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Downregulation of Akt and FAK phosphorylation reduces invasion of glioblastoma cells by impairment of MT1-MMP shuttling to lamellipodia and downregulates *MMPs* expression

Aneta Kwiatkowska [a], Magdalena Kijewska [a], Maciej Lipko [a], Urszula Hibner [b], Bozena Kaminska [a,*]

[a] *Laboratory of Transcription Regulation, Department of Cell Biology, Nencki Institute of Experimental Biology, 3 Pasteur Str., 02-093 Warsaw, Poland*
[b] *University of Montpellier, Centre National de la Recherche Scientifique, Institut de Génétique Moléculaire de Montpellier, 34293 Montpellier Cedex 5, France*

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ABSTRACT

Human malignant glioblastomas are highly invasive tumors. Increased cell motility and degradation of the surrounding extracellular matrix are essential for tumor invasion. PI3K/Akt signaling pathway emerges as a common pathway regulating cellular proliferation, migration and invasion; however, its contribution to particular process and downstream cascades remain poorly defined. We have previously demonstrated that Cyclosporin A (CsA) affects glioblastoma invasion in organotypic brain slices and tumorigenicity in mice. Here we show that CsA impairs migration and invasion of human glioblastoma cells by downregulation of Akt phosphorylation. Interference with PI-3K/Akt signaling was crucial for CsA effect on invasion, because overexpression of constitutively active myr-Akt antagonized drug action. Furthermore, the drug was not effective in T98G glioblastoma cells with constitutively high level of phosphorylated Akt. CsA, comparably to pharmacological inhibitors of PI3K/Akt signaling (LY294002, A443654), reduced motility of glioblastoma cells, diminished MMP-2 gelatinolytic activity and *MMP-2* and *MT1-MMP* expression. The latter effect was mimicked by overexpression of dominant negative Akt mutants. We demonstrate that CsA and LY294002 reduced *MMP* transcription partly via modulation of IκB phosphorylation and NFκB transcriptional activity. Those effects were not mediated by inhibition of calcineurin, a classical CsA target. Additionally, CsA reduced phosphorylation and activity of focal adhesion kinase that was associated with rapid morphological alterations, rearrangement of lamellipodia and impairment of MT1-MMP translocation to membrane protrusions. Our results document novel, Akt-dependent mechanisms of interference with motility/invasion of human glioblastoma cells: through a rapid modulation of cell adhesion and MT1-MMP translocation to membrane protrusions and delayed, partly NFκB-dependent, downregulation of *MMP-2* and *MT1-MMP* expression.

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1. Introduction

The invasion of neoplastic cells into brain parenchyma and fast proliferation are hallmarks of glioblastomas, the most malignant brain tumors [1,2]. In order to penetrate brain parenchyma glioma cells generate actin-rich membrane protrusions with extracellular matrix (ECM) proteolytic activity such as lamellipodia and invadopodia. These dynamic structures penetrate the microenvironment, anchor motile cells by focal adhesions and release proteinases that degrade ECM. Invasiveness and migration are complex processes which are regulated by phosphoinositide 3-kinase (PI3K), downstream Akt kinase and focal adhesion kinase (FAK) signaling pathways [3–5]. Binding of ECM proteins or growth factor receptor activation triggers focal adhesion kinase phosphorylation initiating focal adhesions turnover [6–10] and allows PI 3-kinase recruitment to the membrane and stimulation of Akt signaling [2,6]. PI3K/Akt signaling enhances actin remodeling and formation of membrane protrusions influencing Rac proteins [11], and through the activation of p70S6K modulates cell migration and invasion [12]. Akt is localized at the leading edge of moving cells in actin-rich structures and interacts with actin binding proteins [4,13]. Downregulation of Akt expression (in particular Akt2) with antisense or dominant negative constructs resulted in inhibition of glioma cell invasion *in vitro* [14] and *in vivo* [15]. The expression of matrix metalloproteinases (MMPs)-2 and -9 was inhibited in the rat tumor tissue with reduced Akt2 expression [15].

Local modification of ECM by the peptidases in gliomas involves the plasminogen activators, matrix metalloproteinases and cathepsins. Among various MMPs, the increased expression of the gelatinases MMP-2 and MMP-9 strongly correlates with glioma progression [16–18] and malignancy [19–21]. Physiological levels of MMPs are low and the amount of active enzyme is tightly regulated at several levels that involve regulation of gene expression, activation of

* Corresponding author. Fax: +48 22 822 53 42.
E-mail address: bozenakk@nencki.gov.pl (B. Kaminska).

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zymogens and inhibition of active enzymes by specific inhibitors [20,22]. The expression of many MMPs and their inhibitors TIMPs (tissue inhibitor of metalloproteinases) is regulated by transcriptional and post-transcriptional mechanisms by a variety of growth factors, cytokines and chemokines [23–26]. Membrane bound MT-MMPs, in particular membrane-type MT1- and MT2-MMP, play a major role in activating MMP-2. Newly synthesized MMP-2 is secreted as an inactive pro-enzyme, which is cleaved on the cell surface by membrane-type MT1-MMP (MMP-14) complexed with TIMP-2 [17,19,27,28]. Development of pharmaceutical approaches that affect expression or regulation of MMPs may be beneficial in targeting invasion of glioma cells but specific inhibitors are still to be found.

We have previously demonstrated that cyclosporin A (CsA), a calcineurin inhibitor, affects growth of glioma cells [29–32] and downregulates PI3K/Akt signaling and Akt-dependent phosphorylation of downstream targets [33]. Moreover, at low micromolar concentrations CsA suppresses glioma cell invasion *in vitro*, in organotypic brain slice cultures, and reduces tumorigenicity *in vivo* [34]. We showed that CsA may directly block glioma invasion without affecting cell proliferation or viability.

In the present study we studied molecular mechanisms underlying the inhibitory effect of CsA on migration/invasion of human glioblastoma cells with different alterations of PI3K/Akt signaling pathway and contribution of PI3K/Akt signaling in the regulation of tumor cell migration and invasion. We demonstrate that CsA impairs Akt and FAK signaling that results in reduction of motility and invasion of glioblastoma cells. CsA, as well as pharmacological and genetic inhibitors of PI3K/Akt signaling, reduced invasion and MMP-2 proteolytic activity likely in two mechanisms: by rapid impairment of shuttling MT1-MMP to lamellipodia and delayed downregulation of NFκB-dependent MMP expression. Our findings show for the first time anti-invasive action of CsA and define a complex involvement of Akt signaling into the regulation of cellular motility and invasion.

2. Materials and methods

2.1. Cell cultures

Human glioblastoma cell lines LN229, T98G and U373 (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, MD, USA) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) in a humidified atmosphere of CO_2/air (5%/95%) at 37 °C (Heraeus, Hanau, Germany).

2.2. Reagents

Antibodies recognizing phosphorylated forms of Akt (Thr308/Ser473), IκB (Ser32), FAK (Thr925/Thr397), GSK3β (Ser-9), as well as corresponding anti-total Akt, IκB, FAK, GSK3β, antibodies and horseradish peroxidase-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly MA, USA). For MMPs Western blot, antibodies anti-MT1-MMP from Chemicon Intern. and anti-MMP-2 from Santa Cruz Biotechnology were used. Immunocomplexes were detected using enhanced chemiluminescence detection system (ECL). Cyclosporin A was from Novartis (Novartis Pharma GmbH Nurnberg, Germany), LY294002 was from Cell Signaling Technology (Beverly MA, USA), FK506 (Prograf) from Fujisawa GmbH (Monachium, Germany) and BAY11-7082 was purchased from Sigma Aldrich Inc. (St. Louis, MO). A-443654–Akt inhibitor was a kind gift from Dr. V. Giranda, Abbott Laboratories.

2.3. Western blot analysis

Whole-cell lysates were prepared as previously described [35]. Cells were lysed in a buffer containing 20 mM Tris, pH 6.8, 137 mM NaCl, 2 mM EDTA, 25 mM glycerophosphate, 2 mM NaPPi, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 5 μg/ml leupeptin (Sigma), 5 μg/ml aprotinin (Sigma), 2 mM benzamidine and 0.5 mM DTT (Roche Applied Science). The cell lysates were centrifuged and the protein concentration was evaluated with BCA protein assay (Pierce). Total protein extracts (50 μg of proteins) were mixed with 5 × Laemmli buffer (50 mM Tris–HCl pH 6.8, 100 mM DTT, 10% glicerol), denaturated by boiling for 10 min and equivalent amounts of protein were loaded onto 4–10% Tris glycine/SDS-polyacrylamide gels and electrotransferred to ECL nitrocellulose membranes. The specific antibodies as indicated were used for immunoblots. Primary antibodies were detected with either anti-mouse IgG or anti-rabbit IgG antibody linked to horseradish peroxidase (Sigma). Immunocomplexes were visualized by ECL (Amersham Pharmacia Biotech). Bands intensity was determined by densitometry with BioRad Molecular Imager FX and Quantity One software.

2.4. Measurements of cell viability and proliferation

LN229, T98G and U373 cells (8×10^4 cells/well) were seeded in 24-well plates, incubated overnight and treated with CsA for next 12 or 24 h, respectively. Then MTT metabolism test was performed as previously described [36]. Cell proliferation was determined with BrdU assay. Cells were seeded in 96-well plates (6×10^3 cells/well), transfected with specific plasmids and after overnight incubation at 37 °C. Cells were treated for 2 h with BrdU labeling solution (Cell Proliferation ELISA BrdU assay, Roche Diagnostics GmbH Mannheim, Germany), fixed and incubated with a mouse monoclonal anti-BrdU antibody conjugated with peroxidase. Adding 1 M H_2SO_4 enables photometric detection by Thermo labsystem Multiscan EX at 450 nm.

2.5. Plasmids, transfection and gene reporter assay

LN229 cells (8×10^4 cells/well) were seeded on 24-well plates 24 h before transfection. Cells were transfected using Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA) with the plasmid carrying a firefly luciferase gene under promoter consisting multiple binding sites for NFκB (NF-κB *cis*-Reporting System, Stratagene-Agilent Technologies Company, CA, USA). After cell lysis the luciferase activity was measured using Luciferase Reporter Assay System (Promega Corporation Madison, WI, USA) according to manufacturer's protocol.

The plasmid coding for wtAkt was a kind gift from Dr. T. Franke and was previously described [33,37,38]. Plasmid encoding constitutively active myrAkt was kind gift from Dr. M. Weber [33,39]. LN229 cells were transfected using Cell Line Nucleofector™ Kit V according to manufacturer's protocol. Twenty-four hours after transfection cells were detached with 0.25% trypsin/0.02% EDTA (Sigma), counted and used in the matrigel invasion or BrdU assays.

The plasmids coding for dominant negative Akt mutants: ΔPHAkt and K179MAkt were kind gifts from Dr. T. Franke and have been previously described [37,38]. The K179MAkt construct contains a point mutation within the ATP-binding pocket rendering the kinase inactive and the ΔPHAkt construct has the plecstrin homology domain of Akt deleted activation. The LN229 cells were transfected using Cell Line Nucleofector™ Kit V following manufacturer's protocol. Forty-eight hours later total RNA was isolated and used as a template in RT-PCR.

2.6. Invasion and migration assays

The invasion assay was performed as described [34,40]. Briefly, 24-well tissue culture inserts (12 μm pore size Transwell, Corning, NY, USA) coated with the Growth Factor Reduced Matrigel™ Matrix (BD Biosciences, San Diego. CA, USA) were used. One hundred microliters of the Matrigel™ Matrix (1 mg/ml) diluted in distilled water, was dried under sterile conditions (37 °C) for 5–6 h and reconstituted for 30 min

in 200 μl of culture medium. LN229, U373 and T98G glioblastoma cells were seeded in the number of 1.5×10^4/insert on matrigel-covered membrane in serum-reduced medium (2% FBS) and treated with 1 μM or 5 μM CsA or left untreated. After 12 or 18 h cells were fixed and cell nuclei stained with DAPI (4′,6-Diamidino-2-Phenylindole; 0.01 mg/ml, Sigma). The membranes from Transwell® inserts were cut out and the total number of invading cells that migrated through the Matrigel was determined using Laser Scanning Cytometry (LSC, CompuCyte). The results were confirmed by manual blind counting. All experiments were performed three times, in triplicate.

For a scratch assay, LN229 cells were plated in 6-well plate and cultured overnight in DMEM containing 10% of FBS to reach 90% confluence. Cell monolayer was wounded with a sterile Eppendorf pipette tip (100 μl), washed with PBS, then fresh 2% serum containing medium was added to control plates and supplemented with 5 μM CsA to treated plates. Cells were incubated for 18 h, fixed with 2% paraformaldehyde, stained with DAPI or phalloidin and visualized by fluorescent microscopy. The nuclei of cells migrating to the cell-free area of scratch in randomly selected fields were counted.

For analysis of cell migration using live cell imaging the monolayer of cells was scratched using a sterile pipette tip (10 μl) and after washing with PBS cells were treated with 5 μM CsA, 1 μM A-443654, 30 μM LY294002 or corresponding amounts of DMSO as a control. The cultures were placed in a 37 °C chamber equilibrated with humidified air containing 5% CO_2 throughout the experiment. Time lapse microscopy was performed with a Leica DMIR 2 microscope using a 20× objective. Images were taken with Micromax YHS 1300 camera (pixel of 6.7. μm) at 30 min intervals for 24 h. Pictures were analyzed, and the total distance and the velocity were calculated using Metamorph 6 and Image J.

2.7. Real-time PCR

Total RNA (2 μg) isolated from LN229 cells treated with various inhibitors (CsA, BAY11-7082, LY294002) or transfected with plasmids coding for ΔPHAkt or K179MAkt was used as a template to generate cDNA. Then *MMP-2 and MT1-MMP* genes were amplified using primers (Hs00234422_m1) and (Hs00237119_m1) respectively. TaqMan MGB probe was marked with FAM™ reporter dye at the 5′ end and nonfluorescent quencher at the 3′ end of the probe. As endogenous control 18S (Hs99999901_s1) rRNA was applied. Gene expression quantification was performed using the Applied Biosystem TaqMan® Gene Expression Assay with the following parameters: stage 1 (50 °C for 2 min) 1 cycle, stage 2 (95 °C for 10 min) 1 cycle, stage 3 (95 °C for 15 s, 60 °C for 1 min) for 40 cycles. Data were analyzed by the Relative Quantification ($^{\Delta\Delta}$Ct) method using 7500 System SDS software (Applied Biosystems). The expression of each product was normalized to 18S rRNA and is shown as the ratio of the target gene to 18S gene expression, calculated by $2^{-\Delta\Delta Ct}$.

2.8. Gelatin zymography

LN229 cells were treated with control medium alone or supplemented with 5 μM CsA, 5 μM FK506 or 30 μM LY294002. Conditioned media were collected and samples were prepared in non-denaturating conditions in 5× Laemmli buffer without DDT. Samples were resolved in 8% SDS-PAGE gel containing 2 mg/ml of gelatin (SIGMA–ALDRICH CO, USA). The gels were washed twice in 2.5% Triton X-100 at room temperature before overnight incubation in renaturation buffer (50 mM Tris–HCl pH 7,6; 10 mM $CaCl_2$; 1 μM $ZnCl_2$; 1% Triton X–100 and 0.02% sodium azide). Gels were stained with Coomasie brilliant blue and band intensities were determined densitometrically with BioRad Molecular Imager FX and Quantity One software.

2.9. Immunocytochemistry

LN229 cells were seeded in 8 chamber Polystyrene Vessel Culture slides (BD Falcon) 4×10^4 cells per chamber. The next day monolayer of cells was scratched, cells were washed with PBS and cultured in the presence or absence of 5 μM CsA or 30 μM LY294002 for 6 h. Following fixation with 2% p-formaldehyde for 20 min at room temperature, cells were washed three times with PBS and permeabilized 1 h with 0.5% Triton X-100, followed by 90-min incubation in a blocking buffer (4% BSA, 3% NGS in 0.1% Triton X-100 in PBS). Subsequently, cells were incubated overnight at 4 °C with antibodies against MT1-MMP (Chemicon International, 1:100), phospho-paxilin (Tyr118, Cell Signaling, 1:50) or phospho-ezrin (Tyr354, Santa Cruz Biotechnology, 1:100). Next day cells were washed with 0.1% Triton X-100 in PBS for 5 min and incubated with a secondary antibody labeled with FITC. Cells were also stained with a Rhodamine–phalloidin (1 μg/ml, Sigma) dissolved in PBS. Subcellular localization of MT1-MMP and F-actin distribution pattern were analyzed using confocal microscopy.

2.10. Statistical analysis

Each experiment was performed at least 3 times, on independent passages, usually in triplicates. Data were analyzed by Newman–Keuls test using Statistica software as indicated and are presented as mean ± SEM. $p < 0.05$ was considered statistically significant. Results of time lapse microscopy experiments were analyzed with Wilcoxon test in R software.

3. Results

3.1. Cyclosporin A reduces invasion and motility of LN229 glioblastoma cells

In the present work invasion through matrigel, a matrix extract of non-crosslinked ECM macromolecules was used to evaluate effect of CsA on invasion of human LN229 glioblastoma cells. We developed a modified, quantitative matrigel invasion assay, in which the nuclei of cells migrating through matrigel to the lower surface of the membrane were stained with DAPI. The blue emission of DAPI-stained DNA was measured using laser scanning cytometry (iCys; CompuCyte, Cambridge, MA) with standard filter settings (Fig. 1A). The number of invading LN229 glioblastoma cells significantly decreased in cultures treated for 18 h with 5 μM CsA in comparison with untreated cultures (Fig. 1B). The observed reduction in the number of invading cells was not due to decrease in cell proliferation or cytotoxicity, because CsA applied at such concentrations did not affect the viability of cells, as determined by MTT metabolism test (Fig. 1C). The effect of CsA on cell migration was examined using a "scratch assay". Quantification of cell migrating to cell-free areas along a scratch revealed that the number of cells migrating to cell-free area was reduced to 60% in cultures treated with 5 μM CsA in comparison to controls (Fig. 1D).

3.2. CsA affects glioblastoma invasion/motility by interference with PI3K/Akt signaling pathway

Treatment of cells with 5 μM CsA led to a rapid reduction of the level of phosphorylated Thr308 Akt, starting as early as 30 min after treatment (Fig. 2A). Six hours after treatment with 5 μM CsA the levels of Akt phosphorylated at Thr308 and Ser473 were barely detectable. These results were confirmed by densitometric analysis of immunoblots from 3 independent experiments (Fig. 2A, lower panel).

To study if downregulation of PI3K/Akt signaling pathway by CsA is crucial for this effect, the constitutively active myristylated Akt (myrAkt) or the wild type Akt (wtAkt) were overexpressed in LN229 cells and CsA effect on invasion of transfected cells was analyzed. Cell

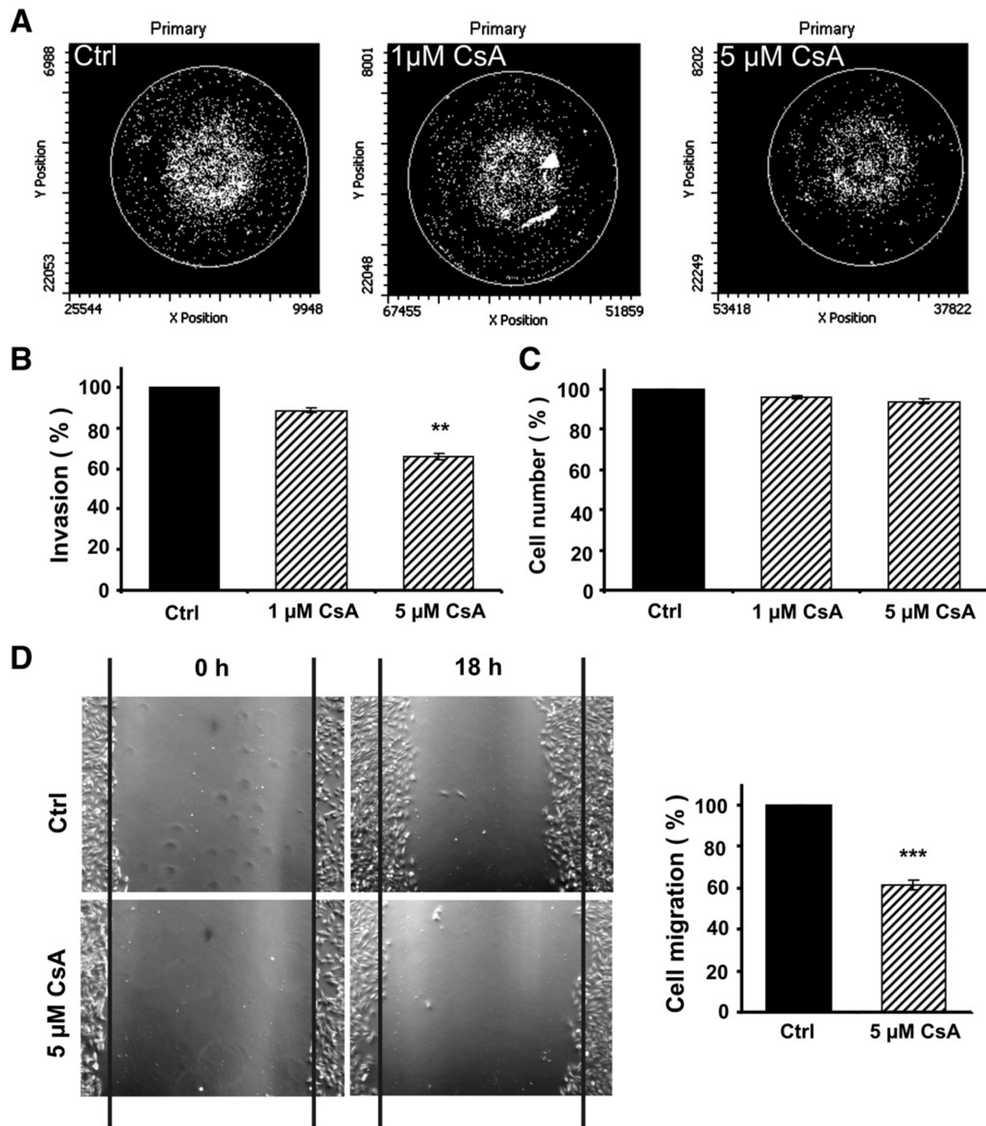

Fig. 1. Cyclosporin A reduces invasion and migration of human glioblastoma LN229 cells. A. LN229 cells plated on a matrigel-coated Boyden chamber were cultured for 18 h in DMEM with 2% FBS in the presence or absence of 1 or 5 μM CsA. Cells migrating through matrigel were fixed, their nuclei stained with DAPI and counted with Laser Scanning Cytometry. Representative LSC histograms are shown. B. Quantification of migrating cells (means ± SEM; 3 independent experiments, each in triplicate; $^{*}p<0.05$ CsA vs. ctrl). C. CsA does not affect cell viability as assessed by MTT metabolism test, means ± SEM; 3 experiments, each in triplicate. D. Cell motility is impaired by CsA as determined with a "scratch" assay. Nuclei of migrating cells were stained with DAPI and counted under the fluorescent microscope with the excitation 330–380 nm. Cells from 16 fields were counted and are presented as means ± SEM, $n=4$ experiments, each in triplicate.

transfection was performed with the Amaxa system resulting in 90% transfection efficiency. Overexpression of myrAkt abolished the inhibitory effect of CsA on cell invasion when compared with of mock or the wild-type Akt transfected cells (Fig. 2B). Cell proliferation of transfected cells was evaluated using BrdU incorporation test to exclude that overexpression of wtAkt or myrAkt affects proliferation of LN229 cells changing the number of cells (Fig. 2C).

Invasion of PTEN-mutated T98G glioblastoma cells and PTEN-null U373 cells, exhibiting constitutively high level of phosphorylated Akt, have been analyzed. Most T98G and U373 cells migrated through matrigel during 12 h and CsA treatment did not affect their invasiveness or cell viability (Fig. 3A). In parallel, the levels of phosphorylated Akt remained unaffected in CsA-treated T98G and U373 cells (Fig. 3B and the supplementary Fig. 1). Thus, overexpression of constitutively active Akt in LN229 cells or persistent Akt phosphorylation in PTEN-deficient cells abolished the inhibitory effect of CsA on glioblastoma invasion.

Live cell imaging technology was employed to analyze cell motility during scratch-induced migration. LN229 glioma cells were incubated with CsA, LY294002—an inhibitor of PI3 kinase, or A443654—an inhibitor of Akt kinase [41]. The inhibitory effect of A443654 on Akt activity was determined indirectly by measuring its ability to reduce GSK-3β phosphorylation (Supplementary data). Quantification of the results showed that cell migration was significantly reduced in CsA, LY294002 or A443654 treated cultures, and CsA was as effective as classical Akt signaling inhibitors. The reduction of speed was constant throughout 24 h of the experiment. The total distances calculated for 200 cells from each of experimental condition are presented in Fig. 4.

3.3. Direct effect of CsA on cell motility and morphology of LN229 glioblastoma cells

Actin assembly at the cell front drives membrane protrusion and initiates a directional cell migration. Microtubules extend forward within protrusions to sustain cell polarity and promote adhesion site turnover. To determine whether reduced cell motility in CsA-treated cultures is associated with cytoskeleton rearrangements, phalloidin staining of F-actin was performed. Several hours after scratch, control

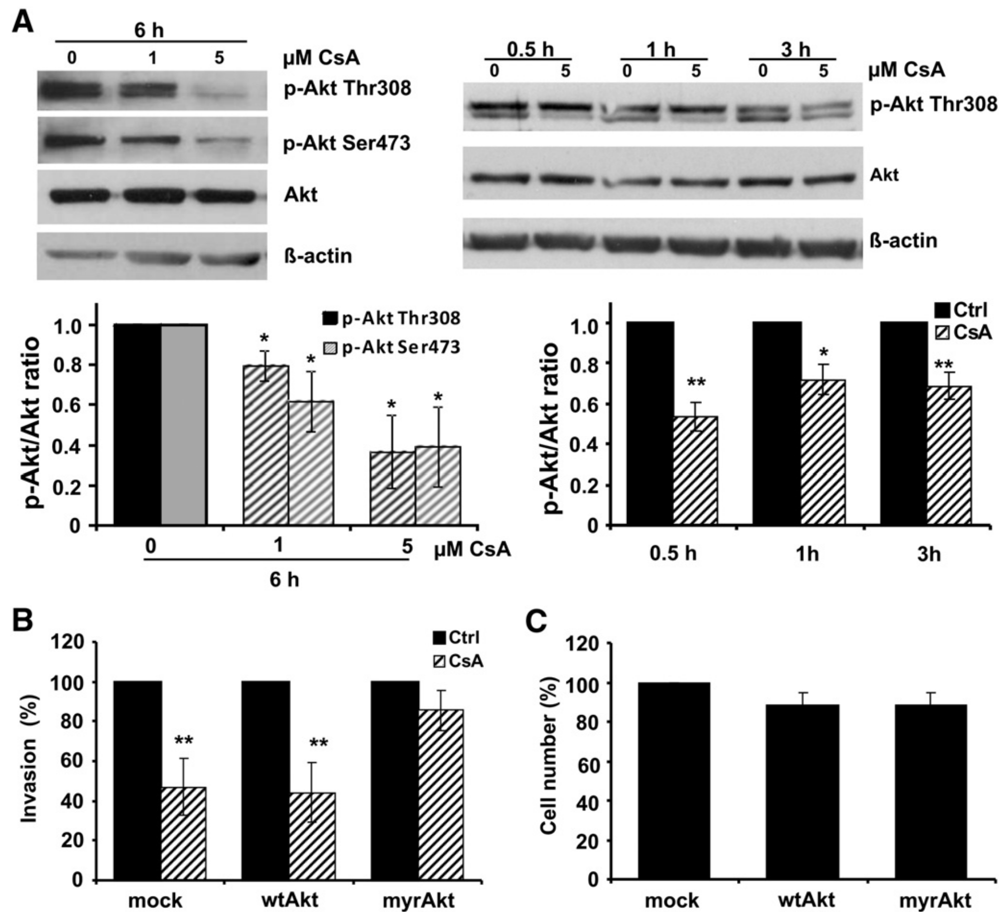

Fig. 2. Decrease of cell invasion in CsA-treated LN229 cultures is due to downregulation of PI3K/Akt signaling. A. LN229 cells were treated with CsA and total protein extracts were collected. Immunoblots were probed with antibody recognizing total or phosphorylated Akt and re-probed with anti-β-actin antibody. Densitometric analysis was performed and phosphorylated/total kinase ratio was calculated (means ± SEM, $n = 3$). B. LN229 cells were detached by trypsinization 24 h after transfection with plasmids encoding GFP, the wild-type Akt (wtAkt) or the constitutively active Akt (myrAkt) and seeded on matrigel covered inserts in DMEM with 2% FBS with or without 5 μM CsA. After 18 h cells invading matrigel were fixed, stained with DAPI and counted with LSC (means ± SEM; $n = 3$, each in triplicate). C. Cell proliferation was determined with a BrdU proliferation test in transfected cells.

cells exhibited elongated, polarized shape with an increased number of actin clusters, mostly at the leading edge (Fig. 5A, indicated by white arrows). Numerous ruffled membrane extensions (lamellipodia), characteristic feature of moving cells, were observed. On the contrary, cells treated with CsA were flattened with visible, stretched actin stress fibers and such phenotype was preserved for several hours (Fig. 5A).

A key regulator of cell movement and focal contact turnover is focal adhesion kinase (FAK) [6,8]. Paxilin, a focal adhesion-associated protein, functions downstream to FAK as an adaptor protein recruiting diverse cytoskeleton and signaling proteins into a adhesion complex (including ezrin that binds to plasma membrane and F-actin). FAK-deficient cells exhibit a refractory cell motility, in part due to enhanced stability of focal adhesions [8,10]. To assess if CsA affects FAK activity, Western blot analysis of FAK autophosphorylation at Tyr397 and subsequent phosphorylation of Tyr925 (reflecting the kinase activity of FAK and its phosphorylation by Src) was performed. As shown in Fig. 5B–C, the phosphorylation of both residues within FAK, which represents FAK activity, was strongly decreased in LN229 cells treated with 5 μM CsA; the level of total FAK was unaffected. Reduction in the FAK activity correlated in time with changes in the levels of phospho-paxilin (Tyr118) after CsA treatment. Detection of phospho-paxilin and phospho-ezrin by immunofluorescence evidenced changes in their localization and disappearance of ezrin-marked lamellipodia in CsA-treated cultures (supplementary Fig. 2).

Deregulation of FAK phosphorylation and activity may lead to dysfunction of adhesion contacts during cell movement. Paxilin is one of the cytoskeletal components of the focal adhesion and a direct substrate of FAK. Immunoblot and densitometry show the reduction of the level of phosphorylated paxilin (Tyr118 in CsA-treated cultures (Fig. 5C). Immunofluorescence for phospho-ezrin (Tyr353), another substrate of FAK and protein abundant in invadopodia, demonstrated diffused staining and disorganization of adhesion contacts in CsA-treated cultures (Supplementary data 2).

3.4. CsA reduces MMP-2 activity and downregulates MMP-2 and MT1-MMP expression in LN229 glioblastoma cells

Elevated levels of MMP-2 expression and activity correlate both with increased invasiveness of glioma cell lines [42] and with tumor grade [43]. Therefore, conditioned media from LN229 cultures untreated or treated for 18 and 24 h with 5 μM CsA or 30 μM LY294002 were collected and processed for gelatin zymography. The effect of CsA on proteolytic activity of MMP-2 was clearly visible (Fig. 6A and B), in contrast to barely detectable pro-MMP-2 or MMP-9 (data not shown). The amount of active MMP-2 was reduced in cultures treated with 5 μM CsA or 30 μM LY294002 (Fig. 6A). Quantification of densitometric results from 3 independent experiments confirmed 30–40% reduction of the MMP-2 gelatinolytic activity in drug-treated cultures 18–24 h after treatment. FK506, a calcineurin inhibitor unrelated to CsA, did not affect activity of MMP-2, suggesting that an inhibitory effect of CsA does not rely on calcineurin inhibition (Fig. 6B).

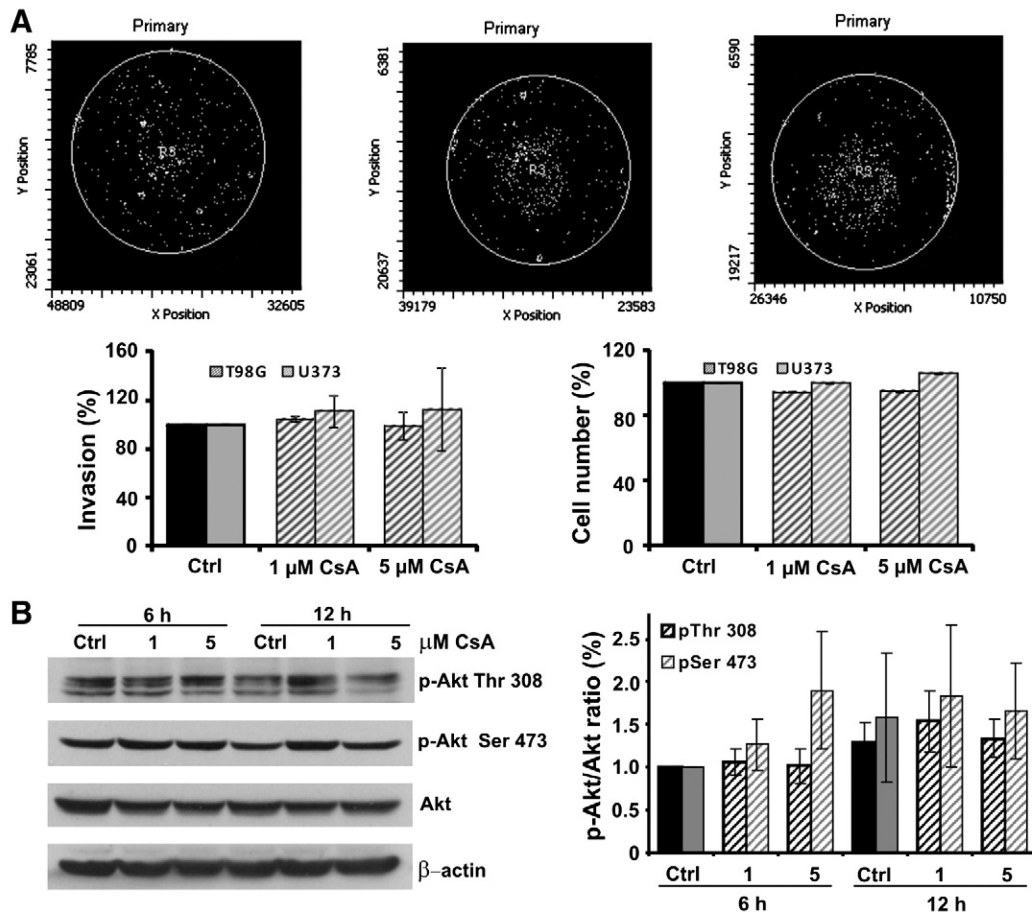

Fig. 3. CsA fails to reduce invasiveness of human T98G glioblastoma cells with constitutively high Akt phosphorylation. A. Upper panel: Representative pictures of T98G cells invading matrigel for 12 h. Cells were fixed, their nuclei stained with DAPI and counted by LSC. Lower panel: Quantification of results of T98G and U373 cell invasion and proliferation (means ± SEM, $n = 3$ experiments, each in triplicate). B. The influence of CsA on viability of glioblastoma cells determined using MTT metabolism test. The levels of total and phosphorylated Akt in T98G cells cultured in the presence or absence of CsA. Ratio of phosphorylated/total kinase calculated after densitometric analysis of immunoblots (means ± SEM, $n = 3$).

Pro-MMP-2 activation occurs by formation of the complex between membrane type 1 MMP (MT1-MMP) and tissue inhibitor of MMP-2 (TIMP-2) followed by processing of pro-MMP-2 by MT-MMP [44]. Since transcription of MMPs may be a rate-limiting factor in MMP-2 regulation, we analyzed the levels of *MMP-2* and *MT1-MMP* mRNA in LN229 cells under various conditions using a quantitative RT-PCR. A significant reduction of *MMP-2* mRNA level was observed 24 and 48 h after treatment, while *MT1-MMP* mRNA level decreased by 40% at later time point. At earlier time points *MMP-2* and *MT1-MMP* mRNA levels were similar in untreated and CsA-treated cells (Fig. 6C and D).

The influence of PI3K/Akt signaling pathway on expression of *MMP-2* and *MT1-MMP* was further analyzed in cells incubated with LY294002 or transfected with plasmids coding for dominant negative Akt mutants (ΔPHAkt or K179MAkt). Treatment with LY294002 or overexpression of dominant negative Akt kinase reduced the levels of both of *MMP-2* and *MT1-MMP* mRNAs, indicating that the expression of these genes is regulated by Akt (Fig. 7A and B). Moreover, treatment of LN229 cells with BAY11-7082 (an irreversible inhibitor of IκBα phosphorylation) for 24 h strongly reduced the expression of *MMP-2* (Fig. 7C) suggesting that it depends on NFκB signaling. An inhibitory effect of BAY11-7082 on *MT1-MMP* mRNA synthesis was observed only 48 h after treatment (data not shown). Results of Western blot analysis corroborate gene expression data and show the reduced level of MT1-MMP in CsA- and LY294002- but not BAY11-7082-treated cultures. The expression of MMP-2 was mainly reduced by LY294002; however, both CsA and BAY11-7082 reduced the MMP-2 level to some extent (Fig. 7D).

3.5. CsA downregulates NFκB activation and transcriptional activity via interference with PI-3/Akt signaling pathway

Correlation between increased Akt phosphorylation and high levels of NFκB activity in malignant gliomas has been reported [45]. The promoter regions of *MMP-9* and *MMP-2* genes contain consensus motifs for NF-κB [20]. Western blot analysis demonstrated that the level of phosphorylated IκB was rapidly downregulated by treatment of LN229 cells with 5 μM CsA in comparison to control cells (Fig. 8A and B). The NF-κB transcriptional activity (determined using a NFκB-luciferase gene reporter assay) was high in untreated cells and was strongly reduced in LN229 cells exposed to 5 μM CsA. FK506 (5 μM) did not significantly affect NFκB transcriptional activity (Fig. 8C). The treatment of cells with 10 and 30 μM LY294002 reduced NFκB-driven transcription, however less efficiently than CsA (Fig. 8D). These results confirm a link between activated Akt and activation of NFκB in LN229 cells.

3.6. MT1-MMP translocation to the cell membrane protrusions is inhibited in CsA-treated cells

The reduction of active Akt levels and decrease of MMP-2 enzymatic activity occur 18 h after CsA treatment, while drug effects on *MMP-2* and *MT1-MMP* expression were observed 24 h later. Therefore, a transcription-independent mechanism of MMP-2 regulation should be considered. Therefore, we compared subcellular localization of MT1-MMP visualized by immunofluorescence with changes in cytoskeleton rearrangements visualized by phalloidin staining of F-actin.

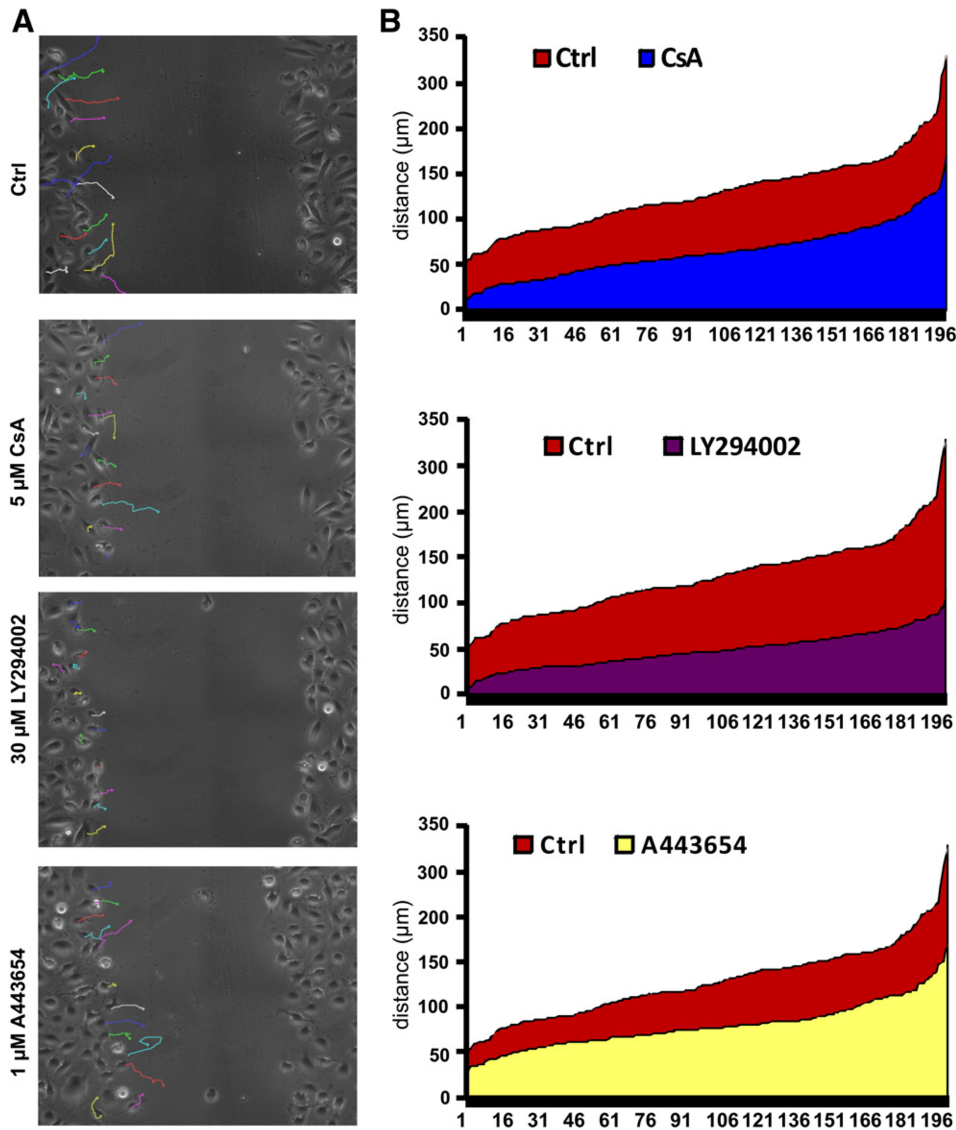

Fig. 4. CsA suppresses migration of LN229 cells through downregulation of PI3K/Akt pathway. Confluent monolayer was scratched with a pipette tip, washed and incubated with medium containing 5 μM CsA, 30 μM LY294002, 1 μM A443664 or DMSO (Ctrl). The cell migration was monitored for 18 h using time lapse live imaging technology. Pictures were taken every 30 min. Tracings of movement of a few cells are shown as an example (colored lines). The graphs represent total distance covered in 24 h by each of 196 cells tested under different conditions ($n = 3$).

Immunostaining revealed abundance of MT1-MMP in lamellipodia of control cells. It is particularly clear after merging MT1-MMP immunofluorescence with F-actin staining, which shows elongated cells with multiple membrane protrusions enriched in actin filaments (Fig. 9). In contrast, cells treated with 5 μM CsA were flatten, more stretched and membrane ruffles were not observed. In CsA-treated cultures MT1-MMP did not localize at membrane protrusions. Similar pattern of MT1-MMP immunostaining was observed in cultures treated with 30 μM LY294002. Taken together, these results demonstrate that CsA modulates directly tumor cell motility by blocking formation of membrane protrusions and translocation of MT1-MMP to the membrane ruffles.

4. Discussion

4.1. CsA reduces glioblastoma invasion via inhibition of PI3K/Akt signaling pathway

In the present study we demonstrated two major findings: (1) the inhibitory effect of CsA on Akt phosphorylation resulting in considerable reduction of migration/invasion of human malignant glioblastoma cells; (2) two mechanisms possibly underlying the effect of CsA: rapid blockade of MT1-MMP shuttling to lamellipodia leading to local modulation of MMP-2 activity, and delayed downregulation of *MMP-2* and *MT1-MMP* transcription.

Several studies have shown that targeting of Akt signaling pathway in malignant glioma cells with antisense, siRNA or small molecule inhibitors results in downregulation of tumor invasion and tumorigenesis [46,47]. However, effects of Akt signaling inhibition on invasion/motility were not separated from its effect on cellular survival/proliferation and downstream mechanisms were poorly characterized. We used CsA at the moderate concentration of 5 μM that did not affect cellular survival/proliferation. We demonstrate that the observed inhibitory effect of CsA on invasion is due to interference with PI3K/Akt signaling pathway because CsA reduced the level of active Akt as genetic or pharmacological inhibitors of PI3K/Akt signaling. Importantly, overexpression of constitutively active kinase Akt (myrAkt) or constitutively high level of active Akt in T98G and U373 cells resulting from deficiency in PTEN function abrogated

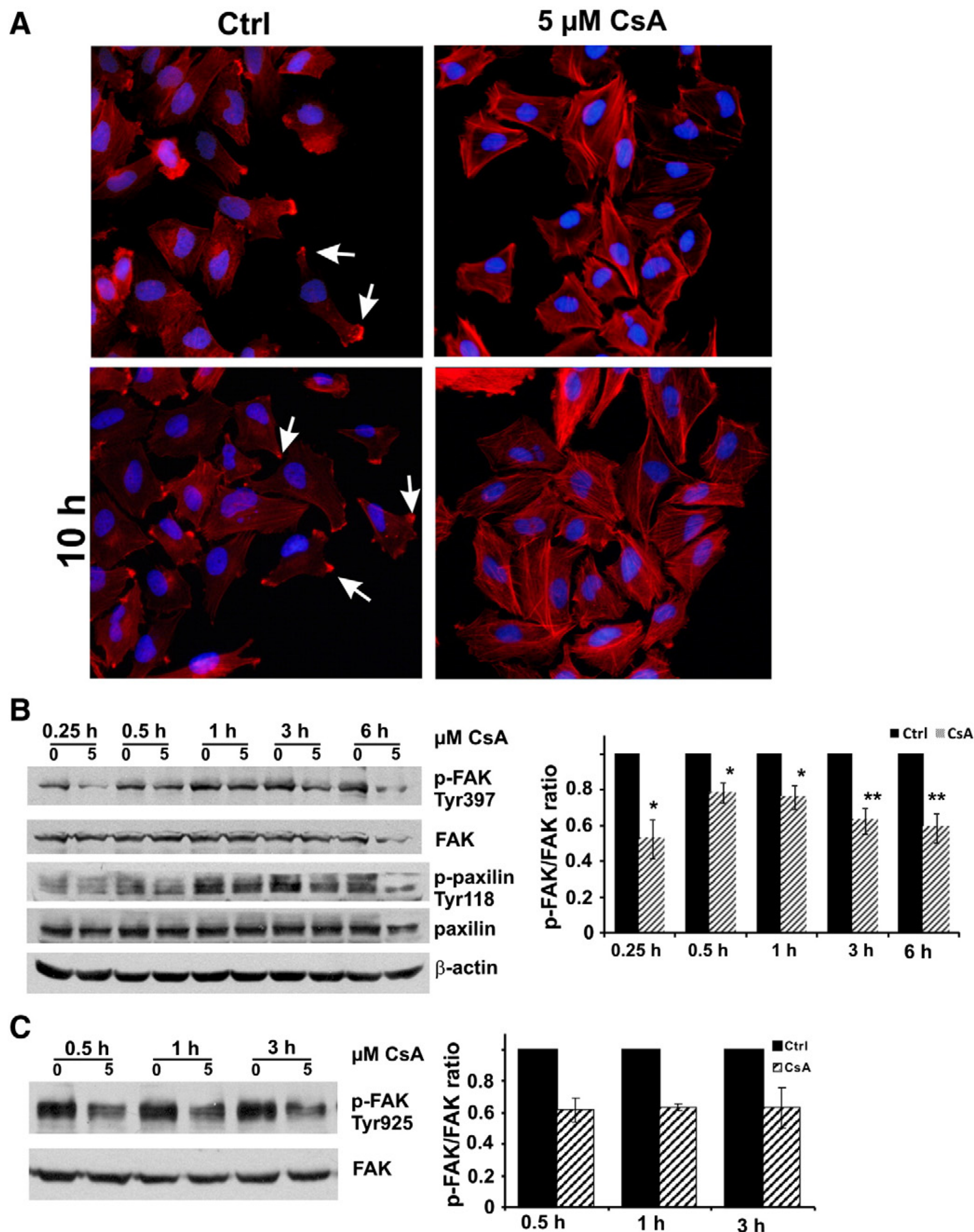

Fig. 5. CsA induces alterations of actin assembly in glioblastoma cells. Confluent monolayer was scratched with a pipette tip, washed, treated with 5 μM CsA and fixed at different time points. (A) F-actin staining with phalloidin was performed to visualize actin organization in cells. Representative pictures of migrating cells are shown. White arrows indicate membrane protrusions enriched in actin mashwork characteristic for motile cells. (B) Immunoblot shows changes in FAK and paxilin phsophorylation in CsA-treated cultures in a representative experiment. B–C Immunoblots were probed with antibodies recognizing phosphorylated and total FAK and paxilin; followed by detection of β-actin on the same blot to ensure equal loading. Densitometric evaluation of immunoblots for total and phospho-FAK is shown (means ± SEM, $n = 3$).

inhibitory effects of CsA on tumor cell invasion. It is a first demonstration of the inhibitory effect of CsA on intrinsic invasion potential and motility of human glioblastoma cells. Up to date, CsA has been shown to attenuate invasion stimulated by calcium ionophore/PMA in U251MG glioblastoma cells, by inhibiting IL-8 production in NFκB-dependent manner [48].

Using several methods we demonstrate that 5 μM CsA reduced invasion of human LN229 glioblastoma cells through matrigel matrix and cell motility in a scratch assay concomitantly with down-regulation of active Akt levels. Similarly, pharmacological inhibitors of PI3K/Akt signaling: LY294002 (PI3K inhibitor) and A443654 (Akt inhibitor) slowed down cell migration and reduction of velocity was constant throughout 24 h of experiment. This is consistent with data showing that reduction of Akt2 expression or activity in glioma cells disturbed migration and invasion due to diminished phosphorylation of girdin and cofillin, two proteins involved in actin filament remodeling [14,15].

We identified two mechanisms of inhibition of tumor invasion and motility: the one mediated by inhibition of PI3K/Akt-dependent translocation of MT1-MMP to membrane protrusions and the second one through downregulation of PI3K/Akt and NFκB-dependent *MMP-2* and *MT1-MMP* transcription. Inhibition of Akt and FAK phosphorylation by CsA or LY294002 resulted in rapid morphological changes: disappearance of lamellipodia, stabilization of adhesion contacts and cell immobilization. The observed changes in cell morphology mimic those observed in FAK-deficient cells, where inhibition of FAK signaling

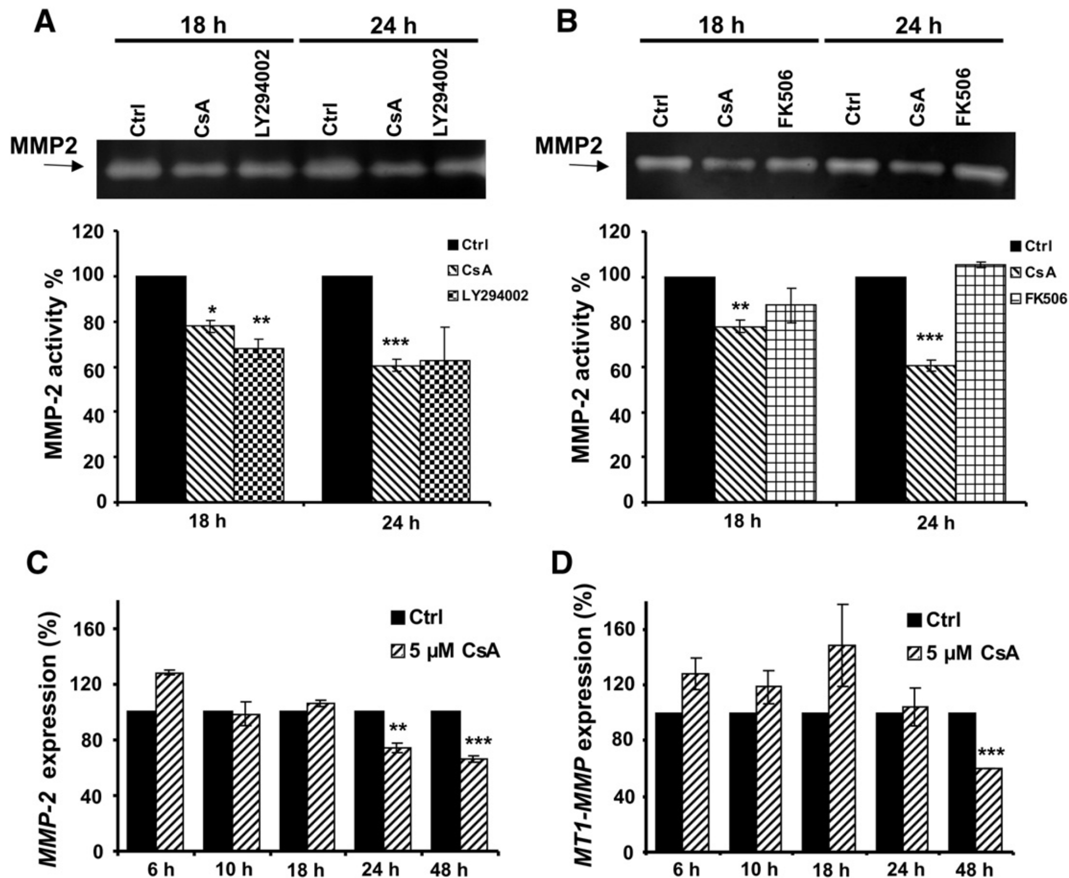

Fig. 6. Reduction of MMP-2 gelatinolytic activity and modulation of *MMP-2 and MT1-MMP* expression by CsA. A–B. Cells untreated or cultured with 5 μM CsA, 5 μM FK506 or LY294002. MMP-2 proteolytic activity was determined by gelatin zymography in conditioned media collected 18 or 24 h after treatment. An active MMP-2 protein (63 kDa) is indicated an arrow; lower panel shows densitometric analysis of the levels of MMP-2 (means ± SEM, $n = 3$). C–D. Relative expressions of *MMP-2* and *MT1-MMP* mRNAs were determined in LN229 cells untreated or treated with 5 μM CsA by a quantitative RT-PCR with TaqMan MGB probes (means ± SEM, three experiments each in triplicate).

prevents disruption of focal adhesions and formation of new ones [8–10]. FAK autophosphorylation on Tyr-397 permits interactions with a number of effectors, including Src-family kinases that phosphorylate two FAK-interacting proteins, Crk-associated substrate (CAS) and paxillin, which results in regulation of Rho-family GTPases and phosphatidylinositol 3-kinase [8–10]. Downregulation of FAK signaling preceding morphological alterations and cell immobilization is consistent with a possible role of FAK in phosphorylation of paxilin at adhesion contacts and modulation its interactions with adaptor proteins such as ezrin.

A second, delayed consequence of CsA treatment is downregulation of MMP-2 activity. While MMP-2 activity was easily detected by gelatin zymography in LN229 cells, we did not detect the activity corresponding to MMP-9. However, some studies described detection of MMP-2 and MMP-9 in glioblastoma cell lines, others studies failed to detect MMP-9 expression (f.e. in T98G cells) [26]. Upregulated expression of MMP-2 and MT1-MMP correlates with increased glioma invasion and tumor grade [18,21]. Involvement of Akt in regulation of MMP-2 and MMP-9 activities has been demonstrated in rat C6 glioma cells, in which transfection of dominant-negative or antisense AKT2 constructs reduced the production of MMP-2 and MMP-9, migration and invasion *in vitro* [14] and in intracranial gliomas *in vivo* [15]. Our results demonstrate that both CsA and LY294002 decreased MMP-2 gelatinolytic activity in human glioblastoma cells.

Activity of MMPs can be regulated at the level of *MMP-2*, *MT1-MMP or TIMP-2* transcription and production, as well as secretion or activation of pro-MMP-2 by MT1-MMP and TIMP-2 at the cell membrane surface [14,17,20,27]. However, NFκB transcription factor has been postulated as a regulator of *MT1-MMP* expression, its participation has been demonstrated only in human dermal and rabbit synovial fibroblasts [49,50]. Studies with interfering antibody antip65 NFκB showed reduction of MMP-9, urokinase-type plasminogen activator activities and invasion of glioblastoma cells [51,52]. Similarly, although Akt can phosphorylate and activate IκB kinase regulating phosphorylation of IκB that is crucial for NFκB activation [53–55], this action was not shown in glioma cells.

We demonstrate that treatment of glioblastoma cells with CsA or LY294002 reduced the level of phosphorylated IκB and NFκB-driven transcription. Thus, we postulate that downregulation of Akt activity by CsA or LY294002 leads to reduction of IκB phosphorylation, its stabilization, inhibition of NFκB transcriptional activity and reduction of NFκB-dependent *MMP-2* target gene expression. The NFκB inhibitor – BAY11-7082 – strongly reduced the *MMP-2* expression in LN229 cells (Fig. 7C). FK506 (an unrelated calcineurin inhibitor) which did not affect NFκB activity, had no effect on MMP-2 activity. It suggests that mechanism of CsA action is calcineurin-inhibition independent.

4.2. Impairment of MT1-MMP shuttling to invadopodia may locally inhibit MMP-2

MT1-MMP is a cell surface activator of pro-MMP-2 and has been implicated in the proteolytic cleavage of many extracellular and membrane-associated substrates. MT1-MMP activation is complex and involves regulation of MT1-MMP expression, trafficking, and/or endocytosis after stimulation by multiple factors [56,57]. The enhanced surface expression of MT1-MMP in lamellipodia-like

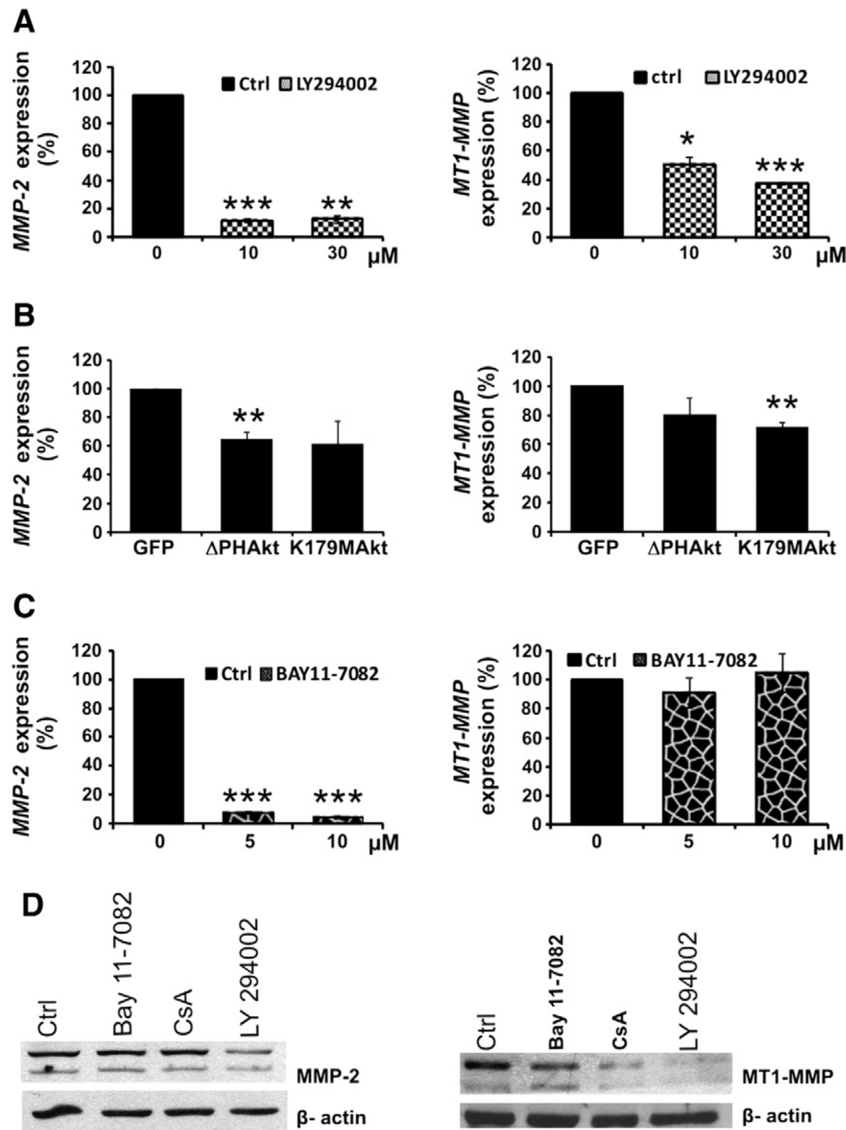

Fig. 7. Contribution of Akt and NFκB in regulation of *MMP2* and *MT1-MMP* expression. Relative expressions of *MMP-2* and *MT1-MMP* mRNAs in LN229 treated for 24 h with 10 or 30 μM LY294002 (A), transfected with plasmids coding for mutated Akt (ΔPHAkt or K179MAkt) (B) or incubated for 24 h with BAY11-7082 (C). Relative expression of *MMP-2* and *MT1-MMP* mRNAs was determined using quantitative RT-PCR with TaqMan MGB probes; data show means ± SEM of three experiments in A, C, and two experiments in B (each in triplicate). (D) Representative immunoblot shows the levels of MMP-2 and MT1-MMP proteins in glioblastoma cells exposed for 48 h to 5 μM CsA, 10 μM BAY11-7082 and 30 μM LY294002; similar results were found in three experiments.

membrane protrusions was observed in control glioblastoma cells contrary to CsA-treated cultures, where MT1-MMP immunofluorescence was diffused (Fig. 9). Moreover, CsA-treated cells did not form cell protrusions. F-actin staining revealed that in contrast to control cells, having multiple membrane protrusions and polarized lamellipodia, treated cells exhibited more stretched cell shape with strong actin stress fibers. A growing evidence demonstrates an importance of MT1-MMP trafficking for local modulation of protease activity in invadopodia [58]. However, overall inhibition of MMP-2 activity by CsA was 40% (in gel zymography assay), disruption of local activity of MMP-2 at invadopodia together with stabilization of adhesion contacts resulting in cell immobilization may produce inhibitory mechanisms sufficiently affecting cell migration and invasion.

5. Conclusions

• We show here that CsA, through a mechanism yet unknown but independent of calcineurin inhibition, downregulates phsophorylation of Akt and FAK, as well as downstream signaling in malignant glioblastoma cells that result in impairment of cell motility/invasion and reduction of MMP-2 activity. • More importantly, this study demonstrates that PI3K/Akt signaling contributes to translocation of MT1-MMP that can locally influence MMP-2 proteolytic activity and facilitate formation of invadopodia. • Furthermore, we established that the expression of MMP-2 is regulated in Akt- and NFκB-dependent manner, while MT1-MMP in Akt-dependent in glioblastoma cells. Understanding of molecular determinants of glioblastoma cell invasion and proven anti-tumor efficacy of CsA in animal model help to move forward to translational research.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.01.020.

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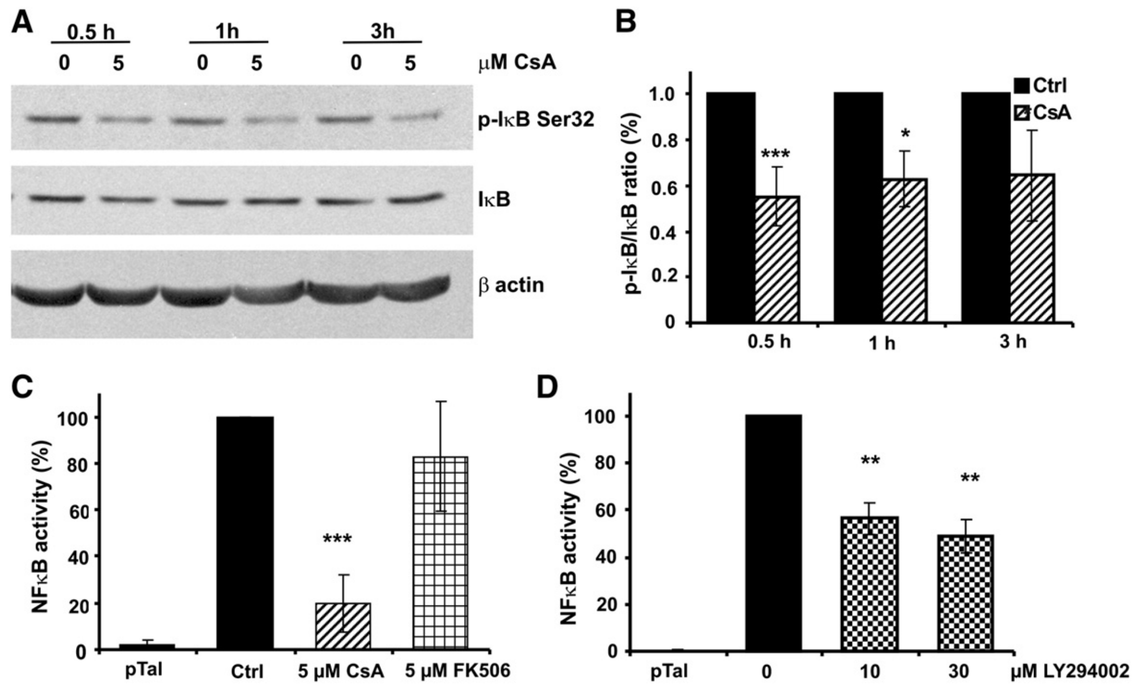

Fig. 8. CsA suppresses transcriptional activity of NFκB via inhibition of PI3K/Akt signaling pathway. A. The levels of phosphorylated and total IκB in total protein lysates from LN229 cells cultured in the presence or absence of 5 μM CsA. B. Densitometric evaluation of immunoblots (means ± SEM, $n=3$). C–D. The expression of NFκB-driven luciferase in LN229 cells treated with 5 μM CsA, 5 μM FK506, 10 or 30 μM LY294002 (means ± SEM, $n=3$).

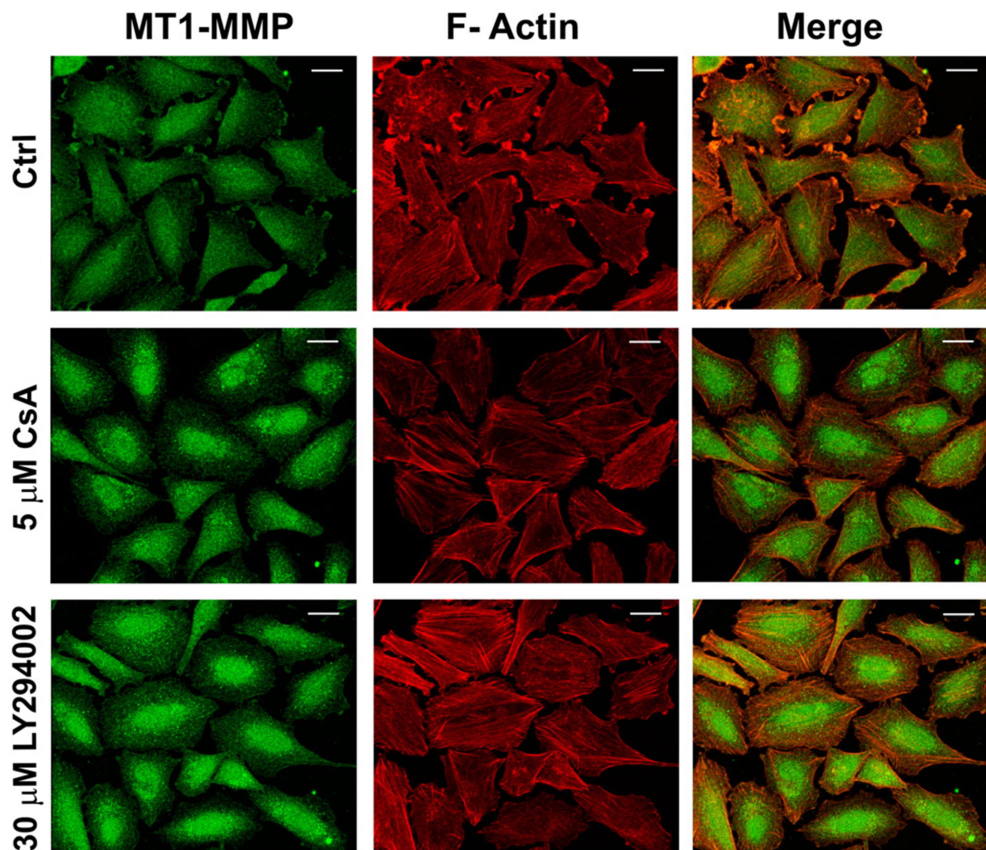

Fig. 9. CsA and LY29400 block formation of membrane protrusions, and translocation of MT1-MMP. Cellular localization of MT1-MMP revealed by immunocytochemistry in LN229 cells treated with 5 μM CsA or 30 μM LY294002 (or DMSO for control cells). Cells were stained with anti-MT1-MMP antibody followed by a secondary antibody conjugated with FITC; F-actin was stained with phalloidin. White arrows indicate membrane protrusions with abundance of short actin filaments and of MT1-MMP protein. Scale bar represents 20 μm.

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References

[1] M. Nakada, S. Nakada, T. Demuth, N.L. Tran, D.B. Hoelzinger, M.E. Berens, Molecular targets of glioma invasion, Cell. Mol. Life Sci. 64 (2007) 458–478.
[2] S.S. Stylli, A.H. Kaye, P. Lock, Invadopodia: at the cutting edge of tumour invasion, J. Clin. Neurosci. 15 (2008) 725–737.
[3] W.G. King, M.D. Mattaliano, T.O. Chan, P.N. Tsichlis, J.S. Brugge, Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation, Mol. Cell. Biol. 17 (1997) 4406–4418.
[4] D. Kim, S. Kim, H. Koh, S.O. Yoon, A.S. Chung, K.S. Cho, J. Chung, Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production, FASEB J. 15 (2001) 1953–1962.
[5] M. Natarajan, T.P. Hecker, C.L. Gladson, FAK signaling in anaplastic astrocytoma and glioblastoma tumors, Cancer J. 9 (2003) 126–133.
[6] D.A. Hsia, S.K. Mitra, C.R. Hauck, D.N. Streblow, J.A. Nelson, D. Ilic, S. Huang, E. Li, G.R. Nemerow, J. Leng, K.S. Spencer, D.A. Cheresh, D.D. Schlaepfer, Differential regulation of cell motility and invasion by FAK, J. Cell Biol. 160 (2003) 753–767.
[7] J. Gu, M. Tamura, R. Pankov, E.H. Danen, T. Takino, K. Matsumoto, K.M. Yamada, Shc and FAK differentially regulate cell motility and directionality modulated by PTEN, J. Cell Biol. 146 (1999) 389–403.
[8] D.D. Schlaepfer, S.K. Mitra, Multiple connections link FAK to cell motility and invasion, Curr. Opin. Genet. Dev. 14 (2004) 92–101.
[9] S.K. Mitra, D.A. Hanson, D.D. Schlaepfer, Focal adhesion kinase: in command and control of cell motility, Nat. Rev. Mol. Cell Biol. 6 (2005) 56–68.
[10] S.K. Mitra, D.D. Schlaepfer, Integrin-regulated FAK-Src signaling in normal and cancer cells, Curr. Opin. Cell Biol. 18 (2006) 516–523.
[11] G. Scita, P. Tenca, E. Frittoli, A. Tocchetti, M. Innocenti, G. Giardina, P.P. Di Fiore, Signaling from Ras to Rac and beyond: not just a matter of GEFs, EMBO J. 19 (2000) 2393–2398.
[12] Y. Qian, L. Corum, Q. Meng, J. Blenis, J.Z. Zheng, X. Shi, D.C. Flynn, B.H. Jiang, PI3K induced actin filament remodeling through Akt and p70S6K1: implication of essential role in cell migration, Am. J. Physiol. Cell Physiol. 286 (2004) C153–C163.
[13] K.Z. Wang, N. Wara-Aswapati, J.A. Boch, Y. Yoshida, C.D. Hu, D.L. Galson, P.E. Auron, TRAF6 activation of PI 3-kinase-dependent cytoskeletal changes is cooperative with Ras and is mediated by an interaction with cytoplasmic Src, J. Cell Sci. 119 (2006) 1579–1591.
[14] P. Pu, C. Kang, J. Li, H. Jiang, Antisense and dominant-negative AKT2 cDNA inhibits glioma cell invasion, Tumour Biol. 25 (2004) 172–178.
[15] P. Pu, C. Kang, J. Li, H. Jiang, J. Cheng, The effects of antisense AKT2 RNA on the inhibition of malignant glioma cell growth in vitro and in vivo, J. Neurooncol. 76 (2006) 1–11.
[16] J.S. Rao, Molecular mechanisms of glioma invasiveness: the role of proteases, Nat. Rev. Cancer 3 (2003) 489–501.
[17] A.T. Belien, P.A. Paganetti, M.E. Schwab, Membrane-type 1 matrix metalloprotease (MT1-MMP) enables invasive migration of glioma cells in central nervous system white matter, J. Cell Biol. 144 (1999) 373–384.
[18] P. Guo, Y. Imanishi, F.C. Cackowski, M.J. Jarzynka, H.Q. Tao, R. Nishikawa, T. Hirose, B. Hu, S.Y. Cheng, Up-regulation of angiopoietin-2, matrix metalloprotease-2, membrane type 1 metalloprotease, and laminin 5 gamma 2 correlates with the invasiveness of human glioma, Am. J. Pathol. 166 (2005) 877–890.
[19] T.E. VanMeter, H.K. Rooprai, M.M. Kibble, H.L. Fillmore, W.C. Broaddus, G.J. Pilkington, The role of matrix metalloproteinase genes in glioma invasion: co-dependent and interactive proteolysis, J. Neurooncol. 53 (2001) 213–235.
[20] I.M. Clark, T.E. Swingler, C.L. Sampieri, D.R. Edwards, The regulation of matrix metalloproteinases and their inhibitors, Int. J. Biochem. Cell Biol. 40 (2008) 1362–1378.
[21] M. Nakada, H. Nakamura, E. Ikeda, N. Fujimoto, J. Yamashita, H. Sato, M. Seiki, Y. Okada, Expression and tissue localization of membrane-type 1, 2, and 3 matrix metalloproteinases in human astrocytic tumors, Am. J. Pathol. 154 (1999) 417–428.
[22] C. Chang, Z. Werb, The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis, Trends Cell Biol. 11 (2001) S37–S43.
[23] S. Philip, A. Bulbule, G.C. Kundu, Matrix metalloproteinase-2: mechanism and regulation of NF-kappaB-mediated activation and its role in cell motility and ECM-invasion, Glycoconj. J. 21 (2004) 429–441.
[24] S. Philip, A. Bulbule, G.C. Kundu, Osteopontin stimulates tumor growth and activation of promatrix metalloproteinase-2 through nuclear factor-kappa B-mediated induction of membrane type 1 matrix metalloproteinase in murine melanoma cells, J. Biol. Chem. 276 (2001) 44926–44935.
[25] C. Yan, D.D. Boyd, Regulation of matrix metalloproteinase gene expression, J. Cell. Physiol. 211 (2007) 19–26.
[26] M. Dziembowska, M. Danilkiewicz, A. Wesolowska, A. Zupanska, S. Chouaib, B. Kaminska, Cross-talk between Smad and p38 MAPK signalling in transforming growth factor beta signal transduction in human glioblastoma cells, Biochem. Biophys. Res. Commun. 354 (2007) 1101–1106.
[27] H.L. Fillmore, T.E. VanMeter, W.C. Broaddus, Membrane-type matrix metalloproteinases (MT-MMPs): expression and function during glioma invasion, J. Neurooncol. 53 (2001) 187–202.
[28] J.L. English, Z. Kassiri, I. Koskivirta, S.J. Atkinson, M. Di Grappa, P.D. Soloway, H. Nagase, E. Vuorio, G. Murphy, R. Khokha, Individual Timp deficiencies differentially impact pro-MMP-2 activation, J. Biol. Chem. 281 (2006) 10337–10346.
[29] G. Mosieniak, I. Figiel, B. Kaminska, Cyclosporin A, an immunosuppressive drug, induces programmed cell death in rat C6 glioma cells by a mechanism that involves the AP-1 transcription factor, J. Neurochem. 68 (1997) 1142–1149.
[30] G. Mosieniak, B. Pyrzynska, B. Kaminska, Nuclear factor of activated T cells (NFAT) as a new component of the signal transduction pathway in glioma cells, J. Neurochem. 71 (1998) 134–141.
[31] B. Pyrzynska, G. Mosieniak, B. Kaminska, Changes of the trans-activating potential of AP-1 transcription factor during cyclosporin A-induced apoptosis of glioma cells are mediated by phosphorylation and alterations of AP-1 composition, J. Neurochem. 74 (2000) 42–51.
[32] B. Pyrzynska, M. Serrano, A.C. Martinez, B. Kaminska, Tumor suppressor p53 mediates apoptotic cell death triggered by cyclosporin A, J. Biol. Chem. 277 (2002) 14102–14108.
[33] I. Ciechomska, B. Pyrzynska, P. Kazmierczak, B. Kaminska, Inhibition of Akt kinase signalling and activation of Forkhead are indispensable for upregulation of FasL expression in apoptosis of glioma cells, Oncogene 22 (2003) 7617–7627.
[34] M. Sliwa, D. Markovic, K. Gabrusiewicz, M. Synowitz, R. Glass, M. Zawadzka, A. Wesolowska, H. Kettenmann, B. Kaminska, The invasion promoting effect of microglia on glioblastoma cells is inhibited by cyclosporin A, Brain 130 (2007) 476–489.
[35] A. Ellert-Miklaszewska, B. Kaminska, L. Konarska, Cannabinoids down-regulate PI3K/Akt and Erk signalling pathways and activate proapoptotic function of Bad protein, Cell. Signal. 17 (2005) 25–37.
[36] K. Szydlowska, M. Zawadzka, B. Kaminska, Neuroprotectant FK506 inhibits glutamate-induced apoptosis of astrocytes in vitro and in vivo, J. Neurochem. 99 (2006) 965–975.
[37] T.F. Franke, S.I. Yang, T.O. Chan, K. Datta, A. Kazlauskas, D.K. Morrison, D.R. Kaplan, P.N. Tsichlis, The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase, Cell 81 (1995) 727–736.
[38] T.F. Franke, D.R. Kaplan, L.C. Cantley, PI3K: downstream AKTion blocks apoptosis, Cell 88 (1997) 435–437.
[39] G. Kulik, A. Klippel, M.J. Weber, Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt, Mol. Cell. Biol. 17 (1997) 1595–1606.
[40] A. Wesolowska, A. Kwiatkowska, L. Slomnicki, M. Dembinski, A. Master, M. Sliwa, K. Franciszkiewicz, S. Chouaib, B. Kaminska, Microglia-derived TGF-beta as an important regulator of glioblastoma invasion—an inhibition of TGF-beta-dependent effects by shRNA against human TGF-beta type II receptor, Oncogene 27 (2008) 918–930.
[41] Y. Luo, A.R. Shoemaker, X. Liu, K.W. Woods, S.A. Thomas, R. de Jong, E.K. Han, T. Li, V.S. Stoll, J.A. Powlas, A. Oleksijew, M.J. Mitten, Y. Shi, R. Guan, T.P. McGonigal, V. Klinghofer, E.F. Johnson, J.D. Leverson, J.J. Bouska, M. Mamo, R.A. Smith, E.E. Gramling-Evans, B.A. Zinker, A.K. Mika, P.T. Nguyen, T. Oltersdorf, S.H. Rosenberg, Q. Li, V.L. Giranda, Potent and selective inhibitors of Akt kinases slow the progress of tumors in vivo, Mol. Cancer Ther. 4 (2005) 977–986.
[42] K. Lampert, U. Machein, M.R. Machein, W. Conca, H.H. Peter, B. Volk, Expression of matrix metalloproteinases and their tissue inhibitors in human brain tumors, Am. J. Pathol. 153 (1998) 429–437.
[43] C. Wild-Bode, M. Weller, W. Wick, Molecular determinants of glioma cell migration and invasion, J. Neurosurg. 94 (2001) 978–984.
[44] Y. Nishida, H. Miyamori, E.W. Thompson, T. Takino, Y. Endo, H. Sato, Activation of matrix metalloproteinase-2 (MMP-2) by membrane type 1 matrix metalloproteinase through an artificial receptor for proMMP-2 generates active MMP-2, Cancer Res. 68 (2008) 9096–9104.
[45] H. Wang, H. Wang, W. Zhang, H.J. Huang, W.S. Liao, G.N. Fuller, Analysis of the activation status of Akt, NFkappaB, and Stat3 in human diffuse gliomas, Lab. Invest. 84 (2004) 941–951.
[46] Q. Shi, S. Bao, L. Song, Q. Wu, D.D. Bigner, A.B. Hjelmeland, J.N. Rich, Targeting SPARC expression decreases glioma cellular survival and invasion associated with reduced activities of FAK and ILK kinases, Oncogene 26 (2007) 4084–4094.
[47] Q. Shi, A.B. Hjelmeland, S.T. Keir, L. Song, S. Wickman, D. Jackson, O. Ohmori, D.D. Bigner, H.S. Friedman, J.N. Rich, A novel low-molecular weight inhibitor of focal adhesion kinase, TAE226, inhibits glioma growth, Mol. Carcinog. 46 (2007) 488–496.
[48] K. Wakabayashi, F. Kambe, X. Cao, R. Murakami, H. Mitsuyama, T. Nagaya, K. Saito, J. Yoshida, H. Seo, Inhibitory effects of cyclosporin A on calcium mobilization-dependent interleukin-8 expression and invasive potential of human glioblastoma U251MG cells, Oncogene 23 (2004) 6924–6932.
[49] Y.P. Han, T.L. Tuan, H. Wu, M. Hughes, W.L. Garner, TNF-alpha stimulates activation of pro-MMP2 in human skin through NF-(kappa)B mediated induction of MT1-MMP, J. Cell Sci. 114 (2001) 131–139.
[50] M.P. Vincenti, C.I. Coon, C.E. Brinckerhoff, Nuclear factor kappaB/p50 activates an element in the distal matrix metalloproteinase 1 promoter in interleukin-1beta-stimulated synovial fibroblasts, Arthritis Rheum. 41 (1998) 1987–1994.
[51] L. Li, C.S. Gondi, D.H. Dinh, W.C. Olivero, M. Gujrati, J.S. Rao, Transfection with anti-p65 intrabody suppresses invasion and angiogenesis in glioma cells by blocking nuclear factor-kappaB transcriptional activity, Clin. Cancer Res. 13 (2007) 2178–2190.
[52] B. Raychaudhuri, Y. Han, T. Lu, M.A. Vogelbaum, Aberrant constitutive activation of nuclear factor kappaB in glioblastoma multiforme drives invasive phenotype, J. Neurooncol. 85 (2007) 39–47.
[53] F. Lefranc, J. Brotchi, R. Kiss, Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis, J. Clin. Oncol. 23 (2005) 2411–2422.

[54] R. Ravi, A. Bedi, NF-kappaB in cancer—a friend turned foe, Drug Resist. Updat. 7 (2004) 53–67.
[55] K.M. Nicholson, N.G. Anderson, The protein kinase B/Akt signalling pathway in human malignancy, Cell. Signal. 14 (2002) 381–395.
[56] E.M. Tam, C.J. Morrison, Y.I. Wu, M.S. Stack, C.M. Overall, Membrane protease proteomics: isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates, Proc. Natl Acad. Sci. USA 101 (2004) 6917–6922.
[57] M.V. Barbolina, M.S. Stack, Membrane type 1-matrix metalloproteinase: substrate diversity in pericellular proteolysis, Semin. Cell Dev. Biol. 19 (2008) 24–33.
[58] R. Poincloux, F. Lizarraga, P. Chavrier, Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia, J. Cell Sci. 122 (2009) 3015–3024.