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 Ct 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× 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 Ct (ΔΔCt) 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'-TGA AAG 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)-α2a (IFN-α), IFN-γ and interleukin (IL)-1β. 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-α2a, 20,000 U/ml for IFN-γ and 10 ng/ml for IL-1β.

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-µm 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.

Table SI. *Functions of analysed miRNAs*

miRNA	Function	Ref
miR-146a miR-146b	Inhibit activation of the NF-kB signalling pathway through targeting of interleukin-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor 6 (TRAF6), Relb and caspase recruitment domain-containing protein 10 (CARD10). The expression is upregulated in the skin of psoriasis and atopic dermatitis (AD) patients.	(27, 28) (9, 10, 29, 30)
miR-155	Known as a proinflammatory miRNA that inhibits suppressor of cytokine signalling 1 (SOCS1) Is a positive regulator of interferon signalling in monocytes, dendritic cells and CD8+ cytotoxic T cells	(23) (8, 23, 32)
miR-125b	Regulate proliferation, differentiation, apoptosis and immune responses in various cell types, including keratinocytes	(7, 9)
miR-125a	Inhibit the expression of pigmentation-related genes	(24)
miR-145	The expression is reduced in cultured pigment cells after induction of pigmentation	(18)
miR-99b	Encoded by the same gene cluster as miR-125b and regulates cell proliferation and cell migration	(33)
miR-199a-3p	Highly expressed in the hair follicles Inhibits caveolin-2 and the AKT/mTOR pathway and thereby inhibits cell differentiation and induces proliferation	(34) (35, 36)
miR-203	Regulates the differentiation and functions of keratinocytes and melanosome transport	(37–39)
miR-511	Regulates function of dendritic cells and macrophages	(40)
miR-223	Upregulated in psoriasis and contact dermatitis	(11, 41)
miR-10a	Regulates cell proliferation, inflammatory responses and plasticity of T cells	(42–44)

Table SII. *Pathway analysis of putative targets of miRNAs dysregulated in vitiligo*

miRNA and targets (n)[a]	Functional group ID, name and genes in the functional group, n[b]	Putative targets in the functional group
miR-99b (40)	HP:0001000, abnormality of skin pigmentation, 39	LIFR, FGFR3, PTPN11
miR-125b	GO:0033059, cellular pigmentation, 54	BCL2, VPS33A, SS18
(221)	HP:0001000, abnormality of skin pigmentation, 268	LIFR, ALDH3A2
	KEGG:04916, melanogenesis, 101	DVL3, MAPK3
miR-155 (438)	GO:0030318, melanocyte differentiation, 28	MEF2A, SOX10, TYRP1
	GO:0042470, melanosome, 109	RAB5C, SDCBP, SYPL1, SYTL2, TMEM33, TYRP1, YWHAE, YWHAZ
	KEGG:04916, melanogenesis, 101	CREB1, GNAS, GSK3B, TCF7L2, TYRP1
miR-199a-3p (321)	GO:0042470, melanosome, 109	SYTL2, TMEM33, YWHAE, NAP1L1, CALU, SYPL1, SLC2A1
	GO:0030318, melanocyte differentiation, 28	ZEB2
	GO:0032400, melanosome localization, 30	VPS33A
	HP:0001000, abnormality of skin pigmentation, 268	STK11, SRD5A3, PDGFRA, KIAA0319L, FOS, ALDH3A2, SPRED1
	HP:0007440, generalized hyperpigmentation, 24	ALDH3A2
miR-145 (377)	GO:0051403, stress-activated MAPK cascade, 246	ARL6IP5, CRKL, DAB2, DUSP6, FOXO1, FZD4, FZD7, HIPK2, MAP2K4, MAP3K11, MAP3K2, MAP4K2, MAP4K4, NRAS, PDCD4, ZEB2, TAOK1, TNFRSF19
	GO:0007254, JNK cascade, 192	CRKL, DAB2, FZD4, FZD7, HIPK2, MAP2K4, MAP3K11, MAP3K2, MAP4K2, MAP4K4, NRAS, PDCD4, ZEB2, TAOK1, TNFRSF19
	KEGG:04350, TGF-beta signalling pathway, 80	ACVR1B, ACVR2A, INHBB, RPS6KB1, SMAD3, SMAD4, SMAD5, SP1, TGFBR2

[a]Number of predicted putative direct targets expressed in the skin shown in parenthesis. [b]Pathways with significant overlap ($p<0.05$) with the predicted targets are presented.

Table SIII. *Vitiligo-associated proinflammatory cytokines*

Cytokine	Association with vitiligo	Ref
Tumour necrosis factor-α	Increased level in the serum, production by peripheral blood mononuclear cells and in the lesional skin of patients with vitiligo	(45)
Interferon-α	Interferon (IFN)-α producing plasmacytoid dendritic cells are found in the skin of patients with active vitiligo	(20)
	Pegylated IFN-α2a and IFN-α2b, which are used in the treatment of chronic C-hepatitis, induce depigmentation at injection sites	(46)
	Vitiligo patches have been observed at the site of application of imiquimod, a TLR7 and TLR8 agonist that enhances the production of IFN-α	(47)
Interferon-γ	Expression is upregulated in lesional skin and peripheral blood mononuclear cells of patients with vitiligo	(19)
	Decreased depigmentation of IFN-γ–/– mice in mouse models of vitiligo	(48–51)
Interleukin-1β	Level is increased in the serum and whole blood of patients with segmental and generalized vitiligo	(52, 53)

Table SIV. *Functions of miR-155 targets*

Target	Functions	Ref
TYRP1	Affects proliferation and apoptosis of melanocytes; regulates melanin synthesis, transport of melanosomes and maintenance of melanosome structure	(54–56)
	Antibodies against TYRP1 are detected in the blood of patients with vitiligo	(57)
	Polymorphisms in miR-155 binding site have been associated with the development of melanoma	(58)
YWHAE	Modulates the function of tyrosine hydroxylase and affects melanogenesis	(59)
SDCBP	The product of SDCBP gene, syntenin, participates in melanin transport	(60, 61)
SOX10	Transcription factor that affects melanocyte differentiation	(62)
SOCS1	Interferon-inducible gene, well-known direct target of miR-155	(23, 32)
		(8, 23)
IRF1	Interferon-inducible gene	(21)
IFITM1	Interferon-inducible gene	(21)