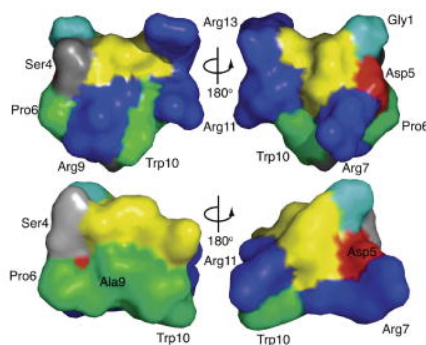


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Silencing of the MT1-MMP/ G6PT axis suppresses calcium mobilization by sphingosine-1-phosphate in glioblastoma cells

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Abstract The contributions of membrane type-1 matrix metalloproteinase (MT1-MMP) and of the glucose-6-phosphate transporter (G6PT) in sphingosine-1-phosphate (S1P)-mediated Ca^{2+} mobilization were assessed in glioblastoma cells. We show that gene silencing of MT1-MMP or G6PT decreased the extent of S1P-induced Ca^{2+} mobilization, chemotaxis, and extracellular signal-related kinase phosphorylation. Chlorogenic acid and (–)-epigallocatechin-3-gallate, two diet-derived inhibitors of G6PT and of MT1-MMP, respectively, reduced S1P-mediated Ca^{2+} mobilization. An intact MT1-MMP/G6PT signaling axis is thus required for efficient Ca^{2+} mobilization in response to bioactive lipids such as S1P. Targeted inhibition of either MT1-MMP or G6PT may lead to reduced infiltrative and invasive properties of brain tumor cells.

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Keywords: MT1-MMP; S1P; Glucose-6-phosphate transporter; Calcium mobilization; Cell migration

1. Introduction

Glioblastoma multiforme is the most commonly occurring primary brain tumor in adults and is highly malignant, displaying increased vascularization, aggressive growth and invasion into surrounding brain tissue [1]. Among the serum-derived lipid and growth factors that exhibit chemotactic influences towards glioblastoma cells, sphingosine-1-phosphate (S1P) is a bioactive lipid that stimulates growth and invasiveness of glioblastoma cell and that signals through a family of five G-protein-coupled receptors termed S1PR(1-5) [2]. The S1P receptor (S1PR) contribution to intracellular calcium (Ca^{2+}) homeostasis and stimulation of glioblastoma cell proliferation

correlated with activation of extracellular signal-regulated protein kinase (ERK) MAP kinase [3,4]. Among the two sphingosine kinase (SphK) isoforms, SphK-1 correlates with short survival of glioblastoma patients [5,6], and is overexpressed in brain tumor-derived endothelial cells [7]. Consequently, the generation of S1P is hypothesized to contribute to the acquisition and the maintenance of the multidrug resistance phenotype in brain tumors as well as to exert chemotactic migration effects in numerous types of cells including ovarian cancer cells [8], HT-1080 fibrosarcoma cells [9], U-87 glioblastoma cells [10] and mesenchymal stromal cells [11]. The molecular players that link the control of S1P-mediated intracellular Ca^{2+} mobilization to cell migration and to extracellular matrix (ECM) degradation remain to be investigated.

Recent evidence from our laboratory identified a crucial signaling axis composed of a cell surface membrane-bound matrix metalloproteinase (MT1-MMP) and of an endoplasmic reticulum (ER)-embedded glucose-6-phosphate transporter (G6PT) which functions as a bioswitch in the regulation of glioblastoma cell survival and migration [12]. Interestingly, genetic deficiencies in G6PT cause a clinical condition where patients have impaired neutrophil chemotaxis and Ca^{2+} flux [13]. These findings, along with G6PT's endoplasmic/sarcoplasmic reticulum Ca^{2+} sequestration function [14], demonstrate that G6PT is not just a G6P transport protein but may also potentially contribute to intracellular Ca^{2+} homeostasis. This explains, in part, why chlorogenic acid (CHL), among the most potent functional inhibitor of G6PT [15], inhibited S1P-induced glioblastoma cell migration as well as the rapid, S1P-induced ERK phosphorylation in glioblastoma cells [16].

In this study, we specifically assessed whether gene silencing of MT1-MMP or G6PT may affect S1P-mediated intracellular Ca^{2+} mobilization and glioblastoma cell migration in response to S1P. We report that an intact, functional MT1-MMP/G6PT signaling axis is required for efficient Ca^{2+} mobilization in response to circulating bioactive lipids such as S1P, suggesting that targeted inhibition of either MT1-MMP or G6PT function may lead to reduced infiltrative and invasive properties of brain tumor cells.

2. Materials and methods

2.1. Materials

Sodium dodecylsulfate (SDS), bovine serum albumin (BSA), PD98059, U73122, Y27632, sphingosine-1-phosphate (S1P), thapsigargin and verapamil were purchased from Sigma (Oakville, ON). TRIZOL

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Abbreviations: CHL, chlorogenic acid; ECM, extracellular matrix; EGCG, (–)-epigallocatechin-3-gallate; ER, endoplasmic reticulum; ERK, extracellular signal-related kinase 1/2; bFGF, basic fibroblast growth factor; G6PT, glucose-6-phosphate transporter; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 MMP; S1P, sphingosine-1-phosphate; S1PR, S1P receptor; SphK, sphingosine kinase

reagent for total RNA extraction was from Life Technologies. The anti-phospho-ERK antibody was from Cell Signaling Technology (Beverly, MA) while the polyclonal anti-ERK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and used for immunoblotting procedures as described previously [16].

2.2. Transfection method and RNA interference

The U-87 glioblastoma cells were transiently transfected with the cDNA constructs encoding either MT1-MMP [17] or G6PT [18], or with 20 nM siRNA (see below) using Lipofectamine 2000 (Invitrogen). The occurrence of MT1-MMP or G6PT specific gene knockdown was evaluated by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) with the One-Step RT-PCR Kit (Invitrogen) and validated by assessing MT1-MMP-mediated proMMP-2 activation with concanavalin-A using gelatin zymography of the conditioned media [11]. Mock transfections of U-87 cultures with pcDNA (3.1+) were used as controls. Small interfering RNAs against MT1-MMP (siMT1-MMP) and G6PT (siG6PT), and mismatch siRNA were synthesized by EZBiolab Inc. (Westfield) and annealed to form duplexes. The sequence of the siMT1-MMP and siG6PT used in this study are as follows: siMT1-MMP: 5'-CCAGAAGCUGAAGGUAGAAAdTdT-3' (sense) and 5'-UUCUACCUUCAGCUUCUGGdTdT-3' (antisense); siG6PT: 5'-CGAAACAUCGACCAAGAdTdT-3' (sense) and 5'-UCUUGGUGCGGAUGUUUCGdTdT-3' (antisense) [12,16].

2.3. Calcium mobilization assay

U-87 glioblastoma cells were cultured in 4-well Labtek chambers (Nalge Nunc). Culture media was then changed to HEPES-buffered saline solution (HBSS) (mM: 121 NaCl, 5.4 KCl, 0.8 Mg₂SO₄, 25 HEPES, 1.8 CaCl₂ and 6.0 NaHCO₃ at pH 7.3) and loaded with 2 μM Fluo-3 AM (Molecular Probes) with an equivalent volume of 20% Pluronic F127 and 5 μM Verapamil for 45 min at 37 °C in the dark. Thereafter, cells were washed with HBSS and the loaded dye was allowed to deesterify for 45 min at room temperature in the dark. Following transfer to a Ca²⁺-free solution (HBSS without CaCl₂), additions were made in an open chamber configuration at room temperature. The cells were examined with a laser scanning confocal (Bio-Rad) Nikon TE300 microscope with an Apochromatic 40X N.A. 1.0 objective lens. Fluorescence was excited by an argon laser at 488 nm and emission was collected with a 515 nm filter. Data were analyzed with Laser Sharp 2.1 T, Time Course 1.0 software. The individual fluorescence intensities of 20–30 cells per field were used to obtain a mean fluorescence for each experiment.

2.4. Analysis of U-87 glioblastoma cell migration

U-87 cell migration was assessed using modified Boyden chambers. The upper surfaces of Transwell inserts (8 μm pore size; Costar, Acton, MA) were pre-coated with 0.2% gelatin. The Transwells were then assembled in a 24-well plate and the lower chamber was filled with serum-free MEM or MEM supplemented with either 1 μM S1P or 20 ng/ml basic fibroblast growth factor (bFGF). Cells were collected by trypsinization, washed and resuspended in serum-free medium; 10⁵ cells were then inoculated onto the upper side of each modified Boyden chamber. The plates were placed at 37 °C in 5% CO₂/95% air, and migration left to proceed for 6 h. Cells that had migrated to the lower surfaces of the filters were fixed with 10% formalin phosphate and stained with 0.1% crystal violet-20% methanol (v/v). Images of at least five random fields per filter were digitized (100× magnification). The average number of migrating cells per field was quantified using Northern Eclipse software (Empix Imaging Inc.). Migration data are expressed as a mean value derived from at least four independent experiments.

3. Results

3.1. Pharmacological inhibition of sphingosine-1-phosphate-induced calcium mobilization

ERK phosphorylation represents, among the early intracellular Ca²⁺-dependent events, an efficient means by which to examine sphingosine-1-phosphate (S1P) effects. U-87 glioblastoma cells were thus exposed for 2 min to various doses of S1P (Fig. 1A) and the extent of ERK phosphorylation found optimal at 1 μM S1P (Fig. 1B). That S1P concentration was then used throughout the remaining study, and the contribution of the S1P G-protein-coupled receptors (S1PRs) intracellular signaling pathways involving the phospholipase C, MAPK and RhoA/ROK assessed [19]. While Ca²⁺ mobilization upon S1P treatment was rapidly and transiently induced in the control condition, that mobilization was inhibited in PD98059 (a MEK kinase inhibitor), Y27632 (a RhoA/ROK inhibitor), and in U73122 (a phospholipase C antagonist)

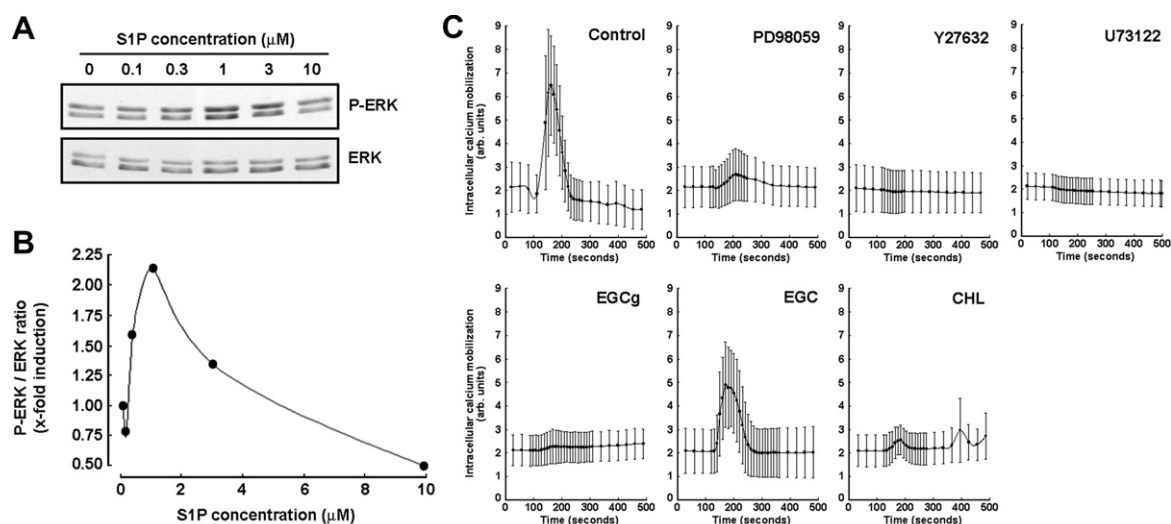


Fig. 1. Pharmacological inhibition of sphingosine-1-phosphate-induced calcium mobilization. (A) Increasing concentrations of sphingosine-1-phosphate (S1P) were used to treat serum-starved U-87 glioblastoma cells for 2 min. Following treatment, 20 μg of cell lysates were loaded on SDS-PAGE and immunoblotting was performed to detect the extent of ERK phosphorylation. (B) Scanning densitometry was used to quantify ERK phosphorylation, and data are represented by a ratio of P-ERK/ERK from one representative experiment. (C) U-87 glioblastoma cell cultures and Ca²⁺ mobilization were induced with 1 μM S1P as described in Section 2. Cells were pre-treated with either vehicle (Control) or 10 μM EGCg, EGC, CHL, PD98059, U73122, or Y27632 for 30 min prior to stimulation with S1P. A representative Ca²⁺ mobilization profile, out of three experiments, is shown.

pre-treated cells (Fig. 1C). This suggested that functional signaling cascades for S1P were required to elicit proper Ca^{2+} mobilization in part through a receptor-dependent mechanism acting via G(i)/ERK pathway in conjunction with activation of Rho- and phospholipase C-mediated signals. When cells were treated with either epigallocatechin-3-gallate (EGCg) or chlorogenic acid (CHL), two diet-derived molecules shown to inhibit, respectively MT1-MMP or microsomal G6P transport functions [20–22], we also observed a decrease in S1P-induced Ca^{2+} mobilization (Fig. 1C). Interestingly, epigallocatechin (EGC) failed to inhibit S1P-induced Ca^{2+} mobilization suggesting a structure–function relationship of the gallate moiety previously shown to target the cooperative functions of MT1-MMP [23]. Following each of the above tested inhibitors, Ca^{2+} release from ER was induced as a control by the addition of 5 μ M Thapsigargin (not shown).

3.2. Gene silencing of MT1-MMP and of G6PT impairs sphingosine-1-phosphate-mediated calcium mobilization

The relevance of each MT1-MMP and G6PT was assessed with respect to Ca^{2+} mobilization by S1P. Gene silencing strategies using specific siRNA was used as previously described [18,24] and efficiency of gene silencing confirmed by RT-PCR (Fig. 2A). MT1-MMP function was efficiently reduced by gene silencing since concanavalin-A, a known MT1-MMP inducer and proMMP-2 activator [25], was unable to trigger proMMP-2 activation (Fig. 2B, siMT1-MMP). In contrast, cells that overexpressed recombinant MT1-MMP were found to activate proMMP-2 into MMP-2 and this effect was amplified by concanavalin-A treatment (Fig. 2B). Gene silencing of G6PT did not affect concanavalin-A's ability to induce proMMP-2 activation. However, transient G6PT cDNA cell transfections lead to decreased proMMP-2 activation by concanavalin-A (Fig. 2B) in agreement with the prosurvival role played by G6PT [12]. When S1P-induced Ca^{2+} mobiliza-

tion was assessed, both siMT1-MMP- and siG6PT-transiently transfected cells showed a significant decrease in Ca^{2+} mobilization (Fig. 2C). On the other hand, cells that overexpressed MT1-MMP demonstrated an increase in Ca^{2+} mobilization by S1P, while those that overexpressed G6PT did not exhibit any changes when compared to mock-transfected cells (Fig. 2C). SiMT1-MMP and siG6PT did not block thapsigargin-induced Ca^{2+} release (not shown). Collectively, this confirms the primary cooperative role of MT1-MMP with S1P, while G6PT, because of its inability to increase S1P-mediated Ca^{2+} mobilization in G6PT-transfected cells, may only play a complementary role in the MT1-MMP/G6PT signaling axis.

3.3. Gene silencing of both G6PT and MT1-MMP suppresses ERK phosphorylation by S1P

We assessed the impact of MT1-MMP and G6PT in S1P-induced ERK phosphorylation. Cells were transiently transfected with siRNA specific for MT1-MMP and G6PT as described in Section 2. Cells were then incubated in the presence of S1P for up to 20 min and the extent of ERK phosphorylation was assessed by Western blotting (Fig. 3A). In agreement with the Ca^{2+} mobilization effects, we found that S1P triggered a rapid but transient phosphorylation of ERK, while silencing of both MT1-MMP and G6PT genes almost completely abolished ERK phosphorylation (Fig. 3B).

3.4. Gene silencing of both G6PT and MT1-MMP suppresses S1P chemotactic effects

S1P is among the most potent chemotactic agents and has been found to induce cell migration in multiple cell models [26]. Given that Ca^{2+} mobilization, as well as ERK phosphorylation, in response to S1P may in part play a role in U-87 cell migration, we assessed the impact of MT1-MMP and G6PT in cell migration. Cells were transiently transfected with siRNA

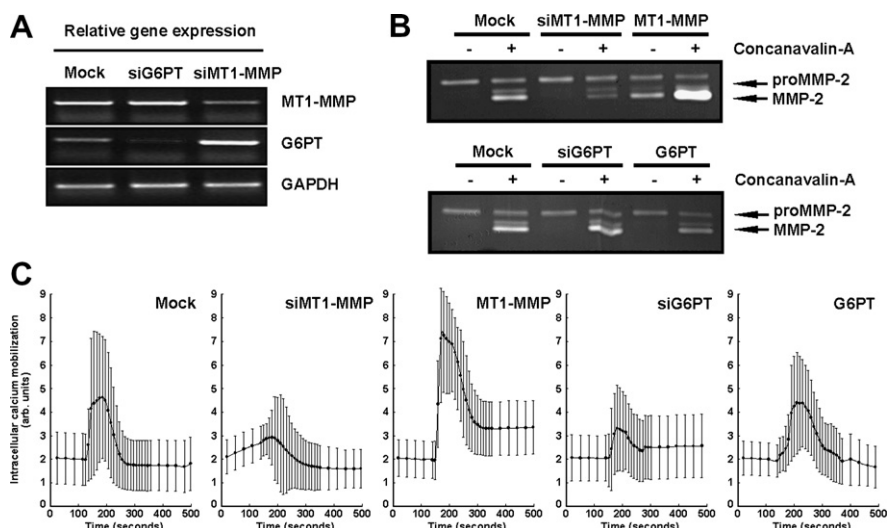


Fig. 2. Gene silencing of MT1-MMP and of G6PT impairs sphingosine-1-phosphate-mediated calcium mobilization. (A) Total RNA was isolated from mock-, siMT1-MMP-, and siG6PT-transfected U-87 cells. The efficiency of gene silencing was assessed using RT-PCR, and cDNA amplicons were visualized on agarose gels stained with ethidium bromide. (B) Serum-starved mock-, siMT1-MMP-, MT1-MMP, siG6PT, and G6PT-transfected U-87 cells were treated in the presence or absence of 10 μ M concanavalin-A for 18 h. Conditioned media were harvested and the extent of proMMP-2 activation was assessed by gelatin zymography as described in Section 2. (C) The above transfected U-87 glioblastoma cell cultures were assessed for Ca^{2+} mobilization induced with 1 μ M sphingosine-1-phosphate (S1P) as described in Section 2. A representative Ca^{2+} mobilization profile, out of three experiments, is shown.

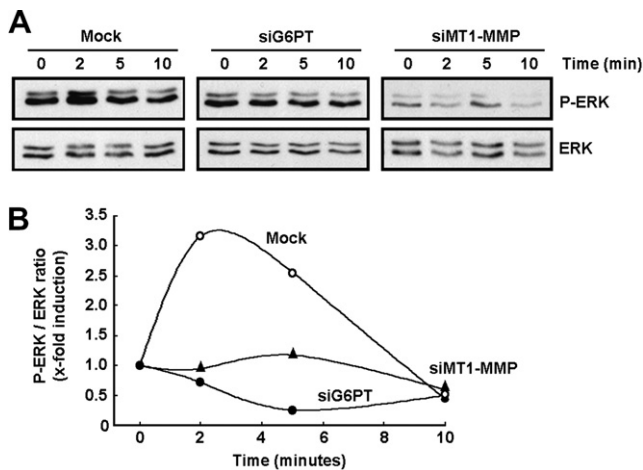


Fig. 3. Gene silencing of both G6PT and MT1-MMP abrogates ERK phosphorylation by sphingosine-1-phosphate. (A) U-87 glioblastoma cells were transiently transfected with siRNA specific for MT1-MMP and G6PT as described in Section 2. Cells were then incubated in the presence of sphingosine-1-phosphate (S1P) for up to 20 min and the expression of phosphorylated ERK (P-ERK) and of total ERK was assessed in cell lysates by Western blotting. (B) Quantification was performed using scanning densitometry, and results are expressed as the ratio of P-ERK/total ERK for each of the conditions. A representative Western blot, out of three experiments, is shown.

specific for MT1-MMP and G6PT and assessed for migration using modified Boyden chambers (Fig. 4A). While basal cell migration (Fig. 4B, white bars) remained relatively unaffected, silencing of both the MT1-MMP and G6PT genes resulted in an overall 55–80% decrease in cell migration in response to S1P (Fig. 4B, black bars). Interestingly, gene silencing of MT1-MMP and of G6PT did not affect U-87 cell migration in response to bFGF (Fig. 4B, grey bars), suggesting that the MT1-MMP/G6PT signaling axis is specifically involved in the S1P-induced chemotaxis but not in non-lipid bFGF-mediated signaling.

4. Discussion

The ER is primarily known as the site of synthesis and folding of secreted, membrane-bound, and some organelle-targeted proteins. It is also a multifunctional metabolic compartment that controls entry and release of Ca^{2+} , sterol biosynthesis, apoptosis and the release of arachidonic acid [27]. As such, metabolic profiling of cell growth and death in cancer is already used in order to identify the changes in glucose utilization for macromolecule synthesis in cancer [28]. Release of Ca^{2+} from ER stores was observed upon S1P activation [29, this study] and was correlated with the activation of the small GTPase Rac [29]. Interestingly, the coordinate modulation of MMP-2 activity and of MT1-MMP expression/processing by Rac1 is consistent with MT1-MMPs role in cell invasion [30]. Among the several brain tumor-derived cell lines tested, G6PT expression was found to be the highest in U-87 glioblastoma cells [16]. This suggests that metabolic adaptative capacity, in part through G6PT, may regulate the invasive phenotype of aggressive cancer cells. Given the ER localization of G6PT and the crucial role that the ER fulfills as a metabolic compartment, intracellular regula-

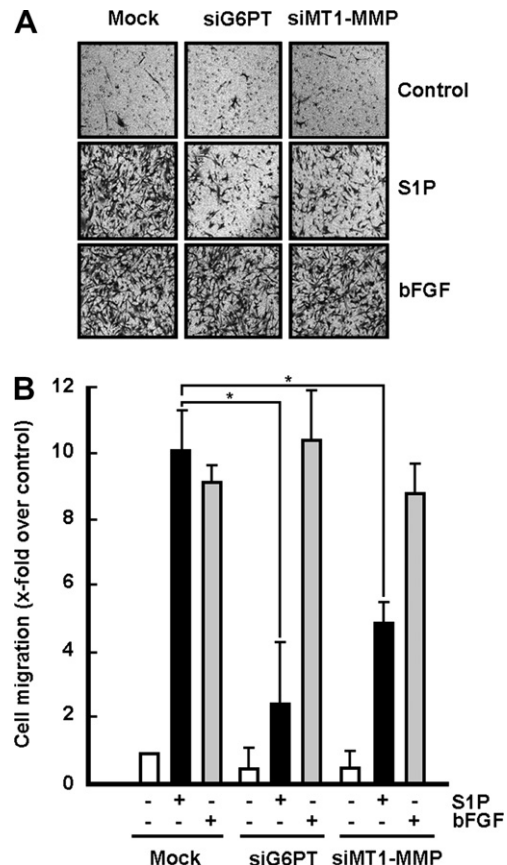


Fig. 4. Gene silencing of both G6PT and MT1-MMP abrogates sphingosine-1-phosphate chemotactic effects. (A) U-87 glioblastoma cells were transiently transfected with siRNA specific for MT1-MMP and G6PT as described in Section 2. Cells were then assessed for migration using modified Boyden chambers in the absence (white bars) or the presence of 1 μ M sphingosine-1-phosphate (S1P, black bars), or 20 ng/ml bFGF (grey bars). Cell migration proceeded for 6 h and was quantified (B). Values shown represent the means \pm S.D. of a representative experiment where 5 random fields per filter were counted for each condition. Probability values of less than 0.05 using Student's unpaired *t*-test were considered significant when S1P- or bFGF-induced cell migration was compared to mock-transfected cells, and an asterisk (*) identifies such significance.

tion of Ca^{2+} flux, cytosolic ATP and G6P levels would be among the parameters that G6PT could modulate in the transformed proliferating cells. We thus propose that G6PT further acts as a mediator in the regulation of cancer cell survival and ECM degradation signaling. In fact, evidence that regulation of G6PT expression may function as a bioswitch enabling cells to promote either migration or cell death processes through MT1-MMP cytoplasmic domain signaling was recently provided [24].

Recent evidence has revealed a molecular link between MT1-MMP and S1P signaling in mesenchymal stromal cell and endothelial cell migration, survival, ECM proteolysis, and cytoskeletal rearrangement [19,24,31,32]. We now highlight a significant and new contribution of the MT1-MMP/G6PT signaling axis that may further regulate S1P activity as a second messenger in mobilizing intracellular Ca^{2+} in tumor cells. Several explanations for this MT1-MMP/S1P cooperative effect have been forwarded. Among these, RhoA signaling, a crucial intermediate step in S1P transduction was

increased in MT1-MMP-transfected cells [21]. More recently, S1P was shown to regulate endothelial cell locomotion by inducing the association of MT1-MMP with the adaptor protein p130Cas at the leading edge of migrating cells [33]. Although the ability of S1P to increase cytosolic Ca^{2+} has been characterized in terms of the individual S1PRs involved [4], specific studies to identify the S1PR that cooperates with MT1-MMP activity may however be challenging. Ca^{2+} mobilization being a hallmark of most S1PRs [34], it can be anticipated that one S1PR would take the functional relay upon another. Design and use of specific siRNA targeting each of the S1PRs (S1P-1 to -5) should shed light on how MT1-MMP cooperates with S1PRs functions in the control of cell migration, cell survival, and ECM degradation. It is known that S1P1 and S1P2 receptors are expressed on glioblastoma cells [35], and that S1P2 is likely responsible for the effect of PLC, Ca^{2+} release and PLD [10].

Altered expression, maturation and trafficking of MT1-MMP to the plasma membrane were observed in diabetic states [36,37], a condition known to upregulate the expression of G6PT [38]. G6PT expression was shown to be down-regulated by MT1-MMP in mesenchymal stromal cells [24]. This is in agreement with the fact that cytoskeleton disorganization, an early step in the activation process of matrix metalloproteinase 2 (MMP-2) by MT1-MMP, is also associated with ER dysfunction and subsequent cell death. Furthermore, recent studies suggest that the microtubule cytoskeleton and the centrosomes (the microtubule cytoskeleton-organizing centers) are essential for the trafficking and the internalization of the membrane-bound matrix metalloproteinase MT1-MMP [39], involved in brain tumor cell invasion, ECM degradation and cell-ECM interaction [40]. Our results suggest that an intact, functional MT1-MMP/G6PT signaling axis is required for efficient Ca^{2+} mobilization processes to take place in response to circulating bioactive lipids such as S1P. Given that such axis regulates cell survival and chemotaxis, targeted inhibition of either MT1-MMP or G6PT function may lead to reduced infiltrative and invasive properties of brain tumor cells. Documenting the Ca^{2+} -mediated roles and potential contribution of G6PT in cancer cells will further help optimize or design new anti-tumor therapies.

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