Loss of microRNA-205 expression is associated with melanoma progression

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In this study, we used formalin-fixed paraffin-embedded melanocytic tumors to demonstrate reproducible alterations in microRNA expression in nevi compared with melanomas using a microarray platform. We validated those results in an independent set of nevi and melanomas by quantitative RT-PCR. miR-205 demonstrated a statistically significant, progressive diminution in expression from nevi to primary melanomas to metastatic melanomas. Enforced miR-205 expression in melanoma cells profoundly impairs cell motility and migration along with significantly decreased F-actin polymerization with only a modest reduction in cell proliferation. Using a xenograft model, melanoma cells overexpressing miR-205 exhibit a reduced migratory capacity compared with control tumor cells. Mechanistically, miR-205 overexpression results in decreased expression of the zinc-finger E-box binding homeobox 2 (ZEB2) mRNA and protein. This coincides with increased expression of E-cadherin mRNA and protein. Furthermore, re-introduction of ZEB2 into melanoma cells overexpressing miR-205 rescues these phenotypic effects and results in a restoration of cell migration and F-actin polymerization with a concomitant reduction in E-cadherin expression. Together, these results provide *in vitro* and *in vivo* evidence for miR-205 as a critical suppressor of melanoma cell migration.

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Melanoma is the most deadly of the common forms of skin cancer, and its incidence is steadily rising. Despite aggressive management strategies ranging from surgical excision to targeted therapies, the consequences are equally dire: approximately 8700 people died of melanoma in 2010—which constituted ~65% of all skin cancer-related deaths.^{1–3} There is therefore a critical need to identify clinically significant biomarkers in melanoma that illuminate biochemical pathways central to the aggressive behavior of this disease and in the process, unveil new opportunities for the design of rational therapeutic interventions in high-risk patients.

microRNAs (miRNAs) are non-coding RNAs of approximately 20–22 nucleotides in length that function in post-transcriptional gene regulatory pathways. There are approximately 1000 miRNAs in the human genome,⁴ and it has been estimated that approximately one-third of all human mRNAs are subject to miRNA regulation.⁵ miRNAs function in a diverse set of biological processes, including differentiation, proliferation

and apoptosis.^{6–8} In addition, alterations in miRNA expression have been described in many different human tumors, and numerous studies have demonstrated that miRNAs function as key pathogenic components impacting cancer cell growth, survival and the capacity to metastasize.^{7,9–13} Furthermore, miRNAs serve as instructive molecular surrogates in the distinction of benign from malignant as well as in prognostic algorithms of outcome in various tumor types.^{14–16} Finally, it is feasible to obtain high-quality miRNA expression data from formalin-fixed paraffin-embedded (FFPE) melanomas—often the only tissue available for retrospective analyses.¹⁷

Despite increasing evidence underscoring miRNAs as key components in melanoma biology, relatively few studies have characterized miRNA expression profiles in patient samples.^{18–26} Even fewer studies have described differences in the miRNA expression profile of benign nevi compared with malignant melanoma.^{19,26} In this study, we performed miRNA profiling

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of clinical samples of benign and malignant melanocytic lesions. This analysis identified a set of miRNAs that distinguish benign nevi from malignant melanoma—a subset of which were validated in an independent set of clinical samples. We have also functionally characterized miR-205, which showed the most significantly diminished expression in primary melanomas and metastatic melanomas compared with nevi. We demonstrate that enforced expression of miR-205 impedes melanoma cell migration and invasion *in vitro* and *in vivo* while exerting only a modest impact on cell proliferation. Expression of miR-205 in melanoma cells results in decreased zinc-finger E-box binding homeobox 2 (ZEB2) mRNA and protein levels, and this correlates with increased levels of E-cadherin mRNA and protein expression. Furthermore, re-introduction of ZEB2 into melanoma cells overexpressing miR-205 reverses the observed defects in cell migration and restores suppression of E-cadherin mRNA and protein expression. Together, our results show a direct role for miR-205 in the progression of malignant melanoma.

MATERIALS AND METHODS

Sample Selection and RNA Extraction

The tissue samples were obtained from archives in the Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania. All tissues were collected according to the guidelines and policies of the Hospital of the University of Pennsylvania Institutional Review Board. We collected 10 specimens of benign nevi with a substantial dermal component, 10 primary melanoma specimens with a substantial dermal component (Clark level IV, Breslow thickness >1.0 mm) and 10 metastatic melanomas from sentinel lymph node specimens with extensive nodal involvement. Four 20- μ m sections per sample were obtained from the selected block, and the area of interest was macrodissected from a glass slide for the extraction of total RNA using the RecoverAll Total Nucleic Acid Isolation system (Ambion, Cat#1975) according to the manufacturer's instructions. The total RNA yield was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Tech, Wilmington, DE, USA). RNA integrity was assessed by Agilent 2100 Bioanalyzer electrophoresis compared with standard reference RNA as previously described for total RNA prepared from FFPE tissue.^{17,27–30}

miRNA Microarrays

Targets were prepared using the miRCURY LNA microRNA Power Labeling Kit (Exiqon #208032-A) for Exiqon miRNA microarrays according to the manufacturer's instructions. Labeling reactions were performed essentially as previously described^{17,29} using equivalent 1 μ g aliquots from each FFPE melanoma sample. The labeled miRNA was applied to miRCURY LNA microRNA array (Exiqon #208002-A) according to the manufacturer's instructions for hybridization and washing.

Data Analysis and Statistical Analysis

Arrays were scanned using Genepix 6.0 software with standard settings. Genepix files (GPR) were imported into Partek Genomics Suite (v6.4, Partek, St Louis, MO, USA), where foreground–background intensity values were extracted for each probe and on-chip probe replicates (quadruplicate spots of a particular miRNA sequence) were averaged. Probes with flagged (technically bad) intensities for >2/3 of the samples were excluded, and any remaining flagged probe intensities were treated as missing data. Values <1 were assigned a value of 1 to facilitate subsequent log₂ transformation. Statistical analysis comparing the three groups to one another was performed by applying one-way analysis of variance (ANOVA) to the set of log₂ intensities for each probe for each sample of the three tissue types (nevus, primary melanoma and metastatic melanoma). In conjunction with the ANOVA, all three pairwise comparisons (PvN, MvN and MvP) were calculated yielding a *P*-value and a fold-change for each. For all *P*-values (ANOVA and pairwise comparisons) a false discovery rate correction was applied using the Benjamini–Hochberg step up method as available in Partek software. miRNA intensities were clustered hierarchically using Euclidean distance and average linkage. Principal component analysis was performed using log₂ intensities for each probe described above.

Real-Time RT-PCR of miRNAs and RT-PCR of mRNAs

Real-time quantitative RT-PCR was carried out on an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) machine using the TaqMan MicroRNA Assay for miR-205 (Applied Biosystems) according to the manufacturer's instructions. Briefly, total RNA template was prepared, and the concentration and integrity were assessed as described above. In all, 10 ng of total RNA was used for reverse transcription using the specific miRNA primers for hsa-miR-205 (Applied Biosystems), and the U6B snRNA (Applied Biosystems) was used as a control. The mean *C_T* was determined from duplicate or triplicate reactions. Delta *C_T* was calculated as *C_T*_{miRNA} – *C_T*_{U6B}. Relative gene expression was calculated as 2^{–(Delta *C_T*)} and multiplied by 10⁵ to ease data presentation as previously described.^{9,29} Unpaired Student's *t*-test and Wilcoxon test were used to assess for a statistically significant difference between the relative miRNA expression levels in melanomas compared with nevi (*P* < 0.03).

Isolation RNA and Quantitative PCR in Cell Lines

Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA, USA) followed by cDNA synthesis using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using the iQ SYBR green supermix (Bio-Rad Laboratories) with specific primers (listed below). cDNA corresponding to 1 μ g of RNA was added to the iQSYBER green supermix and analyzed with iCYCLER (Bio-Rad Laboratories) according to the manufacturer's instructions. The cycling conditions were

40 cycles of 95 °C for 30 s and 56 °C for 30 s. Melting curve analysis was carried out for each PCR reaction to confirm the specificity of amplification. At the end of each phase, fluorescence was used to qualify PCR product. The following primers were used: real-time PCR primer: E-cadherin forward primer (5'-TTCCCTGCGTATACCCTGGT-3'), E-cadherin reverse primer (5'-GCGAAGATACCGGGGACACTCATGAG-3'), Zeb2 forward primer (5'-CGCTTGACATCACTGAAGGA-3'), Zeb2 Reverse primer (5'-CTTGCCACACTCTGTGCATT-3'), β -actin forward primer (5'-TGACTGACTACCTCATGAAGATCC-3') and β -actin reverse primer (5'-GCCATCTCTTGCTCGAAGTCC-3'). To confirm miR-205 is not lost with progressive cell passage, miR-205 expression was measured before every single experiment.

Cell Culture, Plasmids, Transfection, Western Blots and Immunohistochemistry

Human melanoma cell lines (WM35, WM793, WM115A and 1205Lu) were gifts from Meenhard Herlyn at the Wistar Institute. These cell lines were maintained in 2% MCDB medium. Total RNA and protein were isolated as previously described.³¹ A human 293T cell line was kindly provided by Frank Lee at the University of Pennsylvania and was maintained in high glucose DMEM with 10% fetal bovine serum, penicillin/streptomycin (100 units/ml and 100 mg/ml).

PEZX-MR03 vector (HIV based) containing hsa-miR-205 with eGFP reporter gene, c-Cherry lentiviral control vector and eGFP lentiviral control vector were purchased from GeneCopoeia (Rockville, MD, USA). The plasmid was co-transfected into 293T cells with a packing vector (pCMV-dR8.2-dupr and pCMV-VSV), and viral supernatants were collected 72 h post-transfection and used to infect human melanoma cells (WM115A, WM35, WM793 and 1205Lu). After 48 h, cells were incubated in selection medium containing puromycin (1 μ g/ml) for 5 days to create stable cell lines. pCDNA3-Zeb2 plasmid was a gift from Dr Eugene Tulchinsky and Dr Gareth Browne (Department of Cancer Studies and Molecular Medicine, School of Medicine, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester). 1205Lu cells stably expressing miR-205 were transfected with empty pCDNA3 control vector and pCDNA3-Zeb2 plasmid using FuGENE 6 Transfection Reagent (Roche Nutley, NJ, USA) according to the manufacturer's instructions.

Control or PEZX-miR-205 stably infected melanoma cells were seeded on fibronectin pretreated chamber slides. Cells were fixed with glutaraldehyde for 20 min at room temperature and then washed three times with 1% BSA for 5 min. Cells were then treated with 0.3% Triton X-100, 1% BSA and 10% normal donkey serum in PBS at room temperature for 45 min. Phalloidin-TRITC was purchased from R&D systems. Tumor cells were incubated with Phalloidin-TRITC overnight at 4 °C. Cells were then washed three times with PBS containing 1% BSA. Nuclei were counterstained

with DAPI (Vector Laboratories). Cells were imaged with a Leica inverted fluorescence microscope with a Leica camera.

Western blots were performed as previously described³² using monoclonal mouse anti-human E-cadherin antibody (1:500 dilution, Dako) and rabbit polyclonal anti-human ZEB2 IgG polyclonal antibody (Santa Cruz Biotechnology, sc-48789, 1:200 dilution).

Cell Migration and Invasion Assays

Cell migration and invasion were examined using a two-dimensional wound-healing assay and a three-dimensional Boyden chamber assay. For the wound-healing assay, control or PEZX-miR-205 stably infected melanoma cells (1205 Lu, WM35, WM793, WM115A) were grown to confluence as a monolayer, and cell monolayers were 'wounded' by dragging a 1-ml pipette tip across the cells. Cells were washed to remove debris and allowed to migrate until completely confluent. Images were taken at 0 and every 24 h after wounding using a DMI6000 inverted microscope to monitor cell migration, as a function of 'wound' closure. Three replicates each of two independent experiments were performed. Transwell invasion assays were performed in modified Boyden chambers as previously described.^{32,33} Student's *t*-test or one-way ANOVA was used to determine statistical significance ($P < 0.05$).

Cell Proliferation Assay

WM115A, WM35, WM793 and 1205Lu control and infected with mir-205 cells were seeded in 24-well plates at densities of 5×10^4 cells per well and grown for 48 h. The WST-1 assay was used to detect viable proliferative cells. The absorbance at 450 nm was measured using an iQuant Universal Microplate Spectrophotometer (Bio-tek Instruments, Winooski, VT, USA).

Xenograft Tumor Model

Control (1×10^6 cells) and pPEZX-miR-205 stably infected WM115A cells (1×10^6 cells) were mixed and injected subcutaneously into 4- to 5-week-old male athymic *nu/nu* mice (eight mice per group). After 35 days, mice were killed and necropsies performed. Tumors were harvested and frozen sections were prepared for routine and fluorescence microscopy.

Zeb2 Rescue Assay

1205Lu cells stably expressing miR-205 were transfected with empty pCDNA3 control vector and pCDNA3-Zeb2 plasmid as described above. Quantitative RT-PCR and western blots were performed as described above to confirm ZEB2 overexpression in these cells and E-cadherin repression. Wound-healing assays, Boyden chamber assays, cell proliferation assays and F-actin staining assays were performed in these cells as described above.

RESULTS

miRNA Expression Profiles in Nevus and Melanomas

We employed miRNA microarrays to determine the relative miRNA expression levels among 10 samples each of cutaneous nevi, primary cutaneous melanoma and metastatic melanoma (Figures 1a–c). We selected cutaneous lesions with a dermal component of sufficient size as well as large, expansile metastases adequate for macrodissection with the intention of generating a relatively ‘pure’ RNA sample representative of the intended melanocytic proliferation—with minimal contamination from non-melanocytic cell types.

Using the microarray data, miRNA signal intensities were subjected to unsupervised hierarchical clustering analysis. The dendrogram in Figure 1d displays a relative similarity with regards to global miRNA expression profiles among nevi, and these are distinct from both primary and metastatic melanoma. In contrast, primary and metastatic melanomas did not cluster distinctly from one another according to their overall relative miRNA expression profiles. We next identified a subset of 20 miRNAs whose expression is significantly

($P < 0.05$ after corrections for multiple hypothesis testing) downregulated and 4 miRNAs whose expression is significantly upregulated in melanomas (both primary and metastatic) compared with nevi (Table 1). From this analysis, we did not identify miRNAs whose expression was significantly different between primary cutaneous melanoma and metastatic melanoma.

miR-205 Expression Is Progressively Reduced from Nevus to Melanomas in Tissue Samples and Cell Lines

We chose miR-205 for confirmation in the original sample set and validation in an independent set of nevi, primary melanomas and metastatic melanomas using RT-PCR. miR-205 was selected because its relative signal intensity exhibited the most robust relative changes between melanomas and nevi in the microarray analysis. As shown in Table 1, miR-205 exhibited the greatest reduction in expression in metastatic melanomas *versus* nevi (~ 97 -fold reduction, $P = 8.5 \times 10^{-5}$ after corrections for multiple hypothesis testing) and in primary melanomas compared with nevi (~ 12 -fold reduction,

Figure 1 miRNA expression signatures in nevi, primary melanomas and metastatic melanomas. (a) Low power ($\times 2$) view of representative nevus used in the study (inset $\times 40$ reveals maturation in deeper aspects of dermis, H&E stained slide). (b) Low power ($\times 2$) view of representative primary cutaneous melanoma used in the study (inset $\times 40$ reveals marked cytologic atypia in dermal component, H&E stained slide). (c) Low power ($\times 2$) view of representative metastatic melanoma to lymph node used in the study (inset $\times 40$ reveals marked cytologic atypia in the metastasis, H&E stained slide). (d) Hierarchical clustering analysis of miRNA expression intensity data demonstrate that miRNA expression signatures of nevi cluster distinctly from primary and metastatic melanomas. Legend: nevi (blue), primary melanoma (green) and metastatic melanoma (red).

Table 1 Selected miRNAs with altered expression in melanoma

Gene title	P-value (P vs N)	Fold-change (P vs N)	P-value (M vs N)	Fold-change (M vs N)	P-value (M vs P)	Fold-change (M vs P)
hsa-miR-205	0.0493376	−12.104	8.51077E−05	−97.0232	0.839256	−8.0158
hsa-miR-211	0.00736396	−8.19663	0.0131309	−6.07649	0.849974	1.34891
hsa-miR-200b	0.0185648	−8.07371	8.51077E−05	−25.726	0.839256	−3.18639
hsa-miR-203	0.00529666	−7.44928	0.00001102	−25.3981	0.839256	−3.40947
hsa-miR-200a	0.03092	−6.68673	0.00134812	−13.235	0.839256	−1.97929
hsa-miR-455-3p	0.000762182	−6.50457	0.000394133	−6.33887	0.990181	1.02614
hsa-miR-200c	0.00886728	−6.4791	3.80343E−05	−18.0751	0.839256	−2.78976
hsa-miR-141	0.061971	−4.99914	0.000190174	−17.621	0.839256	−3.5248
hsa-miR-125b	0.00736396	−4.73544	0.00172363	−5.50276	0.899347	−1.16204
hsa-miR-455-5p	0.000916754	−4.70947	0.000770988	−4.6509	0.990181	1.01259
hsa-miR-99a	0.0303064	−3.08136	0.0127295	−3.18958	0.982303	−1.03512
hsa-miR-513b	0.00257157	−2.91634	0.011214	−2.33231	0.839256	1.25041
hsa-miR-548b-5p	0.03092	−2.49956	0.00351676	−3.17349	0.839256	−1.26962
hsa-miR-125a-5p	0.03092	−2.47606	0.00672826	−2.86174	0.858657	−1.15576
hsa-miR-29b-1	0.0205583	−2.37898	0.00102653	−3.05175	0.839256	−1.2828
hsa-miR-382	0.10441	−2.23233	0.00672826	−3.16642	0.839256	−1.41844
hsa-let-7c	0.0304529	−2.19184	0.00672826	−2.44723	0.874787	−1.11652
hsa-miR-132	0.0542121	−1.9622	0.00636154	−2.3607	0.839256	−1.20309
hsa-miR-576-5p	0.0732075	−1.92304	0.00805034	−2.29141	0.839256	−1.19156
hsa-miR-193b	0.152299	−1.71539	0.00805034	−2.58925	0.839256	−1.50942
hsa-miR-9	0.00139738	14.8948	0.00434131	10.0727	0.839256	−1.47873
hsa-miR-21	0.000123528	12.1309	0.000311648	8.44255	0.839256	−1.43688
hsa-miR-142-3p	0.0268823	5.80833	0.00469565	7.79193	0.849974	1.34151
hsa-miR-223	0.00736396	3.96147	0.0255421	2.57643	0.839256	−1.53758

Abbreviations: M: metastatic melanoma; N: nevus, P: primary cutaneous melanoma.

The indicated *P*-values are the 'step up' corrected *P*-values (see Materials and methods section).

$P=0.05$, after corrections for multiple hypothesis testing) (Figure 2a). RT-PCR for miR-205 expression in the set of 30 pilot samples confirmed the progressive reduction in expression observed in the microarray platform (Figure 2b, $P=5.0 \times 10^{-4}$). There was a statistically significant reduction in the expression of miR-205 between nevi and primary melanomas (~ 3.8 -fold, $P=5.8 \times 10^{-3}$) and between nevi and metastatic melanomas (~ 10.5 -fold, $P=6.7 \times 10^{-4}$). By RT-PCR, expression of miR-205 is reduced between primary and metastatic melanoma; however, this did not achieve statistical significance (~ 2.8 -fold, $P=0.059$).

Using an independent set of tissues from five nevi, five primary melanomas and five metastatic melanomas, we validated the reduction of miR-205 expression in the melanomas compared with nevi (Figure 2c, $P=9.2 \times 10^{-3}$). There was a statistically significant reduction in the expression of miR-205 between nevi and metastatic melanomas (>10 -fold, $P=7.9 \times 10^{-3}$) and between primary and metastatic melanomas (>10 -fold, $P=7.9 \times 10^{-3}$). In addition, we compared

miR-205 expression in a panel of melanocyte and melanoma cell lines representing all stages of melanocytic tumor progression: foreskin-derived melanocyte cell lines, WM35 (derived from a radial growth phase-only primary melanoma), WM793 (derived from a vertical growth phase primary melanoma), WM115A (derived from a melanoma metastasis) and 1205Lu (derived from a melanoma metastasis).³¹ As shown in Figure 2d, there is a statistically significant reduction in miR-205 expression from foreskin melanocytes to primary melanoma (~ 2 -fold in WM35, $P=3.9 \times 10^{-4}$) and metastatic melanoma (~ 5 -fold in WM115A and 1205Lu). Furthermore, there was a statistically significant reduction in miR-205 expression between primary, radial growth phase-only melanoma (WM35) and metastatic melanoma (~ 2 -fold in WM115A, $P=1.7 \times 10^{-3}$ and ~ 2.5 -fold in 1205Lu, $P=3.8 \times 10^{-4}$). Taken together with the results from patient samples above, these results show a progressive diminution of miR-205 expression in the progression of melanoma.

Effects of miR-205 Overexpression *In Vitro*

To characterize the function of miR-205 in melanoma cells, we examined the effects of enforced expression of miR-205 in human melanoma cell lines. 1205Lu, WM35, WM793

and WM115A human melanoma cell lines were infected with lentiviruses carrying miR-205 with GFP reporter (Figure 3a, left panels) or control viruses carrying Cherry red expression constructs (Figure 3a, right panels). We mixed these cells (1:1) and grew the mixture to a confluent monolayer. We then created an artificial wound in the monolayer and measured the ability of each of these cell lines to migrate across this artificial separation (Figure 3b). 1205Lu melanoma cells overexpressing miR-205 exhibited a significant reduction in their capacity to close an artificial wound compared with controls (Figures 3b and c). This defect is cell autonomous, as cells with impaired migration were not rescued by control cells capable of undergoing migration (Figure 3b). Similar results were obtained for other melanoma cell lines, including WM35, WM793 and WM115A (Figure 3c). This defect in cell migration also correlated with alterations in cell morphology and the actin cytoskeleton structure: cells overexpressing miR-205 exhibited a rounded morphology with fewer cell protrusions and reduced actin polymerization compared with control cells (Figure 3d). In addition, we performed Boyden chamber assays to assess the impact of enforced expression of miR-205 on cell invasion. The results showed a significant deficiency in a panel of melanoma cell lines for invasion in cell lines stably overexpressing miR-205 compared with controls (Figures 4a and b).

To determine whether the observed defects in cell migration and invasion because of enforced expression of miR-205 were attributable to defects in cell proliferation, we performed cell proliferation assays (Figure 4c). miR-205 overexpression exerted no significant effect on the proliferation of WM35 and WM793 melanoma cells and exerted only a modest impact on proliferation of WM115A and 1205Lu melanoma cells. Together, these results argue that overexpression of miR-205 in melanoma cells causes a predominant defect in cell migration/invasion with a lesser effect on cell proliferation in the melanoma cells tested.

Effects of miR-205 Overexpression *In Vivo*

Next, we sought to determine if miR-205 overexpression exerts a similar effect on melanoma cell migration *in vivo*.

Figure 2 Relative signal intensities of differentially expressed miRNAs in melanomas compared with nevi. (a) Relative signal intensities of miR-205 by microarray analysis ($P = 3.5 \times 10^{-4}$, log scale). (b) Relative expression of miR-205 in the original sample set by RT-PCR ($P = 5.0 \times 10^{-4}$). (c) Relative expression of miR-205 in an independent set of nevi, primary melanomas and metastases by RT-PCR ($P = 9.2 \times 10^{-3}$). (d) Relative expression of miR-205 in a panel of melanocytic cell lines: foreskin-derived melanocyte cell lines, WM35 (derived from a radial growth phase-only primary melanoma), WM793 (derived from a vertical growth phase primary melanoma), WM115A (derived from a melanoma metastasis) and 1205Lu (derived from a melanoma metastasis). Legend: nevi (black), primary melanoma (grey) and metastatic melanoma (white). Asterisk (*) indicates statistical significant differences in expression ($P < 0.05$).

We mixed (1:1) WM115A cells infected with control lentiviral vectors with Cherry red report with WM115A cells overexpressing miR-205 with GFP reporter and injected

this mixture into the subcutaneous tissue of male athymic *nu/nu* mice. The resultant tumors were harvested after 35 days of growth and frozen sections were performed to

Figure 3 Overexpression of miR-205 delays wound closure and affects cell actin polymerization *in vitro*. (a) 1205Lu cells were infected with lentivirus carrying Cherry red control vector or pEZ-205, which carries GFP to create stable 1205Lu cells expressing either Cherry red (control, left two panels) or miR-205 together with GFP (right two panels). (b) These cells were mixed together at a 1:1 ratio, and a wound-healing assay was performed. At 48 h, the majority of the cells migrating across the wound were Cherry red control cells (far right panel, merge) ($n = 3$ replicate experiments). (c) Graphical representation of triplicate experiments of wound closure times in three other different melanoma cell lines (WM35, WM793 and WM115A) infected with control or mir-205 vectors as described above ($n = 3$ replicate experiments). Asterisk (*) indicates statistical significant differences in expression ($P < 0.05$). (d) Actin staining with phalloidin demonstrates a reduction in cellular protrusion, a more rounded morphology and a reduction in actin polymerization in 1205Lu cells infected with a virus carrying a mir-205 construct compared with controls expressing GFP ($n = 3$ replicate experiments).

Figure 4 Overexpression of miR-205 impedes melanoma cell invasion *in vitro* and *in vivo*. (a) Boyden chamber assay. 1205Lu cells were infected with viruses carrying control or mir-205 constructs as indicated (see Materials and methods section). Cells migrating through the chamber were stained and counted ($n = 3$). (b) Quantification of the percentage of cell migration through the chamber compared across four different melanoma cell lines (WM35, WM793, WM115A and 1205Lu) infected with control or mir-205 vectors as indicated (experiment repeated three times). (c) Quantification of cell proliferation (as a function of optical density) in the indicated melanoma cell lines (WM35, WM793, WM115A and 1205Lu) infected with control or mir-205 vectors as indicated. There is a modest reduction in cell density after mir-205 overexpression. (d–f) Mouse xenografts were constructed by combining equal mixtures (1:1) of WM115A melanoma cells infected either with lentivirus carrying Cherry red control vector or pEZX-miR-205, which carries GFP and injected into the subcutaneous tissues of 4- to 5-week-old male athymic *nu/nu* mice. Tumors were harvested after 35 days. (d) Routine histologic analysis of the tumors by frozen section reveals a monotonous proliferation of markedly atypical epithelioid cells forming an infiltrative tumor in the subcutaneous tissue. (e) Fluorescence microscopy reveals a biphasic tumor: control cells (labeled in red) exhibit an infiltrative pattern of growth and occupy the peripheral aspects of the lesion, while cells overexpressing miR-205 (labeled in green) accumulate centrally within the tumor. (f) Graphical quantification of the percentage of tumor volume occupied by each cell type in eight different xenografts. *indicates statistically significant differences.

characterize the tumors. Routine microscopic analysis reveals a monotonous proliferation of markedly atypical melanocytes forming an infiltrative subcutaneous tumor (Figure 4d). However, fluorescence microscopy reveals a distinctly biphasic appearance of the tumors. Namely, the cells overexpressing miR-205 (green cells in Figure 4e) cluster together centrally within the tumor and occupy ~30% of the tumor volume. In contrast, the control cells (red cells in Figure 4e) exhibit an infiltrative pattern of growth, occupy a larger volume of the tumor (~70%) and are concentrated toward the peripheral aspects of the lesion (red cells expressing Cherry red, Figure 4e). These data argue that enforced expression of miR-205 impairs melanoma cell migration and to a certain degree, cell proliferation *in vivo*.

Overexpression of miR-205 Coincides with Alterations in the EMT Pathway

miR-205 has a key role in the epithelial-to-mesenchymal transition (EMT) as a silencer of ZEB1/2 transcriptional repressor levels.^{34–36} As miR-205 expression is progressively diminished from nevi to primary melanomas to metastatic melanomas and because enforced expression of miR-205 impairs melanoma cell mobility, we hypothesized that miR-205 expression might impact ZEB1/2 and consequently, E-cadherin expression in melanoma cells. As shown in Figure 5a, we established a panel of cell lines representing all stages of melanoma progression: WM35 (radial growth phase only), WM793 (vertical growth phase primary melanoma), WM115A (metastatic melanoma) and 1205Lu (metastatic melanoma) stably expressing relatively similar levels of

Figure 5 Overexpression of miR-205 negatively regulates ZEB2 mRNA and protein and results in a reciprocal increase in E-cadherin mRNA and protein. (a) Quantitative RT-PCR indicating relative expression of miR-205 in the indicated melanoma cell lines (WM35, WM793, WM115A and 1205Lu) stably infected with control or mir-205 vectors. (b, c) Quantitative RT-PCR indicating relative reduction in ZEB2 mRNA (b) and relative increase in E-cadherin mRNA (c) in the indicated melanoma cell lines overexpressing miR-205 compared with controls (experiments were repeated three times). (d) Western blots using whole cell lysates from the melanoma cell lines indicated demonstrate decreased ZEB2 protein and increased E-cadherin protein in cells overexpressing miR-205 compared with controls (experiments were repeated three times). *indicates statistically significant differences.

miR-205 in comparison with one another, and this was ~300- to 350-fold higher than the respective parent cell line (Figure 5a). There was a significant reduction in ZEB2 mRNA in three of the four human melanoma cell lines overexpressing miR-205 (Figure 5b). Furthermore, there was a significant increase in E-cadherin mRNA detected in all four cell lines stably expressing miR-205 (Figure 5c). All four cell lines also demonstrated a variable decrease in ZEB2 protein expression and a concomitant increase in E-cadherin protein levels by western blotting analysis, and these effects were most pronounced in the metastatic melanoma cell lines (Figure 5d).

Overexpression of Zeb2 Rescues the Phenotypic Effects of miR-205 in Melanoma Cells

Our results show that enforced expression of miR-205 in melanoma cells inhibits cell migration and invasion, and this phenotype coincides with alterations of both ZEB2 and E-cadherin mRNA and protein levels. Given the well-documented relationship between miR-205, ZEB1/2 and E-cadherin,³⁴ we asked whether overexpression of the proposed downstream targets of miR-205 (ZEB2 or E-cadherin) would overcome the phenotypic and biochemical consequences of miR-205 overexpression. To this end, we transfected 1205Lu cells stably overexpressing miR-205 with a plasmid encoding for ZEB2. ZEB2 mRNA and protein levels were restored in these cells with a concomitant repression of E-cadherin mRNA and protein expression (Figure 6b). Furthermore, whereas 1205Lu melanoma cells overexpressing miR-205 exhibited a significant reduction in their capacity to migrate compared with controls—both in wound-healing and Boyden chamber assays, ZEB2 overexpression in those same cells restored their capacity to undergo cell migration and invasion (Figure 6c, wound-healing assays left and middle panels and Boyden chamber assays right panel). However, ZEB2 overexpression did not significantly alter their capacity to proliferate (Figure 6d). Finally, the observed defects in cell

Figure 6 Zeb2 rescues the function of miR-205 in melanoma cells. (a) Quantitative RT-PCR indicating relative expression of miR-205 in 1205Lu cells stably transfected with control or with miR-205 at the various passage times indicated (P2, P5, P8, P11 and P14) (experiment repeated three times). (b) Quantitative RT-PCR demonstrating relative increase in ZEB2 mRNA (left panel) and decrease in E-cadherin mRNA (middle panel) in 1205Lu cells stably overexpressing miR-205 transfected with a control plasmid or a plasmid overexpressing Zeb2 (experiments were repeated three times). Western blots (right panel) using whole cell lysates from the above 1205Lu cells demonstrate increased ZEB2 protein, decreased E-cadherin protein and no change in PCNA in cells overexpressing Zeb2 and miR-205 compared with cells overexpressing miR-205 alone (experiment repeated three times). (c) Cell migration and motility assays. Wound-healing assay demonstrates the 1205Lu cells stably expressing miR-205 transfected with control exhibit wound-healing defects 48 h after introduction of the wound (left panel) that are rescued with overexpression of Zeb2 (right panel) ($n = 3$ replicate experiments). Graphical representation of triplicate experiments of wound closure times in 1205Lu stably expressing miR-205 transfected with control or Zeb2 (middle panel) (experiment repeated three times). Quantification of cell invasion as a function of the percentage of cell migration in Boyden chamber assays. 1205Lu cells stably expressing miR-205 transfected with a control plasmid migrated through the chamber less efficiently than those transfected with a Zeb2 plasmid (right panel) (experiment repeated three times). (d) Cell proliferation assay. OD values was measured under 450 nm in the 1205Lu cells stably expressing miR-205 transfected with control with Zeb2 demonstrate no significant effect of Zeb2 overexpression on cell proliferation (experiment repeated three times). (e) Actin staining with phalloidin demonstrates a reduction in cellular protrusion, a more rounded morphology and a reduction in actin polymerization in 1205Lu cells stably expressing miR-205 transfected with control compared with those cells also transfected with a Zeb2 plasmid (right panel), which resemble wild-type 1205Lu cells (experiment repeated three times). *indicates statistically significant differences.

morphology and the actin cytoskeleton structure in cells overexpressing miR-205 were also rescued by Zeb2 overexpression (Figure 6e).

DISCUSSION

There is a critical need to improve our understanding of the molecular pathogenesis of melanoma. In this study, we have identified a distinct pattern of miRNA expression in nevi compared with melanomas, with confirmatory findings in an independent set of clinical specimens. We demonstrated that

miR-205 was significantly downregulated in melanoma disease progression. *In vitro* and *in vivo* experiments demonstrated that miR-205 impairs melanoma cell migration and invasion in multiple human melanoma cell lines, supporting the functional significance of the dysregulation of this target. Enforced expression of miR-205 coincides with a reduction in ZEB2 and a concomitant increase E-cadherin. Finally, re-introduction of ZEB2 into cells overexpressing miR-205 rescued these phenotypes. Taken together, these findings support the feasibility of and rationale for characterization

of miRNA expression in clinical specimens of this highly aggressive disease and demonstrate miR-205 to be an important regulator of melanoma progression.

We determined miRNA expression profiles of benign and malignant melanocytic tumors using patient samples as the primary resource for this study. We demonstrated that nevi exhibit a miRNA expression signature distinct from malignant melanomas, and we identified 20 miRNAs whose expression is significantly downregulated and 4 miRNAs whose expression is significantly upregulated in melanomas (both primary and metastatic) compared with nevi. Many of the miRNAs we describe as having dysregulated expression in melanomas compared with nevi were also reported by Chen *et al*,¹⁹ including miR-21, miR-125a, miR-125b, miR-132, miR-141, miR-193b, miR-200b, miR-200c, miR-203, miR-205 and miR-211 (overall, our studies share ~30% of the targets overall). Similarly, in a pattern that is nearly identical to our results, Dar *et al*²⁶ reported a progressive diminution of miR-205 expression comparing nevi with primary melanoma to metastatic melanoma. Together, these results provide compelling evidence for dysregulated expression of discrete miRNAs in melanomas and the utility of FFPE specimens for the discovery of informative and biologically relevant biomarkers in melanoma. Of note, Dar *et al*²⁶ reported a significant negative impact mediated by miR-205 on cell proliferation and senescence in melanoma cells. Although we report a relatively modest effect on cell proliferation *in vitro*, the effect of miR-205 on cell proliferation appears to be more profound *in vivo*.

In our microarray analysis, we did not identify miRNAs differentially expressed between primary and metastatic melanoma; this is likely attributable to our selection of bulky primary melanomas with a relatively expansile dermal component in a concerted effort to ensure adequate amounts of melanoma tissue and relatively pure melanocyte-specific RNA preparations. However, this size and/or depth 'exclusion' potentially created a bias for primary melanomas which already possessed a biological propensity for deeper invasion and effectively, metastasis, and therefore exhibited a miRNA expression profile that was relatively similar to that seen in lesions that had already metastasized. We are currently compiling a larger panel of thin primary melanomas (and desmoplastic melanomas, which rarely metastasize) for comparison with melanoma metastases to identify a miRNA 'metastasis signature'.

RT-PCR analysis demonstrated a trend toward progressively diminished expression of miR-205 from nevi to primary to metastatic melanoma. In a limited number of independent validation samples, there was a marked reduction in miR-205 expression in metastatic melanoma compared with primary melanoma and nevi. Furthermore, we characterized miR-205 expression in a series of melanocytic cell lines representing all phases of melanocytic tumor progression: foreskin-derived melanocyte cell lines, WM35 (derived from a radial growth phase-only primary melanoma),

WM793 (derived from a vertical growth phase primary melanoma), WM115A (derived from a melanoma metastasis) and 1205Lu (derived from a melanoma metastasis). Similar to the observations above, there was a progressive diminution of miR-205 expression from normal melanocytes to metastatic melanoma, suggesting a key role for miR-205 in melanoma progression.

Given its robust and progressive decrease in expression from nevi to metastatic melanoma, we chose to characterize the function of miR-205 expression in melanoma cells. Enforced expression of miR-205 impeded melanoma cell migration and invasion in culture as well as in mouse xenografts, while exerting only a modest impact on cell proliferation (more pronounced *in vivo*). In addition, cells with high levels of miR-205 exhibit an epithelial-like morphology (fewer cell protrusions and reduced actin polymerization) compared with control cells, which adopted a mesenchymal-like morphology in culture (increased cellular protrusions and extensions and increased actin polymerization). These morphologic changes are consistent with their differential migratory and invasive properties.

Given the well-described role of miR-205 in EMT pathways,^{34–36} we investigated the effects of miR-205 overexpression on EMT family members and demonstrated a connection between miR-205 overexpression and an associated reduction of ZEB2 mRNA and protein levels with a reciprocal increase on E-cadherin mRNA and protein levels. Re-introduction of ZEB2 into 1205Lu melanoma cells overexpressing miR-205 rescues the observed defects in cell migration and invasion and restores suppression of E-cadherin. Together, these results argue that miR-205 expression in melanocytes functions, at least in part, to preserve E-cadherin expression; in turn, the diminution of miR-205 expression in melanoma progression coincides with loss of E-cadherin expression and a more aggressive phenotype (cell migration and invasion). Of note, the effects of enforced miR-205 expression on ZEB2 and E-cadherin appear most pronounced in the metastatic melanoma cell lines (WM115A and 1205Lu), where miR-205 expression was initially the lowest (Figure 2d). This apparent increased sensitivity to enforced miR-205 expression in metastatic melanoma cell lines (as a function of changes in ZEB2 and E-cadherin mRNA and protein levels) is a potential reflection of the underlying biological and biochemical changes accompanying the elimination of miR-205 expression in the progression of melanoma.

Our results provide the first experimental evidence that miRNA expression is hijacked to promote an EMT in the progression of melanoma. Previous studies have demonstrated that miR-205 and the miR-200 family (including miR-200a, miR-200b, miR-200c and miR-141) directly repress ZEB1/2 in a variety of different cellular contexts, culminating in increased E-cadherin levels.^{34,37,38} Along with others, we describe a progressive diminution in the expression of miR-141, miR-200b, miR-200c and miR-205 in melanomas

compared with nevi.^{19,26} Taken together with our demonstration that enforced expression of miR-205 coincides with elevated levels of E-cadherin and diminished levels of ZEB2, these observations suggest a complex miRNA regulatory network—including miR-205 and the miR-200 family whose cooperative dysregulation is exploited to evoke a reduction in E-cadherin expression in the progression of melanoma, and these changes are likely to be coincident with more aggressive biological behavior.^{34,36,39,40} Consistent with this hypothesis, reduced expression of E-cadherin in melanoma cell lines correlates with an enhancement of the migratory and invasive capacity of these melanocytes in culture,^{39,41} and in studies of melanoma patients, E-cadherin expression was significantly reduced in the tumors of patients with metastases and fatal outcomes compared with the tumors of patients with longer survival.⁴⁰

Alterations in miR-205 expression have been described in many different human cancers, but a consensus as to the function(s) of miR-205 (in particular, whether miR-205 functions as a canonical oncogene or a tumor suppressor) is lacking. miR-205 is upregulated compared with normal tissues in transitional cell carcinoma of the bladder;^{42,43} squamous cell carcinoma of the uterine cervix;⁴⁴ endometrial adenocarcinomas, including the endometrioid⁴⁵ and serous⁴⁶ types; ovarian endometrioid adenocarcinoma;⁴⁷ and non-small cell carcinomas (adenocarcinoma and squamous cell carcinoma) of the lung.⁴⁸ In contrast, miR-205 is downregulated in breast carcinomas;^{49,50} adenocarcinoma of the prostate;^{51,52} renal cell carcinoma;⁵³ and spindle cell carcinomas of the head and neck.⁵⁴ This apparent disparity of function likely reflects the molecular plasticity of miR-205 as a regulator of multiple different gene targets, which would exert potentially pleiotropic effects.

The mechanism of miR-205 downregulation in melanoma is also unclear. Wiklund *et al*⁴³ demonstrate that the miR-205 gene locus (1q32.2) contains nearby CpG-rich gene elements, which undergo progressive hypermethylation in invasive bladder tumors that parallels an observed downregulation of miR-205. These results are consistent with an epigenetic mechanism for miR-205 downregulation, and methylation is a well-documented mechanism of aberrant gene expression in melanoma.⁵⁵ In addition, distinct sets of chromosomal gains and losses have been described in melanoma and are an attractive mechanism for reproducible alterations of global miRNA expression. However, the 1q32 locus was not reported as a commonly deleted region in large-scale comparative genomic hybridization studies of various subtypes of melanoma.⁵⁶ Future studies are warranted to uncover the mechanism of miR-205 reduction in melanoma.

Defining additional components in the molecular pathogenesis of melanoma will illuminate new opportunities for the rational design of targeted therapies. Central to this goal is the identification of clinically meaningful biomarkers from patient samples. In this study, we identified distinct miRNA expression profiles in nevi compared with melanomas, and

validated the downregulation of miR-205 in disease progression. miR-205 controls melanoma cell migration and invasion *in vitro* and *in vivo*, and miR-205 inhibits ZEB2, which coincided with a downregulation of E-cadherin. Together, these results support the utility of miRNA expression profiling as a powerful tool to interrogate clinical samples of melanomas: as a window to clinically and biologically important pathways central to the aggressive behavior of this disease.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Altekruse SF, Krapcho MCL. SEER Cancer Statistics Review, 1975–2007. National Cancer Institute: Bethesda, MD, based on the November 2009 SEER data submission, posted to the SEER website, Available at: http://seer.cancer.gov/csr/1975_2007/.
2. Jemal A, Siegel R, Xu J, *et al*. Cancer statistics, 2010. *CA Cancer J Clin* 2010;60:277–300.
3. Rigel DS, Russak J, Friedman R. The evolution of melanoma diagnosis: 25 years beyond the ABCDs. *CA Cancer J Clin* 2010;60:301–316.
4. Berezikov E, Guryev V, van de Belt J, *et al*. Phylogenetic shadowing and computational identification of human microRNA genes. *Cell* 2005;120:21–24.
5. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;120:15–20.
6. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–297.
7. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857–866.
8. Liu X, Fortin K, Mourelatos Z. MicroRNAs: biogenesis and molecular functions. *Brain Pathol* 2008;18:113–121.
9. Lee EJ, Gusev Y, Jiang J, *et al*. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer* 2007;120:1046–1054.
10. Cortez MA, Ivan C, Zhou P, *et al*. microRNAs in cancer: from bench to bedside. *Adv Cancer Res* 2010;108:113–157.
11. Fabbri M, Calin GA. Epigenetics and miRNAs in human cancer. *Adv Genet* 2010;70:87–99.
12. Ferdin J, Kunaj T, Calin GA. Non-coding RNAs: identification of cancer-associated microRNAs by gene profiling. *Technol Cancer Res Treat* 2010;9:123–138.
13. Le XF, Merchant O, Bast RC, *et al*. The roles of microRNAs in the cancer invasion-metastasis cascade. *Cancer Microenviron* 2011;3:137–147.
14. Lu J, Getz G, Miska EA, *et al*. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834–838.
15. Rosenfeld N, Aharonov R, Meiri E, *et al*. MicroRNAs accurately identify cancer tissue origin. *Nat Biotechnol* 2008;26:462–469.
16. Volinia S, Calin GA, Liu CG, *et al*. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* 2006;103:2257–2261.
17. Liu A, Tetzlaff MT, Vanbelle P, *et al*. MicroRNA expression profiling outperforms mRNA expression profiling in formalin-fixed paraffin-embedded tissues. *Int J Clin Exp Pathol* 2009;2:519–527.

18. Caramuta S, Egyhazi S, Rodolfo M, *et al*. MicroRNA expression profiles associated with mutational status and survival in malignant melanoma. *J Invest Dermatol* 2010;130:2062–2070.
19. Chen J, Feilottter HE, Pare GC, *et al*. MicroRNA-193b represses cell proliferation and regulates cyclin D1 in melanoma. *Am J Pathol* 2010;176:2520–2529.
20. Jukic DM, Rao UN, Kelly L, *et al*. MicroRNA profiling analysis of differences between the melanoma of young adults and older adults. *J Transl Med* 2010;8:27.
21. Philippidou D, Schmitt M, Moser D, *et al*. Signatures of microRNAs and selected microRNA target genes in human melanoma. *Cancer Res* 2010;70:4163–4173.
22. Satzger I, Mattern A, Kuettler U, *et al*. MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. *Int J Cancer* 2009;126:2553–2562.
23. Schultz J, Lorenz P, Gross G, *et al*. MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. *Cell Res* 2008;18:549–557.
24. Segura MF, Belitskaya-Levy I, Rose AE, *et al*. Melanoma microRNA signature predicts post-recurrence survival. *Clin Cancer Res* 2010;16:1577–1586.
25. Worley LA, Long MD, Onken MD, *et al*. Micro-RNAs associated with metastasis in uveal melanoma identified by multiplexed microarray profiling. *Melanoma Res* 2008;18:184–190.
26. Dar AA, Majid S, de Semir D, *et al*. miRNA-205 suppresses melanoma cell proliferation and induces senescence via regulation of E2F1 protein. *J Biol Chem* 2011;286:16606–16614.
27. Nelson PT, Baldwin DA, Kloosterman WP, *et al*. RAKE and LNA-ISH reveal microRNA expression and localization in archival human brain. *RNA* 2006;12:187–191.
28. Nelson PT, Baldwin DA, Scearce LM, *et al*. Microarray-based, high-throughput gene expression profiling of microRNAs. *Nat Methods* 2004;1:155–161.
29. Tetzlaff MT, Liu A, Xu X, *et al*. Differential expression of miRNAs in papillary thyroid carcinoma compared to multinodular goiter using formalin fixed paraffin embedded tissues. *Endocr Pathol* 2007;18:163–173.
30. Liu A, Xu X. MicroRNA isolation from formalin-fixed, paraffin-embedded tissues. *Methods Mol Biol* 2011;724:259–267.
31. Kumar SM, Yu H, Edwards R, *et al*. Mutant V600E BRAF increases hypoxia inducible factor-1 α expression in melanoma. *Cancer Res* 2007;67:3177–3184.
32. Liu S, Yu M, He Y, *et al*. Melittin prevents liver cancer cell metastasis through inhibition of the Rac1-dependent pathway. *Hepatology* 2008;47:1964–1973.
33. Xu Z, Maroney AC, Dobrzanski P, *et al*. The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol Cell Biol* 2001;21:4713–4724.
34. Gregory PA, Bert AG, Paterson EL, *et al*. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 2008;10:593–601.
35. Matsushima K, Isomoto H, Yamaguchi N, *et al*. MiRNA-205 modulates cellular invasion and migration via regulating zinc finger E-box binding homeobox 2 expression in esophageal squamous cell carcinoma cells. *J Transl Med* 2011;9:30.
36. Shimono Y, Zabala M, Cho RW, *et al*. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. *Cell* 2009;138:592–603.
37. Dykxhoorn DM. MicroRNAs and metastasis: little RNAs go a long way. *Cancer Res* 2010;70:6401–6406.
38. Park SM, Gaur AB, Lengyel E, *et al*. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* 2008;22:894–907.
39. Hsu MY, Meier FE, Nesbit M, *et al*. E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. *Am J Pathol* 2000;156:1515–1525.
40. Tucci MG, Lucarini G, Brancorsini D, *et al*. Involvement of E-cadherin, beta-catenin, Cdc42 and CXCR4 in the progression and prognosis of cutaneous melanoma. *Br J Dermatol* 2007;157:1212–1216.
41. Hsu MY, Wheelock MJ, Johnson KR, *et al*. Shifts in cadherin profiles between human normal melanocytes and melanomas. *J Invest Dermatol Symp Proc* 1996;1:188–194.
42. Gottardo F, Liu CG, Ferracin M, *et al*. Micro-RNA profiling in kidney and bladder cancers. *Urol Oncol* 2007;25:387–392.
43. Wiklund ED, Bramsen JB, Hulf T, *et al*. Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer. *Int J Cancer* 2010;128:1327–1334.
44. Wang X, Tang S, Le SY, *et al*. Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. *PLoS One* 2008;3:e2557.
45. Wu W, Lin Z, Zhuang Z, *et al*. Expression profile of mammalian microRNAs in endometrioid adenocarcinoma. *Eur J Cancer Prev* 2009;18:50–55.
46. Hiroki E, Akahira J, Suzuki F, *et al*. Changes in microRNA expression levels correlate with clinicopathological features and prognoses in endometrial serous adenocarcinomas. *Cancer Sci* 2009;101:241–249.
47. Iorio MV, Visone R, Di Leva G, *et al*. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007;67:8699–8707.
48. Markou A, Tsaroucha EG, Kaklamanis L, *et al*. Prognostic value of mature microRNA-21 and microRNA-205 overexpression in non-small cell lung cancer by quantitative real-time RT-PCR. *Clin Chem* 2008;54:1696–1704.
49. Radojicic J, Zaravinos A, Vrekoussis T, *et al*. MicroRNA expression analysis in triple-negative (ER, PR and Her2/neu) breast cancer. *Cell Cycle* 2011;10:507–517.
50. Sempere LF, Christensen M, Silahatoglu A, *et al*. Altered microRNA expression confined to specific epithelial cell subpopulations in breast cancer. *Cancer Res* 2007;67:11612–11620.
51. Gandellini P, Folini M, Longoni N, *et al*. miR-205 exerts tumor-suppressive functions in human prostate through down-regulation of protein kinase C ϵ . *Cancer Res* 2009;69:2287–2295.
52. Majid S, Dar AA, Saini S, *et al*. MicroRNA-205-directed transcriptional activation of tumor suppressor genes in prostate cancer. *Cancer* 2010;116:5637–5649.
53. Majid S, Saini S, Dar AA, *et al*. MicroRNA-205 inhibits Src-mediated oncogenic pathways in renal cancer. *Cancer Res* 2011;71:2611–2621.
54. Childs G, Fazzari M, Kung G, *et al*. Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma. *Am J Pathol* 2009;174:736–745.
55. Schinke C, Mo Y, Yu Y, *et al*. Aberrant DNA methylation in malignant melanoma. *Melanoma Res* 2010;20:253–265.
56. Curtin JA, Fridlyand J, Kageshita T, *et al*. Distinct sets of genetic alterations in melanoma. *N Engl J Med* 2005;353:2135–2147.