non-neoplastic cells (38), several of which can express TSPO. Our experiments demonstrated that GAMs contribute minimally to TSPO expression in gliomas [25]. TSPO down-regulation in GAMs is particularly interesting and seems unique to gliomas confirming the results published by Takaya et al [14]. GAMs are known to increase substantially with tumour grade in astrocytomas and their morphology to be consistent with an activate state but they switch to a M2 phenotype with impairment of their cytotoxicity, antigen presentation and phagocytosis. In contrast, GAMs promote tumour progression by producing a myriad of molecule favouring tumour cell migration, disruption of brain tissue and cell proliferation [31]. Whether down-regulation of TSPO is relevant

to phenotypic change of GAMs needs to be investigated.

Conclusion

Our studies confirmed that TSPO expression increases with tumour grade in astrocytomas but also that some overlap exists between LGA and AA lesions. For the first time, we investigated oligodendrogial tumours and showed increased in anaplastic examples. These results prove the principle that molecular imaging can be effective to stratify patients with glioma in view TSPO targeted treatment with little interference from non-neoplastic cells. The full spectrum of TSPO functions in gliomas remains however to be elucidated [32,33] suggesting a role of this molecule as transporter of drugs into the CNS rather than a direct target for treatment.

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Legends

Figure 1

A **–** Post-contrast T1-weigthed sequences of a case of glioblastoma; B shows co-registered and fused post-contrast T1-weighted MRI (grey scale) and parametric BP_{ND} images (spectrum colour scale) of the

same case; high BP_{ND} areas are indicated wth the white arrow. The colour bar indicates BP_{ND} values. C: Co-registered post-contrast T1-weighted MRI (grey scale) and rCBV images (spectrum colour scale) show different spatial distribution of high BP_{ND} and rCBV foci within the tumour. The white arrow points to an area of high BP_{ND} but low rCBV

D – box plot shows the box-plot of maximal [^{11}C]-(R)PK11195 BP_{ND} in the different grades and types of glioma. The boxes represent interquartile ranges and the whiskers indicate the lowest and highest values that are not outliers. Significant differences were found between LGAs, LGOs, and HGGs. **LGA** = low-grade astrocytoma; **LGO** = low-grade oligodendroglioma; **AA** = anaplastic astrocytoma; **LGOA** = low-grade oligo-astrocytoma; **GBM** = glioblastoma multiforme; **HGG** = high-grade glioma.

E: TSPO tissue expression in two cases of glioblastoma; TSPO is detected with the mouse anti-TSPO antibody (8D7) and goat anti-mouse Alexa Fluor 488 (green)-conjugated antibody. Glioma cells rabbit are identified with an anti-GFAP antibody and goat anti-rabbit Alexa Fluor 555 (red)–conjugated antibody. DAPI (blue) was used to counterstain total cell nuclei.

F: case of low grade astrocytoma that show colocalisation of TSPO and the mutant protein IDH1 in neoplastic cells (IDH1 green; TSPO red; nuclei are counterstained with DAPI)

Figure 2

A: TSPO expression in 30 cases of astrocytoma of different WHO grade is correlated with patient survival; Kaplan-Meier estimates of overall survival; median TSPO expression (percentage of TSPO positive cells) was used to dichotomise the samples into low and high- expression groups. P values in the log rank test. B - patient survival is correlated with *TSPO* gene methylation status. Kaplan-Meier estimates of overall survival; P values in the log rank test.

Figure 3

A - Mitochondrial localization of TSPO in U-87MG human glioma cells is shown with immunofluorescence using MitoTracker (Molecular Probes, Invitrogen Ltd.) (red) and anti-TSPO (monoclonal 8D7, gift from Dr Casellas, Sanofi Montpeller, France) identified with the mouse anti-TSPO antibody Alexa Fluor 488 (green)–conjugated secondary antibody. Cell nuclei were visualised using DAPI (blue). (Bar: 30 μm).

B - Western blot shows TSPO expression in the human glioma cell lines U-87MG, GaMG, DBTRG-05MG, 42-MG-BA and SNB19, proteins were extracted 48 hours after seeding at about 80% confluence. Only the monomeric form of TSPO is identified in these cell lines (B).

C - The efficiency of siRNA-mediated TSPO knock-down in the U-87MG glioma cell line is shown with Western blotting at 72 hours post transfection. Western blotting is performed with mouse anti-TSPO antibody (8D7). Rabbit anti-HSP60 antibody is used as protein loading control. Results of three independent experiments are presented.

D-E - TSPO knock-down does not alter U-87MG cell growth of U-87MG cells in normal (10% FBS) (D) low (0.1% FBS) serum conditions (E). Experiments represent three replicates. At each of the time-points cells were fixed using 10% TCA and quantitated by SRB assay

F – Silencing of *TSPO* gene does not affect the formation of clones of U-87MG cells. Columns represent the percentage of surviving clones. Experiments were performed in triplicate.

G - TSPO knock-down does not impact on the U-87MG cell cycle progression assessed using propidium iodide staining and flow cytometry analysis.. The experiment was conducted three times

Abbreviation: MOCK, mock transfected cells; NT, non-targeting siRNA transfected cells; siTSPO, TSPO Smart Pool siRNA transfected cells

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Disclosure

All authors declare no conflict of interest relevant to this work and manuscript.

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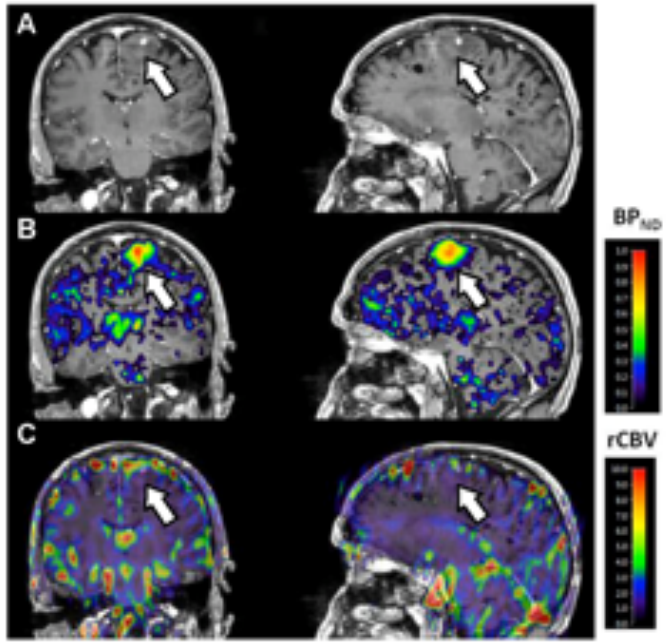
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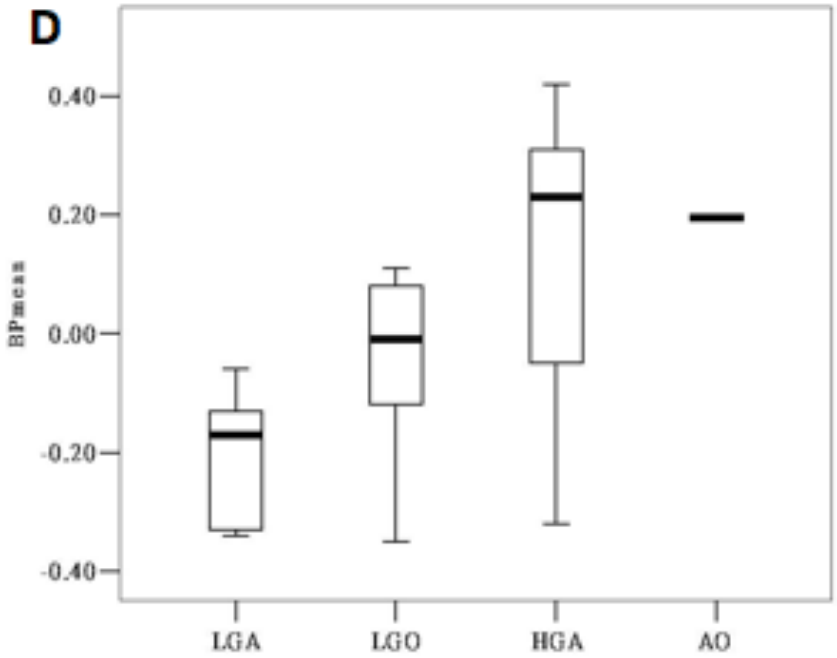
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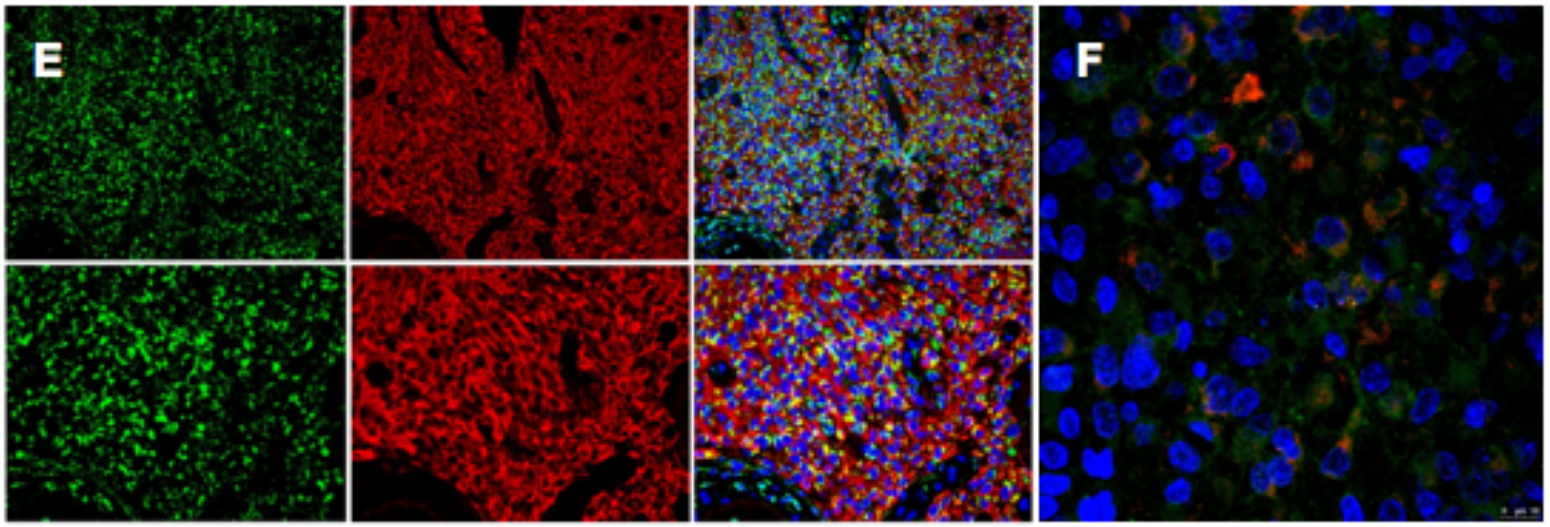
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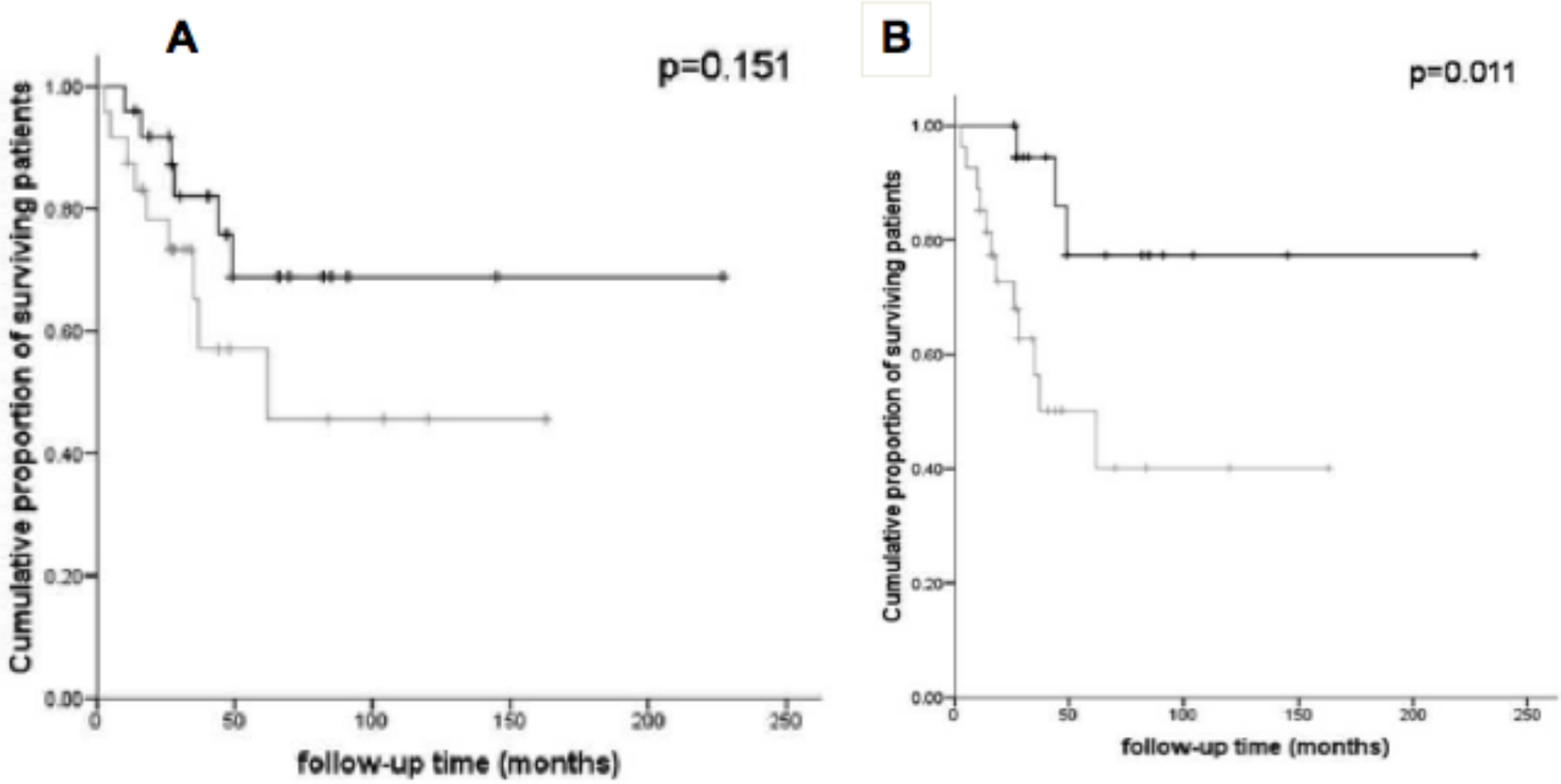
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Cumulative proportion of surviving patients
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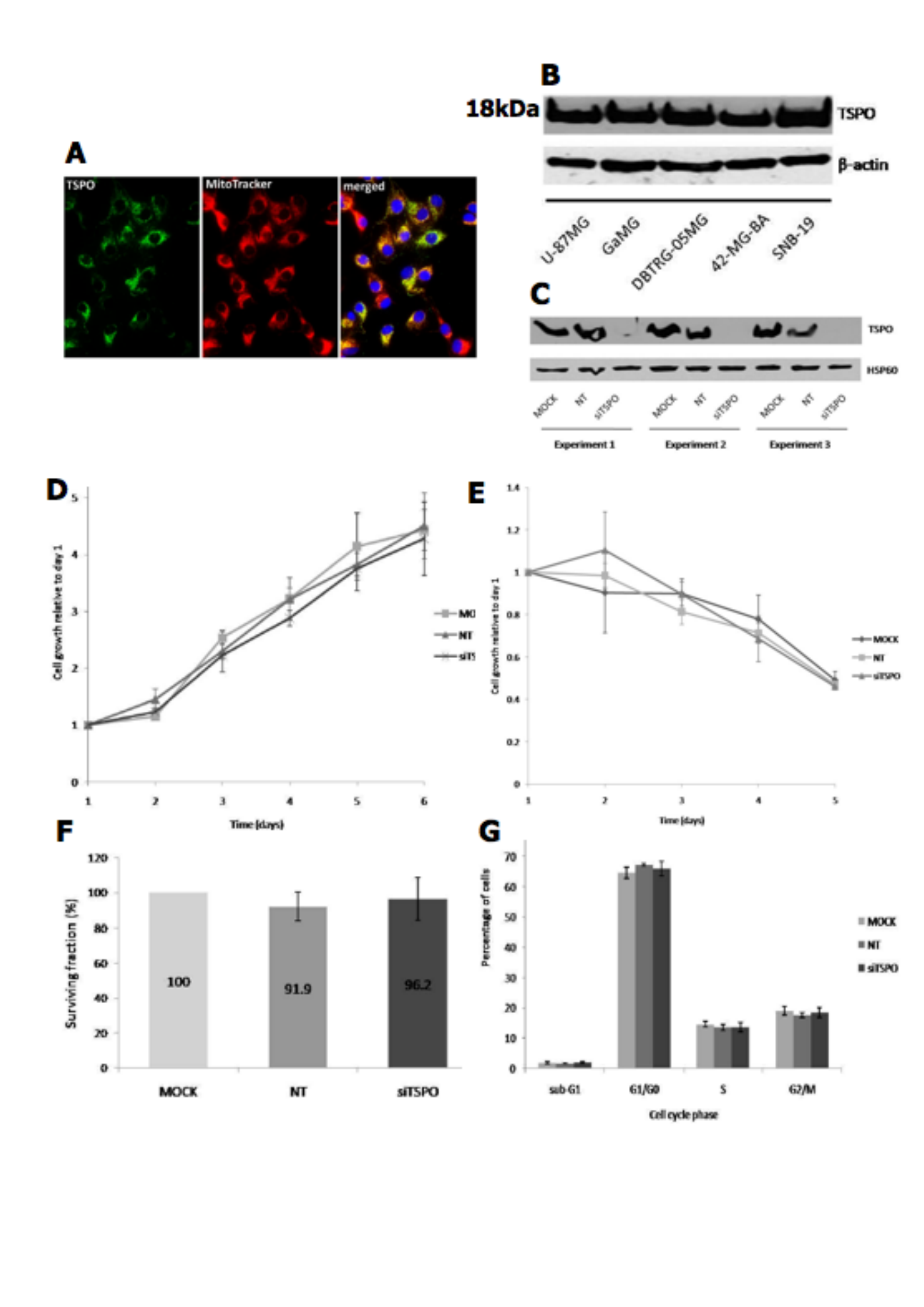
follow-up time (months)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Experiment 1
Experiment 2
Experiment 3
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Cell growth relative to day 1
Time (days)
E
Cell growth relative to day 1
Time (days)
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