Serglycin activates pro-tumorigenic signaling and controls glioblastoma cell stemness, differentiation and invasive potential

Dimitra Manoua, Panagiotis Bourisa, Dimitris Kletsas, Martin Götte, Burkhard Greve, Aristidis Moustakas, Nikos K. Karamanosa and Achilleas D. Theocharis

Abstract

Despite the functional role of serglycin as an intracellular proteoglycan, a variety of malignant cells depends on its expression and constitutive secretion to advance their aggressive behavior. Serglycin arose to be a biomarker for glioblastoma, which is the deadliest and most treatment-resistant form of brain tumor, but its role in this disease is not fully elucidated. In our study we suppressed the endogenous levels of serglycin in LN-18 glioblastoma cells to decipher its involvement in their malignant phenotype. Serglycin suppressed LN-18 (LN-18shSRGN) glioblastoma cells underwent astrocytic differentiation characterized by induced expression of GFAP, SPARCL-1 and SNAIL, with simultaneous loss of their stemness capacity. In particular, LN-18shSRGN cells presented decreased expression of glioma stem cell-related genes and ALDH1 activity, accompanied by reduced colony formation ability. Moreover, the suppression of serglycin in LN-18shSRGN cells retarded the proliferative and migratory rate, the invasive potential in vitro and the tumor burden in vivo. The lack of serglycin in LN-18shSRGN cells was followed by G2 arrest, with subsequent reduction of the expression of cell-cycle regulators. LN-18shSRGN cells also exhibited impaired expression and activity of proteolytic enzymes such as MMPs, TIMPs and uPA, both in vitro and in vivo. Moreover, suppression of serglycin in LN-18shSRGN cells eliminated the activation of pro-tumorigenic signal transduction. Of note, LN-18shSRGN cells displayed lower expression and secretion levels of IL-6, IL-8 and CXCR-2. Concomitant, serglycin suppressed LN-18shSRGN cells demonstrated repressed phosphorylation of ERK1/2, p38, SRC and STAT-3, which together with PI3K/AKT and IL-8/CXCR-2 signaling control LN-18 glioblastoma cell aggressiveness. Collectively, the absence of serglycin favors an astrocytic fate switch and a less aggressive phenotype, characterized by loss of pluripotency, block of the cell cycle, reduced ability for ECM proteolysis and pro-tumorigenic signaling attenuation.

Introduction

Glioblastoma (GBM), which is categorized as brain tumor Grade IV by WHO, constitutes the deadliest form of diffuse gliomas, with the median overall survival of GBM patients being 9.9 to 15 months [1]. Surgical resection and standard chemo- and radio-therapeutic interventions struggle to overcome the complex character of the disease. The heterogeneity of GBM tumors lies in their histological and...
molecular genetic features. Moreover, the existence of tumor stem cells and the invasive cell behavior augment their aggressive nature [2,3]. Extracellular matrix (ECM) carries out regulatory roles for pathophysiological conditions and its composition is modified in various diseases [4–8]. Proteoglycans (PGs) are major macromolecules of the ECM and orchestrate important cell functions [9,10]. In cancer onset and progression, PGs undergo biosynthetic and post-synthetic alterations, thus promoting cell differentiation, proliferation, adhesion, invasion and angiogenesis [11–13]. The most abundant PGs found in the brain ECM and playing essential role in gliomagenesis are substituted with chondroitin sulfate (CS) and heparan sulfate (HS) glycosaminoglycans chains (GAGs) [14,15].

Serglycin was initially mentioned to be involved in the organization of granules and secretory vesicles in hematopoietic cells [16,17], nevertheless it is expressed by a variety of normal cells [18–25]. Over the years, serglycin arose to function as a tumor-promoting PG expressed and secreted by aggressive tumor cells, including breast [26–30], colorectal [28,31], lung [32], hepatocellular [33] and nasopharyngeal cancer cells [34–36] as well as multiple myeloma cells [37,38]. The secreted form of serglycin is incriminated to control tumorigenic properties, such as adhesion, invasion, metastasis, proliferation, epithelial to mesenchymal transition (EMT), drug resistance and stemness, via regulation of various signaling cascades [39,40].

Ser glycin is also associated with glioblastoma [15,41,42]. An early study had detected upregulation of ser glycin mRNA in GBM human samples compared to healthy ones [15]. Furthermore, Roy et al. manifest that high ser glycin expression is in line with low survival of GBM patients. Moreover, high ser glycin, an elevated infiltrating mast cell population and high glioma grade seem to be interrelated [41]. GBM cells can advance the expression of serglycin after co-culturing with mast cells in a distance-dependent way [41] or with cell-cell contact with astrocytes [42], with concomitant acquisition of an enhanced tumor-supportive behavior.

The prominent role of serglycin as a multilevel regulator of proteolytic enzymes and inflammatory mediators is widely appreciated [39,40,43]. Ser glycin can modify not only intracellular features, such as expression and storage, but also secretion and activation of various matrix metalloproteinases (MMPs) and urokinase-plasminogen activator (uPA) [26,44–48]. Concerning inflammatory and angiogenic molecules, ser glycin can modulate their expression and secretion, fostering tumor-promoting characteristics [23,26,37,41,49–52].

Unambiguously, ser glycin accommodates tumor aggressiveness and is a valuable player in the modulation of tumor microenvironment. For this reason, in our study we investigated the role of ser glycin in glioblastoma aggressiveness by suppressing its endogenous levels. Specially, we demonstrated that LN-18 glioblastoma cells with suppressed levels of ser glycin (LN-18\(^{shSRGN}\)) present a glioblastoma to astrocytoma differentiation associated with reduced cell stemness capacity. Further, we discovered that knockdown of ser glycin resulted in attenuation of crucial in vitro and in vivo cell properties, associated with impaired signal transduction. Simultaneously, we demonstrated reduction in the expression and activity of proteolytic enzymes, as well as in the expression and secretion of interleukin 6 (IL-6) and 8 (IL-8) in LN-18\(^{shSRGN}\) glioblastoma cells.

Results

Suppression of endogenous ser glycin regulates LN-18 glioblastoma cell stemness and differentiation

To decipher the involvement of ser glycin in glioblastoma aggressiveness, we transduced ser glycin-expressing LN-18 glioblastoma cells with shRNA lentiviral particles against ser glycin (SRGN) mRNA establishing stable glioblastoma cells with suppressed ser glycin expression, indicated as LN-18\(^{shSRGN}\). We verified the suppression levels using real-time qPCR to be about 99% compared to control scramble lentiviral particles transduced LN-18 cells (LN-18\(^{shSCR}\)) (Fig. 1A). Moreover, the constitutively secreted ser glycin in cell culture supernatants of control LN-18\(^{shSCR}\) was eliminated after the suppression of ser glycin in LN-18\(^{shSRGN}\) glioblastoma cells (Fig. 1B). Following the suppression of ser glycin mRNA in LN-18\(^{shSRGN}\) glioblastoma cells, tremendous morphological alterations were observed (Fig. 1C & D). In particular, ser glycin suppressed LN-18\(^{shSRGN}\) cells appeared as clusters forming tight aggregates, losing the typical aggressive phenotype of individual, spindle-shaped LN-18\(^{shSCR}\) cells. Intracellular localization of the protein core of ser glycin was visualized by immunofluorescence analysis (Fig. 1E). Ser glycin was verified to be substantially decreased in LN-18\(^{shSRGN}\) cells, while in LN-18\(^{shSCR}\) cells had an intense cytoplasmic staining.

Due to overt transition of LN-18\(^{shSRGN}\) cells to a less aggressive morphology, we determined their stem-cell associated characteristics. Suppression of ser glycin in LN-18\(^{shSRGN}\) glioblastoma cells resulted in a significant reduction of the mRNA levels of a variety of self-renewal and pluripotency markers, including SOX2, Oct4, LIF, MSI1, NES, Nanog and KLF4 (Fig. 2A). Concomitant with the reduced expression of glioblastoma cell stemness-associated genes, LN-18\(^{shSRGN}\) cells exhibited elevated expression of the astrocytic...
differentiation marker SPARCL-1, as well as of the regulator of the astrocytic fate SNAIL [53,54] (Fig. 2B). Moreover, the mRNA (Fig. 2B) and protein (Fig. 2C) levels of GFAP, which is a marker of terminally differentiated astrocytes, were found to be markedly increased in LN-18shSRGN cells. ALDH1 is a novel marker for stem cell maintenance, which predicts poor prognosis in glioma patients [55,56]. In LN-18shSRGN glioblastoma cells, serglycin suppression evoked a significant reduction up to 6.5 fold of the ALDH1 activity (Fig. 2D). Suppression of serglycin decreased also the potential of glioblastoma cells to give rise to colonies, as LN-18shSRGN formed only the half colonies compared to LN-18shSCR cells (Fig. 2E). Therefore, serglycin suppression in LN-18shSRGN glioblastoma cells results in a population of clustering cells with poor stemness capacity and increased astrocytic differentiation status.

**Fig. 1.** Serglycin affects morphology of LN-18 glioblastoma cells. (A) Serglycin (SRGN) mRNA levels were determined by real-time qPCR and (B) secreted serglycin was determined by western blot analysis in LN-18 glioblastoma cells transduced with scramble shRNA (LN-18shSCR) and shRNA targeting serglycin (LN-18shSRGN). Secreted form of serglycin was determined in concentrated culture media after 24 h incubation with serum free media. (C) Phase-contrast microscopy images and (D) four areas of higher magnifications reveal the closely packed colony formation of the stably transduced LN-18shSRGN cells which contradicts the spindle-like morphology of control LN-18shSCR cells. Scale bar 200 μM. (E) Immunofluorescence staining for serglycin core protein (green) and nuclei (blue) depicts the distribution of serglycin in LN-18shSCR and LN-18shSRGN cells. Moreover, lower staining of serglycin is observed in LN-18shSRGN cells. Scale bar 25 μM. Statistically significant differences are displayed by asterisks: *** (p < 0.001).
Serglycin suppression reduces the tumorigenic potential of LN-18 glioblastoma cells and disrupts the cell cycle

Despite the established tumor-supportive role of serglycin, little is known concerning its involvement in glioblastoma progression. For this reason, we investigated crucial cellular hallmarks after the suppression of serglycin in LN-18 glioblastoma cells. Particularly, serglycin suppression diminished the proliferation and migration capacity almost by 50% in LN-18shSRGN glioblastoma cells (Fig. 3A & supplementary figure). Similarly, serglycin suppression markedly reduced the invasive potential of LN-18shSRGN cells in collagen type I gels (Fig. 3A & supplementary figure). Afterwards, we examined the effect of suppressed serglycin levels in the regulation of cell cycle in LN-18 glioblastoma cells. LN-18shSRGN cells seemed to be accumulated in G2 phase, with concurrent reduction of the cellular population in the G1 and S phases (Fig. 3B). In accordance with this observation, LN-18shSRGN cells were characterized by reduced mRNA levels of important cell-cycle regulators, including the G1/S specific cyclin D1, the cyclin B1 responsible for the S/G2 checkpoint and the cyclin A1 controlling the G1/S and G2/M transitions (Fig. 3C). Due to the important impact of serglycin suppression in the above features in vitro, we next evaluated the ability of LN-18shSRGN cells to form tumors in vivo. Notably, only one out of seven inoculated sites of SCID mice developed a small tumor from LN-18shSRGN cells, in contrast to LN-18shSCR cells, which developed a significant tumor mass in all cases (Fig. 3D). Taken together these findings, serglycin can control cellular behavior of LN-18 glioblastoma cells by regulating crucial tumor-promoting properties.
Serglycin suppression perturbs the proteolytic and inflammatory balance of LN-18 glioblastoma cells

Tumor microenvironment plays an important role in cancer progression. Mediators such as matrix metalloproteinases (MMPs), and components of the plasminogen activation system participate in ECM remodeling, advancing the aggressiveness of the tumor [57,58]. Hence, we investigated the impact of serglycin suppression in the biosynthesis of several proteolytic enzymes. All the investigated enzymes were

![Graph A: Proliferation Rate, Wound Healing Rate, Invasion Area](image)

| Cell Cycle Phases | LN-18^shSCR | LN-18^shSRGN | p-value |
|-------------------|-------------|--------------|---------|
| G1                | 69.0 ± 4.9% | 35.2 ± 2.5%  | < 0.01  |
| S                 | 14.7 ± 3.9% | 7.4 ± 0.8%   | < 0.01  |
| G2/M              | 16.3 ± 1.0% | 57.4 ± 3.2%  | < 0.001 |

![Graph B: Relative mRNA level](image)

![Graph C: Tumor Volume](image)

Fig. 3. Serglycin suppression diminishes tumorigenic potential and disorganizes the cell cycle of glioblastoma cells. (A) Cell functional properties including proliferation, migration and invasion of control LN-18^shSCR and serglycin-suppressed LN-18^shSRGN cells were evaluated after culturing for 24 h. (B) Cell cycle analysis of LN-18^shSCR and LN-18^shSRGN cells was determined after 24 h incubation with serum free media. (C) The expression of cell cycle regulators CDK6, cyclin A1, B1 and D1 in LN-18^shSCR and LN-18^shSRGN cells was measured by real-time qPCR. (D) Tumor burden in SCID mice injected with LN-18^shSCR and LN-18^shSRGN cells was examined by calculating the tumor volume using the Caliper method. Each bar represents mean ± SD values from seven samples. Statistically significant differences are displayed by the p value or by asterisks: * (p < 0.05), ** (p < 0.01), *** (p < 0.001).
Fig. 4. Serglycin suppression in LN-18 glioblastoma cells attenuates the expression and activity of proteolytic enzymes, both in vitro and in vivo. The mRNA levels of MMPs, uPA and PAI-1 (A) in control LN-18<sup>shSCR</sup> and serglycin suppressed LN-18<sup>shSRGN</sup> cells and (B) in tumor samples developed from LN-18<sup>shSCR</sup> and LN-18<sup>shSRGN</sup> cells after injection in SCID mice were measured by real-time qPCR. The activity of MMP-2, MMP-9 and uPA was assessed by gelatin and casein zymography, respectively, in LN-18<sup>shSCR</sup> and LN-18<sup>shSRGN</sup> cells (C) as well as in tumor samples developed from the inoculation of LN-18<sup>shSCR</sup> and LN-18<sup>shSRGN</sup> cells in SCID mice (D). (E) Quantification of MMP-2, MMP-9 and uPA activities in LN-18<sup>shSCR</sup> and LN-18<sup>shSRGN</sup> cells and tumor samples (F). Statistically significant differences compared to control are displayed by asterisks: * (p < 0.05), ** (p < 0.01), *** (p < 0.001), while non-statistically significant differences by ns (p > 0.05).
including MMP-1, MMP-2, MMP-9, MT1-MMP, as well as uPA and its inhibitor PAI-1 presented substantially decreased mRNA levels after the suppression of serglycin in LN-18shSRGN glioblastoma cells, whereas the expression of their endogenous inhibitors TIMP-1 and TIMP-2 was only moderately decreased (Fig. 4A). Moreover, tumor samples developed from LN-18shSRGN cells exhibited downregulated serglycin mRNA levels, associated with reduced mRNA levels of MMP-1, MMP-9, TIMP-1 and uPA, compared to tumor samples developed by the inoculation of LN-18shSCR cells in mice (Fig. 4B). Zymography assays using specific substrates revealed significant reduction of the enzymatic activity of MMP-2, MMP-9 and uPA in the case of LN-18shSRGN cells, compared to LN-18shSCR cells (Fig. 4C & E). Enzymatic activity levels of the aforementioned molecules were also decreased in tumor sample developed from LN-18shSRGN cells compared to tumors generated by LN-18shSCR cells in vivo (Fig. 4D & F).

Interleukins are essential mediators for the progression of inflammation, a step crucial for glioblastoma pathogenesis [59]. Moreover, a recent study of our lab revealed the regulatory role of serglycin upon IL-8/CXCR-2 signaling activation [26]. To further elucidate the role of serglycin in LN-18 glioblastoma cells, we examined the expression and/or secretion of molecules participating in inflammation. Specially, LN-18shSRGN cells exhibited reduced mRNA levels of IL-6 and IL-8, as well as of the IL-8 receptor CXCR-2 (Fig. 5A). Moreover, secreted protein levels of IL-8 determined by ELISA assay in concentrated cell culture media after 48 h incubation with serum free media. (C) The mRNA levels of IL-6, IL-8 and CXCR-2 in tumor samples developed from LN-18shSCR and LN-18shSRGN cells were measured by real-time qPCR. Statistically significant differences compared to control sample are displayed by asterisks: * (p < 0.05), ** (p < 0.01), *** (p < 0.001), while non-statistically significant differences by ns (p > 0.05).

Serglycin activates pro-tumorigenic signaling

Serglycin can modulate tumorigenic characteristics by activating a variety of signaling pathways [26,27,32,34]. In our study, we examined its involvement in the activation of AKT, ERK1/2, p38 MAPK, SRC and STAT-3 signaling. Our data revealed that the suppression of serglycin in LN-18shSRGN glioblastoma cells caused a significant reduction of the phosphorylation levels of ERK1/2, p38 MAPK, SRC and STAT-3 (Fig. 6A & B). Using specific inhibitors for
Fig. 6. The loss of serglycin attenuates tumorigenic signaling in glioblastoma cells. (A) Western blot analysis of phosphorylated and total forms of the signaling mediators AKT, ERK 1/2, p38 MAPK, SRC and the transcription factor STAT-3 in LN-18shSCR and LN-18shSRGN cell extracts. (B) Quantification of the immunoblot bands presenting the relative ratio of phosphorylated to total forms of signaling molecules in LN-18shSCR and LN-18shSRGN cell extracts. (C) The migratory properties of LN-18shSCR and LN-18shSRGN cells were determined after selective inhibition of downstream signaling molecules as well as after inhibition of the CXCR-2 receptor. LN-18shSCR and LN-18shSRGN cells were treated either with DMSO or specific inhibitors in serum free medium for 24 h and cell migration was measured by using wound healing assay. Statistically significant differences compared to control are shown by asterisks: * (p < 0.05), ** (p < 0.01), *** (p < 0.001), while non-statistically significant differences by ns (p > 0.05).
the intracellular mediators MEK1/2 (U0126), PI3K/AKT (LY294002), p38 MAPK (SB203580), STAT-3 (AG490) and SRC (PP2) as well as for the IL-8 receptor CXCR-2 (SB225002), we further analyzed the role of these signaling pathways in the aggressiveness of LN-18 glioblastoma cells. We examined the migratory capacity of LN-18<sup>shSCR</sup> and LN-18<sup>shSRGN</sup> cells after treatment with the inhibitors for 24 h. Particularly, in serglycin expressing LN-18<sup>shSRGN</sup> glioblastoma cells all inhibitors led to a significant reduction of the migration rate. In contrast, serglycin suppressed LN-18<sup>shSRGN</sup> glioblastoma cells were characterized by reduced migratory capacity compared to control LN-18<sup>shSCR</sup> cells and their migratory potential was not further affected by the inhibition of all above signaling mediators (Fig. 6C). Taken together these findings, it can be concluded that the aggressive behavior of LN-18 glioblastoma cells seems to depend on serglycin availability for the activation of important signaling cascades.

Discussion

This study investigates the autocrine functional role of serglycin in glioblastoma. Accumulated data from this study pinpoint that suppression of serglycin evokes the differentiation of glioblastoma LN-18 cells to an astrocytic phenotype, with less aggressive behavior and pluripotency potential. Moreover, the differentiation of LN-18 cells is associated with reduced tumorigenic properties, while alterations are also observed in multiple effectors participating in ECM remodeling, inflammation, cell cycle regulation and signal transduction.

After the suppression of serglycin, LN-18<sup>shSRGN</sup> cells lose their elongated shape and acquire a less aggressive morphology, characterized by closely packed cellular formations. Furthermore, knockdown of serglycin in LN-18<sup>shSRGN</sup> cells results in decreased mRNA levels of glioblastoma stem cell-related genes, including SOX2, Oct4, LIF, MSI1, NES, Nanog and KLF4, as well as decreased ALDH1 activity. Also, suppressed levels of serglycin are accompanied by enhanced expression of the astrocytic differentiation markers SPARCL-1 and GFAP. Additionally, the colony formation ability is markedly reduced in LN-18<sup>shSRGN</sup> cells. After serglycin suppression in glioblastoma cells, we observed a significant induction of transcription factor Snail, which is an emerging modulator of the glioma stem cell differentiation fate. Actually, it has been shown that Snail depletes the capacity of glioblastoma cells to form gliomaspheres in vitro and to develop tumors in vivo repressing glioma cells stemness. Specifically, ectopic expression of Snail in U298 MG glioblastoma cells results in upregulation of astrocytic markers GFAP and SPARCL-1 and downregulation of glioma stem cell markers SOX2, Oct4, LIF, MSI1 and Nestin [53,54]. Snail is induced by BMP7 signaling activation and promotes astrocytic differentiation in glioma stem cells while suppresses tumor growth in vivo. Snail interacts with SMAD signaling mediators, generating a positive feedback loop of BMP signaling that in turn suppresses TGF-β1 signaling activity by restraining TGFβ1 gene transcription. Our data suggest that the absence of serglycin is associated with limited stemness ability of glioblastoma cells, favoring a differentiation fate. Particularly, serglycin seems to control the mRNA levels of crucial stemness- and differentiation-related markers and potentially the regulation of Snail contribute to this switch.

Moreover, the suppression of serglycin in LN-18<sup>shSRGN</sup> cells reduces the proliferation and migration rate, the invasion area in vitro and the tumor growth in vivo. Also, the majority of LN-18<sup>shSRGN</sup> cells are gathered in G2/M phase, accompanying by significant reduction of the percentage of cells being in G1 and S phases and by decreased mRNA levels of cell cycle regulatory molecules including CCNA1, CCNB1 and CCND1. These findings are in accordance with data presented in our previous studies where cobblestone-shaped epithelial MCF-7 cells after ectopic expression of serglycin obtain a mesenchymal phenotype, followed by alterations in EMT-related genes [26,30]. Furthermore, the ability of breast cancer cells to proliferate, migrate and invade is interrelated with the expression of serglycin. Notably, aforementioned cellular functions are also increased after the overexpression of serglycin in MCF-7 cells [26,27,30] and markedly decreased after the suppression of serglycin in aggressive MDA-MB-231 cells that constitutively secrete high levels of serglycin [26,27]. Of note, MDA-MB-231 serglycin suppressed cells show also reduced metastasis and tumor growth in vivo [27]. Using the online tool GOBO database for breast cancer, the expression of serglycin is associated with genes related to checkpoint and M-phase [26]. Suppression of serglycin in the highly metastatic and serglycin-expressing clone of nasopharyngeal cell line S18 results in decreased tumorigenic potential in vivo and in a more epithelial phenotype [34,36]. Specifically, S18 serglycin suppressed cells resemble the aggregates formed by the low metastatic and low serglycin-expressing clone of nasopharyngeal cell line S26. Moreover, S18 serglycin suppressed cells demonstrate reduced mRNA levels of several stemness-associated genes including Oct4, Nanog, Bmi1, ABCG2, CD44 and SOX2, as well as decreased sphere formation ability. Concerning the role of serglycin in the regulation of cell cycle, serglycin-activated β-catenin enhances the expression of c-Myc and CCND1 in nasopharyngeal cancer cells [34]. Experiments with loss or gain of serglycin expression in non-small cell lung cancer cells reveal that cellular migration, invasion, sphere formation
and primary tumor growth in vivo are similarly affected by serglycin expression. Moreover, overexpression of serglycin in non-small cell lung cancer H1299 cells augments the expression of Nanog whereas suppression of serglycin in H460 cells reduces the mRNA levels of Oct4, SOX2 and Nanog [32]. The presented body of evidence highlights the importance of serglycin for the aggressive phenotype of glioblastoma cells, since its absence is not only connected with reduced stemness ability but also with lower proliferation and cell cycle arrest, decreased migration and invasion rates and outstandingly diminished in vivo tumorigenicity.

ECM proteolytic balance and inflammatory conditions corroborate the aggressive potential of glioblastoma [59–65]. In our study, we investigated the mRNA and/or activity levels of several MMPs, TIMPs, uPA and PAI-1 after the suppression of serglycin in LN-18 shSRGN glioblastoma cells and in the tumors developed in mice. The expression of the majority of these proteases such as MMP-1, MMP-2, MMP-9, MT1-MMP, uPA, TIMP-1, TIMP-2 and PAI-1 are regulated in vitro and in vivo by serglycin expression. Similarly, we have previously shown that serglycin expression directs the protein and mRNA levels of MMP-1, MMP-2, MMP-7, MMP-9, MT1-MMP and uPA in breast cancer cells [26]. Serglycin can handle the biosynthesis, secretion and activation of proteolytic enzymes [16,39,40]. For example, in multiple myeloma the cell surface associated serglycin evokes the adhesion of myeloma cells to collagen I and induces the expression and secretion of MMP-2 and MMP-9 [46]. Likewise, ectopic expression of serglycin in Madin-Darby canine kidney results in increased mRNA and protein levels of MMP-9 and uPA [47]. The enzyme of MMP-9 can interact with serglycin, forming complexes both in vivo and in vitro [45]. Moreover, serglycin can bind MMP-13, with which it co-localizes in cytoplasmic vesicles of chondrocytes [44]. Serglycin shows also a co-distribution with tPA in secretory vesicles of HUVEC [66].

Between different grades of brain tumors, MMP-1, MMP-2, MMP-9 and MT1-MMP are expressed in high levels in glioblastoma and predict poor prognosis of glioblastoma patients [67–71]. MMP-1 seems to be a tumor-associated angiogenic regulator and has a tumor-supportive role, as its overexpression enhances the tumorigenic potential of glioblastoma cells in vivo [72]. On the contrary, its suppression reduces the migratory potential of glioblastoma cells [73]. MMP-2 acquires a significant role in glioblastoma, as its suppression reduces cellular invasion and migration in vitro, as well as angiogenesis and tumorigenicity in vivo [74]. Invasive ability of glioblastoma cells is also induced after Wnt-5-dependent activation of MMP-2 [75]. The expression of MMP-2 and TIMP-1 is interrelated in glioblastoma and the combination of them indicates a poor survival compared to the predictive potential of MMP-2 alone [76]. Concerning MMP-9, its downregulation in glioblastoma cells results in attenuation of cellular migration and invasion in vitro and in vivo [77,78]. Glioblastoma cell invasiveness can be boosted by MMP-9 after the direct activation of its pro-enzyme by uPA [79]. The expression of MT1-MMP is also related to glioblastoma invasive ability [71]. Its inhibition ends in the arrest of glioma cell cycle in G2/M phase, in reduced cell proliferation rate and enhanced sensitivity to temozolomide and radiation [70]. MT1-MMP is also capable to interact with TIMP-2 and to activate proMMP-2 [80–82]. Low tumor-associated or serum TIMP-1 levels have been proposed to predict favorable survival for glioblastoma patients [83,84]. Components of the plasminogen system facilitate cancer dissemination, as plasminogen activators produce plasmin, which in turn participate in the maturation of several MMPs. All the aforementioned enzymes can further enhance ECM proteolysis [85,86]. Inhibition of uPA alone reduces glioma invasion, migration and colony formation, causing actin reorganization and attenuation of PI3K/AKT signaling [87,88]. Synchronous inhibition of uPA receptor and uPA diminishes invasion and anchorage independent growth of glioma cells by interfering with the stemness-associated Notch 1 signaling pathway [89]. Malignant intracranial glioma progression decreases after targeting uPA/uPAR [90] or uPA/uPAR/MMP-9 axis [91]. Considering the importance of the above enzymes in glioma progression, serglycin emerges as a potent regulator of the proteolytic and invasive potential of glioblastoma cells.

It has been also shown that serglycin is induced upon inflammatory conditions and can regulate the expression and storage of a variety of inflammatory mediators, such as CXCR-4, CXCL-4, CXCL-7 and CCL-3 in several cell types [16,40]. After exposing glioblastoma cells to mast cells, which express endogenous serglycin, CXCL-10 and CXCL-12 are upregulated [41]. In MMTV-PyMT mammary carcinoma mouse model, the loss of serglycin is accompanied by the absence of lung metastasis due to low expression of CCL-2, which is a crucial chemokine for the establishment of secondary tumors in the lungs [51]. So, it seems that serglycin participates in the establishment of a prominent pro-inflammatory milieu to facilitate tumor progression.

In our study, serglycin suppression in LN-18 shSRGN glioblastoma cells controls the expression and secretion of IL-6 and IL-8, as well as the expression of CXCR-2, both in vivo and in vitro. Consistently, we have previously demonstrated that serglycin can regulate the expression of IL-8 and the concomitant activation of IL-8/CXCR-2 cascade in breast cancer cells, which in turn participates in cancer cell aggressiveness through...
the signaling mediators PI3K, SRC and Rac [26]. Investigating crucial signaling cascades for tumorigenesis, we demonstrate the decreased phosphorylation of the intracellular mediators ERK1/2, p38, SRC and STAT-3 in LN-18\textsuperscript{shSRGN} cells as well as the reduced expression of IL-6 and IL-8, which closely related to glioma progression. These signaling cascades together with the PI3K/AKT and IL-8/CXCR-2 axis emerge to be overt for the aggressiveness of LN-18 glioblastoma cells. Glioblastoma cells express and secrete IL-6, and its receptors, in high levels compared to low grade astrocytomas [59,64]. IL-6 is important for gliomagenesis in vivo as SRC-transgenic mice which develop spontaneous astrocytomas loss this ability after depletion of IL-6 [92]. Activation of IL-6/STAT-3 signaling increases glioma invasion and migration [93], while inhibition of STAT-3 reverses these aggressive characteristics [94,95]. IL-6 signaling also seems to regulate the fate of glioma stem cells, as knock-down of IL-6 or its receptor reduces their proliferation rate as well as their ability to form neurospheres. Glioblastoma stem cells develop significant tumor masses after injection in mice, a hallmark that is decreased after suppression of IL-6 or direct treatment of the tumor with IL-6 antibody [96]. Bioinformatic and gene expression profile analysis pinpoint IL-8 as a crucial mediator for gliomagenesis, which is expressed in high-grade tumor astrocytes mainly with CXCR-2 and indicates poor prognosis [97]. When glioblastoma cells are incubated with IL-8 their self-renewal capacity and the expression of stemness-related genes are induced. Moreover, knockdown of IL-8 decreases their proliferation ability and their tumorigenic capacity in vivo [65]. Treatment of LN-18 cells with Raparixin-L-lysine, an antagonist for CXCR-1/2, reduces the cellular proliferation and colony formation ability, as well as the spheroid invasion in vitro [98]. The activation of serglycin/CD44 axis emerges to control the cytoskeletal reorganization needed for migration of non-small cell lung cancer cells via induction of phosphorylation of SRC and Rho-family GTPases [99]. The binding of serglycin to CD44 also activates the MAPK/β-catenin signaling, which is responsible for the expression of CD44, c-Myc and CCND1 and the overall plasticity and aggressiveness of nasopharyngeal carcinoma cells [34]. These data support our findings and strengthen the view that serglycin expression triggers multiple tumorigenic signaling cascades in tumor cells to drive their aggressiveness.

To sum up, our study reveals that autocrine serglycin is essential for the aggressive behavior of glioblastoma cells, as its absence leads cells to abolish their cellular plasticity and tumorigenicity in vitro and in vivo, with subsequent establishment of a differentiated astrocytic phenotype. Moreover, suppression of serglycin perturbs the proteolytic, inflammatory and cell-cycle dynamics of glioblastoma cells, while crucial tumor-associated signal transduction cascades are also dysregulated (Fig. 7).

**Experimental procedures**

**Cell culture, transduction and reagents**

The LN-18 glioblastoma cell line was obtained from the American Type Culture Collection (ATCC). Transduction-ready viral particles containing 3 target-specific constructs that encode 19–25 nucleotides short hairpin RNA (shRNA) targeting human serglycin mRNA (SRGNshRNA Lentiviral Particles: sc-44093-V, Santa Cruz Biotechnology, Inc.) were used for serglycin gene silencing. Control shRNA lentiviral particles (SCRshRNA Lentiviral Particles: sc-108080; Santa Cruz Biotechnology, Inc.) containing a shRNA construct encoding a scramble sequence were used as a negative control. Transduction of LN-18 glioblastoma cells with shRNA against human serglycin (LN-18\textsuperscript{shSRGN}) or scramble shRNA (LN-18\textsuperscript{shSCR}) was performed according to manufacturer’s protocol. Selection and maintenance of stably transduced LN-18 cells were performed with puromycin dihydrochloride (sc-108071, Santa Cruz Biotechnology, Inc.) at the optimal concentration of 0.8 μg/mL. Puromycin-resistant cells were routinely cultured in a humidified 95% air/5% CO\textsubscript{2} incubator at 37 °C in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% (v/v) Fetal Bovine Serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine and a cocktail of antimicrobial agents (100 IU/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamycin sulfate and 2.5 μg/mL amphotericin B). All cell culture reagents mentioned, together with the cell detachment solution [0.05% (w/v) trypsin in PBS/0.02% Na\textsubscript{2}EDTA] were purchased from Biosera (France). The specific inhibitors SB225002 of CXCR-2 (iCXCR-2), U0126 of MEK1/2 (iMEK1/2), LY294002 of PI3K/AKT (iPI3K/ AKT), Tyrophostin AG490 of JAK/STAT (iJAK/STAT), PP2 of SRC (iSRC) and SB203580 of p38 MAPK (ip38 MAPK) were all supplied from Sigma-Aldrich.

**Tumorigenicity assay**

An equal number (10\textsuperscript{6}) of LN-18\textsuperscript{shSRGN} or LN-18\textsuperscript{shSCR} cells was inoculated in the back of seven SCID mice. Four weeks later, the animals were sacrificed and the tumors were removed. The tumor volume was calculated with the Caliper method, using the formula tumor volume = 1/2(length x width\textsuperscript{3}) [100,101]. Tumor samples developed from LN-18\textsuperscript{shSCR} cells and from LN-18\textsuperscript{shSRGN} cells were
mechanically homogenized in the presence of liquid nitrogen and stored in −80 °C. All animal studies were conducted according to the institutional guidelines conforming to international standards and the protocols were approved by the relevant committee of the Veterinary Direction, Greek Ministry of Rural Development and Food.

RNA isolation, cDNA synthesis and Real-Time qPCR analysis

LN-18shSRGN or LN-18shSCR cells were seeded in 60 mm dishes and incubated in complete medium for 24 h followed by overnight starvation in serum free medium. Afterwards, cells were incubated with serum free medium for 24 h and then were proceeded for total RNA isolation. LN-18 transduced cells and homogenized tissue samples were subjected to RNA isolation using NucleoSpin® RNA kit (Macherey-Nagel) following the manufacturer's instructions for each type of starting material. Quantification of isolated RNA was determined by absorbance measurements at 260 nm. cDNA synthesis was performed using PrimeScript™ RT Reagent kit (Perfect Real Time PCR) (TAKARA) according to manufacturer's instructions. Real-Time qPCR analysis was conducting using the reaction mixture KAPA SYBR® Fast qPCR kit Master Mix (2x) Universal (KAPABIOSYSTEMS) according to manufacturer's instructions and gene specific primers (supplementary material) in a Rotor Gene Q equipment (Qiagen, USA). Relative quantification of the data was obtained using the ΔΔCt method and

Fig. 7. Suggested model of the associated alterations caused by the knockdown of serglycin in LN-18 glioblastoma cells. The suppression of serglycin results in a less aggressive phenotype of LN-18 glioblastoma cells, with tightly packed cellular morphology and astrocytic differentiation features. Serglycin knockdown is also associated with simultaneous decrease of signaling transduction and gene expression signatures related to ECM proteolysis, stemness ability and cell cycle regulation. Some illustrations are acquired from somersault 1824 library which is covered under the Creative Commons 4.0 license (CC BY-NC-SA 4.0).
the GAPDH gene as normalizer. The fold changes were determined as $2^{-\Delta\Delta Ct}$.

**Immunoblotting**

LN-18<sup>shSRGN</sup> or LN-18<sup>shSCR</sup> cells were seeded in 100 mm dishes and incubated in complete medium for 24 h followed by overnight starvation in serum free medium. Afterwards, cells were incubated with serum free medium for 24 h. Then, the culture supernatants were collected, centrifuged at 3000 rpm for 5 min and concentrated with Vivaspion 6 30 K ultrafiltration devices (Sartorius Biotech.), while the cells were lysed with lysis buffer containing 25 mM Heps, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) glycerol, 1% (v/v) Triton X-100 supplemented with 1× protease inhibitor cocktail (Chemicon, Millipore, CA, #20-201) and 0.5 mM sodium orthovanadate (Sigma-Aldrich, #S6508). Equal amounts of proteins were reduced with β-mercaptoethanol in Laemmli buffer, separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Macherey-Nagel). The membranes were blocked with 5% (w/v) Bovine Serum Albumin (BSA) (Sigma-Aldrich) in TBS pH 7.4 containing 0.05% Tween-20 or LN-18<sup>shSCR</sup> cells (5 × 10<sup>5</sup>) were resuspended in 1 mL assay buffer and kept on ice prior to flow cytometric analysis on a CyFlow Space (Sysmex/Partec) using the 488 nm blue laser for excitation, while the fluorescence emission was measures at 545 nm (BP 527/30 nm). Gates were set by comparing the fluorescence of the DEAB control with that of the analyzed sample.

**Cell colony formation**

LN-18<sup>shSRGN</sup> or LN-18<sup>shSCR</sup> cells were seeded in 35 mm culture dishes (1000 cells/dish) and incubated with DMEM supplemented with 20% (v/v) FBS for 3 days. Media was renewed every 2 days. Colonies with &gt;50 cells were counted.

**Cell cycle analysis**

LN-18<sup>shSRGN</sup> or LN-18<sup>shSCR</sup> cells were seeded in 6 well plates and incubated in complete medium for 24 h followed by overnight starvation in serum free medium. Afterwards, cells were incubated with serum free medium for 24 h. After trypsinization and washing steps, 100 µL of cell suspension was diluted with 900 µL of DAPI (CyStain, Sysmex/Partec). Samples were incubated for 5 min at room temperature and analyzed by flow cytometry on a
Cells were incubated with serum free medium for 24 h followed by overnight starvation in serum free medium. Afterwards, cells were incubated with serum free medium for 24 h. For detecting the cell proliferation, we used the Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Japan), where the reagent WST-1 was added at a ratio of 1:10 and cell proliferation was determined according to manufacturer’s protocol.

LN-18\textsuperscript{shSRGN} or LN-18\textsuperscript{shSCR} cells were seeded in 24 well plates and incubated in complete medium until confluent cell layers. Cells were then starved with serum free medium for 16 h and then scratched using a 100 \mu L pipette tip. Detached cells were removed by washing and cells were incubated for 40 min at 37 °C with serum free media containing 10 \mu M of the cytostatic agent cytarabine (Sigma-Aldrich) and then were photographed with OLYMPUS CKX41 microscope with a color digital camera CMOS (SC30). Afterwards, the cells were incubated for 24 h either under serum free conditions or in the presence of specific inhibitors. Images were captured and wound surface was quantified using Image J Software. The percentage of wound closure was calculated for each condition.

**Collagen type I cell invasion assay**

The invasion assay with collagen type I was performed as described by De Wever et al. [102]. The collagen type I solution with final concentration of 1 mg/mL was prepared as previously described [102] and seeded in 12 well plates. Prior to seeding, LN-18\textsuperscript{shSRGN} or LN-18\textsuperscript{shSCR} cells were treated with serum free media for 16 h and then seeded at density of 6 x 10\(^4\) cells per well on top of collagen type I gels. After 24 h, phase contrast images were captured at 10\(\times\) using the OLYMPUS CKX41 microscope with a color digital camera CMOS (SC30). The evaluation of cell invasion was conducted according to De Wever et al. [102], using Image J Software.

**Gelatin and casein zymography assays**

LN-18\textsuperscript{shSRGN} or LN-18\textsuperscript{shSCR} cells were seeded in 100 mm dishes and incubated in complete medium for 24 h followed by overnight starvation in serum free medium. Afterwards, cells were incubated with serum free medium for 24 h. Then, the culture supernatants were collected, centrifuged at 3000 rpm for 5 min and concentrated with Amicon® Ultra 4 3 K centrifugal filter devices (Millipore). Homogenized tissue samples were subjected to protein extraction using lysis buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.1% (w/v) SDS supplemented with 1x protease inhibitor cocktail (Chemicon, Millipore, CA, #20-201) and 2.5 mM sodium orthovanadate (Sigma-Aldrich, #S6508). The samples were homogenized with lysis buffer (volume was 10x the weight of the sample) and incubated for 4 h at 4 °C under constant agitation. The samples were centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatants were kept and stored at −20 °C. Equal amounts of proteins from the concentrated culture supernatants or the proteins extracted from the tissue samples were incubated in Laemmli buffer for 30 min at 37 °C. For gelatin zymography, proteins were separated by SDS-PAGE in 10% polyacrylamide gels supplemented with 0.1% (w/v) gelatin. After electrophoresis, the gels were washed twice with 0.1 M Tris-HCl pH 7.3/5% (v/v) Triton X-100 and then incubated at 37 °C for 20 h in the same buffer containing 5 mM CaCl\(_2\) and 5% (v/v) Triton X-100. For casein zymography, proteins were separated by SDS-PAGE in 10% polyacrylamide gels supplemented with 2 mg/mL casein and 10 \mu g/mL human plasminogen. After electrophoresis, the gels were washed twice with 0.1 M Tris-HCl pH 8, 50 mM Na\(_2\)EDTA, 0.1 mg/mL NaN\(_3\) buffer, containing 2.5% (v/v) Triton X-100 and then incubated at 37 °C for 20 h in the same buffer without Triton X-100. In both gelatin and casein zymography, gels were stained with 0.25% (w/v) Coomassie brilliant blue R-250 in 43% (v/v) methanol/7% (v/v) acetic acid and destained in 40% (v/v) methanol/7% (v/v) acetic acid. Areas of protease activity were detected as clear bands against the blue background and their density was determined by using Image J Software.

**IL-6 and IL-8 enzyme-linked immunosorbent assay (ELISA)**

LN-18\textsuperscript{shSRGN} or LN-18\textsuperscript{shSCR} cells were seeded in 6 well plates and incubated in complete medium for 24 h followed by overnight starvation in serum free medium. Afterwards, cells were incubated with serum free medium for 48 h. Then, the culture supernatants were collected, centrifuged at 3000 rpm for 5 min and concentrated with Amicon® Ultra 4 3 K centrifugal filter devices (Millipore). The protein levels of interleukins were detected using the Human IL-8 ELISA (ImmunoTools, Germany, #31670089) and the Human IL-6 Standard TMB ELISA Development kit (Peprotech, USA, #900-T16) according to manufacturer’s instructions.
Statistical analysis

For each assay, individual experiments were conducted at least 3 times. Data in diagrams are expressed as mean ± standard deviation (SD). Statistically significant differences were evaluated using an unpaired two-tailed t-test. Statistical analyses and graphs were made using GraphPad Prism 5 (GraphPad Software). Statistically significant differences are indicated by asterisks: * (p ≤ 0.05), ** (p < 0.01), *** (p < 0.001). Non-statistical significant comparisons are displayed as ns (p > 0.05).

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Author contributions

DM performed the main experimental part and prepared the manuscript draft and the figures, PB performed the transduction procedure and supervised part of the experiments, DK performed the experimental animal procedure, MG and BG supervised part of the experiments, AM kindly provided useful materials and analyzed data, NKK analyzed data and drafted the manuscript, ADT conceived, supervised and coordinated the overall project. All authors reviewed the manuscript and approved the final article.

Declaration of competing interest

The authors declare that no conflict of interest exists.

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Appendix A. Supplementary data

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