# miRNA Expression Profiling in Melanocytes and Melanoma Cell Lines Reveals miRNAs Associated with Formation and Progression of Malignant Melanoma

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Although deregulated expression of microRNAs (miRNAs) demonstrably contributes to the development and progression of all types of human cancers, little data are available about the changes in miRNA expression levels in malignant melanoma. In our study, we performed microarray-based miRNA profiling of melanocytes and melanoma cell lines derived from either primary tumors or metastatic melanomas. In addition, we analyzed miRNA expression patterns of melanoma cell clones in which the expression of melanoma specific genes was stably knocked down by antisense techniques. We also generated miRNA expression profiles for two derivatives of a melanoma cell line that differ in their invasive potential. Comparing miRNA expression patterns of melanocytes and subsets of melanoma cell lines, we identified large cohorts of miRNAs associated with malignant transformation as well as with the progression of the disease and with metastatic colonization. Surprisingly, the bulk of miRNAs that deregulated most strongly was not described to be of importance in tumor development before. The results of our study, therefore, not only provide insights into alterations in the miRnomes of melanocytes and melanoma cell lines during melanoma progression but also present a large assortment of miRNAs to be analyzed for their potential as diagnostic markers or targets for therapies in the future.

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## INTRODUCTION

The discovery of a new class of small non-coding RNAs—the so-called microRNAs (miRNAs)—gave rise to a new, highly interesting field of molecular biology. These miRNAs are initially transcribed mainly by RNA polymerase II as capped and polyadenylated primary transcripts, which are called pri-miRs (Cai *et al.*, 2004; Lee *et al.*, 2004; Kim, 2005). After processing of the pri-miR by a microprocessor complex (composed predominantly of the RNAse III enzyme Drosha) and a second RNAse III enzyme known as Dicer, an approximately 18–24 nucleotide duplex is loaded on the miRNA-induced silencing complex (miRISC, also called miRNPs) (Du and Zamore, 2005; Bushati and Cohen, 2007; Peters and Meister, 2007; Rana, 2007). Only one strand of the duplex—the mature miRNA—remains stable on the miRISC and induces post-transcriptional silencing of one or more target gene(s) by binding with imperfect complementarity to a target sequence in the 3′-UTR of a target RNA with respect to a set of general rules that are only incompletely determined experimentally and bioinformatically to date (Doench and Sharp, 2004; Brennecke *et al.*, 2005; Lewis *et al.*, 2005; Grimson *et al.*, 2007; Nielsen *et al.*, 2007). miRNAs mediate post-transcriptional silencing either by destabilization of the target mRNA (Behm-Ansmant *et al.*, 2006; Giraldez *et al.*, 2006; Wu *et al.*, 2006) or by repression of translation (Pillai *et al.*, 2007; Standart and Jackson, 2007); it is not yet clear if the latter occurs at the initiation or after the initiation step (Filipowicz *et al.*, 2008). To date, more than 500 miRNAs have been discovered in the human genome, resulting in an estimated total number of 1,000 possible miRNA genes. Taking into consideration that every miRNA can target several mRNAs, about 30% of genes in the human genome could be regulated by miRNAs (Lewis *et al.*, 2005). It is already widely accepted that miRNAs are involved in the regulation of multiple cellular processes, such as developmental patterning, lineage differentiation, apoptosis, proliferation, and antiviral defense. Owing to the participation of miRNAs in the above-mentioned processes, it becomes clear

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*Abbreviations: as-Snail, antisense Snail; MIA, melanoma inhibitory activity; miRNA, microRNA; NHEM, normal human epidermal melanocyte; qRT-PCR, quantitative real-time PCR*

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that abnormalities in miRNA activity also play an important role in the formation and progression of cancer diseases (Esquela-Kerscher and Slack, 2006; Kloosterman and Plasterk, 2006; Bushati and Cohen, 2007; Chang and Mendell, 2007; Hwang and Mendell, 2007).

As a consequence of the important role of miRNAs in cancer development, several studies comparing miRNA expression profiles of various tumors with corresponding normal tissues have been performed (Lu *et al.*, 2005; Volinia *et al.*, 2006; Zhang *et al.*, 2006; Gaur *et al.*, 2007). Those studies revealed that alterations in miRNA expression profiles can serve as phenotypic signatures of particular types of cancer. Thus, it becomes clear that profiling of miRNA expression harbors a high potential to identify miRNAs that may serve as both diagnostic markers and therapeutic targets in many different tumor types in the future.

Although some data regarding altered miRNA expression in malignant melanoma are included in parts of the above-mentioned studies, there is a lack of studies comparing normal human epidermal melanocytes (NHEMs) with established melanoma cell lines with respect to a large number of known miRNAs. In our study, we performed miRNA expression arrays comparing NHEMs with melanoma cell lines derived from primary tumors and metastases (Giard *et al.*, 1973; Jacob *et al.*, 1998), as well as from different antisense cell clones, which are used as model systems for examining molecular changes in the malignant transformation of melanocytes into melanoma cells in our laboratory (Poser *et al.*, 2001, 2004; Kuphal *et al.*, 2005; Tatzel *et al.*, 2005). Thus, we were able to identify sets of miRNAs with altered expression during malignant transformation as well as in the progression of the disease and in metastasizing events. In addition, we found several miRNAs influenced when genes highly important in malignant melanoma are knocked down by antisense techniques, and we could also show that the miRNA expression profiles of those cell lines exhibit high similarity to the miRnome of cultured NHEMs. The results of our study provide insights into alterations of the miRnome in malignant melanoma cell lines and present a cohort of miRNAs that have to be analyzed for their potential to serve as diagnostic markers or targets for therapies in the future.

## RESULTS

### Comparison of miRnomes of melanocytes and melanoma cell lines

The microarray experiment included pooled NHEMs from two donors, three melanoma cell lines derived from primary melanomas (Mel Ei, Mel Wei, and Mel Juso), four cell lines derived from melanoma metastases (Mel Im, Mel Ju, HMB2, and A375), and HMB2 cell clones stably transfected with an antisense melanoma inhibitory activity (MIA) construct (Tatzel *et al.*, 2005), as well as Mel Im cell clones stably transfected with an antisense Snail (as-Snail) construct (Kuphal *et al.*, 2005). In addition, we analyzed two Mel Im derivatives differing in their invasive behavior (Mel Im hi, highly invasive; Mel Im wi, weakly invasive) (Jacob *et al.*, 1995). For each cell line, analysis was performed in duplicates. The Agilent miRNA microarrays contained 470 human and 64 viral microRNAs from the Sanger database (v 9.1).

First, an unsupervised analysis was performed using melanocytes, the seven tested melanoma cell lines, and the MIA-deficient HMB2 cell clone, HMB2-MIA5, to observe whether the miRNA expression profiles reflect the origins of the cell lines. The miRNA tree well-separated NHEMs and HMB2-MIA5 (which clustered together) from the melanoma cell lines (Figure 1 and Figure S1). In contrast to the origin of the melanoma cell lines, one melanoma cluster consisted of two metastatic cell lines (Mel Im and Mel Ju) and one primary tumor cell line (Mel Juso). The other melanoma cluster comprised two melanoma cell lines derived from primary tumors (Mel Ei and Mel Wei) and one melanoma cell line derived from a metastasis (A375). Another metastatic cell line, HMB2, showed a somewhat distinct expression pattern, resulting in clustering at a distance from the two other melanoma clusters.

In addition, we performed unsupervised analysis of melanocytes, the Mel Im cell line, and its derivatives, Mel Im hi and Mel Im wi, together with the as-Snail cell clones to find out if the as-Snail clones can be distinguished from the other Mel Im cell lines with respect to their miRNA expression profile. The resulting miRNA tree separated the Mel Im cell line, with its derivatives differing in invasive behavior from the melanocytes, which clustered together with the as-Snail clones (Figure 2 and Figure S2).

### Determining miRNAs related to early progression, metastasis, and progression of malignant melanoma

To identify miRNAs deregulated in the transition of melanocytes into melanoma cells, we compared the expression of 461 miRNAs in melanocytes with primary melanoma cell lines. The criterion for considering a miRNA as deregulated was an at least three-fold up- or downregulation, in primary melanoma cell lines compared with NHEMs. Values of $<20\%$ of the mean fluorescence intensity of all signals and miRNAs expressed in only one of the primary melanoma cell lines were also excluded from the analysis. We found 91 miRNAs fulfilling our criteria, of which 77 were upregulated (Table S1) and only 14 were downregulated (Table 1) in primary melanoma cell lines. To narrow the list of upregulated genes, we focused on miRNAs that were


**Figure 1. Unsupervised clustering of melanocytes and melanoma cell lines.** Schematic representation of a miRNA tree generated by the comparison of miRNA expression profiles of melanocytes with melanoma cell lines (see text for more information).

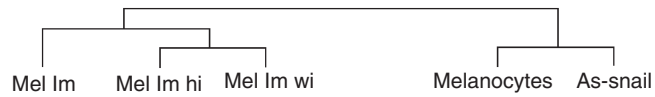


**Figure 2. Unsupervised clustering of melanocytes, melanoma cell line Mel Im, and its derivatives.** Schematic representation of a miRNA tree generated by the comparison of miRNA expression profiles of melanocytes with derivatives of the malignant melanoma cell line Mel Im (see text for more information).

**Table 1. miRNAs found to be upregulated or downregulated in primary tumor cell lines compared with NHEMs and thus associated with early progression**

| miRNAs upregulated during early progression | | |
|---|---|---|
| *hsa-miR-10a* | *hsa-miR-326* | *hsa-miR-526b* |
| *hsa-miR-126* | *hsa-miR-340* | *hsa-miR-545* |
| *hsa-miR-135b* | *hsa-miR-373* | *hsa-miR-550* |
| *hsa-miR-141* | *hsa-miR-379* | *hsa-miR-557* |
| *hsa-miR-145* | *hsa-miR-382* | *hsa-miR-564* |
| *hsa-miR-181a** | *hsa-miR-383* | *hsa-miR-583* |
| *hsa-miR-182* | *hsa-miR-449* | *hsa-miR-622* |
| *hsa-miR-183* | *hsa-miR-454-5p* | *hsa-miR-628* |
| *hsa-miR-18a** | *hsa-miR-504* | *hsa-miR-640* |
| *hsa-miR-196b* | *hsa-miR-507* | *hsa-miR-641* |
| *hsa-miR-200a** | *hsa-miR-517** | *hsa-miR-658* |
| *hsa-miR-200c* | *hsa-miR-518a* | *hsa-miR-662* |
| *hsa-miR-218* | *hsa-miR-518f** | *hsa-miR-758* |
| *hsa-miR-26b* | *hsa-miR-520b* | *hsa-miR-9* |
| *hsa-miR-27b* | *hsa-miR-520d* | *hsa-miR-92b* |
| *hsa-miR-301* | *hsa-miR-520d** | |
| *hsa-miR-30a-3p* | *hsa-miR-525* | |
| **miRNAs downregulated during early progression** | | |
| *hsa-miR-148b* | *hsa-miR-331* | *hsa-miR-489* |
| *hsa-miR-181a* | *hsa-miR-363* | *hsa-miR-503* |
| *hsa-miR-23b* | *hsa-miR-422b* | *hsa-miR-527* |
| *hsa-miR-299-3p* | *hsa-miR-455* | *hsa-miR-595* |
| *hsa-miR-324-5p* | *hsa-miR-485-3p* | |

**Table 2. miRNAs found to be upregulated or downregulated in metastatic melanoma cell lines compared with primary tumor cell lines and thus associated with the metastatic colonization of malignant melanoma cells**

| miRNAs upregulated in metastatic cell lines | | |
|---|---|---|
| *hsa-miR-199a** | *hsa-miR-515-3p* | *hsa-miR-519d* |
| *hsa-miR-302c** | *hsa-miR-517b* | *hsa-miR-520f* |
| *hsa-miR-30a-5p* | *hsa-miR-518b* | *hsa-miR-523* |
| *hsa-miR-425-3p* | *hsa-miR-519b* | |
| **miRNAs downregulated in metastatic cell lines** | | |
| *hsa-miR-30e-3p* | *hsa-miR-514* | |

upregulated more than 10-fold in primary melanoma cell lines or that were not expressed in melanocytes but highly expressed in primary melanoma cell lines. Thus, we were able to identify a cohort of 49 miRNAs strongly upregulated during early progression (Table 1; for mean fluorescence intensity of signals, see Table S1).

Next, we compared miRNA expression levels in primary melanoma cell lines with those in melanoma cell lines derived from metastatic melanomas to identify miRNAs associated with metastatic colonization. In addition to the above-mentioned criteria (three-fold up-/downregulation, 20% of mean intensity, and exclusion of miRNAs only expressed in one of the cell lines), miRNAs considered as deregulated in metastasis had to be expressed at the same level in melanocytes and in primary melanoma cell lines. We found 13 miRNAs deregulated, of which 11 were upregulated and only 2 were downregulated in metastatic melanoma cell lines (Table 2).

Later we wanted to identify miRNAs strongly related to the progression of malignant melanoma, and looked for miRNAs steadily up- or downregulated during early progression and subsequent metastasis. We found six miRNAs upregulated at least three-fold from melanocytes to primary melanoma and again from primary melanoma to metastatic melanoma cell lines (miR-133a, miR-199b, miR-453, miR-520f, miR-521, and miR-551b), and one miRNA whose expression was downregulated to less than one-third from melanocytes to primary melanoma and again from primary melanoma to metastatic melanoma cell lines (miR-190). In addition, comparing the lists of miRNAs related to early progression and the list of miRNAs involved in metastasis, we identified seven miRNAs that were either upregulated during early progression and subsequently downregulated in metastatic colonization (miR-126, miR-29c, miR-506, miR-507, and miR-520d*) or *vice versa* (miR-489 and miR-527).

**Determining miRNAs deregulated in melanoma model systems**

To analyze the role of the transcription factor Snail in malignant melanoma, Mel Im cell clones were created in which Snail expression is stably knocked down by an as-Snail construct (Poser *et al.*, 2001; Kuphal *et al.*, 2004, 2005). In this study, we analyzed the miRnome of those as-Snail cell clones compared with cells of the parental melanoma cell line Mel Im. We found 64 miRNAs differentially expressed in as-Snail clones compared with Mel Im cells when setting the cut-off to three-fold up- and downregulated. The cohort contains 46 miRNAs upregulated and 18 miRNAs downregulated in the as-Snail clones (Table 3).

MIA-deficient melanoma cell clones were generated in our laboratory by stable transfection of HMB2 melanoma cells with an antisense-MIA cDNA expression plasmid (Poser *et al.*, 2004). The MIA-deficient cell clones showed a recovery of pigmentation and a melanocytic growth pattern (Tatzel *et al.*, 2005). cDNA and protein array data displayed that MIA-deficient cell clones also show high similarities in gene expression to melanocytic cells, which enables us to use

**Table 3. miRNAs found to be upregulated or downregulated in as-Snail cell clones compared with cells of the parental Mel Im cell line**

| miRNAs upregulated in as-Snail cell clones | | |
|---|---|---|
| *hsa-miR-125b* | *hsa-miR-206* | *hsa-miR-450* |
| *hsa-miR-126* | *hsa-miR-21* | *hsa-miR-506* |
| *hsa-miR-130a* | *hsa-miR-218* | *hsa-miR-507* |
| *hsa-miR-143* | *hsa-miR-224* | *hsa-miR-526b* |
| *hsa-miR-148b* | *hsa-miR-23a* | *hsa-miR-592* |
| *hsa-miR-15b* | *hsa-miR-23b* | *hsa-miR-596* |
| *hsa-miR-181a** | *hsa-miR-26a* | *hsa-miR-598* |
| *hsa-miR-189* | *hsa-miR-32* | *hsa-miR-606* |
| *hsa-miR-191* | *hsa-miR-324-5p* | *hsa-miR-624* |
| *hsa-miR-191** | *hsa-miR-330* | *hsa-miR-641* |
| *hsa-miR-192* | *hsa-miR-335* | *hsa-miR-660* |
| *hsa-miR-194* | *hsa-miR-362* | *hsa-miR-663* |
| *hsa-miR-195* | *hsa-miR-378* | *hsa-miR-9** |
| *hsa-miR-196a* | *hsa-miR-425-3p* | *hsa-miR-96* |
| *hsa-miR-199a** | *hsa-miR-431* | |
| *hsa-miR-200a** | *hsa-miR-432* | |

| miRNAs downregulated in as-Snail cell clones | | |
|---|---|---|
| *hsa-miR-122a* | *hsa-miR-365* | *hsa-miR-489* |
| *hsa-miR-181d* | *hsa-miR-381* | *hsa-miR-491* |
| *hsa-miR-196b* | *hsa-miR-382* | *hsa-miR-517** |
| *hsa-miR-299-5p* | *hsa-miR-383* | *hsa-miR-539* |
| *hsa-miR-34b* | *hsa-miR-423* | *hsa-miR-551b* |
| *hsa-miR-363* | *hsa-miR-454-5p* | *hsa-miR-758* |

**Table 4. miRNAs found to be upregulated or downregulated in MIA-deficient HMB2 cell clones HMB2-MIA compared with cells of the parental HMB2 cell line**

| miRNAs upregulated in HMB2-MIA cell clones | | |
|---|---|---|
| *hsa-miR-10b* | *hsa-miR-34c* | *hsa-miR-609* |
| *hsa-miR-145* | *hsa-miR-485-3p* | *hsa-miR-640* |
| *hsa-miR-224* | *hsa-miR-539* | *hsa-miR-9** |
| *hsa-miR-335* | *hsa-miR-606* | |

| miRNAs downregulated in HMB2-MIA cell clones | | |
|---|---|---|
| *hsa-miR-126** | *hsa-miR-487a* | *hsa-miR-596* |
| *hsa-miR-141* | *hsa-miR-506* | *hsa-miR-625* |
| *hsa-miR-146a* | *hsa-miR-507* | *hsa-miR-627* |
| *hsa-miR-148a* | *hsa-miR-514* | *hsa-miR-632* |
| *hsa-miR-196a* | *hsa-miR-518d* | *hsa-miR-641* |
| *hsa-miR-203* | *hsa-miR-545* | *hsa-miR-658* |
| *hsa-miR-21* | *hsa-miR-550* | *hsa-miR-660* |
| *hsa-miR-345* | *hsa-miR-551b* | *hsa-miR-767-5p* |
| *hsa-miR-373* | *hsa-miR-565* | *hsa-miR-768-3p* |
| *hsa-miR-374* | *hsa-miR-571* | *hsa-miR-769-5p* |
| *hsa-miR-449* | *hsa-miR-577* | |
| *hsa-miR-454-3p* | *hsa-miR-583* | |

the HMB2/HMB2-MIA cell system as a model system for investigating molecular differences between ''melanocytes'' and melanoma cells. We now also wanted to analyze the miRnome of those cells compared with that of melanocytes. We first compared miRNA expression of the MIA-deficient cell clones with that of cells of the parental HMB2 cell line and found 83 deregulated miRNAs (30 miRNAs upregulated and 53 miRNAs downregulated in HMB2-MIA cells; Table S2). To narrow the list of interesting miRNAs deregulated in the MIA-deficient HMB2 cell clones, we filtered the candidates for miRNAs 10-fold up- or downregulated, and thus identified a cohort of 46 miRNAs (with 12 up- and 34 downregulated miRNAs; Table 4). The fact that more miRNAs are downregulated when MIA is knocked out (about 60% of deregulated miRNAs) fits our observation that more miRNAs are up- than downregulated during early progression. Comparing relative expression levels of 461 miRNAs, we found 311 miRNAs (~70% of all miRNAs) equably expressed in melanocytes and in MIA-deficient cell clones HMB2-MIA (data not shown). Comparing the lists of miRNAs deregulated during early progression (checking NHEMs against primary tumor cell lines) and deregulated comparing MIA-deficient HMB2 cell clones with the parental HMB2 cells revealed 18 miRNAs equably regulated (that is, upregulated in early progression and downregulated when MIA is knocked down or *vice versa*) (Table S3).

In our laboratory, we also use derivatives of Mel Im cells called Mel Im wi and Mel Im hi (Jacob *et al.*, 1995). These two sublines strongly differ in their invasive behavior, with the weakly invasive phenotype (Mel Im wi) harboring only 50% of the invasive potential of parental Mel Im cells, and the highly invasive phenotype (Mel Im hi) showing 250% of the invasive potential of parental Mel Im cells. By continuous propagation in tissue culture for more than 40 passages and repeated analyses, we were able to show that these sublines represent genetically stable phenotypes. To identify miRNAs having an influence on invasiveness of melanoma cells, we compared miRNA expression levels of highly invasive Mel Im cells (Mel Im hi) with a less-invasive Mel Im derivative (Mel Im wi). We found 69 miRNAs to be differently expressed (34 upregulated and 35 downregulated in the more invasive phenotype; Table 5). Comparing this cohort of miRNAs with the lists of miRNAs deregulated in metastatic colonization on the one hand and with the list of miRNAs deregulated in early progression on the other, we found three (miR-133a,

**Table 5. miRNAs found to be upregulated or downregulated in the highly invasive Mel Im derivative (Mel Im hi) compared with an Mel Im derivative with a less-invasive phenotype (Mel Im wi)**

| miRNAs upregulated in highly invasive Mel Im cells | | |
|---|---|---|
| *hsa-miR-122a* | *hsa-miR-21* | *hsa-miR-518f** |
| *hsa-miR-132* | *hsa-miR-28* | *hsa-miR-523* |
| *hsa-miR-133a* | *hsa-miR-302c** | *hsa-miR-526a* |
| *hsa-miR-133b* | *hsa-miR-30e-3p* | *hsa-miR-560* |
| *hsa-miR-15a* | *hsa-miR-31* | *hsa-miR-628* |
| *hsa-miR-15b* | *hsa-miR-362* | *hsa-miR-631* |
| *hsa-miR-17-3p* | *hsa-miR-373** | *hsa-miR-659* |
| *hsa-miR-181b* | *hsa-miR-378* | *hsa-miR-660* |
| *hsa-miR-183* | *hsa-miR-425-5p* | *hsa-miR-662* |
| *hsa-miR-187* | *hsa-miR-489* | *hsa-miR-770-5p* |
| *hsa-miR-200a** | *hsa-miR-493-3p* | *hsa-miR-99b* |
| **miRNAs downregulated in highly invasive Mel Im cells** | | |
| *hsa-miR-10a* | *hsa-miR-330* | *hsa-miR-590* |
| *hsa-miR-134* | *hsa-miR-331* | *hsa-miR-602* |
| *hsa-miR-151* | *hsa-miR-34c* | *hsa-miR-609* |
| *hsa-miR-152* | *hsa-miR-371* | *hsa-miR-625* |
| *hsa-miR-18a* | *hsa-miR-449* | *hsa-miR-629* |
| *hsa-miR-18a** | *hsa-miR-484* | *hsa-miR-650* |
| *hsa-miR-191** | *hsa-miR-506* | *hsa-miR-652* |
| *hsa-miR-204* | *hsa-miR-509* | *hsa-miR-7* |
| *hsa-miR-211* | *hsa-miR-510* | *hsa-miR-766* |
| *hsa-miR-219* | *hsa-miR-519b* | *hsa-miR-92b* |
| *hsa-miR-23b* | *hsa-miR-520b* | |
| *hsa-miR-296* | *hsa-miR-574* | |

miR-302c*, and miR-523) and nine equably regulated candidate miRNAs (upregulated: miR-183, miR-200a*, miR-362, miR-378, miR-518f*, miR-560, and miR-628; downregulated: miR-23b and miR-331), respectively.

**Confirmation of array data by quantitative real-time PCR in NHEMs, melanoma cell lines, and melanoma tissue samples**

To ensure the quality of our data derived from the miRNA microarray experiments, we performed a quantitative real-time PCR (qRT-PCR) analysis on a set of seven miRNAs comparing the expression levels in different pools of NHEMs with three primary melanoma cell lines (Mel Ei, Mel Wei, and Mel Juso) as well as in three metastatic cell lines (Mel Im, Mel Ju, and A375). In addition, we included a set of melanoma tissue samples containing four primary tumor samples and six samples derived from melanoma metastases (lung, skin, brain, small intestine, and two lymph-node metastases) in our analysis to investigate whether observed changes in the expression of some miRNAs *in vitro* also play a role *in vivo* or whether they might be an artifact of long-term melanoma cell lines and cell culture conditions.

A qRT-PCR analysis of miR-17-5p, miR-222, miR-181a, miR-194, miR-22, and miR-373 expressions in melanocytes and melanoma cell lines is in agreement with the data obtained by the miRNA microarray experiments (Figures 3a–e and 4a). miR-17-5p (Figure 3a) serves as an example of a miRNA upregulated during the progression of malignant melanoma and was chosen because it is a miRNA that is already described in the literature in detail (see the section Discussion). The miRNA miR-222 (Figure 3b) was detected to be weakly upregulated during melanoma progression in the microarray experiments but did not pass our very strict selection criteria for identifying the most interesting miRNAs for future analysis. The rationale for our selection criteria is supported by qRT-PCR data obtained by the analysis of melanoma tumor samples in which miR-222 expression also appears only weakly deregulated, for example, in comparison with miR-17-5p. The miRNAs miR-181a (Figure 3c) and miR-194 (Figure 3d) were taken as examples for miRNAs downregulated in melanoma progression. miR-181a is already known to function as a tumor suppressor in glioma cells triggering growth inhibition, inducing apoptosis, and inhibiting invasion (Shi *et al.*, 2008), whereas the only information available for the function of miR-194 is its involvement in intestinal epithelial cell differentiation (Hino *et al.*, 2008). To also examine the expression level of a miRNA not differentially expressed between melanocytes and melanoma cells, we further analyzed miR-22 (Figure 3e). We were able to show that in accordance with the microarray data, miR-22 expression was not shown to be differentially regulated in these qRT-PCR experiments. Our data reveal that the changes in miRNA expression levels detectable in melanoma cell lines can also be observed in melanoma tissue samples, although expression levels are more variable between tissue samples and *x*-fold changes might be higher or lower in tissue samples than in long-term melanoma cell lines.

As mentioned above, we also analyzed the expression level of miR-373 in melanocytes, melanoma cell lines, and melanoma tissue samples. Upregulation of this miRNA in melanoma cell lines as detected in the microarray study was confirmed by qRT-PCR (Figure 4a). miR-373 was of special interest for us, as it is one member of the panel of miRNAs that we found to be equably deregulated in our HMB2/HMB2-MIA melanoma model system during the microarray study (see Table S3). We were able to confirm this deregulation by qRT-PCR as shown in Figure 4b. Looking at the miR-373 expression levels in melanoma tumor samples, we found a diverse expression pattern reflected by the high standard deviation seen in Figure 4a. Two of the four primary tumor samples showed strongly induced expression of miR-373, whereas the remaining samples showed a very low expression level. Accordingly, three of the six melanoma metastases showed strongly induced miR-373 expression, whereas the remaining

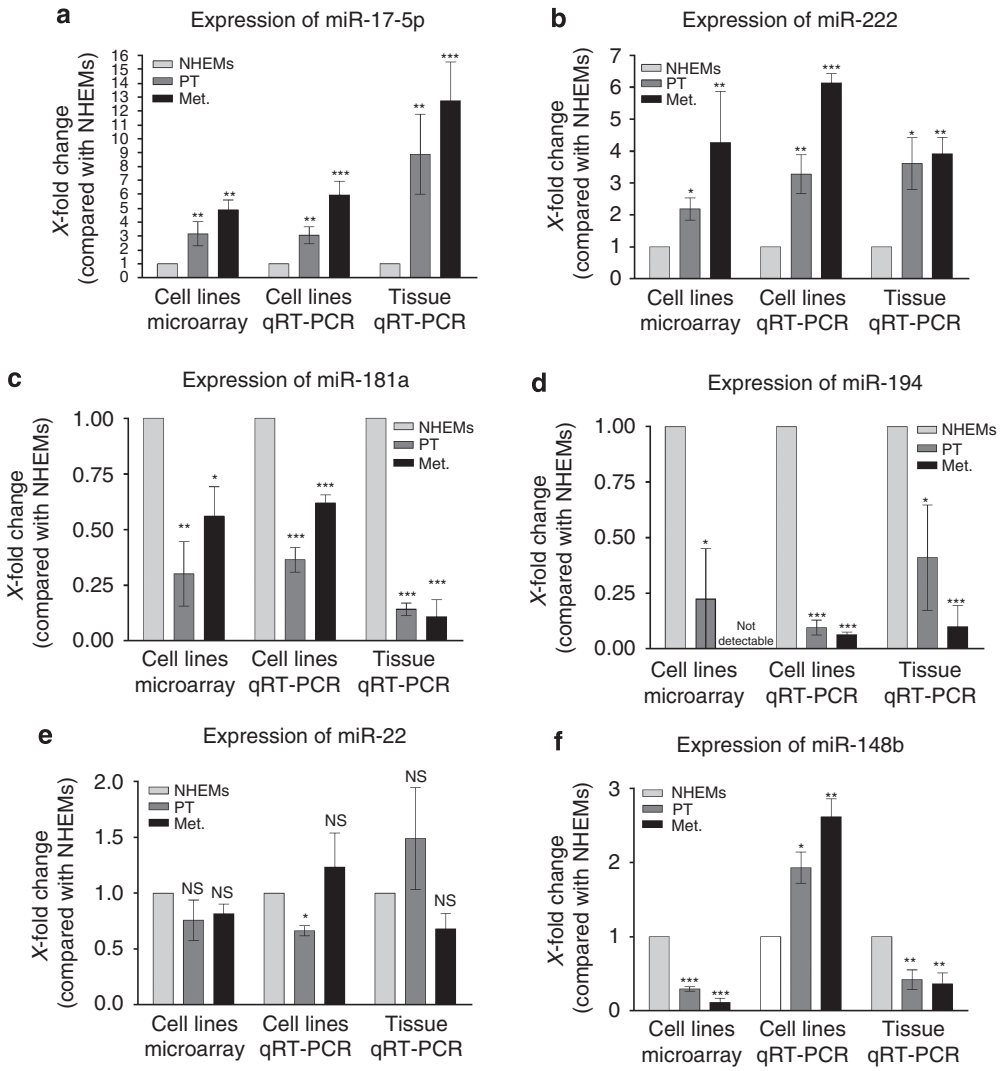


**Figure 3. Comparison of miRNA expression levels detected by miRNA microarrays and qRT-PCR in NHEMs, melanoma cell lines, and melanoma tissue samples.** (**a**) The expression level of miR-17-5p in NHEMs, cell lines derived from primary tumors (PT), and metastases (Met.), and in primary and metastatic melanoma tissue samples. (**b**) The expression level of miR-222 in the same samples. miR-17-5p and miR-222 were chosen as examples for miRNAs upregulated in melanoma. (**c** and **d**) The expression levels of miR-181a and miR-194 in the same panel of samples. These miRs were chosen as examples for miRNAs downregulated in melanoma. (**e**) The expression level of miR-22 in the same panel of samples. This miR was taken as an example of a not differentially expressed miRNA. (**f**) The expression level of miR-148b in the set of samples described above. In contrast to the miRNA microarray experiments, expression of this miRNA was found to be upregulated in our melanoma cell lines by qRT-PCR. *$p \leqslant 0.05$; **$p \leqslant 0.01$; ***$p \leqslant 0.001$.

metastatic tissue samples showed almost no expression of miR-373 at all.

In the panel of seven miRNAs initially chosen for qRT-PCR confirmation of our microarray data, we also had one miRNA—miR-148b—whose expression was shown to be incorrectly detected by the miRNA microarray analysis. In contrast to the microarray data, expression of miR-148b was detected to be upregulated in the melanoma cell lines rather than downregulated (Figure 3f).

### Analysis of the expression of a possible miRNA target gene, Rab38, in melanocytes, melanoma cell lines, and melanoma tissue samples

As a consequence of imperfect base pairing, which is the mechanism by which miRNAs bind to target sequences in the 3′-UTRs of their target gene transcripts, current algorithms for miRNA/target predictions give rise to lists containing up to several hundred possible target genes for each individual miRNA. Searching for target genes for our sets of miRNAs would have very likely resulted in dozens of potential ''target genes'' presumably involved in melanoma progression and would have been of no further help.

To get additional information about the importance of the miRNAs that we detected to be deregulated in melanoma, we decided to analyze the expression of the gene *Rab38*. Rab38 (also called ''melanocyte differentiation antigen RAB38/NY-MEL-1'') is a member of the Rab small G-protein family that regulates intracellular vesicle trafficking, and is involved in the biogenesis of melanosomes resulting in a protein highly expressed in melanocytes and downregulated in melanoma

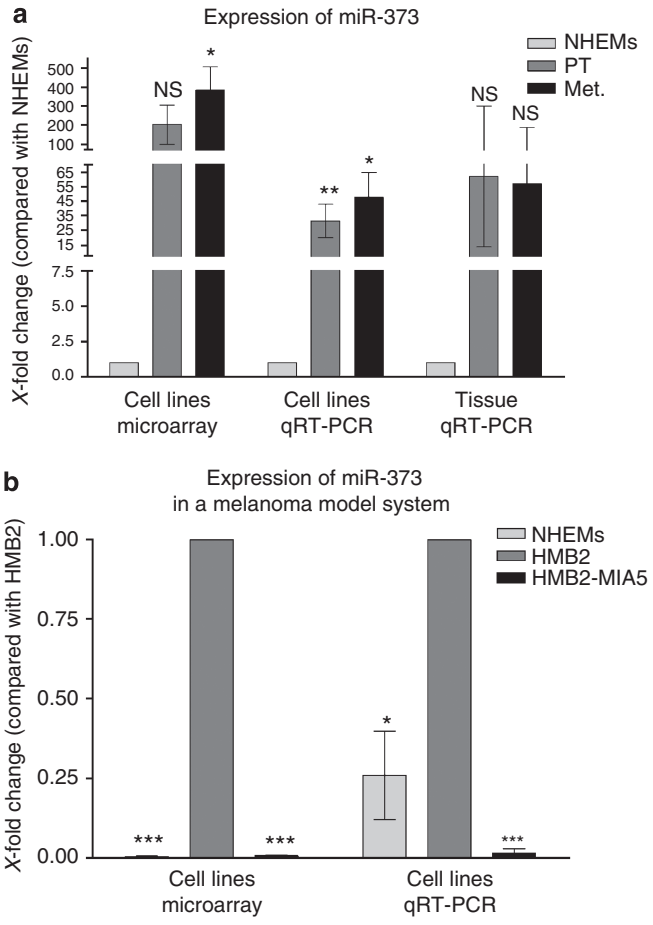


**Figure 4. Expression of miR-373 in NHEMs, melanoma cell lines and tissue samples, and in the melanoma model system.** Comparison of miR-373 expression levels detected by miRNA microarrays and qRT-PCR in normal human epidermal melanocytes (NHEMs), melanoma cell lines, and melanoma tissue samples, and in our HMB2/HMB2-MIA melanoma model system.(**a**) The expression level of miR-373 in NHEMs, melanoma cell lines derived from primary tumors (PT) and metastases (Met.), and in primary and metastatic melanoma tissue samples. (**b**) The expression level of miR-373 in NHEMs, HMB2 metastatic melanoma cells, and MIA-deficient HMB2 cell clone HMB2-MIA5. *$p \leqslant 0.05$; **$p \leqslant 0.01$; ***$p \leqslant 0.001$.

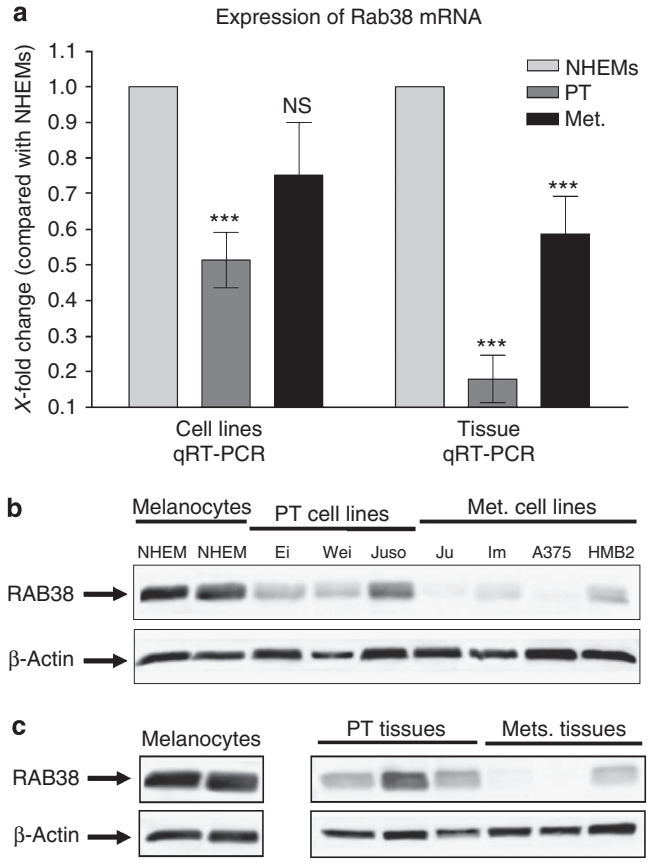


**Figure 5. Expression of Rab38 in melanocytes, melanoma cell lines, and melanoma tissue samples** (**a**) Rab38 mRNA level in NHEMs, melanoma cell lines derived from primary tumors (PT), and metastases (Met.), respectively, and in primary and metastatic melanoma tissue samples as detected by qRT-PCR. (**b**) RAB38 protein expression in melanocytes and melanoma cell lines derived from primary tumors (PT) and metastases (Met.), respectively, as determined by western blotting. β-Actin serves as a loading control. (**c**) RAB38 protein expression in melanocytes and in melanoma primary tumor (PT) and metastatic (Met.) tissue samples. *$p \leqslant 0.05$; **$p \leqslant 0.01$; ***$p \leqslant 0.001$.

cell lines (Jager *et al.*, 2000; Osanai *et al.*, 2005; Wasmeier *et al.*, 2006). In accordance with these findings, we were able to detect reduced levels of Rab38 mRNA in melanoma cell lines derived from primary tumors (about 50% downregulation) and melanoma metastases (about 25% downregulation), compared with normal melanocytes (Figure 5a, left-hand bars). The downregulation of Rab38 mRNA was even stronger in melanoma tissue samples from primary tumors (about 80% downregulation) and melanoma metastases (about 40% downregulation), compared with that from NHEMs (Figure 5a, right-hand bars). Performing western blots with an anti-RAB38 antibody we were able to confirm reduced RAB38 expression on protein level (Figure 5b and c). It is worth mentioning that, in contrast to the mRNA data, it seems there is less RAB38 protein present in metastatic melanoma cell lines and tissue samples than in cell lines and tissue samples derived from primary melanomas—a fact that provides an indication of *Rab38* expression regulation by miRNA-induced repression of translation.

Searching for miRNAs potentially targeting Rab38 mRNA gave rise to a list of 21 miRNAs (search performed using miRGen; Megraw *et al.*, 2007). Looking at our list of miRNAs deregulated in melanoma, 16 of these miRNAs do not appear to be differentially regulated. Two other miRNAs, which are predicted to target Rab38 mRNA, miR-181a and miR-194, were found to be downregulated in melanoma during our studies (data already confirmed by qRT-PCR; Figure 3c and d), and are thus eliminated from the list. One of the three residual miRNAs, miR-506, was shown to be first upregulated from melanocytes to primary melanoma cell lines, but subsequently downregulated from primary tumor to metastatic melanoma cell lines. A regulation in this manner does not fit the observation that the RAB38 protein expression is further downregulated in metastatic melanoma cell lines and tissue samples from melanoma metastases. Of the 21 miRNAs predicted at the beginning of this analysis, only two remain to be analyzed for a potential miRNA/target relationship with

Rab38 mRNA (miR-9 and miR-141), as those miRNAs appeared to be upregulated in cell lines derived from primary tumors as well as in cell lines derived from metastases during our microarray studies.

## DISCUSSION

It is widely accepted that deregulated expression of miRNAs is highly involved in the formation and progression of all types of human cancers (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; Cho, 2007; Cowland *et al.*, 2007; Dalmay, 2008). Several studies showed that alterations in miRNA expression profiles can serve as phenotypic signatures of a particular cancer type. Lu *et al.* (2005) analyzed 217 mammalian miRNAs from 334 tissue samples, and showed that the miRNA profiles reflected the developmental lineage and the differentiation status of tumors. Volinia *et al.* (2006) performed a large-scale miRnome analysis on 540 solid tumor samples, including lung, breast, stomach, prostate, colon, and pancreatic tumors, thus identifying a solid cancer miRNA signature composed largely of over-expressed miRNAs. Zhang *et al.* (2006) analyzed 283 known human miRNA genes in 227 human cancer tissue samples by means of high-resolution array-based comparative genomic hybridization, and showed that genomic loci harboring miRNA genes are often subject to DNA copy number alterations in ovarian cancer, breast cancer, and in melanoma. In addition, they gave evidence for a direct correlation between copy number changes and the expression level of a given miRNA. Gaur *et al.* (2007) examined the expression of 241 miRNAs in normal tissues, and the NCI-60 panel comprised 60 cancer-derived cell lines and showed (1) that the NCI-60 cell lines clustered because of their tissue origin and (2) that the miRNA expression profiles of normal tissue and tumor tissue showed significant differences, resulting in the identification of miRNAs with oncogenic or tumor-suppressing character, respectively.

Although these studies show that miRNA profiling can be successfully employed to detect miRNAs deregulated in cancer formation and progression and may thus be attractive targets for future therapies, for malignant melanoma there has been no study examining large panels of miRNAs comparing NHEMs with established melanoma cell lines derived from primary tumors as well as from metastatic melanomas. Therefore, we performed a microarray analysis on NHEMs and seven malignant melanoma cell lines, three of which were derived from primary melanomas and four of which were derived from metastatic melanomas, to identify cohorts of miRNAs potentially involved in tumorigenesis, progression, and metastatic colonization of malignant melanoma. Performing unsupervised clustering with all miRNAs expressed at least in one cell line gave rise to a miRNA tree, which well-separated NHEMs from melanoma cell lines. The melanoma cell lines derived from primary tumors and metastatic melanomas were not completely separated, but showed a somehow ''mixed'' appearance. In recent publications, the melanoma cell lines used were characterized with respect to their invasive potential (Jacob *et al.*, 1995, 1998). When examining the ability of melanoma cell lines derived from primary tumors and those derived from metastatic melanomas to invade membrane barriers, it was shown that the mean invasive capacity of the metastatic cell lines was significantly higher than that of the primary tumor cell lines. Interestingly, it was shown that in addition to the metastatic cell lines, some of the primary tumor cell lines were also able to invade either collagen or matrigel matrices in some cases. By measuring MMP (matrix metalloproteinase) and TIMP (tissue inhibitor of metalloproteinase) levels, metastatic cell lines showed higher MMP and lower TIMP levels compared with primary tumor cells when considering at the average values. However, in those experiments, not all cell lines derived from primary tumors and metastasis behaved homogenously, but showed differences according to their different properties in the invasion assays. For example, transforming growth factor beta 2 (TGF-β2) levels were significantly higher in supernatants of metastatic cell lines compared with those of primary tumor cell lines as a collective. However, one primary tumor cell line also showed a TGF-β2 level almost as high as that of metastatic cell lines. Taken together, those experiments showed that when collectives of cell lines derived from primary tumors and metastasis were compared, each group behaves as expected, but individual members of each group show specific alterations, which make the collectives a heterogeneous mass. We, therefore, can state that despite the origin of the cell lines (primary tumor or metastasis), every cell line harbors some specific properties and this includes the possibility that some primary tumor cell lines already hold the potential to become metastatic. This fact might be reflected in the miRnomes of the melanoma cell lines so that the unsupervised clustering cannot precisely separate melanoma cell lines derived from primary tumors from cell lines derived from metastatic melanomas, as some intrinsic miRNA expression differences might not be related to tumor stage at all. In addition, clustering can provide evidence to support the cell lines chosen for the analysis, but the aim is, of course, to determine single miRNAs or miRNA clusters, with primary importance given to malignant melanoma development and progression. While investigating groups of cell lines derived from the same origin, we can minimize the effects of individual alterations specific for a single cell line.

In accordance with the results of Volinia *et al.* (2006), who found a large portion of miRNAs upregulated in the types of cancer they examined , the bulk of miRNAs we identified to be deregulated in melanoma cell lines compared with melanocytes was upregulated in the melanoma cell lines.

To narrow the list of miRNAs that could be promising targets for future research, we compared the miRNA expression profiles of melanoma cell clones used as model systems for some steps in melanoma progression with the miRNA profiles of NHEMs and melanoma cell lines. MIA-deficient melanoma cell clones were generated in our laboratory through stable transfection of HMB2 melanoma cells with an antisense-MIA cDNA expression plasmid (Poser *et al.*, 2004). There is a chain of evidence that MIA regulates the attachment/detachment of melanoma cells to the extracellular matrix (Stoll *et al.*, 2001; Bosserhoff *et al.*, 2003;

Bauer *et al.*, 2006), and thus promotes the migratory behavior of melanoma cells (Guba *et al.*, 2000; Bosserhoff *et al.*, 2001). MIA-deficient cell clones show re-established cell–cell contacts, a significantly reduced cell-diameter compared with parental HMB2 cells, a switch from multinuclear to mononuclear appearance of the cells, and re-established dendritic cell protrusions. A recovery of pigmentation was also observed (Tatzel *et al.*, 2005) and additionally relates the function of MIA to melanocytic differentiation and pigmentation. cDNA and protein array data displayed that MIA-deficient cell clones show high similarities in gene expression to melanocytic cells (for example, downregulation of melanoma-specific genes *MT1-MMP, t-PA, integrin β3, SPARC, fibronectin, L1 protein, protein kinase C-α*, and *MCAM*). The most important effect mediated by the knock-down of MIA is a switch from N-cadherin expression back to E-cadherin expression in the MIA-deficient HMB2 cell clones. This seems to revert the switch from E-cadherin to N-cadherin expression in malignant transformation from melanocytes to early melanoma cells, which subsequently lose growth control mediated by keratinocytes—a very early step in melanoma progression. We therefore think that using the HMB2/HMB2-MIA cell system is a powerful model for investigating molecular differences between ''melanocytes'' and early melanoma cells, although the MIA-deficient cell clones are closer to ''re-differentiated'' melanoma cells still containing some tumorigenic characteristics. In addition to examining the transcriptome of MIA-deficient cell clones, we also wanted to analyze the miRnome of those cells compared with that of melanocytes. The MIA-deficient HMB2 cell clones showed a miRNA profile very similar to that of NHEMs (about 70% of all miRNAs are equably regulated in NHEMs and HMB2-MIA), reflected in the fact that both cell lines clustered together when an unsupervised analysis was performed together with the melanoma cell lines. This shows that the HMB2-MIA cell clones, in addition to other melanocytic features, exhibit a miRnome similar to that of melanocytes. By comparing the lists of miRNAs deregulated in early progression and deregulated in HMB2-MIA, we were able to identify 18 equably regulated miRNAs, which were not associated earlier with melanoma or tumor development, in general, and which will now be analyzed bioinformatically and in functional tests.

Mel Im clones stably transfected with an as-Snail plasmid clustered together with NHEMs when compared with other Mel Im derivatives. Snail, a member of the Snail superfamily of zinc finger transcription factors, was shown to be involved in epithelial–mesenchymal transition. An important insight into the invasive potential and metastasis of tumor cells came from the discovery that increased motility and invasiveness of cancer cells are reminiscent of the epithelial–mesenchymal transition that occurs during embryonic development (Zhou *et al.*, 2004). Snail has been shown to convert otherwise normal epithelial cells into mesenchymal cells through the direct repression of E-cadherin expression. This phenomenon has also been described for malignant melanoma development by our group (Poser *et al.*, 2001). In contrast to melanocytes, Snail expression is induced in melanoma cells and represses E-cadherin expression. Lost E-cadherin expression subsequently leads to a loss of control of melanocyte proliferation mediated by keratinocytes and to acquisition of an invasive tumor phenotype. A cDNA-array comparison of paternal Mel Im cells with stable as-Snail clones showed changes in the expression levels of genes involved in epithelial–mesenchymal transition (downregulation of MMP-2, EMMPRIN, SPARC, TIMP-1, t-PA, RhoA, Notch4, N-cadherin, and re-induced expression of E-cadherin) (Kuphal *et al.*, 2005). As upregulation of Snail and subsequently induced downregulation of E-cadherin are indisputably an early event in melanoma formation, we think we are able to identify some miRNAs deregulated in the early progression of melanoma by comparing the miRnome of the as-Snail clones with the miRnomes of primary melanoma cell lines. We were able to identify only a small portion of miRNAs equably regulated in NHEMs and Mel Im as-Snail compared with primary melanoma cell lines, which is now subject to further characterization. There is also a high probability of identifying miRNAs whose expression is directly regulated by Snail comparing the miRNA expression patterns of as-Snail clones with the parental Mel Im cell line. Further studies on those miRNAs may also contribute to the gathering of more knowledge about the regulation of miRNA expression by identifying a cohort of miRNAs whose expression is regulated on a transcriptional level by binding of Snail to the promoter sequences of those miRNAs. Taken together, although miRNAs identified as differentially expressed after the knock down of Snail and MIA cannot be exclusively considered as miRNAs involved in melanoma formation and progression, performing comparisons, including these model systems, may identify miRNAs that seem promising for future analyses.

In addition, when comparing miRNAs differentially regulated in a highly invasive derivative of the Mel Im cell line (in comparison with a Mel Im derivative, which is only weakly invasive) with miRNAs deregulated in cell lines derived from metastatic melanomas, we found a small number of miRNAs equably regulated, which is very likely to be related to metastatic colonization.

Despite the growing knowledge about miRNAs, there are few examples of miRNAs with known functions and target genes in cancer. In our analyses, we found induced expression of miR-373 in primary tumor cell lines and then further upregulation (about two-fold) in metastatic melanoma cell lines. This miRNA was earlier shown to be involved in tumor invasion and metastasis by suppressing the oncogene-induced p53 pathway and cooperating with oncogenic RAS to promote cellular transformation (Voorhoeve *et al.*, 2006; Huang *et al.*, 2008). Although the role of p53 in malignant melanoma is not completely understood, several studies showed an upregulation of the p53 protein in melanoma progression rather than a downregulation (Li *et al.*, 2006), as the case would be if p53 were a target for miR-373 in melanoma cells. Hence, it seems more likely that there are additional targets for this miRNA in melanoma cells that are involved in the progression of the disease and have to be identified in subsequent studies. Another interesting fact about miR-373 is that the analysis of melanoma tissue

samples revealed that there are two sub-populations of melanoma primary tumors and melanoma metastases with regard to its expression level—one sub-population showing strongly induced miR-373 expression and the other sub-population showing almost no expression of miR-373.

Budhu *et al.* (2008) identified several miRNAs that are potentially involved in the metastasis of hepatocellular carcinoma. Comparing our miRNA cohort with their list, we found two miRNAs upregulated (miR-219 and miR-185) and one miRNA downregulated (miR-194) in malignant melanoma. It is worth mentioning that those miRNAs were already deregulated in primary tumor cell lines and then kept at the same level in metastatic melanoma cell lines.

The proven oncogenic miRNA gene clusters, miR-17-92 and miR-106-363, were also included in the large cohort of miRNAs that we found upregulated in malignant melanoma cell lines (He *et al.*, 2005; Landais *et al.*, 2007). All members of the miR-17-92 cluster, which promotes tumor progression by cooperating with MYC and blocking apoptosis (He *et al.*, 2005), were shown to be upregulated in primary tumor cell lines compared with NHEMs, although some members of the cluster were only about two-fold upregulated, and thus were outside our threshold of three-fold upregulation. The expression level of the miR-17-92 cluster is even higher in metastatic cell lines, with an approximately two-fold upregulation compared with primary melanoma cell lines. This shows that the miR-17-92 gene cluster, in addition to other types of cancers, is also involved in the progression of malignant melanoma.

The miRNA gene cluster miR-106-363 shares many similarities with the miR-17-92 cluster described above. We could detect strong upregulation of miR-106a and miR-92, as well as a slight upregulation of miR-19b expression in primary tumor cell lines, with a further increasing level (about two-fold) of those miRNAs in metastatic cell lines. The members of the miR-106-363 cluster are thought to regulate target genes that are involved in cell attachment, cell motility, cell contact inhibition, and also cell proliferation, like *Mylip* (myosin regulatory light chain-interacting protein) and *Rbp1-like* (retinoblastoma binding protein 1 like) (Landais *et al.*, 2007). The members of the RBP1 family cause a profound inhibition of cell proliferation and induced expression of a senescence marker when overexpressed in breast cancer cells (Binda *et al.*, 2006).

In summary, our array experiments revealed that several miRNAs, known to be deregulated in different types of human cancers, may also play important roles in malignant melanoma. Interestingly, the expression of those miRNAs of known importance in processes such as cellular transformation and metastasizing events was often found to be only slightly deregulated in our array experiments (often in the range of two-fold). The corpus of miRNAs that we had selected to be interesting for further examination was deregulated more strongly ($>10$-fold and regulated on the basis of a yes/no decision), implicating that there may be many miRNAs that might have even more dramatic effects on tumorigenesis and metastasis in malignant melanoma than do those miRNAs already described.

In addition, by performing an exemplary search for miRNAs potentially regulating a melanoma-specific gene (Rab38), we were able to give an ancillary application for our lists of miRNAs found to be deregulated in malignant melanoma. Although functional experiments are necessary to clarify whether Rab38 expression is regulated by a miRNA and (if this is the case) to identify the particular miRNA regulating the Rab38 expression, our data imply that the results obtained by our miRNA microarray analyses will likely facilitate the search for miRNAs involved in melanoma-specific gene regulation.

In summary, we were able to define large cohorts of miRNAs associated with the early and late progression of malignant melanoma potentially acting as oncogenes and tumor suppressor genes. It will be a challenging process to relate those miRNAs to their cellular function to unravel the impact of miRNAs in the formation and progression of malignant melanoma, and thus identify the most promising targets for future therapies of this fatal disease.

## MATERIALS AND METHODS

### Cell lines and cell culture conditions

Melanoma cell lines Mel Im (and its derivatives Mel Im hi and wi), Mel Wei, Mel Juso, Mel Ei, Mel Ju, HMB2, and A375, have been described in detail earlier (Jacob *et al.*, 1995, 1998). MIA-deficient HMB2 clone, HMB2-MIA5, and Mel Im cell lines stably transfected with an as-Snail construct were also described in detail earlier (Kuphal *et al.*, 2005; Tatzel *et al.*, 2005). For tissue culture, the cells were maintained in DMEM supplemented with penicillin (400 U ml$^{-1}$), streptomycin (50 μg ml$^{-1}$), L-glutamine (300 μg ml$^{-1}$), and 10% fetal calf serum (Sigma, Deisenhofen, Germany) and split 1:5 every 3 days.

Human primary melanocytes (PromoCell, Heidelberg, Germany) derived from normal skin were cultivated in a melanocyte growth medium, M2 (PromoCell), under a humified atmosphere of 5% $CO_2$ at 37 °C. Cells were used between passages 2–4 and not later than 2 days after trypsinization. For subcultivation or assay, cells were detached with 0.05% of trypsin, 0.04% of EDTA in phosphate-buffered saline. For miRNA microarray experiments, isolated total RNAs from two different charges of melanocytes (passage 2 in each case) were pooled.

### miRNA microarrays

miRNA microarrays were performed using Agilent's miRNA Microarray System (Agilent, Santa Clara, CA) according to the manufacturer's instructions. Briefly, total RNA was isolated from cell pellets using the one-step protocol of the mirVana miRNA Isolation Kit (Ambion, Austin, TX). For each cell line and replicate, 100 ng of total RNA were fluorescence-labeled with Cyanine 3-pCp, and hybridized to oligonucleotide arrays for 24 hours at 55 °C. After washing the microarrays in an ozone-depleted room, the array slides were scanned and analyzed using an Agilent DNA microarray scanner. Microarray images were processed using Feature Extraction Software 8.5 (Agilent) according to the miRNA-v1_95_May07 protocol. As commonly used normalization methods are not appropriate for miRNA microarrays, raw data were used for further analysis after checking the reproducibility between replicates.

Processed data were imported into Microsoft Office Excel 2003 for further analysis. Mean values of sets of cell lines were compared (for example, melanocytes versus primary tumor cell lines or primary tumor cell lines versus metastatic cell lines). miRNAs were considered differentially regulated if at least three-fold up- or downregulation was observed. miRNAs were excluded from further analysis if expressed in only one cell line of a set of cell lines. We also excluded values if the fluorescence signal in a sample was <20% of the mean fluorescence signal intensity in this sample. Additional criteria were applied in the comparison of some sets of cell lines and are referred to in the corresponding part of the Results section. Hierarchical clustering was performed using TIGR MultiExperiment Viewer Version 4.0 (http://www.tm4.org/mev.html).

### qRT-PCR on miRNAs derived from melanocytes, melanoma cell lines, and melanoma tissue samples

Total RNA was isolated from cultured cells and from snap-frozen melanoma tissue samples using the one-step protocol of a mirVana miRNA Isolation Kit (Ambion), as described in detail earlier (Muller and Bosserhoff, 2008).

miRNAs were reverse-transcribed using the RT reaction setup of a mirVana qRT-PCR miRNA Detection Kit (Ambion), and qRT-PCR of reverse-transcribed miRNAs was performed as described in detail earlier (Muller and Bosserhoff, 2008).

### Rab38 qRT-PCR on reverse-transcribed total RNA derived from melanocytes, melanoma cell lines, and melanoma tissue samples

Total RNA was isolated from cultured cells and from snap-frozen melanoma samples, as described above. For each sample, cDNA was generated using a SuperScript II Reverse Transcriptase Kit (Invitrogen, Groningen, The Netherlands).

qRT–PCR was performed on a Lightcycler (Roche, Mannheim, Germany). cDNA template (1 µl), 0.5 µl (20 mM) of forward (hRab38_fwd_97: 5′-AACTTCTCCTCGCACTACCG-3′) and reverse (hRab38_rev_314: 5′-TTCCACTTTGCCACTGCTTC-3′) primer, and 10 µl of Sybr Premix Ex Taq (TaKaRa, Shiga, Japan) in a total of 20 µl were applied to the following PCR program: 30 seconds at 95 °C (initial denaturation); a 20 °C per second temperature transition rate up to 95 °C for 10 seconds, 10 seconds at 61 °C, 20 seconds at 72 °C, 88 °C acquisition mode single, repeated 45 times (amplification). β-Actin was used for normalization.

### RAB38 western blot analysis with protein lysates derived from melanocytes, melanoma cell lines, and melanoma tissue samples

Western blot analysis was performed as described in detail before (Tatzel *et al.*, 2005), using the following antibodies: monoclonal anti-β-actin Clone AC-15 (A5441, Sigma) and anti-human RAB38 mAb Clone 7F1 (H00023682-M02, Abnova, Jhongli City, Taiwan).

### Statistical analysis

In the bar graphs, results are expressed as mean ± SD (range) or percent. Comparison between groups was made using Student's unpaired *t*-test. A *P*-value <0.05 was considered statistically significant. All calculations were made using the GraphPad Prism Software (GraphPad Software Inc., San Diego, CA).

### Statement of institutional approvals

The study was approved by the local ethics committee. The study was conducted according to the Declaration of Helsinki Principles.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

**Table S1.** Extended list of miRNAs up-/downregulated during early progression of malignant melanoma.

**Table S2.** Extended list of miRNAs up-/downregulated in MIA-deficient HMB2 cell clones (HMB2-MIA) compared with a parental HMB2 malignant melanoma cell line.

**Table S3.** Intersection of miRNAs deregulated during early malignant melanoma progression and deregulated in MIA-deficient HMB2 cell clones (HMB2-MIA) compared with a parental HMB2 malignant melanoma cell line

**Figure S1.** Hierarchical clustering of miRNA expression profiles derived from melanocytes and melanoma cell lines using TIGR MultiExperiment Viewer Version 4.0 (http://www.tm4.org/mev.html).

**Figure S2**. Hierarchical clustering of miRNA expression profiles derived from melanocytes and derivatives of malignant melanoma cell line Mel Im using TIGR MultiExperiment Viewer Version 4.0 (http://www.tm4.org/mev.html).

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