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miR-203 Inhibits the Proliferation and Self-Renewal of Esophageal Cancer Stem-Like Cells by Suppressing Stem Renewal Factor *Bmi-1*

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Cancer stem-like cells exist in many malignancies and several stem cell-related genes and microRNAs, such as *Bmi-1* and miR-203, have been identified as cancer stem-like cell regulators using gene microarray or sequencing analysis. Previously, we used side population (SP) sorting to enrich cancer stem-like cells from esophageal squamous cell carcinoma (ESCC) cell line EC9706. Our results demonstrated that EC9706 SP cells shared common features of cancer stem-like cells. In this study, we examined the expression of Bmi-1 and miR-203 in ESCC SP and non-SP (NSP) cells. Our results showed that, when compared with NSP cells, *Bmi-1* was up-regulated and miR-203 was down-regulated in SP cells. During the differentiation from SP to NSP cells, the expression levels of Bmi-1 were gradually decreased. Overexpression of miR-203 resulted in a significant reduction of endogenous Bmi-1 protein level in EC9706 cells. SP and NSP analyses revealed that the SP cell fraction was markedly decreased in miR-203 overexpressed cells. miR-203 overexpressed cells also showed a significant reduction in colony formation, which was resistant to chemotherapeutic drug treatment and tumorigenicity in nude mice. Rescue experiments demonstrated that ectopic expression of Bmi-1 in miR-203 overexpressed cells increased the SP fraction and restored cell proliferation. Taken together, these results indicated that stem renewal factor *Bmi-1* was a direct target of miR-203. The regulation of *Bmi-1* by miR-203 may play an important role in controlling cell proliferation and self-renewal of esophageal cancer stem-like cells. It may also promote the development of new therapeutic strategies and efficient drugs that target ESCC stem-like cells.

Introduction

Esophageal cancer is one of the most common cancers worldwide. It ranks seventh and sixth in terms of cancer incidence and mortality worldwide, respectively [1]. In China, esophageal squamous cell carcinoma (ESCC) is the most common histological subtype, and it accounts for ~90% of all esophageal cancers diagnosed [2]. Despite recent advances in cancer therapeutics, most ESCC patients die from recurrence or delayed metastasis of the cancer later in their life. Understanding the mechanisms underlying carcinogenesis is essential for the development of novel therapeutic strategies that target esophageal malignancies. One possible cause of subsequent therapeutic failure is likely due to residual malignant cells in tumors with a stem-cell-like potential. These small populations of cells that are able to maintain and drive malignancy proliferation and metastasis in variety of cancers have been defined as "cancer stem cells," "cancer stem-like cells," or "tumor-initiating cells" [3].

Molecular markers of cancer stem-like cells have been found in many malignancies, including stem cell-related genes and microRNAs using gene microarray or sequencing analysis. miRNAs, a class of small non-coding RNAs, are identified as a new kind of gene expression regulators through targeting mRNAs for translational repression or cleavage. For instance, miR-203 was first identified as a keratinocyte-specific miRNA in the skin, which was associated with inflammation. In addition to the skin, miR-203 was also expressed in organs that contained squamous epithelium, esophagus, and cervix [4]. The expression of miR-203 was found to be down-regulated in ESCC tissues when compared with normal esophageal epithelium [5]. It was proposed that miR-203 might be a key molecular repressor of the stemness-related genes, such as the transcription factor *SOX2* and *P63* [6–8]. Together, these results implicated that miR-203 could serve as a critical player in stem cell self-renewal and esophageal carcinogenesis.

The Polycomb-group transcriptional repressor *Bmi-1* has emerged as a key regulator in several cellular processes, including stem cell self-renewal and cancer cell proliferation [9]. Many studies have shown that *Bmi-1* expression is up-regulated in various types of cancers, including lung cancer, ovarian cancer, breast cancer, neuroblastoma, leukemia, and

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esophageal cancer [10–14]. Furthermore, several studies indicated that *Bmi-1* was involved in the self-renewal and differentiation of cancer stem-like cells [14,15].

Previously, we employed a side population (SP) sorting method to enrich cancer stem-like cells from ESCC cell line EC9706 [16]. Our results demonstrated that EC9706 SP cells shared certain common features of cancer stem-like cells, such as the ability of self-renewal, highly proliferative and tumorigenic, and expressing several stem cell-related genes and miRNAs, including *Bmi-1* and miR-203. In this study, we investigated the role of miR-203 and *Bmi-1* in esophageal cancer stem-like cells. Our results indicated that *Bmi-1* was the direct target of miR-203, and miR-203 and *Bmi-1* together played important roles in maintaining the proliferation and self-renewal abilities of esophageal cancer stem-like cells.

Materials and Methods

Cell culture

Human ESCC cell lines EC9706 and KYSE150 were maintained in RPMI 1640 (Gibco) culture medium that was supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C and 5% CO_2. EC9706 was established and studied by Han et al. [17]. KYSE150 was a generous gift from Dr. Y. Shimada at Hyogo College of Medicine [18,19].

Tissue specimens

Thirty-two paired tissue specimens (tumor and adjacent normal mucosa) of primary human ESCC were obtained from patients undergoing surgical resection for esophageal cancer subsequent to them providing informed consent. All of the tissues were obtained at the time of surgery and immediately stored in liquid nitrogen until use. This study was approved by the ethics committees of the Chinese Academy of Medical Sciences, Cancer Institute and Hospital.

Analyzing and sorting of cell lines by fluorescence-activated cell sorting

The procedure for SP analysis is based on procedures that were previously described [20] with modification. Cells were detached from plates with trypsin, washed with phosphate-buffered saline (PBS), resuspended in serum-free medium at 1×10^6 cells/mL, and incubated for 90 min at 37°C with Hoechst 33342 (Sigma) at a final concentration of 5 μg/mL either alone or in the presence of 5 μM fumitremorgin C (FTC; Sigma). After incubation, cells were washed with ice-cold PBS and filtered through a 40-μm cell strainer. Propidium iodide (Sigma) was added at a final concentration of 1 μg/mL for 5 min before analysis. Cells were sorted using dual-wavelength analysis with BD FACSVantage SE (Becton, Dickinson and Company) that was equipped with a Coherent Innova 300C laser. The SP gate in the flow cytometry analysis was defined as the diminished area on the dot plot in the presence of FTC. SP and non-SP cells were collected for further studies.

RNA extraction and quantitative real-time polymerase chain reaction

The relative expression levels of miR-203 were measured by a two-step TaqMan assay according to the manufacturer's instruction. The relative expression levels of *Bmi-1* were carried out according to the standard protocol of use with SYRB Premix Ex Taq™ Perfect Real Time system (Takara). Briefly, for miR-203, RNA extraction was the template for reverse transcriptase–polymerase chain reaction (RT–PCR) of miR-203 or the internal control U6 (Applied Biosystems) using the TaqMan micro-RNA reverse transcription kit (Applied Biosystems). Next, TaqMan real-time PCR was prepared by using TaqMan universal master mix (Applied Biosystems) and specific primers for miR-203 or U6. For *Bmi-1*, RNA was reverse transcribed into cDNA using the SuperScript 3 First-strand cDNA Synthesis Kit (Invitrogen). *GAPDH* was used as an internal control. The primer sequences were as follows: Bmi-1: forward, 5′-TCGTTCTTGTTATTACGCTGTTTT-3′ and reverse, 5′-CGGTAGTACCCGCTTTTAGGC-3′. GAPDH: forward, 5′-GAGTCAACGGATTTGGTCGT-3′ and reverse, 5′-GACAAGCTTCCCGTTCTCAG-3′. Fold changes in miR-203 and *Bmi-1* expression were calculated using the 2-ΔΔCt method [21].

Western blot analysis

Cells were harvested and lysed in lysis buffer, and western blot analysis was performed as previously described in reference. Equivalent amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). After blocking, the membranes were incubated with the appropriate diluted primary antibodies, including Bmi-1(Millipore) and β-actin (Sigma). The signal of the target protein was detected by an enhanced chemiluminescence detection system (Pierce) and recorded on film in the linear detection range.

DNA constructs

To express human miR-203 in cell cultures by transfection, its genomic sequence was amplified from genomic DNA by PCR using the following primers: hsa-mir-203-Age I-F: 5′-GAGGATCCCCGGGTACCGGTATATTTGCATGTCGCTATGTG-3′; has-mir-203-EcoR I-R: 5′-ATAAGCTTGATATCGAATTCAAAAAAGGCACAGTCGAGGCTGATC-3′. The resulting fragment was restricted and ligated to the lentiviral vector pGC-FU (GeneChem Co., Ltd.). We generated pGC-FU-GFP lentiviral vector as a control. To overexpress *Bmi-1*, the Bmi-1 vector (pEGFP-N1-Bmi1-GFP, Cat: GCK970038, RefSeq: NM_005180) with empty vector (pEGFP-N1) were purchased from Genechem Co., Ltd. For construction of human *Bmi-1* 3′UTR reporter plasmid, nucleotides +816 to +1713 of human *Bmi-1* cDNA were amplified and cloned into the pIS0 [22] luciferase plasmid. Mutant construct of *Bmi-1* 3′UTR, named Bmi-1 3′UTRmut, which carried a substitution of seven nucleotides within the core binding site of *Bmi-1* 3′UTR, was carried out using MutanBEST Kit (Takara). The primers for *Bmi-1* 3′UTR were as follows: BmiUTR-SacI 5′-GAGCTCGCAGATACCCATAACCTA-3′ and BmiUTR-XbaI 5′-TCTAGACAACACTTACAATGGGACT-3′. The primers for mutation were: 5′-GTAAAGTTTGTCCCCAGTCTGCAAA-3′ and 5′-TTTAGCTACAATTTTCCCATACAAA-3′.

Transfection and infection

Cell transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Lentiviruses were generated by triple transfection of 80% confluent HEK293T cells with modified pGC-FU plasmid and pHelper 1.0 and pHelper 2.0 helper plasmids (Genechem Co., Ltd.). Lentiviruses were harvested in serum-free medium after 3 days. EC9706 cell line was infected at multiplicity of infection 100, for 12 h at 37°C in Dulbecco's modified Eagle's medium supplemented with 2% FBS.

Luciferase reporter assay

The cells of 90% confluence in 96-well plates were transiently transfected with firefly luciferase reporter gene plasmid (100 ng) using LipfectAMINE 2000 (Invitrogen). Transfection efficiency was standardized by cotransfection with 1.0 ng of pRL-SV40 (Promega). The luciferase activity was determined using the Luciferase Assay system (Promega). For each plasmid construct, there independent transfection experiments were performed in triplicate. The fold increase was calculated by defining the activity of the empty pIS0 plasmid as 1.

Sensitivity to chemotherapeutic reagents

Sensitivity to chemotherapeutic reagents was assessed using the MTT assay as previously described [16]. Briefly, 2×10^3 cells per well were seeded on 96-well plates and cultured overnight. Cisplatin (0.5 or 1 μg/mL) was chosen as a representative chemotherapeutic agent and was added to the medium individually. After 48 h in culture, the absorption values were determined using a Bio-Rad enzyme reader (Bio-Rad).

Soft agar and plate colony formation assay

Suspensions of logarithmically growing cells at a density of 200 cells per well were seeded in six-well plates with complete medium for plate colony formation assay. Soft-agar colony formation was assessed using a modified previously described method [23]. Cells were suspended in 0.3% agar/culture medium and plated at a density of 1,000 cells per well in a six-well plate that had been previously coated with 0.5% agar. Fresh medium was added to each well every 3 days. After 3 weeks of incubation, colonies were stained with 0.2% *p*-iodonitrotetrazolium violet (Amersco) and counted.

Xenograft assay in mice

Nude mice and nonobese diabetic/severe combined immunodeficient mice were kept in microisolator cages according to the guidelines of CAMS&PUMC, and all experiments were approved by the animal care committee of CAMS&PUMC. The freshly prepared (1×10^6/each) cells were injected subcutaneously into the left axillary fossa of female mice (3–4 weeks old) in 200 μL PBS. The mice were monitored twice a week for palpable tumor formation and were sacrificed at 4 weeks after transplantation to determine tumor formation. Tumors were fixed in 10% buffered formalin and paraffin embedded; then, the slides were prepared for immunohistochemical staining.

RNA interference synthesis and transfection

Small-interfering RNA (siRNA) and non-silencing control siRNA were synthesized and annealed by Genechem Co., Ltd. The siRNA sequence used for *Bmi-1* was as follows: 5′-CAGUAGAGGAGCCGUCAAAtt-3′ selected by Wu et al. [24]. Cell transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, siRNA was transfected at a concentration of 100 nM, and the cells were collected for further study 48 h after transfection. Silencing was validated by western blot.

Statistical analysis

Statistical software SPSS12.0 was used in data processing and for analyzing the significance among groups. $P<0.05$ was considered statistically significant.

Results

miR-203 and Bmi-1 are inversely expressed in ECSS cancer stem-like cells

To investigate the roles of miR-203 and *Bmi-1* in esophageal cancer stem-like cells, we evaluated miR-203 RNA expression by real-time RT-PCR in SP and non-SP cells of esophageal cell lines EC9706 and KYSE150. As shown in Fig. 1A, miR-203 expression was up-regulated in non-SP cells. Concurrently, with miR-203 up-regulation, *Bmi-1* mRNA and protein expression levels were down-regulated in non-SP cells (Fig. 1B, C).

The SP fraction accounted for 99.3 ± 0.6% when EC9706 SP cells were isolated by cell sorting and analyzed immediately (Fig. 1D). After culturing for 8 days, the SP fraction decreased to 1.5 ± 0.4%, which was the normal level of the SP fraction of EC9706 cells. This result indicated that the SP cells could differentiate to non-SP cells gradually during the culture. Previously, *Bmi-1* was reported to play an important role in the maintenance of the stemness of stem cells and cancer stem-like cells [14]. Therefore, we explored the expression level of Bmi-1 during the process of SP cell differentiation. We collected cell lysates from SP cells that were cultured for 0, 2, 4, 6, and 8 days and performed immunoblotting analysis. We found that the protein expression level of Bmi-1 was significantly decreased during the differentiation process (Fig. 1E). In contrast, the miR-203 RNA expression was up-regulated during the differentiation process (Fig. 1F). These results suggested that the self-renewal factor *Bmi-1* and miR-203 may play critical roles in esophageal cancer stem-like cell maintenance.

Bmi-1 is a direct target of miR-203

The inverse correlation observed between miR-203 and *Bmi-1* in expression suggested that *Bmi-1* might be a direct target of miR-203. Therefore, we used TargetScan4.0 to predict targets of miR-203 in human mRNA transcripts. We found that the 3′UTR of *Bmi-1* mRNA contained a heptamer (7-mer) that perfectly matched miR-203's 5′ seed sequence. Shown in Fig. 2A is the miR-203 binding site at +1442 to +1449 nucleotides of *Bmi-1* 3′UTR. Comparing the sequence for interspecies homology, we found that the miR-203 target sequence of *Bmi-1* 3′UTR is highly conserved among different species.

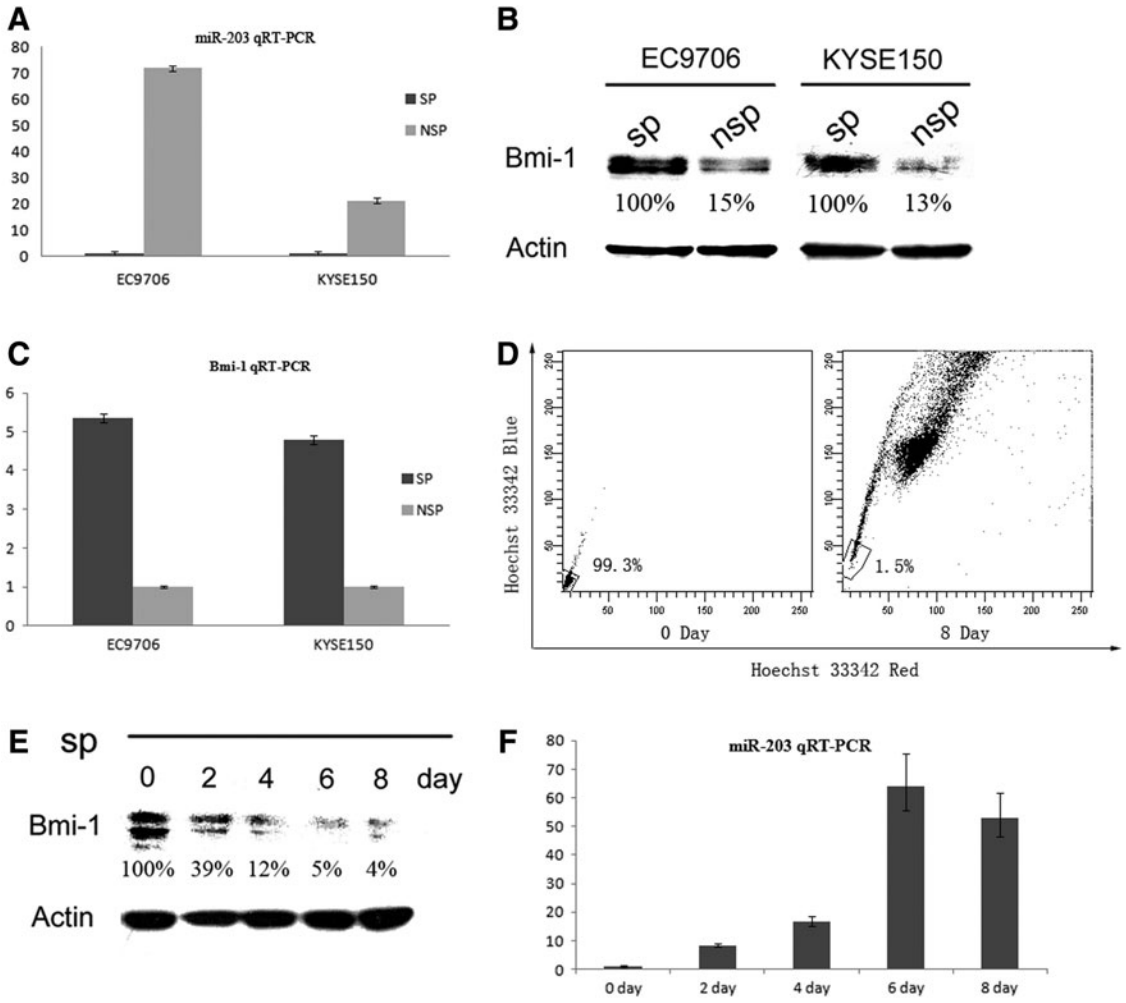

FIG. 1. The expression of miR-203 and *Bmi-1* in esophageal cancer stem-like cells. **(A)** In side population (SP) and non-SP cells of EC9706 and KYSE150 cell lines, miR-203 expression level was measured by real-time PCR, and **(B, C)** Bmi-1 expression level was measured by immunoblotting analysis and real-time PCR. **(D)** SP analysis of sorted SP cells of EC9706 after being cultured for 0 and 8 days. **(E, F)** *Bmi-1* and miR-203 expression level of sorted SP cells of EC9706 after being cultured for 0, 2, 4, 6, and 8 days.

To determine whether *Bmi-1* was a direct target of miR-203, we overexpressed miR-203 in EC9706 cells using lentivirus-mediated transduction. miR-203 gene was amplificated and inserted into the recombinant lentivirus plasmid. Efficiency of lentivirus infection was more than 90% as evidenced by GFP expression 3 days after infection (Fig. 2B), and miR-203 expression was measured by real-time PCR. As shown in Fig. 2C, compared with parental EC9706 and lenti-GPF cells, miR-203 expression was obviously increased in lenti-miR-203 cells. We determined the *Bmi-1* protein level using immunoblotting analysis. We found that *Bmi-1* protein level was significantly down-regulated in lenti-miR-203 cells when compared with controls (Fig. 2D). These results indicated that miR-203 was involved in regulating *Bmi-1* expression.

Next, we constructed pIS0-Bmi-1-3′UTR and pIS0-Bmi-1-3′UTRmut plasmid (Fig. 2E). As shown in Fig. 2F, transfection of lenti-miR-203 cells with Bmi-1-3′UTR plasmid caused about a 30% decrease in the luciferase activity compared with EC9706 and lenti-GFP cells, whereas mutation of the *Bmi-1* 3′UTR binding site weakened miR-203-mediated repression of the luciferase activity under the same conditions. Taken together, these results indicate that miR-203's effect on *Bmi-1* expression was direct and mediated through the 3′UTR at +1442 to +1449 nucleotides binding site of *Bmi-1*.

miR-203 regulates the SP phenotype

Since we found that the expression of miR-203 was down-regulated in SP cells, the results suggested that miR-203 might be involved in regulating esophageal cancer stem-like cells. To this end, we stained EC9706, lenti-GFP, or lenti-miR-203 cells with Hoechst33342 and performed SP analysis. The results showed that the percentage of cells in the SP fraction in lenti-miR-203 cells was 0.2 ± 0.1%, which was significantly lower than that in parental EC9706 cells (1.6 ± 0.4%) or in lenti-GFP cells (2.4 ± 0.6%) (Fig. 3A). These results indicated that overexpression of miR-203 could effectively regulate the SP phenotype of EC9706 cells.

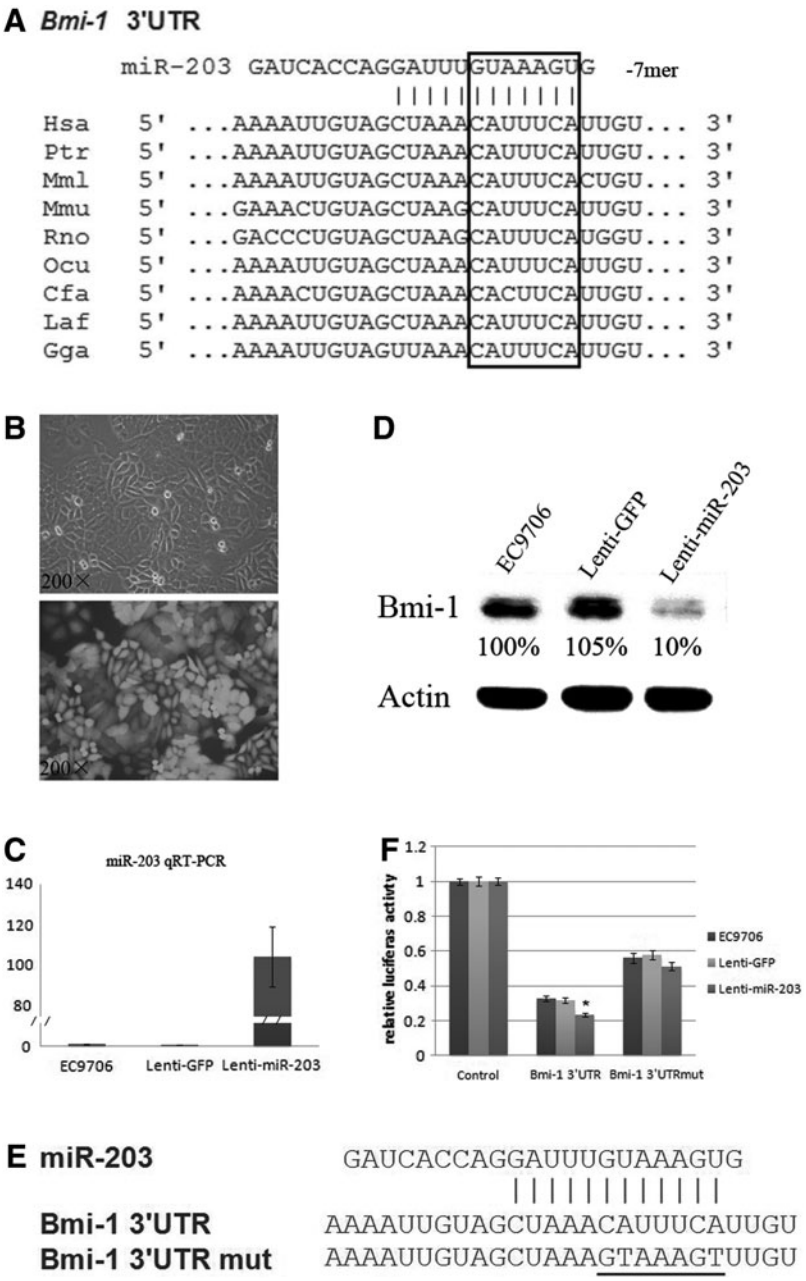

FIG. 2. *Bmi-1* is a direct target of miR-203. **(A)** An miR-203 target site resides at +1442 to +1449 of the *Bmi-1* 3′UTR and is highly conserved in different species. *Upper panel*, sequence of the miR-203 binding site within the *Bmi-1* 3′UTR of nine species. *Lower panel*, sequence alignment of miR-203 with binding sites on the *Bmi-1* 3′UTR. **(B)** Representative graph of EC9706 cells infected with indicated lentivirus (×200). **(C)** After infection of cells with indicated lentivirus for 5 days, miR-203 expression level was measured by real-time PCR and **(D)**, Bmi-1 expression level was measured by immuoblotting analysis compared with EC9706 and lenti-GFP cells. **(E)** Diagram of the luciferase reporter plasmids: plasmid with *Bmi-1* 3′UTR insert (pIS0-Bmi-1-3′UTR) and plasmid with a mutant *Bmi-1* 3′UTR (pIS0-Bmi-1-3′UTRmut) that carried a substitution of seven nucleotides within the miR-203 binding site. **(F)** Luciferase activity assay demonstrates a direct targeting of the 3′UTR of *Bmi-1* by miR-203. EC9706, lenti-GFP, and lenti-miR-203 cells were transfected with plasmids pIS0-Bmi-1-3′UTR and pIS0-Bmi-1-3′UTRmut. pRL-SV40 was used for normalization of transfection efficiency. After 48 h, the luciferase activities were measured. Mutation of the *Bmi-1* 3′UTR binding site weakened miR-203-mediated repression of the luciferase activity under the same conditions. Hsa, *Homo sapiens*; Ptr, *Pan troglodytes*; Mml, *Macaca mulatta*; Mmu, *Mus musculus*; Rno, *Rattus norvegicus*; Ocu, *Oryctolagus cuniculus*; Cfa, *Canis lupus familiaris*; Laf, *Loxodonta africana*; Gga, *Gallus gallus*.

miR-203 affects the resistant ability to chemotherapeutic reagents

To explore the function of miR-203, we performed drug sensitivity assays with EC9706, lenti-GFP, and lenti-miR-203 cells. EC9706, lenti-GFP, or lenti-miR-203 cells were treated with cisplatin, a commonly used chemotherapeutic drug for ESCC, by MTT assay. As shown in Fig. 3B, after exposure to 0.5 and 1 μg/mL cisplatin, the viability of EC9706 or lenti-GFP cells was markedly higher than that of lenti-miR-203 cells ($P < 0.05$). Since EC9706, lenti-GFP, and lenti-miR-203 cells did not differ in their growth rate (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd), lenti-miR-203 cells were less resistant to cisplatin and this suggested that overexpression of miR-203 in ESCC cells affected their chemotherapeutic drug resistance. Given the fact that we previously showed that cancer stem-like cells of EC9076 were more resistant to anticancer drugs [16], our results indicated that miR-203 could affect the resistant ability of esophageal cancer stem-like cells.

miR-203 inhibits the colony formation and tumorigenicity ability of ESCC cells

To specifically determine whether miR-203 represses proliferative potential of EC9706 cells, we tested the tumorigenic ability of EC9706, lenti-GFP, or lenti-miR-203 cells both in vitro and in vivo. We observed that colonies formed by lenti-miR-203 cells were smaller in size and less in number than EC9706 or lenti-GFP cells in both the plate colony

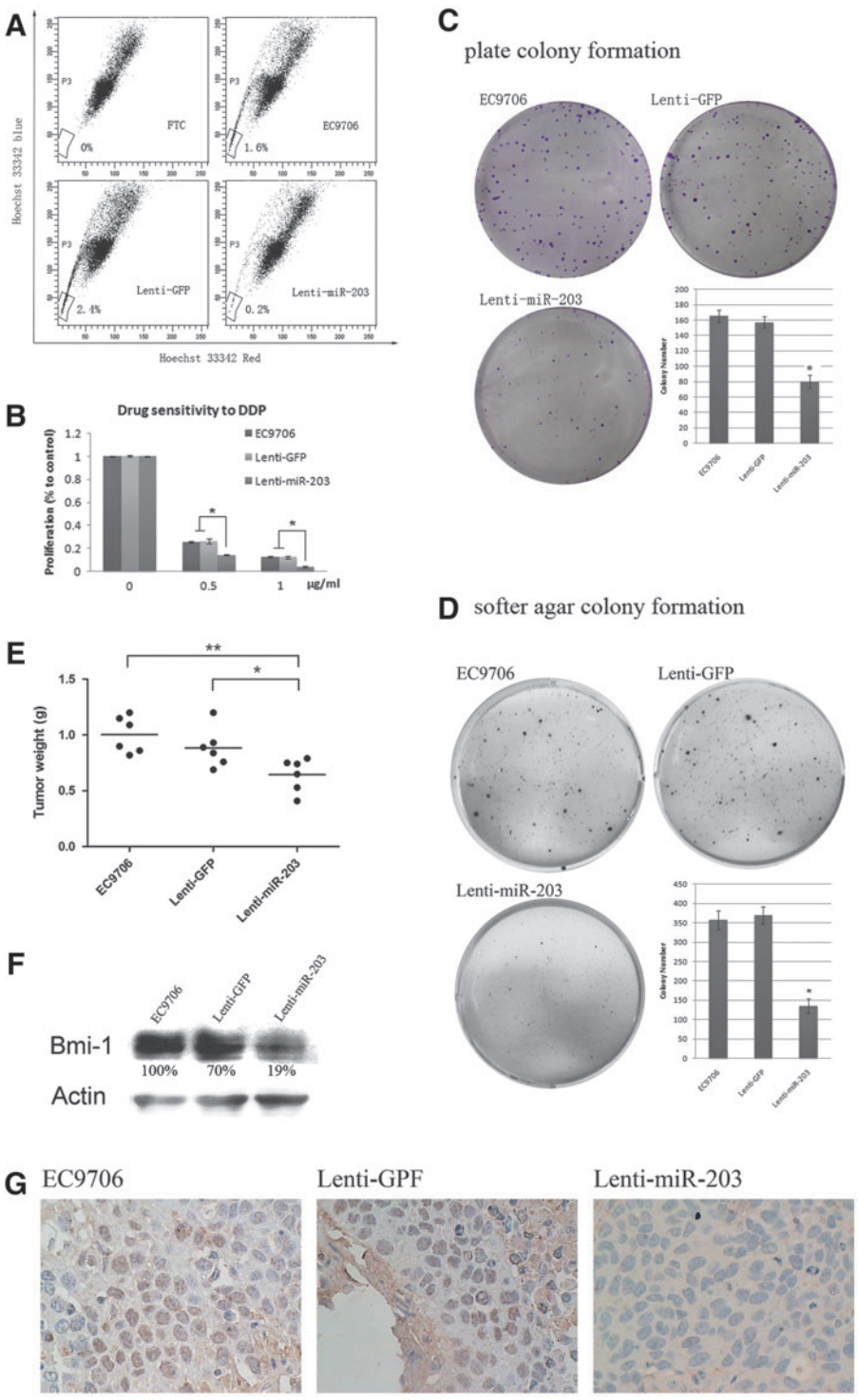

FIG. 3. The effect of miR-203 overexpression on esophageal cancer stem-like cells. **(A)** SP analysis of EC9706, lenti-GFP, and lenti-miR-203 cells. **(B)** Sensitivity of EC0796, lenti-GFP, and lenti-miR-203 cells to cisplatin (DDP). Lenti-miR-203 cells were less resistant to the chemotherapeutic reagents on 48 h of DDP treatment, $*P < 0.05$. **(C, D)** Representative images from plate and softer agar colony formation assay, $*P < 0.05$. **(E)** Tumor weights of lenti-miR-203 cells and control EC9706 and lenti-GFP cells were plotted. The tumorigenicity of lenti-miR-203 cells was significantly lower than that of control cells, $*P < 0.05$, $**P < 0.01$. **(F, G)** Immunoblotting and immunohistochemical staining assays of Bmi-1 from tumor xenografts of EC9706, lenti-GFP, and lenti-miR-203.

formation assay and the softer agar colony formation assay (Fig. 3C, D). Furthermore, in vivo tumorigenic ability was evaluated by injecting EC9706, lenti-GFP, or lenti-miR-203 cells into nude mice, respectively. Four weeks after injection, mice were killed and tumor xenografts were collected. As shown in Fig. 3E, weight measurement showed that tumor xenografts from lenti-GFP or EC9706 cells were much heavier than those from lenti-miR-203 cells. These results indicated that overexpression of miR-203 in ESCC cells inhibited the proliferation both in vitro and in vivo. In addition, we determined the Bmi-1 expression level of the tumors by immunoblotting and immunohistochemistry analyses. Consistently, the result showed that the Bmi-1 expression level was down-regulated in lenti-miR-203 cells in both assays, compared with control cells (Fig. 3F, G).These results suggested that miR-203-Bmi-1 regulation may play a critical role in controlling cell proliferation in esophageal cancer.

miR-203 regulates the esophageal cancer stem-like cells by suppressing Bmi-1

To investigate the critical role of miR-203-Bmi-1 regulation in esophageal cancer stem-like cells in detail, we transfected the pEGFP-N1-Bmi1 plasmid into the lenti-miR-203 cells to rescue the Bmi-1 expression. As shown in Fig. 4A and B, immunoblotting analysis showed that, when compared with control cells without miR-203 overexpression, ecotopic expression of Bmi-1 in lenti-miR-203 cells was successfully restored after Bmi-1 protein expression. SP analysis indicated that the SP fraction was increased after Bmi-1 overexpression in lenti-miR-203 cells (Fig. 4C, $0.4 \pm 0.1\%$ in lenti-miR-203 cells, $0.5 \pm 0.2\%$ in lenti-miR-203 plus control vector, and $4.6 \pm 0.7\%$ in lenti-miR-203 plus Bmi-1 vector). Consistently, Bmi-1-rescued cells restored their proliferative ability in plate colony formation assay (Fig. 4D, E). To further determine the effect of *Bmi-1*, RNA interference was used for silencing endogenous Bmi-1 expression in EC9706 and lenti-GFP (Fig. 4A, B). We found that a loss of Bmi-1 function leads to decreased cell proliferative ability in plate colony formation assay (Fig. 4D, E). These results indicated that *Bmi-1* functioned as a stem renewal factor to be a key regulator factor in the maintenance of cancer stem-like cells in EC9706, and miR-203 regulated the esophageal cancer stem-like cells by suppressing *Bmi-1*.

miR-203 and Bmi-1 are inversely expressed in human ESCC

To determine whether miR-203 and *Bmi-1* were also inversely expressed in human ESCC samples, we measured RNA expression levels of miR-203 and *Bmi-1* in 32 ESCC tumor specimens and their paired normal adjacent tissues by real-time RT-PCR. As shown in Fig. 5A, significant elevations of *Bmi-1* mRNA levels were detected in 69% (22 of 32) of the tumor samples when compared with their normal adjacent tissues ($P < 0.01$). Meanwhile, as shown in Fig. 5B, RNA expression levels of miR-203 exhibited the opposite pattern of *Bmi-1* in this identical cohort of clinical samples. RNA expression levels of miR-203 were significantly downregulated in tumor tissues when compared with their normal adjacent tissues (75%, 24 of 32, $P < 0.05$). These results were consistent with the results obtained from the ESCC cell lines (Fig. 1A, C).

Discussion

To date, cancer stem-like cells have been identified in various malignancies such as breast cancer, pancreatic cancer, brain cancer, and colon cancer. However, only a few studies have focused on ESCCs. In the absence of known surface antigens of ESCC cancer stem-like cells, we

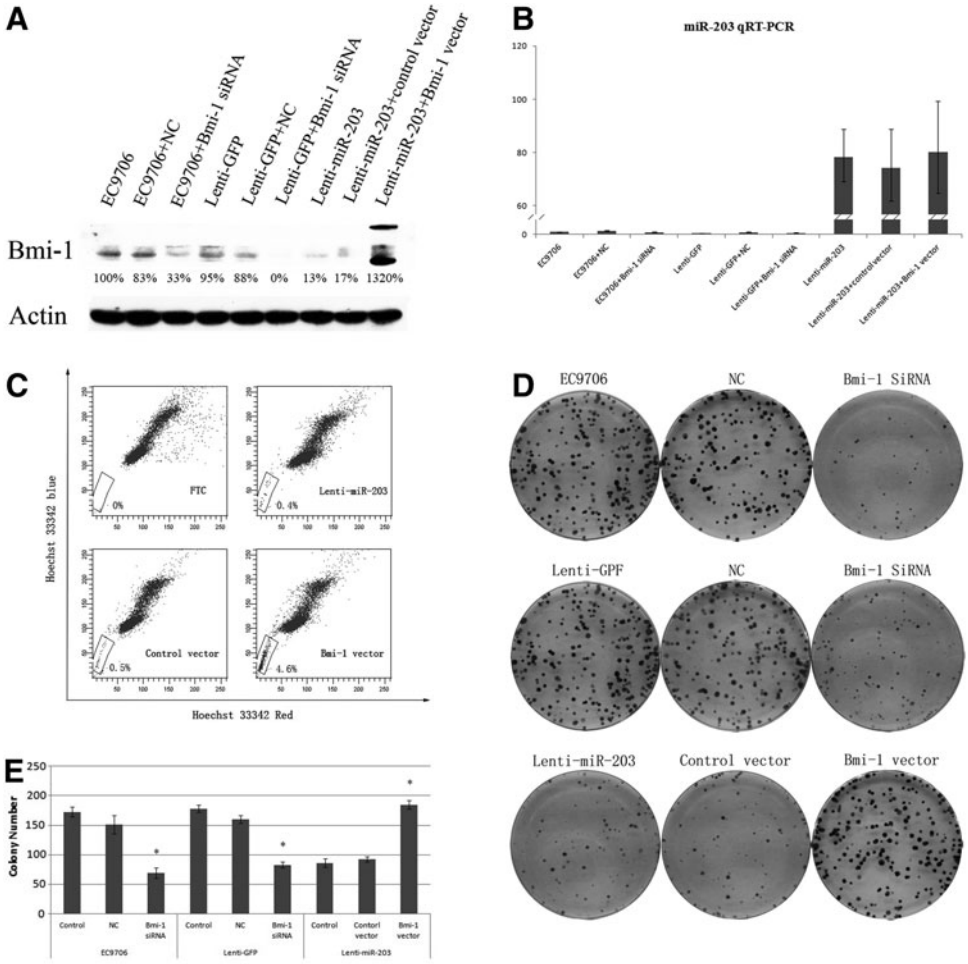

FIG. 4. The effect of rescued Bmi-1 expression on esophageal caner stem-like cells. **(A, B)** The expression of Bmi-1 and miR-203 after transfection or RNA interference. Forty-eight hours after Bmi-1 vector or small-interfering RNA (siRNA) transfection, immunoblotting and real-time PCR analyses was performed. **(C)** SP analysis showed that rescued Bmi-1 expression increased SP fraction in lenti-miR-203 cells. **(D, E)** Representative images from plate colony formation assay, $^*P < 0.05$.

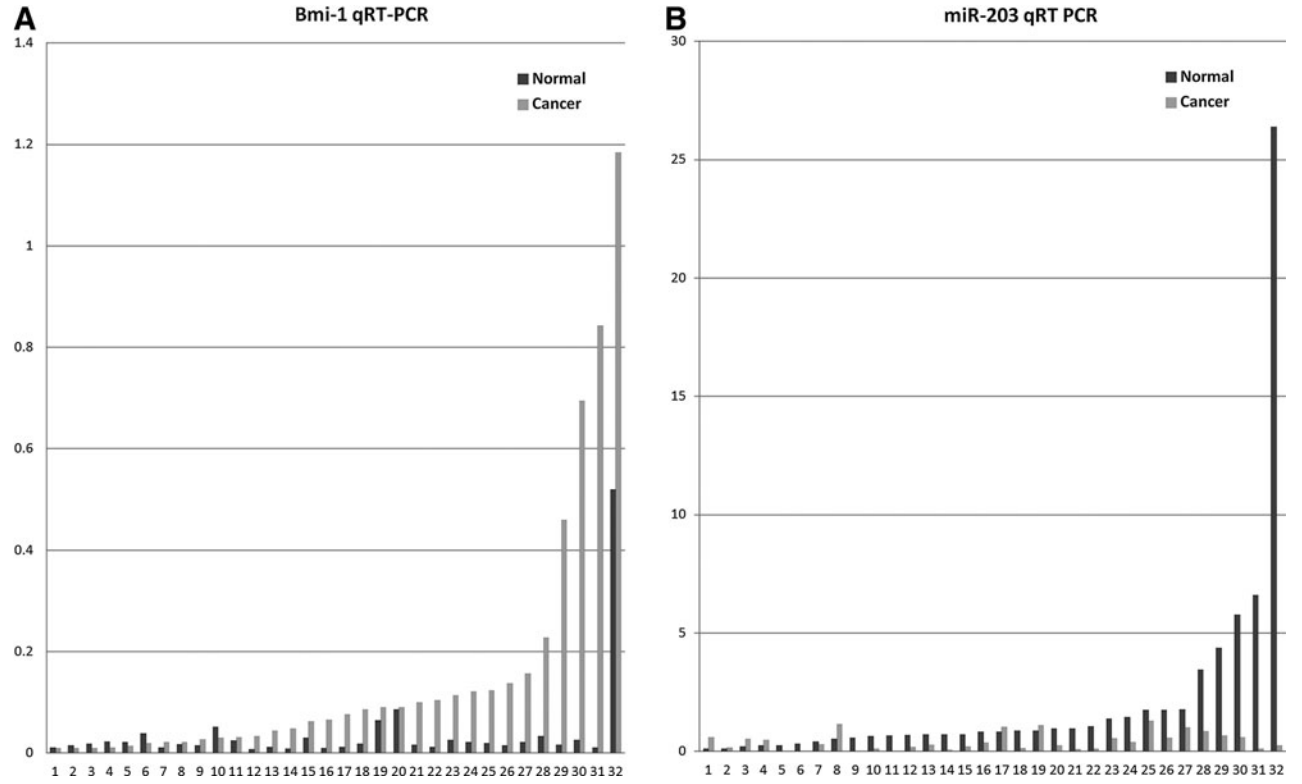

FIG. 5. The RNA expression of *Bmi-1* and miR-203 in human esophageal squamous cell carcinoma (ESCC). **(A, B)** *Bmi-1* and miR-203 expression in 32 paired tissue specimens (cancer and adjacent normal mucosa) of primary human ESCC was measured by real-time PCR. *GAPDH* and U6 were used as internal controls. Student's *t*-test was used to examine the differences of *Bmi-1* and miR-203 levels between cancer and normal tissues.

effectively isolated esophageal cancer stem-like cells using SP sorting [16]. We previously performed a miRNAs microarray comparison between the isolated SP and non-SP (NSP) cell population from the EC9706 cell line. A series of differentially expressed miRNAs were obtained, including miR-203 (data not show). We also analyzed the mRNA expression of stem cell self-renewal factor *Bmi-1* between SP and NSP, and found that *Bmi-1* was highly expressed in SP cells [16]. In this study, we demonstrated that the miR-203 expression was down-regulated in SP cells by real-time PCR, while the Bmi-1 protein level was up-regulated by immunoblotting, which is consistent with the results we obtained from the microarray analysis. In addition, we found that Bmi-1 expression was significantly decreased during the differentiation process from SP to NSP cells. These results indicated that miR-203 and *Bmi-1* played important roles in esophageal cancer stem-like cells.

miR-203, which is down-regulated in many types of cancers, regulates multiple cellular processes that are associated with proliferation, self-renewal, differentiation, and apoptosis [7,25,26]. To explore the role of miR-203 in esophageal cancer stem-like cells, first, we established lenti-miR-203 cells, which were EC9706 cells with overexpression of miR-203. Our results showed that SP cell fractions markedly decreased in lenti-miR-203 cells. Due to the decrease of cancer stem-like cells, lenti-miR-203 cells showed significant reductions in the colony formation, which was resistant to chemotherapeutic drug treatment and tumorigenicity in nude mice.

Since miR-203 and *Bmi-1* were inversely expressed in SP cells, we explored the molecular mechanism underlying the function and found that *Bmi-1* was likely to be a direct target of miR-203. Next, to explore the role of *Bmi-1* in regulation, proliferation, and self-renewal, we first rescued Bmi-1 expression in lenti-miR-203 cells. We observed that the SP fraction increased after the rescue experiment. Bmi-1 overexpression abolished the reduction of cancer stem-like cells by miR-203. Due to the increasing number of cancer stem-like cells, the plate colony formation ability of Bmi-1 overexpression in cells was much higher than in control vector and lenti-miR-203 cells. Consistently, we found that a loss of Bmi-1 function leads to a decrease in the proliferative ability in plate colony formation assay.

Besides the ability to maintain self-renewal, *Bmi-1* is important for cell cycle and apoptosis [27]. The down-regulation of *Bmi-1* can induce cell apoptosis, which is likely to be mediated by repressing the PI3K/Akt signaling pathway [28–30]. PI3K/Akt signaling is associated with chemoresistance in many malignances [31,32] and regulates the SP cell phenotype via ABCG2 translocation [33]. Previously, we demonstrated that PI3K/Akt signaling activity was vital for the maintenance of cells in the SP fraction and required for high tumorigenicity of ESCC cells [34]. In this study, we observed that rescued Bmi-1 expression in lenti-miR-203 cells led to a significant increase in the levels of phospho-Akt, whereas knockdown Bmi-1 expression in EC9706 cells caused down-regulated phospho-Akt and Bcl-2 expression (Supplementary Fig. S2A, B). These results indicated that

Bmi-1 could regulate the activity of PI3K/Akt pathway, thereby affecting the cancer stem-like cells in esophageal cancer.

We examined the miR-203 and *Bmi-1* expression level in 32 primary human ESCC tissues and their paired normal adjacent tissues. We found that 69% of the cancer tissues had a *Bmi-1* level higher than normal tissues, which was in line with the discoveries in melanoma, ovarian cancer, lung cancer, and breast cancer [11,12]. Meanwhile, the expression of miR-203 was significantly down-regulated in tumor tissues (75%). These results were consistent with the previous studies that miR-203 was down-regulated in melanoma, endometrial carcinoma, cervical cancer, and breast cancer [35–38]. Although we could not analyze the association of RNA expression levels of miR-203 and *Bmi-1* with the ESCC clinicopathological features due to the limited tumor samples examined, previous studies showed that *Bmi-1* mRNA expression could be a valuable marker for ESCC progression [39,40] and down-regulation of miR-203 RNA expression could be a potential marker for ESCC prognosis [41,42].

In summary, we demonstrated that stem renewal factor *Bmi-1* was a direct target of miR-203. Regulation of *Bmi-1* by miR-203 played an important role in controlling the cell proliferation and self-renewal of esophageal cancer stem-like cells. Thus, our results provide compelling evidence that miR-203 and *Bmi-1* could be a potential target of the eliminating cancer stem-like cells in ESCC and might promote the development of new therapeutic strategies and efficient drugs which target ESCC stem-like cells.

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Author Disclosure Statement

The authors declare no financial or other conflicts of interest.

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