Identification of miR-145 as a Key Regulator of the Pigmentary Process

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The current treatments for hyperpigmentation are often associated with a lack of efficacy and adverse side effects. We hypothesized that microRNA (miRNA)-based treatments may offer an attractive alternative by specifically targeting key genes in melanogenesis. The aim of this study was to identify miRNAs interfering with the pigmentary process and to assess their functional role. miRNA profiling was performed on mouse melanocytes after three consecutive treatments involving forskolin and solar-simulated UV (ssUV) irradiation. Sixteen miRNAs were identified as differentially expressed in treated melan-a cells versus untreated cells. Remarkably, a 15-fold downregulation of miR-145 was detected. Overexpression or downregulation of miR-145 in melan-a cells revealed reduced or increased expression of *Sox9*, *Mitf*, *Tyr*, *Trp1*, *Myo5a*, *Rab27a*, and *Fscn1*, respectively. Moreover, a luciferase reporter assay demonstrated direct targeting of *Myo5a* by miR-145 in mouse and human melanocytes. Immunofluorescence tagging of melanosomes in miR-145-transfected human melanocytes displayed perinuclear accumulation of melanosomes with additional hypopigmentation of harvested cell pellets. In conclusion, this study has established an miRNA signature associated with forskolin and ssUV treatment. The significant down- or upregulation of major pigmentation genes, after modulating miR-145 expression, suggests a key role for miR-145 in regulating melanogenesis.

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INTRODUCTION

Skin pigmentation is a complex multistep process leading to the distribution of melanin particles throughout the different layers of the epidermis. This provides a photoprotective barrier against UVR as melanin acts as a UV absorbant and exerts antioxidative and radical scavenging activities (Brenner and Hearing, 2008; Yamaguchi and Hearing, 2009). Melanogenesis takes place in specific organelles, called melanosomes, which are produced in the melanocytes of the skin (Raposo and Marks, 2007). During their maturation, melanosomes are transported from the perinuclear region into the dendritic tips of melanocytes and are subsequently transferred to surrounding keratinocytes.

Several key proteins involved in melanogenesis and melanosome transport have been identified. Tyrosinase (TYR) is the rate-limiting enzyme of melanin synthesis and is induced after UVR as part of the tanning response. TYR cooperates with TYR-related protein 1 (TYRP1) and dopachrome tautomerase to synthesize the two main types of melanin: eumelanin and pheomelanin (Costin *et al.*, 2005). *TYR* defects, caused by mutations, are known to cause albinism in humans (Saxena and Verma, 2010). Another critical factor in melanin production is microphthalmia-associated transcription factor (*MITF*), which, besides regulating survival and proliferation of melanocytes, induces the expression of several pigmentation genes (e.g., *TYR* and *TYRP1*) (Vachtenheim and Borovansky, 2010; Levy and Fisher, 2011). The *MITF* promotor is regulated by various other transcription factors, including sex-determining region Y-box (*SOX9*) (Passeron *et al.*, 2007; Wan *et al.*, 2011). Mature melanosomes make short-range movements along actin filaments through the tripartite protein complex consisting of Rab27a-myosin Va-melanophilin. Mutations in any of these pigment transport genes lead to a dysfunction of the tripartite complex, causing accumulation of melanosomes in the perinuclear regions of these mutant melanocytes, because of disruption of their anchoring onto the cell periphery (Wu *et al.*, 2002; Westbroek *et al.*, 2003). Mutations in *myosin VA (MYO5A)*, *RAB27A*, and *melanophilin* (*MLPH*) are a cause of different forms of Griscelli syndrome in humans (GS type I, II, and III, respectively). These patients are characterized by mild hypopigmentation of the skin and hair (Van Gele *et al.*, 2009). A blockade of the intracellular transport of melanosomes can be mimicked *in vitro* by RNA interference-induced silencing of the exon

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Abbreviations: GS, Griscelli syndrome; miRNA, microRNA; MITF, *microphthalmia-associated transcription factor; PT, post-transfection; RT-qPCR, real-time quantitative PCR; siRNA, small interfering RNA; ssUV, solar-simulated UV; TYR, tyrosinase; TYRP1, tyrosinase-related protein 1; 3′UTR, 3′-untranslated region*

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F isoforms of *MYO5A* (Van Gele *et al.*, 2008). However, as more than 150 genes have been identified to affect pigmentation, sole inhibition of one particular gene might not be the most effective strategy to induce hypopigmentation *in vivo* (Bennett and Lamoreux, 2003; Chi *et al.*, 2006). Dysregulation of multiple genes involved in melanosome synthesis and transport, e.g., under the control of one or multiple microRNAs (miRNAs), might offer a more potent approach.

miRNAs are single-stranded noncoding RNA molecules that span over 18–24 nucleotides. They form a large family of regulatory molecules found in all multicellular organisms and are able to inhibit the expression of protein-coding genes. In humans, most miRNAs imperfectly base pair with the 3′-untranslated region (3′UTR) of target messenger RNAs (mRNAs) and inhibit subsequent protein synthesis by either repressing translation or promoting mRNA degradation. miRNAs have proven to be able to regulate multiple genes involved in the same molecular pathway. This enables a single miRNA to have critical roles in a variety of biological processes such as cell proliferation, differentiation, apoptosis, and carcinogenesis (He and Hannon, 2004; Iorio and Croce, 2009; Huang *et al.*, 2010; Tsang *et al.*, 2010; Gandellini *et al.*, 2011). In addition, miRNAs have been associated with different cellular stress responses such as oxidative stress, nutrient deprivation, DNA damage, and oncogenic stress (Leung and Sharp, 2007, 2010).

UVR is the major environmental inducing factor of the melanogenesis process upregulating a network of genes involved in the pigmentation process. Interestingly, plant research reveals that expression patterns of miRNAs change in response to UVB radiation (Zhou *et al.*, 2007; Jia *et al.*, 2009). In addition, irradiation of NIH3T3 cells with a low dose of UVB also results in robust changes of their miRNA expression pattern, in a time-dependent manner (Guo *et al.*, 2009). Identification of miRNAs regulated by UV irradiation, followed by the determination of their target genes and corresponding gene networks, will lead to more insights in the regulation of UV signaling pathways.

In this study, we explore the miRNA expression profiles of melan-a cells responding to the treatment of solar-simulated UV (ssUV) and forskolin. Sixteen differentially expressed miRNAs could be identified in treated melan-a cells versus untreated cells. One of these miRNAs, miR-145, was 15-fold downregulated. This led us to functionally characterize miR-145 and investigate its effect on the expression of genes influencing melanogenesis and melanosome transport in pigment cells.

RESULTS

Identification of miRNAs involved in the pigmentary process

To adequately mimic the physiological pigmentation process and to identify the miRNAs involved, it is necessary to establish a robust *in vitro* model system using ssUV and forskolin treatments on pigment cells. The responses are expected to stimulate the production, processing, and transport of melanosomes, which all have an essential role during melanogenesis. A melan-a mouse cell line (Palmisano *et al.*, 2008) representing normal functioning pigment-producing melanocytes was chosen. Compared with human primary melanocytes, this cell line is known to be significantly more responsive to physiological pigmentation factors, such as α-melanocyte-stimulating hormone and UVR (Lei *et al.*, 2002; Yoon and Hearing, 2003). In addition, by using melan-a cells, a less complex *in vitro* cell culture model was introduced, in contrast to human primary melanocytes that are typically characterized by donor-specific variability. The treatment protocol is schematically depicted in Figure 1a. Three treatments were performed on melan-a cells consisting of a low ssUV dose (60 mJ cm^{-2}) and addition of forskolin (20 μM), a known stimulator of the cAMP pathway. These treatments consequently induced pigmentation, whereby the phenotypic appearance of the cells could be monitored by bright-field microscopy. Increased proliferation of the treated cells with additional production of melanin, including an increased processing/movement of the melanosomes toward the periphery of the melanocytes and into the dendritic tips, was observed (data not shown).

To confirm these observations, quantification of mRNA expression levels of a number of key genes involved in the pigmentation process was determined by real-time quantitative PCR (RT-qPCR) (Figure 1b). A marked upregulation of the key pigmentation genes was observed, when comparing treated versus control samples. Upregulation was 2.2 (±SEM = 0.2)-fold for *Mitf*, 3.4 (±SEM = 0.2)-fold for *Sox9*, 1.7 (±SEM = 0.2)-fold for *Trp1*, and 3.3 (±SEM = 0.2)-fold

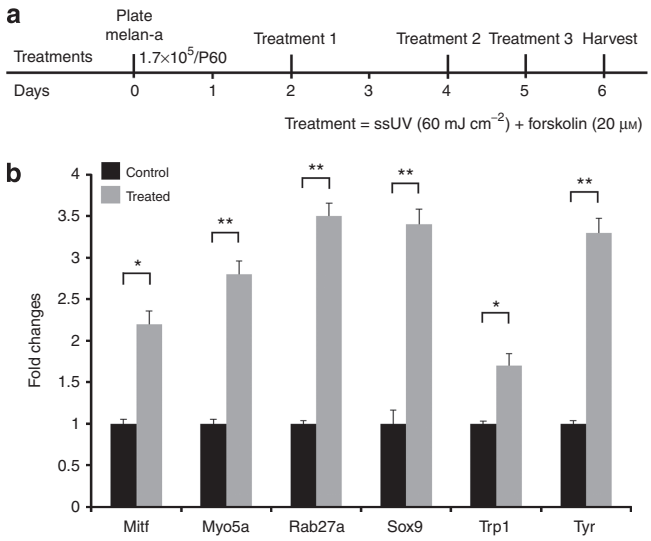


Figure 1. Experimental setup and induction of pigmentation genes at the messenger RNA (mRNA) level. (**a**) Schematic representation of the treatment protocol. (**b**) The mRNA expression levels of several pigmentation genes (*Mitf*, *Myo5a*, *Rab27a*, *Sox 9*, *Trp1*, and tyrosinase (*Tyr*)) were determined by real-time quantitative PCR 24 hours after the last treatment of melan-a cells. The graph shows normalized gene expression levels (fold changes) of all examined genes in the melan-a-treated cells (gray bars) versus nontreated cells (controls, black bars). The mRNA expression level of each gene was significantly increased after treatment. The mean (±SEM) of three independent experiments is shown. All values were compared with their control counterpart using an unpaired *t*-test. *P*-values are indicated with * (<0.05) or ** (<0.001). ssUV, solar-simulated UV.

for *Tyr*. Two genes, *Myo5a* and *Rab27a*, which, together with *Mlph,* form a tripartite complex, were upregulated by 2.8 (±SEM = 0.2)- and 3.5 (±SEM = 0.2)-fold, respectively. On the basis of these data, we conclude that a reliable and reproducible pigmentation assay was obtained.

Subsequently, the expression of 540 miRNAs for each triplicate control sample versus each triplicate treated sample was profiled. The cutoff value for considering a miRNA as deregulated was set at 1.5-fold (mean value) between treated and control samples. This filtering resulted in a miRNA signature for our pigmentation assay, identifying 16 differentially expressed miRNAs. The majority of these miRNAs were uniformly downregulated in treated samples compared with control counterparts. These include miR-125b, miR-139-5p, miR-145, miR-155, miR-193*, miR-206, miR-218, miR-221, miR-222, miR-28, miR-335*, miR-365, and miR-455. In contrast, three miRNAs were upregulated. These included miR-130b, miR-182, and miR-9. We decided to focus on miR-145, considering that treated samples displayed on average a 15-fold downregulation in comparison with their controls (Supplementary Table S1 online).

Modulation of miR-145 expression influences key pigmentation genes

To assess the role of miR-145 in the pigmentation process, overexpression with the aid of pre-miRs (mimics) has been performed in melan-a cells. Three consecutive post-transfection (PT) time points (24, 48, and 72 hours) were monitored. Overexpression of miR-145 was confirmed by RT-qPCR (Figure 2a). In addition, the expression levels of several genes involved during the pigmentation process were also determined. These genes have been subdivided into genes involved in the onset of the pigmentary process (*Sox 9*, *Mitf*, *Tyr*, and *Trp1*), and genes involved in the correct processing and transfer of melanosomes to the periphery (*Fscn1*, *Myo5a*, and *Rab27a*). When observing genes involved in the initiation of melanogenesis, a knockdown effect was observed 72 hours PT. *Sox9* showed a 70% (±SEM = 4), *Mitf* a 60% (±SEM = 3%), *Tyr* a 63% (±SEM = 7%), and *Trp1* a 50% (±SEM = 6%) knockdown of their gene expressions (Figure 2b). With regard to genes involved in processes situated at the periphery of the melanocytes, a sharp decline in *Fscn1* (84% (±SEM = 15%)) as well as *Myo5a* (62% (±SEM = 7%)) gene expressions was detected 24 hours PT. This knockdown persisted after 72 hours, with *Fscn1* showing a 67% decline (±SEM = 7%) and *Myo5a* an 81% (±SEM = 2%) decline. *Rab27a* showed a similar expression pattern but evolved in a time-dependent manner, finally resulting in a 51% (±SEM = 3%) knockdown after 72 hours (Figure 2c).

The effect of miR-145 overexpression on the protein levels of several pigmentation genes was investigated by western blotting. The experiments were performed in triplicate by transfecting pre-miR-145 in melan-a cells compared with scrambled negative controls. Protein samples were collected 72 hours PT. Western blot analysis correlated with the qPCR data, demonstrating knockdown of protein levels of Myo5a by 61% (±SEM = 4%), Rab27a by 25% (±SEM = 1%), Tyr by 65% (±SEM = 5%), and Fscn1 by 20% (±SEM = 4%) (Figure 2d). In addition, downregulation of miR-145 using an anti-miR resulted in upregulation of *Sox9* (41% (±SEM = 1%)), *Mitf* (60% (±SEM = 22%)), *Tyr* (143% (±SEM = 13%)), *Fscn1* (87% (±SEM = 14%)), *Myo5a* (78% (±SEM = 14%)), and *Rab27a* (57% (±SEM = 8%)) gene expression levels, 48 hours PT (Supplementary Figure S1 online). These data provide evidence that miR-145 is a physiological regulator of *Myo5a* and other genes involved in the pigmentation process.

miR-145 targets a binding site of *Myosin Va*

To identify possible relevant target genes of miR-145, we initially used the online miRNA target prediction software, TargetScan (Lewis *et al.*, 2005). This target prediction database generated a list of candidate transcripts with putative miR-145-binding sites. We focused on target genes involved during the pigmentary process/melanogenesis. Myosin Va was identified as a potentially interesting target. For the mouse, TargetScan database lists two proximal binding regions on the 3′UTR. The first predicted target site for miR-145 pairing on *Myo5a* is located at position 123–130 and is a highly conserved 8-mer binding site. The second is located at position 456–462 of *Myo5a* 3′UTR and contains a potential 7-mer binding site for the mature miR-145. To address *Myo5a* as a direct target of miR-145, a luciferase target reporter assay was designed for the two potential *Myo5a*-binding sites located at the proximal region of 3′UTR. The luciferase reporter assay for mouse *Myo5a* confirmed an actual target site for miR-145. Luciferase activity was significantly decreased (32% decrease, ±4% SEM) at one of the two predicted sites (positions 123–130) (Figure 3a). This effect was not observed when the putative miR-145-binding region was replaced with a scrambled sequence (rescue experiment). The sequences of the constructs used during the luciferase reporter assays described above are shown in Supplementary Table S2 online. The second predicted binding site for *Myo5a* did not affect luciferase activity of the reporter assay (data not shown).

Interestingly, human *myosin VA* contains three putative miRNA-binding sites for hsa-miR145, whereby the first one is highly conserved between mammals. To investigate whether miR-145 directly binds to the 3′UTR of human *MYO5A*, we created a wild-type 3′UTR *MYO5A* luciferase reporter construct containing all three putative miRNA-binding sites. Reporter constructs containing single-site mutations for each individual binding sites of miR-145 (Mut1, Mut2, or Mut3) were also engineered by introducing 4-bp mutations on each of the three miRNA seed regions separately located on the 3′UTR of *MYO5A* (Supplementary Figure S2 online). Each construct was used in a luciferase reporter assay in combination with either a pre-miR-145 or a validated pre-miR negative control. The pre-miR-145 significantly reduced the luciferase activities of the wild-type *MYO5A* by 34% (±7% SEM), compared with the negative control (Figure 3b). In addition, the reporter construct (Mut1) containing mutations in the seed region of binding site 1 (position 129–136 of MYO5A 3′UTR) rescued the *MYO5A* reporter repression,

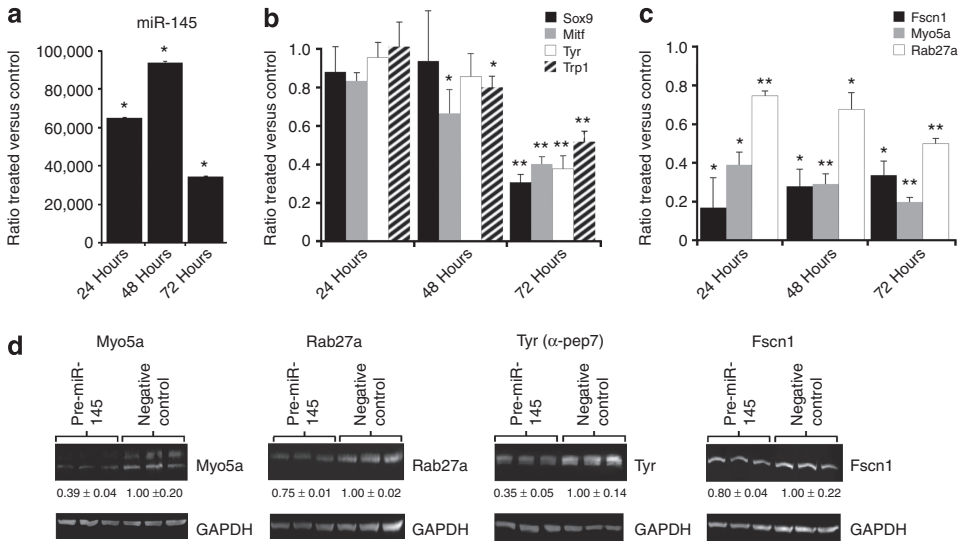


Figure 2. Effect of miR-145 overexpression on the expression of genes involved in the pigmentary process. Melan-a cells were transfected with 50 nM pre-miR-145 or negative controls. The relative expression levels of miR-145 and several pigmentation genes were determined by real-time quantitative PCR. The mean ratio of the treated samples versus the mean ratio of negative controls was plotted for miR-145 and each analyzed gene at different time points. A clear overexpression of miR-145 was observed (**a**). A time-dependent decrease in gene expression levels (ratio <1) was observed for genes involved at the onset of pigmentation (*Sox9*, *Mitf*, tyrosinase (*Tyr*), and *Trp1*) (**b**) and for genes involved in the transport and transfer of melanosomes to the cell periphery (*Fscn1*, *Myo5a*, and *Rab27a*) (**c**). The ratio (treated vs. control) is shown as the mean (± SEM) of three independent experiments. *P*-values were determined with an unpaired *t*-test and are indicated with * (<0.05) or ** (<0.001) (**d**). Western blots and densitometry values ($n=3$, ± SEM) demonstrating downregulation of Myo5a, Rab27a, Tyr, and Fscn1 of pre-miR-145-transfected cells compared with negative controls, 72 hours post-transfection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.

indicating that the first binding site contains an active binding region for miR-145 on *MYO5A*. Mutant reporters for binding site 2 or 3 did not result in a significant rescue of the *MYO5A* reporter repression (data not shown).

Overexpression of miR-145 inhibits melanosome transport and induces depigmentation

To study the effect of miR-145 in melan-a cells, immunofluorescent staining using an antibody against melanosomes (HMB45) and Myo5a was performed. The cells were fixed 5 days PT and fluorescence images were taken. A clear image of perinuclear accumulation of melanosomes was observed in melan-a cells after transfection with miR-145, in contrast to the distribution of melanosomes at the dendritic tips in non-transfected melanocytes (Figure 4a, left panel). Phalloidin staining showed an intact actin cytoskeleton (Figure 4a, right panel).

As influencing pigmentation in human melanocytes or skin, by modulating the expression of miR-145, would be of potential high interest as a dermatological or therapeutical application, we decided to investigate the role of miR-145 in human primary melanocytes. Overexpression of miR-145 in human primary melanocytes (Supplementary Figure 3Sa online) resulted in a time-dependent decrease of the same pigmentation genes studied in melan-a cells (Supplementary Figure 3Sb and c online). For protein analysis, we focused on MYO5A and were able to show that the protein level of MYO5A (58% decrease, ± 3% SEM) was also decreased in pre-miR-145-transfected human pigment cells compared with control cells (Supplementary Figure 3Sd online).

Interestingly, immunostaining of the melanosomes in transfected human melanocytes showed retention of the melanosomes around the nucleus (Figure 4b), similarly seen in melan-a-transfected cells. In addition, equal amount of cell pellets harvested from pre-miR-145-transfected human melanocytes displayed a hypopigmentation effect (reduction of 54%) compared with their control counterparts. This lightening effect increased in a time-dependent manner and started 4 to 5 days PT (Figure 4c).

By performing a successful *in vitro* rescue experiment (cotransfection of anti-miR-145 and small interfering RNA (siRNA) against MYO5A versus siMYO5A, followed by immunohistochemical staining of fixed cells), we were able to provide compelling evidence, next to our luciferase reporter assay, that MYO5A is a target of miR-145 in human melanocytes. The perinuclear effect caused by siMYO5A was restored to wild type after cotransfecton with an anti-miR-145, owing to an enhanced endogenous expression of MYO5A (Supplementary Figure S4 online).

DISCUSSION

The mechanisms by which specific miRNAs may transcriptionally regulate the pigmentation pathway have remained largely elusive. To our knowledge, only two miRNAs have been described to be involved in the pigmentation process. Wu *et al.* (2008) designed a miR-434-5p-targeting TYR in human and mouse melanocytes, and Zhu *et al.* (2010) considered miR-25-targeting MITF as a regulator of pigmentation in Alpaca melanocytes. In this study, a novel miRNA signature associated with ssUV and forskolin has been

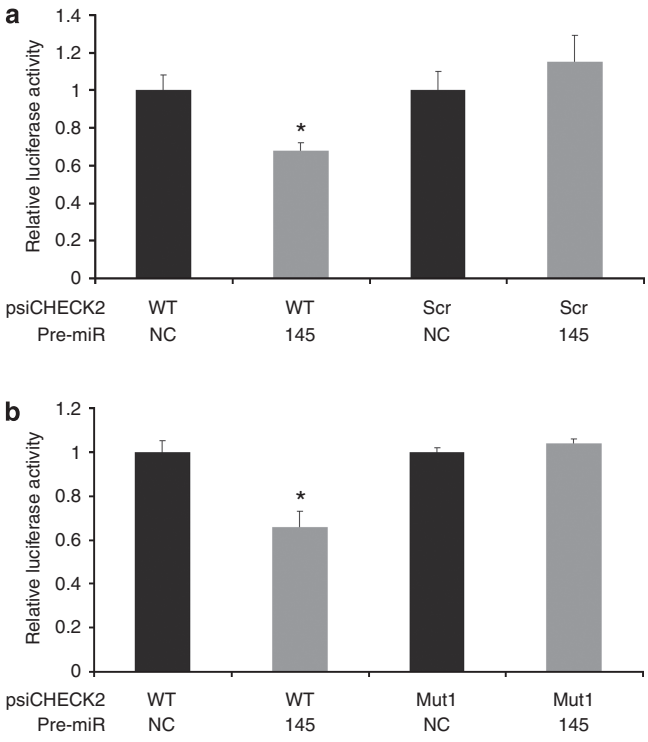


Figure 3. miR-145 regulates *Myo5a* expression by targeting the 3′-untranslated region (3′UTR) of *Myo5a* messenger RNA. (**a**) Relative 3′-UTR luciferase reporter activity for murine *Myo5a*. The luciferase activity of *Myo5a* is significantly decreased in the presence of the miR-145 and the reporter construct containing the first predicted binding site of the *Myo5a* 3′UTR (wild-type (WT) experiment) as compared with transfection with a negative control (NC) pre-miR. The reporter gene activity is rescued when using a scrambled miRNA-binding site (Scr). *$P<0.05$. (**b**) Relative 3′-UTR luciferase reporter activity for human *MYO5A*. The luciferase activity of the MYO5A WT construct (containing the three putative binding sites for miR-145 on the 3′UTR of human *MYO5A*) decreases significantly in the presence of miR-145. Mutagenesis of the first seed region (Mut1) resulted in a rescue effect, indicating that seed 1 is the active binding site for miR-145. Mean levels ($n=3$, ± SEM) of normalized luminescence values in NC and pre-miR-145-transfected cells are depicted. *P*-values were determined by unpaired *t*-test. *$P<0.01$.

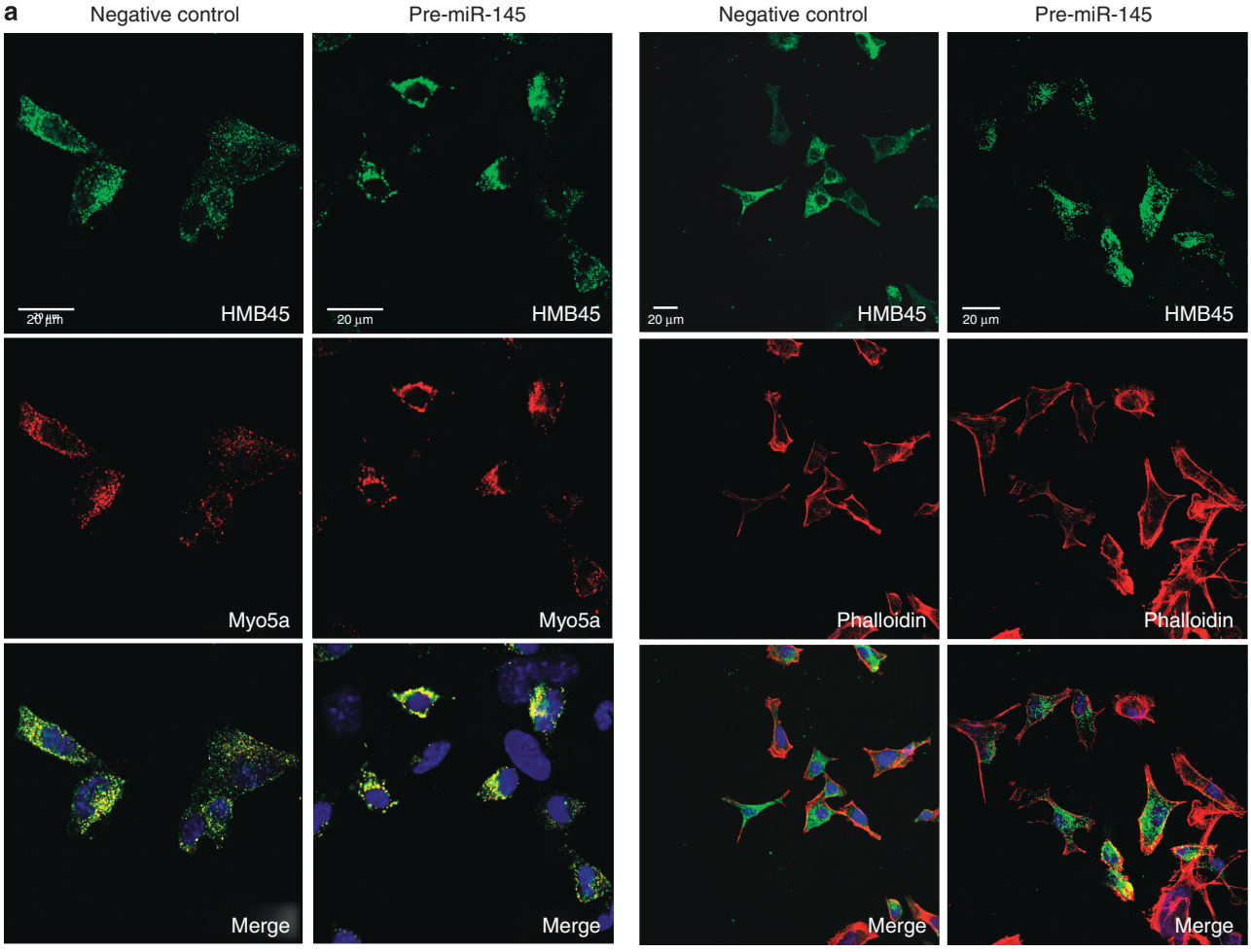
defined consisting of 16 differentially expressed miRNAs potentially involved in the pigmentation process. Genetic evidence that specific miRNA genes add an additional layer of regulation during the pigmentation process is provided. In particular, we focused on miR-145, which showed a marked 15-fold downregulation in mouse melanocytes treated with ssUV and forskolin. Overexpression of miR-145 reduces the expression of pigmentation genes (*Tyr*, *Myo5a*, *Rab27a*, and *Fscn1*), and this correlates with decreased protein levels of Tyr, Myo5a, Rab27a, and Fscn1 in melan-a cells. On the other hand, downregulation of miR-145 resulted in an increased gene expression level of these pigmentation genes, adding extra weight to the importance of miR-145 during the pigmentation process.

Prediction data from TargetScan database for mouse showed that miR-145 could target *Myo5a* on two potential binding sites located at the proximal region of the 3′UTR of *Myo5a,* which are separated by 333 nucleotides. In this study, we were able to demonstrate that miR-145 post-transcriptionally represses the expression of murine *Myo5a* by direct binding to the first of its 3′-UTR-binding sites, whereas the other demonstrated no significant binding. Moreover, we additionally confirmed the direct binding of miR-145 to the 3′UTR of human *MYO5A* by luciferase reporter assays. Parallel to the mouse, we were able to show that the first binding site of *MYO5A* mediates the interaction between miR-145 and *MYOVA* 3′UTR. Several research groups, including ours, showed that Myo5a forms a tripartite complex together with Rab27a and Mlph, responsible for transporting melanosomes along actin filaments (Hume *et al.*, 2002; Wu *et al.*, 2002; Westbroek *et al.*, 2003). Disruption of this tripartite complex, by loss or reduced expression of one of the three members, results in a redistribution of melanosomes from the peripheral to the perinuclear region. This phenomenon is also observed in the melanocytes of GS patients, which are characterized by hypopigmentation of the skin and hair (Van Gele *et al.*, 2009). Interestingly, we observed a similar biological effect by introducing miR-145 into mouse melanocytes. This additionally confirms the direct binding of miR-145 to *Myo5a* in mouse melanocytes. The fact that the protein expression level of Rab27a was also decreased may indicate that the overall stability of the Rab27a–Mlph–Myo5a protein tripartite complex is affected. Previously, similar effects have been observed after knocking down *MYO5A* in human primary melanocytes by use of siRNA (Van Gele *et al.*, 2008). In this study, we also overexpressed miR-145 in primary human melanocytes and found that the same set of genes, as studied in mouse pigment cells, were also decreased. In addition, overexpression of miR-145 resulted in a clear accumulation/retention of melanosomes around the nucleus, and we were able to distinguish a clear hypopigmentation effect on cell pellets from miR-145-transfected melanocytes compared with non-treated controls. The observed hypopigmentation effect is comparable to silencing of *TYR* and appears not solely to be caused by the downregulation of MYO5A by miR-145, followed by aberrant melanosome transport, but has an additive effect by regulating several other key melanogenic genes during the pigmentary process.

Here we found a downregulation of mRNA expression level of *Fscn1*; however, this was less clear at the protein level. To achieve efficient melanosome transport and transfer, an intact subcortical actin network is mandatory. Fscn1 is a key actin cross-linker, providing stiffness to filopodial bundles (Vignjevic *et al.*, 2003, 2006). Malfunction might hinder effective melanosome capturing in melanocytic dendrites, even though in miR-145-overexpressing melanocytes the actin structure seemed intact with the resolution we used. Future research is needed to establish the exact role of FSCN1 in the observed decreased pigmentation after miR-145 transfection.

Besides targeting genes located at the periphery of the melanocyte that are responsible for correct transfer of melanosomes, our results also indicate that miR-145 interferes with transcription factors responsible for the initiation of

melanogenesis. Interestingly, it has been proposed that co-regulation of miRNAs and transcription factors is of particular importance in pigment pathways (Chen *et al.*, 2011). SOX (SRY-type high-mobility group box) proteins are transcription factors that belong to the high-mobility group box superfamily of DNA-binding proteins and have a key role during melanocyte development (Harris *et al.*, 2010). SOX9 belongs to the SOX-E subgroup, which includes SOX8, SOX9, and



SOX10. The structures of these proteins show a high conservation and similar positions of their high-mobility group boxes. Passeron *et al.* (2007) identified cAMP-induced upregulation of SOX9 as a main factor in the differentiation and pigmentation of melanocytes after UVB exposure. SOX9 induces melanogenesis by regulating MITF, dopachrome tautomerase, and TYR promoters. By binding directly to the 3′-UTR region of SOX9, miR-145 may directly regulate the influence of SOX9 on these pigmentary processes. Direct binding of miR-145 to SOX9 was recently demonstrated for mouse mesenchymal stem cells (Yang *et al.*, 2011). Future research will be necessary to address this in pigment cells. Computational analysis by TargetScan also predicts three potential binding regions for SOX10. SOX10 has an essential role in pigment cell development and function. A number of miRNAs potentially bind to the 3′UTR of SOX10. They include miR-155, miR-221, and miR-222. Our miRNA profiling results support the notion that these miRNAs are downregulated during the experimental pigmentation setup, thus enabling SOX10 expression.

The identification of key downstream targets of miR-145 is crucial for understanding the molecular basis of its role in pigmentation. To obtain a more complete overview of the key downstream targets of miR-145, a whole genome–wide gene expression analysis (microarray) should be performed on samples that have been overexpressed or knocked down for this specific miRNA. This could reveal additional regulatory elements involved throughout the pigmentation pathway. Compared with single-target knockdown achieved by siRNAs, miRNAs could provide significant advantages for therapeutic intervention by regulating multiple genes in a single pathway (Seto, 2010). The miRNA network is well coordinated by multiple intracellular regulatory systems, and is considered to be effective, specific, and safe. In contrast to siRNAs, miRNAs are naturally occurring regulatory components that are inherent to the melanocyte machinery. By targeting several genes in a more moderate way compared with siRNAs, miRNAs may enhance the efficacy while decreasing side effects resulting from aggressive single-gene knockdown. As a result, miRNAs may be more suited for therapeutic intervention. However, owing to their pleiotropic effect, a careful mapping of the target genes of the considered therapeutic miRNA is necessary to avoid dysregulation of other pathways resulting in adverse effects.

In conclusion, this study has established, to our knowledge, a previously unreported miRNA signature (16 miRNAs) associated with ssUV and forskolin treatment. Significant down- and upregulation of Myo5a expression, after modulation of miR-145, is provided. In addition, miR-145 has the ability to regulate other major pigmentation genes, suggesting a key role for miR-145 in the transport of melanosomes and the regulation of melanogenesis. We conclude that miR-145 could possibly be a potential therapeutic target in the treatment of skin pigmentation disorders.

MATERIALS AND METHODS

Cell culture and treatment of cells with ssUV and forskolin

Murine melan-a melanocytes (from black mice) were kindly provided by Dorothy Bennett (St George Hospital Medical School, London, UK) and cultured in RPMI 1640 medium (Life Technologies Europe B.V., Ghent, Belgium) supplemented with 10% fetal calf serum, 2 mM L-glutamine, streptomycin (50 $\mu g\,ml^{-1}$), penicillin (50 $U\,ml^{-1}$), and 200 nM 12-*O*-tetradecanoylphorbol-13-acetate. Before initiating and during the experimental setup, 12-*O*-tetradecanoylphorbol-13-acetate was removed from the culture medium. After 48 hours, melan-a cells seeded at a density of 170,000 cells per 60 mm (P60) dish were irradiated with ssUV, followed by addition of 20 μM forskolin, following a time-dependent treatment protocol. Shortly before irradiation, the melan-a medium was removed and replaced by a thin layer of phosphate-buffered saline. After removing the plastic cover of the Petri dish, irradiation was administered using an Oriel solar simulator (Model 91294-1000, Newport, Stratford, CT). Using a UVB probe (SED 240), a dosage of 60 $mJ\,cm^{-2}$ was measured with a research radiometer (IL1700; International Light, Newburyport, MA). After irradiation, phosphate-buffered saline was replaced by fresh RPMI 1640 medium containing 20 μM forskolin on the UV-treated cells. Control melan-a cells were treated identically without the irradiation and addition of forskolin. The time-dependent treatment protocol was initiated 2 days after seeding, applying the first treatment, followed by a second treatment 48 hours later, and a third and final treatment after 24 hours. The cells were finally harvested for miRNA extraction 24 hours after the last treatment. The cells were incubated at a temperature of 37 °C, 99% humidity, and 10% CO_2. Human primary epidermal melanocyte cultures were established as described previously (Naeyaert *et al.*, 1991; Van Gele *et al.*, 2008).

RNA isolation and RT-qPCR

Total RNA, including miRNAs, was extracted from melan-a cells using the miRNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's recommendations. A DNase treatment was performed and first-strand cDNA was generated by reverse transcription using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad, Eke, Belgium). Relative gene expression levels were determined using a SYBR Green I reverse transcription-PCR assay as described by Vandesompele *et al.* (2002a), and the comparative Cq method was used for quantification.

Figure 4. Overexpression of miR-145 induces perinuclear aggregation of melanosomes and reduced pigmentation. The melanosomal marker HMB45 (green) and Myo5a (red) colocalize and accumulate around the nucleus of pre-miR-145-transfected melan-a cells, whereas both markers show a peripheral staining in control-transfected cells (**a**, left panel). In addition, fixed melan-a cells were stained with HMB45 and phalloidin. A normal melanosome distribution is observed in control cells compared with a perinuclear accumulation in the pre-miR-145-transfected melan-a cells (**a**, right panel). The actin cytoskeleton is intact. Nuclei were counterstained in blue with 46-diamidino-2-phenyl indole. (**b**) Overexpression of miR-145 in human primary melanocytes results in a perinuclear accumulation of melanosomes (stained with the melanosomal marker NKI-beteb), whereas a normal melanosomal distribution is present in the control-transfected cells. Bar = 20 μm. (**c**) Harvested pellets of pre-miR-145-transfected primary human melanocytes displaying a hypopigmented phenotype comparable to small interfering RNA (siRNA) tyrosinase–transfected melanocytes. Intensities of the pellets were measured with Fiji software, and the mean values of the two controls were rescaled to 1 and compared against the treated conditions ($n = 3$ measurements ± SEM).

PCR reactions were performed using SYBR Green I master mix (Eurogentec, Ougrée Seraing, Belgium) and were run on a MyiQ iCycler (Bio-Rad). To correct for differences in RNA quantities and cDNA synthesis efficiency, relative gene expression levels were normalized using the geometric mean of three reference genes (*RPL13A*, *UBC*, and *SDHA*) according to Vandesompele *et al.* (2002b).

miRNA profiling

For miRNA cDNA synthesis, RNA was reverse transcribed using the miRNA reverse transcription kit in combination with the murine stem-loop Megaplex primer pools A and B (Life Technologies Europe B.V.), allowing simultaneous reverse transcription of a total of 540 miRNAs and endogenous controls. Briefly, 10 ng of total RNA was supplemented with RT primer mix (10 ×), RT buffer (10 ×), MultiScribe Reverse Transcriptase (50 U μl^{-1}), dNTPs with dTTP (100 mM each), $MgCl_2$ (25 mM), and RNase inhibitor (20 U μl^{-1}) in a total reaction volume of 8 µl. To increase reverse transcription efficiency, a pulsed RT reaction was used (40 cycles of 16 °C for 2 minutes, 42 °C for 1 minute, and 50 °C for 1 second, followed by a final reverse transcriptase inactivation at 85 °C for 5 minutes).

For each cDNA sample, a total of 540 miRNAs and endogenous controls were profiled using a gene maximization PCR plate setup in a 384-well plate. The RT product was diluted 400-fold. PCR amplification reactions were carried out in a total volume of 8 µl, containing 4 µl of TaqMan Master mix, 1 µl of cDNA, and 3 µl of miRNA TaqMan probe and primers (Life Technologies Europe B.V.). Cycling conditions were as follows: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. All PCR reactions were performed on the 7900HT RT-qPCR system (Applied Biosystems, Halle, Belgium). Raw Cq values were calculated using the SDS software v.2.1 (Applied Biosystems) using automatic baseline settings and a threshold of 0.2. miRNA expression data were normalized using the global mean (Mestdagh *et al.*, 2009).

Luciferase reporter assay for murine myosin Va

Dicer-deficient (DLD-1 $Dicer^{ex5}$) cells were seeded in DMEM (Life Technologies Europe B.V.), supplemented with fetal calf serum (10%) at a density of 10,000 cells per well in an opaque 96-well plate (Cummins *et al.*, 2006). Twenty-four hours after seeding, using DharmaFECT Duo (Dharmacon, Thermo Scientific, Erembodegem, Belgium), cells were transfected with 100 ng of a psiCHECK-2 reporter construct (Promega Benelux B.V., Leiden, The Netherlands), either alone or together with a miR-145 mimic or a negative control miRNA (Life Technologies Europe B.V.). Reporter constructs contained one of the two miRNA-binding sites present in the 3′UTR of Myo5a cloned downstream of the Renilla luciferase gene (see also Supplementary Table S2 online). Forty-eight hours after transfection, luciferase assay reporter gene activity was measured using the Dual-Luciferase Reporter assay system (Promega Benelux B.V.) and a FLUOstar OPTIMA microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). Renilla luciferase activities were normalized against Firefly luciferase activities.

Additional information regarding the luciferase assay for human myosin VA, pre-miR/anti-miR transfections, miRNA quantification, western blotting, immunohistochemistry, and quantification of cell pellets is available in the Supplementary Materials and Methods online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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