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MicroRNA-203 Induces Apoptosis by Targeting *Bmi-1* in YD-38 Oral Cancer Cells

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Abstract. *Background/Aim: MicroRNAs (miRNAs) are closely associated with a number of cellular processes, including cell development, differentiation, proliferation, carcinogenesis, and apoptosis. The aim of the present study was to elucidate the molecular mechanisms underlying the tumor suppressor activity of miRNA-203 (miR-203) in YD-38 human oral cancer cells. Materials and Methods: Polymerase chain reaction analysis, MTT assay, DNA fragmentation assay, fluorescence-activated cell-sorting analysis, gene array, immunoblotting, and luciferase assay were carried out in YD-38 cells. Results: miR-203 expression was significantly down-regulated in YD-38 cells compared to expression levels in normal human oral keratinocytes. miR-203 decreased the viability of YD-38 cells in a time- and dose-dependent manner. In addition, over-expression of miR-203 significantly increased not only DNA segmentation, but also the apoptotic population of YD-38 cells. These results indicate that miR-203 overexpression induces apoptosis in YD-38 cells. Target gene array analysis revealed that the expression of the polycomb complex protein gene Bmi-1, a representative oncogene, was significantly down-regulated by miR-203 in YD-38 cells. Moreover, both mRNA and protein levels of Bmi-1 were significantly reduced in YD-38 cells transfected with miR-203. These results indicate that Bmi-1 is a target gene of miR-203. A luciferase reporter assay confirmed that miR-203 suppressed Bmi-1 expression by directly targeting the 3'-untranslated region. Conclusion: miR-203 induces apoptosis in YD-38 cells by directly targeting Bmi-1, which suggests its possible application as an anti-cancer therapeutic.*

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MicroRNAs (miRNAs) are a group of naturally-occurring, small (19-25 nucleotides), non-coding RNAs that are important post-transcriptional regulators of gene expression (1). miRNAs function by binding to the 3'-untranslated region (3'-UTR) of their target genes, and thereby inhibiting translation or inducing mRNA degradation (2). They have been estimated to regulate up to 60% of protein-coding genes (3) and have been shown to control various cellular biological processes, such as cell development, differentiation, proliferation, carcinogenesis, and apoptosis (4-6). In addition, many studies have demonstrated that miRNAs function as oncogenes or suppressive genes and that their aberrant expression contributes to various human diseases such as cancer (7). Further investigation of miRNA involvement in cancer could help us better understand the molecular mechanisms responsible for cancer development and lead to novel strategies for the effective control of cancer (7, 8). Therefore, current studies have focused on the utility of miRNAs as diagnostic and prognostic tools and potential therapeutic targets. However, their biological functions and cellular mechanisms are largely unknown (7, 8).

Many studies have examined the role of *miRNA-203* (*miR-203*) as a tumor suppressor in carcinogenesis. Saini *et al*. reported that *miR-203* regulates cell progression and metastasis in prostate cancer by targeting a cohort of pro-metastatic genes (9). Bo *et al*. reported that *miR-203* suppresses bladder cancer progression by repressing the Bcl-w expression (10). In another study, Takeshita *et al*. showed that miR-203 inhibits cell migration and invasion by regulating LASP1 expression in esophageal squamous cell

carcinoma (11). These studies showed that *miR-203* has anti-tumor activity in several human cancers (9-11). However, the anti-tumorigenesis mechanism and signaling pathway underlying the tumor suppressor activity of *miR-203* in oral cancers are still unclear. Thus, in the present study, the biological functions and mechanisms of the tumor suppressor miRNA function of *miR-203* were investigated, and results showed that *miR-203* functions by repressing the activity of Bmi-1 in oral cancer cells.

Bmi-1, B-cell-specific Moloney murine leukemia virus insertion site 1, is a core member of the polycomb repressive complex 1 (RPC1), RCP1 can modify chromatin structure and regulate the transcription of numerous target genes, including the *INK4a/ARF* locus, which contains the genes encoding $p16^{ink4a}$ and $p19^{arf}$, two proteins that function to suppress proliferation and promote apoptosis (12, 13). *Bmi-1* was originally identified as an oncogene that cooperates with c-Myc during the progression of B-cell lymphoma (14) and it is overexpressed in a variety of human cancers, including breast, colon and gastric cancer (15-17). However, the mechanism underlying *Bmi-1* regulation in cancer cells is largely unknown. The aim of the present study was to determine the biological function of *miR-203* as a tumor suppressor in YD-38 oral cancer cells and the molecular mechanism of *miR-203*-mediated *Bmi-1* repression in oral cancer cells.

Materials and Methods

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 YD-38 was obtained by the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Atlas Biological, Fort Collins, CO, USA) at 37˚C in an atmosphere containing 5% CO_2.

miRNAs and transfection. miR-203 and scrambled *miR-203* mRNA (mimic *miR-203*) were purchased from Ambion (Austin, TX, USA). The *miR-203*, mimic *miR-203*, and psiCHECK-2-promoter-*Bmi-1*-3'-untranslated region (3'-UTR) were transfected into cultured YD-38 cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The experimental cells were incubated with 1 ng/ml miRNA for 2 days.

Cell viability assay. The MTT assay was used to assess the effect of *miR-203* on YD-38 oral cancer cell proliferation. The cells were plated at a density of 5×10^3 cells/well in 96-well plates and allowed to attach to the well overnight. After incubation, the cultured cells were transfected with *miR-203* at defined concentrations (5, 20, and 200 ng/ml) for 24 h or 48 h at 37˚C using Lipofectamine™ 2000. In addition, *miR-203* (200 ng/ml) was transfected into YD-38 cells and incubated for 24 or 48 h as a time-dependent test. The cells were incubated for an additional 4 h with 20 μl of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). To dissolve the formazan crystals the cells were resuspended in 150 μl of dimethyl sulfoxide (DMSO), and the optical density at 495 nm (OD_{495}) of the solution was determined using a spectrometer. The experiments were repeated at least three times.

DNA fragmentation. To evaluate genomic DNA fragmentation as an apoptotic phenomenon in YD-38 cells treated with *miR-203*, a DNA fragmentation assay was performed according to our laboratory method. *miR-203* was transfected into cultured YD-38 cells using Lipofectamine 2000 for 48 h. After transfection, pellets containing 1×10^6 cells were lysed by incubation in lysis buffer (25 mM EDTA, 10 mM Tris-HCL pH 8.0 and 0.5% Triton X-100) for 20 min at 4˚C. After centrifugation at 12,000 × *g* for 30 min at 4˚C, the supernatant was mixed with an equal volume of the phenol:chloroform:isoamyl alcohol (v/v/v, 25:24:1). After centrifugation at 12,000 × *g* for 30 min at 4˚C, the DNA was precipitated with sodium acetate and 70% ethanol, and then dried and dissolved in a deionized water-RNase solution. The purified DNA was then electrophoresed on a 1% agarose gel visualized with ethidium bromide.

Quantitative estimation of apoptosis. Apoptosis was assessed by fluorescence-activated cell-sorting (FACS) using an Annexin V-FITC/7-AAD Kit (BD Biosciences, NJ, USA). The cells were resuspended in a binding buffer (BD Biosciences), Annexin V-FITC and 7-amino-actinomycin D (7-AAD) were added, and the mixture was incubated in the dark for 15 min. The cells were then resuspended in 300 μl of binding buffer and analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data analysis was performed using Cell Quest software (Becton-Dickinson).

Identification of miR-203 target genes. To determine the *miR-203* target genes, we performed a microarray experiment as previously described (18). DNA samples isolated from YD-38 cells overexpressing *miR-203* were run on a DMET microarray (Affymetrix, Santa Clara, CA, USA) using the DMET Plus premier pack kit according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR) and quantitative PCR (qPCR). Total RNA, including mature miRNA, was isolated from cultured cells using the miRNeasy mini kit (Qiagen, Germantown, MD, USA), and the RNA was quantified by spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. The miRNA was reverse transcribed using the miScript Reverse Transcription kit (Qiagen) using 1 μg of total RNA. The TaqMan miRNA assay kit (Life Technologies, Grand Island, NY, USA) was used to examine *miR-203* (5'-GTGAAATGTTTAGGACCACTAG-3') expression by qRT-PCR. The qRT-PCR results, which were recorded as threshold cycle number (Ct), were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (h*GAPDH*-F, 5'-CTTTGGTATCGTGGAAGGACTC-3'; h*GAPDH*-R, 5'-AGTAGAGGCAGGGATGATGT-3'), which was used as an internal control, and the comparative threshold cycle method ($^{\Delta\Delta}$Ct) was used to determine the miRNA expression levels. The levels of *Bmi-1* (h*Bmi-1*-F, 5'-CTTCATTGATGCCACAAC CATAATA-3'; h*Bmi-1*-R, 5'-CTTGGACATCACAAATAGGA CAATAC-3') were measured by qRT-PCR. The deviations in the samples were calculated from three separate experiments. To perform the qPCR, total RNA was isolated 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 (MMLV) reverse transcriptase (Promega, Madison, WI, USA). The expression levels of *Bmi-1* were measured by qPCR and visualized by DNA agarose gel electrophoresis as previously described (18).

Immunoblotting. To determine the level of Bmi-1 in YD-38 cells transfected with *miR-203*, immunoblotting was performed using a previously described method (19). The membrane was probed with a primary antibody against Bmi-1 (Abcam, Cambridge, MA, USA) or β-actin (Santa Cruz, CA, USA) overnight at 4˚C and then incubated with a secondary antibody for 2 h at room temperature.

Plasmid construction and luciferase assays. The 3'-UTR of *Bmi-1* containing the predicted *miR-203* target sites was amplified from human genomic DNA by PCR using nPfu-Forte DNA Polymerase (Enzynomics, Daejeon, Korea) with the following primers: *Bmi-1*-3'-UTR-F, 5'-TACCTGAGACTGTTAAGGAAAA-3'; *Bmi-1*-3'-UTR-R, 5'-TTGGCAAACATTGGTAACTTTT-3'. The PCR product was cloned into the reporter vector psiCHECK-2 (Promega) using *Not*1 and *Xho*1 restriction enzymes (NEB, Ipswich, MA, USA). The recombinant construct was validated by restriction digestion and direct sequencing. For the luciferase activity assay, YD-38 cells were transfected with *miR-203* or scrambled *miR-203* along with psiCHECK-Bmi1-1 or with the empty psiCHECK vector (0.5 μg/well). Firefly and *Renilla* luciferase activity were assessed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. *Renilla* luciferase activity was normalized to the firefly luciferase activity. The transfection experiments were performed at least three times with triplicate samples.

Statistical analysis. The results are expressed as the mean±SEM of at least three separate experiments. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was analyzed by using Student's *t*-test for two groups and one-way analysis of variance for multi-group comparisons. *p*-Values less than 0.05 were considered statistically significant.

Results

miR-203 expression is down-regulated in YD-38 human oral cancer cells. In our previous report, *miR-203* expression was most significantly down-regulated, up to approximately 24-fold, in YD-38 cells compared to the levels in NHOKs in the miRNA microarray analysis (18). The previous microRNA array results were verified by examining the expression pattern of *miR-203* by miRNA qRT-PCR and qPCR using miRNA isolated from both YD-38 cells and NHOKs. qRT-PCR (Figure 1A) and qPCR (Figure 1B) analyses showed that *miR-203* expression was significantly down-regulated in YD-38 cells compared to NHOKs.

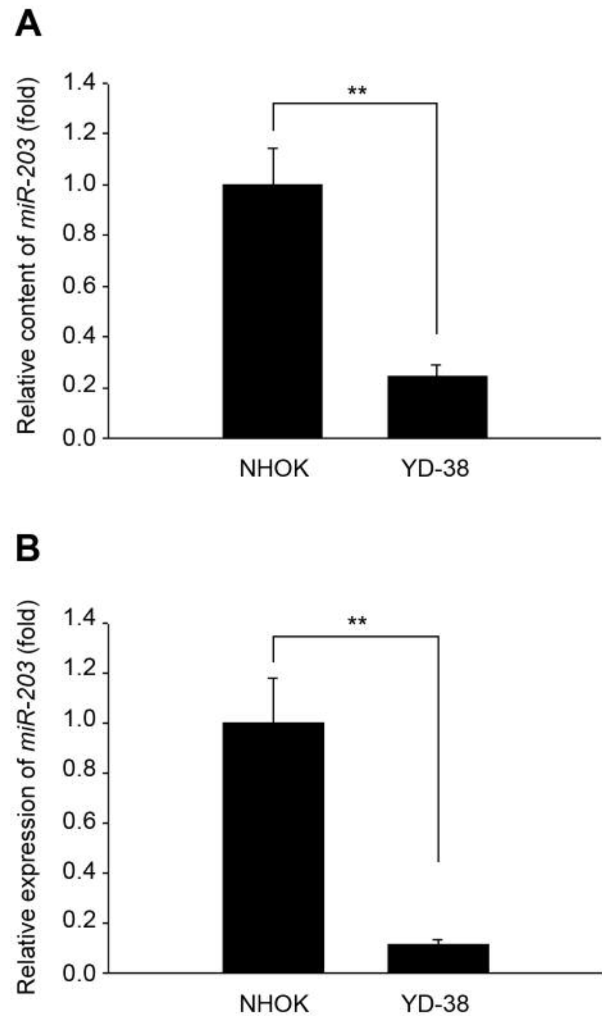

Figure 1. *Down-regulation of miR-203 in YD-38 human oral cancer cells. A: Total RNA from both YD-38 cells and normal human keratinocytes (NHOKs) was isolated. The relative expression of miR-203 in YD-38 cells and NHOKs was assessed by quantitative real time-polymerase chain reaction (qRT-PCR), as described in the Materials and Methods. B: The relative expression of miR-203 in YD-38 cells and NHOKs was assessed by quantitative polymerase chain reaction (qPCR) and 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.*

Overexpression of miR-203 inhibits proliferation and induces apoptosis of YD-38 cells. To investigate the role of *miR-203* in the growth of YD-38 cells, *miR-203* was transfected into YD-38 cells using Lipofectamine 2000, and the cells were monitored for 24 and 48 h. As shown in Figure 2A, up-regulation of *miR-203* did not alter the viability of NHOKs. In contrast, the viability of YD-38 cells transfected with *miR-203* gradually decreased each hour, by approximately 40%, compared to the viability of either non-transfected cells or cells transfected with an empty vector (Figure 2B). The viability of YD-38 cells decreased gradually as the concentration of *miR-203* increased (Figure 2C). To determine whether *miR-203*-induced cell death is associated

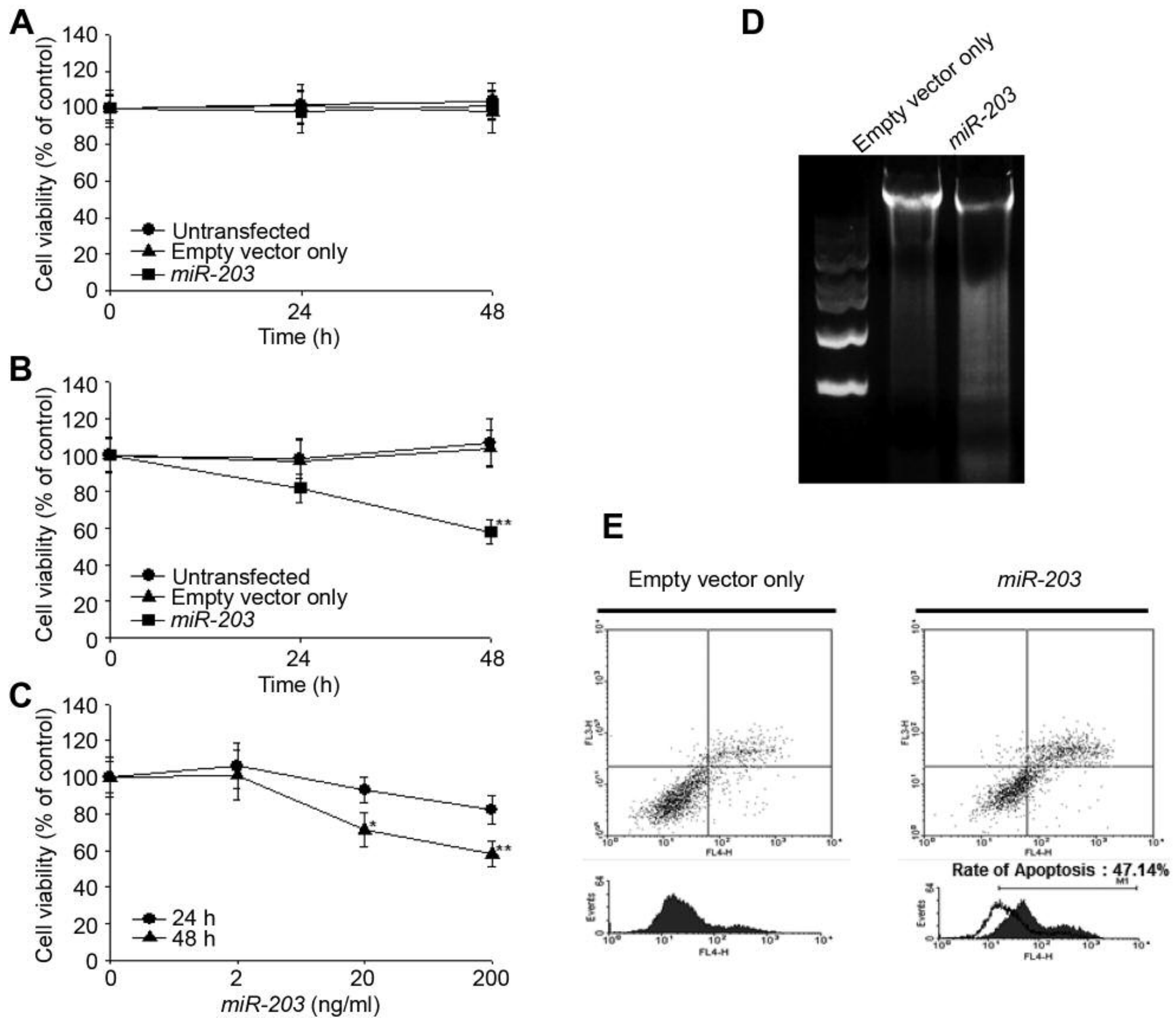

Figure 2. *Overexpression of miR-203 increases cell cytotoxicity and apoptotic cell death in YD-38 cells. The viability of NHOKs (A) and YD-38 cells (B, C) was measured by the MTT assay after cells were transfected with miR-203 following a defined treatment protocol. *p<0.05 vs. control and **p<0.01 vs. control. D: Apoptotic cell death was observed as DNA fragmentation in response to miR-203 transfection. E: The population of apoptotic YD-38 cells was increased after miR-203 transfection.*

with the induction of apoptosis, YD-38 cells were transfected with *miR-203*, and apoptosis-associated DNA fragmentation was assessed. As shown in Figure 2D, in YD-38 cells transfected with *miR-203*, a ladder-like pattern was observed, which is typical of chromatin DNA fragmentation or DNA laddering during apoptosis. In contrast, untreated YD-38 cells showed intact DNA. Similar results were obtained by FACS analysis using Annexin-V-FITC-7-AAD dye. As shown in Figure 2E, the percentage of total apoptotic cells (early and late apoptotic) increased by approximately 26.4% following *miR-203* transfection than the total apoptotic cells obtained when transfected with an empty vector. These results indicate that *miR-203* overexpression induces apoptosis of YD-38 oral cancer cells.

Overexpression of miR-203 down-regulates the expression of Bmi-1 in YD-38 cells. To identify the specific genes that are affected by *miR-203* over-expression, a target gene array was used (DMET Plus Premier Pack kit; Affymetrix) according to the manufacturer's protocol. As shown in Figure 3, *miR-203* overexpression induced the up-regulation of various potential oncogenic genes, such as *anthrax toxin receptor 2, small*

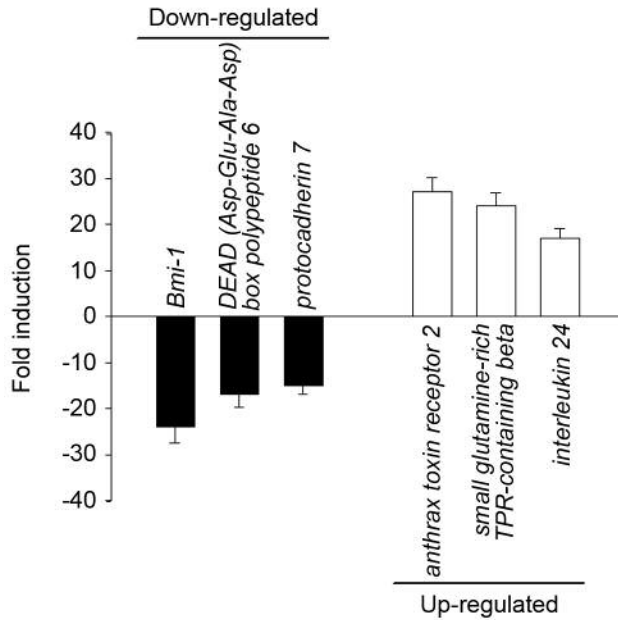

Figure 3. *miR-203 target gene array in YD-38 cells. DNA samples isolated from YD-38 cells overexpressing miR-203 were hybridized to a DMET microarray, stained in an Affymetrix Fluidics Station, and scanned with the Affymetrix GeneChip® Scanner 3000 7G. Data were generated with the Affymetrix GeneChip® and Command console software and analyzed with DMET Console software. Each absolute fold change value represents the mean of three independent experiments with SEM less than±15.9%.*

glutamine-rich tetratricopeptide repeat (TPR)-containing beta, and interleukin 24, whereas *Bmi-1, DEAD (Asp-Glu-Ala-Asp) box polypeptide 6,* and *protocadherin 7* were significantly down-regulated by *miR-203* overexpression in YD-38 cells. miRNAs are generally thought to negatively regulate the expression of their targets through translational repression or mRNA degradation (20). Therefore, we hypothesized that overexpression of *miR-203* might induce apoptosis *via* down-regulation of Bmi-1 in YD-38 cells. Bmi-1 has tumorigenic capacity in various cancer cells (21). To determine whether overexpression of *miR-203* reduces Bmi-1 expression, we transfected *miR-203* into YD-38 cells and analyzed mRNA and protein levels by qPCR, qRT-PCR and immunoblotting.

As shown in Figure 4A and B, both qPCR and qRT-PCR showed that *Bmi-1* expression was significantly reduced in YD-38 cells transfected with 200 ng/ml of pSuper-*miR-203* compared to empty vector-only control cells. In addition, the expression of Bmi-1 protein was significantly reduced following transfection of *miR-203*, whereas the empty vector-only control cells maintained a considerable amount of Bmi-1 protein (Figure 4C), which was in agreement with the qPCR and qRT-PCR results. These results suggested that overexpression of *miR-203* decreased the expression of Bmi-1 at both the transcription and protein levels, indicating that *Bmi-1* is a downstream target gene in YD-38 cells.

Bmi-1 is a target gene of miR-203. To identify the mechanisms underlying *miR-203*-mediated down-regulation of *Bmi-1* in YD-38 cells, the miRNA target prediction programs TargetScan (http://www.targetscan.org/) and miRBase (http://www.mirbase.org/) were used. The predicted interactions between *miR-203* and its target sites in the *Bmi-1* 3'-UTR are illustrated in Figure 5A. As shown in this figure, the *Bmi-1* 3'-UTR is located between bases 1,442-1,449. This sequence is complementary to the seed sequence of *miR-203*, which indicates that *miR-203* may directly target this site. To evaluate *miR-203*-mediated down-regulation of *Bmi-1* in YD-38 cells, a portion of the *Bmi-1* 3'-UTR, which includes the predicted miR-203 binding site, was cloned into the firefly luciferase vector-psiCHECK-2. The psiCHECK-2-control vector was transfected into YD-38 cells to assess basal luciferase activity. As shown in Figure 5B, the luciferase activities of cells were all similar to the basal levels, except in cells transfected with *miR-203* and psiCHECK-2-*Bmi-1*. As expected, the luciferase activity of the reporter containing *Bmi-1* 3'-UTR was notably decreased ($p<0.05$) in *miR-203*-transfected cells. These data suggested that *miR-203* interacts directly with the 3'-UTR of *Bmi-1*, which could be one of the mechanisms that regulate *Bmi-1* expression in YD-38 cells.

Discussion

MicroRNAs play important roles in a wide range of biological cellular processes, such as cell development, proliferation, differentiation, and apoptosis (4-6). Recently, several studies have highlighted the functions of miRNAs in human cancer, showing their roles as oncogenes or tumor suppressors (7). In particular, studies showed that down-regulation of tumor suppressor miRNAs, such as miR-34a (22), miR-145, and miR-133a/b (23), enhanced the oncogenic effects in various cancer cells. *miR-203* was reported to act as a tumor-suppressive microRNA, and its expression was down-regulated in various cancers, such as hepatocellular carcinoma (24), prostate cancer (9), and laryngeal carcinoma cells (25). Moreover, Ding *et al*. described the tumor suppressive effects of *miR-203* in breast cancer, showing that loss of *miR-203* led to increased invasion and metastasis (26). A previous study on the pathophysiological role of *miR-203* in cancer cells showed that the expression of *miR-203* was significantly lower in YD-38 oral cancer cells (Figure 1), similar to that observed in other cancer cells, such as breast cancer cells (27). Therefore, these data suggest that *miR-203* could be clinically useful for developing potent prognostic biomarkers.

Tian *et al*. reported that over-expression of *miR-203* could suppress cell proliferation and induce apoptosis in laryngeal squamous cell carcinoma (25). Therefore, we hypothesized that the viability of YD-38 cells might be reduced and apoptosis might be induced in response to *miR-203*. To test this hypothesis, we induced *miR-203* overexpression in both

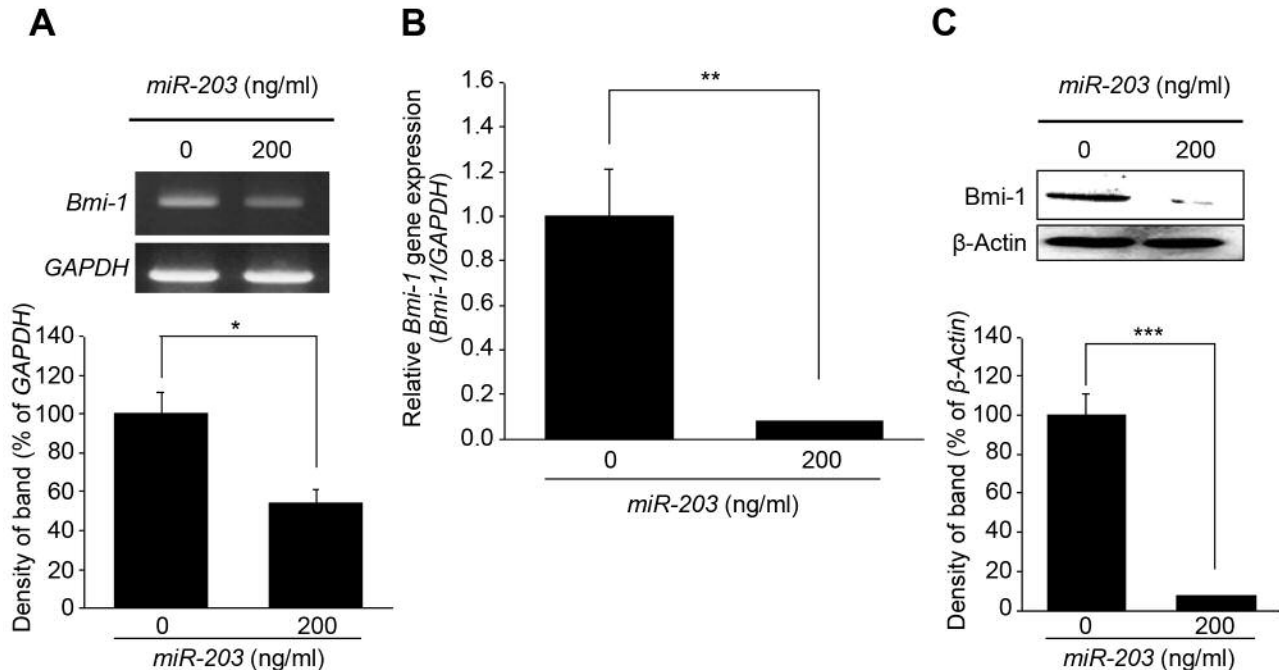

Figure 4. *Overexpression of miR-203 decreased the expression of the oncogene Bmi-1 in YD-38 cells. A: Following miR-203 transfection, the mRNA expression of Bmi-1 was measured by qPCR. The amplified Bmi-1 PCR products were electrophoresed on an agarose gel, and the data are presented as a histogram of the densitometric analysis. B: Following miR-203 transfection, the expression of Bmi-1 was assessed by qRT-PCR. C: Bmi-1 protein expression was quantified by western blotting using an anti-Bmi-1 antibody. Each data point represents the mean±SEM of at least four independent experiments. *p<0.05 vs. control, **p<0.01 vs. control and *** p<0.001 vs. control.*

YD-38 cells and NHOKs. The results showed that cell viability gradually decreased in YD-38 cells in response to *miR-203*, whereas no effect was observed in NHOKs (Figure 2). Furthermore, overexpression of *miR-203* induced a ladder-like pattern of chromatin DNA, a typical characteristic of cell apoptosis, and increased the apoptotic population of YD-38 cells in FACS analysis (Figure 2). These results suggested that miR-203 led to apoptotic cell death and *miR-203* is a potential suppressive miRNA in oral cancer cells. However, the complete mechanism of *miR-203*-induced apoptosis in YD-38 cells is unclear. More research is needed to determine these cellular and molecular mechanisms.

MicroRNAs are believed to elicit their effects by imperfectly base pairing between the seed sequence of miRNAs and the complementary region in the 3'-UTR of their target mRNAs (28). To better understand the role of *miR-203*-induced apoptosis of YD-38 cells, we used a *miR-203* target gene array, and the results (Figure 3) showed that *miR-203* altered the expression patterns of several oncogenes. This study focused on the oncogenes that were down-regulated in response to *miR-203* because miRNAs usually suppress protein expression by inhibiting mRNA translation (28). As shown in Figure 3, potential oncogenes, such as *Bmi-1, DEAD (Asp-Glu-Ala-Asp) box polypeptide 6*, and *protocadherin 7* were down-regulated in YD-38 cells transfected with *miR-203*. DDX6 (also termed *RCK/p54*) encodes *DEAD* (*Asp-Glu-Ala-Asp*) *box polypeptide 6*, which belongs to the DEAD-box RNA helicase family and plays a central role in modulating RNA secondary and tertiary structures (29). Previous studies suggest that *DDX6*, as a proto-oncogene, is highly expressed in most malignant cell lines, and its expression is linked to the regulation of cancer cell growth and differentiation (30, 31). However, the biological function of *DDX6* in many cancers in largely unknown. *Protocadherin 7* is a newly identified breast cancer tumorigenesis- and metastasis-related gene that functions through a cell mechanics-cytoskeleton loop mechanism (32). A functional study revealed that suppression of *protocadherin 7* inhibited breast cancer cell growth, migration, and invasion *in vitro*, whereas overexpression of protocadherin 7 in breast cancer cells enhanced bone metastasis *in vivo* (33). Although *protocadherin 7* is a potential target for breast cancer therapy, its oncogenic activities in various cancers remain largely unknown. Although we are still conducting functional studies of both *DDX6* and *protocadherin 7*, in YD-38 cells, the expression of *Bmi-1* was the most significantly down-regulated by *miR-203*.

Bmi-1 encodes polycomb-group transcriptional repressor complex 1, which is known to be a key regulator of epithelial-

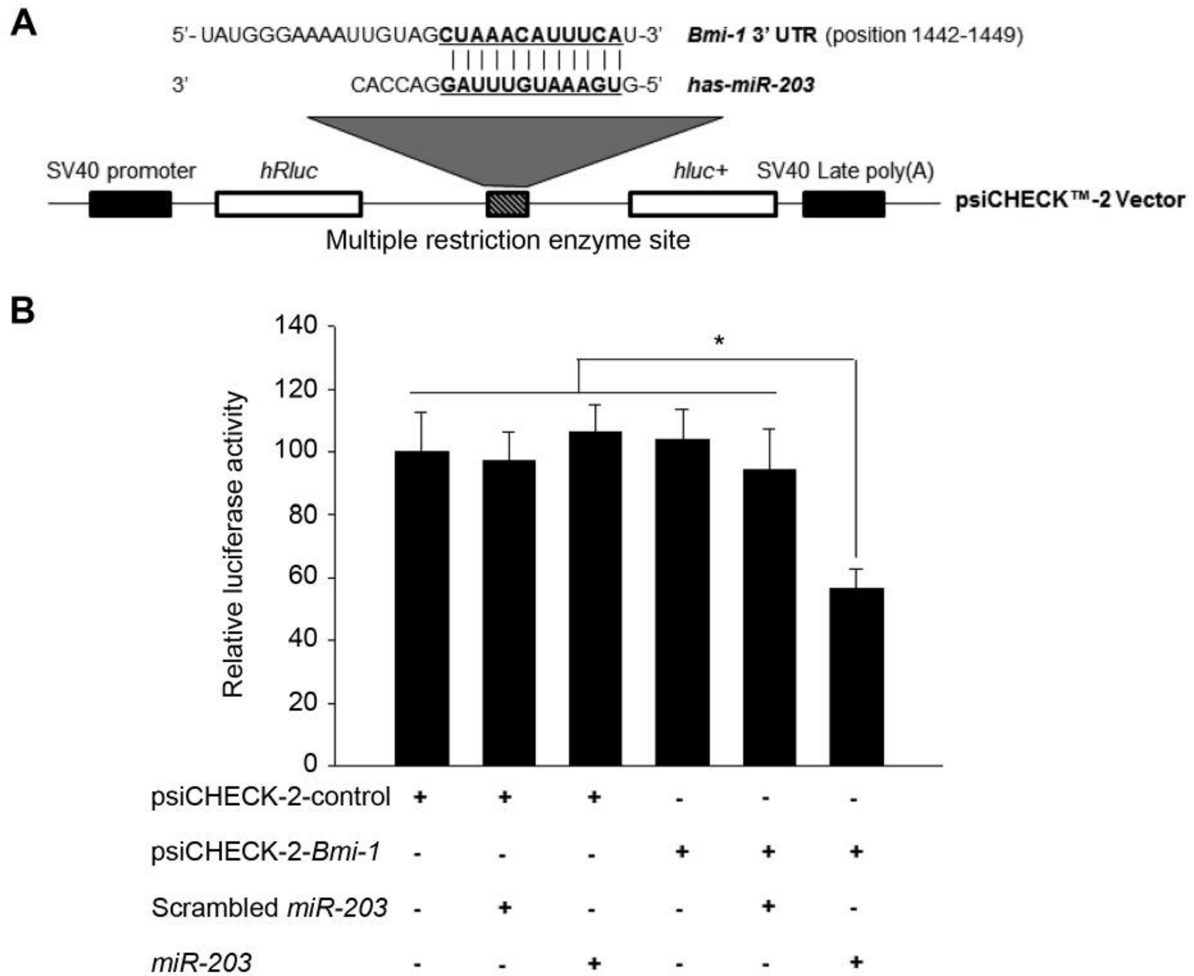

Figure 5. *Bmi-1 is a direct target of miR-203 in YD-38 cells. A: The sequence of the miR-203 target sites in the Bmi-1-3'-UTR. B: The luciferase activity of the Bmi-1 promoter in miR-203-transfected cells. psiCHECK-2-Bmi-1 was cloned as described in the Materials and Methods. Luciferase activity was normalized to total protein. Each data point represents the mean±SEM of at least four independent experiments. *p<0.05 vs. control.*

mesenchymal transition (EMT), cell proliferation, and self-renewal in several human cancers (34). Bmi-1 was first shown to collaborate with c-MYC in tumorigenesis in mice by suppressing the *INK4A* locus, which encodes (both $p16^{Ink4a}$ and $p14^{ARF}$), two proteins that function to suppress cell proliferation and promote apoptosis (14). Several studies have reported that Bmi-1 regulates the *Ink4a/Arf* locus to control cell proliferation and survival and is associated with poor prognosis in pancreatic cancer (35, 36). Bmi-1 can also regulate Twist1, which is an EMT inducer, in cancer stem cells (37) as well as in head and neck cancer cells (38). In breast cancer cells, Bmi-1 activates the WNT pathway by repressing the Dickkopf (DKK) family of WNT inhibitors and increasing c-MYC (39). Recently, several studies have shown evidence for miRNA-mediated regulation of *Bmi-1*. miR-128a increases intracellular reactive oxygen species levels by targeting *Bmi-1*, which inhibits the growth of medulloblastoma cells (40). Both miR-200b and miR-15b regulate chemotherapy-induced EMT by down-regulating Bmi-1 in tongue squamous cell carcinoma (41), and miR-218 inhibits cell proliferation and cell cycle progression and promotes apoptosis by downregulating Bmi-1 in colorectal cancer cells (42). In addition, Saini *et al*. reported that *miR-203* directly down-regulates the expression of survivin, Bmi-1 and ZEB2, which regulate tumorigenesis (9). Based on these results, we hypothesized that the *miR-203*-induced apoptosis in YD-38 cells was related to the suppression of Bmi-1. As depicted in Figure 4, both the mRNA and protein expression of Bmi-1 in YD-38 cells were significantly suppressed by *miR-203*. Although Bmi-1 is an important target of *miR-203*-induced apoptosis in YD-38 human oral cancer cells, the present data also suggest that *miR-203* might regulate other genes.

To determine the *miR-203* binding site in the *Bmi-1* gene, a target gene scan was performed which identified complementary binding sequences located between positions 1,442 and 1,449 in the 3'-UTR (Figure 5A). As shown in Figure 5B, over-expression of *miR-203* decreased *Bmi-1* expression, and *miR-203* directly targeted the 3'-UTR of *Bmi-1*. Together, these data suggest that *miR-203* regulates *Bmi-1* expression by directly targeting the 3'-UTR in YD-38 oral cancer cells.

In conclusion, in the present study, the function of *miR-203* as a tumor suppressor in YD-38 cells was elucidated and showed that it is associated with the suppression of Bmi-1 expression. Further analyses of the mechanism underlying *miR-203*-mediated regulation of cancer progression may contribute to development of new cancer treatments and biomarkers for the diagnosis of cancer.

Conflicts of Interest

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

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