

Original Article

microRNA-200c overexpression inhibits chemoresistance, invasion and colony formation of human pancreatic cancer stem cells

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Abstract: Introduction: Cancer stem cells (CSCs) are believed to be 'seed cell' in cancer recurrence and metastasis. MicroRNAs (miRNAs) have emerged as potential therapeutic candidates due to their ability to regulate multiple targets involved in tumor progression and chemoresistance. The goal of this study was to investigate the role of miRNA-200c (miR-200c) in regulating colony formation, invasion and chemoresistance of human pancreatic cancer stem cells (PCSCs). Methods: PCSCs with CD24⁺CD44⁺ESA⁺ as the marker was sorted from PANC-1 cell line by fluorescence activated cell sorter (FACS). Quantitative real-time PCR (qRT-PCR) assay was used to detect the expression of miR-200c in PCSCs and PANC-1 cells. Transfection of miR-200c mimic into PCSCs was performed to establish miR-200c over-expressed cells. The effects of overexpressing miR-200c on PCSCs were examined by cell colony forming, invasion and survival assays in vitro. Results: Our data showed that CD24⁺CD44⁺ESA⁺ PCSCs (0.5%) were isolated from PANC-1 cells. Expression of miR-200c was significantly reduced in PCSCs compared with PANC-1 cells. In addition, the capability of colony formation, invasion and chemoresistance were markedly increased in PCSCs than that in PANC-1 cells. Adverse results were obtained in miR-200c overexpressing PCSCs transfected with miR-200c mimic. Conclusion: Our study demonstrated that miR-200c overexpression could decrease colony formation, invasion and chemoresistance of PCSCs. It may become a new therapeutic target for gene therapy in patients suffered from pancreatic cancer.

Keywords: Pancreatic cancer, cancer stem cells, microRNA-200c, chemoresistance, invasion

Introduction

Pancreatic cancer is the seventh leading cause of cancer deaths in China and the fourth leading cause of cancer deaths in the United States [1, 2]. The prognosis of patients suffered from pancreatic cancer is one of the worst in all cancer forms, because there has been little progress in cancer diagnosis and treatment strategies during the past decades [3, 4]. It is therefore crucial to conduct an in-depth investigation of the biology of pancreatic cancer. The major advance of tumor biology in recent years has been the discovery of the cancer stem cells (CSCs), which play pivotal roles in cancer progression and treatment resistance in various neoplastic diseases. CSCs may open up new possibilities of generating novel targets, diminishing resistance to chemoradiation and improving therapeutic efficacy [5].

MicroRNAs (miRNAs) comprise a class of 19~23 nucleotides of noncoding RNAs regulating gene expression by targeting translational cleavage or repression [6]. In recent years, a growing number of evidence had demonstrated that miRNA expression levels were directly associated with cancer cell formation, development and pathology [7-9]. With the growing knowledge of relation between miRNAs and cancer, it also has been shown that the sensitivity of cancer cells to anticancer drug was affected by miRNAs [10]. Among them, miR-200c has been shown to be expressed in various carcinoma tissues and its over- or down-expression played essential role in tumor formation or cancer cell apoptosis. However, much less information is available about miR-200c expression in PCSCs, and the functional role of miR-200c in regulating PCSCs is yet to be elucidated.

In the present study, we first isolated PCSCs from PANC-1 cell line. Second, we examined the molecular expressions of miR-200c in both PCSCs and PANC-1 cells. Third, we investigated the difference of colony formation, chemoresistance and invasion between PCSCs and PANC-1 cells. Finally, we explored biological function of miR-200c in PCSCs by overexpressing miR-200c *in vitro*. Our data demonstrated that miR-200c played important roles in regulating the colony formation, invasion and chemoresistance of PCSCs.

Materials and methods

Cell lines and cultures

Human pancreatic cancer cell line (PANC-1) was purchased from the Cell Bank of Chinese Academy of Sciences. Cells were cultured in RPMI 1640 and DMEM medium, respectively, with 10% fetal bovine serum, at 37°C in a humidified incubator with 5% CO₂.

Flow cytometry of CSCs

PANC-1 cells were centrifugated for 5 min after digested by trypsin, then suspended at 10⁶ cells/ml in DMEM containing 2% fetal bovine serum. Cell suspension (1 ml) was added with 20 µl anti CD24-PE, anti CD44-APC and anti ESA-FITC (Abcam, USA), dark incubated at room temperature for 30 min, then centrifugated for 5 min. Cells were cleaned with phosphate-buffered saline (PBS), added with culture solution containing 2% fetal bovine serum and re suspended. CD24⁺CD44⁺ESA⁺ cells were sorted and their proportion was detected by FACSaria II (Becton Dickinson, USA). The process was repeated to ensure the purity > 95%.

Cell transfection

PCSCs in logarithmic growth phase were prepared for cell transfection. Transfection of miRNA-200c mimic and its non-specific control (Invitrogen, USA) were performed according to the manual provided with the siPOR™ NeoFX™ Transfection Agent (Ambion, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with the TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Complementary DNA was reverse-transcribed using a reverse transcription kit

(Invitrogen, USA). Briefly, cDNA were synthesized using a miRNA-200c specific primer in reverse transcription system. The reaction condition was as follows: 16°C 30 min, 42°C 42 min, 85°C 5 min. Quantitative detection of RT products was performed using specific sense and antisense primers of miRNA-200c and Sybergreen I dye. The PCR reaction condition was as follows: 95°C for 5 min, 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s, 40 cycles, to obtain fluorescence intensity. U6 was used as an internal control. The sequence of specific primer for miRNA-200c was 5'-GTCGTATCCAGT GCGTGTCTGTGGAGTCGGCAATGCACTGGATACGA CTCCATC-3'; the sequence of sense primer of miRNA-200c was 5'-GGTAATACTGCCGGGTA-AT-3'; the sequence of antisense primer of miRNA-200c was 5'-CAGTGCGTGTCTGTGGAGT3'. The Ct value was analyzed using the RFQ-PCR (Applied Biosystems Viia7, USA) analysis program. Relative mRNA expression levels were determined by the 2^{-ΔΔCt} method in comparison with control cells.

Cell survival assay and gemcitabine treatment

PANC-1cells and PCSCs transfected with either miR-200c negative control or mimics were suspended in RPMI1640 medium containing 10% FCS and seeded in 96-well plates (1 × 10⁵ cells in 200 µL medium/well). Upon attachment of the cells, different concentrations of gemcitabine were added in triplicate wells of the plates, and concentration gradient is 0.01, 0.1, 1, 5, 10 mol/L. Control wells and zero adjusted wells were also set. After 72 h, 20 µL fresh 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Merk, USA) reagent (5 g/L) was added into each well and the cells were cultured for another 4 h. The medium was discarded carefully and 150 µL DMSO was added. Absorbance at 490 nm was measured in an ELISA reader. The mean absorbance of triplicate wells was set as value of each group. All experiments were done in triplicate wells and repeated at least three times.

Matrigel invasion assay

The invasion capabilities of PANC-1cells and PCSCs transfected with either miR-200c negative control or mimics were measured by the number of cells invading Matrigel-coated Transwell chambers (Becton Dickinson, USA). Transwell inserts were coated with Matrigel (40

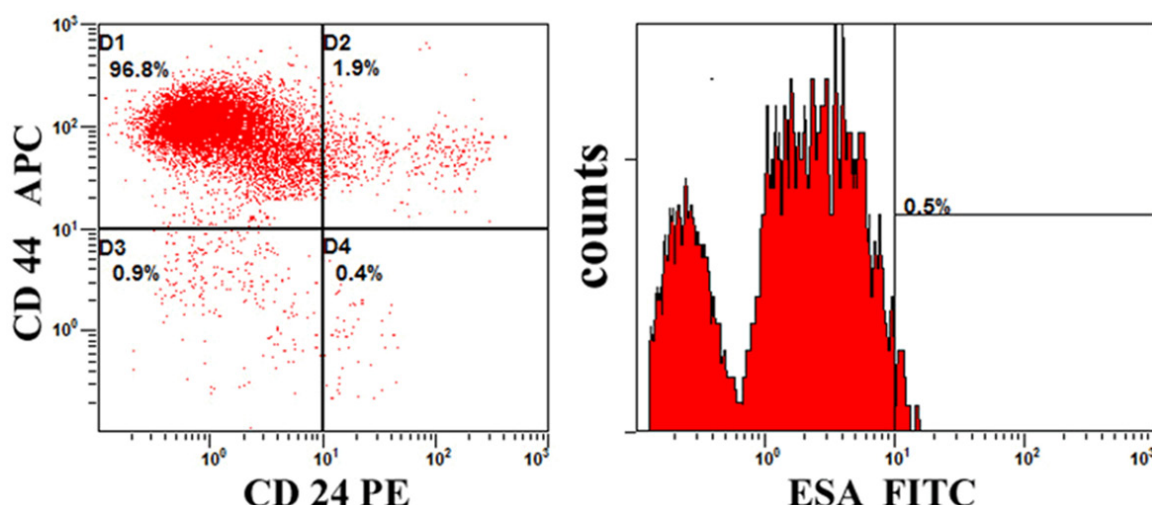


Figure 1. Isolation of CD24⁺CD44⁺ESA⁺ PCSCs. The CD24⁺CD44⁺ESA⁺ PCSCs were isolated from the human pancreatic cancer cell line PANC-1. The CD24⁺CD44⁺ESA⁺ cells accounted for 0.5% of the PANC-1 cell line.

µg/well, Becton Dickinson) and reconstituted with 10% fetal bovine serum-containing medium for 2 h before the experiment. PCSCs were cultured into the upper chambers in 250 µL DMEM supplemented with 10% fetal bovine serum for 72 hours. Culture medium without cells was placed in the lower wells. Cells that invaded into the lower surface of the Matrigel-coated membranes were fixed with 70% ethanol, stained with H&E, and averaged across five random fields at × 200 magnification under a light microscope.

Colony forming assay

The colony formation capabilities of PANC-1 cells and PCSCs transfected with either miR-200c negative control or mimics were investigated. Colonies larger than 75 µm in diameter or containing more than 50 cells were counted as 1 positive colony. About 100 cells per well were added into a six-well culture plate, with three wells per sample. After 2-week incubation, the 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%.

Statistical analysis

Data are presented as mean ± standard deviation, and statistical analyses were performed using Student's t test with a significance level of $P < 0.05$.

Results

Isolation of CD24⁺CD44⁺ESA⁺ PCSCs

As was described in the method section, the CD24⁺CD44⁺ESA⁺ PCSCs (0.5%) were isolated from the human pancreatic cancer cell line PANC-1 using FACS to study the effect of miR-200c overexpression in PCSCs (**Figure 1**).

Correlations of miRNA-200c underexpression with PCSCs chemoresistance, invasion and colony formation

QRT-PCR was used to evaluate the relative miRNA-200c expression levels in both PCSCs and PANC-1 cells. Results showed that miR-200c was significantly underexpressed in PCSCs compared with PANC-1 cells. miRNA-200c reduced its expression by 0.85 fold in PCSCs compared to PANC-1 cells (**Figure 2A**; $P < 0.05$).

Cell survival assay was employed to detect the chemoresistance of both PCSCs and PANC-1 cells. Results showed that after treatment gemcitabine, the survival rates were markedly lower in the PANC-1 cells than the survival rates of PCSCs (**Figure 2B**; $P < 0.05$).

Matrigel invasion assay was used to further investigate the invasion of both PCSCs and PANC-1 cells. Results showed that invasions were significantly increased in PCSCs, as compared to PANC-1 cells (**Figure 2C**; $P < 0.05$).

Colony forming assay was performed to examine the colony forming capability of both PCSCs

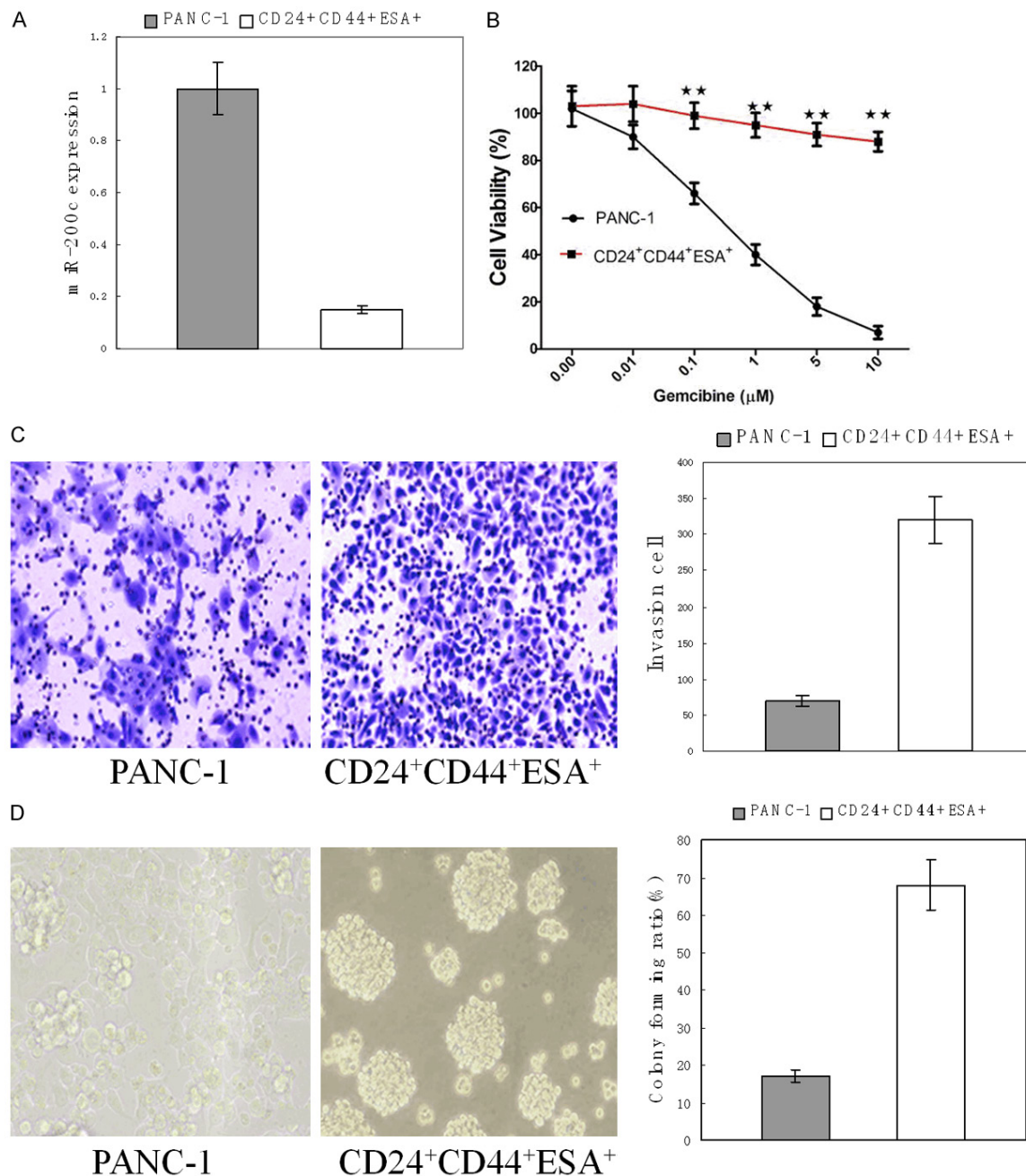


Figure 2. Comparison of miRNA-200c expression, chemoresistance, invasion and colony forming capability between PCSCs and PANC-1 cells. A. The miR-200c expression differences between PCSCs and PANC-1 cells were detected by qRT-PCR; B. Cell survival assay; C. The matrigel invasion assay; D. The plate colony forming assay.

and PANC-1 cells. The plating colony formation rates were significantly higher for the PCSCs than PANC-1 cells (**Figure 2D**; $P < 0.05$).

Overexpression of miRNA-200c increased sensitivity of PCSCs to gemcitabine

To investigate the role of the miR-200c in regulating the sensitivity to chemotherapy, we first

measured the levels of miR-200c expression in PCSCs transfected with the miR-200c negative control and mimics by qRT-PCR. The expression levels of miR-200c, as measured by qRT-PCR, were significantly increased by miR-200c mimics in PCSCs. The cells transfected with the mimics (miR-200c, 200 ng) exhibited 16.8 folds higher level of miR-200c than the cells trans-

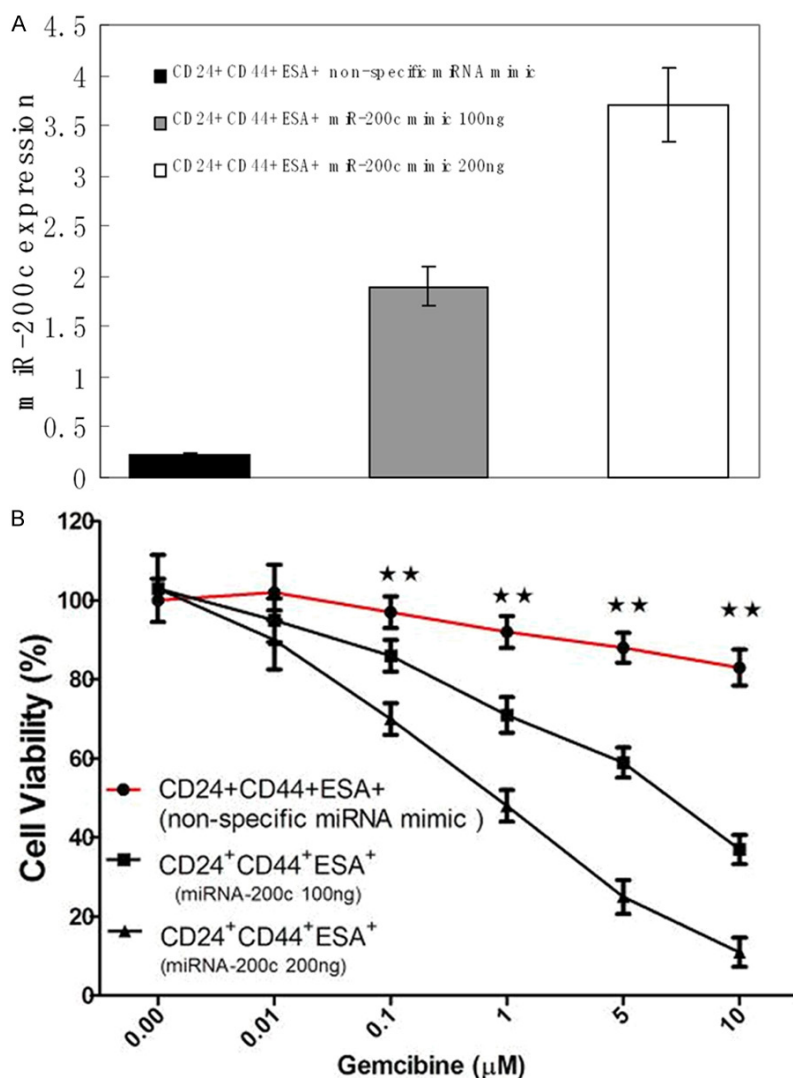


Figure 3. miR-200c enhanced sensitivity of PCSCs to gemcitabine. A. miR-200c expression differences among the PCSCs either transfected with miR-200c mimic (100 ng, 200 ng) or non-specific control were detected by qRT-PCR. B. Effects of miR-200c overexpression on PCSCs gemcitabine chemoresistance.

fectected with the non-specific negative control (Figure 3A; $P < 0.05$), and the cells transfected with the mimics (miR-200c, 100 ng) exhibited 8.6 folds higher level of miR-200c than the cells transfected with the non-specific negative control (Figure 3A; $P < 0.05$).

Then we investigated the cell survival rates of PCSCs after treatment with an anticancer agent gemcitabine. The PCSCs were either transfected with miR-200c mimic (100 ng, 200 ng) or non-specific control. The cell survival rates were evaluated after treatment with gemcitabine. The result showed that after treatment gemcitabine, the survival rates were markedly lower in the cells transfected with

miR-200c mimic than the survival rate of cells transfected with the negative control (Figure 3B; $P < 0.05$).

Overexpression of miRNA-200c inhibited PCSCs invasion

To further investigate the transfection efficiency of miR-200c, we examined the effects of miR-200c on PCSCs invasion with a Matrigel invasion assay. We used PCSCs for these experiments and transfected them with either miR-200c control or miR-200c mimic. The results showed that invasions were significantly decreased in cells transfected with miR-200c mimic, as compared to the control condition after 72 hours (Figure 4, $P < 0.05$).

Overexpression of miRNA-200c decreased PCSCs colony formation

To further characterize the function of miR-200c in the PCSCs, we examined the effects of miR-200c overexpression on the PCSCs with regard to colony forming. The colony forming capability was analyzed by the

plate colony forming assay. Results showed that the colony formation rates were markedly decreased for PCSCs transfected with miR-200c mimic, compared to control (Figure 5, $P < 0.05$).

Discussion

It has been known that CSCs are in general less differentiable, more invasive, more chemoresistant, and result in poor clinical outcomes. Numerous studies of pancreatic cancer have focused on modulating the miR-200 family (including miR-200a, miR-200b, miR-200c, and miR-141). However, it is unknown whether the PCSCs, the “seed cells” in pancreatic cancer,

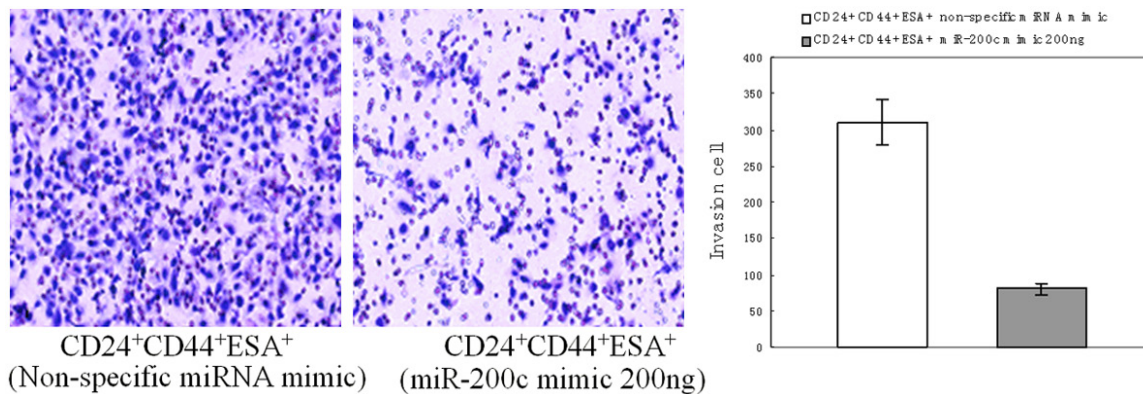


Figure 4. Decreased matrigel invasion with PCSCs was seen with miR-200c mimic transfection compared to control.

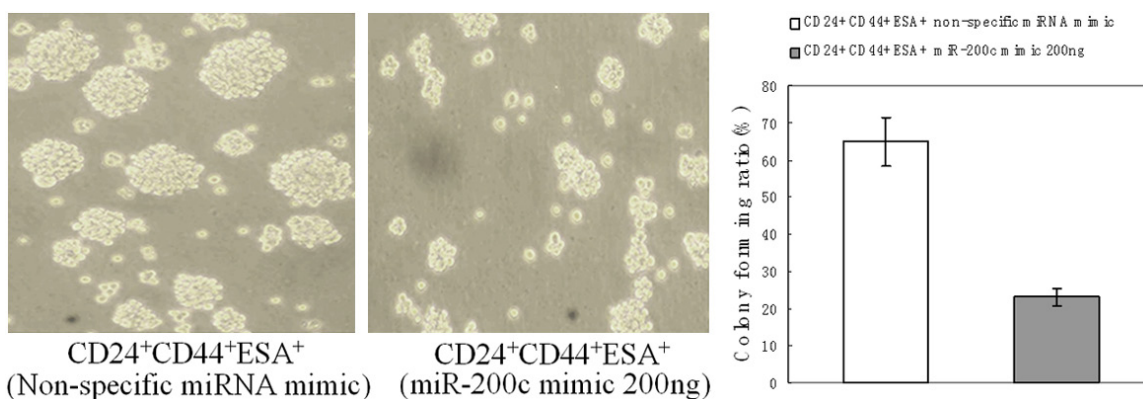


Figure 5. PCSCs transfected with miR-200c mimic reduced the ability of colony formation compared to control.

are closely associated with the miR-200 family expression.

In the present study, we presented the first group of data characterizing the biological functions of miR-200c in PCSCs. The findings from our study demonstrated that the population of the rare CD24⁺CD44⁺ESA⁺ PCSCs (5%) existed in the human PANC-1 cell line, and that the PCSCs showed not only lower expression of miR-200c but also stronger capability of colony formation, invasion and chemoresistance than the PANC-1 cell line. With the stable miR-200c overexpression in the PCSCs, the cells markedly decreased the colony forming capability. It is known that the tumor cell cloning efficiency is correlated positively with the cellular proliferation and self-renewal ability that may be associated with the cell tumorigenesis [11, 12].

Drug resistance in tumor leads to chemotherapy failure. Recent studies had showed that miR-200c and factors in its regulatory pathway were

correlated with chemoresistance [13, 14]. In this study, our data revealed that PCSCs were the chemoresistant “core cells” in pancreatic cancer cells and miR-200c overexpression could increase the sensitivity of PCSCs to gemcitabine. Our findings were in agreement with a recent report that besides the involvement of target genes of miR-200c in the drugresistance of cancer cells, miR-200c also increases the sensitivity of breast cancer MDAMB231 cells to adriamycin (ADR) [14].

Our results also showed that miR-200c acted as a tumor suppressor during PCSCs invasion. The expression of miR-200c was negatively correlated with the invasion of PCSCs. Moreover, our results suggest that decreased miR-200c levels promoted, increased miR-200c levels inhibited PCSCs invasion in vitro. The activity of miR-200c in relation to epithelial-to-mesenchymal transition (EMT)-associated phenotypes has been extensively studied. Previous studies showed that miR-200c inhibits cancer invasion

and metastasis by negatively regulating EMT [15, 16].

In summary, the findings from our experiments demonstrate that miR-200c was significantly underexpressed in PCSCs which were the root of malignant biological characteristics of pancreatic cancer. Overexpression of miR-200c could reduce invasion, increase sensitivity to anti-cancer drug and inhibit colony formation. These findings suggest that the method of enhancing tumor suppressor miR-200c may provide a novel therapeutic approach for patients with pancreatic cancer.

Disclosure of conflict of interest

None.

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References

- [1] Jemal A, Siegel R, Xu J and Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; 60: 277-300.
- [2] Chen W, Zheng R, Zhang S, Zhao P, Li G, Wu L and He J. Report of incidence and mortality in China cancer registries, 2009. *Chin J Cancer Res* 2013; 25: 10-21.
- [3] Hirata K, Egawa S, Kimura Y, Nobuoka T, Oshima H, Katsuramaki T, Mizuguchi T and Furuhashi T. Current status of surgery for pancreatic cancer. *Dig Surg* 2007; 24: 137-147.
- [4] Gillen S, Schuster T, Meyer Zum Buschenfelde C, Friess H and Kleeff J. Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. *PLoS Med* 2010; 7: e1000267.
- [5] Korb E, Ptak-Belowska A, Brzozowski T. Mechanisms promoting physiological cells progression into tumorigenesis. *J Physiol Pharmacol* 2012; 63: 565-570.
- [6] Pillai RS. MicroRNA function: multiple mechanisms for a tiny RNA? *RNA* 2005; 11: 1753-1761.
- [7] Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; 6: 857-866.
- [8] Papagiannakopoulos T, Kosik KS. MicroRNAs: regulators of oncogenesis and stemness. *BMC Med* 2008; 6: 15.
- [9] Palanichamy JK, Rao DS. miRNA dysregulation in cancer: towards a mechanistic understanding. *Front Genet* 2014; 5: 54.
- [10] Hede K. Studies define role of microRNA in cancer. *J Natl Cancer Inst* 2005; 15: 1114-1115.
- [11] Dou J, Li Y, Zhao F, Hu W, Wen P, Tang Q, Chu L, Wang Y, Cao M, Jiang C and Gu N. Identification of tumor stem-like cells in a mouse myeloma cell line. *Cell Mol Biol (Noisy-le-Grand)* 2009; 55 Suppl: OL1151-OL1160.
- [12] Matsui W, Huff CA, Wang Q, Malehorn MT, Barber J, Tanhehco Y, Smith BD, Civin CI and Jones RJ. Characterization of clonogenic multiple myeloma cells. *Blood* 2004; 103: 2332-2336.
- [13] Adam L, Zhong M, Choi W, Qi W, Nicoloso M, Arora A, Calin G, Wang H, Siefker-Radtke A, McConkey D, Bar-Eli M and Dinney C. miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy. *Clin Cancer Res* 2009; 15: 5060-5072.
- [14] Tryndyak VP, Beland FA, Pogribny IP. E-cadherin transcriptional down-regulation by epigenetic and microRNA-200 family alterations is related to mesenchymal and drug-resistant phenotypes in human breast cancer cells. *Int J Cancer* 2010; 126: 2575-2583.
- [15] Korpai M, Kang Y. The emerging role of miR-200 family of miRNAs in epithelial-to-mesenchymal transition and cancer metastasis. *RNA Biol* 2008; 5: 115-119.
- [16] Hurteau GJ, Carlson JA, Spivack SD and Brock GJ. Overexpression of the miRNA has-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. *Cancer Res* 2007; 67: 7972-7976.