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miR-200a-mediated downregulation of ZEB2 and CTNNB1 differentially inhibits nasopharyngeal carcinoma cell growth, migration and invasion

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ABSTRACT

Nasopharyngeal carcinoma (NPC), a highly metastatic and invasive malignant tumor originating from the nasopharynx, is widely prevalent in Southeast Asia, the Middle East and North Africa. Although viral, dietary and genetic factors have been implicated in NPC, the molecular basis of its pathogenesis is not well defined. Based on a recent microRNA (miRNA) microarray study showing miR-200 downregulation in NPC, we further investigated the role of miR-200a in NPC carcinogenesis. We found that the endogenous miR-200a expression level increases with the degree of differentiation in a panel of NPC cell lines, namely undifferentiated C666-1, high-differentiated CNE-1, and low-differentiated CNE-2 and HNE1 cells. By a series of gain-of-function and loss-of-function studies, we showed that over-expression of miR-200a inhibits C666-1 cell growth, migration and invasion, whereas its knock-down stimulates these processes in CNE-1 cells. In addition, we further identified ZEB2 and CTNNB1 as the functional downstream targets of miR-200a. Interestingly, knock-down of ZEB2 solely impeded NPC cell migration and invasion, whereas CTNNB1 suppression only inhibited NPC cell growth, suggesting that the inhibitory effects of miR-200a on NPC cell growth, migration and invasion are mediated by distinct targets and pathways. Our results reveal the important role of miR-200a as a regulatory factor of NPC carcinogenesis and a potential candidate for miRNA-based therapy against NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy arising from the epithelial cells lining the nasopharynx, which is vastly more common in certain regions of Southeast Asia and North Africa than elsewhere. In addition to environmental factors such as high dietary salt intake and cigarette smoking, genetic susceptibility and Epstein-Barr virus (EBV) infection have been reported to be associated with the etiology of NPC [1]. EBV-positive NPC malignancy is associated with a state of persistent latent infection, in which only a few latent genes are expressed [2]. Among these gene products, EBV-encoded latent membrane protein-1 (LMP1) oncogene is considered to play a critical role in NPC development [3]. In this regards, our group has previously demonstrated that suppression of LMP1 by RNA interference inhibits metastasis of NPC cells both *in vivo* and *in vitro* [4,5].

Since NPC is highly sensitive to radiation, the most common and effective treatment of NPC patients is primarily based on radiotherapy, and concurrent adjuvant chemotherapy can also improve survival rates [6]. However, NPC can easily invade local tissues and metastasize to distant organs. Once metastasis occurs, the prognosis of patients is poor. To facilitate the development of new effective treatment strategies, it is important to further elucidate the molecular basis of NPC pathogenesis which is still not well defined at the moment.

Recent advances in cancer biology have strong implications for the alterations of microRNA (miRNA) as one of the most important events in human tumorigenesis. To date, several human miRNAs and EBV-encoded miRNAs have been shown to be deregulated in NPC [7–9]. For example, Lo et al. found that LMP1 protein expres-

Abbreviations: NPC, nasopharyngeal carcinoma; miRNA, microRNA; EBV, Epstein-Barr virus; ZEB2, zinc finger E-box binding protein 2; SIP1, smad interacting protein 1; LMP1, latent membrane proteins 1; EMT, epithelial-to-mesenchymal transition; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide.

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sion is modulated by EBV-encoded miRNAs [8]. Moreover, Sengupta et al. identified eight differentially expressed miRNAs in NPC and demonstrated the involvement of miR-29c in metastasis by regulating mRNAs encoding extracellular matrix proteins [7]. Recently, Chen et al. have shown that miR-200 family is downregulated in NPC by miRNA microarray analysis. They quantified the expression levels of 270 miRNAs in 13 human NPC samples and 9 adjacent normal tissues, and identified 35 miRNAs (including miR-200a and miR-200b) whose expression levels are significantly altered in NPC samples [9]. However, the function of miR-200a in NPC remains unknown.

In this study, we investigated the function of miR-200a in NPC carcinogenesis. In gain-of-function and loss-of-function studies, we found that miR-200a inhibits NPC cell growth, migration and invasion. By *in silico* prediction and experimental validation, we identified zinc finger E-box binding homeobox 2 (ZEB2; also known as SIP1) and β-catenin (CTNNB1) as the functional downstream targets of miR-200a, and showed that they play distinct roles in regulating NPC development.

Material and methods

Cell culture and reagents. The EBV-positive NPC cell line C666-1 was kindly provided by Dr. Dolly P. Huang from The Chinese University of Hong Kong. CNE-1, CNE-2 and HNE1 NPC cell lines were obtained from Sun Yat-Sen University Cancer Center (Guangzhou, China). All cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY). Pre-miR-200a precursor (pre-miR-200a) and anti-miR-200a inhibitors (anti-miR-200a) were purchased from Ambion (Austin, TX).

Real-time quantitative RT-PCR. To quantitate miRNA expression, total RNA was extracted from NPC cell lines with the use of TRIzol reagent (Invitrogen). The isolated total RNA was polyadenylated and reverse transcribed for use in a two-step quantitative RT-PCR using the NCode™ miRNA First-Strand Synthesis and qRT-PCR kits (Invitrogen) according to the manufacturer's instructions. The sequence-specific forward primers for mature miR-200a and U6 internal control were 5′-CGTAACACTGTCTGGTAACGATGT-3′ (24 bp, GC = 45.84%, T_m = 62.2 °C) and 5′-CGCAAGGATGACACG CAAATTCGT-3′, respectively. The amount of miRNA was monitored with SYBR GreenER™ qPCR SuperMix reagent (Invitrogen). The reactions were performed in 96-well plate (ABI) using a preheated real-time instrument (ABI 7900HT). The PCR conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. After cycling, the reaction was maintained at 4 °C until further analysis.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. C666-1 (4×10^3) or CNE-1 cells (5×10^3) were plated onto 96-well plates in 80 μl growth medium and allowed to adhere overnight. The cells were then transfected with 50 nM pre-miR-200a or anti-miR-200a using X-tremeGENE siRNA transfection reagent (Roche, USA). At different time points (24 h, 48 h and 72 h), the culture medium was removed and replaced with culture medium containing 10 μl of sterile MTT dye (5 mg/ml). After incubation at 37 °C for 4 h, the MTT solution was removed, and 150 μl dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Spectrometric absorbance at 570 nm was measured by Multiskan EX microplate photometer (Thermo Scientific, Waltham, MA).

Wound healing assay. Cell culture and transfection conditions were optimized to ensure a homogeneous and viable cell monolayer prior to wounding. One day before transfection, equal numbers of C666-1 cells (5×10^4) or CNE-1 cells (1×10^5) were seeded onto 24-well plates. Cells were then transfected with 50 nM pre-miR-200a or anti-miR-200a using X-tremeGENE siRNA transfection reagent. When the cell confluence reached about 90% at around 48 h post-transfection, an artificial homogenous wound was made onto the monolayer with the use of a sterile plastic 200 μl micropipette tip. After wounding, the debris was removed by washing the cells with serum-free medium. At different time points, cells that migrated into the wounded area or cells with extended protrusion from the border of wound were photographed under an inverted microscope (40× objective) (Leica, Solms, Germany).*In vitro Matrigel invasion assay. In vitro* cell invasiveness was reflected by the ability of cell to transmigrate a layer of extracellular matrix in BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA). C666-1 or CNE-1 cells were transfected with 50 nM pre-miR-200a or anti-miR-200a as mentioned above. At 48 h post-transfection, cells were detached by treatment with 0.25% trypsin–EDTA (Invitrogen), centrifuged and resuspended into serum-free medium. Transfected cells (5×10^4 in 500 μl blank medium) were reseeded into the rehydrated insert. Medium with 10% FBS was added to the lower chamber as chemoattractant. After 20 h incubation, non-invading cells on the upper surface of the membrane were scrubbed gently with a cotton-tipped swab. The invasive cells, which had the ability to push themselves through the 8 μm pores and grow on the lower surface, were fixed with 100% methanol and stained with 1% toluidine blue (Sigma, St. Louis, MO). The inserts were rinsed twice with distilled water and allowed to air dry. The stained invasive cells were photographed under an inverted light microscope (40× objective) and quantified by manual counting in three randomly selected areas. This experiment was performed in triplicate in three independent transfection experiments.

Western blotting. C666-1 or CNE-1 cells were transfected with 50 nM pre-miR-200a or anti-miR-200a for 48 h. Transfected cells were harvested for immunoblot analysis according to our previously described protocol [10]. Rabbit polyclonal antibodies against human ZEB2 (H-260, sc-48789; 1:500 dilution) and GAPDH (FL-335, sc-25778; 1:2500 dilution), and mouse β-catenin monoclonal antibody (E-5, sc-7963; 1:400 dilution) were bought from Santa Cruz Biotechnology (Santa Cruz, CA).

Dual-luciferase reporter assay. The 3′-UTR sequence of CTNNB1 predicted to interact with miR-200a or a mutated sequence with the predicted target sites were inserted into the XbaI and FseI sites of pGL3 control vector (Promega, Madison, WI). These constructs were named pGL3-CTNNB1 and pGL3-CTNNB1-mut. For reporter assay, C666-1 cells were plated onto 24-well plates and transfected with 100 ng of pGL3-CTNNB1 or pGL3-CTNNB1-mut and 50 nM pre-miR-200a or pre-control using Lipofectamine 2000 (Invitrogen). A Renilla luciferase vector pRL-SV50 (5 ng; Promega) was also co-transfected to normalize the differences in transfection efficiency. After transfection for 48 h, cells were harvested and assayed with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Transfection was repeated three times in triplicate.

Transient transfection of C666-1 cells with siRNAs. Small-interfering RNA (siRNA) duplex oligonucleotides targeting human ZEB2 mRNA (siZEB2; 5′-GCAUGUAUGCAUGUGACUUTT-3′) [11] and CTNNB1 mRNA (siCTNNB1; 5′-CGGGAUGUUCACAACCGAATT-3′) [12], and a non-silencing siRNA (siControl; 5′-UUCUCCGAACGUGUCACGUTT-3′) were synthesized and purified by GenePharma Co. (Shanghai, China). One day before transfection, C666-1 cells at 80% confluence were detached and plated onto a 24-well plates at 5×10^4 cells per well. The cells were then transfected by incubation with siZEB2, siCTNNB1 or siControl at a final concentration of 100 nM and Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. At 48 h post-transfection, the decrease in ZEB2 and CTNNB1 mRNA and protein levels

were determined by semi-quantitative RT-PCR (T_m = 55 °C for 30 cycles) and Western blotting as previously described [10]. The primer pairs for ZEB2 were sense 5′-CGCTTGACATCACTGAAGGA-3′ and antisense 5′-CTTGCCACACTCTGTGCATT-3′; and for CTNNB1 were sense 5′-GAAACGGCTTTCAGTTGAGC-3′ and antisense 5′-CTGGCCATATCCACCAGAGT-3′. Cell growth, migration and invasion were also assayed at 48 h post-transfection. Each transfection was performed in triplicate, and the results were confirmed by three independent experiments.

Statistical analysis. Experimental data were presented as the mean ± standard deviation. All statistical analyses were performed using a two-tailed Student's *t* test (SPSS 12.0, SPSS Inc., Chicago, IL), and differences were considered to be statistically significant at *P*-value <0.05.

Results

Expression of miR-200a in NPC cell lines

To examine the effects of miRNA in functional studies, it is important to select a cell culture system that expresses the appropriate level of endogenous miRNAs. Here, we first analyzed the expression level of miR-200a in a panel of NPC cell lines with different degrees of differentiation, including C666-1 (undifferentiation), CNE-1 (high differentiation), CNE-2 and HNE1 (low differentiation) cells. We observed that miR-200a expression was relatively lower in undifferentiated C666-1 cells than in high-differentiated CNE-1 cells (Fig. 1A), suggesting that miR-200a expression may be associated with the degree of NPC cell differentiation. Based on this expression pattern, we therefore chose C666-1 and CNE-1 cells for the following gain-of-function and loss-of-function studies, respectively.

miR-200a suppresses NPC cell growth

miR-200a has been shown to be downregulated in NPC by miRNA microarray analysis [9], revealing its potential role in regulating NPC progression. Since cell growth is an important determinant of tumor progression, we investigated how expression of miR-200a affects NPC cell growth. We transfected NPC cells with synthetic oligonucleotides to overexpress or knock-down miR-200a expression. At different time points (24 h, 48 h and 72 h) after transfection, we confirmed by a two-step quantitative RT-PCR that transfection of pre-miR-200a or anti-miR-200a could, respectively, increase or decrease the expression level of miR-200a in C666-1 or CNE-1 cells, as compared with their respective control groups (Fig. 1B). Subsequently, MTT assay showed that over-expression of miR-200a significantly inhibited C666-1 cell growth, whereas miR-200a inhibition promoted CNE-1 cell growth (Fig. 1C).

miR-200a inhibits NPC cell migration and invasion

Apart from the difference in miR-200a expression level, we also noticed that C666-1 cells are more invasive than CNE-1 cells in our pilot study (data not shown). This prompted us to examine the role of miR-200a in the migration and invasion of NPC cells. By wound healing assay, we found that that over-expression of miR-200a inhibited C666-1 cell migration, whereas its knock-down induced CNE-1 cell migration (Fig. 2A). Consistently, results from *in vitro* Matrigel invasion assay showed that miR-200a over-expression inhibited C666-1 cell invasion, while knock-down of miR-200a in CNE-1 cells enhanced invasion (Fig. 2B). These results indicate that miR-200a inhibits the migration and invasion of NPC cells *in vitro*.

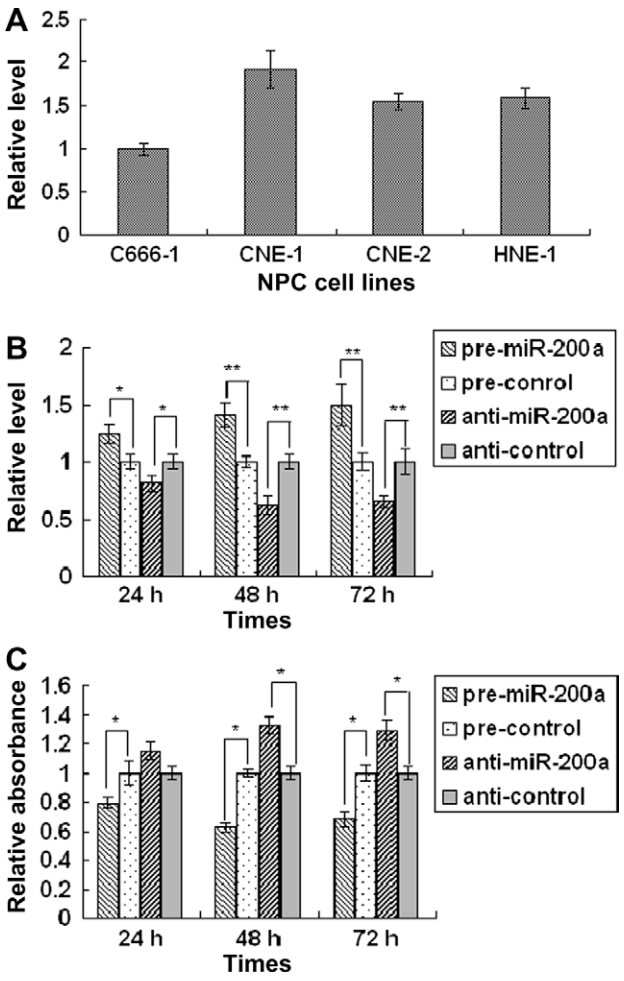

Fig. 1. Effect of miR-200a on NPC cell growth. (A) Quantitative RT-PCR of miR-200a expression in NPC cell lines with different degrees of differentiation. C666-1: undifferentiation; CNE-1: high differentiation; CNE-2 and HNE1: low differentiation. (B) Expression levels of miR-200a after transfecting with pre-miR-200a (in C666-1 cells) or anti-miR-200a (in CNE-1 cells) for 24 h, 48 h and 72 h. (C) Effects of miR-200a over-expression or inhibition on NPC cell growth by MTT assay. $^{*}P < 0.05$; $^{**}P < 0.01$, as compared pre-miR-200a with pre-control groups, or anti-miR-200a with anti-control groups.

miR-200a regulates ZEB2 and CTNNB1 protein expressions

To elucidate the mechanisms by which miR-200a inhibits NPC cell growth, migration and invasion, we attempted to identify the downstream targets of miR-200a by computational prediction. Among hundreds of genes predicted by three major online miRNA target prediction algorithms, namely TargetScan (http://www.targetscan.org), PicTar (http://pictar.mdc-berlin.de) and miRDB (http://mirdb.org/miRDB), we were particularly interested in ZEB2 and CTNNB1, which had been reported to regulate NPC carcinogenesis or act as potential functional targets of miR-200 family [11,13–16]. To validate the prediction, we detected the protein levels of ZEB2 and CTNNB1 in response to the changes in miR-200a expression. We found that over-expression of miR-200a significantly downregulated, whereas knock-down of miR-200a upregulated, the protein expressions of both ZEB2 and CTNNB1 (Fig. 3A).

CTNNB1 is another direct downstream target of miR-200a

While ZEB2 have been reported as a direct functional target of miR-200 family [11,14,15], it is not known whether miR-200a can also directly recognize the 3′-UTR of CTNNB1 mRNA. According

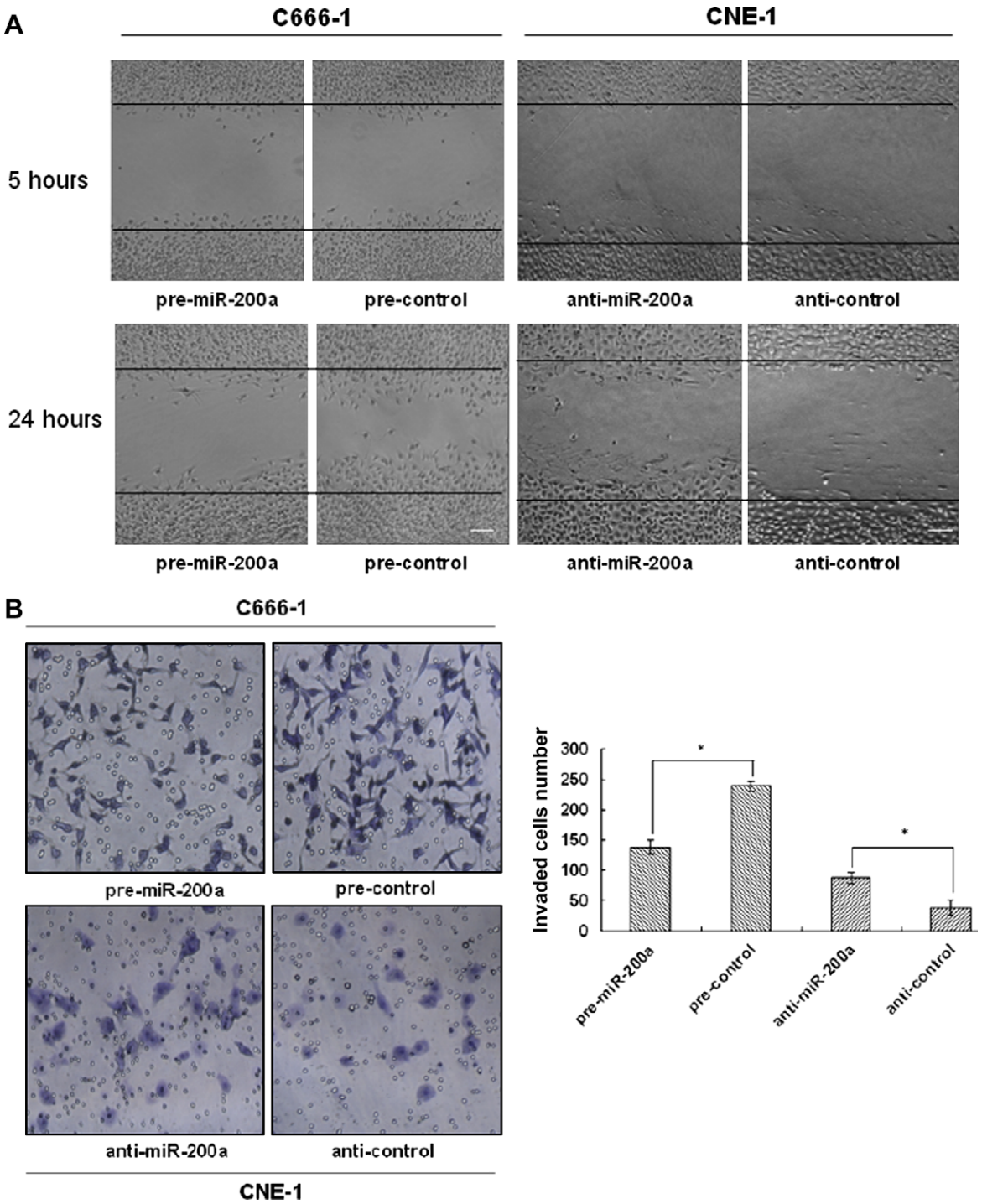

Fig. 2. Effect of miR-200a expression on NPC cell migration and invasion. C666-1 and CNE-1 cells were transfected with pre-miR-200a and anti-miR-200a, respectively. (A) Wound healing assay. Representative images photographed at 5 h (*upper*) and 24 h (*lower*) post-wounding were shown at magnification of 40×. (B) Matrigel invasion assay. The invasive NPC cells were stained and counted under microscope (magnification of 40×) at 20 h after reseeding. $^{*}P < 0.05$, as compared pre-miR-200a with pre-control groups, or anti-miR-200a with anti-control groups. (For interpretation to colours in this figure, the reader is referred to the web version of this paper.)

to the predicted target site from TragetScan, we cloned the 3′-UTR fragment containing this predicted site into the pGL3 luciferase reporter vector (pGL3-CTNNB1). Another 3′-UTR fragment with mutated sequence within the predicted target site were also cloned as control (pGL3-CTNNB1-mut). We found that co-transfection of pre-miR-200a and pGL3-CTNNB1 vector significantly decreased the luciferase activity in C666-1 cells, as compared with the control. However, pre-miR-200a had no effect on the luciferase activity when transfected with pGL3-CTNNB1-mut vector (Fig. 3B). These data show that CTNNB1 mRNA is one of direct targets of miR-200a.

Suppression of ZEB2 and CTNNB1 has different effects on NPC cell growth, migration and invasion

To verify ZEB2 and CTNNB1 as the functional effectors of miR-200a on suppressing NPC cell growth, migration and invasion, we

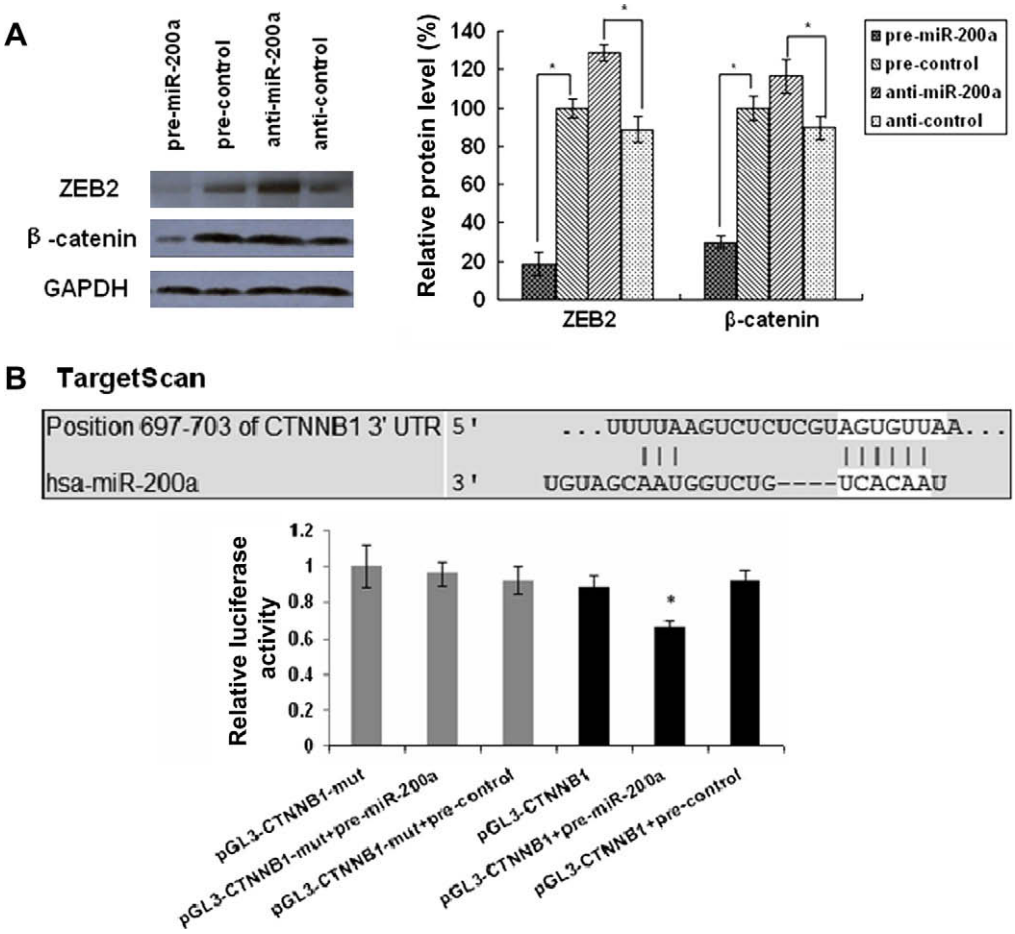

Fig. 3. Targeting of ZEB2 and CTNNB1 by miR-200a. (A) Effect of miR-200a on ZEB2 and CTNNB1 protein expressions. C666-1 and CNE-1 cells were transfected with pre-miR-200a and anti-miR-200a, respectively. At 48 h post-transfection, cell lysates were prepared and the protein levels of ZEB2, CTNNB1 and GAPDH were determined by immunoblot analysis. (B) Effect of miR-200a on CTNNB1 expression by luciferase reporter assay. The potential miR-200a binding site at the 3′-UTR of CTNNB1 mRNA (697–703 nt) was computationally predicted by TargetScan. C666-1 cells were co-transfected with 50 nM synthetic pre-miR-200a (or pre-control) with pGL3-CTNNB1 (or pGL3-CTNNB1-mut) vector. Luciferase activity was normalized by the ratio of firefly and Renilla luciferase signals ($n = 3$; $^{*}P < 0.05$).

knocked down ZEB2 and CTNNB1 by siRNAs and examined its effects in C666-1 cells. Results from RT-PCR and Western blotting showed that the expression levels of ZEB2 and CTNNB1 were decreased, respectively, by siZEB2 and siCTNNB1, as compared with siControl-transfected groups (Fig. 4A and D). Interestingly, we found that suppression of ZEB2 significantly inhibited C666-1 cell migration and invasion (Fig. 4B and C), but not cell growth (data not shown). On the contrary, knock-down of CTNNB1 only inhibited C666-1 cell growth (Fig. 4E), but had no effect on cell migration and invasion (data not shown). These results suggest that the inhibitory effects of miR-200a on NPC cell growth, migration and invasion are mediated by different targets.

Discussion

The miR-200 family is constituted by five miRNAs locating on chromosome 1 (miR-200a, miR-200b and miR-429) and chromosome 12 (miR-200c and miR-141). This miRNA family was first proved to regulate olfactory neurogenesis [17]. Recently, miR-200 family is shown to regulate epithelial-to-mesenchymal transition EMT and cancer cell migration by targeting E-cadherin transcriptional repressors ZEB1 and ZEB2 [11,13–15,18]. In a miRNA microarray analysis, it has been reported that the miR-200 family members, miR-200a and miR-200b, are downregulated in NPC [9]. However, the function of miR-200a in NPC carcinogenesis remains unknown.

In this study, we investigated the role of miR-200a and identified its targets in NPC. We first quantified the expression of miR-200a in a panel of NPC cell lines with various degrees of differentiation. These cell lines were selected to resemble the three microscopic subtypes of NPC: well-differentiated keratinizing type, moderately-differentiated nonkeratinizing type and undifferentiated type which is the most common and is strongly associated with EBV infection in cancerous cells. We found that the endogenous miR-200a expression is relatively low in undifferentiated C666-1 cells, moderate in low-differentiated CNE-2 and HNE1 cells, and high in differentiated CNE-1 cells. This expression pattern raises that possibility that miR-200a is related to NPC differentiation. With this mentioned, NPC clinical data are needed to further examine whether miR-200a is indeed associated with NPC differentiation.

Based on the miR-200a expression level, we rationally chose C666-1 and CNE-1 cells for the subsequent gain-of-function and loss-of-function studies, respectively. Our results support that miR-200a inhibits NPC cell growth, migration and invasion *in vitro*. These findings also suggest that downregulation of miR-200a in NPC is related to its progression. Although a large number of human miRNAs have been reported, many of their mRNA targets remain unidentified. In this study, we used a combined bioinformatics and experimental approach to identify that ZEB2 and CTNNB1 are the two functional downstream targets of miR-200a. This finding is consistent with a recent study published at the time

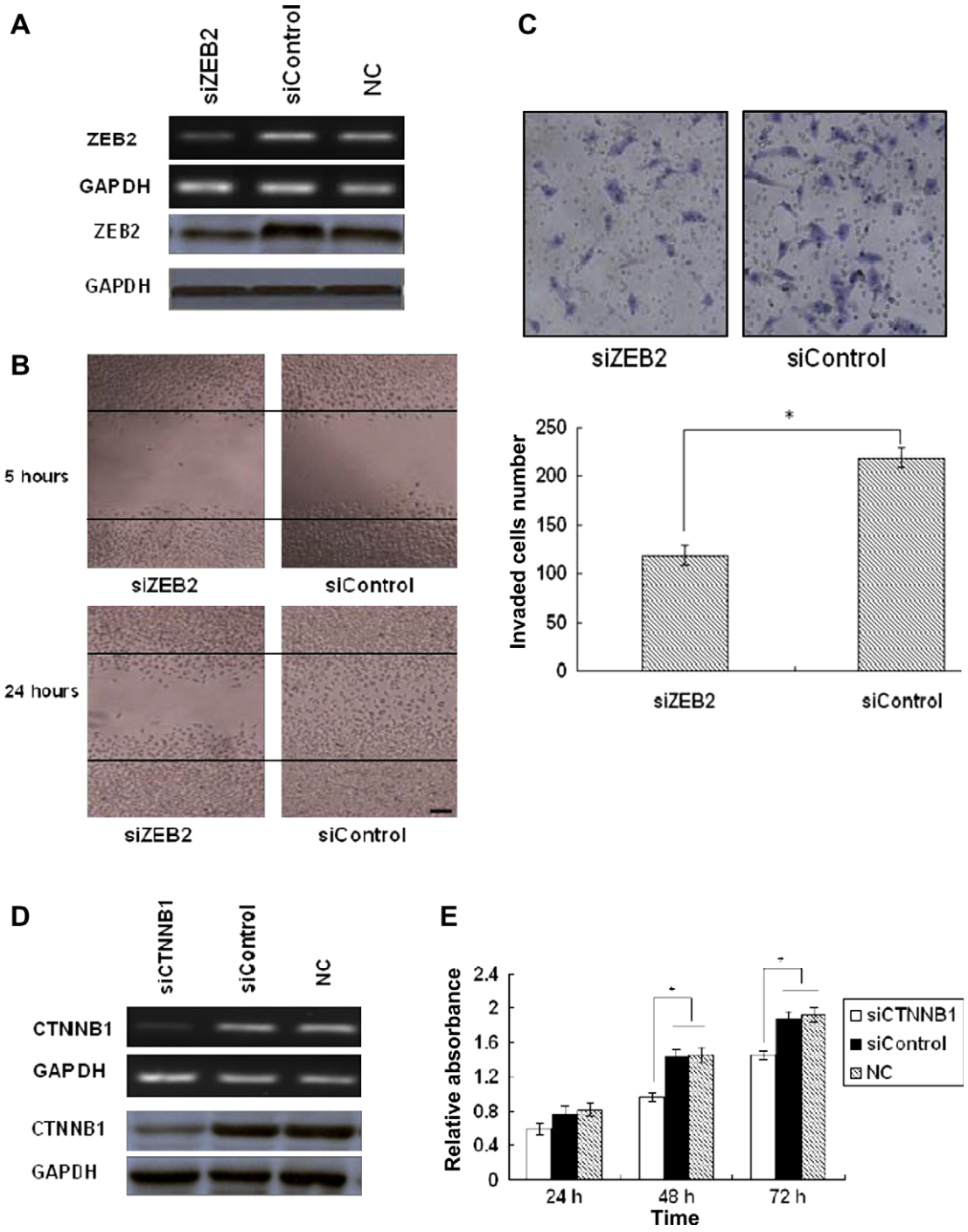

Fig. 4. Effects of siRNA suppression of ZEB2 and CTNNB1 on NPC cell growth, migration and invasion. (A and D) RT-PCR and Western blotting analysis of ZEB2 and CTNNB1 mRNA and protein expression in siZEB2-, siCTNNB1- and siControl-transfected C666-1 cells at 48 h after transfection. GADPH was shown as internal control. NC represents negative control. (B) Wound healing assay. Representative images photographed at 5 h (*upper*) and 24 h (*lower*) post-wounding were shown at magnification of 40×. (C) Matrigel invasion assay. Invasive C666-1 cells were stained and counted under microscope (magnification of 40×) at 20 h after reseeding. $^{*}P < 0.05$, as compared with siControl-transfected group. (E) Time-dependent effect of siCTNNB1 on C666-1 cell growth determined by MTT assay at 24, 48 and 72 h post-transfection. $^{*}P < 0.05$, as compared with siControl-transfected group. (For interpretation to colours in this figure, the reader is referred to the web version of this paper.)

of preparing this paper, which showed the same regulation of ZEB2 and CTNNB1 by miR-200a in meningiomas [19].

ZEB2 is a member of the delta-EF1(TCF8)/Zfh1 family of 2-handed zinc finger/homeodomain proteins [20]. It interacts with receptor-mediated, activated full-length SMADs. ZEB2 has been identified in a large-scale screening of cancer-related genes, with putative role in oncogenic transformation [21]. CTNNB1 encodes β-catenin, which is a key mediator of the canonical Wnt/β-catenin signaling pathway. β-Catenin is an important regulator of NPC development, as its increased level is critical to NPC proliferation. Specifically, nuclear β-catenin level was found to increase in 92% of NPC tumors [16]. We noticed that the expressions of ZEB2 and CTNNB1 were detectable in most NPC cell lines (data not shown). By employing siRNA technology, we found that knock-down of ZEB2 solely inhibits NPC cell migration and invasion, while CTNNB1 suppression only inhibits NPC cell growth. These results

suggest that the inhibitory effects of miR-200a on NPC cell growth, migration and invasion are mediated by different targets, highlighting the potency of miRNAs in orchestrating multiple processes for tumor progression.

To conclude, we report for the first time that miR-200a is an important regulator of NPC carcinogenesis, in which miR-200a inhibits NPC cell growth, migration and invasion by targeting different mRNAs, ZEB2 and CTNNB1. In addition, we unravel the novel functions for ZEB2 and CTNNB1 in supporting the development and progression of NPC, and nominate miR-200a as a potential candidate for miRNA-based therapy against NPC.

Acknowledgments

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