

Appendix SI.

MATERIALS AND METHODS

Patients and control subjects

A case-control study was conducted at the Dermatology Clinic of Tartu University Hospital and was approved by the Research Ethics Committee of the University of Tartu. All participants signed a written informed consent. For miRNA expression analysis with quantitative reverse transcription (qRT-PCR), we included 15 patients with vitiligo (4 males and 11 females; ages ranging from 19–60 years) and 15 control subjects (6 males and 9 females; ages ranging from 25–53 years) in the study. *In situ* hybridization results of 2 representative patients with stable vitiligo (2 females; 18 and 35 years old; skin phototype III) and 2 representative control subjects (2 females; 47 and 27 years old; skin phototypes II). All of the participants were unrelated Caucasian individuals living in Estonia. Patients with vitiligo were collected from the outpatient department of the Dermatology Clinic. The diagnosis of vitiligo was based on the loss of pigmentation with typical localization and depigmented macules on the skin under a Wood's lamp. All patients with vitiligo had non-segmental type vitiligo; 5 of them had active and 12 had stable vitiligo. Active vitiligo was defined as a condition in which the development of new lesions or the extension of old lesions was revealed 3 months before examination. None of the patients had received any treatment for their vitiligo for at least one month before recruitment. Control subjects were recruited from among healthcare personnel, medical students and patients who attended the dermatological outpatient clinic for surgical excision of naevus. None of the control subjects had any chronic skin disease history or vitiligo in their family. Two skin punch biopsy samples (3–4 mm in diameter) were collected from 15 patients with vitiligo, 1 from the marginal zone of the lesional skin and another from non-sun-exposed non-lesional skin, and for *in situ* hybridization, 1 skin punch biopsy sample from the marginal zone of the lesional skin was collected from 2 patients with vitiligo. One skin punch biopsy sample (3–4 mm in diameter) from non-sun-exposed skin was taken from each of the control subjects. The skin samples were instantly frozen in liquid nitrogen or in dry ice and stored at -80°C until RNA extraction. For *in situ* hybridization, skin biopsy specimens were embedded into the Tissue-Tek (Thermo Scientific, Waltham, MA, USA) before freezing.

RNA purification and qRT-PCR

A total RNA from the skin, melanocytes and keratinocytes was isolated using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) or miRNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. For RNA extraction from the skin, the skin biopsy samples were placed in 700 μl of the QIAzol Lysis Reagent (Qiagen) and homogenized by a gentleMACS™ Dissociator (Miltenyi Biotec, Heidelberg, Germany). The concentration and quality of the RNA were assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). Purified total RNA concentration was 27–603 ng/ μl , the mean 260/280 and 260/230 ratios of the RNA samples were 1.99 and 1.4, respectively. miRNA expression levels were analysed with TaqMan miRNA qRT-PCR assays (Life Technologies, Carlsbad, CA, USA) according to the manufacturers' protocol with few modifications. Briefly, miRNA-specific cDNAs were synthesized using TaqMan® MicroRNA Reverse Transcription Kit and 10 ng of total RNA in 5 μl reaction mix, of which 2.5 μl was used per PCR reaction in 10 μl . Each PCR reaction was performed in duplicate

using a ViiA™ 7 Real-Time PCR system (Life Technologies). The relative gene expression levels were calculated using the comparative C_t ($\Delta\Delta C_t$) method. The expression levels were normalized to the expression of let-7a. Let-7a was chosen for the study as one of the most common housekeeping miRNAs, with C_t values between 20.2 and 22.1 across all the skin samples. To measure mRNA levels, cDNA was synthesized from 100 to 900 ng of total RNA using oligo-dT and reagents from Thermo Fisher Scientific. 5 \times HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia) and Via7 were used for qPCR. The relative gene expression levels were normalized to the level of human EEF1A1 and calculated using the comparative C_t ($\Delta\Delta C_t$) method (Life Technologies). qPCR primers were designed with assistance of Primer 3 and were ordered from TAG Copenhagen (Copenhagen, Denmark).

qPCR primers

The following primers were used: *SOCS1* for: 5'-AGC GGA ACT GCT TTT TCG CCC T-3'; *SOCS1* rev: 5'-TGAAAG CGG CCG GCC TGA AA-3'; *IRF1* for: 5'-CAA CTT CCA GGT GTC ACC CA-3'; *IRF1* rev: 5'-CGA CTG CTC CAA GAG CTT CA-3'; *SOX10* for: 5'-CTT CAT GGT GTG GGC TCA G-3'; *SOX10* rev: 5'-TGT AGT CCG GGT GGT CTT TC-3'; *TYRP1* for: 5'-CCG AAA CAC AGT GGA AGG TT-3'; *TYRP1* rev: 5'-TCT GTG AAG GTG TGC AGG AG-3'; *YWHAE* for: 5'-CTT CCA CCA ACG CAT CCT AT-3'; *YWHAE* rev: 5'-ACC CTG CAT GTC TGA AGT CC-3'; *SDCBP* for: 5'-CTG CAG CCA GAA ATG GTC TT-3'; *SDCBP* rev: 5'-AAA CCT CAG GAA TGG TGT GG-3'; *MEF2A* for: 5'-AGC TCC TCA GAG ACC ACC AA-3'; *MEF2A* rev: 5'-GGA GGG GGA GAC TTT GTA GG-3'. The primers for *EEF1A* and *IFITM1* have been published previously (21).

Culture and stimulation of human primary melanocytes and keratinocytes

Human melanocytes harvested from paediatric foreskin (approval number 178/T-19) (26) were cultivated in melanocyte growth medium M2 with supplement mix (PromoCell, Heidelberg, Germany). Melanocytes from 3 different donors were used in the stimulation experiments. Pooled, normal human epidermal keratinocytes (Promocell, Heidelberg, Germany) were cultured as described previously in keratinocyte-SFM medium with supplements (Life Technologies, Grand Island, NY, USA). Both melanocytes and keratinocytes were stimulated for 5, 24 and 48 h with tumour necrosis factor (TNF)- α , interferon (IFN)- α 2a (IFN- α), IFN- γ and interleukin (IL)-1 β . 2×10^4 cells in one 24-well was used per each stimulation. The following cytokine concentrations were used: 10 ng/ml for TNF- α , 20,000 U/ml for IFN- α 2a, 20,000 U/ml for IFN- γ and 10 ng/ml for IL-1 β .

Transfection of melanocytes and keratinocytes

Transfections were carried out in 12-well plates using 3 μl siPORT NeoFX (Life Technologies, Grand Island, New York, USA) and 2×10^4 primary melanocytes per well in 1.1 ml of melanocyte growth medium 254CFM2 supplemented with PMA-free Human Melanocyte Growth Supplement-2 (Life Technologies, Grand Island, NY, USA) or with 2×10^4 primary keratinocytes per well in 1.1 ml of keratinocyte-SFM medium (Life Technologies) according to the manufacturer's protocol. After 24 h, melanocytes and keratinocytes were stimulated with IFN- γ for 48 h when indicated. Transfections were performed at 60 nM of miRIDIAN microRNA hsa-miR-155-5p mimic and miRIDIAN microRNA Mimic Negative Control #1 (GE Healthcare Life Sciences, Fairfield, CT, USA).

miRNA target selection and pathway analysis

Putative targets with a total context score less than -0.15 and/or conserved among vertebrates were selected using Targetscan 6.2 (<http://www.targetscan.org/>) (16). Only the genes expressed in the skin according to the previously published (21) dataset E-MTAB-729 (9966 genes with a mean signal >40.0 in the skin from healthy donors) were subjected to pathway analysis. The pathway analysis was performed with g:Profiler (<http://biit.cs.ut.ee/gprofiler/>) (17). To estimate the significance of the overlap between the target list and indicated functional group, the Fisher's exact test was performed.

In situ hybridization

ISH was optimized and performed on 10- μ m sections of frozen skin biopsy specimens using microRNA ISH Buffer and Controls Kit according to the manufacturer's protocol. For detection of miR-155, miRCURY LNATM Detection Probe for hsa-miR-155 (88072-15) (Exiqon, Vedbaek, Denmark) was used. Prehybridization, hybridization and washings were performed at 50°C. Slides were incubated with alkaline phosphatase-conjugated sheep anti-DIG-AP (1:1500, Roche,

Basel, Switzerland) for 1 h at room temperature. The staining was visualized by adding BM purple AP substrate (Roche). The slides were counterstained with Nuclear Fast Red counterstain (Vector Laboratories, Burlingame, CA, USA). Leica DM5500 B microscope (Leica Microsystems) was used to acquire images.

Statistical analysis

Student's *t*-test was used for statistical analysis when different conditions were compared (Figs 2, 3) and when data were normally distributed. The conformity to a normal distribution was assessed using the Kolmogorov–Smirnov test. In the case of miR-155 in lesional skin of patients with vitiligo (VLS) vs. non-lesional skin of patients with vitiligo (VNLS), miR-145 in skin from control subjects (CS) vs. VLS, miR-99b CS vs. VLS and CS vs. VNLS, the Mann–Whitney *U* test was applied because the data did not follow the normal distribution. As the results of the paired *t*-test for the comparison of non-lesional and lesional skin did not differ significantly from the results gained with Student's *t*-test, we show only the results of the latter. For the data analysis, the Graphpad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used. A *p*-value <0.05 was considered significant.