Tumor and Stem Cell Biology

Cancer Research

Signatures of MicroRNAs and Selected MicroRNA Target Genes in Human Melanoma

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Abstract

Small noncoding microRNAs (miRNA) regulate the expression of target mRNAs by repressing their translation or orchestrating their sequence-specific degradation. In this study, we investigated miRNA and miRNA target gene expression patterns in melanoma to identify candidate biomarkers for early and progressive disease. Because data presently available on miRNA expression in melanoma are inconsistent thus far, we applied several different miRNA detection and profiling techniques on a panel of 10 cell lines and 20 patient samples representing nevi and primary or metastatic melanoma. Expression of selected miRNAs was inconsistent when comparing cell line–derived and patient-derived data. Moreover, as expected, some discrepancies were also detected when miRNA microarray data were correlated with qPCR-measured expression levels. Nevertheless, we identified miRNA-200c to be consistently downregulated in melanocytes, melanoma cell lines, and patient samples, whereas miRNA-205 and miRNA-23b were markedly reduced only in patient samples. In contrast, miR-146a and miR-155 were upregulated in all analyzed patients but none of the cell lines. Whole-genome microarrays were performed for analysis of selected melanoma cell lines to identify potential transcriptionally regulated miRNA target genes. Using Ingenuity pathway analysis, we identified a deregulated gene network centered around microphthalmia-associated transcription factor, a transcription factor known to play a key role in melanoma development. Our findings define miRNAs and miRNA target genes that offer candidate biomarkers in human melanoma. *Cancer Res; 70(10); 4163–73. ©2010 AACR.*

Introduction

MicroRNAs (miRNA) are noncoding ~22 nucleotide short RNAs that typically downregulate expression of their target genes. Nucleotides 2 to 8, the so-called "seed" region of miRNAs, bind to completely or partially complementary regions in the 3′ untranslated region (UTR) of target genes, which are generally present in multiple copies to amplify the regulatory effects of the miRNA (1, 2). To date, 721 human miRNAs have been identified (3), which are thought to regulate at least 30% of human genes. A recent in-depth analysis of human 3′ UTR sequences indicated, however, that even >60% of human protein-coding genes have been under selective pressure to maintain pairing to miRNAs (4).

miRNAs influence most fundamental biological processes by ultimately altering the expression levels of proteins either through interference with mRNA translation or by reducing the stability of the mRNA in the cytoplasm (5). Downregulated target gene mRNAs can be detected by whole-genome microarray technology, keeping in mind that translationally repressed miRNA targets would be missed by such an approach (6).

Given the tremendous regulatory potential of miRNAs and their often tissue-specific and disease-specific expression patterns (7–9), there is increasing evidence that miRNA expression profiles could be indicative of disease risks and burdens, and as such, miRNAs are being assessed as possible biomarkers to aid diagnosis and prediction of different types and stages of cancers, including melanoma (10–12). Melanoma arises from melanocytes, which are pigmented cells present in the basal layer of the epidermis (13). The global incidence of melanoma continues to rise faster than any other malignancy, and despite considerable research efforts, no efficient therapy is available to date. Once melanoma has metastasized, the median 5-year survival rate is <5% (14).

Relatively few miRNA expression profiling analyses have thus far included or focused on melanoma samples, and the available data sets show little agreement with regards to expression patterns of individual miRNAs or the entire miRNome. Using real-time PCR on 241 individual miRNAs, Gaur and colleagues (15) have identified a set of 15 miRNAs that distinguished melanomas from other solid cancers whereas others (16) have described four melanoma-characteristic miRNAs detected by microarray analysis with only miR-335

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common to both studies. Mueller and colleagues (17) have recently described a more detailed analysis of miRNA expression in melanocytes versus seven different melanoma cell lines based on miRNA microarray analysis. In primary melanoma cell lines, a set of 49 miRNAs was found to be strongly upregulated whereas 14 were downregulated; however, most of these had not been shown before to be substantially regulated in melanoma development. miRNA expression in melanocytic nevi derived from formalin-fixed paraffin-embedded (FFPE) samples has recently been investigated by Glud and colleagues, who, among others, have shown that FFPE samples are useful sources for miRNA profiling (18, 19).

Owing to the many factors that differed between these studies (real-time PCR versus miRNA microarray approaches; different microarray platforms and mirBase versions; different sample types and melanoma cell lines) it is not surprising that largely different miRNA sets were considered to be characteristic for melanoma or melanoma progression. However, thus far no miRNAs have been identified that would allow for reproducible and specific distinction between early-stage or late-stage melanoma samples from healthy melanocytes or benign nevi in cell lines and patients.

To evaluate the discrepancies and/or concordances in miRNA expression patterns that might result from different technical approaches on different biological samples, we established miRNA expression profiles in primary human melanocytes [normal human epidermal melanocyte (NHEM)] versus melanoma cell lines by miRNA microarray, real-time miRNA reverse transcription–PCR arrays (RT^2 Profiler), followed by individual quantitative real-time PCR (qPCR) validations. To then correlate cell line–derived expression patterns with melanoma patients, we next analyzed FFPE patient samples including benign nevi, primary melanoma, and metastatic melanoma samples. Cross-correlation of all miRNA expression data revealed that only miRNA-200c was commonly downregulated in different sample types robustly detectable by the various technical approaches. Other miRNAs were only found to be significantly deregulated in either cell lines or patients or could not be reproduced using different detection techniques.

Finally, whole-genome arrays were used on melanoma cell lines and melanocytes to identify possible target genes of some of the differentially regulated miRNAs. We focused on genes commonly deregulated in melanoma and inversely correlated their expression patterns with miRNAs predicted to target such genes, identifying several possible miRNA/target gene pairs likely to play a role in melanoma development. Ingenuity network analysis identified with a high statistical confidence deregulated pathway around the transcription factors microphthalmia-associated transcription factor (MITF), SRY-related HMG box (SOX10), and T-box transcription factor 2.

Materials and Methods

Cell lines and cell culture. In total, nine melanoma cell lines were analyzed: Wm9 (Dr. M. Böhm, Münster, Germany), FM55-M1 (European Searchable Tumor Line Database and Cell Bank), IGR39 and IGR37 (Deutsche Sammlung von Mikroorganismen und Zellkulturen), A375 (American Type Culture Collection), 1102 (Dr. M. Kortylewski, City of Hope, California), MeWo (Dr. Schadendorf, Essen, Germany), and MelIm and MelJuso (Dr. A. Bosserhoff, Regensburg, Germany). All cells were maintained in RPMI 1640 supplemented with 10% FCS, 50 μg/mL penicillin, 100 μg/mL streptomycin, and 0.5 mmol/L L-glutamine. NHEMs from lightly pigmented adult skin (PromoCell) were maintained in serum-free and phorbol 12-myristate 13-acetate–free MGM-M2 medium and were used at passage number 5 or 6. MCF-7 breast cancer cells (Dr. G. Vetter, Luxembourg) were grown in DMEM supplemented with 10% FCS, 50 μg/mL penicillin, and 100 μg/mL streptomycin. All cells were grown in a humidified atmosphere with 5% CO_2 supply and were routinely PCR tested to be *Mycoplasma* negative.

Patient samples. Skin tissue samples from patients with either benign nevi or melanoma were collected at the Dermatology Department of University Hospital of Freiburg (Germany) and histopathologically examined to confirm clinical diagnoses. Upon excision, tissues were fixed in FFPE according to standard dermatohistopathologic techniques. In total, 3 pools of benign nevi (RNAs of two different donors each) and 17 primary and subcutaneous melanoma metastasis patient samples were analyzed (age and gender information is included in Supplementary Table S1). Additionally, four breast cancer FFPE samples from two patients were included in this study. The study was approved by the ethical review board of EK Freiburg (reference 196/09), and written informed consent was obtained from healthy controls and live patients.

Total RNA extraction and quality control. Total RNA of cell lines was extracted using TRIsure (Bioline USA, Inc.) and treated with DNaseI (New England Biolabs) following each manufacturer's instructions. For miRNA and whole-genome microarray analyses, total RNA was extracted using the miRNeasy kit (Qiagen) according to the manufacturer's protocol with additional on-column DNaseI digestion. In FFPE samples, for total RNA extraction, five scalpel-scraped slices of FFPE tissue were pooled and processed using the RT^2-FFPE RNA Extraction kit (SABiosciences) according to the supplied protocol. Quantity and purity of RNA samples were assessed using a NanoDrop ND-100 Spectrophotometer. For FFPE tissues, in particular, when RNA quantity or quality was insufficient, samples were further processed by standard ethanol precipitation and resuspended in appropriate volumes of DEPC-H_2O to achieve total RNA concentrations of >350 ng/μL and absorbance ratios of > 1.8 (260/280) and > 1.7 (260/230).

miRNA and whole-genome microarrays. Total RNA from NHEM, IGR39, and IGR37 cells was subjected to (*a*) genome-wide miRNA expression profiling (miRBase, version 11.0) using the μParaflo microarray technology (LCSciences) and (b) whole-genome expression profiling using GeneChip Human Gene 1.0 ST arrays (Affymetrix). Gene network analyses were performed with the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems). Detailed methodologies

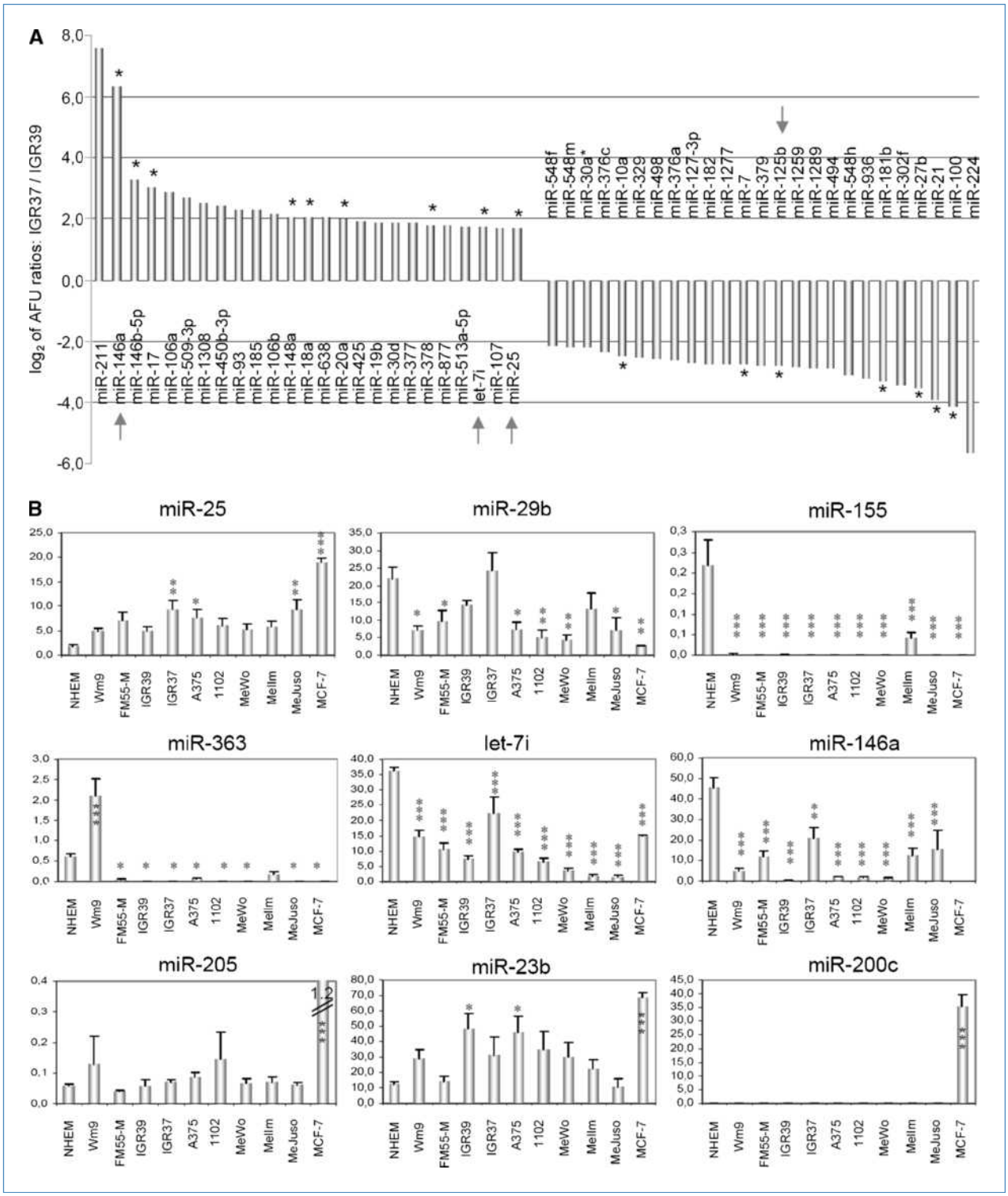

Figure 1. A, top 25 gradually upregulated and downregulated miRNAs in metastatic (IGR37) versus primary (IGR39) melanoma cell lines established from the same patient. AFUs were derived from at least duplicate miRNA microarrays (LCSciences). AFUs of >500 were selected and sorted, and $\log_2$ values of AFU ratios were plotted. miRNAs marked with an arrow were further validated; the ones marked with an asterisk were confirmed by RT^2 Profiler results. B, relative expression of selected miRNAs in primary human melanocytes (NHEM, passage no. 5), a panel of nine melanoma cell lines, and MCF-7 breast cancer cells (relative to SCARNA17 expression). Results are depicted as mean of biological triplicates ± SEM. Statistical analysis was performed using a one-way ANOVA test, comparing results for each cell line to NHEM cells. ***, $P < 0.001$; **, $P = 0.001–0.01$; *, $P = 0.01–0.05$; no star, not significant at $P > 0.05$.

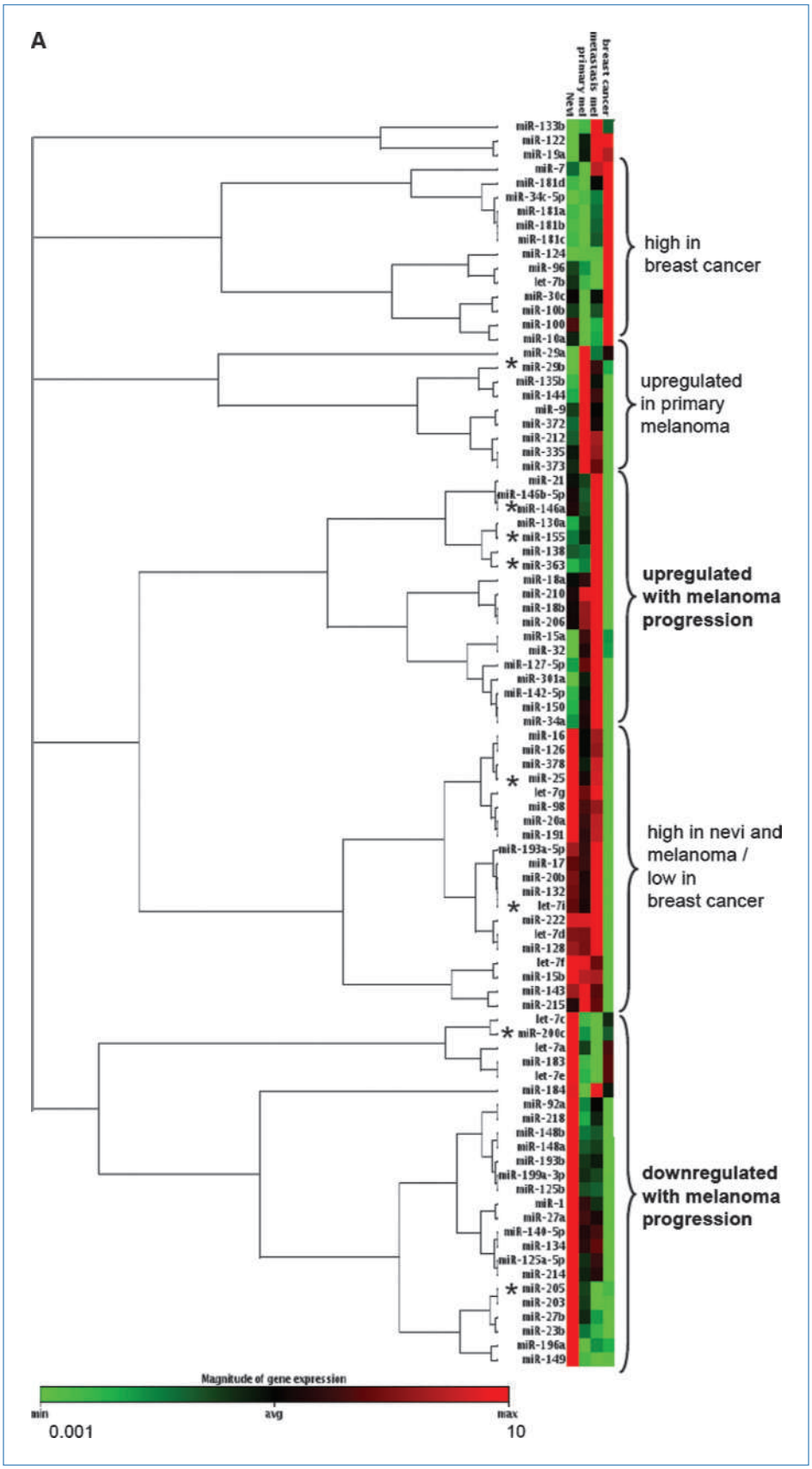

Figure 2. A, miRNA expression profiling in melanoma patients (RT^2 Profiler). Clustergram generated from average miRNA C_t values of individual FFPE patient samples from nevi (3), primary melanoma (7), metastatic melanoma (10), and breast cancer samples (4). For each patient group, average C_t values were normalized to a panel of four different housekeeping genes (Supplementary Table S1). The range of $2^{-\Delta C_t}$ values is given below the color scale. miRNAs that were further validated by qPCR are marked by an asterisk.

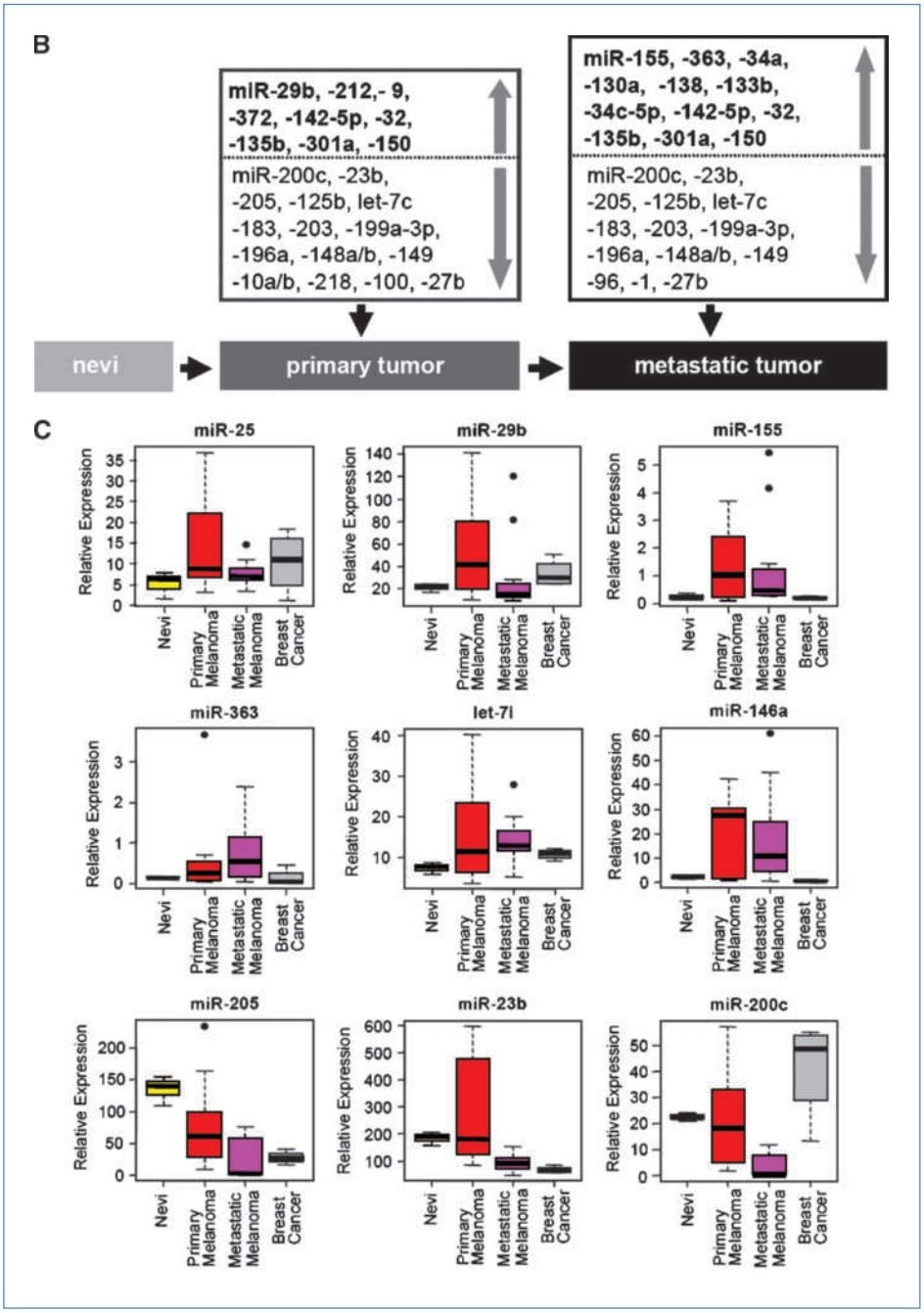

Figure 2. ***Continued.*** B, miRNA signatures for stages of melanoma progression. miRNAs of >2.0-fold upregulated (bold) or downregulated relative to nevi are shown for each patient group. C, box plots showing triplicate qPCR validations for nine selected miRNAs of individually analyzed patient samples (as in A).

and bioinformatic analyses are described in Supplementary Materials and Methods.

Real-time PCR-based miRNA expression profiling. FFPE patient samples were analyzed for the presence and differential expression of a panel of 88 cancer-related miRNAs using cancer RT^2 miRNA PCR arrays (called RT^2 Profiler; SABiosciences) according to the manufacturer's instructions. Data analysis was performed with the web-based software package for the miRNA PCR array system (20).

miRNA and gene expression validation by real-time qPCR. Briefly, 250 and 100 ng of total RNA from cell lines and FFPE tissues, respectively, were reverse transcribed in a 10-μL reaction volume with the miScript System (Qiagen) following the manufacturer's instructions. To quantify mature miRNAs, real-time qPCR was carried out on a CFX96 Detection System (Bio-Rad) using 5 ng RNA input, 2× iQ SYBR Green Supermix (Bio-Rad), and 10× miRNA-specific primer assay (Qiagen). For the detection of cKIT and MITF,

50 ng RNA input, 2× iQ SYBR Green Supermix, and 5 pmol gene-specific primer pairs were used. Thermal cycling conditions for all assays were 95°C for 3 minutes, 40 cycles at 95°C for 10 seconds, and 60°C for 30 seconds, followed by melting curve analyses. RNA input was normalized to endogenous controls: SCARNA17 for miRNAs and TATA-binding protein for protein encoding genes. The $2^{-\Delta C_t}$ method was used to calculate the fold relationships in miRNA or gene expression among the tested samples (21).

miRNA target gene prediction. Three publicly available databases, TargetScanHuman 5.1 (4), DIANA-microT v3.0 (22), and MicroCosm Targets Version 5 (3), were used for miRNA target gene predictions. Predicted target genes in combination with miRNA and whole-genome microarray data were used to visualize possible biological miRNA/mRNA processes correlating to melanoma development and/or progression.

Results

miRNA expression profiling of primary human melanocytes (NHEM) and melanoma cell lines. As a first step, we analyzed the miRNome of two melanoma cell lines from a single patient representing primary (IGR39) and metastatic tumors (IGR37) and compared them to NHEM using LCSciences microarray data and RT^2 Profiler PCR arrays, followed by individual qPCR validations for selected miRNAs (Fig. 1, Supplementary Table S1). Figure 1A depicts the top 25 miRNAs upregulated and downregulated with tumor progression as detected by miRNA microarray. Sixteen miRNAs (asterisk-marked) were also present on RT^2 Profiler assays. Of those, 12 had matching expression ratios (except for miR-10a, miR-7, miR-181b, and miR-21) following tumor progression in cell lines (Supplementary Table S1) and melanoma patients (see Fig. 2A). This suggests that cell lines give some indication of relevant expression patterns, but individual results may change in patient-derived samples. Complete array-based expression profiles of all miRNAs (miRBase version 11.0) served as a first base for selection of miRNAs to be investigated further. Comparison of microarray results with data from the RT^2 Profiler revealed that 66 of the 88 common miRNAs (75%) showed comparable results, with both of the assays being clearly positive [C_t < 30 or arbitrary fluorescence unit (AFU) > 500] or negative.

The expression levels of several miRNAs were further validated by qPCR, extending the cell line panel to nine different melanoma cell lines, NHEMs, and MCF-7 breast cancer cells (Fig. 1B). qPCR results for NHEM, IGR39, and IGR37 correlated with microarray-measured expression levels for all tested miRNAs except let-7i, wherein levels were lower in NHEM compared with IGR39 and IGR37. Figure 1B further illustrates that some miRNAs have considerably different expression levels in individual cell lines, suggesting that profound variations would also be apparent when analyzing the entire miRNomes of such cell lines. Nevertheless, a few robustly regulated miRNAs seemed to be useful candidates to distinguish between NHEMs and melanoma cell lines in general: miR-155 and miR-146a with decreased expression levels in all melanoma lines tested and miR-25 and miR-23b (as well as miR-23a; data not shown), which were consistently upregulated.

miRNA expression profiling of FFPE melanoma patient samples. Due to the small amplicon size of miRNAs, FFPE samples represent useful sources to analyze miRNA expression even when some degree of RNA degradation is apparent (18). In total, 24 FFPE samples were analyzed by RT^2 Profiler arrays (Fig. 2A) followed by qPCR for selected miRNAs (Fig. 2B). The heat map (Fig. 2A) depicts expression values for 88 cancer-relevant miRNAs of four donor groups relative to four different house-keeping genes (Supplementary Table S1). Nevi (consisting of pools of RNAs extracted from five healthy volunteers) showed very similar C_t values across 85% of analyzed miRNAs. The primary melanoma group represents mean expression values from seven individual primary tumors, whereas the metastatic melanoma group consisted of 10 different patient samples. The breast cancer group (four samples from two patients) was included to allow for identification of miRNAs that were distinct between the two cancer types. miRNA-16, miRNA-27a, miRNA-125b, 199a-3p, 199a-21, and 199a-205 were highly expressed in melanoma having greater RNA levels than the four housekeeping genes (U6, SNORD44, SNORD46, and SNORD47), whereas the breast cancer group was characterized by highest expression of miRNA-16, miRNA-100, miRNA-21, and let-7b (Supplementary Table S1). Figure 2B shows that each melanoma stage bore a distinct miRNA signature. Considerable interpatient variability (>2.0 C_t) among the different melanoma patient groups was detected for <10% of the analyzed 88 cancer-related miRNAs (Supplementary Table S1).

qPCR validation on duplicate RNAs extracted from individual FFPE samples revealed a generally good correlation when compared with expression patterns achieved with averaged values for the different patient groups. Figure 2C shows box plots for nine selected miRNAs. Most striking were the levels of miR-200c, miR-205, and miR-23b, which were strongly downregulated in melanoma patients when compared with nevi. miR-200c is further interesting, as it allows for discrimination between melanoma and breast cancer samples.

miR-363, miR-146a, and miR-155 were clearly upregulated in all melanoma patients but not in breast cancer samples. Interestingly, the latter two were consistently low or undetectable in cell lines. In this context, it has recently been suggested that miR-155 was a negative regulator of melanoma proliferation, as its expression was downregulated or lost in the majority of melanoma cell lines (23). Here, we also monitored an almost complete loss of miR-155 in our panel of cell lines (Fig. 1B); however, miR-155 was clearly detectable in all of the 17 patient-derived samples, indicating that the loss of miR-155 expression may be a tissue culture–related phenomenon. Taken together, miR-155, miR-205, miR-146a, and miR-23b emerged as useful markers to distinguish between melanoma cell lines and patient samples relative to human melanocytes and nevi, respectively.

Correlation of expression levels of selected miRNAs with their predicted target genes. Whole-genome cDNA arrays

were performed on melanoma cell lines and melanocytes to obtain a pattern of transcriptionally regulated candidate genes that could serve as possible targets of some of the identified differentially regulated miRNAs. Scatter plots with highlighted genes known to be important for melanoma development and/or progression are shown in Fig. 3. Interestingly, melanoma-associated antigen C2, a predicted (although nonconserved) target of miR-200a, miR-200b, and miR-200c family, was strongly expressed in most melanoma cell lines but not in MCF-7 breast cancer cells, whereas inversely miR-200c expression was undetectable in all tested melanomas (Figs. 1B and 2A; Supplementary Fig. S1). Expression of the receptor tyrosine kinase cKIT, a confirmed target of miR-221/miR-222 (24), was augmented in IGR37, which showed correspondingly low levels of miR-221. An inversely and perfectly correlated expression pattern of miR-221 and cKIT was also confirmed for all nine melanoma cell lines but interestingly not for the MCF-7 cell line (Supplementary Fig. S1). miR-23a and miR-23b, upregulated in melanoma cell lines, are predicted to target many genes, of which several were found to be reciprocally downregulated in this data set [MITF (Supplementary Fig. S1), TYR (tyrosinase), TRPM1 (transient receptor potential cation channel 1), MLANA (Melan-A), and others]. All of these genes have previously been implicated in melanoma development (25–27). We further validated and confirmed the array-based expression levels for four additional genes [CDKN1B (p27), CDK6, E-cadherin, and N-cadherin] for NHEM, IGR39, and IGR37 (Supplementary Fig. S2).

Using differentially expressed gene lists of NHEM and IGR39, Ingenuity analysis identified 33 pathways with statistical scores of >10 (score = –log (*P* value) from Fisher's exact test analysis). Network no. 3 "skin development and function" (score = 37) was selected based on statistical and biological relevance for the study (Fig. 4A). We then prompted Ingenuity to include miRNAs that were predicted to target the strongly regulated and melanoma-relevant genes. The resulting network shows some of the known key players in melanoma development and progression with *MITF* regulating the expression of several genes known to be involved in pigmentation and skin development, such as TYR, tyrosinase-related protein 1, SILV (melanocyte protein Pmel 17), and TRPM1, whose expression is inversely correlated with melanoma metastasis (25).

To evaluate the robustness of the interactions predicted by the software, we analyzed gene levels of MITF and its tentative targeting miRNA-23a and miRNA-23b, as well as four genes that have previously been shown to be regulated by MITF (Fig. 4B; refs. 26, 28, 29). In IGR39 cells, wherein miR-23a/miR-23b expression was highest, MITF levels were almost undetectable. Correspondingly, the expression patterns of three of the four tested and potentially MITF-regulated genes (miR-146a, let-7I, and cKIT) matched the expression trends of the transcription factor, indicating that they could indeed be regulated by MITF. miRNA-363, which is also regulated by MITF (28), was however an exception as such that IGR37 had reproducibly undetectable levels of miR-363.

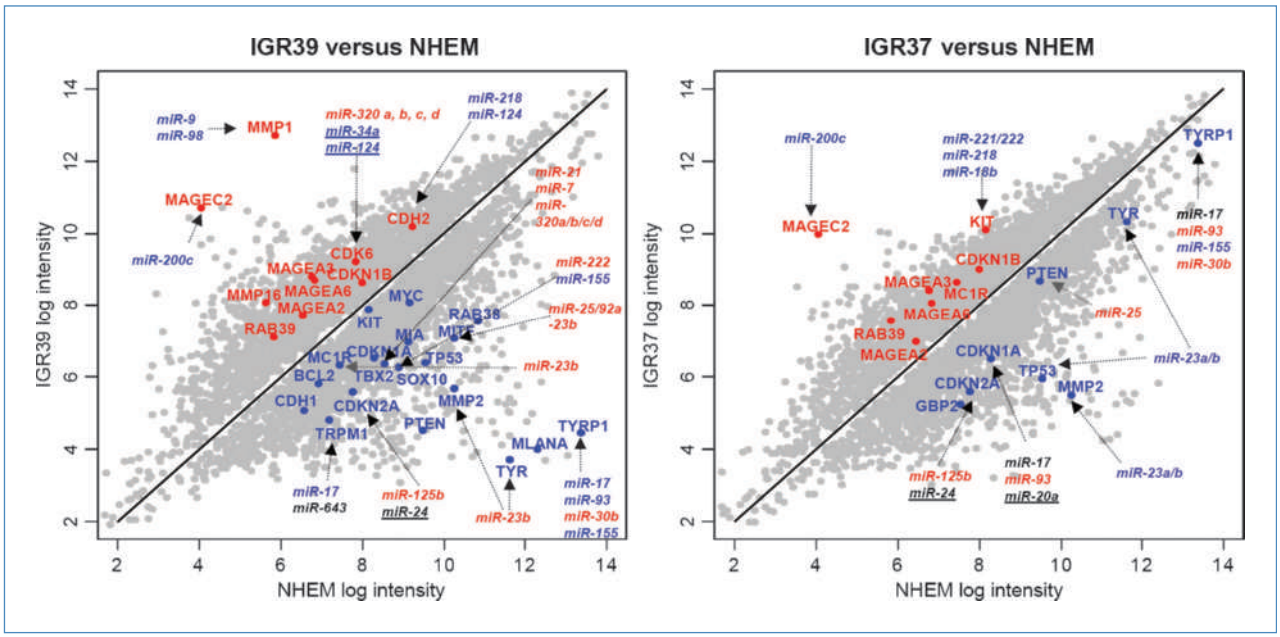

Figure 3. Mapping of potential miRNA targets in gene expression profiles. Scatter graphs were generated by plotting normalized log expression data obtained with IGR39 or IGR37 cells against those found with NHEM control cells. Each gray dot in the graphs corresponds to the expression value of a particular gene found to be significantly differentially expressed (FDR < 0.05). Selected genes previously implicated in melanoma are highlighted in red or blue (upregulated or downregulated in melanoma versus melanocytes, respectively). miRNAs computationally predicted to target some of the highlighted genes are depicted in italics: red or blue (upregulated or downregulated in the respective melanoma cell line versus melanocytes, respectively), or black (unchanged expression). Underlined are miRNAs that have been experimentally confirmed to regulate the respective target genes. For some genes, no targeting miRNA was predicted by Targetscan or MicroCosm Targets.

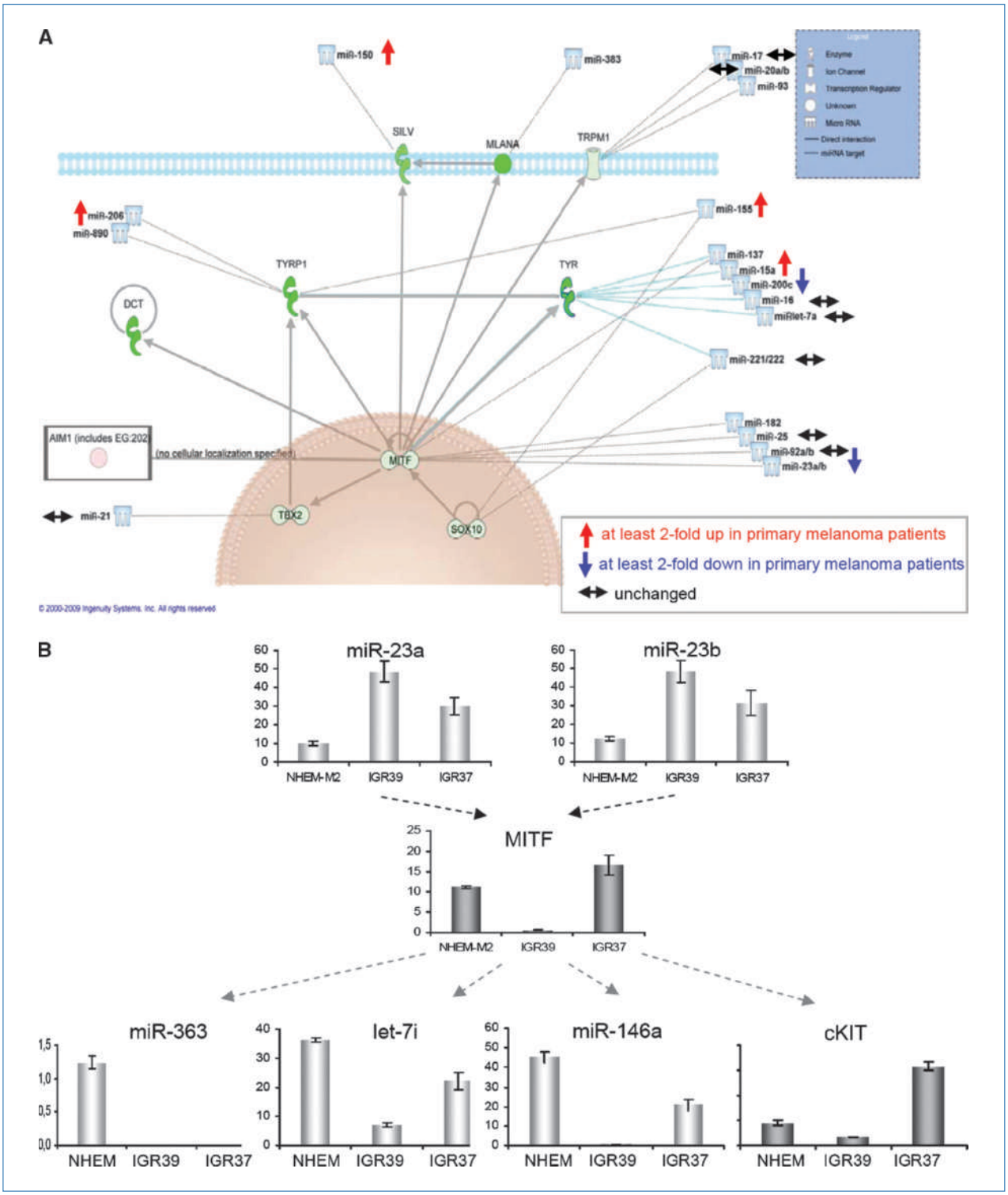

Figure 4. A, miRNA target map illustrating parts of the "skin development and function" network, which IPA identified to be highly significant in our experimental data set (IGR39 versus NHEM). Central to this network is MITF and its predicted interactions with pigmentation-relevant and melanoma-relevant proteins together with their subcellular localization. The node color intensity indicates the expression level of genes: red, upregulated; green, downregulated in the comparison IGR39 versus NHEM. IPA-predicted miRNAs and their relative expression levels in our primary melanoma patients are also shown (red, up; blue, down). B, relative expression levels of MITF of biological triplicates of NHEM, IGR39, and IGR37. Inverse expression levels for miR-23a and miR-23b, which are predicted to target MITF, are shown above. Expression patterns for miRNA-363, miRNA-146a, let-7i, and cKIT, suggested to be regulated by MITF, are shown in the bottom, all of which (except miR-363) show MITF-concordant expression trends.

Taken together, computational pathway analysis proved to be a useful tool to select key-regulated genes. Inversely correlated expression patterns of such selected genes and their predicted miRNAs in an extended number of biological samples can point to regulatory relationships between miRNAs and their tentative target genes.

Discussion

Recent years have seen a tremendous number of miRNA profiling studies aiming at identifying unique miRNA signatures for many diseases, for different stages thereof, or for prediction of disease risks (9, 11). These efforts have been paralleled by continuous discovery of new miRNAs and by rapidly changing developments and improvements in miRNA detection techniques. Given the different technical approaches applied to miRNA profiling (various microarray chemistries and real-time PCR quantifications) together with the plethora of different biological samples that are being profiled, it is not surprising that there is often little correlation between data sets coming from different laboratories. Here, we have addressed this question by comparing miRNA expression patterns in well-characterized melanoma cell lines with patient-derived samples of different melanoma stages by applying microarray and qPCR profiling techniques.

A key issue for comparability or lack thereof of different expression data sets is the selected reference line or tissue, whose basal miRNA expression levels will determine the calculated fold expressions in the tested samples. We have observed that primary human melanocytes (NHEM), which are commonly used as calibrators for melanoma studies, considerably change their absolute levels of several important miRNAs with increased passage number (data not shown). Therefore, great care should be taken in choosing negative controls for comparative and consecutive studies. In this context, we noted very little agreement between our miRNA expression patterns of NHEM melanocytes and melanoma cell lines with a recent study by Mueller and colleagues (17), who have used Agilent miRNA microarrays. Of the 76 listed miRNAs associated with melanoma development and progression, we only identified six to be correspondingly regulated in our data set (miR-27b, miR-92b, miR-10a, miR-182, miR-26b, and miR-379), with the latter four being only weakly expressed. This indicates that the use of different profiling platforms together with the negative control cells and likely even their passage number may result in strikingly different conclusions drawn from the data. On the other hand, our miRNA expression profiles of FFPE-extracted nevi was in good agreement with results recently reported by Glud and colleagues, who used Invitrogen arrays to profile miRNA expression in 15 nevi (18). Taking into account the different array platforms and miRBase versions that were used, we found a remarkable 81% (68 of 84) of miRNAs to be similarly expressed in both studies. Although FFPE samples represent useful sources for miRNA profiling, it is important to keep in mind that variable handling conditions as well as lengths and conditions of storage may directly influence the degree of RNA degradation, which in turn could affect expression levels of some miRNAs.

Another source for intrinsic data variability stems from the various biological sources that are used in miRNA profiling studies. Several miRNAs, such as miR-155, miR-146a, miR-23b, and miR-205, showed divergent expression patterns in cell lines and patient samples, indicating tissue culture–induced adaption processes that likely lead to the loss or upregulation of certain miRNAs, which are not present in patient material. Nonetheless, we identified miR-25 to be upregulated in our samples, and this was in line with a recent observation made in melanoma (30). miR-200c was reproducibly downregulated in patients relative to nevi and was absent in normal human melanocytes and cell lines. The loss or reduced expression of miR-200c in melanoma cell lines has also been reported previously (15). The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) is clearly emerging as a key regulator of differentiation in various cell types, and a marked downregulation with tumor progression has been noted for several cancers (31–33). The loss of E-cadherin expression in our melanoma cell lines is consistent with absent miR-200c expression, which releases repression of the transcription factor ZEB1, which in turn suppresses E-cadherin gene transcription (34). In doing so, the miR-200 family becomes an important regulator of epithelial-to-mesenchymal transition (EMT), a key process for initializing metastasis, and might therefore represent one of the most promising miRNA candidates for therapeutic intervention (35). Interestingly, downregulation of miR-205 has also been implicated in the EMT processes (33). Reduced amounts of miR-205 with tumor progression were detected in all of our melanoma patients, although melanoma cell lines showed slightly increased expression of this miRNA.

Olson and colleagues showed that miR-25 was upregulated 2.6-fold in hyperproliferative stages of pancreatic tumors whereas miR-146a was downregulated 8.4-fold in metastatic lesions, a pattern that we have also observed in melanoma patient samples (36). It is noteworthy that miRNA-15b has recently been suggested to be a prognostic marker for melanoma progression (37). Although melanomas have indeed high levels of miR-15b, we also detected these elevated levels in nevi, questioning the usefulness of this miRNA as a biomarker for melanoma.

Taken together, miRNA-205, miRNA-200, and let-7 family (-125b, -146a, -155, -21, -25, -23a, -29b) have repeatedly been shown to become deregulated with tumor progression in general and in melanomas in particular (this study and refs. 12, 38). Although this is by no means an exclusive list, it certainly contains some of the most promising miRNAs for therapeutic intervention or to be further evaluated for their biomarker potential in larger patient cohorts.

Having identified several miRNAs that could be of value for the understanding of melanoma development, we set out to find potential target genes by analyzing mRNA expression patterns in primary melanocytes (NHEM) and two melanoma cell lines. As expected, many genes previously linked to melanoma development were found to be deregulated,

and several were differentially regulated when comparing IGR39 cells (primary melanoma) with the cell line derived from a metastatic tumor of the same patient (IGR37; Fig. 3). For example, cKIT was slightly downregulated in IGR39 whereas its levels were augmented with disease progression in IGR37. It has been shown that miR-221/miR-222 downregulates cKIT and p27 in melanomas, thus favoring a malignant phenotype (30), and another study suggested that melanoma subgroups exist, which overexpress mutated and active cKIT (39). qPCR for miR-221 and cKIT confirmed this by a perfect inversely correlated expression pattern on all our melanoma cell lines (Supplementary Fig. S1).

Inverse correlation of miRNA expression with their tentative target genes is a useful approach to reduce the generally extensive number of computationally predicted target genes for further analysis. In combination with examination of biological pathways targeted by the deregulated miRNAs, Ingenuity analysis indicated that the regulatory network around MITF was most prominent in melanoma cell lines (Fig. 4A). MITF is critically involved in regulation of melanocyte growth, maturation, apoptosis, and pigmentation (40, 41). Over 20% of metastatic melanomas have been found to carry genetic alterations in the MITF pathway (MITF or SOX10 mutations; ref. 42), and amplification of MITF has been linked to poor patient survival (26). By nucleosome mapping and chromatin structure analysis, Ozsolak and colleagues have identified several miRNA promoters that were occupied by MITF (28). Of those, miR-146a, miR-221, and miR-363 were experimentally confirmed to be regulated by MITF. qPCR analysis of candidate miRNA-23a and miRNA-23b levels predicted to target MITF, as well as of cKIT and miRNAs, which are transcriptionally regulated by MITF, supported the central part of the predicted pathway (Fig. 4B).

Although altered expression of the miRNome has been well documented in many cancers, it remains to be shown how single and/or groups of miRNAs or the whole of the miRNome drives or allows for neoplastic transformation. A key prerequisite to these analyses is, however, an accurate and standardized quantification of the miRNA expression levels in a sufficient number of clinical samples and matching cell lines, as effects seen in one cell line or patient might be quite different from those found in another, in which other potential target genes and also other modulators of miRNA activity may be present. This is even more so as minute changes in miRNA levels may have profound consequences for the cell (2). Accurate and extensive measurements of miRNA levels together with improved computational target gene predictions and pathway analyses will surely be necessary before miRNAs make their way into the clinic as robust biomarkers and/or as therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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