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miR-330-5p Targets Tyrosinase and Induces Depigmentation

Journal of Investigative Dermatology (2014) **134,** 2846–2849; doi:10.1038/jid.2014.231; published online 26 June 2014

TO THE EDITOR

There is increasing evidence that microRNAs (miRNAs), small noncoding RNAs, are involved in regulating melanogenesis. Various proteins, including TYR, DCT, MELANA, and TYRP1, whose mRNAs are potentially targeted by miRNAs orchestrate this process. miRNAs regulate gene expression: in general, they inhibit protein synthesis either by repressing translation or by destabilizing/degrading mRNAs by imperfect base pairing to the ''seed match'' region in the 5′UTR, CDS, or 3′UTR of the mRNA

Abbreviations: MITF, microphthalmia-associated transcription factor; mRNA, microRNA; NHEM, normal human epidermal melanocyte

Accepted article preview online 26 May 2014; published online 26 June 2014

(Fabian *et al.*, 2010). To date, 1,872 precursor miRNAs have been identified in humans, giving rise to 2,578 mature miRNAs (mirbase release 20). Melanogenesis, stimulated by UVR, includes synthesis of melanin, transport of melanosomes, and transfer to surrounding keratinocytes. UVR downregulates miR-145 in immortalized murine melanocytes. Overexpression of miR-145 seems to target myosin-5a and interferes with melanosome transport (Dynoodt *et al.*, 2013). Microtubule-based transport of melanosomes appears to be negatively regulated by hsa-miR-203 targeting KIF5B in melanoma cells and melanocytes (Noguchi *et al.*, 2014). Moreover, hsa-miR-125b targets TYR and DCT, thereby affecting melanin levels in a pigmented human melanoma cell line and in darkly pigmented melanocytes in the absence of external stimuli (Kim *et al.*, 2014). Wu *et al.* (2008) claimed that anti-tyrosinase miRNA expression system, which is based on Pol-II-directed intronic mmu-miR-434-5p overexpression, mediates skin whitening *in vitro* and *in vivo*. Injection of miR-675-3p mimics into the skin of C57BL/6 J mice reduces melanogenesis *in vivo*, mainly by targeting microphthalmia-associated transcription factor (MITF). Interestingly, miR-675 was detected in media from keratinocyte cultures, probably released from exosomes (Kim *et al.*, 2013). These observations identify miRNAs as promising tools for treatment of pigmentation disorders as reviewed elsewhere (Yaar, 2013). However, some pigmentation genes are more suitable targets than others for miRNA-based strategies. For example, MITF-M controls the expression of various genes involved in melanogenesis, and its expression is essential to melanocytes (Goding, 2000; Levy *et al.*, 2006). Abolishing MITF-M expression leads to an irreversible loss of melanocytes

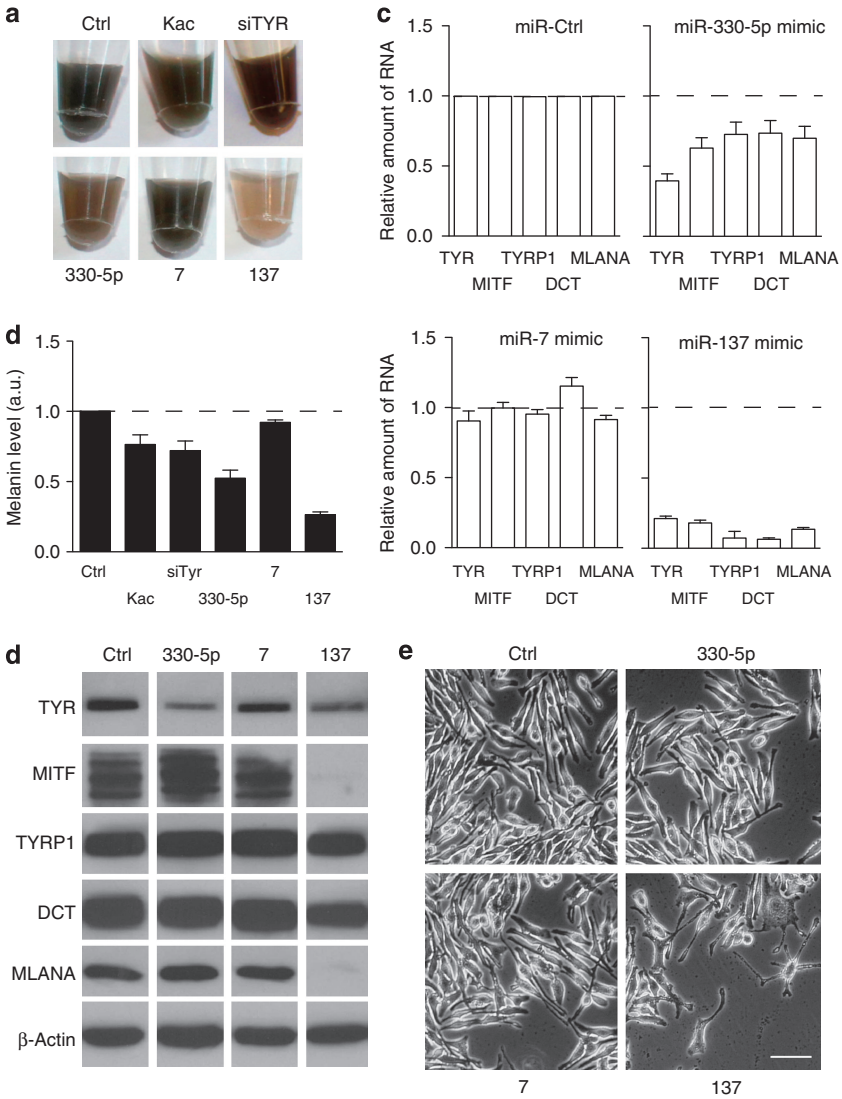

Figure 1. Depigmenting effects of miR-330-5 and miR-137 in MNT-1 cells. (**a**) Highly pigmented human melanoma cells (MNT-1) were treated with miRNA mimics for 12 days. (**b**) Melanin levels after 12 days of treatment were quantified by spectrometry. (**c**) mRNA expression levels of pigmentation genes were assessed by quantitative real-time reverse-transcriptase–PCR and are reported relative to the values for control mimic (miR-Ctrl)–treated MNT-1 cells. (**d**) Western blot analysis. (**e**) Morphological changes were observed after treatment of MNT-1 cells with miR-137 mimics. Scale bar = 50 μm (see Supplementary Information online for additional information). a.u., arbitrary units; MITF, microphthalmia-associated transcription factor.

(Steingrimsson *et al.*, 2004) and hence should be avoided.

We decided to identify miRNAs that may act on pigmentation levels without affecting the survival/proliferation of melanocytes (Supplementary Figure S1 online). As a miRNA target, we chose tyrosinase (TYR): it is the key and rate-limiting enzyme for production of melanin by hydroxylation of a monophenol and conversion of an o-diphenol to the corresponding o-quinone, a precursor of melanin. Impaired tyrosinase production leads to type I oculocutaneous albinism, a complete or partial absence of pigment in the skin, hair, and eyes, underlining the importance of TYR in the pigmentation process. We used *in silico* prediction (mirDIP) to identify candidate miRNAs targeting TYR but not MITF in humans. The four best miRNAs, candidates for TYR, based on their standardized score were miR-450b-5p, miR-1208, miR-326, and miR-330-5p (Supplementary Table S1 online). However, miR-450-5p, miR-1208, and miR-326 were also predicted to target MITF and were therefore excluded (Supplementary Figure S2 online). By contrast, miR-330-5p was an attractive candidate, predicted to target TYR and also MLANA (with less confidence) but not MITF. It has been already shown that miR-330-5p could affect E2F1 and SP1 in prostate cancer cells (Lee *et al.*, 2009; Mao *et al.*, 2013). In melanoma cells, E2F1 and SP1 are not modified, as shown by quantitative real-time reverse-transcriptase–PCR (Supplementary Figure S3 online). We tested whether miR-330-5p did indeed target TYR and act on melanin levels: we overexpressed miR-330-5p using mimics in highly pigmented human melanoma cells (MNT-1) and normal melanocytes for a period of 12 days. As controls, we used classic depigmenting agent Kojic acid (Deo *et al.*, 2013), small interfering RNA against TYR, and miR-137/miR-7 mimics. Prediction score for miR-7 acting on MITF is low, whereas miR-

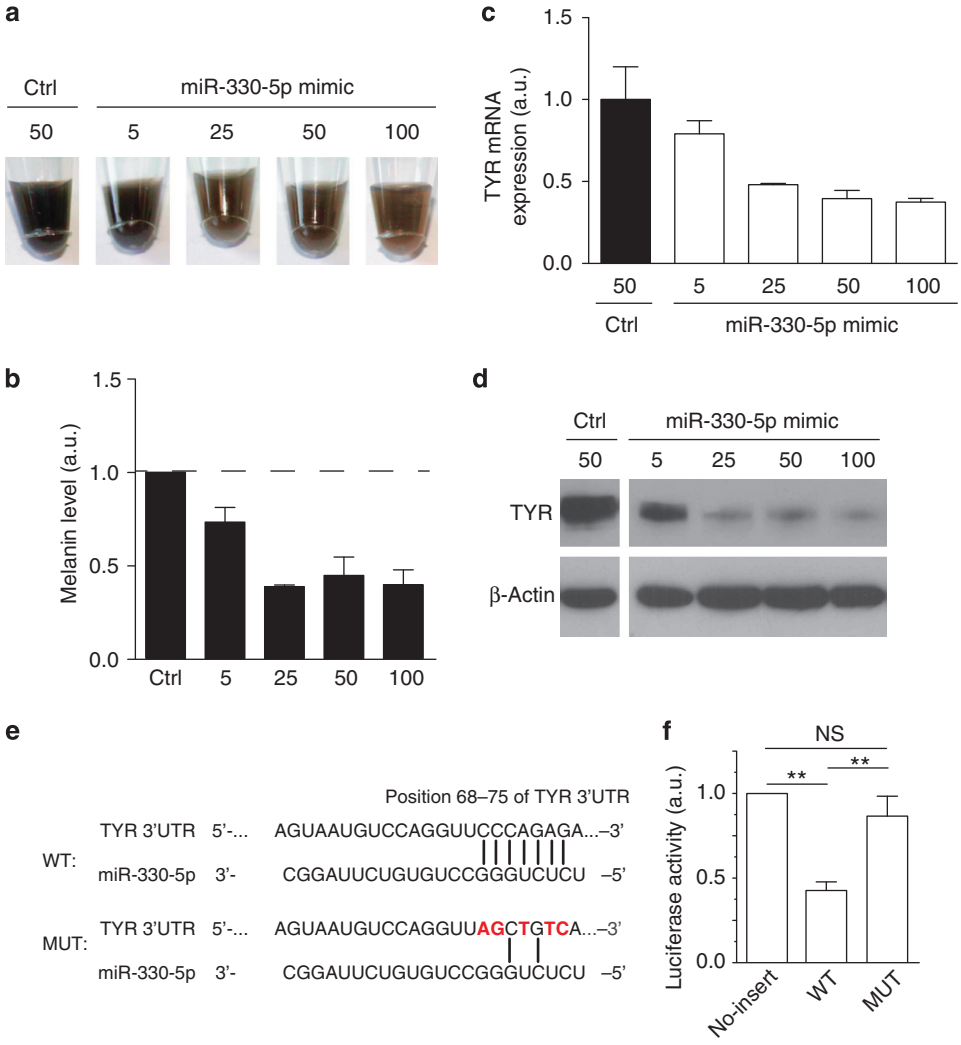

Figure 2. miR-330-5p efficiently targets tyrosinase. (**a**) Pigmentation of MNT-1 cells decreased proportionally with increasing concentrations of miR-330-5p mimics (5–100 nM). (**b**) Melanin was quantified by spectrometry. (**c**) The abundance of tyrosinase mRNA is inversely proportional to the concentration of miR-330-5p mimics. (**d**) The tyrosinase protein concentration is inversely proportional to the concentration of miR-330-5p mimics. (**e**) Schematic representation of the miR-330-5p binding site in the 3′UTR of tyrosinase (TYR). (**f**) Luciferase reporter activity assays showing that miR-330-5p acts on the TYR 3′UTR. Values reported are means, and error bars represent the standard deviation (NS, not significant; $^{**}P<0.01$ Mann–Whitney test) (see Supplementary Information online for additional information). a.u., arbitrary units; MUT, mutant; UTR, untranslated region; WT, wild type.

137 scored the highest (Supplementary Table S1 online).

Melanin levels of MNT-1 cells were significantly reduced after treatment with miR-330-5p and miR-137 mimics (Figure 1a and b). Overexpression of miR-330-5p resulted in significant reduction of tyrosinase mRNA and protein levels, as predicted (Figure 1c and d), whereas MITF, TYRP1, DCT, and MLANA expression levels remained unaltered. Lack of effect of miR-330-5p on MLANA was not surprising as the standardized score was low. TYR expression in normal human epidermal melanocytes (NHEMs; Supplementary Figure S4 online) and in two (501Mel and T1) independent melanoma cell lines (Supplementary Figure S5 online) was similarly affected by miR-330-5p. As expected, overexpression of miR-7 did not detectably affect pigmentation or the expression of pigmentation genes (Figure 1). As predicted, miR-137 overexpression efficiently reduced MITF mRNA and protein levels and therefore pigmentation, consistent with published data showing that MITF is a major target of miR-137 in melanoma cells (Bemis *et al.*, 2008). Transgenic mice overexpressing miR-137 develop a range of coat color changes from dark black to light colors, mainly due to reduced MITF levels (Bemis *et al.*, 2008; Dong *et al.*, 2012). However, miR-137 overexpression seemed to affect survival/morphology of MNT-1 cells and NHEMs (Figure 1 and Supplementary Figure S4 online), as previously reported (Luo *et al.*, 2013). In our conditions, miR-125b acted on cell survival and level of pigmentation in either MNT-1 cells or NHEM (data not shown). We found that the effects of miR-330-5p on TYR mRNA/protein abundance and on overall pigmentation were dose dependent (Figure 2 a–d). The amount of endogenous miR-330-5p in culture is very low in all tested melanoma cell lines and in NHEM. In this respect, the potential use of miR-330-5p is limited to the treatment of hyperpigmentation disorders. Mechanistically, miR-330-5p is predicted to bind to positions 68–75 of the TYR 3′UTR (seed match region; Figure 2e). To test this, we inserted this region into a luciferase reporter vector: miR-330-5p overexpression significantly reduced luciferase activity (Figure 2f and Supplementary Figure S6 online). Mutations in the seed match region rescued the luciferase activity, suggesting that the predicted binding region is valid (Figure 2f and Supplementary Figure S6 online). Mimic control treatment has no effect (Supplementary Figure S6 online). In a nonstressful environment, the presence of miR-330-5p mimic for 12 days does not markedly affect the proliferation/survival of cells *in vitro*.

In summary, we show that miR-330-5p is a potent negative regulator of TYR, but not of MITF, in pigmented melanoma cells and normal melanocytes, and that sustained overexpression of miR-330-5p induces depigmentation without affecting cell morphology, proliferation, or survival. These properties qualify miR-330-5p as a strong candidate for the development of treatments for hyperpigmentation-related disorders.

CONFLICT OF INTEREST

GS, FM, and CM are employees of CHANEL Parfum Beauté. LL declares the receipt of a grant from CHANEL. The other authors state no conflict of interest.

ACKNOWLEDGMENTS

The work was supported by CHANEL Parfum Beauté, Paris, France.

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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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