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Sphingomyelin synthase 1 regulates Neuro-2a cell proliferation and cell cycle progression through modulation of p27 expression and Akt signaling

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Abstract

Sphingomyelin synthase (SMS) is a key enzyme involved in the generation of sphingomyelin (SM) and regulation of cell growth and survival. However, the effects of SMS on neuronal cell proliferation and cell cycle progression are not completely elucidated. In this study, we examined the direct effects of SMS1 in regulating cell cycle progression and proliferation of Neuro-2a cells that exhibit neuronal characteristics. Neuro-2a cells transfected with SMS specific shRNA expressed significantly lower levels of SMS1. RNA interference-mediated depletion of SMS1 in Neuro-2a cells caused a significant decrease in SM levels. Decreased SMS1 levels resulted in reduced proliferation rate and morphological changes including neurite like out growth. Also, silencing of SMS1 induced cell-cycle arrest as shown by the increased percentage of cells in G0/G1 and decreased proportion of cells in S-phase. These changes were accompanied by up-regulation of cyclin-dependent kinase inhibitor p27, and decreased levels of cyclin D1 and phospho-Akt. Nuclear accumulation of p27 was also evident in SMS1 deficient cells. Furthermore, loss of SMS1 inhibited the migratory potential of Neuro 2a cells in association with decreased levels of matrix metalloproteinases. These results indicate that SMS1 plays an important role in mediating the key signaling pathways that are involved in the tight coordination of multiple cellular activities, including neuronal cell proliferation, cell cycle progression, and migration, and therefore may have significant implications in neurodegenerative diseases.

Keywords

Sphingomyelin synthase; Neuro-2a; Cell cycle; Migration

Introduction

The synthesis and metabolism of lipids is critical not only in cell membrane function but also in the regulation of cell proliferation and differentiation with implications in health and diseases [1–7]. Sphingomyelin synthase (SMS) is an important enzyme that controls growth

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and survival through the biosynthesis of sphingomyelin (SM) and diacylglycerol (DAG) from ceramide [8–12]. Many studies have linked SMS activity to enhanced cell proliferation and the suppression of apoptosis [11–18]. However, over expression of SMS1 is shown to increase the rate of apoptosis and inhibition of growth [19, 20]. Sphingolipids including SM are important regulators of various cellular processes including proliferation, migration, apoptosis, differentiation, angiogenesis, and inflammatory responses that are associated with various diseases [3, 6–12]. Particularly, a number of reports implicate the contribution of lipid metabolism and cell-cycle regulating proteins in cerebro-cardiovascular diseases including ischemic stroke [3, 6, 23]. A tumor cytotoxic agent tricyclodecan-9-yl-xanthogenate (D609) is reported to inhibit SMS activity [23–26]. We have reported that D609 inhibits proliferation and cell cycle progression of microglia BV2 cells possibly through SMS inhibition [27]. Furthermore, bFGF stimulated astrocyte proliferation is associated with SMS activity [15]. Despite these reports, the direct effects of SMS on cell cycle progression and proliferation of neuronal cells is not elucidated.

Cell proliferation and differentiation are tightly controlled by cyclin-dependent kinases (Cdks) whose activities are modulated by interactions with cyclins and Cdk inhibitors p27 and p21 [28–31]. Independent of its Cdk inhibitory function, P27 is also shown to regulate the fundamental cellular processes, including cell differentiation and migration [30–35]. Indeed, p27 inhibits cancer cell migration by binding to microtubule destabilizing protein [36, 37]. Particularly, it is increasingly clear that p27 participates in the regulation of neuronal cell cycle, migration, and differentiation as shown in neuronal and neuroblastoma cells [38–41]. Many of the above mentioned functions including cellular proliferation and survival are regulated by the phosphatidylinositol 3-kinase (PI3 K)/Akt signaling pathway. Most notably, activation of Akt signaling promotes DNA synthesis and cell cycle progression in a variety of cell types including neuroblastoma cells [42–45]. Indeed, RNA interference-mediated depletion of PI3K induces cell cycle arrest [45]. Interestingly, Akt signaling inhibits p27 transcription or accelerates p27 degradation in different cell types. Akt activation is also linked to impaired nuclear import of p27, and Akt inhibition restores nuclear p27 expression [46–48]. Recently, it has been shown that cytoplasmic retention of p27 promotes cell migration [48–50]. Tight regulation of neuronal cell migration is critical for proper establishment of neuronal functional networks and thus, disruption of neuronal migration can lead to neurological disorders [40]. It is well established that the process of cell migration is regulated by metallo-proteinases (MMP) including MMP2 and 9 that act intrinsically as important signaling molecules, and their dysregulated expression can lead to pathological conditions [51–53]. Taken together, these studies have greatly increased our understanding of cellular functions, but the role of lipid synthesizing enzymes in regulating these processes has received relatively little appreciation. Specifically, the role of SMS1 in regulating these signaling pathways and functions in cells of neuronal origin is not completely elucidated.

In this study, we examined the direct effects of SMS1 on cell proliferation, cell cycle progression, and migration of Neuro-2a, the cells of neuronal origin derived from mouse brain. Here, we have demonstrated that inhibition of SMS1 expression by RNA interference significantly reduces the SM levels in association with cell cycle arrest, inhibited cell proliferation, and migratory potential of Neuro-2a cells. At the molecular level, SMS1

deficiency results in increased levels of p27, and decreased levels of cyclin D1, phospho-Akt, and MMP, the regulators of important signaling pathways.

Materials and Methods

Cell culture and establishment of Neuro-2a cells expressing SMS1-ShRNA: Neuro-2a, a mouse neuroblastoma cell line used in this study was obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells are derived from the brain and show neuronal like characteristics. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL) containing 10% fetal calf serum (FCS) and 1% penicillin and streptomycin (Life Technologies, Gaithersburg, MD) and were grown in a 5% CO_2 incubator at 37°C. To silence the expression of SMS1 in Neuro-2a cells, we used shRNA plasmid containing 29-mer shRNA sequence in GFP vector targeted against the mouse SMS1 gene expression. Non-effective 29-mer scrambled shRNA cassette in pGFP-V-RS Vector (Origene, Rockville, MD) was used as a control. GFP was used to monitor the efficiency of transfection. Cells were transfected with either control or SMS1-shRNA plasmid using lipofectamine 2000 (Life Technologies, Gaithersburg, MD). After selection with puromycin (1 μg/ml), cells were used for further studies.

Total RNA Isolation and Semi quantitative Reverse Transcription (RT)-PCR

Total RNA was extracted using the RNAeasy extraction kit according to the manufacturer's protocol (Qiagen, Inc., Valencia, CA). The cDNAs synthesized from total RNA (2 μg) using M-MLV reverse transcriptase and Oligo (dT) primer (Invitrogen) were used as templates for the RT-PCR reaction. The primer set used for SMS1 amplification was 5′-AACGGCATGATCCTTGTAGG - 3′ and 5′-ATAAGCCACCTCCAGCAATG -3′, MMP2 amplification was 5′-GCTCCACCACATACAACTTTGA and 5′-TCGGGACAGAATCCATACTTCT, Actin amplification using the primer set 5′-TGTTACCAACTGGGACGACA -3′ and 5′-GGGGTGTTGAAGGTCTCAAA-3′ was used as the control. PCR amplifications were carried out in 30 cycles of denaturation (94 °C, 1 min), annealing (58 °C, 30 s), and extension (72 °C, 1 min), and the products were analyzed on 1% agarose gels.

Measurement of sphingomyelin (SM) levels

Cell pellets were re-suspended in 0.4 mL saline, and aliquots were taken for protein measurements. Total lipids were then extracted from the cell lysates into chloroform/methanol (1:2 by volume) containing 0.01% butylated hydroxytoluene (BHT). After concentrating under a stream of nitrogen, total lipid extracts were applied to Whatman Partisil LK-5 silica gel thin-layer chromatography (TLC) plates with pre-concentration zone (Fisher Scientific, Pittsburg, PA). Sphingomyelin (SM) was separated by development of the TLC in chloroform/ethanol/water/triethylamine (30:35:7:35) and identified using an authentic standard (Sigma, St. Louis, MO). The SM bands were scraped into 1.5 mL of methanol containing 0.01% BHT, 30 μL of sulfuric acid, and 10 nmol of hepta-decanoic acid as an internal standard. A corresponding blank region of the TLC was collected to correct for any background. SM was converted to fatty acid methyl esters by heating at 100° C for 2 hr. The methyl esters were extracted into hexane and quantitated on a Hewlett

Packard 6890 gas chromatograph (GC) equipped with a capillary column (HP cross-linked FFAP) and an auto sampler.

Cell proliferation assay

Cell proliferation was evaluated by a colorimetric assay that measures mitochondrial reductase catalyzed reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). The number of actively proliferating viable cells was determined by using MTT assay kit (Biotium, Hayward, CA) according to the manufacturer's protocol. Briefly, 5×10^3 cells per well were plated in triplicate on 96 well plates and cultured for 18 hours in a 37°C incubator with 5% CO_2 The absorbance of released purple formazan that indicates cell viability was measured at 570 nm in a microtiter plate reader (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA). Optical density values were determined and the results were presented as mean values +/− SD of triplicates.

Cell cycle analysis

The cell cycle parameters for Neuro-2a cells expressing either control or SMS1-ShRNA were determined by flow cytometry of propidium iodide-stained cells. Briefly, from each group, 2×10^6 cells were washed in PBS containing 5 mM EDTA and fixed in 70% ethanol at 4°C overnight. Cells were then resuspended in PBS containing 2% FBS and 5 mM EDTA and were stained with 50 μg/ml propidium iodide and 1 mg/ml RNase A in PBS for 2 hr at room temperature. Cells were then analyzed by flow cytometry (FACSCalibur, BD Biosciences, Fullerton, CA) to determine the cell cycle distribution. The percentage of cells present in G0/G1, S and G2/M phases was determined using the ModFit software package (Verity Software House, Topsham, ME).

Scratch -induced cell migration assay and Trans well cell invasion Assay

Cell migration was analyzed as previously described [54]. *In vitro* injury was induced in SMS1-shRNA transfected, and control-shRNA transfected cell monolayers by creating 1–2 linear scratches of ±0.2 mm wide, after which detached cells were removed and fresh medium was added. Cells were photographed at 2 and 24 hours after wounding using a phase-contrast microscope interfaced with a digital camera. Trans well invasion assay was used for the quantitative measurement of Neuro-2a cells migration. In this assay, Neuro-2a cells transfected with either control or SMS1-shRNA plasmids (1×10^5) were placed in upper side of Boyden chambers containing matrigel-coated Bio coat cell culture inserts (BD Biosciences, Bedford, MA) with 8 μm pores. The lower chamber was filled with 500 μl of serum containing culture medium. Cells were incubated for 24 hours at 37 °C, after which non-migrated cells on the upper surface were removed from the membranes. The migrated cells attached to the lower surface were stained with cresyl violet and extracted in 200 μl of 0.2 m sodium acetate buffer. Optical density values at 540 nm correlating with cell migration were plotted. Results are presented as mean values ± SD of triplicates.

Western blot analysis

Total protein was extracted from SMS1-shRNA and Control-shRNA transfected cells using RIPA buffer (Boston Bioproducts, Ashland MA) containing protease inhibitor cocktail.

(Sigma Aldrich, St. Louis, MO). Twenty μg of total protein was separated by SDS-PAGE and probed with respective antibodies, 1:1000 for phospho/total Akt, p27, cyclin D1 (Cell Signaling, Danvers, MA); 1:500 for Actin, (Sigma Aldrich, St. Louis, MO) followed by incubation with secondary antibody conjugated to horseradish peroxidase at room temperature for 1 hour. Signals were developed with chemiluminescence using an ECL kit (Thermofisher). Total Akt and actin were used as loading controls. The NIH Image J software program was used to quantitate the expression levels. The experiment was repeated twice and the data are presented as mean values +/− SD.

Immunofluorescence

Cells grown on cover slips were fixed with 3.7% paraformaldehyde for 15 min at room temperature. Cells were then blocked and permealized by incubating in PBS containing 2% milk and 0.1% Triton X-100 for 1 hour. Cells were incubated with the primary antibody for 1 hour at 37°C. After cells were washed three times with PBS, they were incubated with Alexa Fluor-conjugated secondary antibody for 1 hour at 37°C. Subsequently, cells were washed three times with PBS, stained with nuclear stain DAPI for 5 minutes, washed three times with PBS, and mounted on a microscope slide with Fluoromount (Diagnostic BioSystems, Pleasanton, CA). Specimens were observed and images were acquired using a Keyence, BZ-9000 fluorescence microscope (IL, USA).

Motility pathway focused gene expression profiling by real-time -PCR (qRT-PCR)

A PCR mouse cell motility array (SA Biosciences, Frederick, MD) that profiles the expression of 84 genes that regulate cell motility was used according to the manufacturer's protocol. Briefly, the cDNA generated from 2 μg of total RNA was combined with SYBR green qPCR master mix. Equal aliquots of this mixture were added to each well of the PCR array plates containing pre-dispensed gene specific primer sets. qRT-PCR analysis was performed in an Applied Biosystems Prism 7000 Sequence Detection system and analyzed using GeneAmp 5700 SDS software. Relative quantification was performed using standard curves generated for each gene-specific primer pair. The values obtained from each set of gene-specific primers were normalized to endogenous control genes and used to determine relative expression levels. Levels of MMP2 mRNA were further validated using PCR assay.

Statistical analysis

A 2-tailed, non-paired Student's *t* test was used to predict statistical significance of the comparison between 2 means. The data is presented as means ± SD. Results were considered significant at $P \leq .05$ at 95% confidence level.

Results

Establishment of Neuro-2a cells deficient in SMS1 expression

To define the functional role of SMS1 in neuronal cells, we established Neuro-2a cells that express SMS1 specific small interfering RNA using SMS-shRNA-GFP plasmids. Using polymerase chain reaction, we confirmed that SMS1 mRNA levels were significantly decreased in SMS1-shRNA transfected Neuro-2a cells. However, there was no alteration in SMS1 mRNA levels in Neuro-2a cells transfected with control plasmid encoding for

scrambled control-shRNA. We used beta actin as an internal control, which remained the same in both cell lines (Figure 1A). Quantification of images using NIH image J software demonstrated more than 70% decrease in SMS1 mRNA levels in SMS1-shRNA transfected Neuro-2a as compared to control cells (Figure 1B). Immunofluorescence staining further confirmed the decrease in SMS1 protein in SMS1-shRNA transfected Neuro-2a as compared to control cells (Figure 1C). We then examined if decreased SMS1 expression resulted in the decreased levels of sphingomyelin (SM), an important byproduct of SMS1 activity. In SMS1-shRNA transfected cells, SM levels were significantly decreased (about 30%) as compared to cells transfected with control-shRNA plasmid (Figure 1D).

Silencing of SMS1 exerts morphological changes and anti-proliferative effect

We monitored the effects of SMS1 silencing on growth and morphological features of Neuro-2a cells. Neuro-2a cells transfected with scrambled control-shRNA exhibited an amoeboid-like structure and grew in clusters with round morphology with short cytoplasmic processes. On the other hand, SMS1-ShRNA transfected cells exhibited smaller cell bodies with longer and branched axon like processes resembling neuronal like cells (Figure 2A). Furthermore, SMS1 silencing in Neuro-2a cells induced about 30% growth arrest as shown by MTT proliferation assay (Figure 2B).

SMS1 silencing inhibits cell cycle progression of Neuro-2a cells

We next explored the functional significance of SMS1 expression loss in Neuro-2a cells. Proliferation inhibition is preceded by cell cycle arrest. Indeed, flow cytometric analysis of the propidium iodide (PI) stained cells following the SMS1 silencing revealed significant alterations in the distribution of cells in different phases of the cell cycle. Transfection of control-shRNA did not significantly alter cell populations in G0/G1, S, and G2/M phases (Figure 3A). SMS1-shRNA transfected Neuro-2a cells demonstrated cell cycle arrest with 77% percent of cells in G1 phase, while about 55% percent of the control-shRNA transfected cells were in G1 phase. The percentage of cells in G2 phase decreased from about 18% (control) to 11 percent in SMS1-shRNA transfected cells. The percentage of cells in S phase decreased by about 15% in SMS1 deficient cells as compared to control-shRNA transfected cells (Figure 3B). These results suggest that SMS1 silencing may block cell cycle progression of Neuro-2a cells by arresting them at G1 phase of the cell cycle.

SMS1 silencing in Neuro-2a cells alters the expression of key cell cycle regulating proteins p27 and cyclin D1

To gain further insight into the consequences of SMS1 loss on key cell cycle regulators, Neuro-2a cell lysates were subjected to western blot analysis using p27, cyclin D1, and phospho, and total Akt antibodies. CDK inhibitor p27 is an important regulators of the G1 cell cycle phase. The levels of p27 protein were significantly increased by SMS1 silencing. In contrast, cyclin D1 levels were significantly decreased in Neuro-2a cells after SMS1 silencing as compared to control cells (Figure 4A). Quantification and normalization of p27 and cyclin D1 protein levels relative to beta actin demonstrated a 2–3 fold increase in p27 and a 2–3 fold decrease in cyclin D1 in SMS1-ShRNA transfected cells vs. cells transfected with control-shRNA (Figure 4B). Furthermore, we observed that silencing of SMS1 led to alterations in cellular localization of p27 protein. Decreased SMS1 resulted in a remarkable

increase in nuclear p27 as seen by immunofluorescence staining (Figure 4C). These results demonstrate that Neuro-2a cell cycle arrest is induced by SMS1 knock down, and that p27 is involved in this process. The PI3-Akt pathway regulates p27 and plays a key role in cell proliferation, cell cycle, and survival in many cells. Hence we examined whether SMS1 silencing inhibited the activation of Akt in Neuro-2a cells. As shown in Figure 4D, the expression of phospho-Akt decreased significantly in SMS1-shRNA transfected cells. Total Akt protein expression was unaffected. Quantification and normalization of phospho-Akt protein levels relative to total Akt demonstrated about a 3 fold decrease in Phos-Akt in SMS1-shRNA transfected cells vs. cells transfected with control-shRNA. These results indicate that SMS1 knock down mediated functions are associated with the regulation of key cell cycle and survival proteins.

SMS1 silencing inhibits the migratory potential of Neuro-2a cells

Migratory potential is an integral part of many physiological processes and is regulated by p27 and Akt. Thus, we next examined the role of SMS1 in migratory properties of Neuro-2a cells using the scratch assay and the Boyden chamber assay that measures the migration of cells through a matrigel-coated membrane. In the scratch assay, control Neuro-2a cells migrated to fill the scratched area after 24 hours, indicating their strong migratory potential. However, silencing of SMS1 greatly reduced this migratory potential as indicated by an unfilled scratched area after 24 hours (Figure 5A). Furthermore, the ability of SMS1 silenced Neuro-2a cells to invade through matrigel-coated filters was significantly reduced (30%) when compared with control cells (Figure 5B). These results demonstrate that SMS1 expression is an important regulator of neuronal cell migration.

SMS1 is associated with the expression of motility genes including matrix metalloproteinase (MMPs) in Neuro-2a cells

Motility regulating genes play critical roles in cell migration, cell cycle progression, and inflammatory responses. To examine whether the effects of SMS1 silencing on Neuro-2a cells are mediated through the regulation of these gene expression, we screened a mouse cell motility pathway-specific PCR array (Figure 6A). RNAs from control-shRNA transfected Neuro-2a and SMS1-shRNA transfected cells were analyzed in parallel. Interestingly, SMS1 silencing resulted in a greater than 3 fold down regulation of 23 genes. Particularly MMP2 and MMP14 showed 57.8 and 6.5 fold decreases respectively (Figure 6B). MMP9 levels were also decreased by 1.9 fold. In contrast, nine genes that showed up-regulation were changed by only 1.5- to 3-fold (data not shown). Since MMP2 showed significantly decreased levels, and its established role in cell migration and survival, its mRNA and protein levels were further validated. RT–PCR analysis confirmed the significant decrease in MMP2 mRNA levels in SMS1 silenced cells (Figure 6 C, D) and immunofluorescence staining confirmed markedly decreased levels of MMP2 protein in SMS1 silenced Neuro-2a cells (Figure 6E). These results suggest that SMS1 expression in Neuro-2a cells may be involved in growth regulation at least in part through MMP signaling.

Discussion

In this study, we have demonstrated that small interfering RNA mediated decrease in sphingomyelin synthase 1 (SMS1) expression leads to decreased sphingomyelin (SM) levels in association with inhibited cell proliferation and cell cycle arrest of Neuro-2a cells. Furthermore, SMS1 deficiency decreases the migratory potential of these cells. At the molecular level, we have shown that effects of SMS1 silencing is mediated through alterations in the levels of p27, PI3K/Akt, and MMP2, the key signaling molecules that govern cell cycle progression and migration. These findings reinforce the importance of lipid synthesizing enzymes not only in cell membrane formation, but also in regulation of fundamental physiological processes. Our current studies may lead to further understanding of sphingomyelin synthases in neuronal cell growth, and the underlying molecular mechanisms in neurodegenerative diseases.

Much progress has been made in sphingolipid biology. However, large gaps still remain in our knowledge of the cellular and molecular mechanisms controlled by the enzymes that regulate sphingolipids. SMS1 is the key enzyme responsible for bulk production of SM from ceramide, the key lipid molecule [8–12]. Although the concept of ceramide as a signaling and regulatory factor is well accepted, the specific role of SMS1 is only now emerging. Indeed, growing evidence supports a role for SM, a byproduct of SMS, in modulating the signal transduction and fundamental biological functions, including cell growth and viability [1–22]. Given these crucial roles, understanding the role of the enzymes that regulate the balance of SM is of importance. Our previous work has shown that exposure of microglia to D609 that blocks the SMS activity, inhibits cell proliferation by inducing cell cycle arrest. D609 also inhibited the proliferation of neural progenitor cells [27 and 55]. Furthermore, SMS blocker D609 conferred neuro-protection in a rat model of ischemia [23]. However, the direct involvement of SMS1 in neuronal cell growth and cell cycle regulation remains speculative. Hence, we investigated further the specific role of SMS1 in neuronal cells. We used the Neuro-2a cell line that is of neuronal origin, and has been extensively used as a cell culture model to study neuronal differentiation, axonal growth, and signaling pathways [38, 39, 56, 57].

Our current finding that SMS1 deficiency inhibits cellular proliferation, is consistent with other studies reporting involvement of SMS1 in enhancing cell proliferation and suppressing apoptosis [13–18, 13, 58]. On the other hand, over-expression and activation of SMS1 by 2-hydroxyoleic acid is shown to increase the rate of apoptosis and inhibition of cellular growth [19, 20]. The possible explanation for these differential effects of SMS and SM is that many of these studies were conducted using various cell types. Continued work in each cell type should increase our clear understanding of the role of SMS in key physiological functions. Furthermore, SMS1 silencing resulted in morphological changes in Neuro-2a cells. Since shapes of cells are associated with cell cycle distribution and migratory properties, SMS1 may be involved in regulating these functions. Collectively, these data strongly agree that maintaining balanced levels of SMS is critical for normal cellular functions, including cell cycle regulation. Indeed, in SMS deficient Neuro-2a cells, we showed up regulation of a key cell cycle molecule, p27 concomitant with a decrease in cyclin D1 protein. Interestingly, p27 was predominantly expressed in the nuclei of the SMS-shRNA transfected cells.

Interestingly, previous studies have shown that phosphatidylinositol 3 (PI3) kinase/Akt mediated phosphorylation of p27 retains it in the cytoplasm and reduces its nuclear import. Akt also contributes to cell-cycle regulation by inhibiting p27 transcription or accelerating p27 degradation in different cell types [46–50]. In support of these observations, SMS1 deficient Neuro-2a cells showed decreased Akt activation in association with increased p27. Thus, our studies thus define a novel mechanism linking SMS1, and Akt activation with modulation of p27 expression and localization in Neuro-2a cells. Specifically, how SMS1 regulates Akt signaling remains to be elucidated. Presumably, SMS1 may mediate extracellular stimuli and growth factor signaling linked to Akt activation that promote cell cycle progression. Of note, previous study has demonstrated a link between basic fibroblast growth factor stimulation, and increased SMS1 activity [15]. In addition, SMS deficiency potentiates the cytokine receptor CXCR4 dimerization and signal transduction [59], and platelet-derived growth factor, as well as pro-inflammatory cytokines such as TNF-α, have been shown to activate sphingosine kinase, which regulates cellular viability and inflammatory responses [4–7]. These results raise the intriguing possibility that SMS1 plays a key role in regulating cytokine and growth factor mediated signaling pathways. However, it is yet to be determined whether SMS1 regulates cytokine and growth factor signaling in neuronal cells.

Neuronal cell migration is a dynamic process that is central to proper distribution and establishment of the neural network, disruption of which can lead to neurological disorders. Although molecular control of neuronal migration is well understood, how cell cycle and migration are coordinated at the molecular level is not clear. In this study, we examined the role of SMS1 in Neuro-2a cells using two different approaches. In the scratch assay, we observed inhibited migration as indicated by larger gap in wound/gap closure in SMS1-ShRNA transfected cells. It is possible that cell proliferation rate and viability can also contribute to decreased gap closure. However, the Boyden chamber assay clearly demonstrated inhibited cell migration in association with SMS1 silencing. Our current studies have found that SMS1 deficiency mediated inhibition of the migratory potential of Neuro-2a cells is associated with decreased levels of phospho-Akt and increased levels of p27, particularly in the nucleus. Indeed, Akt activation is shown to modulate p27 mediated cell migration. Matrix organization facilitated by matrix metalloproteinases are shown to contribute significantly to migratory processes. In support of this, our current study using motility gene PCR array screening showed down regulation of MMP family members MMP-2, -9, and -14 in SMS1 deficient Neuro-2a cells, suggesting that MMPs potentially participate in SMS1 mediated cell migration. Indeed, accumulating evidence demonstrates that sphingomyelin, a major component of microvesicles that also secrete MMPs, confer cellular migratory properties [59]. It is interesting that SMS1 is reported to exert negative effects on the migratory potential of mouse embryonic fibroblast [60]. This discrepancy is probably attributable to the difference in cell culture models, where in lipid metabolism and pathways are likely controlled differently. Thus, alterations in tightly regulated SMS1 expression and activity can lead to dysregulated signaling pathways that may have significant implications in health and disease. Indeed, the impact of sphingolipid-mediated signaling is increasingly being appreciated in the pathogenesis of cerebro-vascular and other diseases [1–7]. Further studies are certainly warranted for identifying the involvement of

SMS1 in neurodegenerative diseases. Overall, our current study may provide important insight for exploring further the role of SMS1 in regulating neuronal cell growth and survival under pathophysiological conditions.

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Abbreviations

SMS1	Sphingomyelin Synthase 1
D609	tricyclodecan-9-yl-xanthogenate
SM	Sphingomyelin
Cdk	cyclin-dependent kinase
MMP	Matrix metalloproteinase

References

1. Zeidan YH, Hannun YA. Translational aspects of sphingolipid metabolism. Trends Mol Med. 2007; 13:327–336. PMID 17588815. [PubMed: 17588815]
2. Corda D, De Matteis MA. Lipid signalling in health and disease. FEBS J. 2013; 280(24):6280. [PubMed: 24286187]
3. Li Z, Fan Y, Liu J, Li Y, Huan C, Bui HH, Kuo MS, Park TS, Cao G, Jiang XC. Impact of sphingomyelin synthase 1 deficiency on sphingolipid metabolism and atherosclerosis in mice. Arterioscler Thromb Vasc Biol. 2012; 32:1577–1584. [PubMed: 22580896]
4. Fyrst H, Saba JD. An update on sphingosine-1-phosphate and other sphingolipid mediators. Nat Chem Biol. 2010; 6:489–497. [PubMed: 20559316]
5. Ponnusamy S, Meyers-Needham M, Senkal CE, Saddoughi SA, Sentelle D, Selvam SP, Salas A, Ogretmen B. Sphingolipids and cancer: ceramide and sphingosine-1-phosphate in the regulation of cell death and drug resistance. Future Oncol. 2010; 10:1603–1624. [PubMed: 21062159]
6. Liu J, Huan C, Chakraborty M, Zhang H, Lu D, et al. Macrophage sphingomyelin synthase 2 deficiency decreases atherosclerosis in mice. Circ Res. 2009; 105:295–303. [PubMed: 19590047]
7. Assi E, Cazzato D, De Palma C, Perrotta C, Clementi E, Cervia D. Sphingolipids and brain resident macrophages in neuroinflammation: an emerging aspect of nervous system pathology. Clin Dev Immunol. 2013; 2013:309302. [PubMed: 24078816]
8. Hampton RY, Morand OH, Hannun YA. Sphingomyelin synthase and PKC activation. Science. 1989; 24(4933):1050. [PubMed: 2555921]
9. Hannun YA, Luberto C, Argraves KM. Enzymes of sphingolipid metabolism: from modular to integrative signaling. Biochemistry. 2001; 40:4893–4903. [PubMed: 11305904]
10. Futerman AH, Hannun YA. The complex life of simple sphingolipids. EMBO Rep. 2004; 5:777–782. [PubMed: 15289826]
11. Tafesse FG, Ternes P, Holthuis JC. The multigenic sphingomyelin synthase family. J Biol Chem. 2006; 281:29421–29425. [PubMed: 16905542]
12. Li Z, Hailemariam TK, Zhou H, Li Y, Duckworth DC, Peake DA, Zhang Y, Kuo MS, Cao G, Jiang XC. Inhibition of sphingomyelin synthase (SMS) affects intracellular sphingomyelin accumulation and plasma membrane lipid organization. Biochimica et Biophysica Acta (BBA). 2007; 1771:1186–1194.

13. Luberto C, Hannun YA. Sphingomyelin synthase, a potential regulator of intracellular levels of ceramide and diacylglycerol during SV40 transformation. Does sphingomyelin synthase account for the putative phosphatidylcholine-specific phospholipase C? J Biol Chem. 1998; 273:14550–14559. [PubMed: 9603970]
14. Burns TA, Subathra M, Signorelli P, Choi Y, Yang X, Wang Y, Villani M, Bhalla K, Zhou D, Luberto C. Sphingomyelin synthase 1 activity is regulated by the BCR-ABL oncogene. J Lipid Res. 2013; 54:794–805. [PubMed: 23160178]
15. Riboni L, Viani P, Bassi R, Giussani P, Tettamanti G. Basic fibroblast growth factor-induced proliferation of primary astrocytes. Evidence for the involvement of sphingomyelin biosynthesis. J Biol Chem. 2001; 276:12797–12804. [PubMed: 11278937]
16. Separovic D, Hanada K, Maitah MY, Nagy B, Hang I, Tainsky MA, Kraniak JM, Bielawski J. Sphingomyelin synthase 1 suppresses ceramide production and apoptosis post-photodamage. Biochem Biophys Res Commun. 2007; 358:196–202. [PubMed: 17467659]
17. Separovic D, Semaan L, Tarca AL, Awad Maitah MY, Hanada K, Bielawski J, Villani M, Luberto C. Suppression of sphingomyelin synthase 1 by small interference RNA is associated with enhanced ceramide production and apoptosis after photodamage. Exp Cell Res. 2008; 314:1860–1868. [PubMed: 18374917]
18. Tafesse FG, Huitema K, Hermansson M, van der Poel S, van den Dikkenberg J, Uphoff A, Somerharju P, Holthuis JC. Both sphingomyelin synthases SMS1 and SMS2 are required for sphingomyelin homeostasis and growth in human HeLa cells. J Biol Chem. 2007; 282:17537–17547. [PubMed: 17449912]
19. Ding T, Li Z, Hailemariam T, Mukherjee S, Maxfield FR, Wu MP, Jiang XC. SMS overexpression and knockdown: impact on cellular sphingomyelin and diacyl glycerol metabolism, and cell apoptosis. J Lipid Res. 2008; 49:376–385. [PubMed: 17982138]
20. Martin ML, Liebisch G, Lehneis S, Schmitz G, Alonso-Sande M, Bestard-Escalas J, Lopez DH, García-Verdugo JM, Soriano-Navarro M, Busquets X, Escribá PV, Barceló-Coblijn G. Sustained activation of sphingomyelin synthase by 2-hydroxyoleic acid induces sphingolipidosis in tumor cells. J Lipid Res. 2013; 54:1457–1465. [PubMed: 23471028]
21. Luberto C, Kraveka JM, Hannun YA. Ceramide regulation of apoptosis versus differentiation: a walk on a fine line. Lessons from neurobiology. Neurochem Res. 2002; 27:609–617. [PubMed: 12374196]
22. Taniguchi M, Okazaki T. The role of sphingomyelin and sphingomyelin synthases in cell death, proliferation and migration-from cell and animal models to human disorders. Biochim Biophys Acta. 2014; 1841(5):692–703. [PubMed: 24355909]
23. Adibhatla RM, Hatcher JF. Protection by D609 through cell-cycle regulation after stroke. Mol Neurobiol. 2010; 41:206–217. [PubMed: 20148315]
24. Meng A, Luberto C, Meier P, Bai A, Yang X, Hannun YA, Zhou D. Sphingomyelin synthase as a potential target for D609-induced apoptosis in U937 human monocytic leukemia cells. Exp Cell Res. 2004; 292:385–392. [PubMed: 14697345]
25. Li Z, Hailemariam TK, Zhou H, Li Y, Duckworth DC, Peake DA, Zhang Y, Kuo MS, Cao G, Jiang XC. Inhibition of sphingomyelin synthase (SMS) affects intracellular sphingomyelin accumulation and plasma membrane lipid organization. Biochimi Biophysic Acta. 2007; 1771:1186–1194.
26. Adibhatla RM, Hatcher JF, Gusain A. Tricyclodecan-9-yl-xanthogenate (D609) mechanism of actions: a mini-review of literature. Neurochem Res. 2012; 37:671–679. [PubMed: 22101393]
27. Gusain A, Hatcher JF, Adibhatla RM, Wesley UV, Dempsey RJ. Anti-proliferative effects of tricyclodecan-9-yl-xanthogenate (D609) involve ceramide and cell cycle inhibition. Mol Neurobiol. 2012; 45:455–464. Erratum in: Mol Neurobiol. 2012 45(3): 465. [PubMed: 22415444]
28. Galderisi U, Jori FP, Giordano A. Cell cycle regulation and neural differentiation. Oncogene. 2003; 22:5208–5219. [PubMed: 12910258]
29. Coqueret O. New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol. 2003; 13:65–70. [PubMed: 12559756]
30. Lim S, Kaldis P. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. Development. 2013; 140:3079–3093. [PubMed: 23861057]

31. Godin JD, Nguyen L. Novel functions of core cell cycle regulators in neuronal migration. Adv Exp Med Biol. 2014; 800:59–74. [PubMed: 24243100]
32. Desdouets C, Bréchot C. p27: a pleiotropic regulator of cellular phenotype and a target for cell cycle dysregulation in cancer. Pathol Biol (Paris). 2000; 48:203–210. [PubMed: 10858954]
33. Nguyen L, Besson A, Heng JI, Schuurmans C, Teboul L, Parras C, Philpott A, Roberts JM, Guillemot F. p27Kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev. 2006; 20:1511–1524. [PubMed: 16705040]
34. Larrea MD, Wander SA, Slingerland JM. p27 as Jekyll and Hyde: regulation of cell cycle and cell motility. Cell Cycle. 2009; 8:3455–3461. [PubMed: 19829074]
35. Tury A, Mairet-Coello G, DiCicco-Bloom E. The cyclin-dependent kinase inhibitor p57Kip2 regulates cell cycle exit, differentiation, and migration of embryonic cerebral cortical precursors. Cereb Cortex. 2011; 21:1840–1856. [PubMed: 21245411]
36. Schiappacassi M, Lovat F, Canzonieri V, Belletti B, Berton S, Di Stefano D, Vecchione A, Colombatti A, Baldassarre G. p27Kip1 expression inhibits glioblastoma growth, invasion, and tumor-induced neoangiogenesis. Mol Cancer Ther. 2008; 7:1164–1175. [PubMed: 18483304]
37. Baldassarre G, Belletti B, Nicoloso MS, Schiappacassi M, Vecchione A, Spessotto P, Morrione A, Canzonieri V, Colombatti A. p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion. Cancer Cell. 2005; 7:51–63. [PubMed: 15652749]
38. Matsuo T, Seth P, Thiele CJ. Increased expression of p27Kip1 arrests neuroblastoma cell growth. Med. Pediatr. Oncol. 2001; 36:97–99. [PubMed: 11464914]
39. Georgopoulou N, Hurel C, Politis PK, Gaitanou M, Matsas R, Thomaidou D. BM88 is a dual function molecule inducing cell cycle exit and neuronal differentiation of neuroblastoma cells via cyclin D1 down-regulation and retinoblastoma protein hypophosphorylation. J Biol Chem. 2006; 281:33606–33620. [PubMed: 16893893]
40. Frank CL, Tsai LH. Alternative functions of core cell cycle regulators in neuronal migration, neuronal maturation, and synaptic plasticity. Neuron. 2009; 62:312–326. [PubMed: 19447088]
41. Besson A, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. Dev. Cell. 2008; 14:159–169. [PubMed: 18267085]
42. Engelman JA. Targeting PI3K signaling in cancer: opportunities, challenges and limitations. Nat. Rev. Cancer. 2009; 9:550–562. [PubMed: 19629070]
43. O' Neill C. PI3-kinase/Akt/mTOR signaling: impaired on/off switches in aging, cognitive decline and Alzheimer's disease. Exp Gerontol. 2013; 48:647–653. [PubMed: 23470275]
44. Qiao J, Paul P, Lee S, Qiao L, Josifi E, Tiao JR, Chung DH. PI3K/AKT and ERK regulate retinoic acid-induced neuroblastoma cellular differentiation. Biochem Biophys Res Commun. 2012; 424:421–426. [PubMed: 22766505]
45. Reagan-Shaw S, Ahmad N. RNA interference-mediated depletion of phosphoinositide 3-kinase activates forkhead box class O transcription factors and induces cell cycle arrest and apoptosis in breast carcinoma cells. Cancer Res. 2006; 66:1062–1069. [PubMed: 16424042]
46. Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J, Arteaga CL. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat Med. 2002; 8:1145–1152. [PubMed: 12244301]
47. Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. Nat Med. 2002; 8:1153–1160. [PubMed: 12244302]
48. Kelly-Spratt KS, Philipp-Staheli J, Gurley KE, Hoon-Kim K, Knoblaugh S, Kemp CJ. Inhibition of PI-3K restores nuclear p27Kip1 expression in a mouse model of Kras-driven lung cancer. Oncogene. 2009; 28:3652–3662. [PubMed: 19648963]
49. Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, Fusco A, Santoro M. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. Nat Med. 2002; 8:1136–1144. [PubMed: 12244303]

50. Nadeem L, Brkic J, Chen YF, Bui T, Munir S, Peng C. Cytoplasmic mislocalization of p27 and CDK2 mediates the anti-migratory and anti-proliferative effects of Nodal in human trophoblast cells. J Cell Sci. 2013; 15:445–453. [PubMed: 23230143]
51. Chen Q, Jin M, Yang F, Zhu J, Xiao Q, Zhang L. Matrix metalloproteinases: inflammatory regulators of cell behaviors in vascular formation and remodeling. Mediators Inflamm. 2013; 2013:928315. [PubMed: 23840100]
52. Lenglet S, Montecucco F, Mach F. Role of matrix metalloproteinases in animal models of ischemic stroke. Curr Vasc Pharmacol. 2013; 2013 PMID 24188490.
53. Malemud CJ. Matrix metalloproteinases (MMPs) in health and disease: an overview. Front Biosci. 2006; 11:1696–1701. Review. [PubMed: 16368548]
54. Wesley UV, Vemuganti R, Ayvaci ER, Dempsey RJ. Galectin-3 enhances angiogenic and migratory potential of microglial cells via modulation of integrin linked kinase signaling. Brain Res. 2013; 1496:1–9. [PubMed: 23246924]
55. Kalluri HS, Dempsey RJ. D609 inhibits the proliferation of neural progenitor cells. Neuroreport. 2010; 21:700–703. [PubMed: 20568314]
56. Munoz JP, Sanchez JR, Maccioni RB. Regulation of p27 in the process of neuroblastoma N2A differentiation. J. Cell. Biochem. 2003; 89:539–549. [PubMed: 12761887]
57. Phan CW, David P, Naidu M, Wong KH, Sabaratnam V. Neurite outgrowth stimulatory effects of culinary-medicinal mushrooms and their toxicity assessment using differentiating Neuro-2a and embryonic fibroblast BALB/3T3. BMC Complement Altern Med. 2013; 13:261. [PubMed: 24119256]
58. Shakor AB, Taniguchi M, Kitatani K, Hashimoto M, Asano S, Hayashi A, Nomura K, Bielawski J, Bielawska A, Watanabe K, Kobayashi T, Igarashi Y, Umehara H, Takeya H, Okazaki T. Sphingomyelin synthase 1-generated sphingomyelin plays an important role in transferrin trafficking and cell proliferation. J Biol Chem. 2011; 286:36053–36062. [PubMed: 21856749]
59. Kim CW, Lee HM, Lee TH, Kang C, Kleinman HK, Gho YS. Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. Cancer Res. 2002; 62:6312–6317. [PubMed: 12414662]
60. Asano S, Kitatani K, Taniguchi M, Hashimoto M, Zama K, Mitsutake S, Igarashi Y, Takeya H, Kigawa J, Hayashi A, Umehara H, Okazaki T. Regulation of cell migration by sphingomyelin synthases: sphingomyelin in lipid rafts decreases responsiveness to signaling by the CXCL12/CXCR4 pathway. Mol Cell Biol. 2012; 32:3242–3252. [PubMed: 22688512]

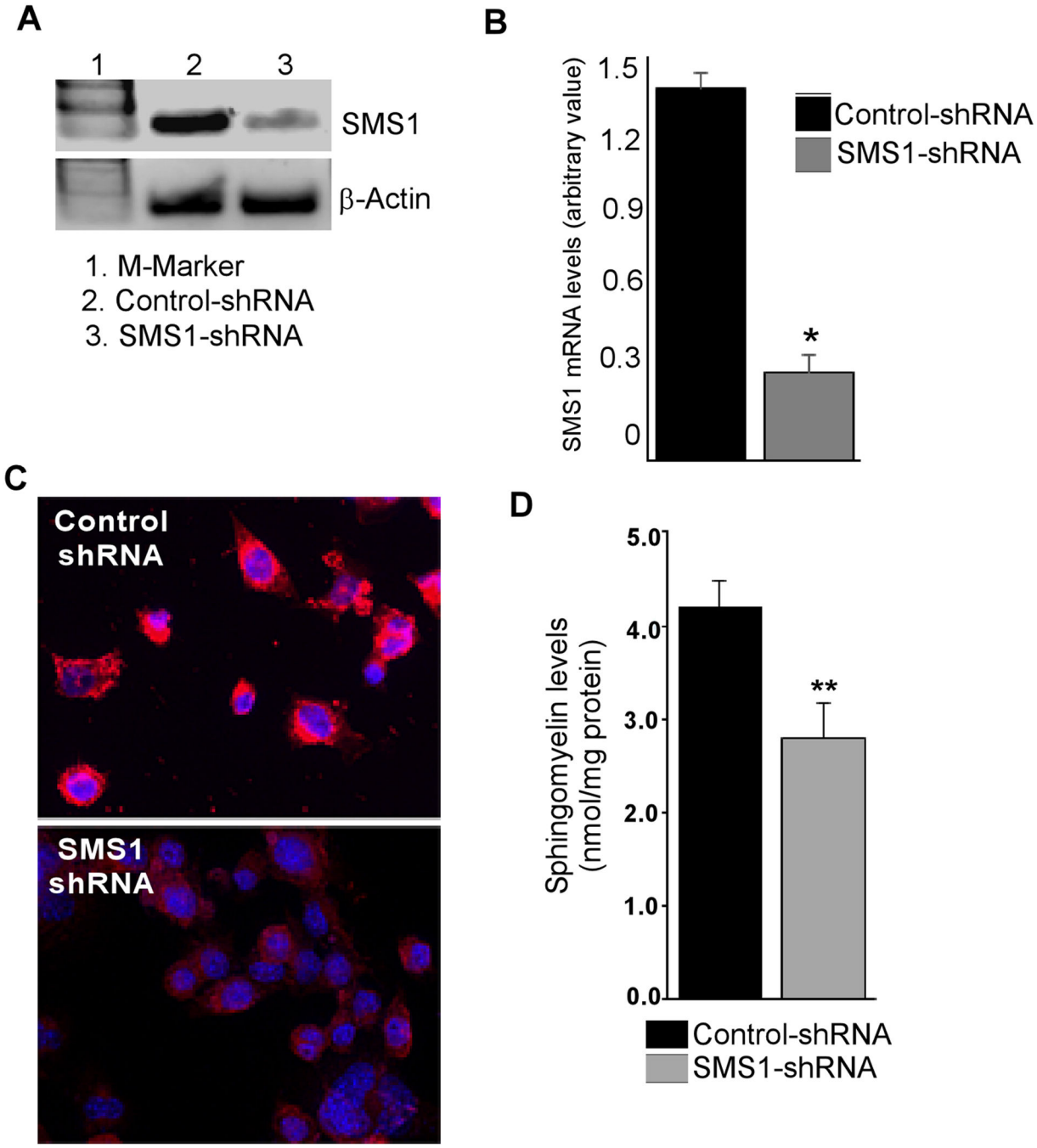

Figure 1. Sphingomyelin synthase (SMS) is down regulated in SMS1-shRNA-transfected Neuro-2a cells

(A). Cells were transfected with SMS1- or scrambled control-shRNA as described in Material and Methods. Puromycin selected cells were harvested, and the SMS1 expression was determined by RT-PCR. (B). Quantification and normalization of SMS1 to beta actin demonstrated significant decrease (about 70%) of SMS1 mRNA levels in SMS1-shRNA transfected Neuro-2a cells as compared to control-shRNA transfected cells. * $P<0.05$ (C). Decrease in SMS1 protein in SMS1-shRNA transfected Neuro-2a cells was further confirmed by immunofluorescence staining. (D). In SMS1-shRNA transfected cells,

sphingomyelin (SM) levels were significantly decreased (about 30%) as compared to cells transfected with control-shRNA plasmid (D). ** P<0.01; Scale bar −100 μm

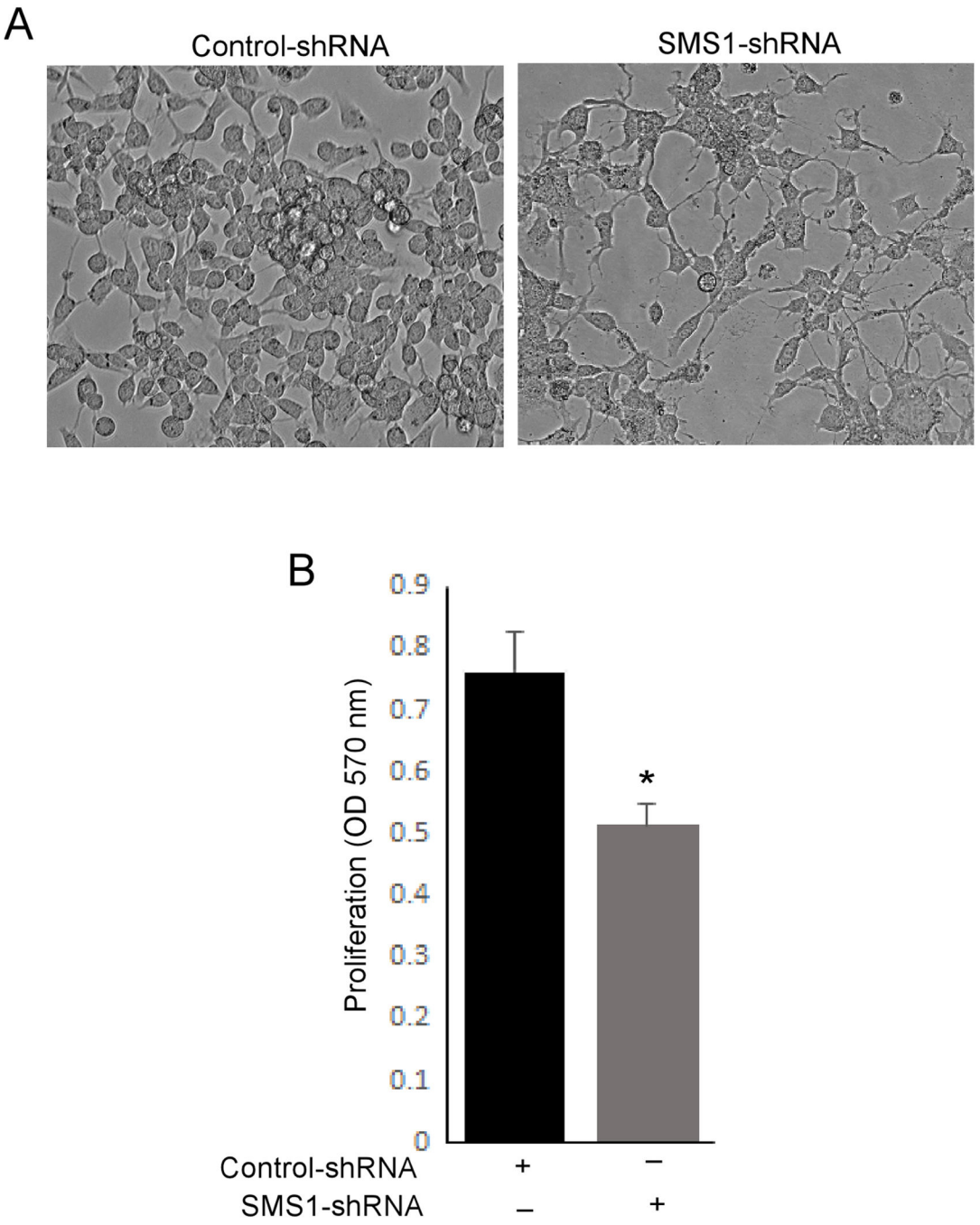

Figure 2. SMS1 silencing exerts morphological changes and anti-proliferative effect

Effects of SMS1 silencing on growth and morphological features of Neuro-2a cells were observed. (A). Neuro-2a cells transfected with control-shRNA grew in clusters with round morphology and short cytoplasmic processes. On the other hand, SMS1 silencing resulted in smaller cell bodies with elongated and branched neurite like extensions. (B). SMS1 knockdown in Neuro-2a cells induced about 30% growth arrest as shown by MTT proliferation assay. * P<0.05; Scale bar −100 μm

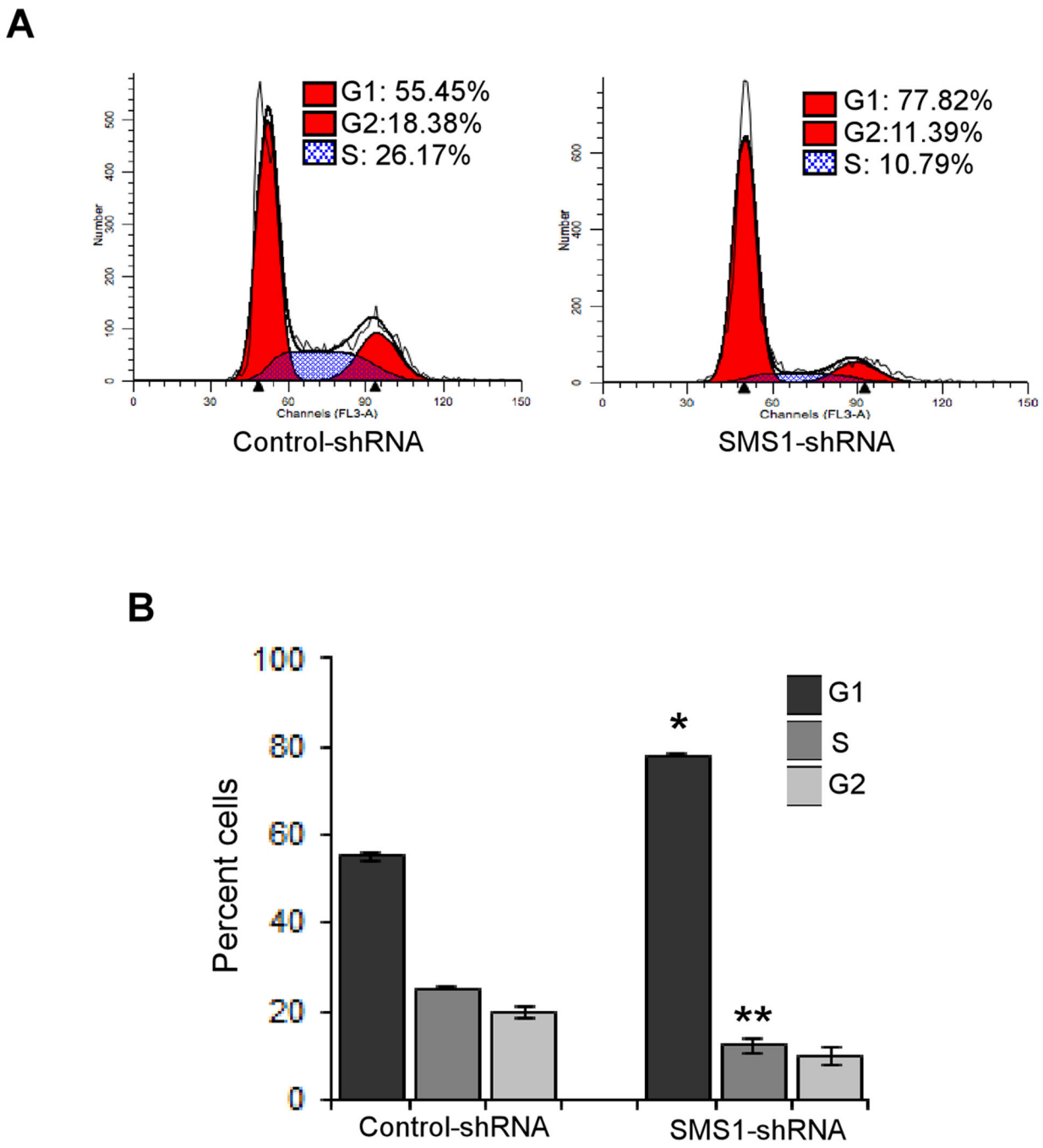

Figure 3. The effects of SMS1-shRNA on cell cycle progression of Neuro-2a cells

Neuro-2a cells transfected with either control-shRNA or SMS1-shRNA were assessed for cell cycle progression. Number of propidium iodide stained cells at each stage of the cell cycle were measured by flow cytometry (first peak was G0/G1, the intervening trough was S phase, and the second peak represented cells in G2/M) and analyzed by MODFIT software. (A) Representative DNA histogram from control-shRNA and SMS1-shRNA transfected Neuro-2a cells. (B). Quantification of the percentage of cells in each phase of cell cycle. SMS1 knockdown induced a significant increase in cells arrested in G1 phase and a decrease

in cells arrested in S phase as compared to control-shRNA transfected cells. * P<0.05, ** p<0.01.

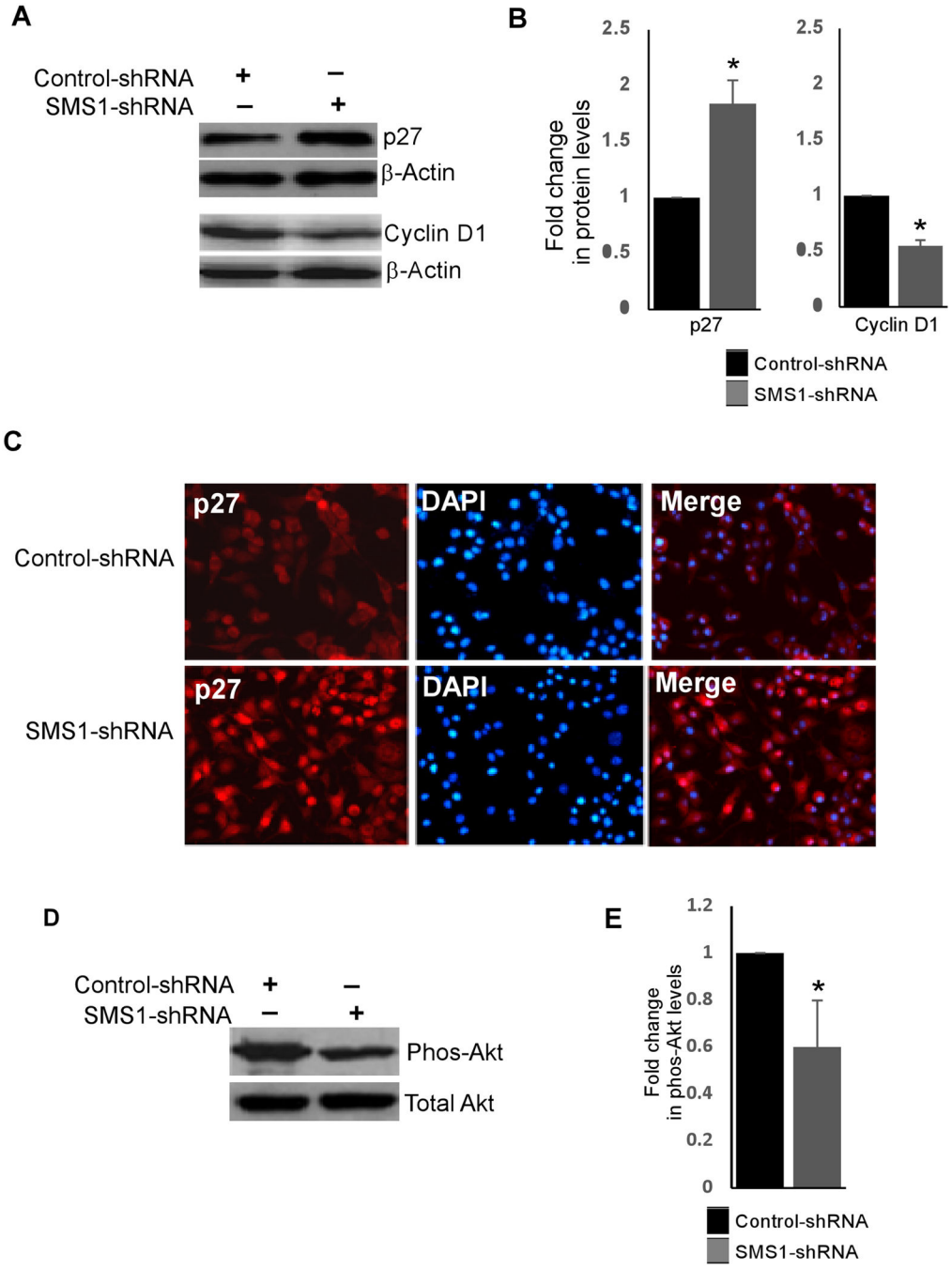

Figure 4. SMS1 silencing in Neuro-2a cells alters the levels of p27 and cyclin D1 proteins

Neuro-2a cells lysates were subjected to western blot analysis using p27, cyclin D1, actin, and Phos- and total Akt antibodies. (A). Representative western blots showing significantly increased levels of p27 and decreased levels of cyclin D1 in Neuro-2a cells transfected with SMS1-shRNA as compared to control-shRNA transfected cells. (B). Quantification and normalization of p27 and cyclin D1 protein levels relative to beta actin demonstrated a 2–3 fold increase in p27 and 2–3 fold decrease in cyclin D1 in SMS1-shRNA transfected cells as compared to cells transfected with control-shRNA (C). Immunofluorescence staining

showing alterations in cellular localization of p27 protein. SMS1 silencing resulted in a remarkable increase in nuclear p27. (D). Representative western blots showing significantly decreased levels of phos-Akt in Neuro-2a cells transfected with SMS1-shRNA as compared to control-shRNA transfected cells. Total Akt levels remained unchanged. (E). Quantification and normalization of phos-Akt protein levels relative to total Akt in Neuro-2a cells. * $P<0.05$; Scale bar −100 μm

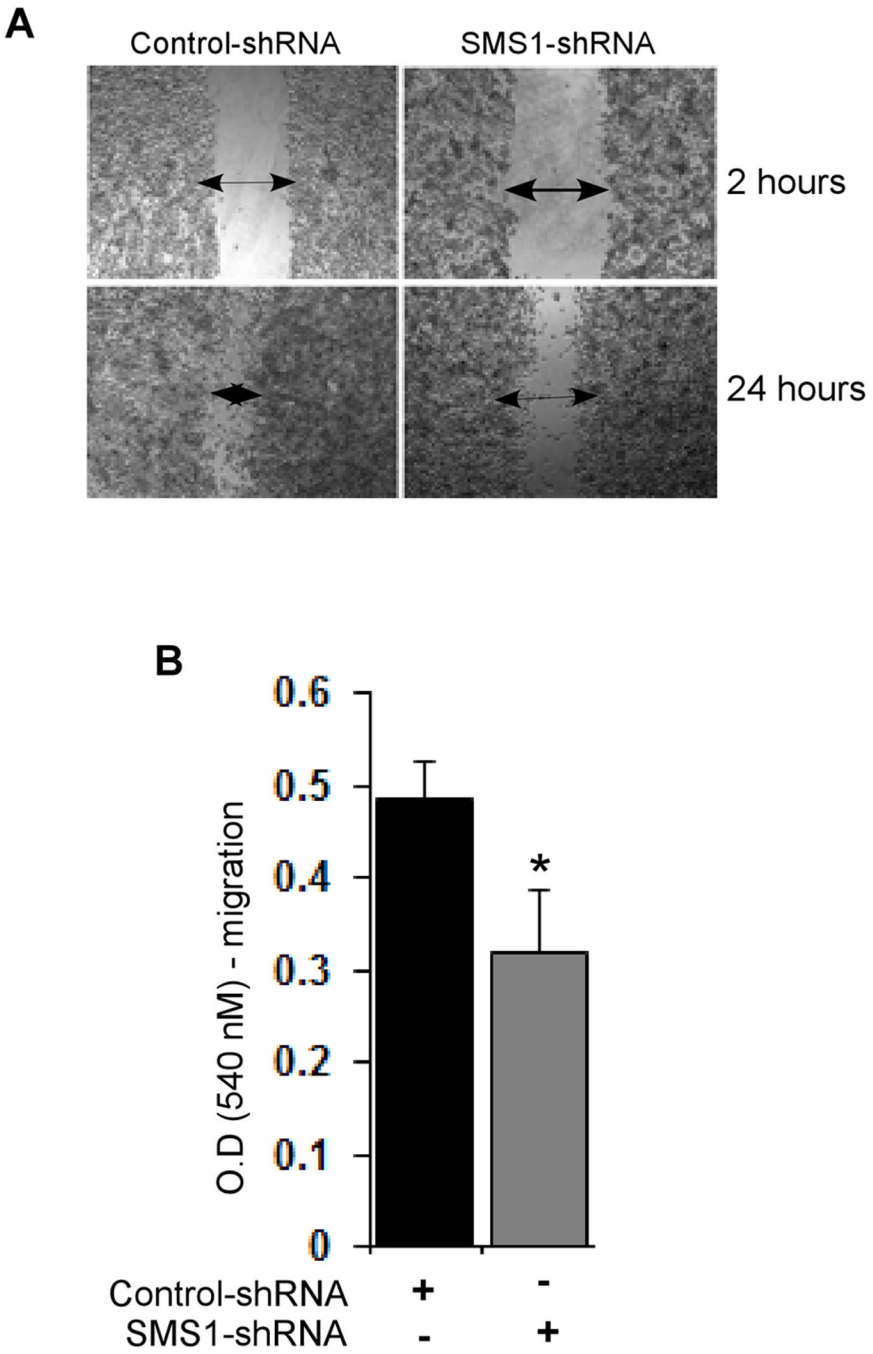

Figure 5. SMS1 silencing inhibits migratory potential of Neuro-2a cells

(A). The scratch induced migration assay. Control Neuro-2a cells migrated to fill the scratched area after 24 h, indicating their strong migratory potential. However, silencing of SMS1 greatly reduced this migratory potential as indicated by larger unfilled scratched area after 24 h. (B). Quantitation of migratory potential by the Boyden chamber assay that measures the invasion of cells through a matrigel-coated membrane. The ability of SMS1 silenced Neuro-2a cells to invade through matrigel-coated filters was significantly reduced

(about 30%) when compared with controls-hRNA transfected cells. * $P<0.05$; Scale bar −100 μm

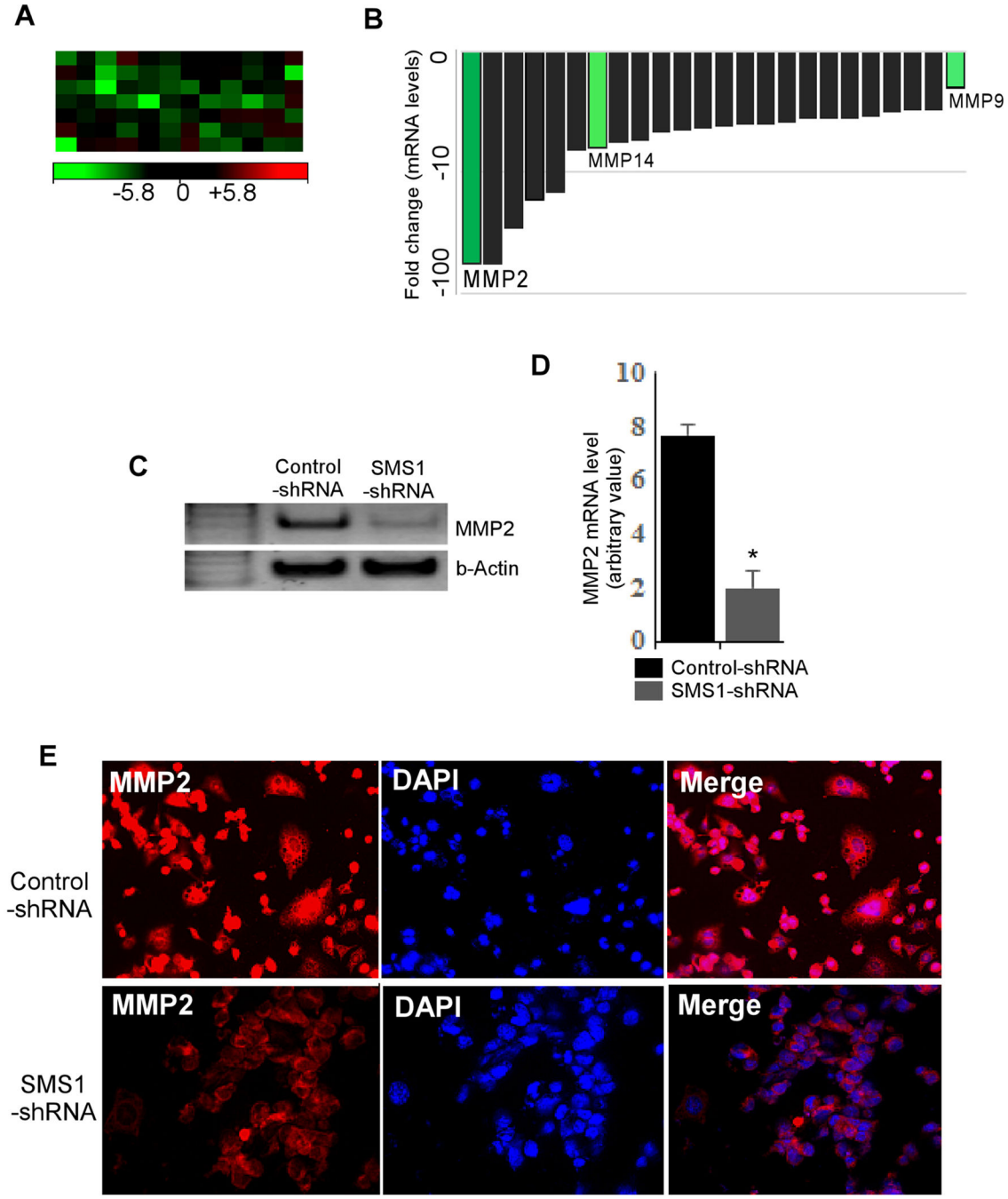

Figure 6. SMS1 silencing down regulates matrix metalloproteinase (MMPs) in Neuro-2a cells

(A). The effects of SMS1 silencing on motility related gene expression in Neuro-2a cells were examined using a motility pathway-specific PCR array as described in the materials and methods section. Total RNAs from control-shRNA transfected Neuro-2a and SMS1-shRNA transfected cells were analyzed in parallel. (B). SMS1 silencing resulted in down regulation of 23 genes that were down regulated more than threefold. Particularly MMP2 and MMP14 showed 57.8 and 6.5 fold decrease respectively. MMP9 levels were also decreased to 1.9 fold. (C). Validation of MMP2 mRNA levels by RT-PCR analysis

confirmed the significant decrease in MMP2 mRNA levels in SMS1 silenced Neuro-2a cells. (D). Quantification and normalization of MMP2 to beta actin demonstrated 6–7 fold decrease in MMP2 levels in SMS1 silenced Neuro-2a cells as compared to control-shRNA transfected cells. (E). Immunofluorescence staining showing markedly decreased levels of MMP2 protein in SMS1 silenced Neuro-2a cells as compared to control-shRNA transfected cells. Scale bar −100 μm