Expression of miRNAs as Important Element of Melanoma Cell Plasticity in Response to Microenvironmental Stimuli

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Abstract. *Background: Melanoma cells form monolayers in serum-containing media, however, in serum-free media they form anchorage-independent spheroids. We investigated miRNAs differentially expressed between these culture types and identified those that possibly control the plasticity of melanoma cells. Materials and Methods: The expression of miRNAs in melanoma cells was evaluated with microarrays, and certain miRNAs were validated with real-time PCR. Several bioinformatic tools were used to assess the involvement of identified miRNAs in cancer-related pathways, and to compile the results of mRNA microarray data from the same melanoma cells. Results: A total of 19 miRNAs were differentially expressed between monolayers and spheroids. miRNAs up-regulated in spheroids modulated cell motility and migration, whereas those up-regulated in monolayers suppressed melanogenesis. Conclusion: The present study identified those miRNAs that participate in the regulation of melanoma cell plasticity.*

Metastatic melanoma is an aggressive tumor highly refractory to chemotherapy and has a very poor prognosis. The median survival time of patients with metastatic melanoma is 6 months and the 5-year survival rate is only 15% (1). One of the factors that accounts for chemotherapy resistance of metastatic melanoma tumors is their excessive heterogeneity manifested by the presence of diverse cell sub-populations within one tumor. These sub-populations are characterized by specific transcriptional signatures that are reversible *e.g.* in response to distinct signals from the microenvironment (2). The pervasive phenotypic plasticity of melanoma cells is driven by reversible changes of expression of several factors promoting the switch between different phenotypes of melanoma cells (3).

Melanoma cells grown in stem cell medium (SCM) form anchorage-independent spheroids with a high capacity to differentiate along the mesenchymal lineage and enhanced expression of human embryonic stem cell pluripotency markers, such as sex determining region Y-box (SOX) 2, Nanog transcription factor and octamer-binding transcription factor 4 (4). Spheroids contain a sub-population of cells exhibiting self-renewal capacity, however, when serum is introduced into the medium, a reduction in the number of cells with this property is observed, and melanoma cells gain a proliferative phenotype and exhibit adherent growth (5, 6).

The mechanisms of microenvironment-dependent molecular changes responsible for this melanoma phenotypic switch are still elusive, however, epigenetic modulation of gene expression, and changes in miRNA levels are considered to be involved. MiRNAs are small non-coding RNA molecules that possess the ability to down-regulate gene expression by binding to target mRNAs. They have been found to control the expression of more than 30% of human genes, and at least 60% of human transcripts are miRNA targets (7). These regulatory RNA molecules are already recognized as important modulators of many signaling pathways and their aberrant expression frequently leads to pathogenic states, including cancer. For example, miRNAs dysregulated in breast cancer preferentially target key pathways associated with oncogenesis, including the mitogen-activated protein kinase (MAPK) pathway, p53 signaling and transforming growth factor-beta (TGFβ) signaling (8). Down-regulation of miR-768-3p in V-Raf murine sarcoma viral oncogene homolog B $BRAF^{V600E}$ melanoma cells, mediated by activation of the MAPK/ERK kinase (MEK) pathway, enhances eukaryotic translation initiation factor 4E protein production and promotes proliferation (9). miRNA expression profiles can be altered by changes in the levels of regulatory molecules, including transcription factors. Knocking-down microphthalmia-associated transcription factor (MITF) substantially changed the miRNA profile in melanocytes (10). The overexpression of hypoxia-inducible factor 1-alpha in TGFβ1-expressing melanoma cells triggered the up-regulation of four miRNAs, whose down-modulation arrests the cell cycle (11). The dysregulation of miRNAs in melanoma directly or indirectly influences the intercellular exchange of various proteins and genetic material *via* exosomes, and exosomal transfer of

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Table I. *Primers used in the real-time PCR validation of miRNA microarray expression.*

Primer	Sequence
miR-1915-3p	5'-CGACGCGGCGGGAAAA-3'
miR-1234	5'-TGACCACCCACCCCACAA-3'
miR-940	5'-AAAAGCTGGGTTGAGAGGGT-3'
miR-320c	5'-GCCCCCGCTCCCCAAA-3'
RNU6B	5'-TGACACGCAAATTCGTGAAG-3'
RPS17	F: 5'-AATCTCCTGATCCAAGGCTG-3'
	R: 5'-CAAGATAGCAGGTTATGTCACG-3'

RNU6B: Small nuclear RNA U6B; RPS17: ribosomal protein S17; F: forward primer; R: reverse primer.

Table II. *miRNAs differentially expressed in DBMC cells grown in stem cell medium (SCM) compared to cells grown in serum-supplemented medium. Results from six DMBC cell lines were averaged.*

miRNA	FDR	Fold change	Log_2 fold change	*p*-Value
Up-regulated in cells grown as spheroids in SCM				
miR-1301	0.00083	2.755	1.46	1.83E-06
miR-182-5p	0.019	6.910	2.79	0.00029
miR-191-5p	0.019	2.480	1.31	0.00034
miR-1915-3p	0.022	1.858	0.89	0.00044
miR-378d	0.011	2.052	1.03	7.07E-05
miR-3934	0.049	1.613	0.69	0.002
miR-4767	0.031	3.104	1.63	0.00083
miR-542-3p	0.016	10.733	3.42	0.00022
Up-regulated in cells grown as monolayers in serum-containing medium				
miR-1234	0.032	1.613	0.69	0.001
miR-1246	0.032	2.25	1.17	0.001
miR-192-5p	0.026	4.170	2.06	0.00057
miR-193a-5p	0.034	6.190	2.63	0.001
miR-3171	0.035	2.900	1.53	0.001
miR-3195	0.037	1.592	0.67	0.002
miR-320c	0.032	1.757	0.81	0.00096
miR-4769-3p	0.011	3.247	1.70	9.77E-05
miR-5701	0.011	3.117	1.64	0.00012
miR-575	0.030	1.815	0.86	0.00072
miR-940	0.007	1.661	0.73	3.51E-05

FDR: False-discovery rate.

miRNAs may contribute to melanoma progression not only between closely localized cells, but also in distant tissues (12).

Several studies have demonstrated a link between this phenotypic switch and alterations in miRNA levels. Lung adenocarcinoma cells in three-dimensional culture were characterized by higher expression of tumor-suppressive miRNAs (*e.g.* miR-200), and lower expression of oncogenic miRNAs (*e.g.* miR-21) compared to those in two-dimensional cultures (13). Mammospheres derived from breast cancer cells differed in the expression of 17 miRNAs compared to cells from monolayers, and target transcripts of these miRNAs were involved in several key signaling pathways (14). The overexpression of miR-888 significantly reduced the ability of breast cancer cells to adhere, and increased their potential for migration and invasion (15). miR-888 directly targets E-cadherin and other genes involved in the adherens junction pathway (16). Finally, polycomb ring finger protein, a stem cell renewal factor, is targeted by miR-203 in esophageal cancer stem-like cells, and its overexpression significantly reduced colony formation of these cells (17).

In the present study, we evaluated the changes in miRNA expression between melanoma cells grown as anchorage-independent spheroids in SCM and cells grown as monolayers in medium supplemented with serum. As each miRNA is able to potentially target hundreds of transcripts, many in-silico methods combining different algorithms for miRNA target prediction can be used to identify significant miRNA-mRNA interactions (18-22). Thus, the obtained results of miRNA expression were combined with results of another study conducted in our laboratory, identifying differential expression of mRNA in melanoma cells cultured in SCM and serum-containing medium (23).

Materials and Methods

Cell culture characteristics. Melanoma cells were obtained during surgical interventions. The melanoma cell populations used, all derived from nodular melanoma specimens, were the following: DMBC2, DMBC8, DMBC9, DMBC10, DMBC11 and DMBC12 (Department of Molecular Biology of Cancer). Histopathological analyses were performed to confirm the melanocytic origin of tumor samples. The study was approved by the Ethical Committee of the Medical University of Lodz (approval number RNN/84/09/KE), and written informed consent was obtained from all patients. Clinical characteristics of melanoma samples used in this study, and the procedure of isolation of melanoma cells have been published elsewhere (6, 24, 25).

Cell culture. DMBC populations (300,000 cells) were grown in low-adherent flasks in SCM composed of: Dulbecco's modified Eagle's medium (DMEM)/F12 (Lonza, Basel, Switzerland) supplemented with B27 (Gibco, Paisley, UK), 10 ng/ml basic fibroblast growth factor and 20 ng/ml epidermal growth factor (BD Biosciences, San Jose, CA, USA), insulin (10 μg/ml), heparin (1 ng/ml), and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml fungizone B). For monolayer growth, cells were transferred to DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and antibiotics. Both types of cultures were run in parallel for at least three weeks (with medium exchanged twice a week) at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were then harvested and used for the study.

Total RNA isolation. Total RNA was extracted using miRvana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Briefly, cells were dissolved in Lysis/Binding Solution, miRNA Homogenate Additive was added

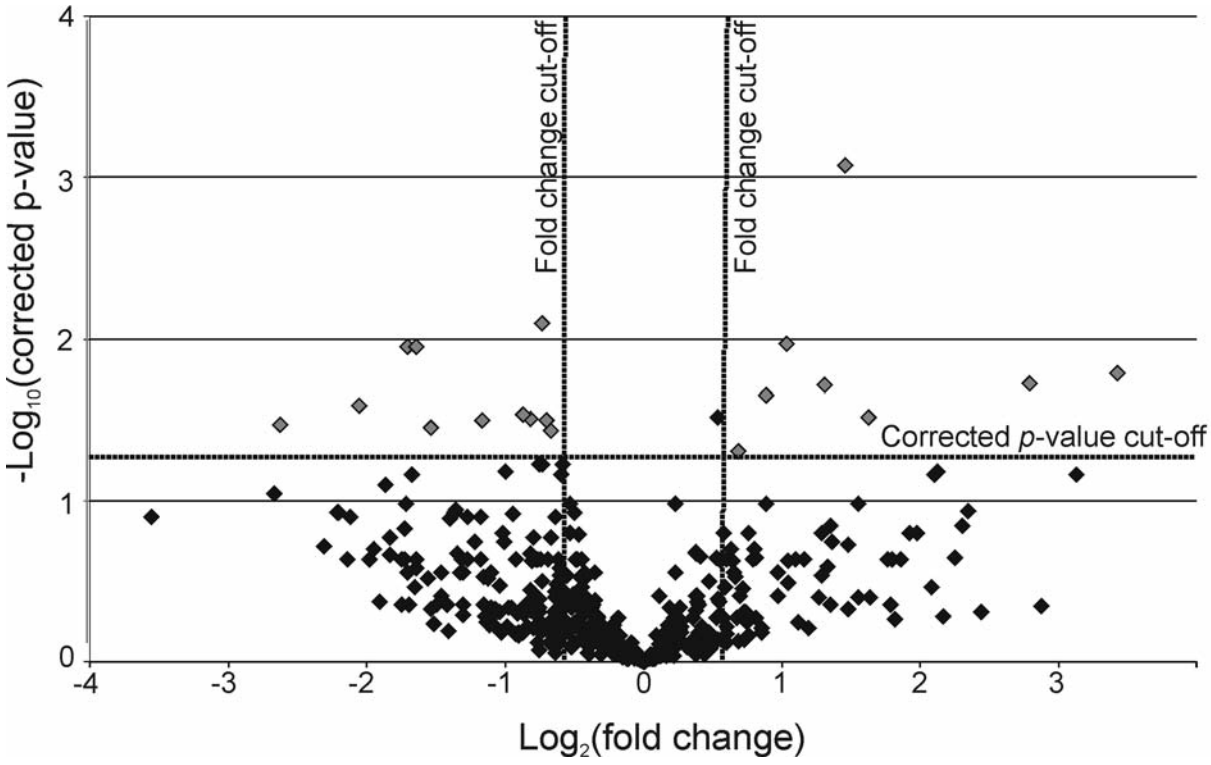

Figure 1. *Differential expression of miRNAs in DMBC cells grown in stem cell medium (SCM) compared to cells grown in serum-containing medium. Volcano plot comparing the expression of miRNAs in cells grown in SCM and serum supplemented medium. p-Values were derived from corrected t-test. Corrected p-value cut-off=0.05, fold change cut-off=1.5. miRNAs significantly up-regulated in cells grown in SCM are located in the top right panel of the graph, whereas miRNAs significantly up-regulated in monolayers are located in the top left panel.*

and after incubation on ice for 10 min, the mixture was subjected to organic extraction with acid/phenol/chloroform mixture (5 min), and the aqueous phase was mixed with 98% ethanol and passed through a glass-fiber filter. RNA particles bound to the filter were washed, extracted with 100 μl of 95˚C Elution Buffer and stored at −80˚C. The quality of the isolated RNA was evaluated using NanoQuant Infinite M200 Pro (Tecan Austria GmbH., Grodig, Austria) and Bioanalyzer 2100 with Total RNA Nano Kit (Agilent Technologies, Santa Clara, CA, USA). Samples with an A260/A280 ratio of between 1.90 and 2.10, *18S/28S* rRNA ratio ≥1.8 and RNA integrity number ≥8.50 were used for subsequent microarray experiments.

Microarray analysis of miRNA expression. The microarray miRNA analysis was carried-out at the Department of Physiological Sciences, Warsaw University of Life Sciences. Total RNA (100 ng) was end-labeled with cyanine 3-pCp (Lumiprobe, Hannover, Germany) following the manufacturer's recommendations using Agilent's miRNA Complete Labeling and Hybridization kit (Agilent). Labeled miRNA was hybridized to Agilent's Human 8×60k miRNA microarrays, custom-designed with eArray platform (AMADID 041036) based on Sanger miRBase v.18 (26). One array covers 1887 human miRNA probes in 30 copies, and spike-in controls. After the hybridization, microarray images were captured using an Agilent DNA Microarray Scanner with default settings for miRNA microarrays. Scanned TIFF images were processed using Agilent Feature Extraction Software version 10.10.1.1. Raw fluorescent intensity signals were subjected to bioinformatics analysis with GeneSpring Software version 12.5 (Agilent). miRNA expression data were filtered with detected/non-detected flag, and quantile-normalized according to the manufacturer's suggestions.

Statistical significance of differences in miRNA expression was determined using an unpaired t-test followed by Benjamini-Hochberg false discovery rate (FDR) correction (27). miRNAs with a fold change of 1.5 or greater and a FDR ≤0.05 were considered to be differentially expressed between cells grown in SCM and cells grown in serum-supplemented media.

Real-time polymerase chain reaction (RT-PCR)validation of miRNA microarray expression. Four selected miRNAs were subjected to expression validation with RT-PCR. Firstly, isolated total RNA containing miRNA was subjected to cDNA synthesis with NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 1000 ng of total RNA containing miRNA was incubated with Reaction and Enzyme mix for 1 h at 37˚C in 20 μl. After reaction termination (5 min at 95˚C) , cDNA samples were stored at −20˚C and diluted 10-fold prior to the qRT-PCR reaction. qRT-PCR reaction was carried out using the Rotor-Gene 3000 Real-Time DNA analysis system (Corbett Research, Mortlake, Victoria Australia) and NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kit Universal

Table III. *Kyoto Encyclopedia of genes and genomes (KEGG) pathways of target transcripts of miRNAs up-regulated in cells grown in stem cell medium (SCM) and in cells grown in serum-supplemented medium.*

KEGG number	KEGG pathway	No. of transcripts	*p*-Value	Benjamini correction
Cells grown in SCM				
hsa05215	Prostate cancer	4	0.00712	0.402
hsa05214	Glioma	3	0.0323	0.694
hsa05218	Melanoma	3	0.0402	0.627
hsa04810	Regulation of actin cytoskeleton	4	0.0708	0.734
hsa04114	Oocyte meiosis	3	0.0874	0.732
Cells grown in serum-supplemented medium				
hsa05215	Prostate cancer	9	3.07E-05	0.0026
hsa05200	Pathways in cancer	15	1.59E-04	0.0068
hsa05210	Colorectal cancer	8	1.61E-04	0.0046
hsa05213	Endometrial cancer	6	7.67E-04	0.0163
hsa05223	Non-small cell lung cancer	6	9.12E-04	0.0155
hsa04722	Neurotrophin signaling pathway	8	0.00171	0.0242
hsa05214	Glioma	6	0.00183	0.0223
hsa04510	Focal adhesion	10	0.00192	0.0205
hsa04916	Melanogenesis	7	0.00259	0.0244
hsa04910	Insulin signaling pathway	8	0.00278	0.0236

(Invitrogen). Five nanograms of cDNA, 200 nM miRNA-specific forward primers, and 200 nM of universal miRNA reverse primer (NCode™ VILO™ miRNA cDNA Synthesis Kit) were mixed with SYBR GreenER Supermix in a total volume of 10 μl. Standard cycling program included 2 min at 50˚C for uracil DNA glycosylase incubation, 2 min at 95˚C for enzyme activation, and 40 cycles of 15 s at 95˚C followed by 1 min at 60˚C. The miRNA expression values were normalized to expression of small nuclear RNA U6B (*RNU6B*) and ribosomal protein s17 (*RPS17*) genes. To calculate the miRNA expression ratio between melanoma cells grown in medium with and without serum, the $2^{-\Delta\Delta Ct}$ method was used (28). The complete sequences of primers are listed in Table I.

Bioinformatics analysis. The impact of all differentially expressed miRNAs on melanoma cells was investigated with the Database for Annotation, Visualization and Integrated Discovery (DAVID) (29, 30). Two sets of putative gene targets for miRNAs found to be up-regulated in cells grown in SCM and in cells grown in serum-supplemented media, respectively, were provided with DIANA-microT-CDS 5.0 tool, which recognizes miRNA recognition elements located in both the 3'-UTR (untranslated region) and coding sequence regions (20). The microT threshold of sensitivity was set to 0.7 (default). Both lists were limited to transcripts targeted by at least three differentially expressed miRNAs and introduced to DAVID to identify the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations that were under-represented in cells grown in SCM and in cells grown in serum-supplemented medium, respectively. Modified Fisher's exact test (EASE) with additional Benjamini-Hochberg correction was used in DAVID (29).

Two programs for identifying miRNA-target mRNA interactions were used: miRTrail and miRTar (21, 22). Venn diagram and visualization with Cytoscape web application (Gladstone Institutes, San Francisco, CA, USA) of potential miRNA-target mRNA interactions for degree constraints of 1 and 2 ware prepared with MiRTrail. This used the algorithm of sequence complementarity and thermodynamic stability of miRNA-mRNA duplex and showed the significance of interactions between differentially expressed miRNAs and their differentially expressed targets assessed in the previous study (23). miRTar identifies miRNA target sites in mRNA by applying diverse algorithms to limit false-positive results. miRTar performed a gene set-enrichment analysis for miRNA-regulated gene sets to identify putative roles of these miRNAs in biological processes and pathways. The list of differentially expressed miRNAs was introduced to the 'miRNAs to Metabolic Pathway' mode in order to assess their involvement in two KEGG pathways: melanogenesis and wingless-int (WNT) signaling. With default parameters of miRNA-mRNA duplex (miminum free energy ≤14 kcal/mol; alignment score ≥140), the list of target mRNAs linked to miRNAs across the particular pathway with its computed *p*-value was obtained.

Results

Microarray profiling revealed 19 miRNAs differentially expressed between melanoma cells grown as spheroids in SCM and as monolayers in serum-containing medium. The composition of culture medium significantly impacts melanoma cell morphology, therefore, we were interested whether this is also reflected by alterations of miRNA expression. For this purpose, self-designed microarray chips, based on Sanger miRNA database v.18 (26), and Agilent's platform were applied. We analyzed six pairs of DMBC melanoma cultures grown side by side in SCM or serum-containing medium. Out of 1,887 human miRNAs, only 444 were found in the majority of DMBC melanoma cultures. A volcano plot prepared for these miRNAs revealed that 19 were differentially expressed in cells grown under the two tested conditions (Figure 1). Eight miRNAs (*miR-1301, miR-182-5p, miR-191-5p, miR-1915-3p, miR-378d, miR-3934, miR-4767, miR-542-3p*) were up-regulated in cells grown in SCM, whereas eleven miRNAs (*miR-1234, miR-1246, miR-192-5p, miR-193a-5p, miR-3171, miR-3195, miR-320c, miR-4769-3p, miR-5701, miR-575, miR-940*) were up-regulated in cells grown as monolayers in serum-containing media (Table II).

miRNAs up-regulated in cells grown as spheroids or monolayers might modulate diverse molecular and cellular processes. To analyze the influence of selected miRNAs on gene expression, we applied *in silico* methods for predicting their target transcripts (Figure 2). Using DIANA-microT-CDS 5.0 miRNA target-prediction tool with a sensitivity threshold of 0.7, a total of 4,668 and 4,841 mRNAs were identified as

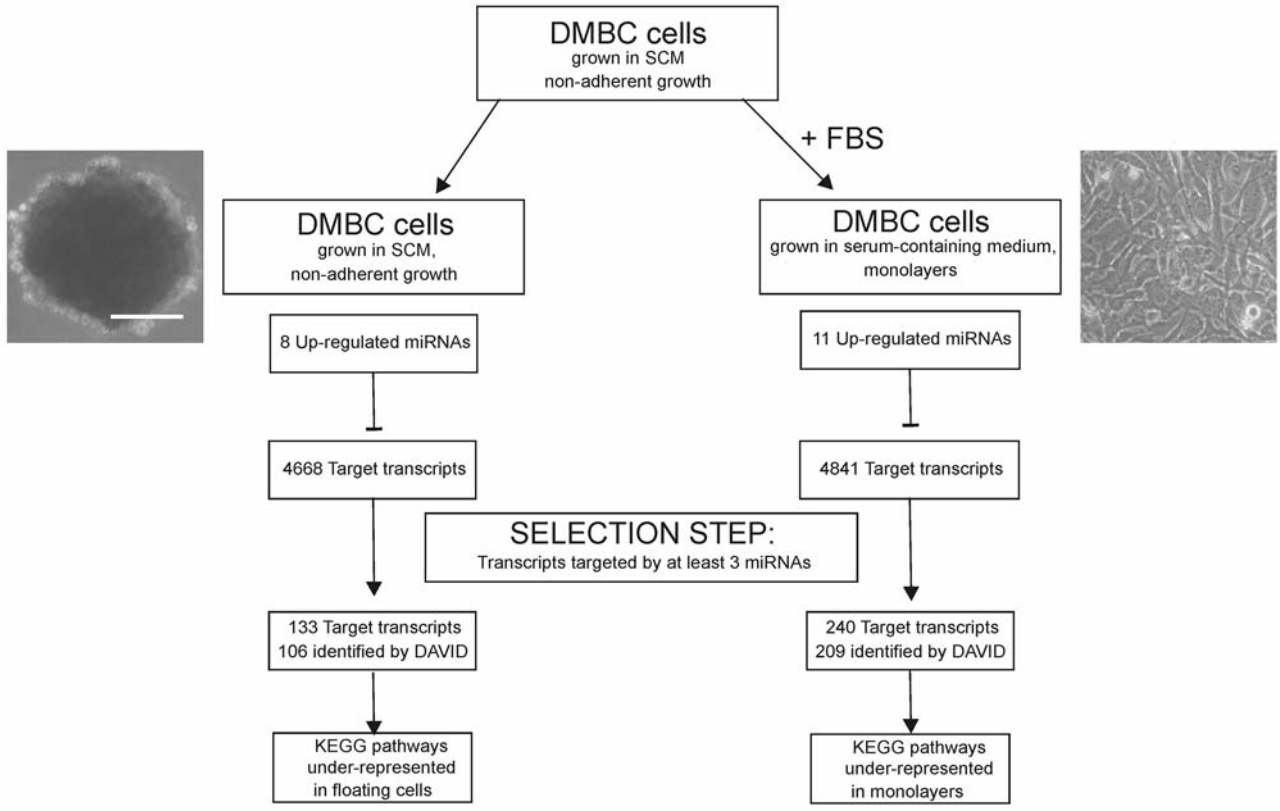

Figure 2. *Bioinformatics analysis of transcripts targeted by miRNAs up-regulated in cells grown in stem cell medium (SCM) and serum-containing medium. A flow chart describing the main assumption of the analysis. Targets for miRNAs up-regulated in DMBC cells grown in SCM and in serum-supplemented medium were identified and only transcripts regulated by at least three miRNAs were subjected to further analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified for transcripts targeted by miRNAs up-regulated in cells grown in SCM and cells grown in serum-supplemented medium. Examples of anchorage-independent spheroids (left) and monolayers (right) are shown. Scale bar, 100 μm.*

being up-regulated in cells grown in SCM and in serum-containing medium, respectively. As single hit/seed predictions might not correlate well with transcript repression (33), we focused on transcripts that were collectively regulated by several miRNAs, provoking their powerful down-modulation. When transcripts targeted by at least three different miRNAs were considered, the initial lists were limited to 133 and 240 mRNAs potentially repressed in cells grown in SCM and in serum-supplemented medium, respectively. Using the DAVID tool, 106 transcripts were successfully annotated as potential targets of eight miRNAs up-regulated in cells grown in SCM and 209 transcripts were selected as potential targets of eleven miRNAs up-regulated in cells grown in serum-containing medium (Figure 2). Using the KEGG pathways database, we linked targets of miRNAs up-regulated in cells grown in SCM with only five pathways, including Melanoma and Regulation of Actin Cytoskeleton, among others. Conversely, targets of miRNAs up-regulated in monolayers were involved in Pathways in Cancer, Focal Adhesion or Melanogenesis. The most significant KEGG pathways for target transcripts of miRNAs up-regulated in cells grown under the two test conditions are shown in Table III.

Transcripts that were controlled by the greatest number of miRNAs are listed in Table IV. Among transcripts targeted by the greatest number of miRNAs up-regulated in monolayers, cAMP responsive binding protein (*CREB5*) targeted by seven miRNAs, and protein tyrosine phosphatase, non-receptor type 4 (*PTPN4*) and myocyte enhancer factor 2C (*MEF2C*) both targeted by six miRNAs were found. Argonaut protein 1 (*AGO1*) and lysine k-specific methyltransferase 2C (*KMT2C*) were each targeted by five miRNAs up-regulated in spheroids.

Altered miRNA expression correlates with the micro-environment-dependent differential expression of mRNAs in melanoma cells. We used transcription profiles for melanoma cells [grown either in SCM or in serum-containing media (23)] from a parallel study conducted in our laboratory to determine the possible contribution of miRNAs in the regulation of melanoma phenotype. We used the list of differentially expressed mRNAs containing 857 transcripts

Table IV. *Transcripts targeted by the largest number of miRNAs up-regulated in cells grown in stem cell medium SCM and in cells grown in serum-supplemented medium.*

Transcript	Transcript name	No. of miRNAs	Up-regulated miRNAs
Cells grown in SCM			
AGO1	Argonaut RISC catalytic component 1	5	*miR-1301, miR-182-5p, miR-1915-3p, miR-378d, miR-542-3p*
KMT2C	Lysine (K)-specific methyltransferase 2C	5	*miR-1301, miR-182-5p, miR-1915-3p, miR-3934, miR-542-3p*
SLC7A6	Solute carrier family 7 (amino acid transporter light chain, y+L system), member 6	4	*miR-1301, miR-1915-3p, miR-378d, miR-3934*
SLC33A1	Solute carrier family 33 (acetyl-CoA transporter), member 1	4	*miR-1301, miR-182-5p, miR-1915-3p, miR-3934*
NTRK3	Neurotrophic tyrosine kinase, receptor, type 3	4	*miR-1301, miR-182-5p, miR-3934, miR-542-3p*
SHPRH	SNF2 histone linker PHD/RING helicase, E3 ubiquitin protein ligase	4	*miR-1301, miR-182-5p, miR-378d, miR-542-3p*
CDK5RAP3	CDK5 regulatory subunit associated protein 3	4	*miR-1301, miR-182-5p, miR-1915-3p, miR-3934*
MMP24	Matrix metallopeptidase 24 (membrane-inserted)	4	*miR-182-5p, miR-1915-3p, miR-3934, miR-542-3p*
MAPRE2	Microtubule-associated protein, RP/EB family, member 2	4	*miR-182-5p, miR-1915-3p, miR-3934, miR-542-3p*
ARL10	ADP-ribosylation factor-like 10	4	*miR-182-5p, miR-1915-3p, miR-378d, miR-542-3p*
PTCHD1	Patched domain containing 1	4	*miR-182-5p, miR-1915-3p, miR-3934, miR-542-3p*
Cells grown in serum-supplemented medium			
CREB5	cAMP responsive element-binding protein 5	7	*miR-1246, miR-192-5p, miR-3171, miR-320c, miR-5701, miR-575, miR-940*
PTPN4	Protein tyrosine phosphatase, non-receptor type 4	6	*miR-1246, miR-192-5p, miR-320c, miR-4769-3p, miR-5701, miR-940*
MEF2C	Myocyte enhancer factor 2C	6	*miR-1246, miR-192-5p, miR-320c, miR-5701, , miR-575, miR-940*
MMP16	Matrix metallopeptidase 16	6	*miR-1246, miR-193a-5p, miR-3171, miR-320c, miR-4769-3p, miR-5701*
AR	Androgen receptor	6	*miR-1246, miR-193a-5p, miR-320c, miR-4769-3p, miR-575, miR-940*
KMT2C	Lysine (K)-specific methyltransferase 2C	6	*miR-1234, miR-1246, miR-193a-5p, miR-320c, miR-4769-3p, miR-5701*

RISC: RNA-induced silencing complex; SNF2: sucrose non fermentation 2; PHD/RING: specific zinc finger domain; CDK5: cyclin-dependent kinase 5; RP/EB: retinitis pigmentosa/end binding.

up-regulated and 949 transcripts down-regulated by at least two-fold in cells grown in SCM, and integrated them with the list of 19 miRNAs that were differentially expressed. To assess the miRNA-target interactions, the miRTrail bioinformatic tool was used, which employs an algorithm based on sequence complementarity and thermodynamic stability of miRNA-mRNA duplex (21).

We obtained a Venn diagram that depicts the relationship between the 19 miRNAs differentially expressed in either microenvironment and their potential targets. As shown in Figure 3A, 127 miRNA-mRNA pairs were found. When miRNA-target interactions were visualized with the Cytoscape web application with a prediction *p*-value threshold of 0.01, only four out of 19 input miRNAs had differentially expressed targets. Among these *miRNAs, miR-193a-5p, miR-575*, and *miR-940* were up-regulated in cells in monolayers, and *miR-542-3p* was up-regulated in spheroid cells grown in SCM. The graph in Figure 3B depicts all 127 interactions between these four miRNAs and their potential targets. In order to only focus on the most significant interactions, we increased the miRNA degree constraint to 2 (the minimum number of target genes that interacts with each miRNA) which led to the identification of mRNAs that were potentially down-regulated in monolayers and spheroids (Figure 3C).

Our study on melanoma transcriptome profiles indicates that the microenvironment may substantially influence the WNT signaling pathway and melanogenesis (23). In order to determine a possible involvement of the 19 miRNAs selected in the present study in either of these two pathways, we used the web-based miRTar tool (22). Using the 'miRNAs to Metabolic Pathway' prediction workflow, we uploaded a set of eight miRNAs up-regulated in spheroids and chose the WNT signaling pathway for mRNA target prediction. We identified four miRNAs (*miR-542-3p, miR-182-5p, miR-*

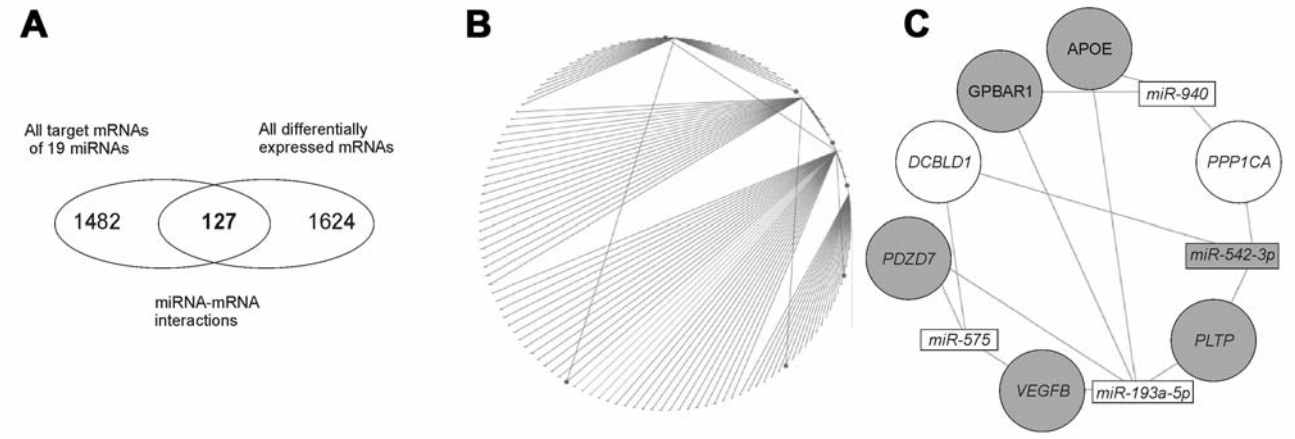

Figure 3. *Correlation between mRNAs and miRNAs differentially expressed in DMBC cells grown as spheroids and in monolayers. A: Venn diagram prepared in miRTrail tool depicting all mRNAs with unaltered expression that were targets of 19 selected miRNAs, differentially expressed mRNAs that are not targets of the selected miRNAs, and pairs of miRNAs and mRNAs differentially expressed in SCM and serum-containing medium. B: A subnetwork of the top four miRNAs that passed the p-value threshold of 0.01 with all 127 affected mRNA targets. C: A subnetwork of the top four miRNAs with a degree constraint of a target mRNA of 2. Grey represents up-regulated and white represents down-regulated miRNAs and their targets. APOE: Apolipoprotein E, PPP1CA: protein phosphatase 1, catalytic unit, PLTP: phospholipid transfer protein, VEGFB: vascular endothelial growth factor B, PDZD7: PZD domain containing 7, DCBLD1: discoidin, GPBAR1: G protein-coupled bile acid receptor 1.*

1915-3p and *miR-1301*) that were directly involved in regulating the expression of several genes belonging to the WNT signaling pathway. Since miRNAs negatively regulate gene expression, targets of miRNAs up-regulated in spheroids might be down-regulated in melanoma cells building these three-dimensional structures. By uploading a list of differentially expressed genes obtained in our previous study that were annotated to WNT signaling pathway (23), we found several genes whose expression was lowered at least two-fold in spheroids when compared to monolayers [*e.g.* frizzled class receptor 8 (*FZD8*), *WNT3* and T-cell transcription factor 7 (*TCF7*), shown in bold in Table V].

When melanogenesis was chosen for mRNA-target prediction, nine miRNAs were directly linked to this KEGG pathway. Six of them up-regulated in monolayers (*miR-320c, miR-3195, miR-1234, miR-575, miR-193a-5p* and *miR-940*) potentially targeted several genes encoding proteins involved in pigmentation [*e.g.* tyrosinase (*TYR*) and tyrosinase-related protein 1 (*TYRP1*), shown in bold in Table VI]. Indeed, their expression was reduced in the presence of serum (23).

Several miRNAs were chosen for in vitro validation by real-time RT-PCR. The expression of 4 (*miR-940, miR-1915-3p, miR-320c* and *miR-1234*) out of 19 miRNAs identified in this study was confirmed with qRT-PCR. miR-940 is a member of a sub-network designed with miRTrail tool and directly impacts apolipoprotein E (*APOE*), G protein-coupled bile acid receptor 1 (*GPBAR1*) and protein phosphatase 1, catalytic unit (*PPP1CA*) genes. Moreover, this miRNA, together with *miR-320c* and *miR-1234*, target several important genes involved in melanogenesis, a pathway that was substantially suppressed in monolayers grown in the presence of serum. *miR-1915-3p* targets genes from the WNT signaling pathway. Mean values of at least 3 biological replicates obtained for DMBC melanoma cells were compared with the single microarray results. In agreement with the microarray data, *miR-1915-3p* was significantly up-regulated in cells in spheroids, whereas *miR-1234*, *miR-940* and *miR-320c* were up-regulated in cells in monolayers (Figure 4A). When expression of each miRNA was averaged among the six DMBC cultures, the overall Pearson's correlation coefficient between both methods was 0.99 (Figure 4B).

Discussion

The aim of the present study was to evaluate the differential expression of miRNAs in melanoma cells grown in the presence of different stimuli. Melanoma cells grown in serum-free medium frequently form three-dimensional melanospheres (6). Sphere-forming capacity is a typical feature of cancer cells with stem-like properties. Melanospheres grown in SCM are highly heterogeneous and contain a subset of poorly-differentiated cells characterized by increased potential for self-renewal and decreased proliferation, and a subset of highly pigmented differentiated cells (23). In addition, melanoma cells are characterized by high phenotypic plasticity and can reversibly change their phenotype (4-6). It has already been shown for other types

Table V. *Genes of the wingless (WNT) signaling pathway found to be targets of miRNAs up-regulated in spheroids. Genes in bold were suppressed in spheroids according to Hartman et al. (23). p-Values are given for the interaction of miRNA and target transcripts of the WNT pathway.*

miRNA	*p*-Value	Target gene	No. of genes/no. of genes in pathway
miR-542-3p	1.65e-12	*VANGL1, TCF7L1, SFRP1, PRKCB, PPP2CA, NFAT5, FZD7, DVL1*	8/182
miR-182-5p	1.90e-24	*WNT5B, WNT5A, SMAD4,* ***RUVBL1****, PRKCB, PPP3R1, PPP2R5A, MAPK10, LRP6, FZD3, FZD1, DKK2, CXXC4, CSNK2A2, CAMK2B, BTRC*	16/182
miR-1915-3p	9.77e-110	*WNT9A, WNT8B, WNT8A, WNT7B, WNT7A, WNT5B, WNT4,* ***WNT3A****,* ***WNT3****, WNT2B, WNT2,* ***WNT11****, WNT10A, WNT1, VANGL2, VANGL1, TCF7L1, SOX17, SMAD3, SMAD2 SFRP5, SFRP1,* ***RUVBL1****, ROCK2, RAC1, PRKX, PRKCG, PRKCA, PRKACB, PRKACA, PPP3CB, PPP2R5D, PPARD,* ***PLCB2****, NLK, NKD2, NFATC4, NFATC2, NFATC1, NFAT5 MAPK9, MAPK10, LEF1, FZD7, FZD5, FZD10, FRAT2, FRAT1, FOSL1, FBXW11, EP300, DVL3, DKK4, DAAM2, CTNNBIP1, CTNNB1, CTBP2, CTBP1, CSNK1A1, CREBBP CHP2, CHD8, CCND3, CCND2, CAMK2G, CAMK2D, CAMK2A, AXIN2, AXIN1, APC2,*	70/182
miR-1301	1.94e-116	*WNT9B, WNT9A, WNT8B, WNT8A, WNT5B, WNT5A, WNT4,* ***WNT3****, WNT2B,* ***WNT11****,* ***WNT10B****, WNT10A, WNT1, WIF1, VANGL2, VANGL1, TCF7, TBL1XR1, SMAD4, SMAD2 SFRP2, SFRP1,* ***SENP2****,* ***RUVBL1****, ROCK2, ROCK1, RHOA, RAC3, RAC2, RAC1, PRKCB, PRKCA, PRICKLE2, PPP3CB, PPP3CA, PPP2R5D, PPP2R5C, PPP2R5B, PPP2R5A, PPARD PORCN, PLCB3, PLCB1, NLK, NFATC4, NFATC3, NFATC1, NFAT5, LRP5, JUN, GSK3B,* ***FZD8****, FZD6, FZD5, FZD4, FZD1, FRAT1,* ***FOSL1****, EP300, DAAM2 CTNNBIP1, CTNNB1, CTBP2, CSNK2B, CSNK2A1, CSNK1E, CREBBP, CHD8, CCND3, CCND2, CAMK2G, AXIN1, APC2, APC*	74/182

of cancer cells that phenotypic changes are linked to alterations in miRNA expression (13, 34). In the present study, we identified 8 miRNAs that were consistently up-regulated in melanoma cells grown as three-dimensional spheroids in SCM and eleven that were up-regulated in cells grown as monolayers in serum-containing medium considering a fold-change of 1.5 or more (35-37).

The analysis of transcripts that could be simultaneously targeted by five different miRNAs revealed that expression of *AGO1* and *KMT2C* might be diminished in cells grown in SCM. AGO1 interacts with RNA polymerase II and regulates genes that are implicated in oncogenic pathways, including cell-cycle progression, cell growth and survival (38). KMT2C possesses histone methylation activity and regulates the expression of homeotic genes causing oncogenic transformation (39). The high proliferative potential of cells in monolayers might, at least, be partially mediated by down-modulation of *PTPN4*, targeted by 6 miRNAs up-regulated in these cells. PTPN4 negatively regulates cell-cycle progression and proliferation *via* inhibiting crk-like protein (40). Another transcript targeted by the 6 miRNAs up-regulated in monolayers is *MEF2C*, therefore its expression in these cells might be reduced. Together with *SOX10* transcription factor, *MEF2C* positively regulates the expression of melanocyte pigment genes (41). Our previous study indicated that melanogenesis is one of the most enriched KEGG pathways in melanospheres grown in SCM, with 22 genes annotated to this pathway (23). Two genes highly up-regulated in melanospheres and involved in melanin synthesis, *TYR* and *TYRP1* (23), might be targeted by miRNAs overexpressed in monolayers: *miR-320c* (*TYR* and *TYRP1*) and *miR-193* (*TYRP1*). Moreover, the expression of these 2 genes is dependent on MITF. In the present study, MITF was identified by two target-predicting tools, DIANA microT and miRanda, as being negatively regulated by 4 miRNAs highly expressed in monolayers: *miR-320c, miR-940, miR-1246* and *miR-5701*. Cells grown in monolayers in serum-supplemented medium are characterized by lower melanin synthesis and with 25 up-regulated genes that belong to the WNT signaling pathway (23). *FZD8*, *WNT3*, RuvB-like AAA ATPase 1 (*RUVBL1*) and *TCF7* are positive regulators of this signaling pathway, whereas sentrin-specific protease 2 (*SENP2*) and *WNT11* negatively regulate WNT signaling. All genes listed in Table V are targeted by miRNAs up-regulated in melanospheres. Although cells in monolayers overexpress both positive and negative regulators of this pathway, the final effect towards the blockade of WNT/β-catenin signaling is mediated by a strong up-regulation of the inhibitor Dickkopf 1 (*DKK1*) (23).

Using miRTrail (21), we combined data for differential expression of miRNAs and mRNAs in cells grown in different microenvironments and identified the significant pairs of interacting molecules. The results revealed direct interactions of four miRNAs (*miR-940, miR-575, miR-542-3p* and *miR-*

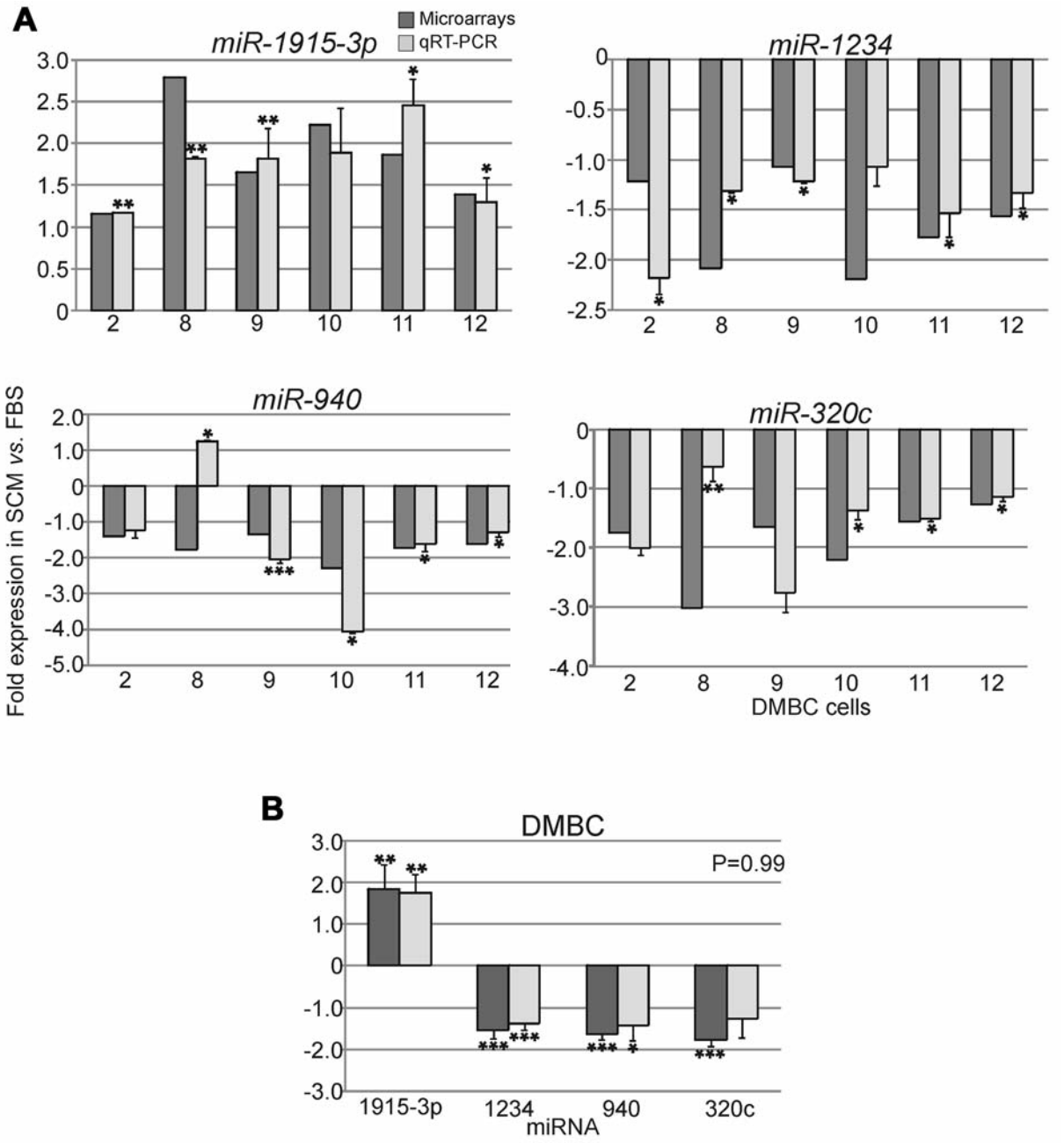

Figure 4. *Validation of miRNA expression with RT-PCR. A: Four miRNAs differentially expressed between melanoma cells grown in SCM or serum-containing medium were subjected to RT-PCR analysis, and results performed in at least three biological replicates were compared under their normalized microarray data. B: Expression of four selected miRNAs was averaged among six different DMBC populations and Pearson's correlation coefficient between microarray and RT-PCR was calculated. The data present the mean±standard deviation and Student's t-test was used to determine significant differences between the mean values of expressed miRNAs. $^{*}p<0.05$,$^{**}p<0.01$ and $^{***}p<0.005$. DMBC under the graph is for Department of Molecular Biology of Cancer, where melanoma populations were isolated and cultured, and numbers indicate the particular patient-derived melanoma population.*

193a-5p) with seven target transcripts [*APOE*, vascular endothelial growth factor *(VEGF)B*, *PDZD7, DCBLD1, PLTP, PPP1CA* and *GPBAR1*], identified recently in our laboratory as being differentially expressed in melanospheres and cells in monolayers (23). According to the Cytoscape web graph (Figure 3C), both *miR-193a-5p* and *miR-940*, up-regulated in monolayers, target *APOE*. This secretory protein is an anti-angiogenic, and metastasis-suppressive factor in melanoma

Table VI. *Genes of the melanogenesis pathway found to be targets of miRNAs up-regulated in cells grown in monolayers. Genes in bold were suppressed in monolayers according to Hartman et al. (23). p-Values are given for the interaction of miRNA and target transcripts of the WNT pathway.*

miRNA	*p*-Value	Target gene	No. of genes/no. of genes in pathway
miR-3137	4.41e-4	*MAP2K2, MAP2K1*	2/112
miR-192-5p	1.88e-7	*TCF7, FZD4, CALM1, ADCY7*	4/112
miR-1246	7.74e-11	*PRKACA, PLCB1, KRAS, KITLG, GNAQ, CALM2*	6/112
miR-320c	5.16e-28	*WNT5A,* ***TYRP1****,* ***TYR****, TCF7L2, TCF7L1, TCF7, PRKCG, MAPK1, GNAI2, FZD5, EP300, EDNRB, CREB3L3,* ***CREB3L2****, CALM3, ADCY6*	16/112
miR-3195	1.68e-31	*WNT7B, WNT6, WNT4, TCF7, PRKCG, MAPK3, MAP2K2, GNAS,* ***FZD9****, FZD2, FZD10, FZD1,* ***CREB3L2****, CAMK2A, CALML3, ADCY9, ADCY5, ADCY4*	18/112
miR-1234	9.15e-37	*WNT7A, WNT6, WNT3, WNT2B, WNT2, WNT1, TCF7, PRKX, PRKCG, PRKCA, POMC, PLCB3, MAPK3, FZD2, EP300, CREBBP,* ***CREB3L2****, ADCY6, ADCY3, ADCY2* ***ADCY1***	21/112
miR-575	1.57e-38	*WNT8A, WNT5B, WNT5A, WNT4,* ***WNT10B****, TCF7L1, RAF1, PRKCA, PLCB3, PLCB1, MAP2K1, GNAI2, DVL2, DVL1, CTNNB1, CREBBP, CREB3L3, CAMK2G, CAMK2D, CALM3 ADCY4, ADCY2,*	22/112
miR-193a-5p	1.24e-45	*WNT9B, WNT8B, WNT5B, WNT16, WNT11, WNT1,* ***TYRP1****, PRKX, PRKCG, PRKACA, PLCB1, HRAS, GNAS,* ***FZD7****, FZD4, FZD1, EDNRB, CREB3L4,* ***CREB3L2****, CREB3L1 CAMK2G, CAMK2B, CAMK2A, ADCY5, ADCY4,* ***ADCY1***	26/112
miR-940	3.13e-86	*WNT7B, WNT6, WNT5B, WNT3A, WNT2B, WNT2, WNT1,* ***TYRP1****, TCF7L1, TCF7, RAF1, PRKCG, PRKCB, PRKCA, PRKACA, PLCB3, PLCB2, MC1R, MAPK3, GNAS GNAQ, FZD8,* ***FZD7****, FZD6, FZD5, FZD4, EP300, EDNRB, DVL3, DVL1, CREBBP, CREB3L4, CREB3L3,* ***CREB3L2****, CREB3L1, CREB3, CAMK2G, CAMK2B, CAMK2A, CALML3 CALM3, ADCY9, ADCY7, ADCY6, ADCY4, ADCY3, ADCY2,* ***ADCY1***	48/112

cells (42). It suppresses invasion and metastasis through recruitment to low-density lipoprotein receptor-related protein (LRP) 1 and LRP8 receptors, thus inhibiting WNT signaling pathway in monolayers. Moreover, *miR-940* is a mediator of cell survival and tumor resistance to cytotoxic drugs, acting *via* the MAPK1 pathway (43). *miR-193a-5p* is a member of the p63/p73 regulatory circuit, and its inhibition in squamous cell carcinoma compromised cell viability and enhanced chemosensitivity in response to cisplatin (44). *VEGFB*, also targeted by *miR-193a-5p* and additionally by *miR-575*, is up-regulated in spheroids (23). This gene is usually overexpressed in metastatic melanomas (45). *miR-575*, described as a tumor suppressor, was down-regulated in several different types of cancer (46), and its expression significantly increased in cells after exposure to ionizing radiation (47). *miR-542-3p*, a tumor suppressor in neuroblastoma, reduced tumor growth and invasive potential (48). It also targeted survivin and its ectopic expression in adenocarcinoma and inhibited proliferation by inducing cell-cycle arrest (49).

MiRNAs can also mediate cell communication among distant cells through their release in exosomes (12). The work of Xiao *et al*. identified several miRNAs as being up-regulated in exosomes from the A375 melanoma cell line compared to exosomes released from normal melanocytes, and these miRNAs were linked to cellular development, cell death, proliferation, tumor growth and motility (50). In the present study, *miR-1246, miR-182-5p, miR-378, miR-320c, miR-193a-5p* and *miR-940* were identified as being differentially expressed in spheroids compared to cells growing in monolayers.

Conclusion

The results presented herein suggest that the reversible switch of melanoma cell phenotype might be mediated by miRNAs. The present study showed a differential expression of miRNAs in melanoma cells grown in the presence of extracellular stimuli and identified miRNAs that are linked to different phenotypes of melanoma cells. We identified

possible targets of miRNAs up-regulated in melanoma cells growing as spheroids in SCM and those up-regulated in cells grown as monolayers in medium supplemented with serum. Moreover, using the results of our transcriptome analysis (23), we found several possible miRNA-mRNA interactions that might contribute to different phenotypes of melanoma cells.

Competing Interests

The Authors declare that they have no competing interests.

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