

## Suppression of Oral Carcinoma Oncogenic Activity by microRNA-203 via Down-regulation of SEMA6A

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**Abstract.** *Background/Aim:* The purpose of this study was to elucidate the molecular mechanism underlying regulation of semaphorin-6A (SEMA6A) involving microRNA-203 (miR-203) as a tumor suppressor in YD-38 human oral cancer cells. *Materials and Methods:* miRNA arrays, polymerase chain reaction analyses, MTT assays, immunoblotting, and luciferase assays were carried out in YD-38 cells. *Results:* MiRNA microarray results showed that expression of miR-203 was significantly down-regulated in YD-38 cells compared to normal human oral keratinocytes. The viability of YD-38 cells was reduced by miR-203 in time- and dose-dependent manners. Overexpression of miR-203 increased the nuclear condensation of YD-38 cells and activated the apoptotic signaling pathway by up-regulating pro-apoptotic factors, such as BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist killer (BAK), and the active forms of caspase-9, caspase-3, and poly-(ADP-ribose)-polymerase (PARP). Furthermore, target gene array analyses revealed that the expression of class 6 semaphorin A (SEMA6A) was down-regulated by miR-203 in YD-38 cells. Both the mRNA and protein levels of SEMA6A were reduced in YD-38 cells transfected with miR-203. Luciferase activity assay confirmed that miR-203 directly targets the SEMA6A 3'-untranslated region to suppress gene expression. *Conclusion:* Our results indicate that miR-203 induces the apoptosis of YD-38 human oral cancer cells by directly targeting SEMA6A, suggesting its potential application in anticancer therapeutics.

MicroRNAs (miRNAs) are endogenous 19-25 nucleotide

non-coding sequences (1) and are important post-transcriptional, sequence-specific regulators of gene expression that act on the 3'-untranslated regions (UTR) of mRNA target genes, causing translation inhibition and mRNA degradation (2). miRNAs have been profiled according to their typical mechanism of transcriptional regulation in a variety of cells and cellular systems (1). They may regulate >90% of human genes (3) and control various cellular biological processes such as cell development, proliferation and differentiation, and apoptosis (4). In addition, although a large number of recent studies have demonstrated alterations in miRNA function as oncogenes or suppressor genes and that their aberrant expression contributes to human diseases such as cancer, their biological functions and cellular mechanisms remain largely unknown (5).

Many studies have examined the biological functions of miRNA-203 (miR-203) as a tumor suppressor in carcinogenesis. Saini *et al.* reported that miR-203 suppresses prostate cancer cell progression and metastasis by targeting a cohort of pro-metastatic genes (6). Jin *et al.* (7) and Wang *et al.* (8) reported that miR-203 inhibits cell proliferation and migration by targeting protein kinase C- $\alpha$  (PKC $\alpha$ ) or survivin expression in lung cancer cells (7, 8). In another study, Bueno *et al.* demonstrated that genetic and epigenetic silencing of miR-203 enhanced expression of the oncogenes abelson murine leukemia viral oncogene homolog 1 (ABL1) and breakpoint cluster region protein (BCR)-ABL1 in specific hematopoietic malignancies (9). In contrast, the anti-tumorigenic function and signaling pathway of miR-203 in oral cancer cells remain unclear.

Semaphorins are secreted or transmembrane proteins initially identified based on their role in axon guidance and regulation of cell motility, immune response, cell differentiation, morphology, angiogenesis, and tumor progression (10). Semaphorins contain a conserved extracellular domain of ~500 amino acids (the Sema domain) that is shared with their receptors plexins (11). In the mammalian system, they are subdivided into five different

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*Key Words:* miR-203, tumor suppressor, oral cancer cells, SEMA6A, apoptosis.

subfamilies (semaphorins 3-7) based on specific structural properties (12). Emerging evidence suggests that the expression of specific semaphorins is important in tumor development or progression (12). However, some studies found that semaphorins are overexpressed in cancer, whereas others are down-regulated (13-16).

The transmembrane protein SEMA6A, the first identified class 6 semaphorin (17), is a membrane-bound protein and functions as a guidance cue for nerve axons and migrating cells in the developing central nervous system (12). It was shown that SEMA6A regulates the formation of lamina-specific axon projections in the hippocampus (18), as well as formation of the cortical spinal tract (19). Class 6 semaphorins have also been implicated in cancer development (12). One study showed that in melanoma cells, depletion of SEMA6A alters the cytoskeleton, impairs anchorage-independent growth, and affects motility and invasive activities (20). In addition, SEMA6A mRNA expression was increased in several renal tumor tissue samples and renal cell carcinoma cell lines, whereas its recombinant soluble extracellular domain inhibited angiogenesis induced by growth factors and tumor cell lines (10, 21). However, the mechanism underlying SEMA6A regulation in cancer cells, including oral cancer cells, is largely unknown.

Here, we examined the biological function of miR-203 as a tumor suppressor in YD-38 human oral cancer cells. Furthermore, the molecular mechanism underlying the miR-203-mediated effect on SEMA6A expression in YD-38 human oral cancer cells was investigated.

## Materials and Methods

**Materials.** 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies to cleaved caspase-3, cleaved caspase-9, cleaved poly (ADP-ribose) polymerase (PARP), BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist killer (BAK), and  $\beta$ -actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other analytical reagents purchased were of analytical grade.

**Cell line and cell cultures.** Normal human oral keratinocytes (NHOKs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and maintained according to the manufacturer's instructions. The human oral cancer cell line derived YD-38 cells was provided by the Korean Cell Line Bank (Seoul, Korea) and cultured according to the supplier's instructions. Briefly, YD-38 cells were grown in RPMI-1640 containing 10% fetal bovine serum. The cells were maintained as monolayers in plastic culture plates at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Plasmid construction.** To induce overexpression of miR-203, we generated a pSuper-miR-203 construct as previously described (22). To verify miR-203-induced SEMA6A gene expression using a luciferase assay, the 3'-UTR of SEMA6A containing the predicted

target sites of miR-203 was amplified from human genomic DNA by proofreading the Phusion High-Fidelity PCR master mix (New England BioLabs® Inc., Ipswich, MA, USA) with the following primers: SEMA6A-3'-UTR-F, 5'-CGAGCCCCGGGACTCCCAGG GGGAGGGGGTTCAGGT-3'; SEMA6A-3'-UTR-R, 5'-TCAGCG GCCGCTCGTTTTTCCCTTTTTTCT-3'. The amplified SEMA6A-3'-UTR, 2,997 bp in size, was digested with *Sma*I and *Not*I. The digested SEMA6A-3'-UTR was inserted into the multiple cloning regions downstream of the *hRluc* gene in psiCHECK™-1 (Promega, Madison, WI, USA). The clones were confirmed by sequencing.

**Transfection and cell viability assay.** The MTT assay was applied to estimate the effect of miR-203 on YD-38 cell proliferation. The cells were plated at a density of 5×10<sup>3</sup> cells/well in 96-well plates and allowed to attach to the wells overnight. After incubation, the cultured cells were transfected with pSuper-miR-203 (Oligoengine, Seattle, WA, USA) at 2, 20, or 200 ng/ml for 24 or 48 h at 37°C in a dose-dependency test using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In addition, 200 ng/ml of pSuper-miR-203 was transfected into the YD-38 cells and incubated for 24 or 48 h as a time-dependency test. Next, the cells were incubated for an additional 4 h in 20  $\mu$ l of 5 mg/ml MTT. To dissolve the formazan crystals transformed from MTT, the cells were resuspended in 150  $\mu$ l dimethyl sulfoxide and the optical density (OD) of the solution was determined using a spectrometer at a wavelength of 495 nm.

**Affymetrix miRNA array and data pre-processing.** Total RNAs from both YD-38 cells and NHOKs were isolated with a miRNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The miRNA array was scanned using an Affymetrix GeneChip Platform (Affymetrix, Santa Clara, CA, USA). Total RNA was subjected to a tailing reaction (2.5 mM MnCl<sub>2</sub>, ATP, and polyA polymerase; incubation for 15 min at 37°C), followed by ligation of the biotinylated signal molecule to the target RNA sample and addition of stop solution. Each sample was hybridized to a GeneChipH miRNA Array at 48°C and 60 rpm for 16 h, washed, stained on a Fluidics Station 450, and scanned with a GeneChip® Scanner3000 7G (Affymetrix). Image data were analyzed with miRNA QC Tool (ThermoFisher scientific, Waltham, MA, USA) software for quality control.

**Identification of miR-203 target genes.** DNA samples isolated from YD-38 cells overexpressing miR-203 were evaluated on a DMET microarray (Affymetrix) using DMET Plus Premier Pack kits according to the manufacturer's protocol. Hybridized DMET arrays were washed and stained in Affymetrix fluidic stations and scanned with the Affymetrix GeneChip® Scanner 3000 7G. Data were generated with Affymetrix GeneChip® Command console software and analyzed with DMET Console software.

**Evaluation and quantification of apoptosis.** To evaluate chromatin condensation as an apoptotic phenomenon in YD-38 cells transfected with miR-203, cells were seeded at a density of 2×10<sup>3</sup> cells/well in a 4-well chamber slide and incubated overnight. Next, 200 ng/ml of pSuper-miR-203 was transfected into the cultured YD-38 cells using Lipofectamine™ 2000 for 48 h. After incubation, the cells were fixed with 1% paraformaldehyde and washed three times with phosphate-buffered saline. For nuclear staining, YD-38 cells

were incubated with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; Roche Diagnostics, Basel, Switzerland) for 15 min. Apoptotic cells were quantified by assessing the characteristic nuclear changes of apoptosis using fluorescence microscopy (Eclipse TE200; Nikon Instruments, Tokyo, Japan).

**Quantitative real-time polymerase chain reaction (qRT-PCR) and quantitative PCR (qPCR).** Total RNA, including mature miRNA, was isolated using an miRNeasy mini kit (Qiagen), and the RNA was quantified by spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Reverse transcription of miRNA was performed using a miScript Reverse Transcription kit (Qiagen) with 1 µg of total RNA. TaqMan miRNA assays kits (Life Technologies, Carlsbad, CA, USA) were used to examine *miR-203* (5'-GTGAAATGTTTAGGACCACTA-3') expression by qRT-PCR according to the manufacturer's instructions. The qRT-PCR results, which were recorded as threshold cycle numbers (Ct), were normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (forward primer sequences: 5'-CTTTGGTATCGTGAA GGACTC-3'; reverse primer sequences: 5'-AGTAGAGGCAGG GATGATGT-3') used as an internal control, and the comparative threshold cycle method ( $\Delta\Delta Ct$ ) was used to determine miRNA expression levels. Furthermore, the level of *SEMA6A* (forward primer sequences: 5'-TGCCCATATGCCAAACATGCC-3'; reverse primer sequences: 5'-TCTCCGTAATCCACGGCTTGAACA-3') induction was measured by PCR. The deviations of the samples were determined from four separate experiments.

For qPCR, total RNA was isolated from YD-38 cells using TRIzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's protocol. Reverse transcription was carried out with 1 µg of total RNA and Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA). The expression levels of *SEMA6A* were measured by qPCR and visualized by DNA agarose gel electrophoresis. Differences in expression are presented as a histogram after densitometric analysis using a VersaDoc™ imaging system (Bio-Rad, Hercules, CA, USA). *GAPDH* was used as an internal standard.

**Immunoblotting.** To determine the levels of activated *SEMA6A*, cleaved caspase-3, cleaved caspase-9, cleaved-PARP, BAK, and BAX in YD-38 cells transfected with pSuper-*miR-203*, the proteins were extracted, electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes for immunoblotting using primary antibodies against *SEMA6A*, cleaved caspase-3, cleaved caspase-9, cleaved PARP, BAK, BAX, and  $\beta$ -actin as previously described (23).

**Luciferase assays.** *miR-203* and scrambled *miR-203* were purchased from Ambion (Austin, TX, USA). The *miR-203*, scrambled *miR-203*, and psiCHECK-1-promoter-*SEMA6A*-3'-UTR were transfected into cultured YD-38 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. The experimental group of YD-38 cells was treated with 1 ng/ml miRNAs for 2 days. Next, the cells were harvested and lysed for luciferase assays 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. *Renilla*/firefly luciferase activity was calculated for each reaction.

**Statistical analysis.** All experiments were performed at least four times. The results are presented as the mean±SEM. Statistical

significance was analyzed by using Student's *t*-test for two groups and one-way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA). Differences with values of  $p < 0.05$  were considered significant.

## Results

***miR-203* expression was significantly down-regulated in YD-38 cells.** To identify YD-38-related miRNAs, we performed a miRNA microarray on YD-38 and NHOK cells (n=4 per cell line) using an Affymetrix GeneChip and the data were analyzed, as described previously (24). miRNAs differentially expressed in YD-38 cells and NHOKs were identified (Figure 1A). We focused on *miR-203* because its expression was significantly down-regulated by approximately 24-fold in YD-38 cells compared to NHOKs. The microRNA array results were verified by examining the expression pattern of *miR-203* by miRNA qRT-PCR using miRNA isolated from both YD-38 cells and NHOKs (Figure 1B). qRT-PCR analysis showed that *miR-203* expression was significantly down-regulated in YD-38 cells by up to 95% compared to NHOKs (Figure 1C).

***Overexpression of miR-203* inhibits cell proliferation and induces apoptosis of YD-38 cells.** To determine whether overexpression of *miR-203* affects the cell proliferation of YD-38 cells, 200 ng/ml of pSuper-*miR-203* was transfected into YD-38 cells using Lipofectamine™ 2000. After 48 h, cell proliferation was assessed in an MTT assay. As shown in Figure 2A, up-regulation of *miR-203* did not alter the viability of NHOKs. In contrast, the proliferation of YD-38 cells transfected with 200 ng/ml of pSuper-*miR-203* gradually decreased with time and was reduced by approximately 30% compared to cells treated with either the non-transfected or empty vector-only (Figure 2B). Moreover, the cell proliferation of YD-38 cells decreased with increasing concentrations of pSuper-*miR-203*, as shown in Figure 2C.

Cell morphology was also assessed by DAPI staining, which detects nuclear condensation. The number of cells with the typical morphology of apoptosis was increased when *miR-203* was overexpressed in YD-38 cells by at least 3.5-fold more than both the un-transfected and empty vector cells, as shown in Figure 2D. Otherwise, cells remained intact with no chromatin condensation in the untransfected control nor in YD-38 cells transfected with the empty vector.

Next, in order to evaluate the apoptotic signaling pathways involved in apoptosis induced in *miR-203*-overexpressing YD-38 cells, we measured the expression of apoptotic signaling pathway-related proteins by western blotting in YD-38 cells treated with 200 ng/ml of pSuper-*miR-203* for 48 h. Expression of the pro-apoptotic factors BAX, BAK, and cleaved caspase-9 was significantly

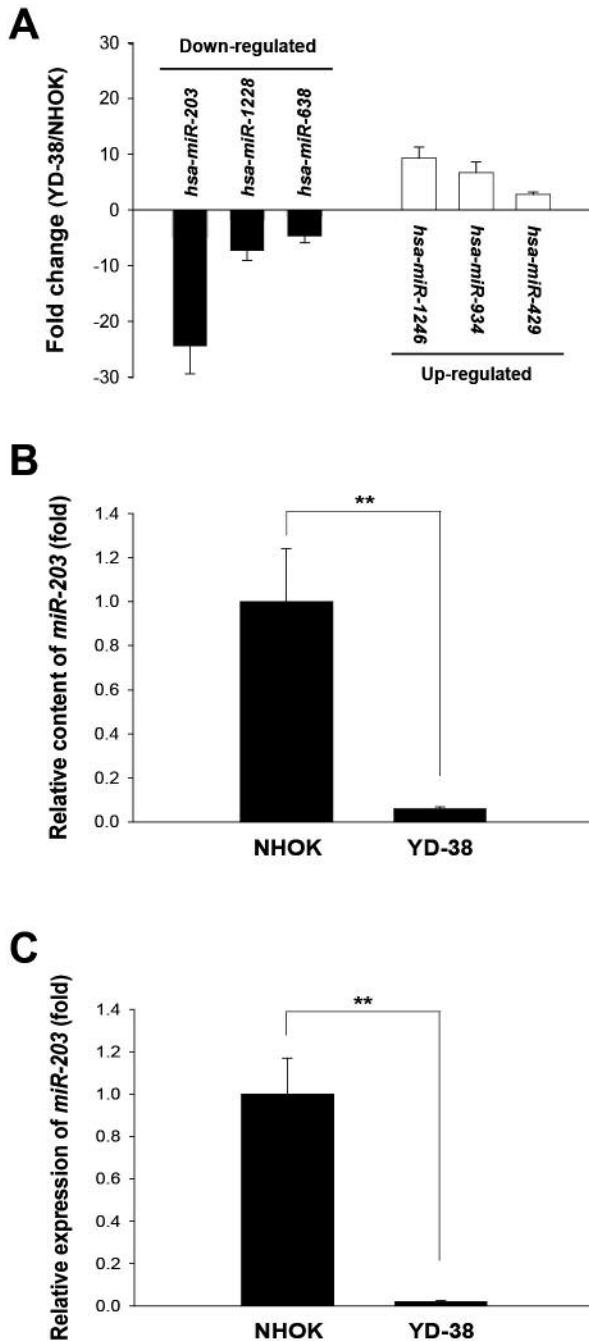


Figure 1. Expression of miR-203 is down-regulated in YD-38 cells. A: Total RNA from both YD-38 cells and NHOKs was isolated, scanned using an Affymetrix GeneChip Platform, stained on Fluidics Station 450, and scanned on a GeneChip® Scanner3000 7G. Each absolute fold change value represents the mean of four independent experiments. B: The relative expression of miR-203 in YD-38 cells and NHOKs was assessed by quantitative real time-polymerase chain reaction, as described in the Materials and Methods. C: The relative expression of miR-203 in YD-38 cells and NHOKs was assessed by quantitative polymerase chain reaction is shown as a histogram after densitometric analysis. Each data point represents the mean±SEM of at least four independent experiments. \*\*p<0.01 vs. control.

increased when *miR-203* was overexpressed (Figure 2E). Therefore, we analyzed the expression of cleaved caspase-3 and its downstream target molecule PARP. As shown in Figure 2E, cleaved caspase-3 was significantly upregulated in YD-38 cells treated with *miR-203*. Subsequently, the level of cleaved PARP, which functions downstream of caspase-3 (25, 26), was significantly increased by *miR-203* treatment in YD-38 cells (Figure 2E). Taken together, these data suggested that overexpression of *miR-203* inhibited cell proliferation and induced the apoptosis of YD-38 cells.

*Overexpression of miR-203 regulates the expression of SEMA6A in YD-38 cells.* To identify *miR-203* target genes in YD-38 cells, a target gene array was performed using DMET Plus Premier Pack kits (Affymetrix) according to the manufacturer’s protocol. As shown in Figure 3, *miR-203* overexpression induced down-regulation of *SEMA6A*, ATP-binding cassette sub-family E member 1 (*ABCE1*), and myeloid/lymphoid or mixed-lineage leukemia translocated to 4 (*MLLT4*), whereas interleukin 24 (*IL24*), sel-1 suppressor of lin-12-like (*SEL1L*), and ADAM metalloproteinase with thrombospondin type 1 motif 6 (*ADAMTS6*) were significantly up-regulated by *miR-203* overexpression in YD-38 cells. We are currently evaluating alterations in the expression of other genes associated with *miR-203*. The mRNA expression of *SEMA6A* is elevated in several renal tumor tissue samples, suggesting that *SEMA6A* is a promising biomarker for the detection and diagnosis of cancer (10, 21). Therefore, we hypothesized that overexpression of *miR-203* induces apoptosis *via* down-regulation of *SEMA6A* in YD-38 cells. To further confirm that *miR-203* inhibits *SEMA6A* expression in YD-38 cells, pSuper-miR-203 constructs were transfected into YD-38 cells and the mRNA and protein levels of *SEMA6A* were analyzed.

As shown in Figure 4A and B, both qPCR and qRT-PCR showed that *SEMA6A* expression was significantly reduced, by approximately 40%, in YD-38 cells transfected with 200 ng/ml of pSuper-*miR-203* compared to in empty vector-only cells.

In addition, *SEMA6A* protein expression was notably reduced by *miR-203*, as shown in Figure 4C. A histogram of densitometric analysis normalized to expression of β-actin showed that the level of *SEMA6A* protein expression was reduced compared to that in empty vector-transfected cells. Together, these results suggest that *miR-203* reduced the expression of *SEMA6A*, indicating that *SEMA6A* is a downstream target gene in YD-38 cells.

*SEMA6A is directly targeted by miR-203.* To determine the mechanism of *miR-203*-mediated down-regulation of *SEMA6A* in YD-38 cells, we performed target gene scanning using TargetScan (<http://www.targetscan.org/>) and miRBase (<http://www.mirbase.org/>) and found that *SEMA6A* was a

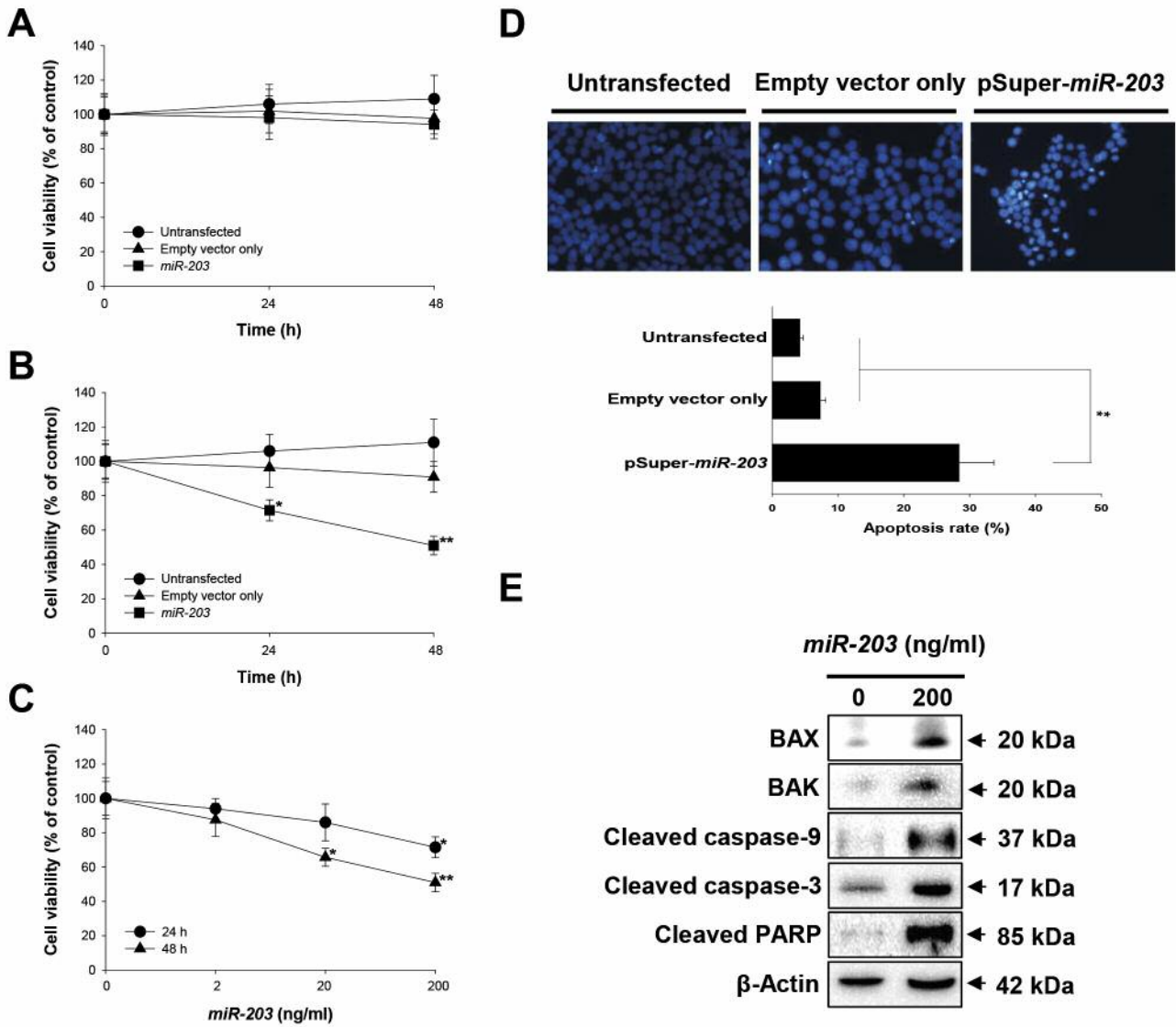


Figure 2. Overexpression of *miR-203* increases cytotoxicity via apoptotic cell death in YD-38 cells. Cell viability of NHOKs (A) and YD-38 cells (B, C) was measured after *miR-203* transfection over time and at different concentrations. Cell cytotoxicity was assessed by MTT assay after *miR-203* transfection into YD-38 cells. D: Nuclear morphology was observed using 4',6-diamidino-2-phenylindole (DAPI) staining after transfection with 200 ng/ml of pSuper-*miR-203*. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells. E: Overexpression of *miR-203* induced apoptosis of YD-38 cells. YD-38 cells were transfected with 200 ng/ml of pSuper-*miR-203*. Cell lysate was prepared and analyzed by immunoblotting as described in the Materials and Methods. \* $p < 0.05$  vs. control and \*\* $p < 0.01$  vs. control.

predicted target gene. Furthermore, *SEMA6A* contains a potential complementary binding sequence located from nucleotide 1,155 to 1,161 in its 3'-UTR, indicating that *miR-203* can directly target this site. Therefore, we cloned 2,997 base pairs (full-length) of the *SEMA6A* 3'-UTR into the firefly luciferase based-psiCHECK-1 vector. In addition, a psiCHECK-1-empty vector was transfected into YD-38 cells to assess the basal level of luciferase activity (Figure 5A). As shown in Figure 5B, the relative luciferase activity of the

reporter containing the 3'-UTR of *SEMA6A* was notably reduced in *miR-203*-transfected cells by approximately 1.9-fold compared to control in YD-38 cells. In contrast, the psiCHECK-1-empty vector plus pSuper-*miR-203* and psiCHECK-1-*SEMA6A* plus pSuper-empty vector led to similar basal levels of luciferase activity. These results suggest that *SEMA6A* is a novel and specific target gene of *miR-203* and *miR-203* may regulate *SEMA6A* expression in YD-38 cells.

## Discussion

In the present study of the pathophysiological role of miR-203 in cancer cells, we demonstrated that the expression of *miR-203* was most significantly down-regulated in YD-38 cells compared to that in NHOKs (normal tissues) in the present study (Figure 1). Therefore, these data suggest that *miR-203* may be clinically useful for developing prognostic biomarkers. These data also suggest that down-regulation of *miR-203* is closely associated with oral cancer progression.

Recently, miRNA research associated with cancer therapies has suggested that overexpression of tumor suppressor miRNAs such as *miR-124* in gastric cancer (27), *miR-494-3p* in prostate cancer (28), *miR-23a/b* in lymphoma (29), and *miR-205* in oral cancer (30) can inhibit proliferation and induce apoptosis of cancer cells. More recently, Tian *et al.* reported that overexpression of *miR-203* suppressed proliferation and induced apoptosis of laryngeal squamous cell carcinoma (31). Therefore, based on our results and those of previous studies, we hypothesized that overexpression of *miR-203* could induce apoptosis of YD-38 cells by altering cellular responses. As shown in Figure 2, cell viability gradually decreased in YD-38 cells transfected with *miR-203* in both time- and dose-dependent manners. Furthermore, overexpression of *miR-203* increased nuclear condensation in YD-38 cells, as shown by DAPI staining (Figure 2D). Moreover, *miR-203* up-regulated or activated the pro-apoptotic factors, BAX, BAK, and cleaved caspase-9 (25, 26), as shown in Figure 2E. The levels of cleaved caspase-3 and cleaved PARP were significantly increased in YD-38 cells transfected with *miR-203* (Figure 2E). Finally, as a typical phenomenon of apoptosis, activated PARP induced DNA fragmentation *via* single-strand DNA breakage (25, 26) in the nucleus of YD-38 cells. These data suggest that *miR-203* led to apoptotic cell death in YD-38 cells. However, the complete mechanisms of *miR-203*-induced apoptosis in YD-38 cells remain unclear. Further studies are needed to determine these cellular and molecular mechanisms.

miRNAs are thought to elicit their effects by base-pairing complementation between the seed sequence of miRNAs and 3'-UTR of their target mRNAs, which is a key determinant of miRNA-target recognition (32). Therefore, we used a *miR-203* target gene array to explore the molecular biological mechanism of *miR-203*-induced apoptosis in YD-38 cells. The results of the *miR-203* target gene array in YD-38 cells revealed that overexpression of *miR-203* altered the expression patterns of some oncogenes. Although *miR-203* changed the expression of some tumor-suppressor genes, we focused on down-regulated oncogenes in YD-38 cells transfected with *miR-203* because miRNAs typically suppress protein expression by affecting mRNA translation

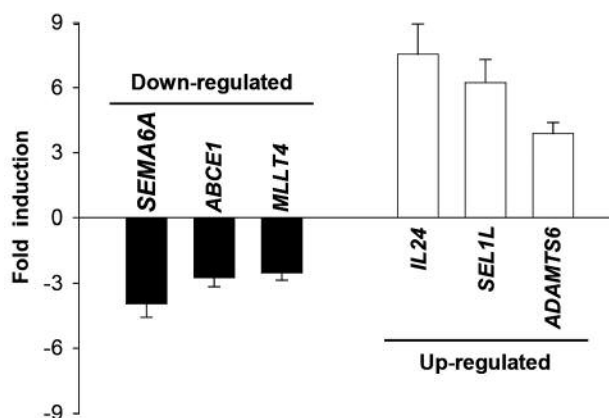


Figure 3. *Semaphorin 6A (SEMA6A)* is a target gene of *miR-203* in YD-38 cells. DNA samples isolated from YD-38 cells with overexpression of *miR-203* were run on a DMET microarray, stained in the Affymetrix fluidic stations, and scanned with Affymetrix GeneChip® Scanner 3000 7G. Data were generated with Affymetrix GeneChip® Command console software and analyzed with the DMET Console software. Each absolute fold-change value represents the mean of four independent experiments. *ABCE1*: ATP-binding cassette sub-family E member 1; *MLLT4*: myeloid/lymphoid or mixed-lineage leukemia translocated to 4; *IL24*: interleukin 24; *SEL1L*: *sel-1* suppressor of *lin-12*-like; *ADAMTS6*: ADAM metalloproteinase with thrombospondin type 1 motif 6.

or stability through interactions with the 3'-UTR of their target mRNAs (32). As shown in Figure 3, *SEMA6A*, *ABCE1*, and *MLLT4* were significantly down-regulated in YD-38 cells transfected with *miR-203*, whereas *IL24*, *SEL1L*, and *ADAMTS6* were significantly up-regulated. *ABCE1* plays an essential role in lung cancer progression and metastasis (33) and *MLLT4* regulates cancer cell migration in colorectal carcinogenesis (34). Additionally, *IL24* functions as a tumor suppressor in breast cancer (35), and *SEL1L* also has a tumor-suppressive role in breast cancer (36). Furthermore, *ADAMTS6* suppresses tumorigenesis by inhibiting cell migration and invasion in breast cancer (37). However, their oncogenic or tumor-suppressive activities remain largely unclear in different types of cancer. Although we are still conducting functional studies for both *ABCE1* and *MLLT4* in YD-38 cells, the expression of *SEMA6A* was most significantly down-regulated by *miR-203* in YD-38 cells (Figure 3).

Semaphorins comprise a large family of conserved glycoproteins that regulate cell motility, immune response, cell differentiation, morphology, angiogenesis, and tumor progression (12). Additionally, semaphorins and their receptors are involved in multiple functions during development and in adult organisms, particularly in the nervous system, immune system, and various pathological conditions (38). A recent study observed miRNA-mediated

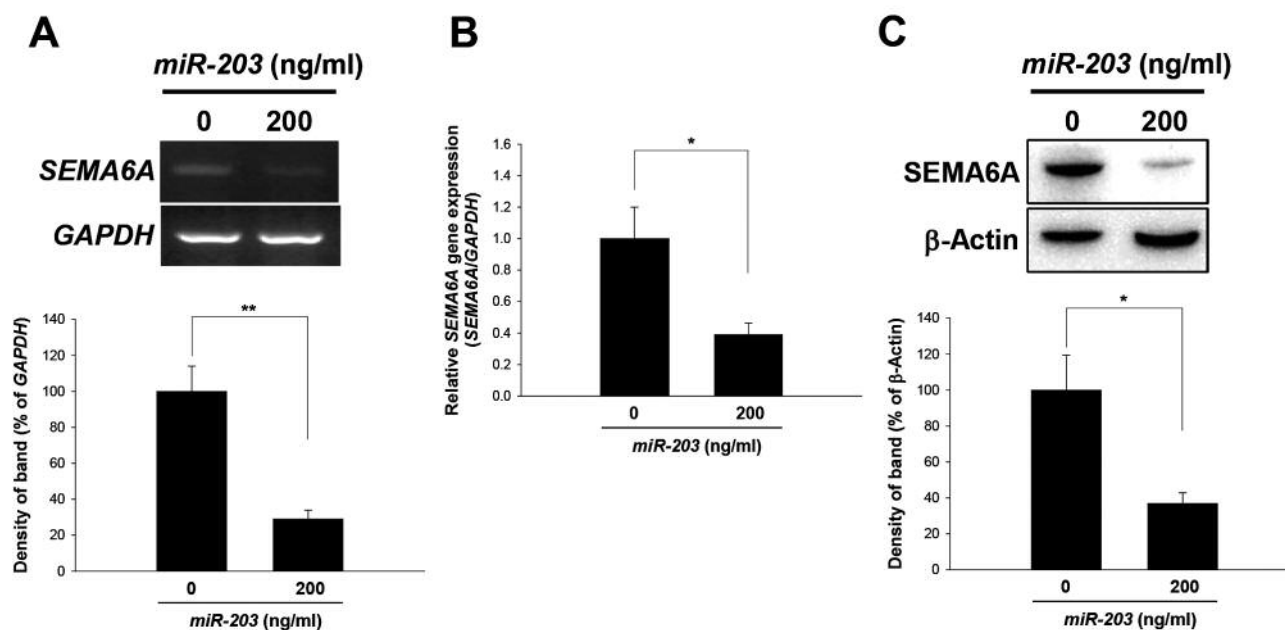


Figure 4. Overexpression of miR-203 reduces expression of the oncogenic gene semaphorin 6A (*SEMA6A*) in YD-38 cells. A: The expression of *SEMA6A* was measured by quantitative polymerase chain reaction (PCR) after miR-203 transfection. The amplified PCR products of *SEMA6A* were electrophoresed on agarose gels and the data presented as a histogram after densitometric analysis. B: The expression of *SEMA6A* was assessed by quantitative real-time PCR after miR-203 transfection into YD-38 cells. C: *SEMA6A* protein expression was quantified by western blotting using *SEMA6A*-specific antibody. Each data point represents the mean  $\pm$  SEM of at least four independent experiments. \* $p < 0.05$  vs. control and \*\* $p < 0.01$  vs. control.

regulation of *SEMA6A*. Wolter *et al.* reported that *SEMA6A* up-regulation facilitated by miR-346 silencing may promote glioma growth (39). However, the mechanism underlying *SEMA6A* regulation related to miRNAs in cancer cells is unclear. In the present study and based on the results of previous studies (10, 21, 39), we hypothesized that miR-203-induced apoptosis of YD-38 cells is related to the suppression of *SEMA6A*. As shown in Figure 4, both mRNA induction and protein expression of *SEMA6A* were significantly suppressed by miR-203 transfection in YD-38 cells. These data suggest that *SEMA6A* is indeed a target oncogene of miR-203 in YD-38 cells.

However, miRNAs are thought to elicit their effects by silencing the expression of their target genes by directly interacting with their own regulatory region, such as the promoter (40) or 3'-UTR (32). Therefore, to identify the miR-203 binding site in the *SEMA6A* gene, we performed target gene scanning and identified potential complementary binding sequences from positions 1,155 to 1,161 in its 3'-UTR as shown in Figure 5A. Next, to verify whether miR-203 can suppress the expression of *SEMA6A* in YD-38 cells, we generated the psiCHECK-1-*SEMA6A* construct, which contained potential complementary

binding sequences for miR-203 in the 3'-UTR of *SEMA6A*. The miR-203 directly targets the 3'-UTR of *SEMA6A*, as its overexpression suppressed luciferase activity (Figure 5B). In contrast, the overexpression of mimic miR-203 did not affect *SEMA6A* expression. These results suggest that miR-203 down-regulates *SEMA6A* directly via targeting of its 3'-UTR.

In conclusion, we found that miR-203 functions as a tumor suppressor associated with suppression of *SEMA6A* in YD-38 human oral cancer cells. Further analyses of the mechanism underlying the regulation of cancer progression by miR-203 will contribute to the development of new oral cancer treatments and provide a new marker for the diagnosis of cancer.

### Conflicts of Interest

None of the Authors have any conflict of interest in regard to this study.

### Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2016R1D1A1A09916949).

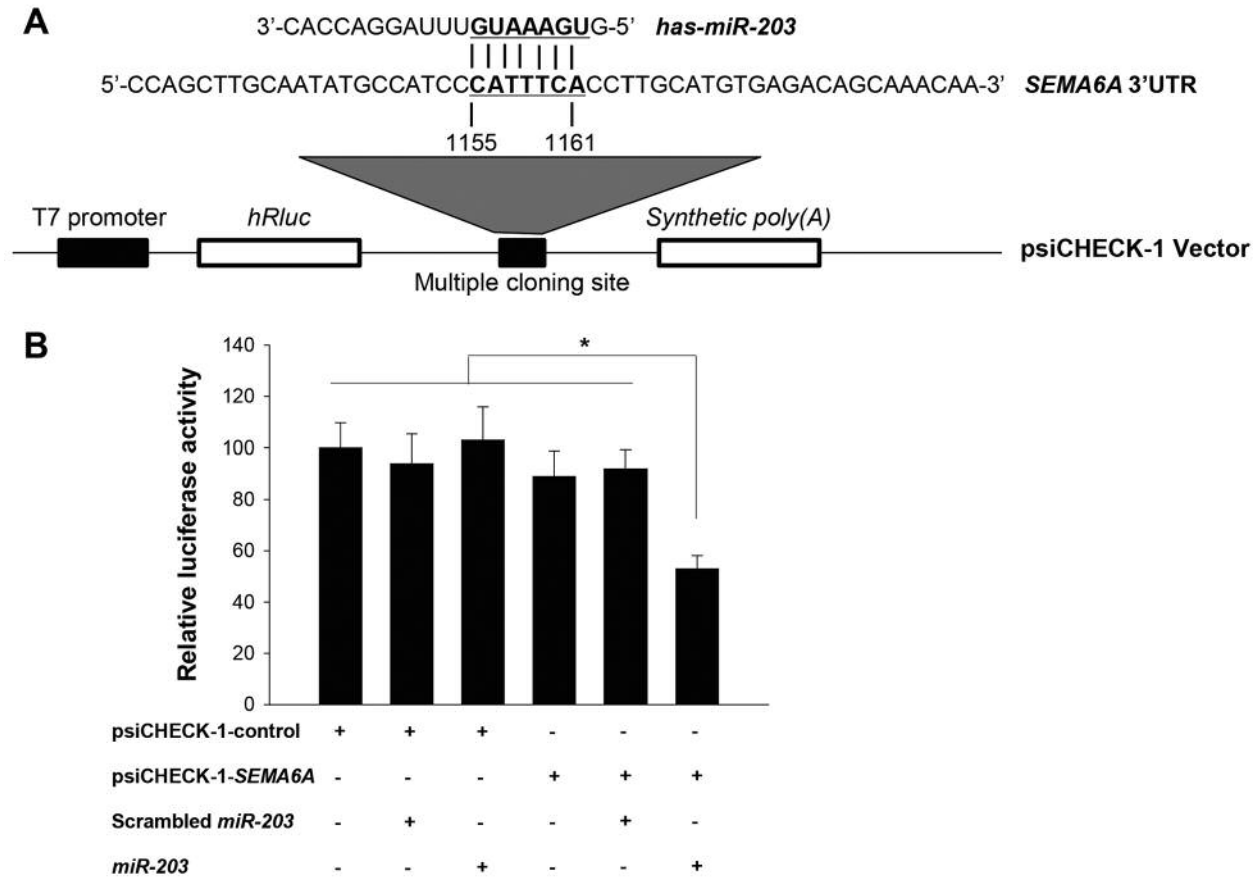


Figure 5. *Semaphorin 6A (SEMA6A)* is a direct target of *miR-203* in YD-38 cells. A: The sequence of the *miR-203* target sites on the *SEMA6A*-3'-UTR. B: The luciferase activity of the *SEMA6A* promoter against *miR-203*. psiCHECK-2-*SEMA6A* was cloned as described in the Materials and Methods. Luciferase activity was normalized to total protein. Each data point represents the mean±SEM of at least four independent experiments. \* $p < 0.05$  vs. control.

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Received August 11, 2017  
 Revised September 1, 2017  
 Accepted September 6, 2017