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Sphingosine Induces Apoptosis and Down-regulation of *MYCN* in PAX3–FOXO1-positive Alveolar Rhabdomyosarcoma Cells Irrespective of *TP53* Mutation

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Abstract. *Background/Aim: Rhabdomyosarcoma is the most common type of pediatric soft-tissue sarcoma. Among the subsets of this disease, alveolar rhabdomyosarcoma (ARMS) expressing paired box 3 (PAX3) and forkhead box O1 (PAX3–FOXO1) fusion oncoprotein has the worst prognosis. The goal of this study was to investigate the chemotherapeutic effects of sphingosine on PAX3–FOXO1-positive ARMS cells [tumor protein p53 (TP53)-mutated RH30 and TP53 wild-type RH18 cells]. Materials and Methods: The proliferation, cell death, apoptosis, cell cycle, and MYCN proto-oncogene (MYCN) expression of RH30 and RH18 cells were determined. Results: Sphingosine inhibited the growth and caused cell death in a dose-dependent manner in both cell lines. Sphingosine triggered cell death by inducing apoptosis without affecting the cell cycle. MYCN expression was down-regulated within 2 and 4 h of sphingosine treatment in both RH30 and RH18 cells. Conclusion: Sphingosine exerts antiproliferative and pro-apoptotic effects via MYCN down-regulation independently of TP53 mutation status in PAX3–FOXO1-positive ARMS cells.*

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Rhabdomyosarcoma (RMS) is the most common type of pediatric soft tissue sarcoma and accounts for approximately 50% of all soft-tissue sarcomas (1). RMS occurs predominantly in the sites of skeletal muscle lineage. RMS is categorized into two subtypes based on histological observations (2); embryonal (ERMS) and alveolar (ARMS). Between these two subtypes, ARMS has the worse prognosis and is associated with a higher frequency of metastasis (3, 4). ARMS is characterized by the translocation t(2;13)(q35;q14) or t(1;13)(q36;q14) that lead to paired box 3–forkhead box O1 (PAX3–FOXO1) and paired box 7–forkhead box O1 (PAX7–FOXO1) gene fusions, respectively. PAX3–FOXO1 is present in about 55% of ARMS harboring overexpression of this fusion gene at both RNA and protein levels. PAX7–FOXO1 is present in 22% of ARMS, and the remaining 23% of ARMS is fusion-negative (3, 5). PAX3–FOXO1-positive ARMS is the most clinically intractable subtype among RMS types (2, 3, 6, 7). Mutation of tumor protein p53 (TP53) is infrequent in RMS, occurring in fewer than 10% of patients (8), and inactive TP53 is more common in fusion-negative RMS (9).

Sphingolipids are practically ubiquitous and are found in all eukaryotic cell membranes, some prokaryotes, and in foods such as dairy and soy products (10, 11). Animal studies showed complex dietary sphingolipids (*e.g.* sphingomyelin, dihydrosphingomyelin, glucosylceramide, lactosylceramide, and ganglioside GD_3), reduced aberrant colonic foci in CF1 mice (12-17) and reduced tumors in all regions of the intestine in multiple intestinal neoplasia (Min) mice with truncated adenomatous polyposis coli (17, 18). These anti-tumorigenic effects of sphingolipids against colon carcinogenesis could be the result of the conversion of complex sphingolipids to sphingolipid metabolites. Sphingolipid metabolites, such as ceramide (acylated form of sphingosine) and sphingoid bases (sphingosine,

sphinganine), function as second messengers in various signal transduction pathways to regulate cellular function. For example, sphingolipid metabolites were found to inhibit cell proliferation and induce apoptosis (programmed cell death) in human cancer cells (19-24). Many of these previous studies focused on the effects of ceramide, but a few have examined the action of sphingosine (24, 25). Interestingly, sphingosine is a metabolic precursor to sphingosine-1-phosphate (S1P), but the two have opposing action on cells (*i.e.* induction of apoptosis by sphingosine *versus* inhibition of apoptosis by S1P, respectively) (25, 26). While it was previously shown that S1P is a pro-metastatic factor in RMS (27), little is known about the effects of sphingosine, particularly in PAX3–FOXO1-positive ARMS.

In the current study, we examined whether antiproliferative and pro-apoptotic effects of sphingosine in PAX3–FOXO1-positive ARMS cells would differ according to *TP53* mutation status. Previously, we and others identified that the up-regulation of *MYCN* oncogene at both mRNA and protein levels is a distinguishable feature and a potential direct target of PAX3–FOXO1-positive ARMS in comparison to ERMS (28-30). Here, we tested whether the regulation of *MYCN* expression could mediate the effects of sphingosine on proliferation and apoptosis.

Materials and Methods

Cell culture and assessment of cell proliferation. RH30 and RH18 ARMS cells expressing PAX3–FOXO1 fusion oncoprotein were provided by Dr. Frederic G. Barr at the National Cancer Institute of the National Institutes of Health (Bethesda, MD, USA). RH30 cells carry mutations in *TP53* gene, while RH18 cells exhibit wild-type *TP53*. RH30 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 1% penicillin/streptomycin (P/S; Invitrogen) and 1% antibiotic-antimycotic (AM; Invitrogen). RH18 cells were maintained in DMEM with 15% FBS, 1% P/S, and 1% AM. Cell cultures at 80% confluence were trypsinized and cells were seeded at a density of 4×10^4 per well in 6-well plates and cultured in 2 ml of the appropriate medium (as described above) for 24 hours. The media were then switched to DMEM/1%FBS/1%PS/1%AM and the cells were treated with sphingosine at different concentrations for 1, 3, and 5 days. Total nucleic acid concentrations were determined as described previously (31).

Sphingosine treatment. D-Sphingosine (Cat# S7049; Sigma, St. Louis, MO, USA) was prepared in ethanol as 50 mM stock concentration, kept at −20˚C and used only for 2 months. Prior to each experiment, 50 mM sphingosine was freshly diluted in 50% ethanol and delivered to the cells at different concentrations of sphingosine in 0.05% ethanol. Cells treated with 0.05% ethanol served as controls.

RNA extraction and real time quantitative polymerase chain reaction (QPCR) analysis. Total RNA was extracted using Trizol (Invitrogen, Grand Island, NY, USA). Corresponding cDNA was synthesized and was quantified by QPCR on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously (28). The expression of *MYCN* gene was normalized to the expression of 18S ribosomal RNA (18S rRNA). The 18S rRNA [Assay ID#: Hs99999901_s1; ThermoFisher Scientific (Applied Biosystems)] Taqman gene-expression assay was used and its sequences are not disclosed by Applied Biosystems. The sequences of the forward and reverse primers and probes for *MYCN* were reported previously (28).

Cell death, apoptosis, and cell-cycle analysis via flow cytometry. Floating dead cells were collected in FBS (HyClone), and adherent cells were detached using TrypLE Express no Phenol red [ThermoFisher Scientific (Gibco), Carlsbad, CA, USA]. Cells were incubated with calcein AM or propidium iodide (PI) viability dyes (eBioscience, San Diego, CA, USA) to distinguish between dead and live cells. Apoptotic cells were detected with annexin V–allophycocyanin (APC) (eBioscience). For cell-cycle analysis, cells were fixed with ethanol and then stained with PI (50 μg/ml) as described previously (31). Cells were then determined *via* fluorescence-activated cell sorting (FACS) Canto II (BD Biosciences, San Jose, CA, USA) and the data were quantified using the FlowJo program (Tree Star Inc., Ashland, OR, USA).

Statistical analysis. Data for total nucleic acid assay at different concentrations of sphingosine and multiple culture periods were analyzed by two-way ANOVA. One-way ANOVA was applied for the data of total nucleic acid and cell death analyses at different concentrations of sphingosine for a single culture period (24 hours). After application of two-way ANOVA or one-way ANOVA, the significance of differences in the means between control and treatment groups for specific culture periods was evaluated by multiple comparisons using Holm-Sidak method. Differences between the groups were considered significant at $p<0.05$, and the analyses were carried out using Sigma plot version 12.0 program (Systat Software, Inc., San Jose, CA, USA).

Results

Sphingosine inhibited cell growth and caused death of PAX3–FOXO1-positive ARMS cells. Effects of sphingosine on the growth and death of PAX3–FOXO1-positive ARMS cells, RH30 and RH18, were determined using the total nucleic acid assay. RH30 and RH18 cells carry mutated and wild-type *TP53* tumor-suppressor gene, respectively (32-34). Total nucleic acids in control RH30 cell cultures doubled within 1 day and increased up to 10-fold in 5 days (Figure 1A). In comparison, total nucleic acids in control RH18 cultures doubled within 3 days and increased up to 3-fold in 5 days (Figure 1B), indicating that RH18 cells grew more slowly than RH30 cells. The cells were treated with sphingosine at different concentrations (0 to 15 μM) for 0-5 days (Figure 1). Sphingosine caused a dose-dependent inhibition of cell growth in both cell lines (Figure 1A and B). The half-maximal inhibitory concentration (IC_{50}) of sphingosine against RH30 and RH18 cells were 3.55 μM (Figure 1C) and 2.66 μM (Figure 1D), respectively.

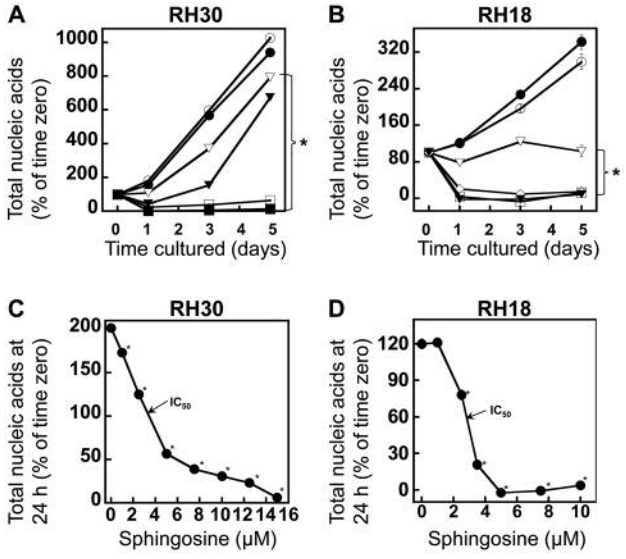

Figure 1. *Sphingosine inhibited growth and caused death of RH30 and RH18 cells. A: Sub-confluent RH30 cells were cultured with sphingosine at 0 (○), 1 (●), 2.5 (▽), 5 (▼), 10 (□), and 15 μM (■) for 1, 3, and 5 days. B: Sub-confluent RH18 cells were cultured with sphingosine at 0 (○), 1 (●), 2.5 (▽), 3.5 (◇), 5 (▼), and 10 μM (□) for 1, 3, and 5 days. *Means at each time of cells treated with sphingosine at 2.5 to 15 μM differed significantly from the corresponding controls at p<0.001 by two-way ANOVA. C, D: For determination of the half-maximal inhibitory concentration (IC_{50}), RH30 cells (C) and RH18 cells (D) were cultured with sphingosine at different concentrations (1 to 15 μM) for 24 h. *Significantly different at p<0.001 by one-way ANOVA compared to the corresponding control and different sphingosine concentrations. Total nucleic acids were measured as an index of cell number and results are expressed as a percentage of the quantity at zero hour (mean±SEM, n=3, except n=6 for time zero). Where an error bar is not seen, it lies within the dimensions of the symbol.*

Sphingosine caused cell death by inducing apoptosis without cell-cycle arrest. Dead cells were detected using PI (Figure 2) and calcein AM (Figure 3) viability dyes *via* flow cytometry. RH30 and RH18 cells were treated with sphingosine for 12 h at 5 μM and 3.5 μM, respectively (Figure 2). Sphingosine significantly increased the population of dead RH30 cells by about 5-fold and of RH18 cells by 4-fold (Figure 2). In addition, sphingosine significantly increased the percentage of dead RH30 cells in a dose-dependent manner (Figure 3).

We further examined whether sphingosine causes the death of PAX3–FOXO1-positive ARMS cells by inducing apoptosis. Annexin V-APC-positive cells were quantified to detect apoptotic cells *via* flow cytometry (Figure 4). Our flow cytometric experiments using annexin V demonstrated that treatment with sphingosine at 5 μM for RH30 cells and 3.5 μM for RH18 cells for 12 h significantly increased the numbers of annexin V-positive apoptotic cells by 5-fold (Figure 4). The effects of sphingosine (3.5 μM for 12 h) on the cell cycle in RH30 cells were also analyzed *via* flow cytometry. The percentage of RH30 cells in G_0/G_1, S, and G_2/M (mean±SEM) were: control: 40.5±1.0, 38.9±1.4, 18.1±3.2; and sphingosine: 41.2±1.1, 38.7±1.3, 17.3±3.3. This indicates that the induction of apoptosis by sphingosine was independent of cell-cycle arrest, as there were no significant differences in the distribution of cells in G_0/G_1, S, and G_2/M stages between control and the sphingosine treatment.

Sphingosine down-regulated MYCN oncogene expression within 2 hours. We and others previously identified that up-regulation of *MYCN* expression is a potential therapeutic target of PAX3–FOXO1 in ARMS (28-30). In the current study, we explored whether the regulation of *MYCN* expression is a mechanism by which sphingosine inhibits growth and induces apoptosis in PAX3–FOXO1-positive ARMS cells. The cells were treated with sphingosine at the approximate IC_{50} (3.5 μM for RH30 cells and 2.5 μM for RH18 cells) for 2 and 4 hours. Total RNA was then extracted, converted to cDNA and the expression of *MYCN* was determined using Taqman QPCR assay (Figure 5). Significant down-regulation of *MYCN* expression was observed even with 2 hours of sphingosine treatment. At 4 hours of sphingosine treatment, *MYCN* expression was further reduced by 7-fold in RH30 cells (Figure 5A) and by 1.5-fold in RH18 cells (Figure 5B).

Discussion

Sphingolipids have gained attention as potential chemotherapeutic and chemopreventive agents against some forms of cancer. Most studies addressed the role of ceramide, an acylated form of sphingolipid metabolites, in the apoptosis of major types of cancer cells (19-22, 24, 35, 36), but the effects of the sphingolipid metabolite sphingosine have been explored to a lesser extent. Furthermore, the effects of sphingolipids on ARMS have not been studied extensively. To our knowledge, currently only two prior studies examined the effects of sphingosine and sphingosine-1-phosphate on RMS cell lines (27, 37). In particular, Phillips *et al*. (37) demonstrated that sphingosine mediates apoptosis in RMS cell lines.

In the present study, we found that sphingosine inhibited cell growth and induced apoptosis without affecting cell-cycle progression in both RH30 and RH18 PAX3–FOXO1-positive ARMS cells. Our cell proliferation data indicate that RH30 cells grow faster than RH18 cells. The slower proliferation rate of RH18 cells might be associated with a growth-inhibitory function of the wild-type tumor suppressor gene *TP53* (38, 39). Mechanisms by which sphingosine inhibits proliferation and induces apoptosis in some types of cancer cells have been studied. For example, sphingosine induced

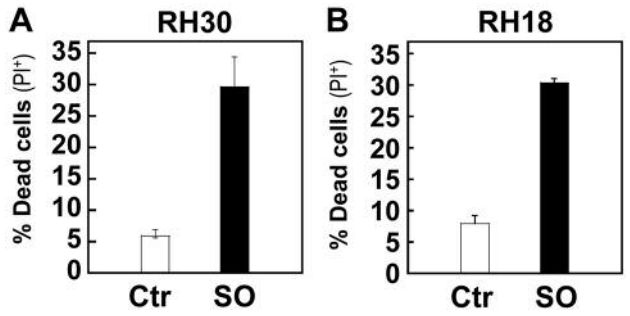

Figure 2. *Sphingosine induced cell death in RH30 and RH18 cells. Sub-confluent RH30 and RH18 cells were cultured in the absence (Ctr) or the presence of 5 μM or 3.5 μM sphingosine (SO), respectively, for 12 h and were stained with propidium iodide (PI). Dead cells were identified as the PI-positive cell population using flow cytometric analysis (mean±SEM, n=2).*

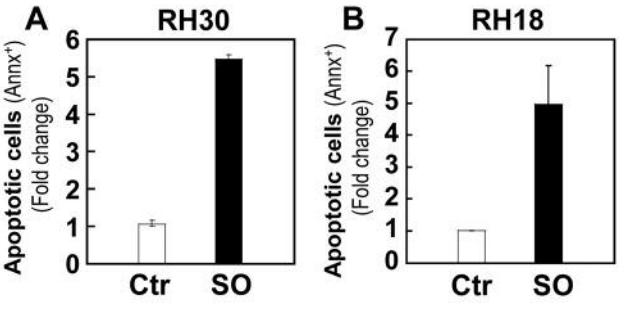

Figure 4. *Sphingosine induced apoptosis of RH30 and RH18 cells. Sub-confluent RH30 and RH18 cells were cultured in the absence (Ctr) or the presence of 5 μM or 3.5 μM sphingosine (SO), respectively, for 12 h and were stained with annexin V (Annx). Apoptotic cells were identified as the annexinV-positive cell populations (Annx+) with flow cytometry (mean±SEM n=2). Where an error bar is not seen, it lies within the dimensions of the symbol.*

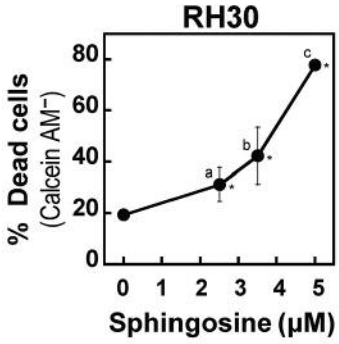

Figure 3. *Sphingosine induced cell death in RH30 cells. Sub-confluent cells were cultured with sphingosine at different concentrations (2.5, 3.5, and 5 μM) for 12 h and were stained with calcein AM. The percentage of dead cells (calcein AM-negative population) was determined via flow cytometric analysis (mean±SEM, n=3). *Significantly different from the control at p<0.025 by one-way ANOVA. Different superscript letters (a, b, c) indicate significant differences between sphingosine concentrations. Where an error bar is not seen, it lies within the dimensions of the symbol.*

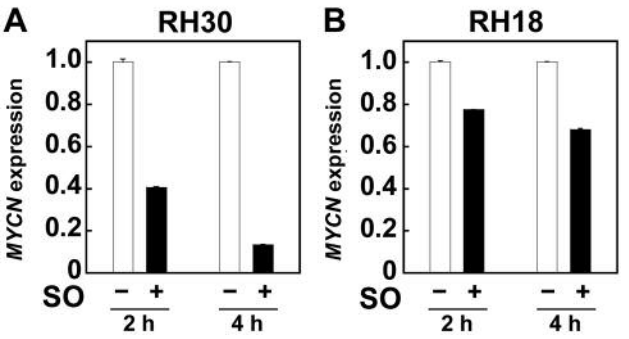

Figure 5. *Sphingosine down-regulated the expression of MYCN in RH30 and RH18 cells. Sub-confluent RH30 and RH18 cells were cultured with and without sphingosine (SO) at 3.5 μM and 2.5 μM, respectively, for 2 and 4 h. Total mRNA was isolated and the expression of MYCN gene was determined using Taqman real-time quantitative polymerase chain reaction. The relative expression levels of MYCN gene were normalized by 18S rRNA as an endogenous control transcript. Data represent fold changes normalized to the corresponding control at specific times (mean±SD, n=3 for A; n=6 for B). Where an error bar is not seen, it lies within the dimensions of the symbol.*

apoptosis by repressing AKT activation in colon (31) and liver (40) cancer cells. Previously, we and others identified *MYCN* as a potential target gene of PAX3–FOXO1 in ARMS (28-30). Here, we examined whether the regulation of *MYCN* oncogene expression is a potential mechanism by which sphingosine inhibits proliferation and induces apoptosis in PAX3–FOXO1-positive ARMS cells. We demonstrate that sphingosine at IC_{50} significantly down-regulated *MYCN* expression within 2 hours, which occurs earlier than significant inhibition of proliferation and induction of apoptosis. Recent studies showed that sphingosine kinase 2 (SK2), which phosphorylates sphingosine to S1P, promoted colon cancer cell proliferation and invasion by enhancing MYC expression (41). Furthermore, pharmacological and siRNA inhibition of SK2 was found to down-regulate *MYC* (c-Myc) and inhibit growth in multiple myeloma and prostate cancer (42, 43). Since sphingosine and S1P are known to have opposite effects on apoptosis (25, 26), our findings are thus consistent with a mechanism in which sphingosine inhibits proliferation and induces apoptosis by down-regulating *MYCN* expression.

To elucidate potential interactions of MYCN with *TP53*, we examined effects of sphingosine in PAX3–FOXO1-positive ARMS cells differing in TP53 status (mutant *TP53 versus* wild-type). Although *MYCN* down-regulation was observed in both RH30 and RH18 cells within 2 h, the extent of *MYCN* down-regulation was greater in RH30 cells than in RH18 cells. We speculate that this might be due to the presence of the wild-type TP53 in RH18 cells. It was reported that *MYCN* can regulate *TP53* transcription, expression, and activation in human neuroblastoma cells (40, 44). For example, high TP53 expression was positively correlated with MYCN expression and amplification in immunohistochemical analysis of neuroblastoma tumors (45). *MYCN* knockdown reduced *TP53* expression in *MYCN*-amplified neuroblastoma cell lines. MYCN bound directly to a motif close to the transcriptional start site within the *TP53* promoter (45). Taken together, the different extent of *MYCN* down-regulation based on the *TP53* mutation status in our study implies a feedback mechanism in which wild-type TP53 regulates *MYCN* expression. Alternatively, sphingosine may induce apoptosis by both TP53-dependent and TP53-independent mechanisms in PAX3–FOXO1-positive ARMS. This may be dependent on the interactions among *TP53, MYCN*, and other proliferation/ apoptosis-related oncogenes or tumor-suppressor genes, as well as the mutation status of these genes. To establish a direct causative relationship for the *MYCN* down-regulation with sphingosine-induced apoptosis in PAX3–FOXO1-positive ARMS, future studies could test the effects of sphingosine by overexpressing *MYCN* in RH30 and RH18 cells to see whether the antiproliferative and pro-apoptotic effects of sphingosine are attenuated.

Since RMS is often diagnosed in very young children, there is a growing effort to reduce the reliance on radiotherapy when treating RMS in order to avoid any potential long-term side-effects (4). Our data suggest that sphingosine inhibits the growth of PAX3–FOXO1-positive ARMS cells in a dose-dependent manner and is a potent inducer of apoptosis by down-regulating *MYCN* expression in cells harboring either mutated or wild-type *TP53*. In conclusion, we provide one of the first findings that sphingosine treatment may be efficacious against ARMS, irrespective of *TP53* mutation status.

Acknowledgements

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