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Original Article

miR-181a and miR-203 inhibit migration and invasion of laryngeal carcinoma cells by interacting with ATF2

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Abstract: MicroRNAs (miRNAs) have been recognized to modulate the progression of tumorigenesis by serving as oncogenes or tumor suppressors. Despite the involvement of miR-181a and miR-203 in several cancers as has been substantiated, their roles in laryngeal carcinoma (LC) remain unclear. In this study, the abundances of miR-181a, miR-203 and activating transcription factor 2 (ATF2) mRNA in LC cell lines were detected by RT-qPCR. Western blot was performed to detect the protein levels of N-cadherin, E-cadherin and ATF2. Cell migration and invasion ability were assessed by Trans-well assay. The putative binding sites between miR-181a or miR-203 and ATF2 were predicted using Bioinformatics software and further validated by Dual-Luciferase reporter and RNA immunoprecipitation (RIP) assays. Results showed reduced abundances of miR-181a and miR-203 in LC cell lines. Introduction of miR-181a or miR-203 reduced cell migration and invasion, which was further confirmed by the reduction of N-cadherin and increase of E-cadherin in LC cells. ATF2 was identified to be a potential target of miR-181a and miR-203. Absence of ATF2 overturned the stimulatory effects of anti-miR-181a and anti-miR-203 on cell migration and invasion in LC cells. Our findings suggested that miR-181a and miR-203 attenuated cell migration and invasion ability by directly targeting ATF2 in LC, providing novel insight into the regulatory mechanisms of miR-181a and miR-203 in LC.

Keywords: Laryngeal carcinoma, miR-181a, miR-203, activating transcription factor 2, migration, invasion

Introduction

Laryngeal carcinoma (LC) is a prime leading cause of head and neck carcinoma worldwide with a fast-growing morbidity and mortality [1, 2]. In 2018, about 13150 new cases were diagnosed in the United States, leading to an estimated 3710 deaths from LC [3]. Although great advances in surgery and radiotherapy have been achieved over the past years, the prognosis for patients with advanced LC remains dispiriting [4]. Therefore, clarification of the detail molecular mechanisms underlying LC progression will help to develop novel targets for LC diagnosis and therapy.

MicroRNAs (miRNAs) are a class of conservative, endogenous, non-protein coding RNAs ranging from 21-23 nucleotides in length [5]. Reliable evidence has confirmed the central regulatory roles of miRNAs in gene expression by base-pairing with the 3'-UTR of downstream target mRNAs [6]. Functionally, miRNAs are implicated in a variety of cellular processes, covering cell differentiation, proliferation, apoptosis, invasion, and migration [7]. Abnormal expression of miRNAs is associated with tumorigenesis and has the potential to serve as a prospective diagnostic and therapeutic strategy for multiple cancers [8], including LC [9]. miR-181a is dysregulated in many types of solid tumors and acts as an oncogene or tumor suppressor. It is worth noting that miR-181a was significantly downregulated in NSCLC [10], oral squamous cell carcinoma (OSCC) [11], while upregulated in gastric cancer [12], breast cancer [13], and colorectal cancer [14]. However, little is known about the biological roles of miR-181a in LC. miR-203, known as a tumor suppressor, is lowered expression in various cancers, like prostate cancer [15], bladder cancer [16], and lung cancer [17]. Recent researches have demonstrated the involvement of miR-

203 in the progression of malignancies. For instances, lowered expression of miR-203 was responsible for cell proliferation, invasion, and migration in renal cancer [18]. Likewise, the function of miR-203 in pancreatic cancer was fully consistent with that in renal cancer [19]. In LC, miR-203 was obviously downregulated and re-expression of miR-203 inhibited tumorigenesis by regulation of cell proliferation and apoptosis [20]. However, the effects of miR-203 in LC cell invasion and migration are uncertain.

Here, we investigated the roles of miR-181a and miR-203 in the development of LC. According to our results, notable losses of miR-181a and miR-203 were observed in LC cell lines. Moreover, activating transcription factor 2 (ATF2) was directly targeted by miR-181a and miR-203. Exogenous restoration of miR-181a or miR-203 reduced cell invasion and migration by suppressing ATF2.

Materials and methods

Cell culture

Normal Human Oral Keratinocyte NHOK cells were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Human LC cell lines Hep-2, AMC-HN-8 and TU-177 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and TU-212 cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All LC cells were grown in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) during this study. NHOK cells were grown in indicated keratinocyte-serum free medium (KSFM, Thermo Fisher, Waltham, MA, USA) as a control.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

For the analyses of miR-181a, miR-203, and ATF2 mRNA expressions, total RNA in LC cells was extracted using Trizol reagent (Thermo Fisher) according to the manufacturer's protocols, followed by the detection of RNA purity using a Spectrophotometer (NanoDrop, Wilmington, DE, USA). Reverse transcription of miRNA or mRNA was performed using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher) or M-MLV Reverse Transcriptase (Thermo Fisher). Then, the relative expression of miRNA or mRNA was determined by TaqMan Advanced miRNA Assay reagents (Thermo Fisher) or SYBR™ Select Master Mix (Thermo Fisher), respectively. Results were calculated by $2^{-\Delta\Delta Ct}$ method with U6 snRNA and GAPDH as housekeeping genes to normalize miRNA or mRNA. All primers for miRNA and mRNA were obtained from GenePharma Co., Ltd (Shanghai, China).

Cell transfection

miR-181a mimics (miR-181a), miR-203 mimics (miR-203) and their matched control (miR-NC), miR-181a inhibitor (anti-miR-181a), miR-203 inhibitor (anti-miR-203) and negative control (anti-miR-NC), si-RNA targeting ATF2 (si-ATF2) and scrambled control (si-NC) were purchased from GenePharma Co., Ltd. Hep-2 cells were seeded into 6-well plates, followed by the transfection of above oligonucleotides using Lipofectamine 2000 reagent (Thermo Fisher) referring to the manufacturer's instructions.

Trans-well assay

For migration assay, transfected cells resuspended in serum-free RPMI-1640 medium were seeded into the upper chamber (Corning, New York, USA), and complete medium containing 10% FBS was added into the bottom chamber. After incubation for 24 h, cells remaining on the upper membrane were carefully removed, and those migrating to the basal side of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet (Sigma, St. Louis, MO, USA) for 30 min. Finally, migrated cells in random three visual fields were photographed and counted under a microscope (Olympus, Tokyo, Japan). Besides, the chambers coated with matrigel (BD, San Jose, CA, USA) were employed for invasion assay as mentioned above.

Western blot assay

Total proteins were harvested from transfected cells using RIPA lysis buffer (Thermo Fisher) in the presence of phosphatase inhibitor cocktail (Thermo Fisher), and then quantified by BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Equal

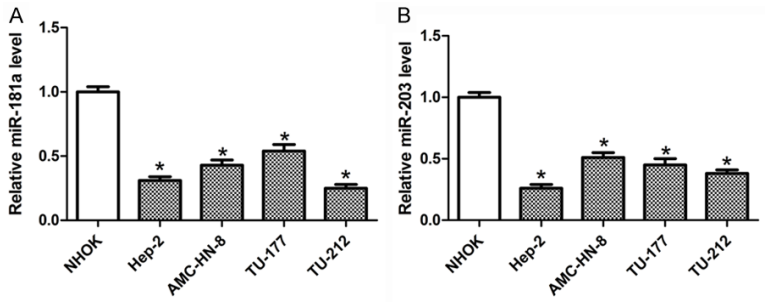

Figure 1. miR-181a and miR-203 were downregulated in LC cell lines. The expression of miR-181a (A) and miR-203 (B) in NHOK and four LC cells (Hep-2, AMC-HN-8, TU-177, and TU-212) was measured by RT-qPCR.

amounts of protein were divided by SDS-PAGE gel, and electro-transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocked with 5% non-fat milk, the membranes were hatched overnight at 4°C with primary antibodies against N-cadherin, E-cadherin, ATF2, and β-actin (Abcam, Cambridge, UK). Next, HRP-conjugated secondary antibodies (Abcam) were further added for another 1.5 h incubation. Protein bands were visualized by Pierce™ ECL Western Blotting Substrate (Thermo Fisher) and quantified using Bio-Rad Image Lab software (Bio-Rad, Hercules, CA, USA).

Luciferase reporter assay

Partial sequences of ATF2 3'-untranslated regions (3'-UTR) containing the putative binding sites of miR-181a were cloned into psiCHECK-2 luciferase vector (Promega, Madison, WI, USA) to generate the wild-type ATF2 construct (ATF2-WT). After that, mutant ATF2 construct (ATF2-MUT) containing mutant miR-181a binding sites was generated by using KOD-plus-mutagenesis kit (Toyobo, Osaka, Japan). Wild-type (ATF2-wt) or mutant ATF2 reporter (ATF2-mut) containing the binding sites of wild-type or mutant miR-203 were cloned parallelly as above. 293T cells (2×10^5) were seeded into 96-well plates and co-transfected with luciferase reporter and miR-181a, miR-203 or corresponding controls using Lipofectamine 2000. At 48 h post-transfection, cells were harvested for luciferase activity assay using Dual-Luciferase Reporter Assay System (Promega).

RNA immunoprecipitation (RIP)

Ago2 immunoprecipitation was employed using Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore) to probe the correlation between miRNA and mRNA. In brief, Hep-2 cells transfected with miR-181a, miR-203, or miR-NC were lysed using RIP Lysis Buffer. Afterwards, cell lysate was added to protein A/G magnetic beads which were incubated with anti-Argonaute2 (Ago2, Millipore) or anti-IgG antibody (Millipore) overnight at 4°C. After washing, the protein in immunoprecipitated complex was removed with proteinase K buffer and total RNA was extracted for the detection of ATF2 by RT-qPCR assay.

Statistical analysis

All quantitative data are shown as mean ± standard deviation (SD) with three independent experiments. Statistical analyses were carried out using SPSS 20.0 (SPSS Inc., Chicago, IL, USA) with Student's t-test and one-way ANOVA to estimate significant differences between groups. $P < 0.05$ was considered significant.

Results

miR-181a and miR-203 are impaired in LC cells

To clarify the roles of miR-181a and miR-203 in LC, RT-qPCR assay was initially performed to detect the two miRNAs' expression. As presented in **Figure 1A**, a strong reduction of miR-181a was observed in four LC cell lines (Hep-2, AMC-HN-8, TU-177, TU-212) compared to NHOK cells. Likewise, the expression of miR-203 was also lessened in LC cells, particularly in Hep-2 cells with severest loss (**Figure 1B**). Thus, Hep-2 cells were selected for the following functional studies.

miR-181a and miR-203 inhibited the migration and invasion of LC cells

In the present study, we observed enforced abundances of miR-181a and miR-203 in Hep-2 cells after transfected with miR-181a or miR-203 mimics (**Figure 2A**), indicating that miR-181a or miR-203 mimics could be used for the following gain-of-function experiments. Given that migration and invasion were required for

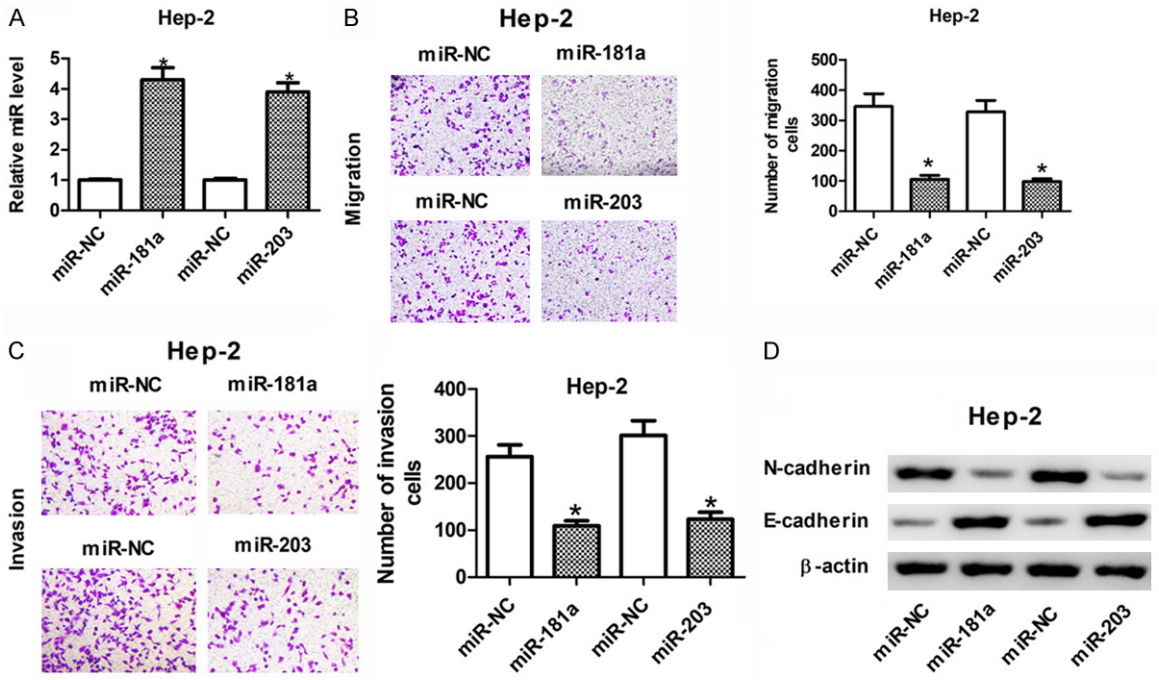

Figure 2. Upregulation of miR-181a or miR-203 inhibited cell migration and invasion in Hep-2 cells. Hep-2 cells were transfected with miR-181, miR-203, or relative control. About 48 h after transfection, the expression of miR-181a and miR-203 were determined by RT-qPCR (A). Cell migration (B) and invasion ability (C) were assessed by Trans-well assay. The protein levels of N- and E-cadherin were measured by western blot (D).

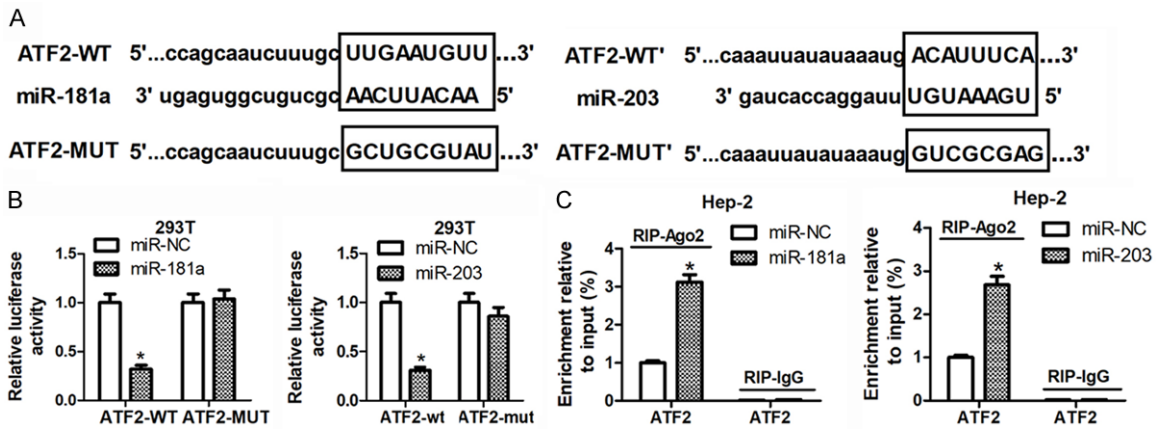

Figure 3. ATF2 was identified to be a direct target of miR-181a and miR-203. A. The putative binding sites of miR-181a or miR-203 within the 3'-UTR fragments of ATF2. B. 293T cells were co-transfected with miR-181a, miR-203, or matched controls and luciferase reporter carrying the wild-type or mutated 3'-UTR of ATF2, followed by the detection of luciferase activity by Dual-Luciferase reporter assay. C. Hep-2 cells were transfected with miR-181a, miR-203, or respective controls, then RIP analysis was used to evaluate the enrichment degree of ATF2 in IgG or Ago2 immunoprecipitation complex.

tumor progression, we addressed the roles of miR-181a and miR-203 in LC. As shown in **Figure 2B**, cell migration ability was markedly suppressed in Hep-2 cells after the transfection of miR-181a or miR-203. Similarly, overexpression of miR-181a or miR-203 also attenuated cell invasion ability (**Figure 2C**). E- and N-cadherin, known as calcium-dependent cell adhesion molecules, are major landmarks of tumor metastasis [21]. Alterations of E- and N-cadherin are closely involved in the transfer of epithelial tumors from a non-invasive to inva-

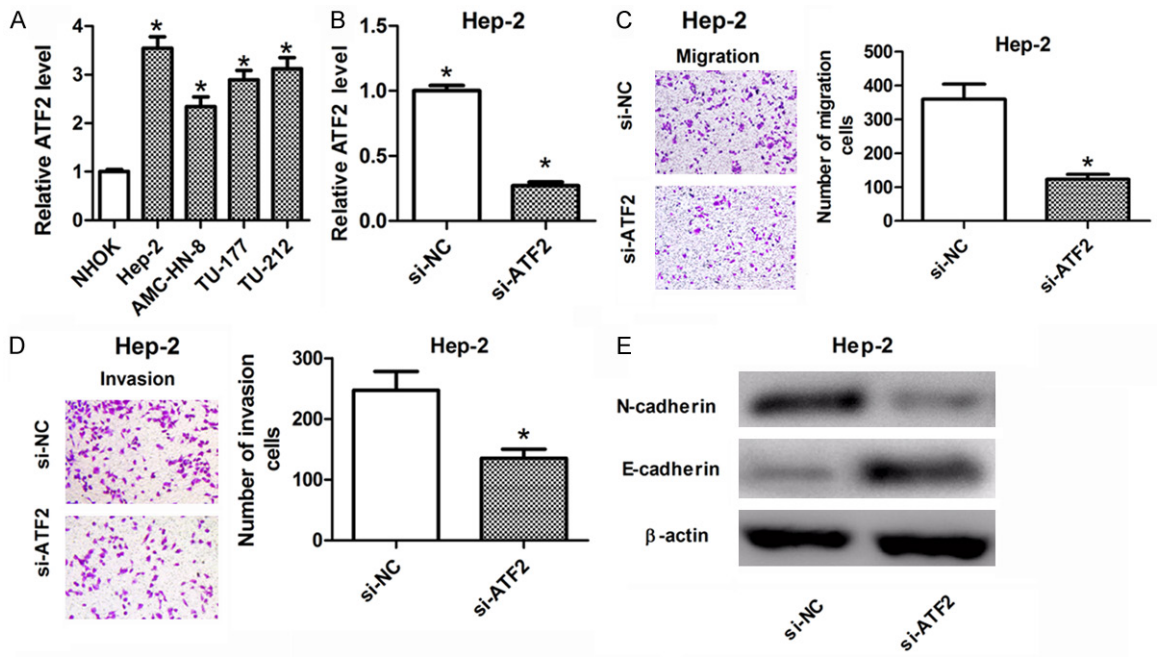

Figure 4. miR-181a or miR-203 silencing inhibited cell migration and invasion in Hep-2 cells. (A) The mRNA expression of ATF2 in NHOK and four LC cells was measured by RT-qPCR. Hep-2 cells were transfected with si-ATF2 and si-NC. About 48 h after transfection, the mRNA expression of ATF2 was determined by RT-qPCR (B). Cell migration (C) and invasion (D) tests were carried out by Trans-well assay. The protein expressions of N- and E-cadherin were measured by western blot (E).

sive status [22]. Next, the expression levels of E- and N-cadherin in miR-181a or miR-203-upregulated LC cells were determined by western blot assay. Results revealed that addition of miR-181a and miR-203 resulted in the downregulation of N-cadherin and upregulation of E-cadherin in Hep-2 cells (**Figure 2D**), denoting the inhibitory effects of miR-181a and miR-203 on cell migration and invasion.

ATF2 was a direct target of miR-181a and miR-203

Having confirmed that miR-181a and miR-203 were required for LC progression, we expected to explore a potential target gene. Bioinformatics software revealed the existence of putative binding regions of miR-181a and miR-203 within the 3'UTR fragments of ATF2 (**Figure 3A**). To further demonstrate this prediction, Dual-Luciferase and RIP assays were carried out to probe the interplay between miR-181a or miR-203 and their target gene ATF2. As displayed in **Figure 3B**, elevated expression of miR-181a or miR-203 attenuated the luciferase activity of wile-type ATF2 reporters ATF2-WT and ATF2-wt in 293T cells, but no substantial alteration of mutant ATF2 reporters ATF2-MUT and ATF2-mut. Next, RIP assay revealed that ATF2 could be highly enriched by Ago2 antibody in miR-181a or miR-203-upregulated Hep-2 cells compared to miR-NC group, but IgG antibody failed to pull down ATF2 (**Figure 3C**). These findings suggested that ATF2 was a potential target of miR-181a and miR-203.

Knockdown of ATF2 inhibited migration and invasion of LC cells

ATF2 expression was first detected by RT-qPCR and results exhibited an elevated expression of ATF2 mRNA in all four LC cell lines compared to NHOK cells (**Figure 4A**), indicating that ATF2 might be required for LC progression. For the following loss-of-function experiments, si-ATF2 was transiently transfected into Hep-2 cells to interfere ATF2 (**Figure 4B**). Functionally, ATF2 depletion remarkably reduced cell migration and invasion ability, reflected by the decreased number of migrated (**Figure 4C**) and invaded cells (**Figure 4D**), as well as the lowered expression of N-cadherin, and enhanced abundance of E-cadherin (**Figure 4E**). Altogether, these findings suggested that ATF2 deficiency suppressed cell migration and invasion in Hep-2 cells.

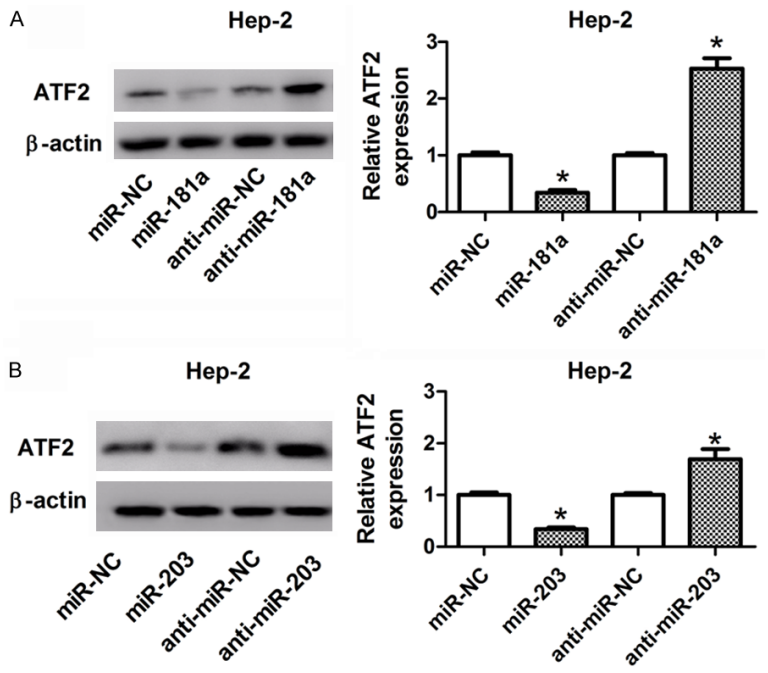

Figure 5. miR-181a and miR-203 suppressed ATF2 expression by direct complementary binding sites. A, B. Hep-2 cells were transfected with miR-181a, anti-miR-181a, miR-203, anti-miR-203, and matched controls. At 48 h post-transfetion, ATF2 expression at protein level was detected by western blot assay.

ATF2 was negatively modulated by miR-181a and miR-203

In this study, we probed the regulatory impacts of miR-181a and miR-203 on ATF2 expression in Hep-2 cells by western blot assay. Results displayed that overexpression of miR-181a or miR-203 suppressed, while reduction of miR-181a or miR-203 induced the expression of ATF2 protein in Hep-2 cells (**Figure 5A**, **5B**).

miR-181a or miR-203 impeded cell migration and invasion by targeting ATF2 in LC

To further investigate the functional interaction between miR-181a or miR-203 and ATF2, rescue experiments were performed in anti-miR-181a or anti-miR-203-transfected Hep-2 cells through silencing ATF2. Trans-well assay revealed that absence of miR-181a or miR-203 evidently stimulated cell migration (**Figure 6A**, **6B**) and invasion (**Figure 6C**, **6D**), which was reversed following ATF2 knockdown. Western blot analysis showed that downregulated ATF2 strikingly abrogated the stimulatory effect of anti-miR-181a or anti-miR-203 on Ncadherin expression, as well as the inhibitory effect on E-cadherin expression (**Figure 6E**, **6F**). These results indicated that overexpression of miR-181a or miR-203 markedly reduced cell migration and invasion by directly targeting ATF2.

Discussion

Although radical surgery and radiotherapy have been extensively used in the treatment of LC, local recurrence and tumor metastasis was also considered to be major challenges [23]. An increasing amount of evidence has confirmed the regulatory effects of miRNAs on the expression of special protein-coding RNAs [24], leading to the malignant progression of a range of human cancers, including LC [25]. For example, enforced abundances of miR-9 [26] and miR-23a [27] in LC tumor tissues were associated with the poor prognosis of patients. However, reduction of miR-149 or miR-101 foreboded a short overall survival, metastasis, and an advance clinical stage in LC patients [28, 29]. These findings propose the underlying roles of miRNAs as diagnostic or therapeutic markers for LC.

In this study, we provided the prior evidence that miR-181a and miR-203 expressions were obviously decreased in LC cells, suggesting the potential correlation between miR-181a or miR-203 and LC progression. Accumulated evidence has generally validated the central roles of miR-181a and miR-203 in tumorigenesis. Other than miR-203, miR-181a exhibits pro- or anti-tumor effects in diverse cancers. For example, miR-181a acted as an oncogene by inducing cell proliferation and repressing apoptosis in cervical cancer [30]. Inversely, in NSCLC, lowered expression of miR-181a was significantly associated with severe histological grade and advanced TNM stage. The addition of miR-181a markedly impeded cell proliferation, invasion, and colony formation through repressing CDK1 [31]. Additionally, elevated miR-181a expression evidently retarded the growth of oral squamous cell carcinoma (OSCC)

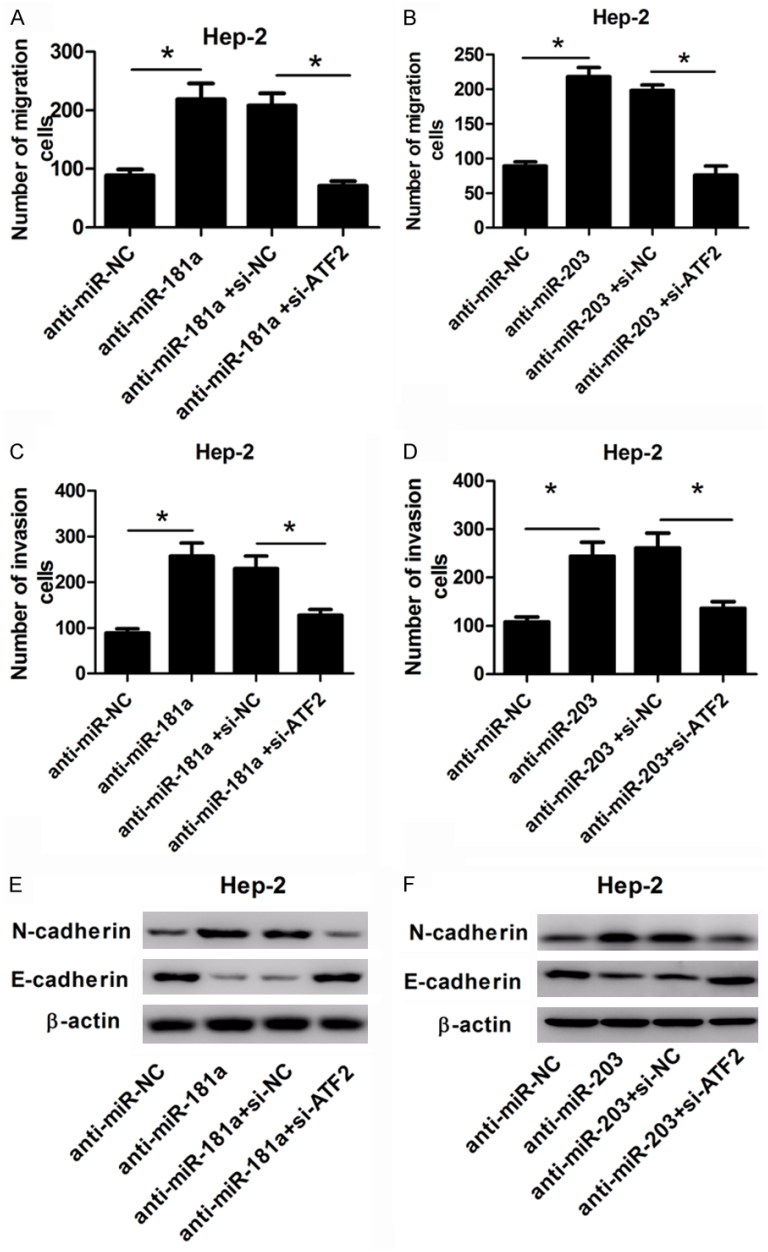

Figure 6. The stimulatory effects of anti-miR-181a and anti-miR-203 on cell migration and invasion were abrogated following ATF2 knockdown. Hep-2 cells were transfected with anti-miR-181a, anti-miR-203, or matched controls or combined with si-ATF2 or si-NC. About 48 h post-transfection, cell migration (A and B), invasion (C and D), and protein levels of N-cadherin and E-cadherin (E and F) were measured by trans-well assay or western blot.

by directly targeting K-ras [11]. In line with previous reports, our study also demonstrated the anti-tumor role of miR-181a in LC progression through regulation of cell migration and invasion.

miR-203, located on human chromosome 12, is generally recognized as a tumor suppressor. Dysregulation of this miRNA was observed in a range of human cancers. As reported by Zhang et al., exogenous restoration of miR-203 dramatically delayed tumor growth of esophageal cancer by downregulation of target gene Ran [32]. Xiang et al. indicated that the inhibitory effects of miR-203 on cell proliferation, adhesion, invasion, as well as tumor growth in prostate cancer were partially reversed by Rap1A [33]. In addition, miR-203 was recognized to serve as a tumor suppressor by affecting cell proliferation, apoptosis, invasion, and migration [20, 34]. These findings were fully consistent with our research that miR-203 inhibited migration and invasion of LC cells.

ATF2 is a member of the basic helix-loop-helix (b-ZIP) transcription factor family [35]. Recent studies indicate that ATF2 plays a key role in the occurrence and development of various cancers. For instance, elevated expression of ATF2 was related to the poor prognosis and aggressive pathological characteristics of renal cancer patients [36]. Moreover, enforced expression of ATF2 weakened the inhibitory effects of miR-204 on cell proliferation, invasion, and migration in human glioblastoma [37]. Here, we aimed to investigate whether miR-181a and miR-203 alleviated LC progression by targeting ATF2. As a result, ATF2 was identified as a target of miR-181a and miR-203. Restoration experiments further revealed that ATF2 knockdown abrogated anti-miR-181a or anti-miR-203-mediated pro-migration and pro-invasion effects in LC cells.

In sum, our study indicated that miR-181a and miR-203 attenuated cell migration and inva-

sion by co-targeting ATF2 in LC. These data uncover that miR-181a and miR-203 may be a therapeutic target for LC.

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Disclosure of conflict of interest

None.

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