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miR-203 inhibits melanoma invasive and proliferative abilities by targeting the polycomb group gene BMI1

Xiao Chang [a,1], Yong Sun [b,1], Siqi Han [c], Wei Zhu [a], Haiping Zhang [a,*], Shi Lian [d,*]

[a] Department of Dermatology and Venereal Disease, Xuanwu Hospital, Capital Medical University, Beijing 100053, China
[b] Department of Burn and Plastic Surgery, Huai'an First People's Hospital, Nanjing Medical University, Huai'an 223300, China
[c] Department of Medical Oncology, Jinling Hospital, Nanjing 210002, China
[d] Department of Dermatology and Venereal Disease, Capital Medical University, Beijing 100069, China

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ABSTRACT

Metastasis is the major problem in malignant melanoma, posing a therapeutic challenge to clinicians. The investigation of the underlying mechanism driving this progress remains a large unmet need. In this study, we revealed a miR-203-BMI1 axis that regulated melanoma metastasis. We found significantly deregulation of miR-203 and up-regulation of BMI1 in melanoma, particularly in metastatic melanoma. An inverse correlation between the levels of miR-203 and BMI1 was further observed in melanoma tissues and cell lines. We also identified BMI1 as a downstream target gene of miR-203, which bound to the 3′UTR of BMI1. Overexpression of miR-203 was associated with decreased BMI1 expression and impaired cell invasion and tumor sphere formation activities. Re-expression of BMI1 markedly rescued miR-203-mediated suppression of these events. Taken together, our results demonstrated that miR-203 regulated melanoma invasive and proliferative abilities in part by targeting BMI1, providing new insights into potential mechanisms of melanoma metastasis.

1. Introduction

Cutaneous melanoma is an aggressive malignancy accounting for 80% of skin cancer-related deaths with still increasing incidence [1]. Although surgical excision is an effective therapy against localized disease, the median survival of patients with metastatic melanoma, following treatment with radiation and chemotherapy, is only 6–9 months historically [2,3]. The investigation of potential therapeutic targets that mediate the progress of melanoma metastasis remains a large unmet need.

microRNAs (miRNAs) are short non-coding RNAs (18–22 nt) that regulate gene expression through the endogenous RNA interference machinery [4]. Cumulative evidence indicates that miRNAs may function as tumor promoters or suppressors, regulating a wide range of biologic processes such as proliferation, apoptosis, invasion and stem cell maintenance [5]. Many miRNAs have been reported to be involved in tumor development via regulation of target networks [6,7]. In particular, miR-203 has been extensively studied in several cancers regulating cell proliferation, invasion and stemness [8,9]. Recently, miRNAs have been implicated as key regulators influencing the development of melanoma [6,10]. However, the functions of miR-203 and its target gene in regulating melanoma development are poorly understood.

BMI1 belongs to the Polycomb Group (PcG) gene family that functions as transcriptional repressors regulating cancer development. Recent studies have revealed that BMI1 is required for the self-renewal of cancer stem cells [11]. Aberrant BMI1 overexpression has been detected in several human cancers and associated with poor prognosis [12,13], highlighting the importance of BMI1 in tumor development. However, the expression of BMI1 in melanoma and its function in regulating melanoma metastasis are poorly understood.

In this study, we found deregulation of miR-203 and up-regulation of BMI1 in melanoma, particularly in metastatic melanoma. Overexpression of miR-203 was associated with decreased BMI1 expression and impaired cell invasion and tumor sphere formation activities, which were further rescued by re-expression of BMI1, suggesting the potential role of miR-203-BMI1 axis in controlling melanoma metastasis.

* Corresponding authors at: Department of Dermatology and Venereal Disease, Xuanwu Hospital, Capital Medical University, 45 Changchun Street, Western District, Beijing 100053, China (H. Zhang). Department of Dermatology and Venereal Disease, Capital Medical University, 10 Xitoutiao, YouAnMen, Beijing 100069, China (S. Lian).

E-mail addresses: zhanghaiping_2000@163.com (H. Zhang), lianshi_2020@163.com (S. Lian).

[1] Xiao Chang and Yong Sun contributed equally to this work and are joint first authors.

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2. Materials and methods

2.1. Tissue specimens, cell lines and transfection

The collection of tumor specimens from melanoma patients was approved by our Institutional Review Board (IRB). A panel of melanoma cell lines (A375, A2058, SKMEL13, HT144 and SKMEL5) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in DMEM (Hyclone, Logan, UT, USA) containing 10% heat inactivated fetal bovine serum (FBS; Hyclone) and 100 Units/ml penicillin, 100 mg/ml streptomycin (Hyclone). Control-miRNA (pre-miR-control), pre-miR-203 (Applied Biosystems, Foster City, CA, USA), pcDNA3.1 control and pcDNA3.1-BMI1 were transfected into cells using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

2.2. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from tissues and cell lines using TRIzol reagent (Invitrogen). To examine the expression of miRNAs, specific stem-loop RT primers (Applied Biosystems, Carlsbad, CA, USA) was used to obtain the cDNA, and then subjected to TaqMan microRNA assay (Applied Biosystems) using primers specific for miR-203 according to the manufacturer's protocol. U6 was used as endogenous control. Relative mRNA expression was analyzed as previously described [14]. cDNA was obtained using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). The gene expression was validated using GoTaq qPCR Master Mix with SYBR green (Promega) with GAPDH as an internal control. Primers for genes were obtained from a pre-validated source, PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

2.3. Western blot and immunohistochemistry (IHC)

For Western blot analysis, whole-cell lysates were prepared in RIPA buffer with protease inhibitors (Sigma, St. Louis, MI, USA), and subjected to Western blot using BMI1 antibody from Abcam (Cambridge, MA, USA). Equal loading was verified using an anti-GAPDH antibody (Abcam). For IHC, the tissue samples were cut in 4-μm sections. After antigen retrieval, the sections were incubated with anti-BMI1 (Abcam) overnight, followed by a HRP-labeled second antibody. The staining was photographed under an inverted light microscope (Olympus, Tokyo, Japan).

2.4. Dual luciferase assay

For reporter assays, A375 and A2058 cells were transiently transfected with pGL3-BMI1-3'UTR luciferase reporter vector and pre-miR-203, using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured 48 h post-transfection using the Dual-Luciferase system (Promega, Madison, Wisconsin). Mutations in the miR-203 seed-matching sequences were made with the QuikChange Mutagenesis Kit (Stragene, La Jolla, CA, USA).

2.5. In vitro invasion assay

Cell invasion assay was performed using transwell chamber (Corning, New York, NY, USA) coated with Matrigel (BD, Bedford, MA, USA). Briefly, 1×10^5 cells were plated on the top chamber in serum-free medium. Medium supplemented with serum was used as a chemoattractant in the bottom chamber. After incubation for 48 h, the non-invading cells were removed from the upper surface of the membrane with cotton swabs. The invaded cells on the lower membrane surface were fixed in 100% methanol, stained with 0.1% crystal violet and counted under a microscope (Olympus, Tokyo, Japan). The assays were conducted three independent times.

2.6. Tumor sphere-forming assays

For assessing the sphere-forming ability, 5×10^3 cells were seeded in 6 well ultra-low attachment plates (Corning) in DMEM medium containing 20 ng/ml epidermal growth factor (EGF; R&D Systems, Minneapolis, MN, USA), 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems), 10 mM HEPES and B-27 supplement (Invitrogen). Sphere size and number was measured after 10 days of seeding.

2.7. Statistical analysis

Data are expressed as mean ± s.e.m. The significance was assessed by *t*-test or one-way ANOVA using GraphPad Prism program version 5 (GraphPad Software, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-203 was down-regulated and inversely correlated with BMI1 expression in melanoma

Since miR-203 was predicted to be potential direct regulator of BMI1 by the web based target analysis tool TargetScan 5.1 (http://www.targetscan.org), we firstly analyzed the expression of miR-203 and BMI1 in 5 normal skin tissues (NS), 8 primary (PM) and 11 metastatic (MM) melanoma tissues by qRT-PCR. Compared to normal skin tissues, expression of miR-203 was significantly deregulated in PM and MM (Fig. 1A). It was demonstrated that MM had lower expression of miR-203 than that of PM, suggesting the relation of miR-203 to melanoma metastasis. In contrast, the expression of BMI1, which is associated with tumor aggressiveness and stemness, was up-regulated in melanoma (Fig. 1B and C). And the greatest elevations of BMI1 levels were found in MM tissues, consistent with the results from reanalysis of two previously published datasets (GSE8401 and GSE46517) (Fig. 1D and E), indicating that BMI1 may be associated with metastasis in melanoma. Furthermore, a strong negative correlation between miR-203 and BMI1 expressions ($r = -0.470$, $P < 0.05$) was found in melanoma tissues (Fig. 1F). Follow-up analysis for miR-203 and BMI1 expression was done in a panel of melanoma cell lines, and found similar inverse relation between miR-203 and BMI1 levels (Fig. 1G).

3.2. miR-203 inhibited the expression of BMI1 by binding to 3′UTR

To further validate targeting of BMI1 by miR-203, we investigated if the miR-203 directly interacted with the 3′UTR of BMI1 by a dual-luciferase reporter assay (Fig. 2A). A reporter plasmid harboring a mutated miR-203 binding site was used as a control. Overexpression of miR-203 significantly suppressed luciferase activity of BMI1 wild-type 3′UTR reporter constructs in both A375 and A2058 cells (Fig. 2B), while the suppressive effect of pre-miR-203 was abrogated with mutant BMI1 3′UTR. This prompted us to study the expression of BMI1 in ectopic miR-124 expressed cells revealing a significant down-regulation (Fig. 2C). These results suggesting that BMI1 was indeed a direct downstream functional target of miR-203.

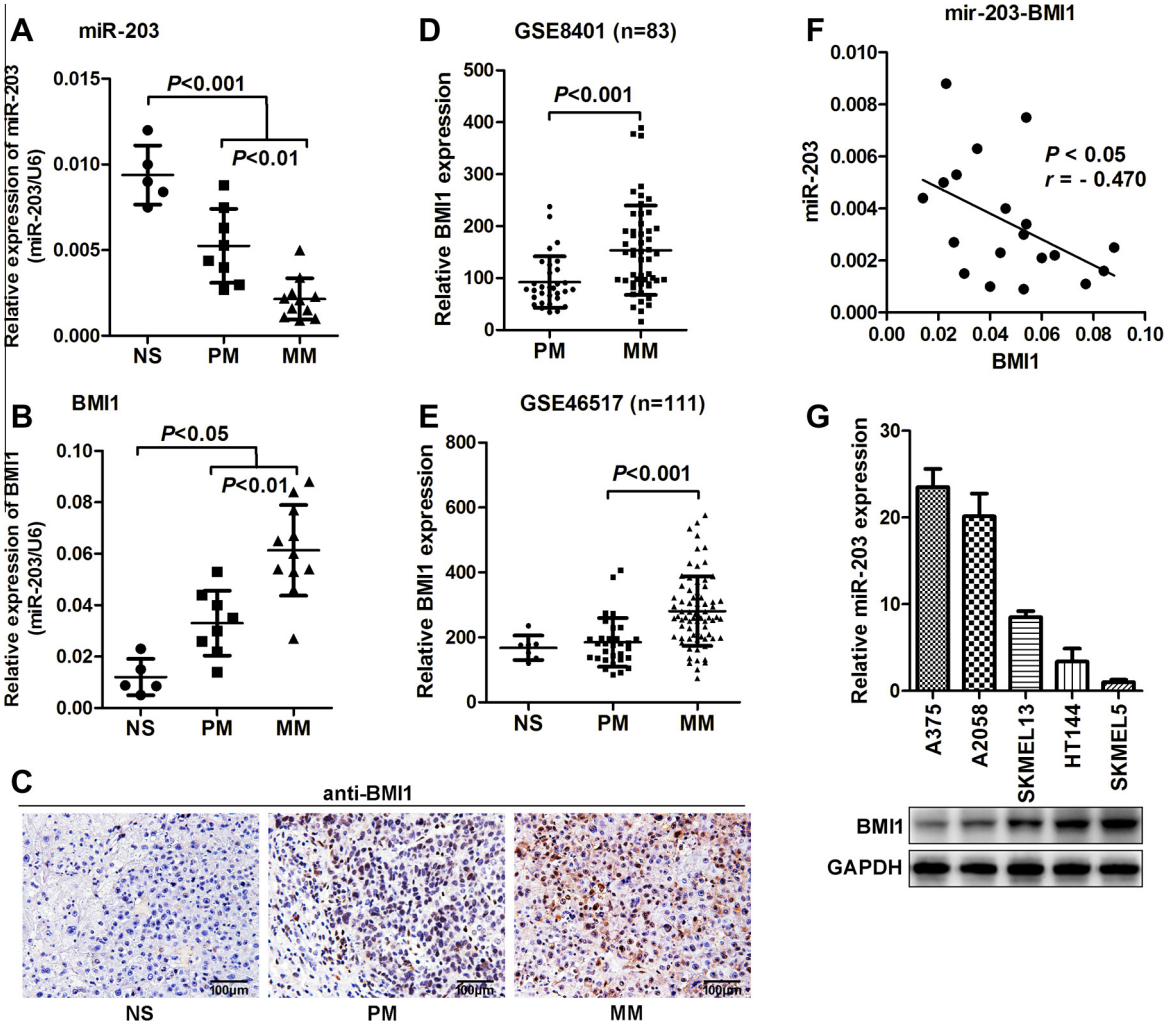

Fig. 1. miR-203 was down-regulated and inversely correlated with BMI1 expression in melanoma. (A) The expression of miR-203 in normal skin tissues (NS), primary (PM) and metastatic (MM) melanoma tissues was measured using qRT-PCR. U6 snRNA was used as a loading control. (B) qRT-PCR detecting the expression of BMI1 in NS, PM and MM tissues. (C) Immunohistochemical (IHC) staining of the BMI1 expression. Micrographs shown represent the range of staining observed in tissues. (D) Reanalysis of BMI1 expression from two previously published datasets GSE8401 and (E) GSE46517. (F) The correlation of BMI1 and miR-203 levels in melanoma tissues was shown. (G) The expression of miR-203 and BMI1 in melanoma cell lines were examined by real-time PCR and Western blot, respectively.

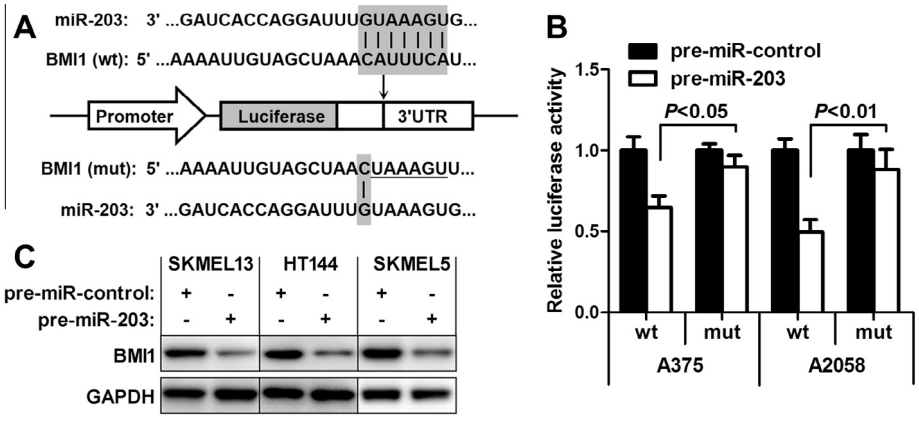

Fig. 2. miR-203 inhibited the expression of BMI1 by binding to 3′UTR. (A) Schematic representation of the human BMI1 3′UTR showing the highly conserved miR-203 binding site, and the pGL3 reporter vectors carrying the wide type or mutated BMI1 3′UTR are indicated. (B) Luciferase reporter vector was co-transfected into A375 and A2058 cells along with pre-miR-203, and the luciferase activity was measured 48 h post-transfection. (C) The expression of BMI1 in SKMEL13, HT144 and SKMEL5 cells transfected with pre-miR-203 were examined by Western blot.

3.3. miR-203 suppressed invasion and tumor sphere formation of melanoma cells

Previous studies have demonstrated that BMI1 could regulate cell invasion and proliferation [15,16]. To evaluate whether the tumor-suppressive function of miR-203 were consistent with BMI1 deregulation in melanoma development, the effects of miR-203 on cell invasion and tumor sphere formation were measured. Overexpression of miR-203 by transfection of pre-miR-203 markedly suppressed the invasive ability of HT144 and SKMEL5 cells (Fig. 3A and B). It has been reported that sphere forming capacity reflected the self-renewal and proliferative abilities [17]. We also determine the formation of tumor sphere by melanoma cells, and found a smaller number of tumor sphere in ectopic miR-124 expressed cells (Fig. 3C and D). Thus, miR-203 may play a suppressive role in melanoma invasion and proliferation.

3.4. Re-expression of BMI1 markedly rescued miR-203-mediated suppression of melanoma invasion and tumor sphere formation

To investigate whether miR-203 suppressed melanoma progression through directly and negatively regulating BMI1, an BMI1 ectopic expression vector (pcDNA3.1-BMI1) was used. As shown in Fig. 4A, BMI1 was re-expressed in cells with pre-miR-control/203 transfection. Overexpression of BMI1 by transfecting pcDNA3.1-BMI1 led to markedly enhancement of the cellular invasion (Fig. 4A) and tumor sphere formation activities (Fig. 4B). While cells expressed high levels of miR-203 exhibited a decrease in invasion and sphere formation (Fig. 3), re-expression of BMI1 in A375 and A2058 cells, as confirmed by Western blot analysis (Fig. 4A), restored the capacities of invasion (Fig. 4B) and tumor sphere formation (Fig. 4B).

4. Discussion

Metastasis is a multistage process that occurs frequently in melanoma, accounting for the vast majority of morbidity and mortality associated with melanoma [18]. There are currently no FDA-approved therapeutic options that markedly improve overall survival (OS) of patients with the metastatic tumor [19]. Despite much work has been made to uncover the etiology and pathogenesis of melanoma, identifying the underlying mechanisms that mediate melanoma metastasis remains a large unmet need. It has been generally considered that miRNAs function as specific post-transcriptional regulators of gene expression that impacts melanoma development [4,5]. Researchers have shown that miR-203 is drastically deregulated in many human cancers, including melanoma [6,10]. However, the expression of miR-203 in metastatic melanoma and its functions underlying the malignant behavior of transformed melanocytes remain unclear.

In this study, we examined the expression of miR-203 in normal skin, primary and metastatic melanoma tissues, also found a significantly deregulation of miR-203 in melanoma compared with normal skin tissues (Fig. 1A). Notably, the expression of miR-203 was further reduced in metastatic melanoma, suggesting that miR-203 may play an important role in melanoma metastasis. Furthermore, the underlying mechanisms of cancer metastasis are still only partly unraveled. Cell invasive and proliferative

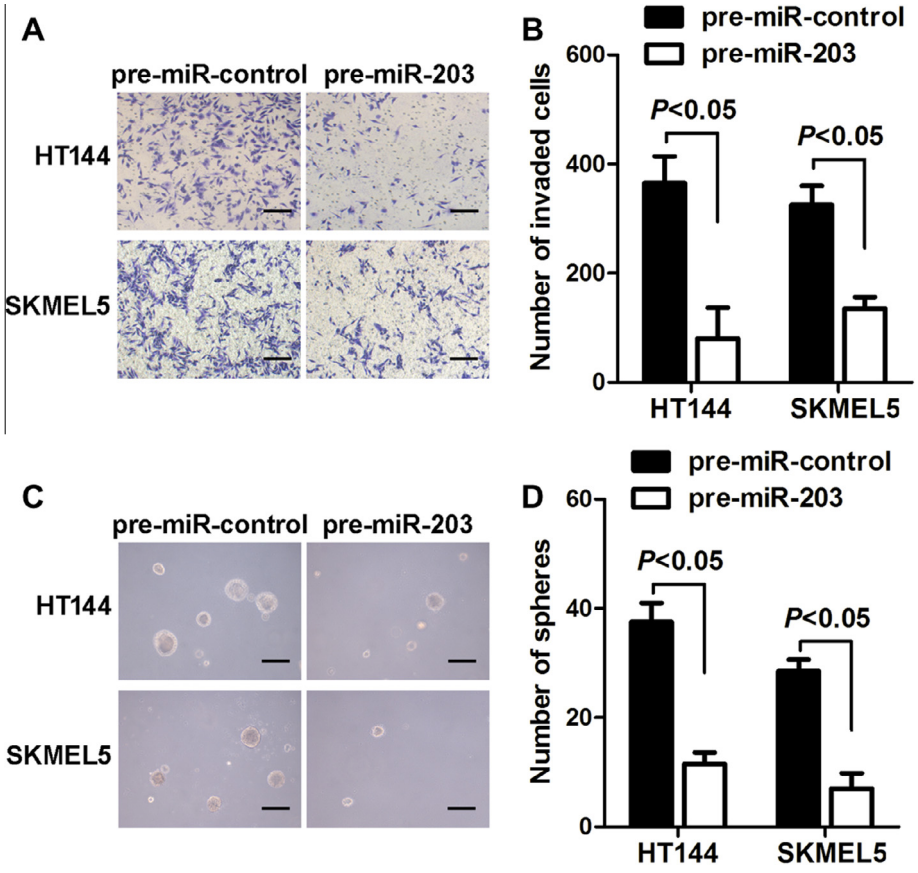

Fig. 3. miR-203 suppressed invasion and tumor sphere formation of melanoma cells. (A) Cell invasion was determined in HT144 and SKMEL5 cells transfected with pre-miR-203. (B) The invaded cells were counted and average numbers were calculated. (C) The morphology of tumor spheres formed by the cancer stem cells from melanoma cells was shown. (D) The number of tumor spheres was further measured.

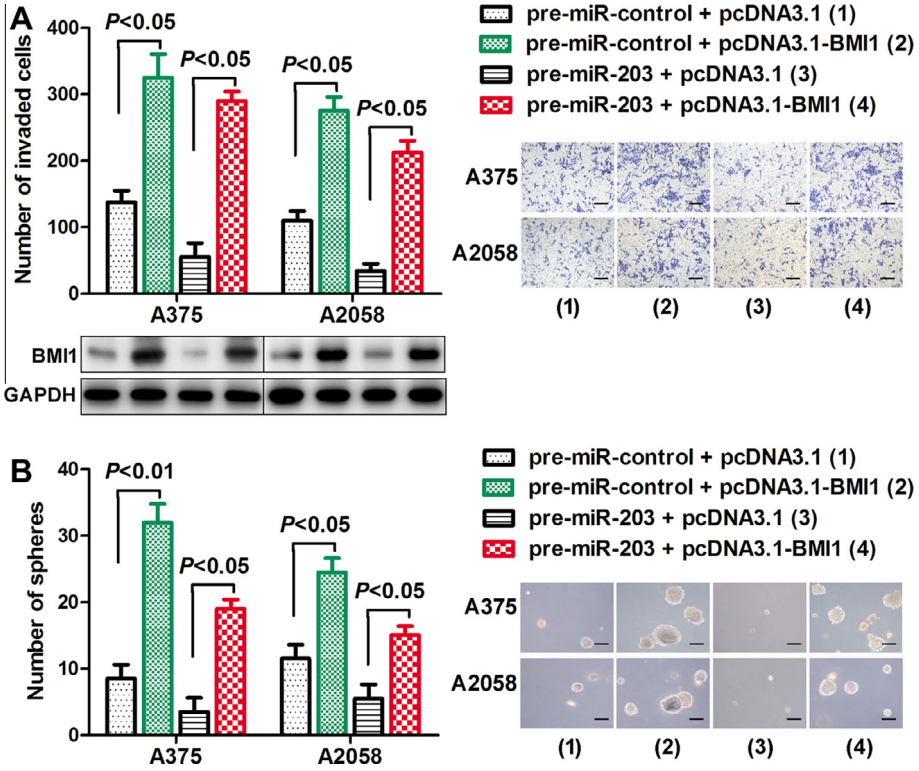

Fig. 4. Re-expression of BMI1 markedly rescued miR-203-mediated suppression of melanoma invasion and tumor sphere formation. (A) A375 and A2058 cells were transfected with pre-miR-203 and subsequently with BMI1 expression vector, and the expression of BMI1 were analyzed by Western blot. Cell invasion was analyzed by Matrigel invasion assays. The invaded cells were summarized in the bar graph and representative images was shown. (B) Melanoma cells were treated as described above, and the formation of tumor spheres was measured, and representative images was shown.

abilities seem to contribute to the oncological phenomena [20,21]. Cumulative evidence indicates that miRNAs are implicated in regulating these events, providing new sight into the understanding cancer development and metastasis [22]. We found that overexpression of miR-203 dramatically decreased the cell invasive and proliferative abilities (Fig. 3), suggesting that miR-203 may suppress melanoma metastasis partly by impairing these progresses.

We further identified BMI1 as a downstream target gene of miR-203, which bound to the 3′UTR of BMI1 and subsequently decreased BMI1 expression. A significant inverse correlation between the levels of miR-203 and BMI1 was also observed in melanoma tissues and cell lines. BMI1 is a member of polycomb group epigenetic gene family that is overexpressed in diverse cancers, promoting cell proliferation, invasion and stem cell self-renewal [11,12]. However, there are few reports about the expression and functions of BMI1 in the metastasis of melanoma. Our data showed that BMI1 was significantly elevated in melanoma, particularly in metastatic melanoma, suggesting that BMI1 may be involved in melanoma metastasis. Indeed, we observed a significant increase in cell invasion and the formation of tumor spheres after instantaneously overexpressing BMI1 (Fig. 4). Notably, re-expression of BMI1 markedly rescued miR-203-mediated suppression of these events, indicating that miR-203 regulates melanoma invasion and proliferation in part by blocking BMI1-associated pathways.

In conclusion, our results proposed a novel mechanism regulating melanoma metastasis by miR-124-BMI1 axis, providing opportunities for developing new treatment strategies.

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References

[1] A.J. Miller, M.C. Mihm Jr., Melanoma, N. Engl. J. Med. 355 (2006) 51–65.
[2] D.L. Cummins, J.M. Cummins, H. Pantle, M.A. Silverman, A.L. Leonard, A. Chanmugam, Cutaneous malignant melanoma, Mayo Clin. Proc. 81 (2006) 500–507.
[3] H.J. Gogas, J.M. Kirkwood, V.K. Sondak, Chemotherapy for metastatic melanoma: time for a change?, Cancer 109 (2007) 455–464
[4] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[5] V. Ambros, The functions of animal microRNAs, Nature 431 (2004) 350–355.
[6] K. Boll, K. Reiche, K. Kasack, N. Morbt, A.K. Kretzschmar, J.M. Tomm, G. Verhaegh, J. Schalken, M. von Bergen, F. Horn, J. Hackermuller, MiR-130a, miR-203 and miR-205 jointly repress key oncogenic pathways and are downregulated in prostate carcinoma, Oncogene 32 (2013) 277–285.
[7] C. Luo, P.W. Tetteh, P.R. Merz, E. Dickes, A. Abukiwan, A. Hotz-Wagenblatt, S. Holland-Cunz, T. Sinnberg, B. Schittek, D. Schadendorf, S. Diederichs, S.B. Eichmuller, MiR-137 inhibits the invasion of melanoma cells through downregulation of multiple oncogenic target genes, J. Invest. Dermatol. 133 (2013) 768–775.
[8] X. Zhou, G. Xu, C. Yin, W. Jin, G. Zhang, Down-regulation of miR-203 induced by *Helicobacter pylori* infection promotes the proliferation and invasion of gastric cancer by targeting CASK, Oncotarget (2014).
[9] S.Y. Ju, S.H. Chiou, Y. Su, Maintenance of the stemness in CD44(+) HCT-15 and HCT-116 human colon cancer cells requires miR-203 suppression, Stem Cell Res. 12 (2014) 86–100.
[10] J. Kozubek, Z. Ma, E. Fleming, T. Duggan, R. Wu, D.G. Shin, S.S. Dadras, In-depth characterization of microRNA transcriptome in melanoma, PLoS ONE 8 (2013) e72699.
[11] I.K. Park, S.J. Morrison, M.F. Clarke, BMI1, stem cells, and senescence regulation, J. Clin. Invest. 113 (2004) 175–179.
[12] L. Jiang, J. Wu, Y. Yang, L. Liu, L. Song, J. Li, M. Li, Bmi-1 promotes the aggressiveness of glioma via activating the NF-kappaB/MMP-9 signaling pathway, BMC Cancer 12 (2012) 406.
[13] J. Silva, V. Garcia, J.M. Garcia, C. Pena, G. Dominguez, R. Diaz, Y. Lorenzo, A. Hurtado, A. Sanchez, F. Bonilla, Circulating Bmi-1 mRNA as a possible prognostic factor for advanced breast cancer patients, Breast Cancer Res. 9 (2007) R55.
[14] G. Li, L. Zhao, W. Li, K. Fan, W. Qian, S. Hou, H. Wang, J. Dai, H. Wei, Y. Guo, Feedback activation of STAT3 mediates trastuzumab resistance via upregulation of MUC1 and MUC4 expression, Oncotarget 5 (2014) 8317–8329.

[15] Y. Tu, X. Gao, G. Li, H. Fu, D. Cui, H. Liu, W. Jin, Y. Zhang, MicroRNA-218 inhibits glioma invasion, migration, proliferation, and cancer stem-like cell self-renewal by targeting the polycomb group gene BMI1, Cancer Res. 73 (2013) 6046–6055.
[16] J. Godlewski, M.O. Nowicki, A. Bronisz, S. Williams, A. Otsuki, G. Nuovo, A. Raychaudhury, H.B. Newton, E.A. Chiocca, S. Lawler, Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal, Cancer Res. 68 (2008) 9125–9130.
[17] L.E. Barrett, Z. Granot, C. Coker, A. Iavarone, D. Hambardzumyan, E.C. Holland, H.S. Nam, R. Benezra, Self-renewal does not predict tumor growth potential in mouse models of high-grade glioma, Cancer Cell 21 (2012) 11–24.
[18] I. Bedrosian, M.B. Faries, D.T. Guerry, R. Elenitsas, L. Schuchter, R. Mick, F.R. Spitz, L.P. Bucky, A. Alavi, D.E. Elder, D.L. Fraker, B.J. Czerniecki, Incidence of sentinel node metastasis in patients with thin primary melanoma (< or =1 mm) with vertical growth phase, Ann. Surg. Oncol. 7 (2000) 262–267.
[19] J.M. Ko, D.E. Fisher, A new era: melanoma genetics and therapeutics, J. Pathol. 223 (2011) 241–250.
[20] L. Yang, Y.F. Ping, X. Yu, F. Qian, Z.J. Guo, C. Qian, Y.H. Cui, X.W. Bian, Gastric cancer stem-like cells possess higher capability of invasion and metastasis in association with a mesenchymal transition phenotype, Cancer Lett. 310 (2011) 46–52.
[21] N. Tapon, U. Ziebold, Invasion and metastasis: stem cells, screens and survival. Conference on Invasion and Metastasis, EMBO Rep. 9 (2008) 1078–1083.
[22] A. Aigner, MicroRNAs (miRNAs) in cancer invasion and metastasis: therapeutic approaches based on metastasis-related miRNAs, J. Mol. Med. (Berl.) 89 (2011) 445–457.