

OVEREXPRESSION OF MICRORNA-200c IN CD44+CD133+ CSCS INHIBITS THE CELLULAR MIGRATORY AND INVASION AS WELL AS TUMORIGENICITY IN MICE

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Cancer stem cells (CSCs) are believed to be responsible for drug resistance, metastasis of tumors. To investigate the biological characteristics of CD44+CD133+CSCs with over- expressing microRNA-200c (miR-200c), and to provide evidences for miR-200c as a tumor suppressor to treat melanoma. CD44+CD133+CSCs were isolated from the mouse melanoma B16F10 cell line by using immune magnetic activated cell sorting. The lentivirus miR-200c was transduced into the cells, and the effect of miR-200c overexpression on the biological characteristics of B16F10 CD44+ CD133+CSCs was analyzed by a series assays. The stable overexpression of miR-200c in B16F10 CD44+CD133+CSCs obviously resulted in downregulation of zinc-finger E-box binding homeobox 1 expression, reduction of the cell proliferation, colony forming, cell migratory and invasion ability in vitro as well as tumorigenicity in vivo compared with those of the B16F10 cells and B16F10 non-CD44+ CD133+CSCs. These findings suggest that the miR-200c overexpression as a novel strategy to target therapy of melanoma CSCs.

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INTRODUCTION

Melanoma is a very highly malignant skin tumor and metastasis is responsible for about 90% of melanoma related mortality. There is no cure in advanced stage melanoma with routine treatment. Therefore, the investigation of the pathogenesis of melanoma and search for new ways to treat melanoma are necessary for improving the survival of patients with melanoma (16, 21, 29).

It is known that tumor ablation, radiotherapy, and chemotherapy, individually or in combination with, are currently the mostly applied therapies for treating melanoma patients. Although these therapies are effective in an initial phase of treatment, the local invasive and metastatic melanoma often acquired resistance to routine treatment, and the prognosis is poor. Intensive research has shown that a small number of cells in tumor tissue represent a distinct tumor subpopulation known as cancer stem cells (CSCs) that play a crucial role in tumor initiation, progression, metastasis, tumor therapy resistance and relapse (8, 32). Latest therapeutic approaches based on CSCs have suggested that developing novel strategies by focusing biological characteristics on the CSCs may allow for the discovery of effective methods to eradicate seeds of malignant tumor cells by specifically targeted therapy (1, 27,28.).

A functional role of the MicroRNAs (miRNAs) mediate the post-transcription regulation in neoplasia and metastasis is becoming clear, and miR-200 family has received much attention for potentially regulating tumor progression (12). Our previous study indicated that the disturbance of epithelial-mesenchymal transition (EMT)-miR-200c-zincfinger E-box binding homeobox 1 (ZEB1) feedback loop promoted the melanoma proliferation and invasive metastasis (14). Accumulated researches further confirm that the ZEB1 is a direct target of miR-200c regulation, and that miR-200 family, ZEB1 and ZEB2 mainly regulate the cellular epithelial (epithelium) and interstitial (mesenchyma) state conversion to finish the typical phenotype changes of EMT in the process of tumor cell growth (15, 17). A recent study showed the miR-137 may act as a tumor suppressor by directly targeting carboxyl-terminal binding protein 1 to inhibit EMT and inducing apoptosis of melanoma cells (11). Recently, a report gave another discovery that breast epithelial cells can gain CSC characteristics through EMT program, and residual breast cancer cells following chemotherapy are enriched in EMT and CSC-like features (25). These studies suggest that the EMT may also be involved in the reprogramming of differentiated tumor cells into CSCs through induction of the EMT program (20, 25).

In this study, we isolated the CD44+CD133+CSCs from the B16F10 murine melanoma cell line, and transducted the lentivirus miR-200c expression vector into the CD44+ CD133+CSCs. The study shows the miR-200c overexpression in CD44+CD133+ CSCs markedly inhibit the cell proliferation and invasion ability in vitro as well as tumorigenicity in mice. Therefore, manipulating miR-200c may represent a promising alternative therapeutic strategy for targeting treatment of CD44+CD133+CSC mediated metastasis through inhibiting EMT program.

MATERIALS AND METHODS

Cells and Mice

B16F10 murine melanoma cell line is syngeneic in C57BL/6 mice, obtained from the Cellular Institute of China in Shanghai. Cells were cultured at 37 °C in 5% CO2 atmosphere in RPMI 1640 supplemented with 10% fetal bovine serum that contained 100Uml-1 penicillin G sodium and 100 mg ml-1 streptomycin sulfate. C57BL/6 mice of 5-6 week of age were obtained from the Yang-

zhou University of China. All mice were housed under the pathogen-free condition and the experiments were performed in compliance with the guidelines of the Animal Research Ethics Board of Southeast University.

Isolation of CD44+CD133+CSCs, transduction of lentivirus miR-200c and production of stable expression colonies

CD44+CD133+CSCs were isolated from the B16F10 murine melanoma cell line by using the magnetic-associated cell sorting (MACS) method as described previously (4, 10). Briefly, CD44+subsets were isolated by using the rabbit antimouse CD44 antibody coupled to magnetic microbeads (Miltenyi Biotec., Germany); the resulting cells were then depleted of CD133–subsets by using the rabbit antimouse CD133 antibody coupled to magnetic microbeads (Miltenyi Biotec., Germany). The resulting CD44+CD133+cells were labeled 'B16F10 CSCs'(34), and the other cells labeled 'B16F10 non-CSCs'.

To generate the miR-200c expression lentivirus vector, we amplified an insert (full-length mouse miR-200c) by PCR from B16F10 DNA. The lentivirus miR-200c was produced from the transient transfection of the HEK293T cells with pHAGE-CMV-miR-200c-IZsGreen, psPAX2, and pMD2.G plasmid DNAs plus Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Forty-eight hours after the co-transfection, the lentivirus-bearing supernatants were collected and passed through a 0.45-mm filter. CD44+CD133+ CSCs were infected with the pHAGE-CMV-miR-200c-IzsGreen lentivirus, and were selected by the IzsGreen expression (6). The stable expression colonies were selected by limiting the dilution assay (18).

RNA Isolation and quantitative RT-PCR

Total cellular RNA was isolated from the different cells by using a Qiagen RNeasy Kit (Qiagen, CA). The sequences of the primers are as follows: miR-200c-primer forward, 5'-GA AGATCTGGAGCAGGAGATCTGC-CGCTTC-3; reverse, GGAATTCAGAGCCACCCTT AACTCGG; ZEB1 forward, 5'-TGAGCACACAGGTA-AGAGGCC-3'; reverse, 5'-GGCT TTTCCCCAGAGT-GCA-3'; β-actin forward, 5'-GCCCTGAGGCTCTTTTC-CA -3'; reverse, 5'-TTACGGATGTCAACGTCA-3'; U6: forward, 5'-CTCGCTTCG GCAGCACATA GG-3'; reverse, 5'-AACGCTTCACGAATTTGCGTAGGAG-3'; URP Universal Reverse Primer, 5'-CCGGCAGGGTCC-GAGGT-3'. The gRT-PCR analysis was performed on an ABI step one plus real-time system (Applied Biosystems, USA) (33).

Western blot

Total cell lysates were prepared and analyzed by using the western blot method as described before (12,31). Briefly, 1×106 different cells were collected and lyzed in a protein extraction buffer according to the manufacturer's protocol. The PVDF membrane was blocked with 4% dry milk in the Tris-buffered saline with Tween-20 for 1 h at 20°C, and was incubated with the rabbit antibody specific to mouse ZEB1 (Santa cruz Biotechnology, CA, USA) for overnight at 4 °C. The membrane was then incubated with the goat anti-rabbit fluorescence secondary antibody for 1 h at 20°C, and the subsequent steps were performed according to the Western Blot Kit's protocol (Pierce Company). Immunoreactive bands were detected by the Odyssey scanning instrument (LICOR Odyssey, USA).

Detecting activity of the luciferase gene linked to the 3'UTR of ZEB1

The pGL3 firefly luciferase reporter plasmids with the wild-type or mutated 3'UTR sequences of ZEB1 were transiently transfected into human embryo kidney 293T cells along with 25 nM miR-200c mimics or negative control and a Renilla luciferase reporter for normalization. Luciferase activities were measured after 48 hr. The mean of the results from the cells transfected by pGL3 control vector was set as 100%(26).

Cell proliferation

2×103 B16F10 cells, B16F10 cells infected with lentivirus miR-200c, B16F10 CSCs, B16F10 CSCs infected with lentivirus miR-200c, B16F10 non-CSCs, B16F10 non-CSCs infected with lentivirus miR-200c suspension were respectively seeded into 96-well plates, and were assayed for proliferative activity in triple wells. To assay cell viability, these cell suspensions were mixed with 0.4% trypan blue (Sigma) in day 1, day 2, day 3, day 4, day 5, day 6 and day 7 after the incubation, and then the mean value of the viable count was counted using a Neubawer hemocytometer chamber (25, 31).

Colony forming assay

Colonies larger than 75μ m in diameter or containing more than 50 cells were counted as 1 positive colony according to our previous reports (2, 10). About 500 cells per well were added into a six-well culture plate, with three wells per sample. After10-day incubation, cells were washed twice with PBS and stained with the Giemsa solution. The plate clone formation efficiency was calculated as (number of colony /number of cells inoculated)×100%.

Wound-Healing Assay

Cells were grown to confluence and were wounded by dragging a 1-mL pipette tip across their monolayer. Cells were washed to remove any cellular debris and were then allowed to migrate for 0, 24, and 48 hours in a humidi-fied 5% CO2 incubator at 37°C. Images were taken, using a DMI6000 inverted microscope (Leica Microsystems GmbH, Germany), at 0, 24, and 48 hours after the wounding procedure (2, 24).

Matrigel invasion assay

The Matrigel invasion assay was done using the BD Biocoat Matrigel Invasion Chamber (pore size: 8 mm, 24-well; BD Biosciences, USA) following the manufacturer's protocol (7, 30). From five randomly selected fields, the cells that had invaded through the membrane to the lower surface were counted under a light microscope.

Animal experiment

Thirty-six C57BL/6 mice (5-6 week-old) were ordered from the Animal Center of Yang Zhou University of China and were raised at the Experimental Animal Center, Southeast University. All the animal experiments were performed in compliance with the guidelines of the Animal Research Ethics Board of Southeast University. Thirtysix mice were randomly divided into six groups of equal size: control of B16F10 cells, B16F10 cells infected with lentivirus miR-200c, B16F10 CSCs without infection of lentivirus miR-200c, B16F10 non-CSCs, B16F10 CSCs infected with lentivirus miR-200c and B16F10 non-CSCs infected with lentivirus miR-200c. Each mouse was subcutaneously (s.c.) injected in the back with 5×104 above mentioned cells, and tumor formations in mice were monitored every three days. Evaluation was also done based on the tumor volume and survival rates. A mouse was sacrificed when any of its tumors was over 1.5 cm in the largest diameter (12, 13).

Statistical analysis

Statistical comparisons of the results between groups were performed using the Student's t-test method. P < 0.05 was considered significant statistically.

RESULTS

miR-200c and ZEB1 expression in lentivirus infected B16F10 CSCs

As was described in the method section, the CD44+CD133+CSCs were isolated from the B16F10 murine melanoma cell line using MACS and were then infected with lentivirus miR-200c. Fig. 1 A-D represent B16F10 CSCs-miR-200c and B16F10 non-CSCs-miR-200c, observed under a light microscope and a fluorescence mi

croscope. To identify the interaction of miR-200c-ZEB1 feedback loop in B16F10 non-CSCs and B16F10 CSCs, we detected the expression of miR-200c and ZEB1 by qRT-PCR and western blot after the cells were stably selected by using a single clone screening method. It was found that the miR-200c expression was lower in B16F10 CSCs than B16F10 non-CSCs but its expression was obvious increased in the cells infected with lentivirus miR-200c, which was were statistically significant (p < 0.05 and p<0.01) (Fig.1E). It was also found that the ZEB1 expression was higher in B16F10 CSCs than B16F10 non-CSCs whereas its expression was significant decreased in the cells infected with lentivirus miR-200c (Fig.1 F) after these cells were infected with lentivirus miR-200c. This coincides with changed expression of ZEB1mRNA and protein (Fig.1 G and Fig.1 H). The differences were statistically significant (p<0.01 or p<0.005).

To determine whether miR-200c really could directly downregulate ZEB1 expression, we performed double-luciferase reporter assay with the firefly luciferase containing plasmid PGL-3.0 as the reference. The result indicated that miR-200c reduced the activity of renilla luciferase that was fused to the wild-type 3'UTR of the ZEB1 mRNA but not to a mutant 3' UTR (Fig.1 I). The altered nucleotides of ZEB1-mutant 3' UTR were introduced in miR-200c 'seed-pairing' recognition site. The results demonstrated



Figure 1. Detection of the miR-200c and ZEB1 expression in lentivirus miR-200c infected B16F10 CSCs by qRT-PCR and western blot. B16F10 CSCs and B16F10 non-CSCs stably infected with lentivirus miR-200c were observed under a light microscope (A, B) and a fluorescence microscope (C, D). The expression differences of miR-200c (E) and ZEB1 (F) between the B16F10 CSCs and B16F10-miR-200c-CSCs, and between the B16F10 non-CSCs and the B16F10-miR-200c-non-CSCs were respectively analyzed by qRT-PCR. G. The expression of ZEB1 was examined by western blot in B16F10 CSCs, B16F10-miR-200c-CSCs, B16F10 non-CSCs and B16F10-miR-200c-non-CSCs. H. The expression difference of ZEB1 was qualified. I. Luciferase activity of the wild-type or mutant ZEB1 3'UTR reporter gene in HEK293T cells transfected with the miR-200c mimics or miRNA negative control while PGL-3.0 plasmid was transfected as endogenous control. Statistically significant differences are indicated by asterisk for **p < 0.01 and ***p < 0.005.

that there exists interaction of miR-200c-ZEB1 feedback loop in B16F10 non-CSCs and B16F10 CSCs, and that the downregulation of ZEB1 by miR-200c depends directly on the recognition site in the ZEB1 3'UTR.

Effect of miR-200c overexpression on colony formation and cellular motility of B16F10 CSCs

To assess the function of miR-200c in the B16F10 CSCs, we examined the effects of miR-200c overexpression on the B16F10 CSCs based on cellular proliferation, colony forming, cellular migration and invasive capacity. Fig.2A shows that the proliferation of B16F10 cells, B16F10 CSCs, and B16F10 non-CSCs, detected by MTT assay, was higher than homologous B16F10-miR-200c cells, B16F10 CSCs-miR-200c, and B16F10 non-CSCs miR-200c respectively, in particular the B16F10 CSCs on 7 days, which was statistically significant between the B16F10 CSCs and the B16F10 CSCs-miR-200c (p<0.005), and between the B16F10 CSCs and the B16F10 non-CSCs (p<0.01) (Fig.2B).

The colony forming capacity was analyzed by the plate colony forming assay (Fig.2C). It was found that the plat-

ing colony formation rate was around 47% for B16F10 CSCs, 34% for B16F10 cells and 13% for B16F10 non-CSCs whereas the colony formation rate was only around 11% for B16F10 CSCs-miR-200c, 12% for B16F10-miR-200c cells, and 8% for B16F10 non-CSCs-miR-200c when measured at 10 days after incubation in the plate media. The differences were statistically significant (p<0.05 and p<0.01) as is shown in Fig.2D.

To evaluate the role of miR-200c overexpression on the cell motility capacity of B16F10 CSCs, cell migration ability was assessed with the wound healing assay. Fig.3A displays the overexpression of miR-200c clearly resulted in a significant reduction in the migration of B16F10-miR-200c cells, B16F10 CSCs-miR-200c and B16F10 non-CSCs-miR-200c, respectively in comparison the control cells (B16F10 cells, B16F10 CSCs and B16F10 non-CSCs, which were not infected with lentivirus-miR-200c). There were a significant differences between the B16F10 cells and the B16F10 -miR-200c (p < 0.05), and between the B16F10 CSCs and the B16F10 non-CSCs and the B16F10 non-CSCs and the B16F10 cells.



Figure 2. The lentivirus miR-200c infected B16F10 CSCs reduced the ability of cell proliferation and colony forming *in vitro*. A. The cell proliferation ability of the different cells *in vitro* was detected by MTT assay. B. The difference of cell proliferation was statistically analyzed on the day 7. C. The plate colony forming assay. 1,2,3,4,5 and 6 represent B16F10 cells, B16F10-CSCs, B16F10 non-CSCs, B16F10-miR-200c, B16F10-miR-200cCSCs and B16F10-miR-200c-non-CSCs, respectively. D. The plate colony forming rate was statistically analyzed. *p < 0.05, **p < 0.01 and *** p < 0.005.

Meanwhile, cell invasive capacity was studied using the transwell invasive assay (Fig.3C). The miR-200c overexpression led to fewer B16F10 CSCs with lentivirus miR-200c (mean \pm SD: 18.2% \pm 1.82%), in the bottom of the chamber insert, than the B16F10 CSCs without infection of lentivirus miR-200c (69.62% \pm 12.65%), which was statistically significant (p<0.01). The other cells infected with lentivirus miR-200c showed the similar downregulated invasive capacity as is shown in Fig.3D. These results indicate that the miR-200c overexpression markedly decreased the proliferation, colony forming and motility of B16F10 CSCs in vitro.

Overexpression of miR-200c decreased B16F10 CSC growth in mice

Because the miR-200c overexpression exhibited significant effects on the colony forming, migration and invasion of B16F10 CSCs in vitro, we sought to know whether the miR-200c overexpression could affect the establishment and progression of melanoma in vivo mouse model. The representative tumor pictures in Fig.4A were taken from the mice injected with aforementioned different cells when the tumor bearing mice were photographed on Day 35. In the tumorigenicity experiment, we found that the mice injected with 5×104 B16F10 cells or B16F10 CSCs or B16F10 non-CSCs all generated tumors in 20 days (arrowhead, top panel), of which the mouse injected with 5×104 B16F10 CSCs formed the biggest tumor size among the 3 tumors, whereas no tumor was found in the mice injected with 5×104 B16F10-miR-200c cells or B16F10-miR-200c CSCs or B16F10-miR-200c non-CSCs (bottom panels) throughout the 40-day observation. Figure 4 B shows that the growth curves of the tumors in the mice injected with the different cells. It was found that although 6 mice injected with 5×104 B16F10 cells all developed tumors in 30 days the tumor sizes in B16F10 cell group mice were smaller than that of B16F10 CSC group mice. Whereas 6 mice with equal injection of 5×104 B16F10 non-CSCs, only 4 out of the 6 mice developed tumors after 40 days into the observation; the tumor sizes of these 4 mice were also smaller than those of the B16F10 CSC group mice. There were a significant differences in tumor sizes between the B16F10 cells and the B16F10 non-CSCs (p < 0.05), and between the B16F10 cells and the B16F10-CSCs (p<0.01), and the B16F10 non-CSCs and the B16F10 CSCs (p<0.005). The tumor sizes were not shown here. The result suggested that the stable miR-200c overexpression in the cells infected with lentivirus miR-200c obviously inhibited the B16F10 cell tumorigenicity and progression of melanoma in mice.



Figure 3. The lentivirus miR-200c infected B16F10 CSCs inhibited the ability of cellular migratory and invasion *in vitro*. A. The wound healing assay. B. The statistical analysis for the wound healing assay. C. The transwell invasion assay. D. The statistical analysis for the transwell invasion assay. *p < 0.05 and **p < 0.01.



Figure 4. The miR-200c overexpression decreased tumorigenicity and progression of B16F10 CSCs in mice. A. The representative images show the tumor growth in the mice injected with the 5×10^4 B16F10 cells, B16F10-miR-200c cells, B16F10 CSCs, B16F10-miR-200c-CSCs, B16F10 non-CSCs, and B16F10-miR-200c non-CSCs on day 35. B. The tumor growth dynamic state in mice injected with the above cells. *p < 0.05, **p < 0.01 and ***p < 0.005.

DISCUSSION

Human malignant melanoma is a highly aggressive and drug-resistant cancer that contains cancer cell subsets with self-renewing CSCs capable of spawning more differentiated tumor cell progeny (5, 23). The malignant melanoma initiating cells, a novel type of CSCs, based on selective expression of the chemoresistance mediator ABCB5, are required for tumorigenesis and neoplastic progression of melanoma. Systemic administration of a monoclonal antibody directed at ABCB5 molecule induced antibody-dependent cell-mediated cytotoxicity in ABCB5+CSCs (22). Emerging data from investigation of melanoma cell lines indicates that the loss of miRNA-205 expression is associated with melanoma progression but using a xenograft model, melanoma cells overexpressing miR-205 exhibit a reduced migratory capacity compared with control tumor cells (17). It was also found that the miR-200 family have been shown to suppress EMT and their down-regulation expression in some tumors promote invasion and metastasis (12). However, it is unknown whether the melanoma CSCs, the "seed cells" in melanoma, could be closely associated with the miR-200 family expression.

In the present study, we focused on the role of miR-200c in melanoma CSCs and hypothesized a potentiality role for miR-200c in the regulating melanoma CSC progression. Depending on our previous report that the B16F10 CD44+CD133+cells have some biological properties of cancer stem-like cells (10), we therefore selected the B16F10 CD44+CD133+ cells (B16F10 CSCs) to investigate whether overexpression of miR-200c in B16F10 CSCs could inhibit the cellular migratory and invasion capacity and tumorigenicity.

The findings from our study demonstrated that the miR-200c expression was lower whereas the ZEB1 expression was higher in B16F10 CSCs than the control cells. With the stable miR-200c overexpression in B16F10 CSCs, the cellular proliferation and colony forming capacity as well as cellular motility were markedly reduced compared with the control cells. It is possible that miR-200c directly bound to the recognition site in the ZEB1 3'UTR to inhibit the ZEB1 expression that resulted in decrease of B16F10 CSC capacity. Our another study, which gave a support to our current study, showed that overexpression of miR-200c inhibited tumorigenicity and metastasis of ovarian CD117+CD44+CSCs by inhibiting the ZEB1 expression and regulating the EMT (3).

Given that the tumor cell cloning efficiency is correlated positively with the cellular proliferation and self-renewal capacity that may be associated with the tumorigenicity (9,19). We further used an allogeneic graft mouse model to confirm the relationship between the cellular proliferation and self-renewal capacity and the B16F10 cell tumorigenicity. We found from the tumorigenicity experiment that the B16F10 CSCs possess stronger tumorigenicity than B16F10 cells in C57BL/6 mice, whereas the tumorigenicity of B16F10 non-CSCs was weaker than that of the B16F10 cells, which is accordant with our previous report (10). However, while these various cells were stably infected with lentivirus miR-200c the cell tumorigenicity in mice was almost completely inhibited, which was reflected in no tumour development in mice throughout the 40-day observation. Notably, the results suggested that the B16F10 CSCs have the ability to self-renew, as well as to fleetly develop tumors in mice, and that the miR-200c overexpression not only effectively decreased the colony forming but also markedly inhibited the tumorigenicity in our establishment mouse model.

In current study, we know that it would be clear to test whether miR-200c overexpression could result in reduction in B16F10 CSC invasion and metastasis in melanoma-bearing mice. Further investigation of the mechanism of miR-200c overexpression in inhibiting the tumorigenicity of B16F10 CSCs still is a necessary for the strategy targeting treatment melanoma.

In conclusion, this preliminary study represents the first attempt to demonstrate that the stable overexpression of miR-200c in B16F10 CD44+CD133+CSCs significantly led to downregulation of ZEB1 expression, and that the low miR-200c expression was associated with melanoma progression. Importantly, overexpression of miR-200c in B16F10 CD44+CD133+CSCs not only availably reduced the colony forming but obviously inhibited the tumorigenicity in establishment mouse model. These findings may provide preliminary evidences for miR-200c as a tumor suppressor to target therapy of CD44+CD133+CSCs in human melanoma.

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