miR-204-5p and miR-3065-5p exert antitumor effects on melanoma cells

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Abstract. MicroRNA (miR)-204-5p was previously identified to be downregulated in melanoma compared with melanocytic nevi. This observation prompted a functional study on miR-204-5p and the newly-identified miR-3065-5p, two miRNAs suggested to be tumor-suppressive oncomiRs. Application of miR-204-5p mimics or inhibitors resulted in a decrease or increase, respectively, in melanoma cell proliferation and colony formation. miR-204-5p mimics hindered invasion, whereas miR-204-5p inhibitors stimulated cancer cell migration. Modulation of miR-3065-5p led to a decrease in melanoma cell proliferation, altered cell cycle distribution and increased expression levels of its target genes HIPK1 and ITGA1, possibly due to functional modifications identified in these cells. miR-204-5p and miR-3065-5p demonstrated antitumor capacities that may need to be taken into account in the development of melanoma treatment approaches.

Introduction

Melanoma is an aggressive malignancy with the highest mortality rate among types of skin cancer, affecting 1.5/100,000 individuals in Central and Eastern Europe and 2.3/100,000 in Germany (2012) (1). Melanoma is characterized by marked genetic and phenotypic heterogeneity. Activating mutations in the B-Raf proto-oncogene, serine/threonine kinase, NRAS proto-oncogene, GTPase or KIT protocol-oncogene receptor tyrosine kinase genes in melanoma were identified to be important targets for treatment by specific inhibitors, although acquired resistance compromises their efficacy (2,3). The search for novel molecular therapeutic targets is one of the most challenging objectives in melanoma studies.

MicroRNAs (miRNAs), endogenous single-stranded RNA molecules 18-24 nucleotides in length, serve a regulatory role in numerous physiological and pathological processes (4,5). Therefore, miRNAs have great potential as targets for the development of novel therapeutics (6).

miR-204-5p was previously identified to be downregulated in human melanoma compared with melanocytic nevi (7). This observation is in agreement with several other studies investigating the involvement of miR-204-5p in the regulation of cell proliferation in colorectal (8) and thyroid cancer (9), and oral squamous cell carcinoma (10). miR-204-5p is involved in various signaling cascades, including Signal transducer and activator of transcription 3 (11) and Wnt/β-catenin signaling (12). One of the co-partners in the mediation of the effects of miR-204-5p is considered to be miR-3065-5p, a relatively newly-identified miRNA, for which limited functional data are available (13). Therefore, the aim of the present study was to determine the effects of miR-204-5p and miR-3065-5p on the biological behavior of melanoma cells, and to investigate the possibility of targeting matched miRNAs for the modulation of melanoma cell function.

Materials and methods

Ethical approval. The present study was approved by the Krasnoyarsk State Medical University Local Ethics Committee (protocol no. 70/2016, issued on June 6, 2016, Krasnoyarsk, Russia).

Tissue samples. Samples from primary melanomas (n=12; 5 males and 7 females; median age, 56 years; age range, 32-81 years) and benign melanocytic tumors (n=9, 4 males and 5 females; median age, 36 years; age range 14-65 years) were obtained from patients from the Krasnoyarsk Regional Oncology Center (Krasnoyarsk, Russia). Samples were taken during the period from June 2016 to October 2016. Skin biopsies were obtained after getting written informed consent. The tissues were resected and immediately immersed in 1xRNAlater® stabilization solution (cat. no. AM7020; Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at room

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temperature for 1 to 2 h for transportation. Subsequently, all samples were stored at -20˚C prior to use. The diagnosis was confirmed by certified pathologists using a light microscope (Leica DM2500; Leica Microsystems, Germany) of the Krasnoyarsk Pathological Anatomy Bureau. Melanoma was staged in accordance with the American Joint Committee on Cancer 2009 guidelines (14).

Cell culture. The human melanoma BRO cell line was obtained from the Research Institute of Fundamental and Clinical Immunology (Novosibirsk, Russia). The melanoma SK-MEL1 cell line (ATCC® HTB-67™) was obtained from the A.N. Sysin Research Institute of Human Ecology and Environmental Health at the Ministry of Health of the Russian Federation (Moscow, Russia). The cells were cultured in RPMI-1640 with L-glutamine (Gibco; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2 in a CO_2-incubator (Sanyo MSO-5AC; Sanyo Electric Co., Ltd., Osaka, Japan).

RNA isolation. For total RNA isolation, tissue samples were homogenized with liquid nitrogen and then suspended in 200 μl digestion buffer of the RecoverAll™ Total Nucleic Acid Isolation kit (cat. no. AM1975; Ambion; Thermo Fisher Scientific, Inc.). RNA was isolated according to the manufacturer's protocol, and then eluted with 50 μl nuclease-free water. miRNA concentration was estimated by a Qubit® 2.0 fluorimeter (Invitrogen; Thermo Fisher Scientific, Inc.) with the use of Qubit® microRNA Assay kit (ref. Q32880; Thermo Fisher Scientific, Inc.).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

miRNA expression assay. RT-qPCR was performed using the TaqMan™ miRNA Assay (cat. no. 4427975; Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction mixture for RT, which consisted of 4 μl RNA from each sample, 0.4 μl 5X primers from the TaqMan™ miRNA assay, 2 μl 50X OT buffer, 1 μl DTT, 1 μl dNTPs mix and 0.5 μl revertase from the Moloney Murine Leukemia Virus (MMLV) RT kit (cat. no. SK021, Evrogen, Moscow, Russia), was incubated at 37˚C for 30 min. Subsequently, 2 μl cDNA was added to the PCR cocktail containing 8 μl 2.5-fold RT-PCR reaction mixture with ROX reference dye (Syntol, Moscow, Russia), 1 μl 20X primers/probe mix from the TaqMan™ miRNA Assay and 9 μl de-ionized water. U6 small nuclear RNA and RNU6B were used as endogenous controls (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction was performed on a Step One™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following temperature cycling protocol: Preheating at 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles: Denaturation step at 95˚C for 15 sec and annealing and elongations step at 60˚C for 1 min with 6-carboxyfluorescein/ROX detection. Data were analyzed using the ΔΔCq method, as previously described (15).

Target gene expression assay. To estimate the expression levels of target genes, RT of total RNA was performed using the MMLV RT kit (Evrogen) in a mixture containing 4 μl RNA sample, 0.5 μl random primers, 2 μl 50X OT buffer, 1 μl DTT, 1 μl dNTPs mix and 0.5 μl revertase. The 2 μl cDNA was used for PCR with primers specific to the genes under investigation (cat. no. 4331182, Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the aforementioned cycling protocol for the miRNA expression assay.

Cell transfection and efficiency of detection. The BRO and SK-MEL1 cells were transfected 24 h prior to starting the subsequent experiments with specific anti-miR™ miRNA inhibitors for miR-204-5p (mature miRNA sequence: UUC CCUUUGUCAUCCUAUGCCU) and miR-3065-5p (mature miRNA sequence: UCAACAAAAUCACUGAUGCUG GA) (both 5 nmol lyophilized pellet, Assay IDs, AM11116 and AM18328, respectively; cat. no. AM17000; Ambion; Thermo Fisher Scientific, Inc.) and with mirVana® miRNA mimics in an additional experimental series (5 nmol lyophilized pellet, Assay IDs, MC11116 and MC18328, respectively; cat. no. 4464066, Ambion; Thermo Fisher Scientific, Inc.). When cells reached a final concentration of 1-6x10^5 cells/ml, transfection experiments were performed using 1.5 μl Lipofectamine® RNAiMAX Transfection Reagent (cat. no. 13778150; Invitrogen; Thermo Fisher Scientific, Inc., USA)/500 μl cells. Anti-miR or mimic solutions were added to the cells containing culture medium RPMI-1640 with L-glutamine (Gibco; Thermo Fisher Scientific, Inc.) to a final concentration of 25 nM. The medium was replaced every 24 h after transfection. To estimate the gain- or loss-of-function effect of microRNA, anti-miR™ miRNA Inhibitor Negative Control #1 (5 nmol lyophilized pellet, cat. no. AM17010, Ambion; Thermo Fisher Scientific, Inc.) and mirVana™ miRNA Mimic Negative Control #1 (5 nmol lyophilized pellet, cat. no. 4464058, Ambion; Thermo Fisher Scientific, Inc.) negative controls were used.

To evaluate the transfection efficiency, the effects of anti-miR™ hsa-let-7c miRNA inhibitor (cat. no. 4392431; Ambion; Thermo Fisher Scientific, Inc.) and hsa-miR-1 mimic (cat. no. 4464062; Ambion; Thermo Fisher Scientific, Inc.) positive controls were measured and compared with those of the respective negative controls. For this purpose, the cells were transfected using 1.5 μl Lipofectamine® RNAiMAX Transfection Reagent (cat. no. 13778150; Invitrogen; Thermo Fisher Scientific, Inc.) with anti-miR or mimic positive controls; anti-miR™ hsa-let-7c miRNA Inhibitor Positive Control (5 nmol lyophilized pellet, cat. no. 4392431, Ambion; Thermo Fisher Scientific, Inc.) and mirVana™ miRNA Mimic miR-1 Positive Control (5 nmol lyophilized pellet, cat. no. 4464063, Ambion; Thermo Fisher Scientific, Inc.) and incubated at 37˚C with 5% CO_2 for 48 h. Subsequently, RNA was isolated from the cells and the changes in levels of the High-mobility group AT-hook 2. (HMGA2, Taqman® Gene Expression Assays, cat. no 4331182, Applied Biosystems™; Thermo Fisher Scientific, Inc.) for the inhibitors and Twinfilin actin-binding protein 1 (TWF1, Taqman® Gene Expression Assays, cat. no. 4331182, Applied Biosystems™; Thermo Fisher Scientific, Inc.) for the mimics were estimated by PCR using a 2.5x reaction mixture for PCR (cat. no. M431, Syntol, Russia). Thermocycling conditions were as follows: Preheating at 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of, denaturation at 95˚C for 15 sec and annealing and elongations at 60˚C for 1 min. Data

were analyzed using the ΔΔCq method (15). The expression of HMGA2 is specifically downregulated by hsa-let-7c (16); therefore, effective inhibition of let-7c leads to a decrease in HMGA2 mRNA level in the cells. Similarly, TWF1 expression is regulated by miR-1 and its effective transfection into the cells leads to downregulation of TWF1 (17). To perform qPCR, the isolated RNA was first converted to cDNA by RT with the Reverta kit (cat. no. K3-1-100, AmpliSens, Moscow, Russia) and then amplified with TaqMan primers for HMGA-2 (assay ID Hs00171569_m1; cat. no. 1287202; Applied Biosystems; Thermo Fisher Scientific, Inc.) or for TWF1 (assay ID Hs00702289_s1; cat. no. 4331182; Applied Biosystems; Thermo Fisher Scientific, Inc.) and endogenous control β-actin (assay ID Hs01060665_g1; cat. no. 4331182; Applied Biosystems; Thermo Fisher Scientific, Inc.).

Cell apoptosis detection. The melanoma SK-MEL1 and BRO cell lines were transfected with relevant inhibitors or mimics and incubated in 24-well plates at 37˚C in 5% CO_2 for 48 h. The cells were then detached with 0.25% trypsin solution in Hank's Balanced Salt Solution (HBSS; Gibco; Thermo Fisher Scientific, Inc.), washed twice with cold PBS and stained with the Annexin V-fluorescein isothiocyanate/7-Aminoactinomycin D (7-AAD) kit (cat. no. IM3614, Immunotech, Inc.; Beckman Coulter, Inc., Quebec, Canada) according to the manufacturer's protocol. The proportion of viable (Annexin V^-/7-AAD^-), early apoptotic (Annexin V^+/7-AAD^-), apoptotic (Annexin V^+/7-AAD^+) and necrotic (Annexin V^-/7-AAD^+) cells was detected on Cytomics FC-500 (Beckman Coulter, Inc., Brea, CA, USA) using CXP Software (version 2.2; Beckman Coulter, Inc.). The experiment was performed in triplicate.

Analysis of cell cycle by flow cytometry. Cell cycle analysis was performed using propidium iodide staining. Transfected SK-MEL1 and BRO cells were incubated in 24-well plates at 37˚C in 5% CO_2 for 48 h. Thereafter, the cells were washed twice with PBS, then treated with RNase A (100 μg/ml) for 30 min, and stained with propidium iodide (100 μg/ml) for 30 min at 37˚C in the dark. The proportion of cells in each phase was detected using a Cytomics FC-500 flow cytometer (Beckman Coulter, Inc.) using CXP Software (version 2.2).

Cell viability/cell proliferation assay

MTT assay. Melanoma cells transfected with miRNA inhibitors or mimics were cultured at 37˚C with 5% CO_2 for 24 h, then detached with 0.25% trypsin solution in HBSS (Gibco; Thermo Fisher Scientific, UK), and placed in 96-well plates at a final concentration of $3x10^4$ cells/ml. An MTT assay was performed at 24, 48, 72 and 96 h following transfection. For this purpose, the culture medium was replaced and 10 μl MTT solution/well was added. The cells with MTT were incubated for 4 h at 37˚C with 5% CO_2. The cells were then washed, and 200 μl DMSO/well was added followed by incubation at room temperature for 10 min. Absorbance of stained supernatants was measured with Efos-9305 spectrophotometer (Shvabe Photosystems, Moscow, Russia) at a wavelength of 560 nm. Cell viability/proliferation was directly proportional to the absorbance rate. The experiment was performed in triplicate.

Fluorescence microscopy. A total of 150 μl transfected cells at a concentration of $5x10^4$ cells/ml were placed in 96-well plates and incubated at 37˚C in 5% CO_2. The cells were stained using the CyQUANT® Direct Cell Proliferation Assay (cat. no. 35011; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, followed by addition of NucBlue® Live reagent from Ready Probes® Cell Viability Imaging kit (Blue/Red) (cat. no. R37610, Molecular Probes; Thermo Fisher Scientific, Inc.). Following 30 min of incubation at room temperature, fluorescence microscopy (magnification, x460) was performed using the Floid® Cell Imaging Station (Floid Software, version no. 22809; Thermo Fisher Scientific, Inc.). The nuclei of proliferating cells were stained green and blue, while non-proliferating live cell nuclei were stained dark blue.

Migration and invasion assay. Migration and invasion experiments were performed with CytoSelect™ 24-Well Cell Migration and Invasion assay kit (cat. no. CBA-100-C, Colorimetric Format; Cell Biolabs, Inc., San Diego, CA, USA). The 24-well migration and invasion plates contained polycarbonate membrane inserts with 8 μm pores (kit component, item no. 10001). The inserts of the invasion assay had an additional uniform layer of dried basement membrane matrix solution on the upper membrane surface (kit component, item no. 11001). Melanoma cells transfected with miRNA mimic or inhibitor were incubated at 37˚C in 5% CO_2 and then detached with 0.25% trypsin solution in HBSS (Gibco; Thermo Fisher Scientific, Inc., UK), washed with PBS and suspended in FBS-free medium RPMI-1640 to a final concentration of $1x10^5$ cells/ml. Following this, the cells were placed inside each insert, while the lower well of the migration plate was filled by the medium containing 10% FBS. Following a 22-h incubation at 37˚C in 5% CO_2, the cells in the interior of the inserts were mechanically removed and the rest were dissolved in the extraction solution (kit component, item no. 11003-C) of the assay for 10 min and transferred to a 96-well plate for optical density evaluation at a wavelength 560 nm using the Efos-9305 spectrophotometer (Shvabe Photosystems). Relative migration and invasion activity was determined by the ratio of the mean absorbance in the test cells vs. the mean absorbance in the negative control cells. The experiments were performed in triplicate.

Colony formation analysis. The ability for colony formation was examined 24 h following transfection of the cells with miR-204-5p and miR-3065-5p inhibitors and mimics. Melanoma cells were detached with 0.25% trypsin solution and placed in a 6-well culture plate containing 2 ml culture medium RPMI-1640 with L-glutamine (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS at a concentration of 10^3 cells/well. The cells were then incubated for 7-10 days at 37˚C with a 5% CO_2 until they formed visible colonies containing ≥50 tumor cells. The culture medium was changed every 3 days. Following the formation of the colonies, they were washed twice with PBS, then fixed with 70% ethanol for 10 min at room temperature, then stained with 0.05% crystal violet solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min at room temperature. The stained cells were washed four times with running water, air-dried at room temperature and the number of colonies in each well was

counted by the naked eye. The experiment was performed in triplicate.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of miRNA target genes. DIANA-mirPath v.3.0 was used for the KEGG pathway analysis (http://www.genome.jp/kegg; 01/01/2018) of miRNA signature. miRNA targets were predicted based on DIANA-microT-coding sequence. The threshold ($P \leq 0.05$) was calculated using the Fisher's exact test. To predict the gene potential targets of miR-3065-5p and miR-204-5-p, three different algorithms [TargetScan (version 7.0; http://www.targetscan.org), miRDB (version 5.0; http://mirdb.org/miRDB) and miRTarBase (version 4.5; http://mirtarbase.mbc.nctu.edu.tw/)] were applied to identify the validated targets of hsa-miR-3065-5p and miR-204-5-p. Subsequently, genes associated with carcinogenesis for each of the miRNAs were selected.

Enrichment analysis. A Gene Ontology term enrichment analysis (http://www.geneontology.org; 14/12/2017) was performed to identify the biological processes, molecular functions and cellular components associated with the target genes of miR-3065-5p and miR-204-5p. The KEGG pathway enrichment analysis was used to identify the miRNA targets associated with signaling pathways in melanoma. $P<0.05$ was considered to indicate a statistically significant difference. The PANTHER™ software classification system (version 10.0; Protein ANalysis THrough Evolutionary Relationships; www.pantherdb.org) was used to provide interpretation of the biological functions of the validated targets of miR-204-5p and miR-3065-5p.

Statistical analysis. Statistical analysis for all experiments was performed using non-parametric Mann-Whitney U-tests for two independent groups (negative control group and experimental group) with Statistica 6.1 software (StatSoft, Inc., Dell, Round Rock, TX, USA). The data are presented as the mean ± standard error of mean. miR expression levels data in melanoma, melanocytic nevi and melanoma cell lines were compared using Kruskal-Wallis H test. Multiple comparisons of mean ranks were conducted for all groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-204-5p and miR-3065-5p expression levels in melanocytic lesions and melanoma cells. miR-204-5p and miR-3065-5p expression was evaluated in skin samples obtained from patients with melanoma and melanocytic nevi, and in the melanoma BRO and SK-MEL1 cell lines. The miR-204-5p levels were identified to be decreased in the BRO melanoma cells compared with those in the melanocytic nevi ($P=0.023$, respectively; Fig. 1). The expression level of miR-3065-5p did not differ between the groups analyzed.

Bioinformatics analysis of miR-204-5p and miR-3065-5p targets and associated pathways. To determine the functional role of miR-204-5p and miR-3065-5p in melanoma cells, bioinformatics analysis was performed using the aforementioned databases. A total of 235 target genes were identified for

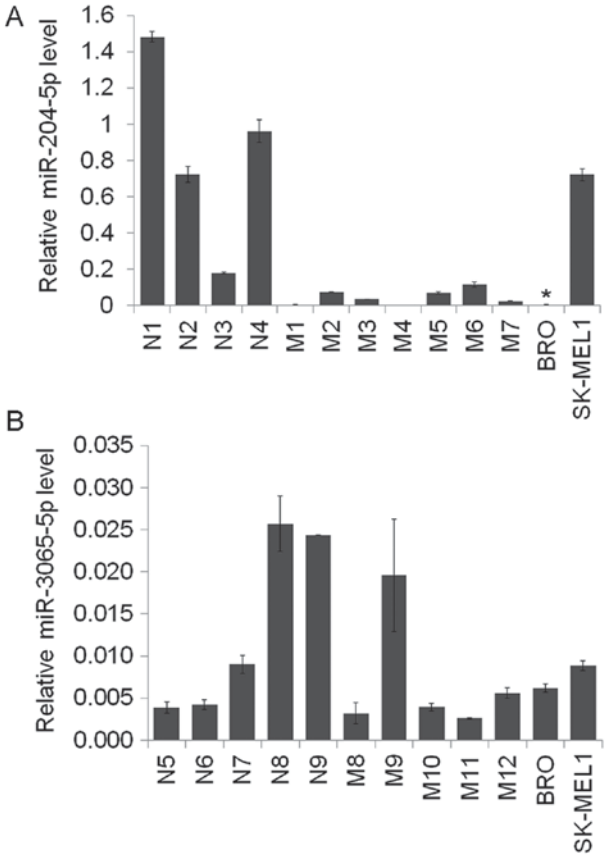

Figure 1. Expression of (A) miR-204-5p and (B) miR-3065-5p in melanocytic nevi, melanoma tissues and the melanoma BRO and SK-MEL1 cell lines. The miR-204-5p level differed between BRO melanoma cells and melanocytic nevi group (*$P<0.03$). N1-N9 melanocytic nevi group; M1-M12 melanoma group. miR, microRNA.

miR-204-5p. Among those, 36 were selected as being associated with carcinogenesis (Table I). Of those, the predicted targets were: Negative apoptosis regulator B-cell lymphoma 2 (Bcl-2), transcription factor Sex Determining Region Y-Box 4 (SOX4), and Forkhead box C1 (FOXC1). These genes were predicted using three databases: TargetScan, miRDB, miRTarBase. The primary signaling pathways associated with miR-204-5p were steroid biosynthesis (hsa00100), cytochrome P450 xenobiotic metabolism and apoptosis (hsa04210). The 184 genes corresponding with miR-3065-5p were detected to be associated with 48 altered biological processes, among which 13 target genes were associated with tumor growth and progression (Table II). One of those is Homeodomain-interacting protein kinase 1 gene (HIPK1), which participates in apoptosis, angiogenesis and cell proliferation (18,19). The other miR-3065-5p gene target was ITGA1 (integrin receptor subunit α1), which may regulate tumor growth and angiogenesis (20). miR-3065-5p was associated with the following signaling pathways in accordance with DIANA-mirPath v.3.0: Ubiquitin-mediated proteolysis (hsa04120), protein processing in the endoplasmic reticulum (hsa04141), lysine degradation (hsa00310), transcriptional dysregulation in cancer (hsa05202) and adherens junctions (hsa04520).

Effect of the application of miR-204-5p inhibitors and mimics on melanoma cell apoptosis, migration, invasion and colony

Table I. microRNA-204-5p target genes and their biological functions.

Target gene symbol	Biological processes involved
AKAP1	Protein binding
ANKRD13A	Cell migration
AP1S1	Protein transporter activity
AP1S2	Protein binding
ARHGAP29	Rho protein signal transduction
ATP2B1	Calcium ion transmembrane transport
Bcl-2	Apoptosis
Bcl-2L2	Negative regulation of apoptosis
BDNF	Collateral sprouting, nervous system development, synapse assembly
CDH2	Cell migration, cell adhesion, β-catenin binding, adherent junction organization
CDX2	Negative regulation of transcription from RNA polymerase II promoter
COL5A3	Collagen binding, cell-matrix adhesion
CREB5	Protein binding
EDEM1	Protein binding
ELOVL6	Fatty acid metabolism
EZR	Cadherin binding involved in cell-cell adhesion
FARP1	Negative regulation of phosphatase activity
FOXC1	Cell migration, cell proliferation, negative regulation of mitotic cycle
HAS2	Hyaluronic acid biosynthesis
HMX1	Negative regulation of transcription, DNA-templated
ITPR1	Calcium ion transport, response to hypoxia, signal transduction
M6PR	Transmembrane signaling receptor activity
MAP1LC3B	Non-motor microtubule binding protein
MEIS1	Protein binding
MEIS2	Protein binding, negative regulation of neuron differentiation
RAB22A	Protein binding, endosome organization
RUNX2	Protein binding
SERINC3	Innate immune response, defense response to virus, L-serine transport
SIRT1	Protein binding, p53 binding, metal ion binding, keratin filament binding, cellular response to tumor necrosis factor, necrosis factor, cellular triglyceride homeostasis, cholesterol homeostasis, chromatin organization, angiogenesis, UV-damage excision repair, positive regulation of apoptotic process, regulation of mitotic cell cycle
SOX4	Negative regulation of cell proliferation
TCF12	Protein binding
TCF4	Positive regulation of transcription, DNA-templated transcription, positive regulation of neuron differentiation
TGFBR1	Transforming growth factor beta-activated receptor activity, angiogenesis, apoptotic process, epithelial to mesenchymal transition, positive regulation of cell migration, apoptotic process, epithelial to mesenchymal transition, positive regulation of cell migration, positive regulation of cell proliferation, transforming growth factor beta receptor signaling pathway, protein serine/threonine kinase activity
TGFBR2	Transforming growth factor beta-activated receptor activity
TRPM3	Cation transport
USP47	Proliferation, cell growth

formation. To investigate the functional role of miR-204-5p in melanoma, its specific inhibitors or mimics were transfected in BRO and SK-MEL1 melanoma cells. Transfection efficiency analysis revealed that the mimics and inhibitors of the studied miRNAs resulted in respective up- or downregulation of the target molecules (Fig. 2). miR-204-5p expression alterations caused by application of either inhibitors or mimics resulted in decreased cell viability/proliferation, as measured by the MTT assay in BRO and SK-MEL1 melanoma cells (Fig. 3). The miR-204-5p inhibitors exerted no effect on the rate of BRO

Table II. microRNA-3065-5p target genes and their biological functions.

Target gene symbol	Biological processes involved
EPT1	Phosphatidylethanolamine biosynthesis
HIPK1	Apoptosis, positive regulation of angiogenesis, positive regulation of cell proliferation
ITGA1	Negative regulation of cell proliferation, negative regulation of epidermal growth factor receptor signaling pathway, cellular extravasation, cell-matrix adhesion, activation of MAPK activity
MYBL1	Positive regulation of transcription, DNA-templated
MYEF2	DNA binding
NUFIP2	RNA binding
PDP2	[Pyruvate dehydrogenase (lipomide)] phosphatase activity, metal ion binding
RAB1A	Cell-cell adhesion, positive regulation of glycoprotein metabolic process
SPATA13	Guanyl-nucleotide exchange factor activity
SYPL1	Chemical synaptic transmission
WASF3	Actin binding
ZMYM6	Multicellular organism development
PCDH9	Regulation of adhesion molecules, calcium ion binding

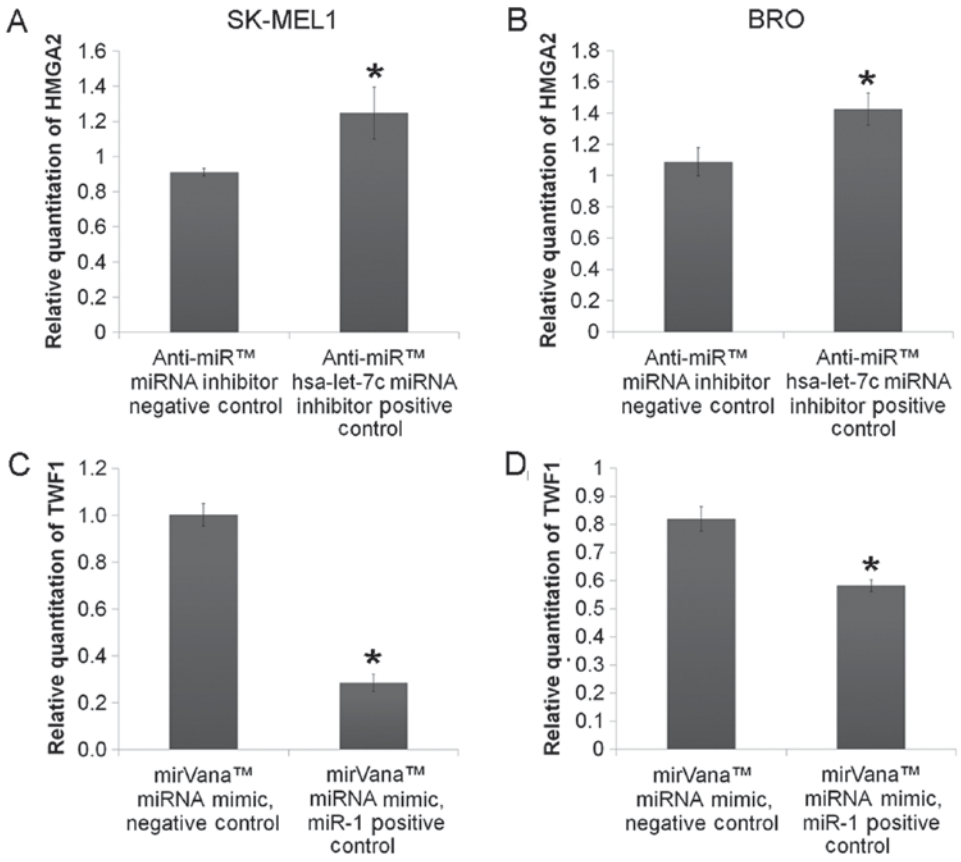

Figure 2. Transfection efficiency. The transfection efficiency was determined by quantitative polymerase chain reaction. Cells transfected with (A) miR-204-5p and (B) miR-3065-5p inhibitor displayed elevated levels of HMGA2 mediated by let-7c Anti-miR™ Inhibitor. (C) miR-204-5p and (D) miR-3065-5p mimic application resulted in TWF1 expression decrease by miRNA Mimic miR-1 Positive Control application. *P<0.05 vs. negative control. miR, microRNA; TWF1, Twinfilin actin-binding protein 1; HMGA2; High-mobility group AT-hook 2.

melanoma cell apoptosis. Application of miR-204-5p mimics led to a decrease in the number of surviving BRO melanoma cells, but did not affect the ratio of apoptotic and necrotic cells (Fig. 4).

Application of either miR-204-5p inhibitors or mimics resulted in the increase of the percentage of cells at the G1 stage and the decrease of those at stage S-G2 among SK-MEL1 melanoma cells (Fig. 4), and miR-204-5p inhibitor significantly

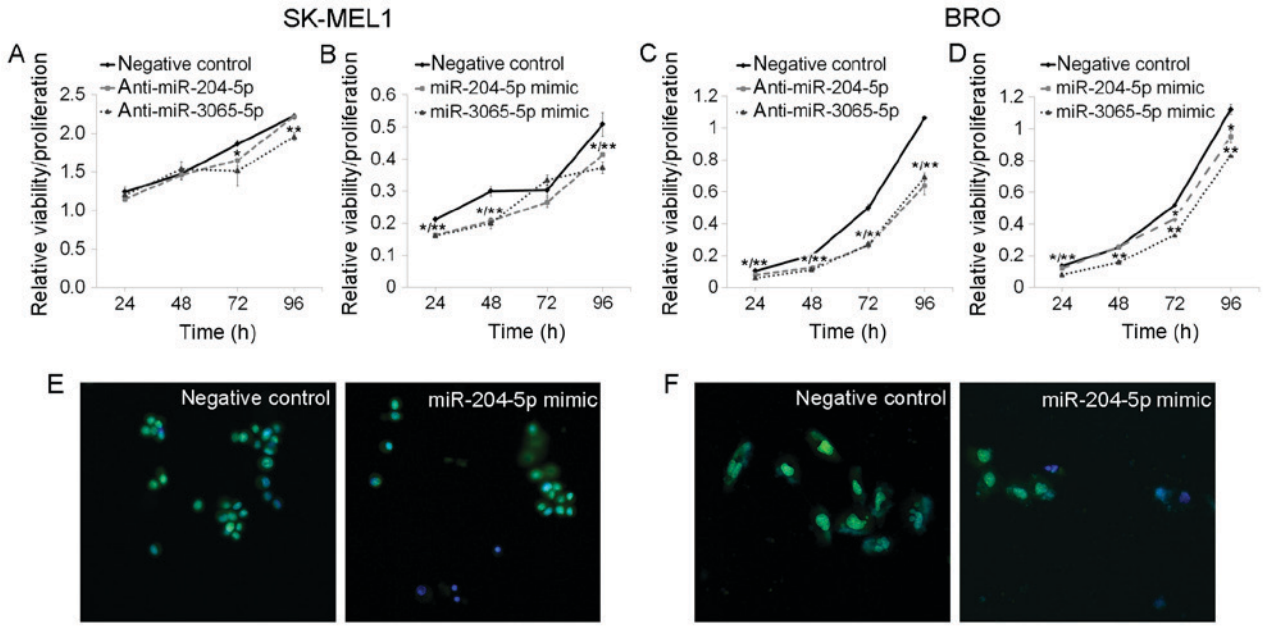

Figure 3. Cell viability/proliferation assay in melanoma BRO and SK-MEL1 cells following modulation of miR-204-5p and miR-3065-5p levels. MTT assay (96 h) in SK-MEL1 cells following transfection with miR-204-5p and miR-3065-5p (A) inhibitors and (B) mimics revealed decrease of cell viability/proliferation. MTT assay in BRO cells 96 h following transfection with miR-204-5p and miR-3065-5p (C) inhibitors and (D) mimics revealed a stable decrease in cell viability/proliferation. (E) CYQUANT cell proliferation assay in SK-MEL1 cells 96 h following transfection with miR-204-5p mimics. (F) CYQUANT cell proliferation assay in BRO cells 96 h following transfection with miR-204-5p mimics. The nuclei of non-proliferating cells are colored blue, whereas proliferating cells express green fluorescence. miR-204-5p mimic transfection decreased the number of proliferating cells compared with the negative control. *P<0.05 vs. negative control. miR, microRNA.

decreased S-G2 stage in BRO melanoma cells (Fig. 4). miR-204-5p downregulation resulted in 1.54-fold increase of cell migration but did not affect the invasion of BRO melanoma cells (Fig. 5). Transfection of the cells by mimics did not alter these characteristics (P>0.05) but reduced their colony formation ability (Fig. 6). Melanoma SK-MEL1 is a low-adhesion cell line, which becomes slightly invasive *in vitro* (21); therefore, its migration, invasion and colony formation were not estimated.

Effect of miR-3065-5p inhibitor and mimic application on melanoma cell apoptosis, migration, invasion and colony formation. miR-3065-5p expression modulation by inhibitors or by mimics led to an apparent decrease of cell viability/proliferation in BRO and SK-MEL1 melanoma cells (Fig. 3). Apoptosis analysis demonstrated that all transfected cells had live and apoptotic cell ratios similar to negative controls (P>0.05). miR-3065-5p inhibition did not affect the cell cycle of either cell line, but miR-3065-5p mimics reduced the number of SK-MEL1 cells at the S-G2 phase (from 24.06±0.64 to 20.95±0.57%; P=0.0495) and increased the cell population at the G1-phase (from 74.87±0.72 to 78.21±0.54%; P=0.0495; Fig. 4). miR-3065-5p inhibition stimulated BRO melanoma cell migration, whereas miR-3065-5p upregulation exerted the opposite effect (Fig. 5). It was also identified that miR-3065-5p inhibitor or mimic application promoted invasion of BRO melanoma cells, whereas miR-204-5p mimics induced suppression of BRO melanoma cell invasive ability (Fig. 5). Upregulation of miR-3065-5p caused the change in the colony number of BRO cells (Fig. 6).

Effects of miR-204-5p and miR-3065-5p on target gene expression. To elucidate the molecular mechanisms underlying the involvement of miR-204-5p and miR-3065-5p in melanoma cell biological behavior, the effect of these miRNAs on the expression of their target genes was investigated. Bcl-2, Transforming growth factor β receptor 1 (TGFβR1) and SOX4 gene expression levels were evaluated following performing gain- and loss-of-function experiments for miR-204-5p, and HIPK1 and ITGA1 for miR-3065-5p. The inhibition of miR-204-5p in BRO melanoma cells was identified to decrease the level of Bcl-2, while stimulation of miR-204-5p exhibited no effect on Bcl-2 expression. Conversely, Bcl-2 expression was decreased in melanoma SK-MEL1 cells following miR-204-5p mimic transfection, and remained stable following specific miR-204-5p inhibitor application. The mRNA levels of TGFβR1 were downregulated following the application of the inhibitor and mimic of miR-204-5p in BRO melanoma cells, and following miR-204-5p mimic transfection in SK-MEL1 melanoma cells. miR-204-5p inhibition did not affect TGFβR1 expression in SK-MEL1 cells. No alterations in SOX4 expression were observed following miR-204-5p inhibitor and mimic application in either cell line (Fig. 7).

Melanoma cell transfection by miR-3065-5p inhibitors and mimics led to a downregulation of HIPK1 in BRO melanoma cells and upregulation in SK-MEL1 melanoma cells. The ITGA1 level was downregulated by miR-3065-5p inhibition and upregulated by its mimics in BRO melanoma cells. On the contrary, overexpression of ITGA1 was observed following miR-3065-5p knockdown in SK-MEL1 cells, while transfection with miR-3065-5p mimics did not affect ITGA1 expression (Fig. 7).

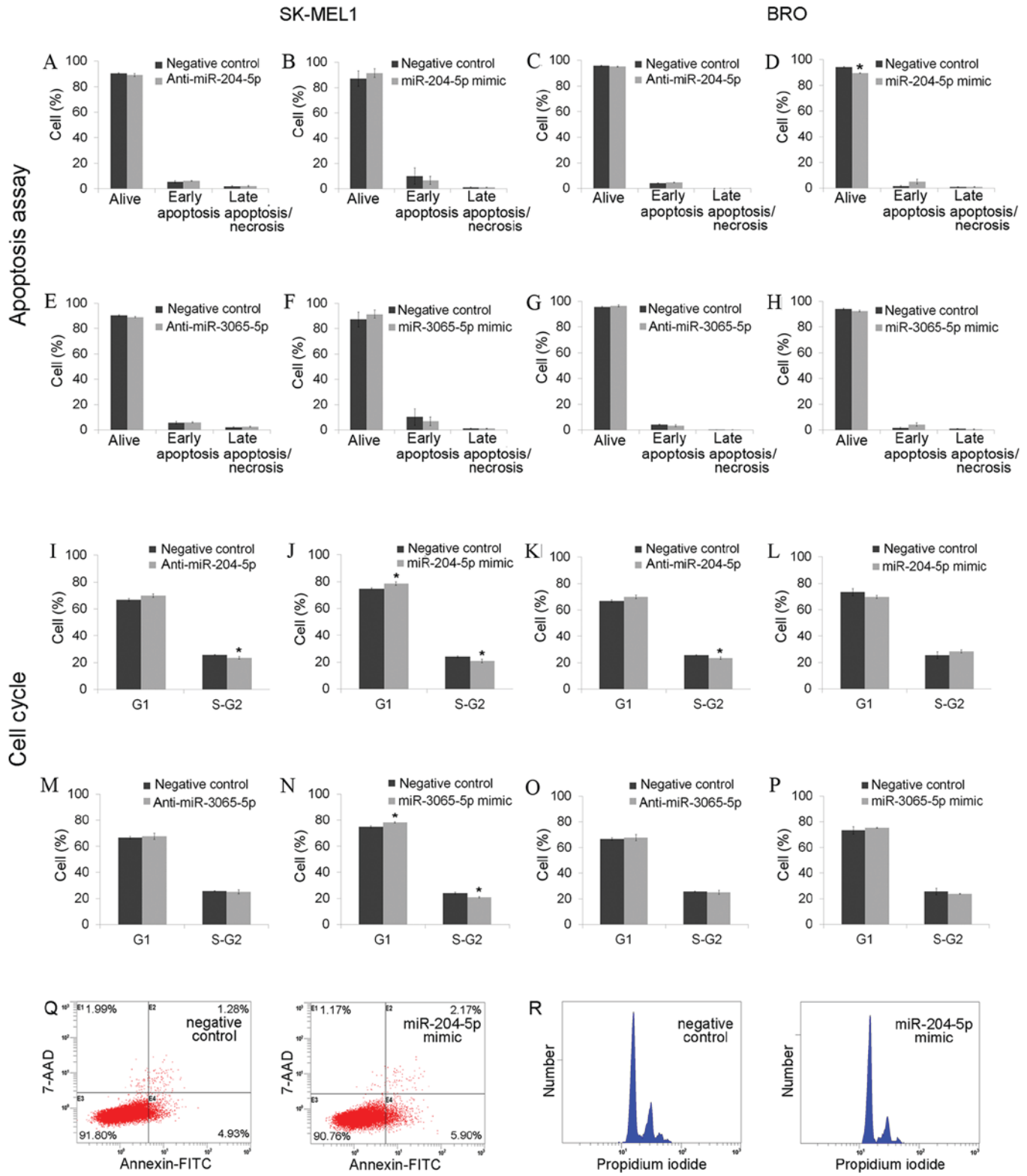

Figure 4. Apoptosis and cell cycle assay. (A) miR-204-5p inhibitor transfection does not reduce apoptosis in SK-MEL1 melanoma cells. (B) miR-204-5p mimic transfection does not alter apoptosis in SK-MEL1 melanoma cells. (C) miR-204-5p inhibitor transfection does not modulate apoptosis in BRO melanoma cells. (D) miR-204-5p mimic decreased percentage of alive BRO melanoma cells. (E) Application of miR-3065-5p inhibitor in SK-MEL1 melanoma cells does not reduce the level of apoptosis. (F) Application of miR-3065-5p mimic in SK-MEL1 melanoma cells does not reduce the level of apoptosis. (G) Application of miR-3065-5p inhibitor in BRO melanoma cells does not alter the level of apoptosis. (H) Application of miR-3065-5p mimic in BRO melanoma cells does not alter the level of apoptosis. (I) miR-204-5p inhibitor transfection in SK-MEL1 cells resulted in S-G2-phase-positive cell decrease. (J) miR-204-5p mimic transfection in SK-MEL1 cells resulted in G1-phase-positive cell increase and S-G2-phase-positive cell decrease. (K) miR-204-5p inhibitor transfection in BRO cells resulted in S-G2-phase-positive cell decrease. (L) miR-204-5p inhibitor transfection in BRO cells does not affect the phases of the cell cycle. (M) miR-3065-5p inhibitor transfection in SK-MEL1 cells does not affect the phases of the cell cycle. (N) miR-3065-5p mimic transfection in SK-MEL1 cells resulted in G1-phase-positive cell increase and S-G2-phase-positive cell decrease. (O) miR-3065-5p inhibitor transfection in BRO cells does not modulate the phases of the cell cycle. (P) miR-3065-5p mimic transfection in BRO cells does not affect the phases of the cell cycle. (Q) Histograms of Annexin V/7-AAD assay in BRO melanoma cells. (R) Histograms of cell cycle assay based on propidium iodide staining in SK-MEL1 cells. *P<0.05 between miRNA modulated cells vs. negative control. miR, microRNA; FITC, fluorescein isothiocyanate; 7-AAD, 7-Aminoactinomycin D.

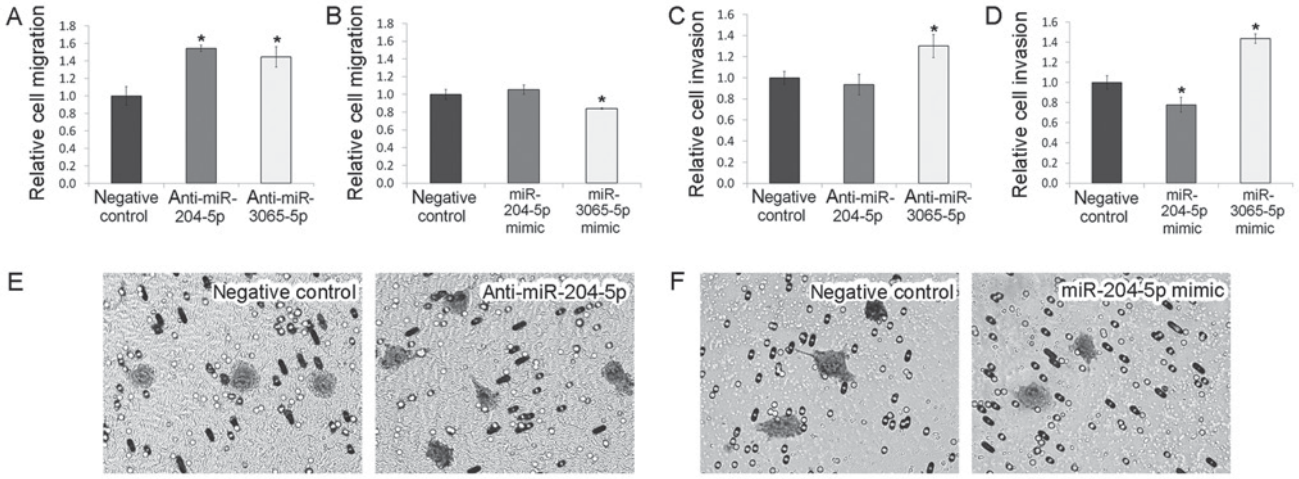

Figure 5. Cell migration and invasion in BRO melanoma cells. (A) miR-204-5p and miR-3065-5p inhibition promoted BRO melanoma cell migration. (B) Application of miR-3065-5p mimics diminished BRO melanoma cell migration, and application of the miR-204-5p mimic did not exert an effect. (C) miR-3065-5p inhibitor transfection increased BRO melanoma cell invasion. (D) miR-204-5p mimic transfection suppressed BRO melanoma cell invasion, whereas miR-3065-5p mimic application promoted cell invasion. *P<0.05 between microRNA modulated cells vs. negative control. (E) Cell migration assay with BRO melanoma cells following miR-204-5p inhibitor application. (F) Cell invasion assay with BRO melanoma cells following miR-204-5p mimic application. miR, microRNA.

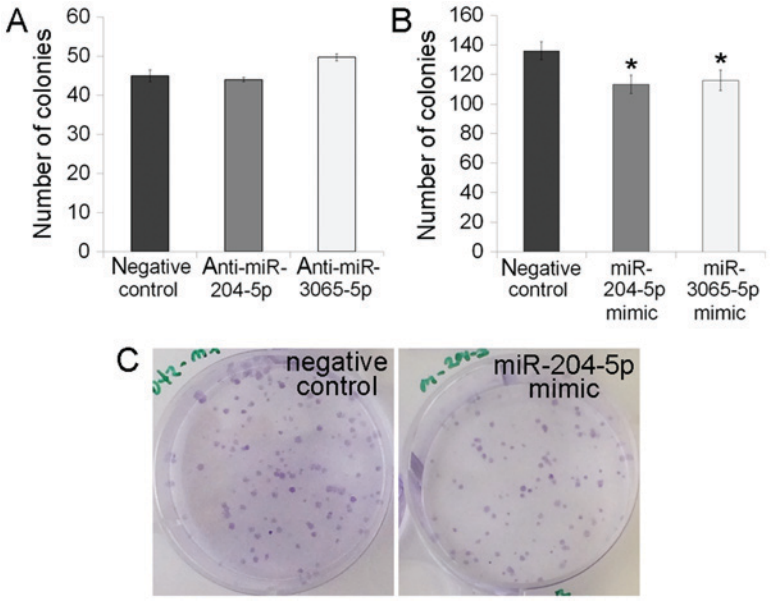

Figure 6. Colony formation assay in BRO melanoma cells. (A) Application of miR-204-5p and miR-3065-5p inhibitors did not affect the rate of colony formation. (B) Application of miR-204-5p mimic and miR-3065-5p mimics decreased the colony-forming ability *P<0.05 between microRNA modulated cells vs. negative control. (C) BRO melanoma cell colonies visualized following crystal violet staining. A decrease in the number of colonies was observed following the application of miR-204-5p mimics compared with the negative control. miR, microRNA.

Discussion

miR-204-5p was identified to be downregulated in melanoma BRO cells compared with that in melanocytic nevi. This result is concurrent with previous studies, which demonstrated that this miRNA functioned as a tumor suppressor and had diminished expression in oral squamous cell carcinoma (10), colorectal cancer (8) and prostate cancer (22). The miR-204-5p mimic/inhibitor and miR-3065-5p mimic/inhibitor application lead to a decrease of melanoma cell viability. This observation may be explained by the numerous targets of miRNA modulation that are presently under study, which may ultimately trigger several signaling pathways. More prominent cell viability inhibition in comparison with the negative control was observed in melanoma BRO cells following anti-miR-204-5p/anti-miR-3065-5p inhibition, as demonstrated by a >3-fold decrease in the MTT assay results, which also allows exclusion of unspecific results. The up- and downregulation of miR-204-5p resulted in downregulation of the expression of its target gene TGFβR1 in the BRO melanoma cell line, and downregulation of TGFβR1 following miR-204-5p mimics application in SK-MEL1 cells. TGFβR1 is a key component of TGFβ signaling, which is implicated in several physiological and pathological processes,

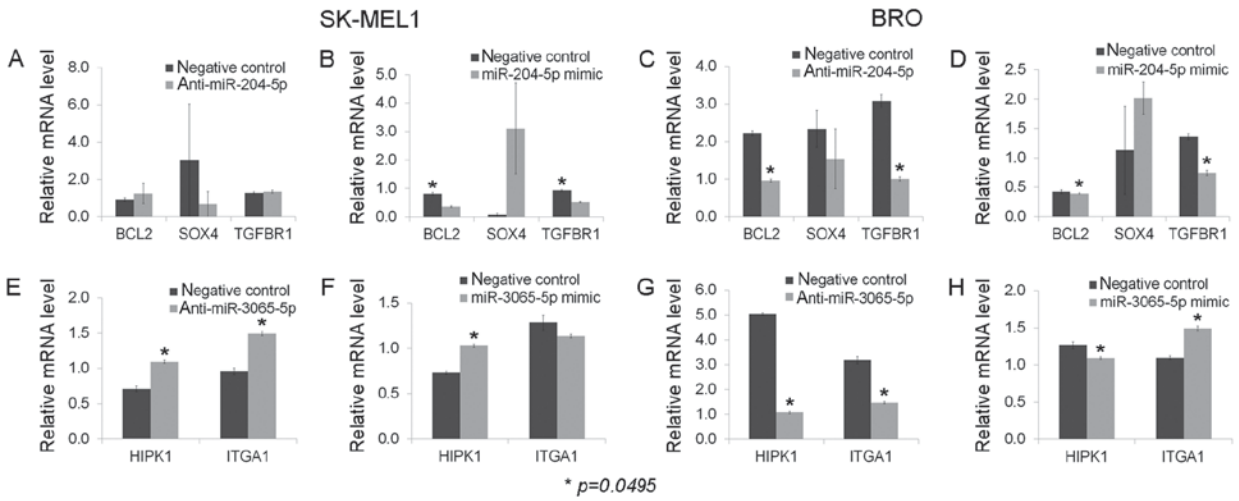

Figure 7. miR-204-5p and miR-3065 target gene expression analysis. (A) miR-204-5p inhibitor application exerted no effect on Bcl-2, SOX4 and TGFβR1 expression in SK-MEL1 cells. (B) miR-204-5p mimics decreased Bcl-2 and TGFβR1 expression in SK-MEL1 cells. (C) miR-204-5p inhibitors decreased Bcl-2 and TGFβR1 expression in BRO melanoma cells. (D) miR-204-5p inhibitors decreased TGFβR1 expression in BRO melanoma cells. (E) miR-3065-5p inhibitors induced HIPK1 and ITGA1 expression in SK-MEL1 melanoma cells. (F) miR-3065 mimics upregulated HIPK1 expression in SK-MEL1 cells. (G) miR-3065-5p inhibitors downregulated HIPK1 and ITGA1 levels in BRO melanoma cells. (H) miR-3065-5p mimics induced HIPK1 downregulation and ITGA1 upregulation in BRO melanoma cells. miR, microRNA; Bcl-2, B-cell lymphoma 2; SOX4, Sex Determining Region Y-Box 4; TGFβR1, Transforming growth factor β receptor 1.

including wound healing, epithelial-to-mesenchymal transition and tumor-suppressor activities in pre-malignant melanocytes (23). TGFβ specifically interacts with its type I receptor, promoting advanced melanoma growth and progression (24). Therefore, the decrease in cell proliferation observed following miR-204-5p expression level modulation in the metastatic melanoma cell lines BRO and SK-MEL1 may be mediated by TGFβ signaling. The colony assay indicated that the miR-204-5p and miR-3065-5p mimics reduced the colony formation, corresponding with the cell cycle assay results. The colony forming assay was based on the number of colonies counted, which was possible when the number of cells was ≥50. Therefore, the cells, depending on the type, were grown from 7-10 days up to 2-3 weeks. Transient transfection with mimics and inhibitors was performed using Lipofectamine® RNAiMAX. The application of a transfection reagent allowed the sustenance of mimics or inhibitor molecules in cells for several days, usually for 3-5 days in accordance with the manufacturer's protocol. Within 3-5 days of the effect of the inhibitors/mimics, the colony formation was analyzed. Cells continued to grow when there was no effect of the mimic/inhibitor, but if the modulators evidently affected cells growth, a difference between control and experimental groups was observed.

We performed similar experiments with BRO melanoma cells following miR-106a inhibitor application, and indicated that evaluation of the number of colonies was possible on the 6-7th day of experimentation (25). The same approaches were described in several previous studies examining miRNAs (26,27).

miR-204-5p acts as a tumor suppressor by targeting SOX4 in renal cell carcinoma (28) and T-cell acute lymphoblastic leukemia (29). In the present study, the SOX4 mRNA level was not altered in the miR-204-5 gain- and loss-of-function experiments, while certain biological effects, which may be associated with SOX4, were observed in melanoma cells. This may be due to the repressive action of miRNA on translation, but not on mRNA degradation (30,31). It may also be hypothesized that SOX4 was not triggered by miR-204-5p in the melanoma cells studied. Bcl-2, which was identified as an additional miR-204-5p target gene, is a crucial negative apoptosis regulator (32). Its downregulation following the application of miR-204-5p inhibitors was identified in BRO cells, and following the application of miR-204-5p mimics in SK-MEL1 cells. As apoptosis was not triggered by miRNA modulation, the observed Bcl-2 dysregulation may be implicated in non-apoptotic functions, including cell survival, in melanoma cells (33).

miR-3065 mimic and inhibitor application modulated HIPK1 levels in BRO and SK-MEL1 cells, but in different ways: The miR-3065 mimics and inhibitors upregulated HIPK1 levels in SK-MEL1 cells, but downregulated HIPK1 levels in BRO cells. HIPK1 has been identified as one of the nuclear protein kinases, participating in the regulation of several processes, including cell growth and apoptosis (18,19). In colorectal cancer cell growth, the suppressive action of HIPK1 was mediated through the p53 signaling pathway (34). HIPK1 is considered to possess both pro-tumorigenic and onco-suppressive properties (34), explained by the possible bidirectional role of this protein, depending on the oxidative status of the cell. This may explain the oppositely directed alterations of HIPK1 observed in BRO and SK-MEL1 cells, which may differ in oxygen supply, due to their distinct origins: SK-MEL1 cells originated from melanoma metastases in regional lymph nodes and are subjected to less oxidative stress compared with BRO melanoma cells, which are derived from melanoma metastases to visceral organs (35).

miR-3065 modulation altered the migration and invasion levels and modulated ITGA1 gene expression in BRO melanoma cells. This glycoprotein belongs to the integrin family and regulates cell-cell adhesion (36); therefore, alterations of its expression may facilitate cell contact and migration. It was

demonstrated that ITGA1 polymorphisms are associated with a high risk of gastric cancer development and progression (37). ITGA1 is a key component of Ras/mitogen-activated protein kinase kinase/extracellular signal-related kinase signaling activation, which regulates cell proliferation in melanoma, as previously described (38). In the present study, ITGA1 expression was modulated by miR-3065-specific mimic and inhibitor application in BRO melanoma cells: Anti-miR-3065-5p led to ITGA1 downregulation, whereas miR-3065-5p mimics increased ITGA1 expression. ITGA1 expression levels were the most clearly modulated among the target genes evaluated in the present study, with >2-fold downregulation following specific miR-3065-5p inhibitor application, suggesting that this gene is the most likely target of miR-3065-5p.

Among other miR-204-5p target genes, CDH2 and FOXC1 should also be under consideration. CDH2 is a gene that codes N-cadherin, which is broadly expressed in melanoma cells, serving as a promotor of melanoma cell invasion and metastasis via triggering several signaling cascades and transcription factors (39). N-cadherin-overexpressing melanoma cells lose contacts with neighboring keratinocytes and demonstrate increased adhesion and motility features (40). FOXC1 is a member of Forkhead box family transcription factors. FOXC1 overexpression was previously identified to activate proliferation, migration, invasion and colony formation of melanoma cells by targeting PI3K/AKT signaling pathway (41). Therefore, we hypothesized that the inhibition of melanoma cell migration following miR-204-5p inhibitor application and the decrease of the invasion rate by melanoma cells following miR-204-5p mimic application may be accounted for by miR targeting FOXC1, which requires additional clarification. Our observation is in line with an additional miR-204-5p functional study on laryngeal squamous cell carcinoma cells, where miR-204-5p mimic and FOXC1 siRNA application suppressed cancer cell migration and invasion (42). miR-204-5p upregulation in glioma cells suppresses glioma cells growth, migration and invasion by triggering RAB22A, which belongs to RAS proteins superfamily (43). miR-204-5p was demonstrated to be downregulated in several malignancies including renal cell carcinoma and gastric cancer (44,45). Our results correspond to the aforementioned data that suggest that miR-204-5p functions as cancer suppressor in various malignancies, including melanoma, with the targeting of several genes implicated in carcinogenesis.

Target genes for microRNAs investigated in the present study were selected by application of three computational tools for microRNA target prediction and estimated functionally by qPCR. However, the luciferase assay may have provided the correct determination of more specific interactions between the microRNA and its targeting site in the mRNA. The absence of luciferase assay data is a limitation of the present study.

miR-204-5p and miR-3065-5p were previously described as miRNAs with tumor-suppressor properties, although their effects are exerted through triggering different signaling pathways and target genes (13). In the present study, their suppressor effects on cell viability and proliferation, and migration and invasion capacity of melanoma cells was observed, to determine whether they may be of value as an anticancer treatment option.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

NP, AK, MA, and AM performed the research. TR designed the study. NP, AK, MA, AS and TR analyzed the data. NP, AK, MA and TR wrote the paper.

Ethics approval and consent to participate

The present study was approved by the Krasnoyarsk State Medical University Local Ethics Committee (protocol no. 70/2016, issued on June 6, 2016, Krasnoyarsk, Russia). Written informed consent was obtained from all patients.

Consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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