

Silencing of *PROS1* induces apoptosis and inhibits migration and invasion of glioblastoma multiforme cells

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Abstract. Glioblastoma multiforme (GBM) is an aggressive brain tumor and most patients have poor prognosis. Despite many advances in research, there has been no significant improvement in the patient survival rate. New molecular therapies are being studied and RNA interference (RNAi) therapy is one of the promising approaches to improve prognosis and increase survival in patients with GBM. We performed a meta-analysis of five different microarray datasets and identified 460 significantly upregulated genes in GBM. Loss-of-function screening of these upregulated genes using LN18 cells was performed to identify the significant target genes for glioma. Further investigations were performed using siRNA in LN18 cells and various functional assays were carried out on the selected candidate gene to understand further its role in GBM. We identified *PROS1* as a candidate gene for GBM from the meta-analysis and RNAi screening. Knockdown of *PROS1* in LN18 cells significantly induced apoptosis compared to siPROS1-untreated cells ($p < 0.05$). Migration in cells treated with siPROS1 was reduced significantly ($p < 0.05$) and this was confirmed with wound-healing assay. *PROS1* knockdown showed substantial reduction in cell invasion up to 82% ($p < 0.01$). In addition, inhibition of *PROS1* leads to decrease in cellular proliferation by 18%. Knockdown of *PROS1* in LN18 cells caused activation of both of the extrinsic and intrinsic apoptotic pathways. It caused major upregulation of *FasL* which is important for death receptor signaling activation and also downregulation of *GAS6* and other members of *TAM* family of receptors. *PROS1* may play an important role in the development of

GBM through cellular proliferation, migration and invasion as well as apoptosis. Targeting *PROS1* in GBM could be a novel therapeutic strategy in GBM treatment.

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults with a median survival rate of only 15 months (1,2). GBM originates from the glial cells which subsequently evolved into tumors known as glioma (3). It is the deadliest primary brain tumor and is classified as grade IV astrocytoma by WHO criteria (1,4,5). The standard treatment for GBM consists of surgical removal, radiation followed by chemotherapy. Unfortunately, these treatments only gave minor improvements to the patients' survival particularly for the recurrent GBM (4).

GBM is resistant towards treatment due to the heterogeneous nature of the disease. These are contributed by the dysregulation of the core signaling pathways such as the *ErbB*, *MAPK*, *mTOR* and *p53* signaling pathways (6-9). It was shown that the pathogenesis of GBM requires alteration of multiple genetic pathways and each of the primary and secondary GBMs has a unique combination of these genetic changes (5). Primary or *de novo* GBM frequently showed loss of heterozygosity (LOH) at 10q (70% of cases), *EGFR* amplification (36%), *p16INK4a* deletion (31%) and *PTEN* mutations (25%) (10). Mutations in the *IDH1* and *IDH2* genes are common in astrocytomas, oligodendrogliomas, oligoastrocytomas and secondary glioblastoma with prevalence of 50-80% of cases (11). In addition, *TP53* and *PTEN* mutations are common in primary and secondary gliomas with a frequency of 28 and 65% for *p53*, 25 and 4% for *PTEN* respectively (10). Secondary GBM is generally initiated from diffuse astrocytomas. Some common molecular lesions associated with secondary GBM are *TP53* mutations (60-65%) and gain of 7q arm (21-50%) with *MET* gene gain of function (47% in primary and 44% in secondary glioblastoma) being affected significantly and associated with poor prognosis (12). There are also reports on the involvement of aberrant intrinsic and extrinsic apoptotic pathways and the overexpression of anti-apoptotic proteins such as *FLIPs*, *BCL2* and *survivin* which contribute to apoptotic resistance (13-17).

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The therapeutic strategy in GBM could be improved by targeting the multiple pathways involved. RNA interference (RNAi) is one of the attractive approaches and may result in the post-transcriptional knockdown of the genes of interest. Significant impact in *in vitro* experiments using RNAi has allowed the implementation of therapeutic approach using RNAi gene therapy *in vivo* (18). One of the major problems in GBM therapy is the difficulty for the drugs to cross the blood-brain barrier (BBB) hindering maximal drug distribution to the tumor site. To date, there are a few strategies being used to efficiently deliver siRNA through the BBB. The RNAi-based nanomedicine platform has been introduced at the pre-clinical stage (18). Based on spherical nucleic acid gold nanoparticle conjugates, which are densely packed, highly oriented siRNA duplexes targeting the oncoprotein *Bcl2Like12* (*Bcl2L12*) were used to neutralize the oncogene expression in GBM (19). There are many delivery systems that form complexes with siRNA including PEGylated immunoliposomes that carry siEGFR, recombinant adeno-associated virus carrying siHec-1 and lentiviral vectors carrying siTRAIL (20-22). An example of a molecular target that has made to clinical trial using siRNA and showed promising results is Tenascin-C (TN-C), which is overexpressed in the extracellular matrix (ECM) of GBM. It has been shown that dsRNA targeting TN-C mRNA could reduce the tumor size significantly and increase the survival rate by 11% (23,24).

Materials and methods

Meta-analysis of microarray datasets. We performed a meta-analysis on five microarray datasets from a cancer microarray database using an integrated data-mining platform, the Oncomine Research Edition (25). Data were filtered based on data source, cancer, the type of datasets and analysis. Candidate genes were selected based on the median rank and $p < 0.05$. Candidate genes obtained from meta-analysis were then screened using synthetic lethal RNAi screening and the hits were selected based on their significant values in viability reduction. The human glioblastoma LN18 (TP53-mutant) cells were transfected with pooled siRNA (SMARTpool™; GE Dharmacon, Lafayette, CO, USA) targeting against 460 genes and cultured for 48 h according to the manufacturer's protocol. The media were changed after 48 h post-transfection and incubated for another 48 h. The cells were then prepared for viability measurement using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corp., Madison, WI, USA). The experiment was performed in triplicate.

Cell culture. LN18 cells were maintained in T-75 flasks and allowed to grow in 15 ml of Dulbecco's modified Eagle's medium (DMEM) (ATCC, Manassas, VA, USA) and supplemented with 10% fetal bovine serum (J R Scientific, Inc., Woodland, CA, USA) until 80% confluency. The cells were incubated under 5% CO₂ condition. Generally, the doubling time for LN18 cells was <24 h. Cells were harvested by removing media and cells were then washed with 5 ml of 1X Dulbecco's Phosphate-Buffered Saline (Gibco, Grand Island, NY, USA) and trypsinised using 1X Trypsin EDTA 0.25% (J R Scientific, Inc.).

Preparation of siRNA. ON-TARGETplus SMARTpool™ of *PROS1* siRNA (GE Dharmacon) consisting of four different siRNA sequences were used in this experiment. The siRNA sequences used were: i) GCAUGGAAGUGAAUUAUAA; ii) GCAACAGGCUUCACAAGUC; iii) UAUUAGAGCUCACUCAUGU; and iv) GAAGAGUUGUGAGGUUGUU. Lyophilized *PROS1* siRNA was resuspended with 1X siRNA buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA). A total of 25 nM final concentration of *PROS1* siRNA and non-targeting siRNA were used with DharmaFECT2. All functional assays were performed 48 h post-transfection.

RNA extraction and qPCR. RNeasy kit (Qiagen, Hilden, Germany) was used to isolate total RNA from cells. The quality and quantity of the isolated RNA were determined using NanoDrop (Thermo Fisher Scientific, Inc.). Briefly, 100 ng of RNA were used to generate cDNA using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). qPCR was conducted using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.) on a Rotor-Gene 3000 (Corbett Life Science/Qiagen, Inc., Valencia, CA, USA) platform. The *PROS1* primers used were: forward, 5'-TGCTGGCGTGTCTCCTCCTA-3' and reverse, 5'-CAGTTCTTCGATGCATTCTCTTTCA-3'. The expression of *PROS1*-related genes such as *GAS6*, *RhoA*, *FasL*, *Tyro-3*, *Axl*, and *Mertk* was also quantified using qPCR and results were calculated based on the $\Delta\Delta C_t$ method (26). *ACTB* gene was used as the reference gene. Primer sequences are shown in Table I.

Viability assay. The CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corp.) which is based on the quantification of ATP present in the viable cells was used for viability assay. Cells were cultured for 24 and 48 h post-transfection. Subsequently, CellTiter-Glo® buffer was added onto the CellTiter-Glo® substrate, which was then loaded into the samples. The luminescent signal was captured at 570 nm using SpectraMax® L Luminescence Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Cellular viability was calculated based on the normalization between treated (siPROS1) vs. non-targeting siRNA cells from three independent experiments.

Proliferation assay. Proliferation assay was performed using the bromodeoxyuridine (BrdU) incorporation method (Millipore Corp., Billerica, MA, USA). Transfected cells were cultured for 24 h in the presence of BrdU which was incorporated into newly synthesized DNA strand of the proliferating cells. The cells were then fixed, and incubated with anti-BrdU monoclonal antibody (Millipore Corp.) for 1 h. Goat anti-mouse IgG peroxidase was added onto the well. Incorporation of BrdU in the proliferating cells leads to colorimetric changes from clear to blue which was measured using Varioskan Flash Multimode Reader (Thermo Fisher Scientific Oy, Vantaa, Finland) at 450 nm wavelength.

Migration assay. The effect of *PROS1* gene silencing on tumor cell invasion was investigated using QCM™ 3 μ m 24-well Chemotaxis Cell Migration Assay kit (Millipore Corp.). Cells were seeded in the 24-well inserts at a density of 1×10^4

Table I. Primer sequences used for qPCR analysis.

Gene	Primer sequence (5'→3')
<i>PROS1</i>	F TGCTGGCGTGTCTCCTCCTA R CAGTTCTTCGATGCATTCTCTTCA
<i>Tyro-3</i>	F CACGGTAGAAGGTGTGCCAT R TGGTAACAGGTTTCAGGGGGA
<i>Axl</i>	F TTAGTGCTACGCGGAATGGG R TGTCCATTAGCACCTCTGGG
<i>Mertk</i>	F GTCCATCTGTCCATCCGTC R CCTCAGTGATAGCTCTACGCC
<i>Gas6</i>	F ACGACCCCGAGACGGATTA R GCGAAGCCTGAGTTTTTGGT
<i>FasL</i>	F CCTTGGTAGGATTGGGCCTG R CTGTGTGCATCTGGCTGGTA

F, forward; R, reverse.

cells/well in serum-free media for 24 h and allowed to migrate through the membrane towards the media. The migrated cells were then lysed and the fluorescent signal was quantified using Varioskan Flash Multimode Reader (Thermo Fisher Scientific Oy). We also performed wound healing scratch assay in order to observe the cellular motility in si*PROS1*-treated LN18 cells. Wound closure was observed at 0, 24, 48 and 72 h post-scratching. These assays were performed in three independent replicates.

Invasion assay. The role of *PROS1* in cell invasion was investigated using QCM™ 24-well Cell Invasion Assay kit (Millipore Corp.). The cells were cultured overnight in serum-free media and allowed to invade through the ECM. The cells were harvested and lysed prior to fluorometric quantification using Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Inc.). The invasion assay was carried out in three independent replicates.

Apoptosis assay. Apoptosis was determined using the ssDNA Apoptosis ELISA kit (Millipore Corp.). In total, 5×10^3 LN18 cells were grown overnight in a 96-well plate. Subsequently, the cells were transfected either with si*PROS1* or non-targeting siRNA for 48 h. Cells were then prepared for apoptosis measurement according to the manufacturer's protocol and the signal was measured using ELx800 TC models 95 Microplate Reader (Biotek Instruments, Inc., Winooski, VT, USA).

Cell cycle analysis. Cell cycle assay was performed using 1×10^6 LN18 cells that were transfected with si*PROS1* or non-targeting siRNA. Cells were harvested using a standard protocol as indicated in the Cycletest™ Plus DNA Reagent Kit protocol (BD Biosciences, Mississauga, ON, Canada). Subsequently, cells were washed three times with wash buffer. Cells were then suspended in solution A containing trypsin. Solution B with trypsin inhibitor and RNase were then added into the cell suspension. Finally, solution C which contained propidium iodide (PI) was added. Flow cytometric analysis was performed using BD FACSAria™ (BD Biosciences,

Franklin Lakes, NJ, USA). Data were analysed using ModFit LT software (Verity Software House, Inc., Topsham, ME, USA). The percentage of arrested cells was measured by the percentage of hypodiploid cells accumulated at the G0/G1, S, G2/M checkpoints of the cell cycle.

Western blotting. Protein expression of *PROS1* was assessed using western blotting. Cells were treated with si*PROS1* and proteins were harvested and extracted using radioimmunoprecipitation assay (RIPA) buffer. A total of 50 µg protein was loaded onto the Mini-PROTEAN® Precast Gels (Bio-Rad Laboratories, Inc.), and then transferred onto the Immobilon transfer membranes (Millipore Corp.). Membranes were then incubated with SuperBlock® (Thermo Fisher Scientific, Inc.) for 1 h at room temperature. After that, membranes were incubated overnight with *PROS1* mouse monoclonal antibody (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C. The membranes were then washed three times with TBST. Membranes were then incubated with goat anti-mouse secondary antibody conjugated to alkaline phosphate (1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Prior to protein detection, the membranes were washed three times. Finally, proteins were detected using Pierce ECL and SuperSignal substrate (Thermo Fisher Scientific, Inc.). β-actin was used as an internal control.

Protein array. Protein array was conducted using the Human Apoptosis Array kit (RayBiotech, Norcross, GA, USA). Protein samples were extracted from 48 h post-transfection according to the manufacturer's protocol. The quantity of the protein isolated was determined using BCA Protein Assay (Thermo Fisher Scientific, Inc.). Briefly, protein was loaded into the chamber slides coated with 43 different types of apoptosis antibodies. Subsequently, the slides were washed and the membranes were incubated with a cocktail of biotin-conjugated anti-apoptotic protein antibodies. The membranes were incubated with HRP-streptavidin prior to signal detection.

Statistical analysis. All data were expressed as the mean ± SD of three independent experiments. Significant differences were defined as $p < 0.05$. All statistical analyses were performed using Microsoft Excel and the Statistical Package for the Social Sciences (SPSS) software.

Results

***PROS1* as a novel candidate for GBM therapy.** Meta-analysis on five microarray datasets (Bredel Brain 2, Lee Brain, Liang Brain, Shai Brain, and Sun Brain) identified 460 upregulated genes based on the median rank and $p < 0.05$. All datasets were normalized between cancers vs. normal tissues. These 460 genes were used as candidates for RNAi screening. Based on the SSMD and kMAD analyses, 212 hits were identified. After selection, *PROS1* was identified as a target gene for validation since the role of *PROS1* in GBM has not been documented (Fig. 1).

***PROS1* silencing decreases *PROS1* mRNA and protein expressions.** The efficiency of *PROS1* silencing was assessed using qPCR. The results showed that ~80% of the *PROS1* gene was

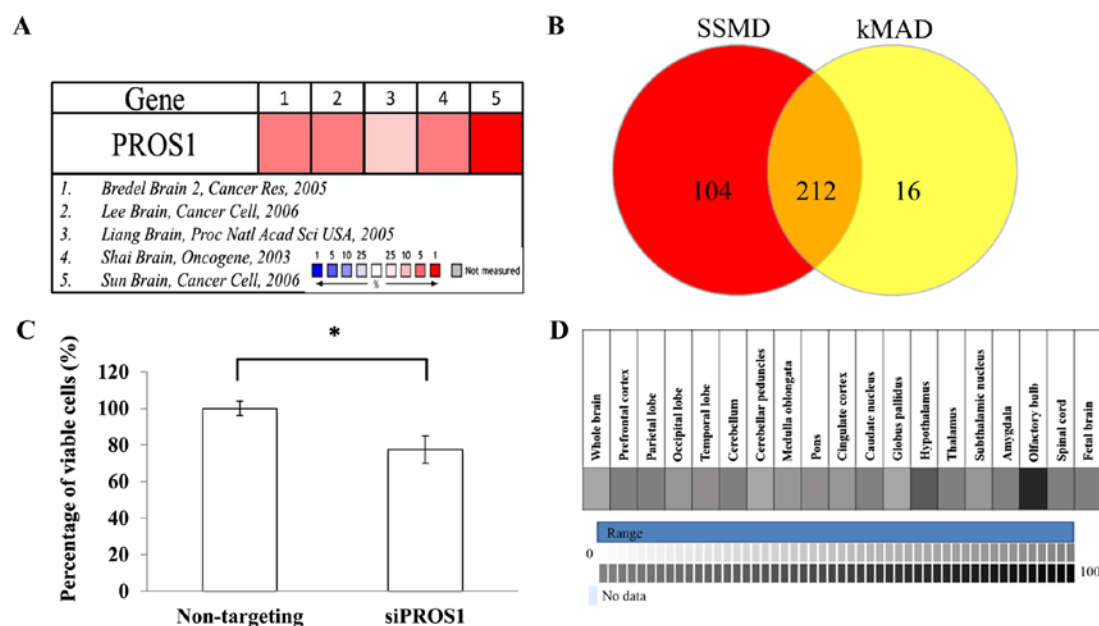


Figure 1. Meta-analysis and RNAi screening identified *PROS1* as a novel candidate for GBM therapy. (A) Meta-analysis of *PROS1* expression on five micro-array datasets from Oncomine database. (B) A total of 212 hits overlapped by using SSMD and kMAD methods. (C) High-throughput RNAi screening shows that *PROS1* gene silencing significantly reduced cell viability in GBM cells. * $P < 0.05$. (D) The expression of *PROS1* in different parts of the brain. RNAi, RNA interference; GBM, glioblastoma multiforme.

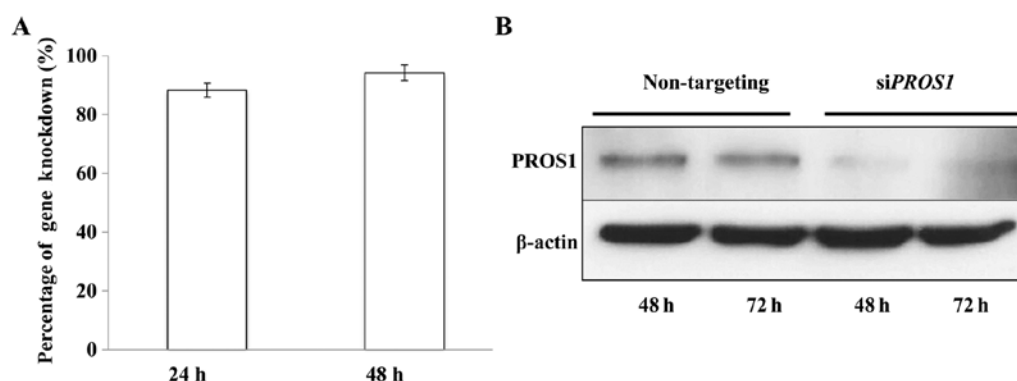


Figure 2. *PROS1* gene silencing results in reduced *PROS1* protein expression. (A) The efficiency of *PROS1* gene knockdown was 80% at 24 h and increased up to ~100% at 48 h. (B) Western blotting of *PROS1* was performed to determine the knockdown efficiency at protein level. The results showed that the *PROS1* protein expression was depleted 48 h post-transfection.

knocked down after 24 h and it increased up to 100% after 48 h of transfection. This was further confirmed at protein level via western blotting at 48 and 72 h post-silencing as the expression of the *PROS1* protein was reduced significantly compared to control (Fig. 2).

***PROS1* silencing reduces cell viability and cell proliferation.** Cell viability assay was conducted using CellTiter-Glo® Luminescent Cell Viability Assay to determine the effect of *PROS1* gene silencing. The number of viable cells was reduced in a time-dependent manner. The quantification of proliferating cells by BrdU showed that the proliferation signal was decreased in si*PROS1* treatment (18%) compared to the control (Fig. 3).

***PROS1* silencing inhibits LN18 cell migration.** Migration was reduced significantly ($p < 0.05$) in si*PROS1*-treated LN18 cells.

The scratch assay demonstrated the inhibition of the migratory potential of the 24-h post-scratch si*PROS1*-treated LN18 cells. Surprisingly, the size of wound scratch remained up to 72 h. These data suggest the possible role of *PROS1* in GBM cell migration (Fig. 4A and B).

***PROS1* silencing reduces GBM cell invasion.** Cell invasion assay was performed to study whether *PROS1* suppression could influence the invasion of LN18 cells. The results showed that the invasion of LN18 cells through the ECM was inhibited with si*PROS1*-treated cells up to 82% ($p < 0.01$) compared to the control group (Fig. 4C).

***PROS1* silencing significantly induces cell death through apoptosis.** ELISA-based assay was conducted to determine the mode of cell death in si*PROS1*-treated LN18 cells. Apoptosis was increased compared to the control at 48 h post-transfection

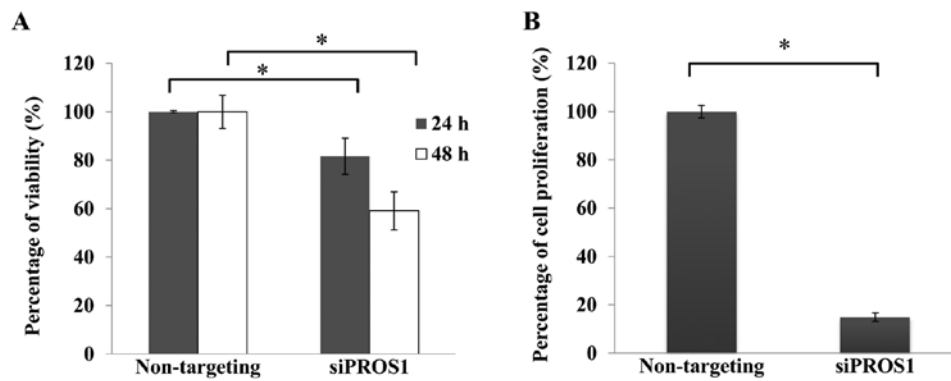


Figure 3. *PROS1* gene silencing significantly reduces viability and cell proliferation. (A) *PROS1* gene silencing resulting in reduced GBM cell viability up to 40% at 48 h post-transfection. * $P < 0.05$. (B) Treatment of GBM cells with siPROS1 showed decreased cell proliferation at 48 h post-transfection by BrdU assay. GBM, glioblastoma multiforme; BrdU, bromodeoxyuridine.

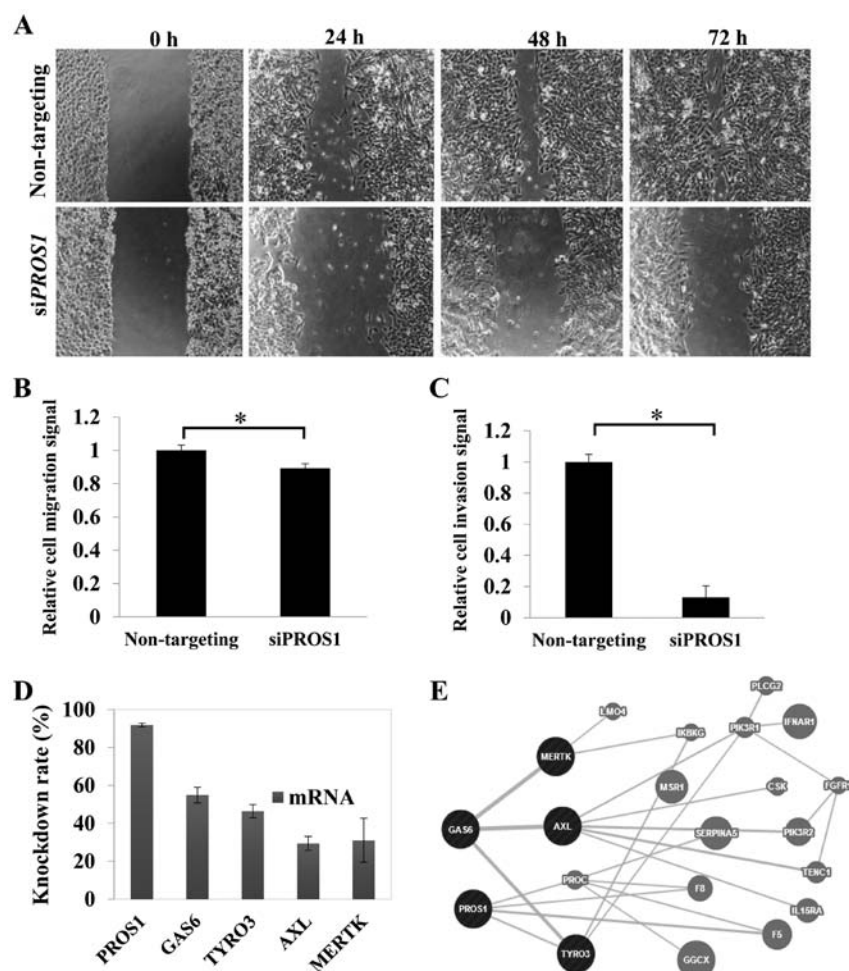


Figure 4. *PROS1* silencing in GBM cells exhibits decreased migratory potential and may become a potential target for cell invasion. Silencing of *PROS1* gene showed a significant reduction in cell migration and invasion in GBM cells at 48 h post-transfection. (A) The morphological observation of *PROS1* gene silencing effects on wound healing assay in GBM cells shows a clear gating in siPROS1 treatment scratched cells at 0-72 h compared to control. (B) *PROS1* gene silencing significantly reduces migratory potential through membrane in GBM cells. (C) *PROS1* gene silencing significantly controls cancer invasiveness by reducing the invasion capability in GBM cells. * $P < 0.05$. (D) *PROS1* gene silencing reduced the mRNA expression of *Gas6* and *TAM* receptors. (E) Mapping of physical interaction between *PROS1*, *GAS6* and *TAM* receptors was generated by GeneMANIA. GBM, glioblastoma multiforme.

($p < 0.05$) (Fig. 5A). There was no evidence of cell cycle arrests identified from the cell cycle assay (data not shown). Further validation was conducted using protein array to elucidate the relevant pathways involved in this process.

PROS1 gene silencing leads to decreased expression of *Tyro-3*, *Axl* and *Mertk*. qPCR was performed to study the effect of *PROS1* silencing on its related interacting genes from the TAM family of receptor tyrosine kinases which include

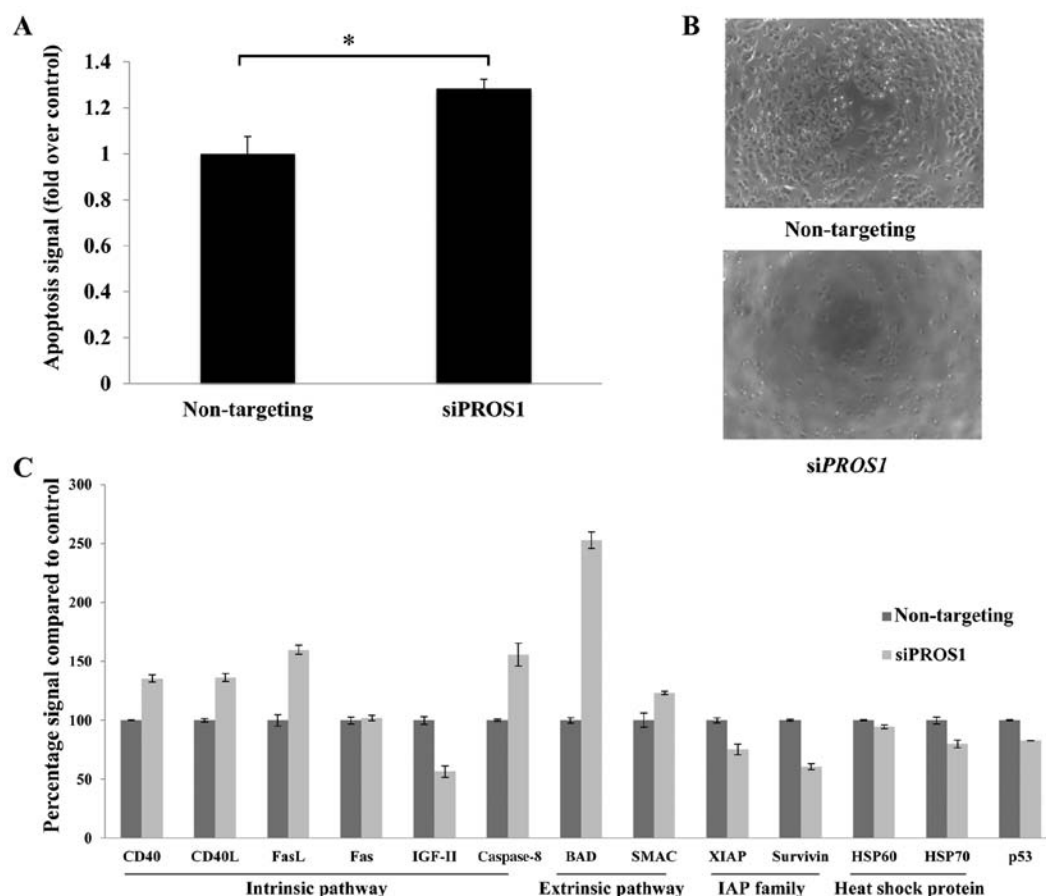


Figure 5. The *PROS1* gene silencing significantly induces cell death through apoptosis by activating both intrinsic and extrinsic pathways. (A) Silencing of *PROS1* resulted in an apoptosis signal at 48 h post-transfection. * $P < 0.05$. (B) si*PROS1* treatment induced formation of apoptotic bodies. (C) Most significant hits induced by *PROS1* gene silencing in apoptosis array indicate the activation of both intrinsic and extrinsic pathways.

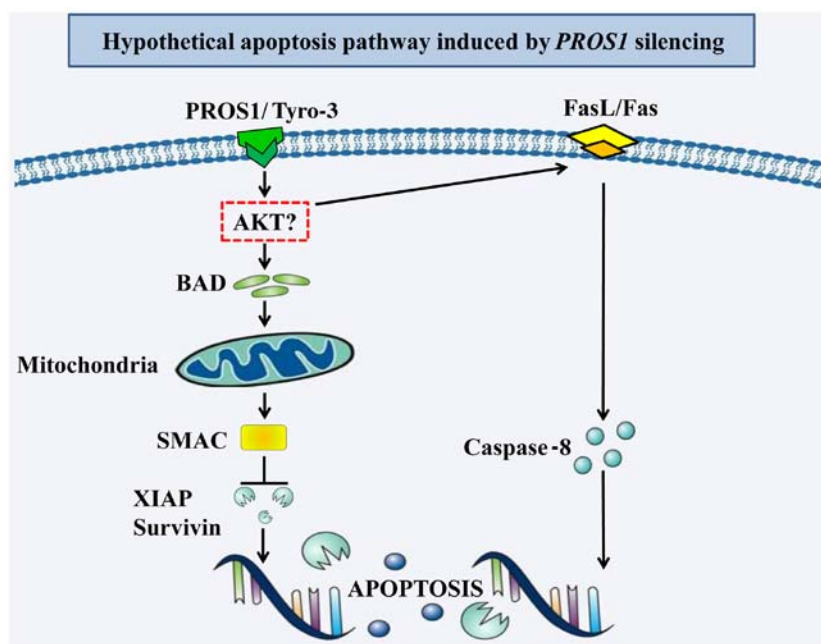


Figure 6. The working model of intrinsic and extrinsic pathways induced by *PROS1* gene silencing. In the intrinsic pathway, *PROS1* silencing activates BAD which is a BCL2-associated antagonist of cell death and then activates the mitochondrial pathway by releasing SMAC protein. The overexpression of SMAC will suppress the expression of IAP family which consists of XIAP and survivin and then induce apoptosis via downstream of apoptosis caspases. In the extrinsic pathway, an overexpression of FasL/Fas activated caspase-8 and induced apoptosis via downstream of apoptosis caspases. In the presence of *PROS1*, AKT will be activated and then phosphorylated BAD protein. The activation of AKT also suppresses the expression of FasL/Fas and then blocks the caspase-8 activation. Previously it was shown that the activation of AKT promotes overexpression of apoptosis inhibitors like BCL2 and IAP family and also suppresses the expression of pro-apoptotic proteins like BAD and BAX. IAP, inhibitor of apoptosis protein; FasL, Fas ligand.

GAS6, *Tyro-3*, *Axl*, and *Mertk*. This was performed at 48 h post-transfection. The level of *GAS6* was reduced to 68.7% compared to the control ($p < 0.05$). *PROS1* gene silencing also reduced the expression of the tyrosine kinases, especially the *Tyro-3* where the expression was 50% reduced compared to the control. The expression of *Axl* and *Mertk* genes was reduced to 70.6 and 69%, respectively (Fig. 4D and 4E).

Discussion

The main aim of this study was to understand the functional role of *PROS1* in GBM by performing silencing experiments coupled with various functional assays. *PROS1* was identified as a potential gene target for GBM from our meta-analysis using five microarray datasets and the loss-of-function RNAi screening of 460 upregulated genes. *PROS1* is a vitamin K-dependent plasma protein and is known to be involved in the anticoagulant cascade. It acts as a cofactor for anticoagulant protease in the blood coagulation system known as the activated protein C (APC) (27). *PROS1* shares ~43% of amino acid identity with *GAS6*, a γ -carboxyglutamic acid (Gla)-containing protein, which stimulates cell proliferation through activation of the *Axl* receptor tyrosine kinase (28-30). *PROS1* and *GAS6* are ligands for *Axl* together with *Tyro-3* and *Mertk* and were reported to be overexpressed in haematological malignancies and solid tumors, suggesting that these molecules activate important autocrine-based oncogenic signaling events in cancer cells (31-34). Overexpression of TAM receptors mediates multiple oncogenic phenotypes in GBM such as *in vitro* proliferation, anchorage-independent growth, xenograft growth, resistance to apoptosis, autophagy, invasion and migration as well as activation of the downstream PI3K and MAPK survival pathways (33). Inhibition of *Mertk* and *Axl* by gene knockdown in astrocytoma cells enhanced apoptosis and improved chemosensitivity towards conventional chemotherapeutic agents such as temozolomide, carboplatin and vincristine (35).

Initially *PROS1* was thought to be the ligand for the TAM receptors. However, Stitt *et al* have shown that *PROS1* has a higher affinity for the *Tyro-3* receptor and can transform NIH 3T3 cells in an autocrine manner (36). One of the important findings that changed the perspective for *PROS1* was that the anticoagulant factor played an important role in activating *Tyro-3* activity as its expression was upregulated in cultured Schwann cells and astrocytes following nerve injury (36). This activation of intracellular signaling cascades by specific cell-surface receptors would promote cell proliferation for tissue repair and growth. *PROS1* was found to be highly expressed in high-grade prostate cancers suggesting that it has an important role in the regulation of cancer cell survival (29). Knockdown of *PROS1* by shRNA was reported to significantly reduce the number of cancerous cells in a time-dependent manner (37). Indeed, this is in agreement with our findings where silencing of *PROS1* using siRNA significantly reduced cell viability of GBM cells by >40%. This was also supported by the reduction of Brdu proliferative signals.

PROS1 is also involved in the phagocytosis of apoptotic cells in the immune, nervous, and reproductive systems through interaction with *Tyro-3* (38). During hypoxia or ischemia, *PROS1* protects neuron cells and inhibits apoptosis

by inhibiting Fas ligand (FasL) production and inhibiting FasL-dependent caspase-8 activation within the extrinsic apoptotic pathway (39). Wang *et al* showed that *Tyro-3* silencing affected several important signaling pathways including *PI3K/AKT*, *Wnt/ β -catenin*, *ERK/MAPK*, *PAK/JNK*, *JAK/Stat* and *TGF- β* as well as the *retinoic acid receptor (RAR)* activation (40). We showed that silencing *PROS1* led to a significant increase in apoptotic signals and this result was validated using protein array. Silencing of *PROS1* caused significant activation of the apoptotic pathways by upregulation of CD40, CD40L, Fas, FasL and caspase-8 of the intrinsic pathway as well as BAD and SMAC of the extrinsic pathway. Interestingly, it significantly reduced the expression of the inhibitor of apoptosis protein (IAP), XIAP and survivin. *PROS1* silencing also led to the downregulation of *Tyro-3*, *Axl*, *Mertk* and *Gas6* gene expressions, suggesting that the GBM cells might undergo apoptosis through the TAM receptor interaction. However, there are some limitations in terms of the number of protein markers available in our protein array which hindered the identification of other downstream apoptosis proteins involved.

Another study on castration-resistant prostate cancer cells showed that the addition of human purified *PROS1* increased the migration of these cells (29). Furthermore, the high-throughput wound healing screening on the epithelial cells revealed the involvement of TAM receptors in cell migration (41). Our results are consistent as we showed that *PROS1* gene silencing delayed the wound enclosure in GBM cells and significantly reduced the capability of cells to migrate. This might be due to the involvement of the extracellular domains of TAM receptors that contain adhesion molecule-like motifs which controls cell-cell contacts and actin cytoskeleton regulation (42). Our results showed that silencing of *PROS1* expression also led to a significant reduction in cell invasion through the ECM in GBM cells. These findings suggest that *PROS1* may provide a survival advantage for advanced stage cancer like prostate and GBM by controlling cancer cell migration and invasion.

In summary, we showed that silencing *PROS1* reduces survival, migration and invasion of GBM cells (as detailed in Fig. 6). It also activates apoptosis in GBM cells by activating the intrinsic and extrinsic apoptotic pathways. Further validation using *in vivo* studies are needed to enhance our understanding on the mechanistic role of *PROS1* in GBM cells. This will hopefully allow the development of *PROS1* gene therapy as a possible approach to increase patient survival and improve the treatment of GBM patients.

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