

Anti-oncogenic MicroRNA-203 Induces Senescence by Targeting E2F3 Protein in Human Melanoma Cells[§]

Received for publication, November 16, 2011, and in revised form, February 9, 2012. Published, JBC Papers in Press, February 21, 2012, DOI 10.1074/jbc.M111.325027

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Background: MicroRNA-203 is down-regulated, and its exogenous expression inhibits cell growth in human melanoma cells.

Results: MicroRNA-203 induced senescence by cell cycle arrest through targeting E2F3.

Conclusion: MicroRNA-203 is a novel senescence-associated microRNA in melanoma cells.

Significance: This study has revealed the relationship between senescence and carcinogenesis in melanoma cells with respect to dysregulation of anti-oncogenic microRNA-203.

MicroRNAs regulate gene expression by repressing translation or directing sequence-specific degradation of their complementary mRNA. We recently reported that miR-203 is down-regulated, and its exogenous expression inhibits cell growth in canine oral malignant melanoma tissue specimens as well as in canine and human malignant melanoma cells. A microRNA target database predicted E2F3 and ZBP-89 as putative targets of microRNA-203 (miR-203). The expression levels of E2F3a, E2F3b, and ZBP-89 were markedly up-regulated in human malignant melanoma Mewo cells compared with those in human epidermal melanocytes. miR-203 significantly suppressed the luciferase activity of reporter plasmids containing the 3'-UTR sequence of either *E2F3* or *ZBP-89* complementary to miR-203. The ectopic expression of miR-203 in melanoma cells reduced the levels of E2F3a, E2F3b, and ZBP-89 protein expression. At the same time, miR-203 induced cell cycle arrest and senescence phenotypes, such as elevated expression of hypophosphorylated retinoblastoma and other markers for senescence. Silencing of *E2F3*, but not of *ZBP-89*, inhibited cell growth and induced cell cycle arrest and senescence. These results demonstrate a novel role for miR-203 as a tumor suppressor acting by inducing senescence in melanoma cells.

Malignant melanoma is one of the most common skin cancers in humans (1). The incidence of malignant melanoma continues to rise more rapidly than that of all other malignancies except for lung cancer (2). Malignant melanoma is a spontaneous, highly aggressive neoplasm that can readily metastasize to other organs. In recent studies on malignant melanoma, both MAPK and PI3K/Akt signaling pathways have been a matter of focus, and their signalings are known to be constitutively activated through multiple mechanisms (3). BRAF (v-Raf murine

sarcoma viral oncogene homolog B1) inhibitors, MEK inhibitors, and ipilimumab (an anti-CTLA-4 (cytotoxic T-lymphocyte antigen 4) monoclonal antibody) are promising new therapies for human malignant melanoma (4). However, safer and more effective therapies are to be desired.

MicroRNAs (miRNA or miR)² have emerged recently as a large group of short (18–25 nucleotides), noncoding, small RNA molecules that negatively regulate gene expression (5–7). Over 1300 miRNAs are predicted to exist in humans (miRBase; available on the World Wide Web). Nevertheless, the latest study indicates that these previous estimates of conserved miRNAs might be inflated (8). Although the specific biological functions of most miRNAs remain largely unknown, these molecules are believed to constitute a large gene regulatory network that can modulate the expression of up to 30% of total cellular proteins. There is increasing experimental evidence supporting the role of miRNAs in the regulation of a range of physiological responses, including development (9), cellular apoptosis (10), differentiation (11), proliferation (12), and cancer (13).

The irreversible cell cycle arrest known as senescence can be induced by a number of factors, including telomere attrition, known as replicative senescence; oxidative stress; oncogene expression; and DNA damage signaling (14). Generally, senescence can be divided into two subsets (*i.e.* replicative senescence termed telomere-initiated senescence and senescence resulting from various stresses, which is known as stress-induced premature senescence) (14). Replicative senescence can be triggered by p53 and its transcriptional target p21^{Cip1} (p53/p21 pathway) and/or by the retinoblastoma (Rb) tumor suppressor and its main upstream inducer, the Cdk inhibitor p16^{INK4a} (p16/Rb pathway). On the other hand, premature cellular senescence can be triggered by several harmful stimuli, such as a lack of optimal culture conditions, the exposure of

[§]This article contains supplemental Fig. 1.

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²The abbreviations used are: miRNA or miR, microRNA; Rb, retinoblastoma; SA- β -Gal, senescence-associated β -galactosidase; HEM, human primary epidermal melanocyte.

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supraphysiologic oxygen, or oncoproteins without an apparent loss of telomere function. Cells reaching senescence in culture, whether via replicative or premature senescence, can be identified by the presence of shorter telomeres and markers, such as senescence-associated β -galactosidase (SA- β -Gal) activity and DNA damage response proteins (14).

Several miRNAs have been shown to be involved in the regulation of pathways involved in cellular senescence, and they exert negative effects on cell cycle progression (14–16). The main senescence pathways associated with miRNAs are the p53/p21 and p16/Rb pathways (15). Especially, many studies have focused on the miR-34a/SIRT1/p53 interaction (17). Overexpression of sirtuin 1 (SIRT1), the mammalian homolog of Sir2, can delay cellular senescence and extend the cellular life span (18, 19). Therefore, down-regulation of SIRT1 leads to cellular senescence. Also, it has been suggested that ZBP-89, a Krüppel-type zinc finger transcription factor that binds to GC-rich sequences, induces senescence by inhibiting p16 expression in human lung cancer (20). Recently, it was reported that miR-205 in human melanoma cells induces senescence by targeting E2F1 (21). The E2F family of transcription factors controls cell cycle progression (16). E2F3 of the E2F family encodes two protein products (E2F3a and E2F3b) that are alternative splicing variants (22). E2F1, -2, and -3a facilitate cell cycle progression. On the contrary, E2F3b is classified as a repressor E2F, and it negatively controls the cell cycle (23). Recent studies reported that E2F1 to -3 are targets of several miRNAs, such as miR-34a (24). Therefore, senescence associated with miRNA/E2F interaction is also important.

Recently, we reported that miR-203 and -205 are down-regulated in human and canine melanoma cells and that the ectopic expression of miR-203 and -205 inhibits their cell growth (25). Here, we show that miR-203 induced senescence in human melanoma Mewo and A2058 cells and discuss the mechanism of senescence induced by ectopic miR-203 expression. Our data suggest anti-oncogenic miR-203 to be a newly recognized senescence-associated miRNA.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Viability—Human malignant melanoma cell lines Mewo and A2058 were purchased from the Health Science Research Resources Bank (Osaka, Japan), and the cells were maintained according to the manufacturer's protocol. The number of viable cells was determined by performing the trypan blue dye exclusion test. Normal human primary epidermal melanocytes (HEMs), which were purchased from ScienCell Research Laboratories (Carlsbad, CA), were cultured as recommended by the manufacturer.

Cell Transfection with miRNA or siRNA—Mewo or A2058 cells were seeded into 6-well plates at a concentration of 0.5×10^5 cells/well the day before transfection. The mature type of miR-203 (Applied Biosystems, Foster City, CA) was used for the transfection of the cells, which was achieved by using cationic liposomes, Lipofectamine RNAiMAX (Invitrogen), at a concentration of 5, 10, or 20 nM, according to the manufacturer's Lipofection protocol. Short interfering RNA (siRNA) for both E2F3a and E2F3b or ZBP-89 (1, 5, or 10 nM) was also used for transfection of Mewo cells. The sequences of these siRNAs

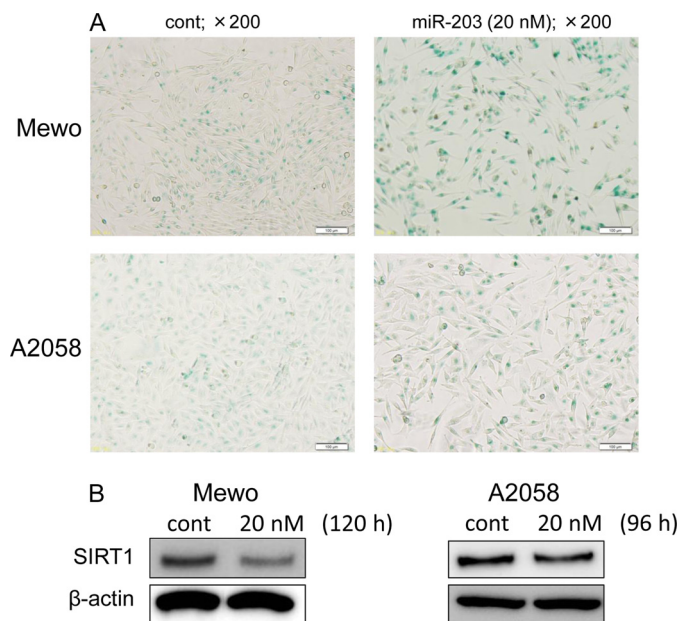


FIGURE 1. A, photographs illustrating the effect of the ectopic expression of miR-203. Cells were evaluated for SA- β -Gal activity at 120 h (Mewo) or at 96 h (A2058) after the transfection with miR-203 at 20 nM (magnification, $\times 200$). B, SIRT1 expression levels following the ectopic expression of miR-203 at the concentration of 20 nM. Total protein from the cells was obtained at 120 h (Mewo) or at 96 h (A2058) after the transfection. Error bars, S.D.

were 5'-UAACCUUGAUUCUCUGAAUCCUCG-3' (siR-E2F3; Invitrogen) and 5'-UUCACAAUGGAGCUGAAGUCCACUC-3' (siR-ZBP-89; Invitrogen). The sequence of the non-specific control miRNA used (Hokkaido System Science, Hokkaido, Japan) was 5'-GUAGGAGUAGUGAAAGGCC-3'.

Quantitative RT-PCR Using Real-time PCR—Total RNA was isolated from cells by the phenol/guanidium thiocyanate method with DNase I treatment. For determination of mRNA expression levels, total RNA was reverse transcribed with a PrimeScript[®] RT reagent kit (TaKaRa, Otsu, Japan). Real-time PCR was then performed with primers specific for E2F3 or ZBP-89 by using SYBR[®] Premix Ex Taq[™] (TaKaRa). The sequences of the primers used in this study were as follows: E2F3-sense-1996, TGTGCAATCAGGTGTCTCTC; E2F3-antisense-2350, TTCATCCAGAAGGCTGTGGA; ZBP-89-sense-4129, CAGCTAGATGAGTGATCGGA; ZBP-89-antisense-4325, CCCAACGCATAACTCAAACC. The primer for E2F3 used in this study can amplify both E2F3a and E2F3b. GAPDH was used as an internal control. The relative expression level of mRNA was calculated by the $\Delta\Delta C_t$ method.

Western Blotting—Total protein was extracted from whole cells by the procedure described previously (26). Protein contents were measured with a DC Protein Assay Kit (Bio-Rad). Ten micrograms of lysate protein for Western blotting was separated by SDS-PAGE using polyacrylamide gels and electroblotted onto a PVDF membrane (PerkinElmer Life Sciences). Details of the method used after blotting were described earlier (26). The antibodies used in this study were anti-human E2F3 rabbit polyclonal antibody (sc-878), which can detect both E2F3a and E2F3b (23), anti-human ZBP-89 mouse monoclonal antibody, anti-human E2F1 mouse monoclonal antibody, and anti-human PARP-1 mouse monoclonal antibody (Santa Cruz

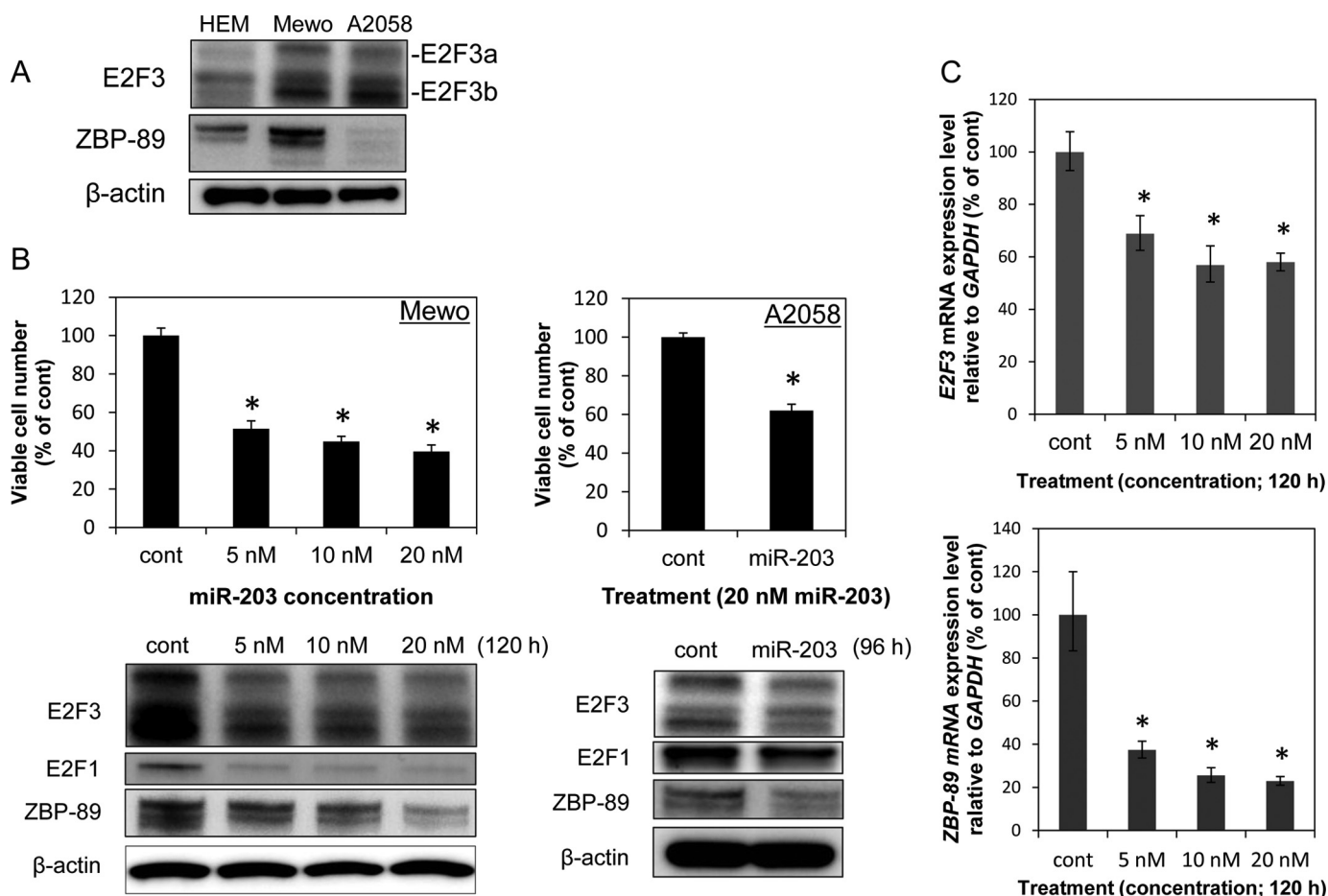


FIGURE 2. *A*, comparison of E2F3a, E2F3b, and ZBP-89 expression levels between HEM and Mewo cells or A2058 cells. *B*, effects on cell viability and the expression levels of E2F3 and ZBP-89 by miR-203 introduced at a concentration of 5, 10, or 20 nM for Mewo cells and 20 nM for A2058 cells. Viable cell counting and protein extraction were performed at 120 h (Mewo) or at 96 h (A2058) after the transfection. *C*, effects of the transfection of Mewo cells with miR-203 at 5, 10, or 20 nM on the expression levels of *E2F3* (top graph) and *ZBP-89* (bottom graph) mRNAs in the cells, as assessed by using real-time PCR at 120 h post transfection. The expression levels of mRNAs were calculated by the $\Delta\Delta C_t$ method and normalized to the level of *GAPDH* mRNA. *, $p < 0.01$. A p value was determined for the difference between the cells transfected with nonspecific control miRNA (HSS) and those transfected with various concentrations of miR-203. Error bars, S.D.

Biotechnology, Inc., Santa Cruz, CA); anti-human SIRT1 rabbit monoclonal antibody (Epitomics, Burlingame, CA); and anti-human IRS2 (insulin receptor substrate 2) rabbit polyclonal antibody, anti-human hyperphosphorylated Rb (Ser-795) rabbit polyclonal antibody, anti-human total Rb rabbit monoclonal antibody, anti-human c-MYC rabbit monoclonal antibody, anti-human phosphorylated p38 rabbit monoclonal antibody, anti-human p38 rabbit monoclonal antibody, anti-human phosphorylated ERK1/2 rabbit monoclonal antibody, anti-human ERK1/2 rabbit monoclonal antibody, anti-human phosphorylated Akt rabbit polyclonal antibody, and anti-human Akt rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA), all of which were properly diluted with TBS-T containing 2% bovine serum albumin and 0.01% sodium azide. The loading control was prepared by reincubating the same membrane with anti-human β -actin antibody (Sigma).

Staining for Senescence-associated β -Galactosidase—The cells were seeded into 6-well plates at a concentration of 0.5×10^5 cells/well the day before transfection. After the transfection with miR-203, siR-*E2F3* or siR-*ZBP-89*, Mewo cells were incubated for 120 h (for miR-203) or 96 h (for siR-*E2F3* and siR-*ZBP-89*), and A2058 cells were incubated for 96 h after the

transfection with miR-203. SA- β -Gal staining was performed by using the Senescence β -galactosidase staining kit (Cell Signaling Technology) according to the manufacturer's protocol.

Flow Cytometric Analysis—Mewo cells were seeded into 6-well plates at a concentration of 0.5×10^5 cells/well the day before transfection. After the transfection with miR-203, siR-*E2F3*, or siR-*ZBP-89*, the cells were incubated for 96 h. For flow cytometric analysis (FACSCalibur, BD Biosciences), the cells were prepared by using the BD CycletestTM Plus-DNA reagent kit (BD Biosciences) according to manufacturer's protocol. The G_0/G_1 and G_2/M ratios were calculated by using analysis software (CellQuest, BD Biosciences).

Assay for Luciferase Activity—We constructed the sensor vector by joining the regions with a possible binding site from the 3'-UTR of human *E2F3* or *ZBP-89* to a luciferase reporter pMIR-control vector (Ambion, Foster City, CA) to examine the target sequence recognized by miR-203 (Fig. 3). Based on the NCBI database, the sequences of the 3'-UTR of human *E2F3a* and *E2F3b* are the same (Fig. 3). Also, to generate the sensor vectors with one or two mutations in the binding site for miR-203, we mutated seed regions from CATTTC A to CATGTCA (mt1) or CATGACA (mt2, for *E2F3* 3'-UTR) and from

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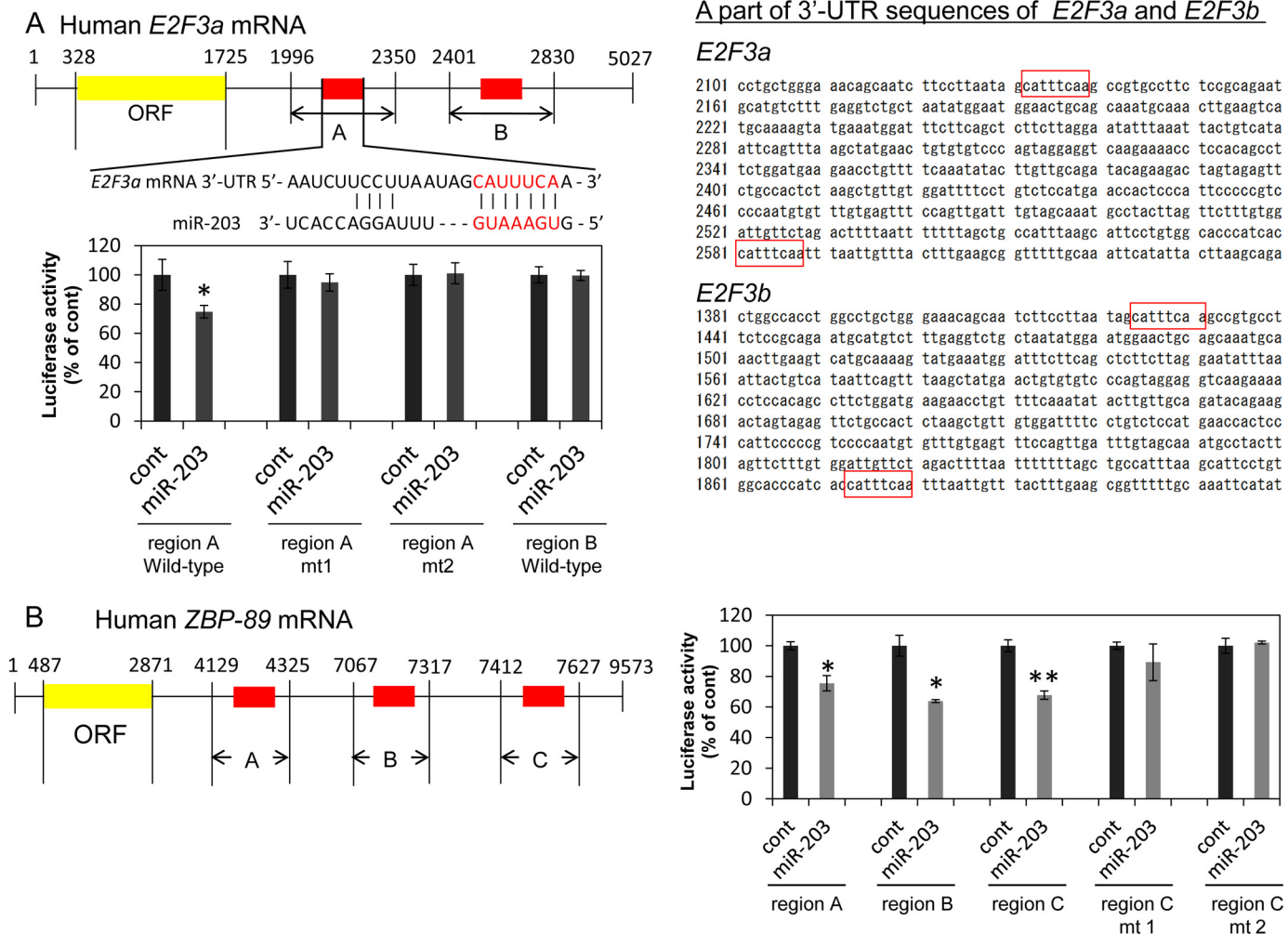


FIGURE 3. Analysis of the target regions for miR-203 in *E2F3* and *ZBP-89* mRNAs. *A*, homology in the 3'-UTR of human *E2F3* mRNA and mature miR-203. The region including miR-203 binding sites (red box) in *E2F3a* and *E2F3b* mRNAs completely matched (middle panel). *B*, regions of the 3'-UTR of human *ZBP-89* mRNA complementary to mature miR-203. The red boxes indicate the predicted binding sites for miR-203. The bar graph shows luciferase activities after co-transfection with control or miR-203 and each of the sensor vectors having the indicated 3'-UTR of *ZBP-89*. *, $p < 0.05$; **, $p < 0.01$. A p value was determined for the difference in luciferase activity between the cells transfected with nonspecific control siRNA (Dharmacon) and those transfected with miR-203. Error bars, S.D.

CATTTC~~A~~A to CATTACA (mt1) or CATTAGA (mt2, for *ZBP-89* 3'-UTR; PrimeSTAR[®] mutagenesis basal kit, TaKaRa). The sensor vectors with mutations were submitted to the Life Science Research Center (Gifu University) for DNA sequencing. The cells were seeded in 12-well plates at a concentration of 0.5×10^5 cells/well the day before the transfection. The sensor vector (concentration $0.5 \mu\text{g}/\text{well}$) and 40 nM miR-203 or nonspecific control miRNA (Dharmacon, Tokyo, Japan) was used for the co-transfection of the cells by using cationic liposomes Lipofectamine RNAiMAX. Forty-eight hours after the co-transfection, luciferase activities were measured by using the Dual-Glo[™] luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Assay for *E2F3a* Overexpression—Eukaryote pIRES-*E2F3a* expression vector was generated by inserting the open reading frame of *E2F3a* cDNA into the *Cl*I/*E*c*o*RI site of the pIRES1 *neo* vector (Clontech). Mewo cells were seeded into 6-well plates at a concentration of 0.5×10^5 /well and transfected with $0.5 \mu\text{g}/\text{well}$ of pIRES1 *neo* as a control vector or pIRES-*E2F3a*

expression vector by using Lipofectamine RNAiMAX at 2 days after the transfection with 20 nM miR-203. The effects manifested by *E2F3* overexpression were assayed at 5 days after the transfection with plasmids.

Statistics—Each examination was performed in triplicate. The cell count analysis and the expression levels of mRNAs in the cells transfected with miR-203 and those transfected with nonspecific control miRNA were compared by using Student's *t* test. A p value less than 0.05 was considered to be statistically significant.

RESULTS

Ectopic Expression of miR-203 Induced Senescence in Mewo and A2058 Cells—As shown in Fig. 1A, the number of cells positive for SA- β -Gal activity, indicating senescence, was markedly greater in Mewo and A2058 cells transfected with miR-203 than in those transfected with the control miRNA. Additionally, the expression level of SIRT1 was significantly decreased in Mewo cells and slightly decreased in A2058 cells

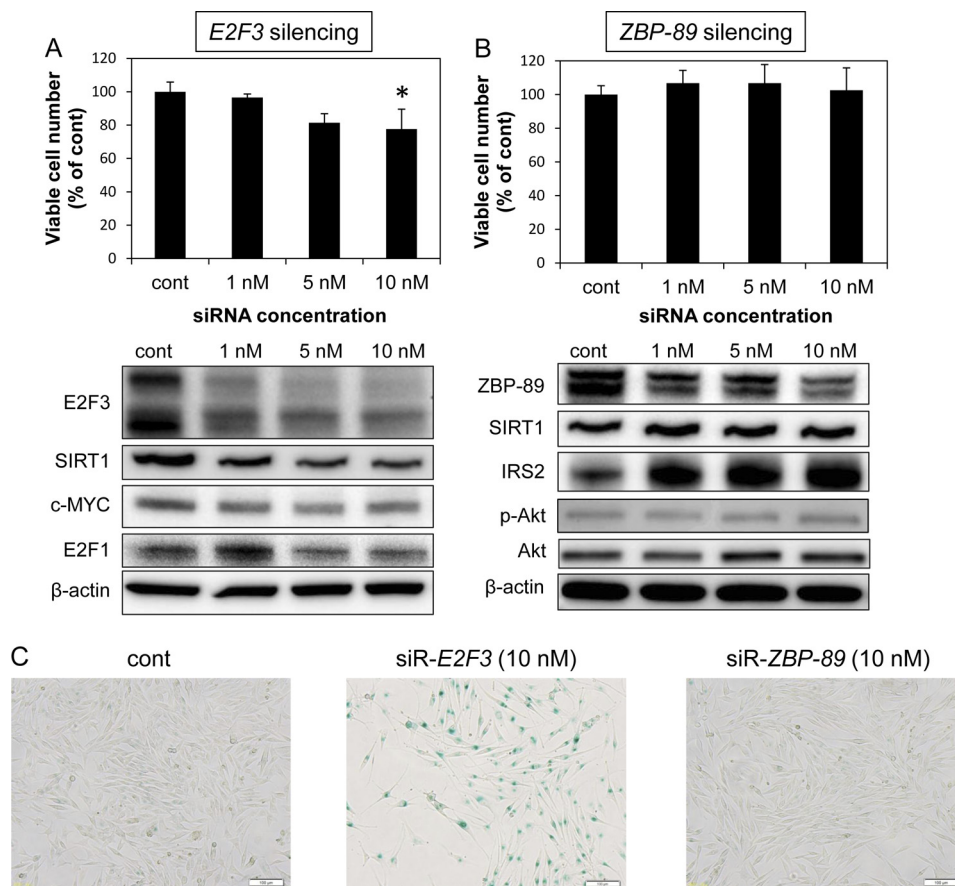


FIGURE 4. *A*, bar graph shows the effects of transfection of Mewo cells with siR-*E2F3* at a concentration of 1, 5, or 10 nM on cell growth. The bottom panels show the expression levels of *E2F3* and the target genes of *E2F3* after *E2F3* silencing. *, $p < 0.05$. A p value was determined for the difference between the cells transfected with nonspecific control miRNA (HSS) and those transfected with siRNA at the indicated concentration. *B*, bar graph shows the effects of transfection of Mewo cells with siR-*ZBP-89* at a concentration of 1, 5, or 10 nM on cell growth. The bottom panels show the expression levels of *ZBP-89* and *SIRT1*, a marker of senescence. In *A* and *B*, viable cell counting and protein extraction were performed at 96 h after the transfection. *C*, photographs illustrating the effect of the ectopic expression of 10 nM siRNAs. Cells were evaluated for SA- β -Gal activity (magnification, $\times 200$). Error bars, S.D.

by the ectopic expression of miR-203 compared with that of the control (Fig. 1B).

E2F3 and ZBP-89 Are Targets of miR-203—We hypothesized that the target involved in the senescence was *E2F3* or *ZBP-89* mRNA based on the database (TargetScan) results. Therefore, we examined the expression levels of *E2F3a*, *E2F3b*, and *ZBP-89* in Mewo, A2058, and HEM cells. As a result, the expression levels of those proteins in Mewo cells were markedly up-regulated compared with those in HEM cells (Fig. 2A). The expression level of *E2F3a* and *E2F3b* in A2058 cells was also markedly up-regulated compared with that in HEM cells. On the other hand, the *ZBP-89* expression level in A2058 cells was down-regulated compared with that in HEM cells. Furthermore, the ectopic expression of miR-203 in Mewo cells significantly and dose-dependently decreased the expression levels of *E2F3a*, *E2F3b*, and *ZBP-89* proteins (Fig. 2B) and those of their mRNAs (Fig. 2C), as well as resulting in a significant decrease in the number of viable cells (Fig. 2B). Likewise, the expression levels of *E2F3* and *ZBP-89* proteins in A2058 cells were decreased by the transfection with miR-203 (Fig. 2B). And also, the cell growth of A2058 cells was suppressed by the treatment (Fig. 2B). Additionally, the expression level of *E2F1* in Mewo and A2058 cells was also slightly decreased (Fig. 2B).

Next, we sought to confirm that *E2F3* and *ZBP-89* were target genes of miR-203 by using Mewo cells. There are two and three predicted binding sites for miR-203 in the 3'-UTR region of human *E2F3* and *ZBP-89* mRNA, respectively (Fig. 3, A and B). As expected, compared with those of the control, the luciferase activities of the wild-type pMIR-*E2F3* region A and pMIR-*ZBP-89* regions A, B, and C were significantly inhibited after the introduction of miR-203 into the Mewo cells (Fig. 3, A and B). However, the luciferase activity of the wild-type pMIR-*E2F3* region B was not inhibited by the ectopic expression of miR-203 (Fig. 3A). Mutations of the *E2F3* 3'-UTR-binding site in region A and the *ZBP-89* 3'-UTR-binding site in region C abolished the ability of miR-203 to regulate luciferase expression (Fig. 3, A and B). These results demonstrate that *E2F3* and *ZBP-89* were potential targets of miR-203.

Down-regulation of E2F3, Not ZBP-89, Resulted in Senescence—We examined the effects of siRNA-mediated *E2F3* or *ZBP-89* knockdown on cell viability and senescence to validate the role of *E2F3* or *ZBP-89* in Mewo cells. As shown in Fig. 4A, *E2F3* silencing significantly inhibited cell growth in a dose-dependent manner. However, the effect of *E2F3* silencing on the cell growth was smaller than that of miR-203 transfection. On the

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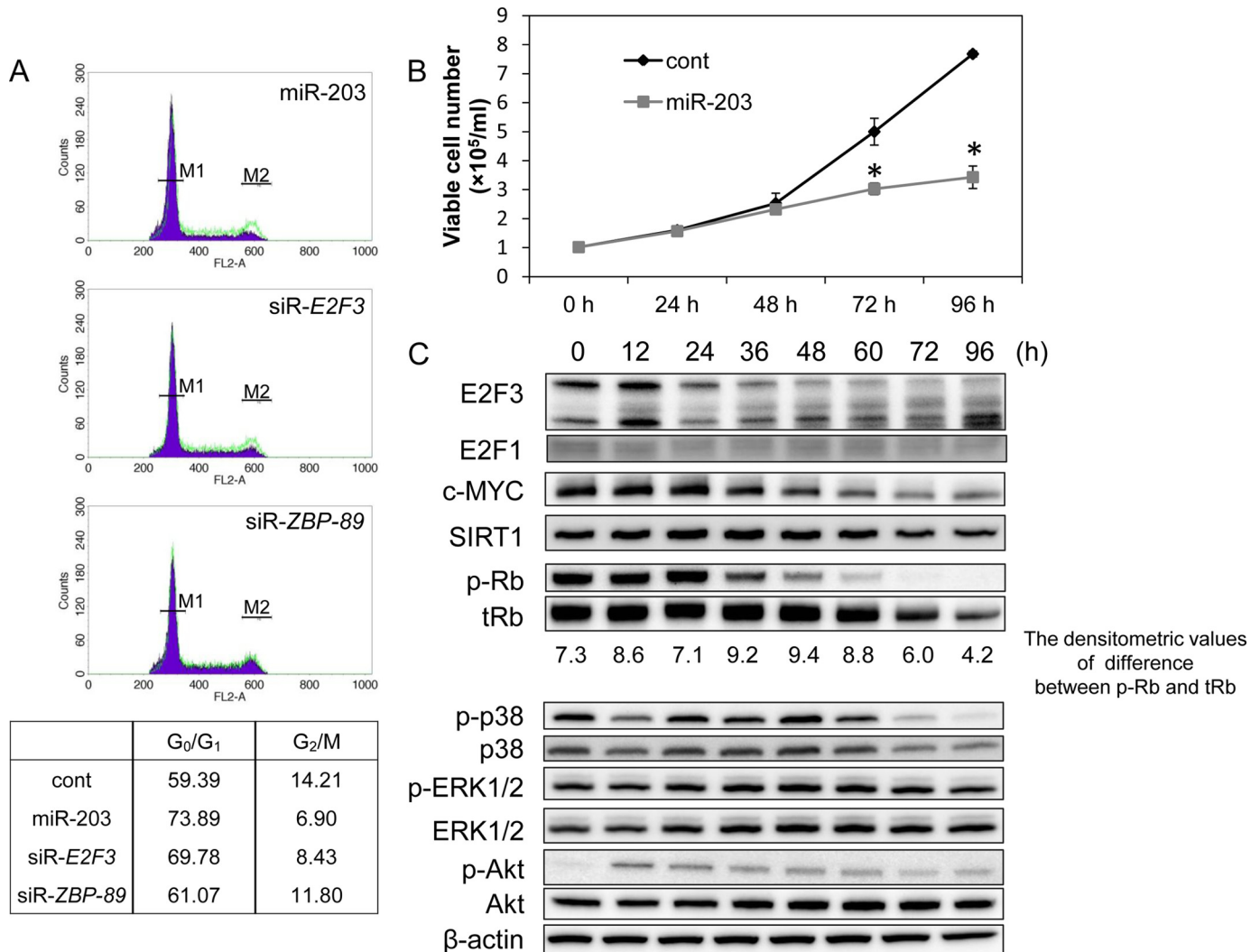


FIGURE 5. *A*, flow cytometric detection of G₀/G₁ and G₂/M ratios of Mewo cells transfected with nonspecific control miRNA (HSS), miR-203, siR-*E2F3*, or siR-*ZBP-89* at a concentration of 20 nM. The assay was performed at 96 h after the transfection. *M1* and *M2* on the histograms show the spike of G₀/G₁ and G₂/M, respectively. The green line in the histograms indicates the control. The values in the lower table are shown as percentages of gated events. *B*, cell growth curve of Mewo cells transfected with nonspecific control miRNA (HSS) or miR-203 (20 nM). The number of viable cells was counted every 24 h. The transfection was done at 0 h. **p* < 0.01. The *p* value was determined for the difference between the cells transfected with nonspecific control miRNA and those transfected with miR-203. *C*, protein expression levels of *E2F3* and its target genes (*SIRT1* and *c-MYC*), senescence-associated genes (hyperphosphorylated Rb (*p-Rb*) and total Rb (*tRb*)), and cell proliferation associated genes (phosphorylated p38 (*p-p38*), *p38*, phospho-ERK1/2 (*p-ERK1/2*), and *ERK1/2*) in Mewo cells transfected with miR-203 (20 nM). Protein extraction was performed every 12 h after the transfection. Error bars, S.D.

other hand, *ZBP-89* silencing did not inhibit cell growth (Fig. 4*B*). Also, *E2F3* silencing induced senescence, because *SIRT1* expression was reduced and SA- β -Gal activity was markedly increased (Fig. 4, *A* and *C*, respectively). Furthermore, the expression levels of *c-MYC* and *E2F1* were also decreased by *E2F3* silencing. However, *ZBP-89* silencing did not result in senescence, because the *SIRT1* expression level was not decreased and the SA- β -Gal activity was not increased (Fig. 4, *B* and *C*). Additionally, the expression level of *IRS2*, which is an activator of insulin/IGF-1 signaling, and that of phospho-Akt were slightly up-regulated by *ZBP-89* silencing (10 nM; Fig. 4*B*).

Effect of miR-203 on Regulators of Cell Cycle and Cell Proliferation—As shown in Fig. 5*A*, flow cytometric analysis revealed that the G₀/G₁ ratio was increased and the G₂/M ratio was decreased by the induction of miR-203 or siR-*E2F3* into the Mewo cells. These findings indicate that cell cycle arrest was

induced by either of the RNAs. However, *ZBP-89* silencing did not lead to cell cycle arrest.

The cell growth was significantly inhibited at 72 h after the transfection with miR-203 (Fig. 5*B*). Consistent with the results of the flow cytometric analysis and cell growth inhibition, the expression levels of cell cycle regulators, such as *E2F3* and *c-MYC*, which interact with each other, were down-regulated starting at 36 h after the transfection with miR-203 and gradually decreased more after that (Fig. 5*C*). Inversely, the expression level of hypophosphorylated Rb, which was expressed as the difference between hyperphosphorylated Rb and total Rb, was increased at 36 and 48 h after the transfection. On the other hand, the expression levels of *SIRT1* and phospho-p38 were significantly reduced at 72 h. However, phospho-ERK1/2 expression remained almost unchanged. Although the phospho-Akt expression level was up-regulated at 12 h, it gradually decreased thereafter.

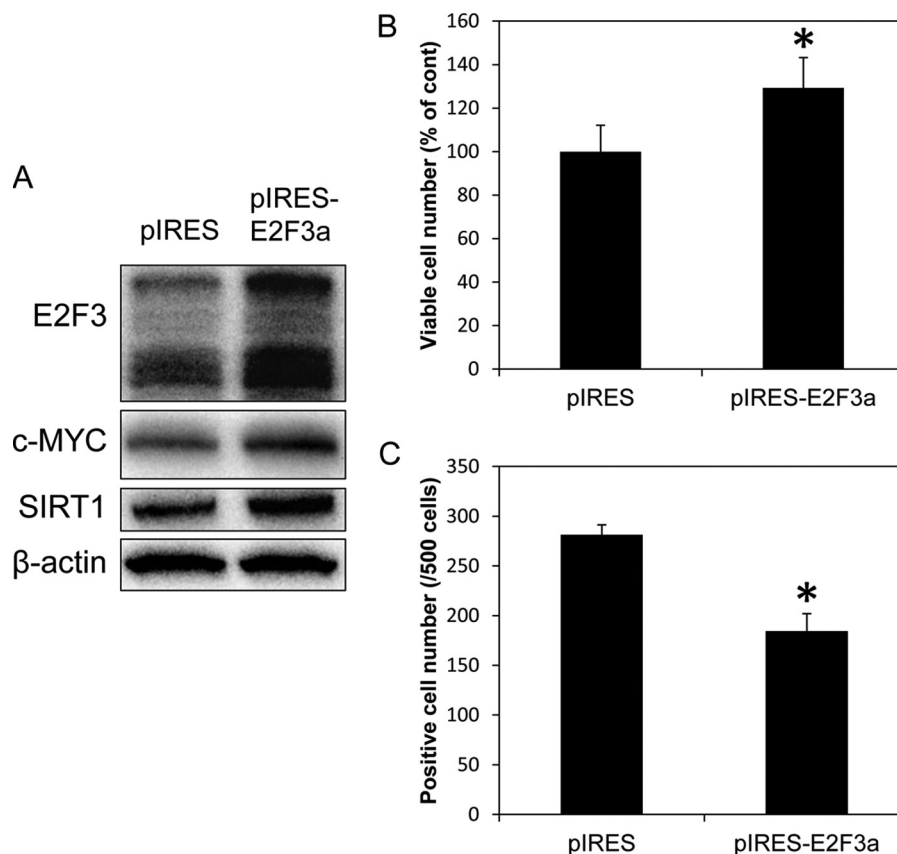


FIGURE 6. *A*, expression levels of E2F3, c-MYC, and SIRT1 after introducing pIRES-E2F3a vector or pIRES1 *neo* control vector into Mewo cells. *B*, number of viable cells among miR-203-transfected Mewo cells incubated with pIRES1 *neo* control vector or pIRES-E2F3a vector. *C*, number of cells positive for SA- β -Gal staining after the introduction of pIRES1 *neo* control or pIRES-E2F3a vector into Mewo cells. pIRES indicates pIRES1 *neo* control vector. *, $p < 0.05$. The p value was determined for the difference between the cells transfected with the pIRES1 *neo* control vector and those transfected with the pIRES-E2F3a vector. Error bars, S.D.

E2F3a Overexpression Rescued Cells from Senescence Induced by miR-203—Overexpression of E2F3a in Mewo cells transfected with miR-203 resulted in up-regulation of c-MYC and SIRT1, significantly increased the number of viable cells, and significantly decreased the number of SA- β -Gal-positive cells (Fig. 6, A–C).

DISCUSSION

This study demonstrated that the ectopic expression of miR-203, which functions as anti-oncomir in human and canine malignant melanoma cells (25), induced cellular senescence by targeting *E2F3* in human melanoma Mewo and A2058 cells. Also, miR-203 affected MAPK p38 and PI3K/Akt. A previous study of ours showed that miR-205 as well as miR-203 is down-regulated in human and canine malignant melanoma cells (25). Chromosome region 1q32.2, in which the miR-205 gene is located, is often deleted in melanomas (27). On the other hand, the miR-203 gene is found in chromosome region 14q32.33 (miRBase), and the deletion of this region in melanoma cells has not been reported to our knowledge.

First, we focused on *E2F3* and *ZBP-89* as target genes of miR-203 involved in the senescence mechanism, as suggested by the database (TargetScan). We verified that, as expected, both genes were targets of miR-203 based on the results of the luciferase activity assay. We considered that miR-203 targeted both *E2F3a* and *E2F3b*, because their protein expression levels were

decreased by the ectopic expression of miR-203 and the predicted miR-203 binding sites were present in both *E2F3a* and *E2F3b* 3'-UTRs. As a result, the key regulator in cellular senescence elicited by the ectopic expression of miR-203 was *E2F3*, not *ZBP-89* (Fig. 4). E2F3a overexpression in Mewo cells rescued them from the cell cycle arrest and cellular senescence caused by miR-203. When the E2F3a expression vector was introduced into cells, both E2F3a and E2F3b proteins were unexpectedly overexpressed. Although the reason for this finding is not yet clear, our data indicate that acceleration of the cell cycle by E2F3a was superior to suppression of it by E2F3b in Mewo cells.

E2F1–3a play a central role in the control of cell cycle progression by regulating the timely expression of genes required for DNA synthesis and mitosis at the G₁/S phase boundary (16). Many proteins crucial for cell cycle progression are E2F targets, such as cyclin A, cyclin E, c-MYC, SIRT1, and Rb (28–31). In this study, the expression levels of SIRT1 and c-MYC also were decreased by the ectopic expression of miR-203 or *E2F3* silencing. These findings suggest that *SIRT1* and *c-MYC* were targets of E2F3, as shown in previous studies (31, 32). Furthermore, the E2F1 expression level was decreased by the introduction of miR-203 into Mewo or A2058 cells. This finding indicates that c-MYC regulates E2F1 expression, as reported earlier by O'Donnell *et al.* (33), because E2F1 is not a direct target gene of

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miR-203, and *E2F3* silencing also decreased E2F1 expression (Fig. 4A). Our data suggest that the down-regulation of E2F3 by miR-203 resulted in cell cycle arrest. On the other hand, our data indicate that the suppressive effect of miR-203 on cell growth resulted from the down-regulation of c-MYC expression, because the *E2F3* silencing showed a smaller inhibitory effect on cell growth than ectopic miR-203 expression, and the effect on cell growth of c-MYC silencing in our previous study was almost as much as that of ectopic miR-203 expression in this study (34). Unfortunately, *E2F3a*- or *E2F3b*-specific siRNA could not be generated, because siRNA for specific sequences of each gene had off-target effects. We could not clarify whether the senescence resulted from the cell cycle arrest or the down-regulation of SIRT1. To resolve this question, we must examine the expression level of acetylated p53 in Mewo cells, which have mutant p53 (35), when the cells are transfected with miR-203. The level of induction of senescence in A2058 cells by miR-203 was smaller than that in Mewo cells. This finding may be associated with the smaller suppressive effect of miR-203 on E2F3 expression in A2058 cells than that in Mewo cells (Fig. 2B) and the lack of Rb protein in A2058 cells (supplemental Fig. 1).

Although there is a relationship between cell cycle arrest and apoptosis, apoptotic cell death was neither biochemically nor morphologically observed in Mewo or A2058 cells transfected with miR-203 (supplemental Fig. 1).

It has been reported that ZBP-89 forms a complex with histone deacetylase 3 (HDAC3) and that this complex induces senescence by inhibiting p16 expression (36). We consider the reason why ZBP-89 silencing failed to induce senescence to be the lack of p16 protein in the Mewo cells (data not shown). ZBP-89 expression is reportedly elevated in some tumor tissues and cell lines, and ZBP-89 plays a role at the early stage of gastric cancer development (36). However, it has also been reported that ZBP-89 arrests cell proliferation through its interactions with p53 and p21 and initiates apoptosis (36). These findings suggest that the ZBP-89 gene functions as both an oncogene and anti-oncogene. Additionally, ZBP-89 binds to the neuronal *IRS2* promoter and inhibits the activation of *IRS2* (37). Our data showed that the introduction of siR-ZBP-89 into Mewo cells enhanced their *IRS2* and phospho-Akt expression levels. This finding indicates that ZBP-89 silencing in Mewo cells activated insulin/IGF-1 signaling, resulting in enhanced cell proliferation and cell survival. Therefore, ZBP-89 in Mewo cells possibly functions as a tumor suppressor.

In conclusion, miR-203 was shown to be a novel tumor suppressor in human melanoma cells. The present demonstration of previously uncharacterized biological functions of miR-203, with the ability to control cell proliferation and to induce senescence in melanoma cells, points to its significance as a unique type of tumor suppressor in melanoma cells.

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