Little is known about the functions of microRNAs (miRNAs) in skin pigmentation disorders. The aim of this study was to investigate the expression and potential role of miRNAs in vitiligo. Of 12 studied miRNAs with proven functions in cell proliferation, differentiation, immune responses and melanogenesis, miR-99b, miR-125b, miR-155 and miR-199a-3p were found to be increased and miR-145 was found to be decreased in the skin of patients with vitiligo. Combined pathway and target analysis revealed melanogenesis-associated targets for miR-99b, miR-125b, miR-155 and miR-199a-3p. In situ hybridization analysis demonstrated increased expression of miR-155 in the epidermis of patients with vitiligo. Correspondingly, miR-155 was induced by vitiligo-associated cytokines in human primary melanocytes and keratinocytes. When overexpressed, miR-155 inhibited the expression of melanogenesis-associated genes and altered interferon-regulated genes in melanocytes and keratinocytes. In conclusion, this study demonstrates that the expression of miRNAs is dysregulated in the skin of patients with vitiligo and suggests that miR-155 contributes to the pathogenesis of vitiligo. Key words: vitiligo; miRNA; skin; melanocytes; keratinocytes.

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Vitiligo is a common acquired, idiopathic disease, characterized by the destruction of melanocytes (1). Although there are rarely any physical symptoms other than depigmented macules on the skin, patients with vitiligo, especially dark-skinned patients, have an impaired quality of life (2, 3). Many theories, e.g. autoimmunity, cytotoxic metabolites, neural and genetic theories, have been proposed to explain the mechanisms of pigmentation loss. In addition to melanocyte dysfunction, keratinocyte alteration plays a role; keratinocytes in depigmented epidermis are more vulnerable to apoptosis and produce lower amounts of melanogenic mediators than normal skin (4). However, the exact mechanism underlying this pigmentary disorder remains unknown (1) and this has held back progress in its treatment (5).

MicroRNAs (miRNAs) are approximately 22 nucleotide-long non-coding gene-regulatory RNA molecules that inhibit gene expression through translational repression or mRNA turnover. In mammalian cells, in most cases, miRNAs bind with partial complementarity to sequences in the 3’ untranslated region (3’UTR) of target messenger RNAs (mRNAs) for the suppression of gene expression. Each miRNA has multiple targets and each mRNA is simultaneously regulated by multiple miRNAs (6).

miRNAs have been intensively investigated in humans for more than 10 years and have been found to regulate most of the cellular processes, including cell proliferation, differentiation, development, signal transduction, metabolism, apoptosis and immune responses (7). For instance, miR-155 is known to target the suppressor of cytokine signalling (SOCS1) and thereby activates interferon signalling in CD8+ cytotoxic T cells (8). miRNA dysregulation is associated with the pathogenesis of various inflammatory diseases, including different inflammatory skin disorders, such as psoriasis (9), atopic dermatitis (7, 10) and allergic contact dermatitis (11). Aberrant expression of several miRNAs in the skin and serum of patients with vitiligo has been demonstrated by microarray analysis (12, 13). Using quantitative reverse transcription-PCR (qRT-PCR), dysregulation of miR-224-3p, miR-4712-3p and miR-3940-5p has been shown in peripheral blood mononuclear cells of patients with vitiligo (14). A single nucleotide polymorphism, rs11614913 in miR-196-a-2, has been found to be associated with vitiligo (15). However, little is known about the role of miRNAs in the pathogenesis of vitiligo.

The aim of this study was to investigate the potential role of previously known melanocyte-, keratinocyte-, immunity-, cell proliferation-, differentiation- and apoptosis-associated miRNAs in vitiligo. We measured their expression in the lesional and non-lesional skin of patients with vitiligo and in cultured unstimulated and
cytokine-treated melanocytes and keratinocytes, and assessed the possible targets of the dysregulated miRNAs.

MATERIALS AND METHODS

Patients

A total of 17 patients with non-segmental vitiligo (5 with active and 12 with stable vitiligo) and 17 control subjects were included in the study. All participants were unrelated Caucasian individuals living in Estonia. The study was approved by the research ethics committee of the University of Tartu. A detailed description of the patients is provided in Appendix SI1.

Cell culture experiments and miRNA and mRNA expression analysis

Human melanocytes from paediatric foreskin were cultivated in melanocyte growth medium M2 with supplement mix (Promocell, Heidelberg, Germany). Pooled, normal human epidermal keratinocytes (Promocell, Heidelberg, Germany) were cultured in keratinocyte-serum-free medium (SFM) with supplements (Life Technologies, Grand Island, New York, USA). In situ hybridization (ISH) was performed on 10-µm sections of frozen skin biopsy specimens using miR-155, miRCURY LNA™ Detection Probe for hsa-miR-155 (88072-15) (Exiqon, Vedbaek, Denmark) according to the manufacturer’s protocol. The transfection protocol, RNA purification, qRT-PCR and ISH are detailed in Appendix SI1.

miRNA target selection, pathway analysis and statistics

Putative targets were selected using Targetscan 6.2 (http://www.targetscan.org/) (16). The pathway analysis was performed with g:Profiler (http://biit.cs.ut.ee/gprofiler) (17). For statistical analysis, Fisher’s exact test, Student’s t-test and Mann-Whitney U test were applied. Details are given in Appendix SI1.

RESULTS

To investigate the potential role of miRNAs in vitiligo, we selected 12 miRNAs with known functions in the regulation of cell proliferation and differentiation (miR-10a, miR-99b, miR-125a, miR-125b and miR-199a-3p), immune responses (miR-146a, miR-146b, miR-155, miR-223 and miR-511), skin homeostasis (miR-203) and melanogenesis (miR-145) (7, 9, 18) (see also Appendix SI1 and Table SII)1. The expression of the selected miRNAs was detectable by qRT-PCR in the skin from control subjects. The most abundantly expressed miRNA was miR-125b, whose expression level was approximately 3,800 times higher than that of miR-511, the miRNA with the lowest expression level (mean threshold cycle 29.5) (Fig. 1A). From this set of miRNAs, we found miR-99b, miR-155, miR-199a-3p, miR-125b and miR-145 to be dysregulated in skin from patients with vitiligo compared with skin from control subjects, and miR-146b to be differentially expressed in the lesional compared with non-lesional skin from patients with vitiligo (Fig. 1B). miR-99b expression levels were increased in vitiligo lesional (p < 0.001) and non-lesional skin (p < 0.01) compared with the control subjects. Highly significant upregulation of miR-155 (p < 0.01), miR-199a-3p (p < 0.01), significant upregulation of miR-125b (p < 0.05) and downregulation of miR-145 (p < 0.05) expression was detected exclusively in vitiligo lesional skin compared with the control subjects. In the cases of miR-155 (p < 0.05), miR-199a-3p (p < 0.05) and miR-146b (p < 0.05), the expression levels were higher in vitiligo lesional skin than in vitiligo non-lesional skin. The expression levels of dysregulated miRNAs did not differ statistically significantly between patients with active and stable vitiligo (data not shown). There were no statistically significant differences in the expression levels of miR-10a, miR-125a, miR-146a, miR-203, miR-223 and miR-511 between the groups (data not shown).

To analyse whether the differentially expressed miRNAs, miR-99b, miR-125b, miR-145, miR-155 and miR-199a-3p, can influence cellular processes associated with vitiligo, we performed pathway analysis for conserved and the best-scored miRNA targets expressed in the skin. Four miRNAs, miR-99b, miR-125b, miR-155 and miR-199a-3p, were found to have predicted direct targets belonging to either the gene ontology (GO) or human phenotype (HP) ontology groups or to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to melanocyte differentiation, melanogenesis, melanosome structure and localization, and skin pigmentation (Table SII). Putative miR-145 targets included genes involved in the regulation of stress-activated mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and transforming growth factor (TGF)-β signalling pathways. All 5 of the differentially expressed miRNAs also had putative targets belonging to functional groups that might indirectly influence cellular processes in vitiligo, including abnormalities in skin physiology, inflammatory abnormalities of the skin, programmed cell death, tight junction interactions, cell growth and cell cycle progression (data not shown).

We next focused our experiments on miR-155, as it was predicted to target multiple important melanogenesis-associated genes (Table SII) and has previously been shown to contribute to the activation of interferon signalling (8). We first performed in situ hybridization on skin samples from patients with vitiligo and control individuals. The expression of miR-155 was detected in stratum basale, where melanocytes and proliferating keratinocytes are located, as well as in stratum spinosum of the epidermis of patients with vitiligo. In line with RT-qPCR results (Fig. 1B), no signal in one donor and faint positive signal of miR-155 was detected in the epidermis of control skin (Fig. 2A).

1http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-2394
We next studied whether the cytokines, which are known to be associated with the pathogenesis of vitiligo, (1, 19, 20) influence the expression of miR-155 in melanocytes and keratinocytes. We treated human melanocytes and keratinocytes with tumour necrosis factor alpha (TNF-α), interferon (IFN)-γ, IFN-α and interleukin (IL)-1β and measured the relative expression of miR-155 using qRT-PCR. In melanocytes, the expression of miR-155 was significantly upregulated by TNF-α, IFN-γ, IFN-α and IL-1β after 24 h ($p<0.001$, $p<0.01$, $p<0.05$ and $p<0.01$, respectively) and 48 h ($p<0.001$, $p<0.01$, $p<0.01$ and $p<0.001$, respectively) of stimulation (Fig. 2B). In keratinocytes, the expression of miR-155 was significantly upregulated by TNF-α and IFN-α ($p<0.001$ and $p<0.001$, respectively) after 24 h of stimulation and then downregulated ($p<0.01$) at the 48 h time-point. In response to IFN-γ, miR-155 was upregulated at both 24 h ($p<0.01$) and 48 h ($p<0.05$) time-points. Significant upregulation by IL-1β was only observed after 48 h of stimulation ($p<0.001$) (Fig. 2C).

To test whether miR-155 may influence the development of vitiligo by targeting of melanogenesis-associated genes and modulating the interferon-regulated genes in melanocytes, we performed miR-155 overexpression experiments in melanocytes and keratinocytes. We first transfected human primary melanocytes with miR-155 and the control mimic (Fig. 3A), stimulated the cells with IFN-γ or left unstimulated, and measured relative expression of selected putative miR-155 targets and interferon-regulated genes with RT-qPCR. The melanogenesis-associated genes, such as tyrosinase-related protein 1 (TYRP1), syndecan binding protein (SDCBP), tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation

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**Fig. 1.** Relative expression of miRNAs in the skin of control subjects and patients with vitiligo. (A) The results in control skin are represented as boxes with whiskers showing the minimum and maximum and are shown relative to the level of miR-511 (=1). (B) Selected individual miRNA levels in control subjects (CS=1) and lesional skin (VLS) and non-lesional skin (VNLS) of patients with vitiligo. Mean ± standard error of the mean (SEM) is indicated. *$p<0.05$; **$p<0.01$; and ***$p<0.001$.

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**Fig. 2.** Expression of miR-155 in the skin, melanocytes and keratinocytes. (A) Lesional skin from 2 patients with vitiligo and the skin from 2 control subjects was used for in situ hybridization with miR-155-specific probe. Red colour indicates nuclear fast red staining, blue colour miR-155 expression, bar = 50 μm. (B, C) The expression of miR-155 in tumour necrosis factor alpha (TNF-α), interferon (IFN)-γ, IFN-α and interleukin (IL)-1β-treated (B) melanocytes and (C) keratinocytes is shown relative to the expression levels in unstimulated cells at each indicated time-point. Results are displayed as the mean ± standard error of the mean (SEM). Data are from 3 different stimulations. *$p<0.05$; **$p<0.01$; and ***$p<0.001$. 

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**Fig. 3.** Relative expression of miR-155 in melanocytes and keratinocytes. (A) Lesional skin from 2 patients with vitiligo and the skin from 2 control subjects was used for in situ hybridization with miR-155-specific probe. Red colour indicates nuclear fast red staining, blue colour miR-155 expression, bar = 50 μm. (B, C) The expression of miR-155 in tumour necrosis factor alpha (TNF-α), interferon (IFN)-γ, IFN-α and interleukin (IL)-1β-treated (B) melanocytes and (C) keratinocytes is shown relative to the expression levels in unstimulated cells at each indicated time-point. Results are displayed as the mean ± standard error of the mean (SEM). Data are from 3 different stimulations. *$p<0.05$; **$p<0.01$; and ***$p<0.001$. 

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MicroRNA-155 in vitiligo, melanocytes and keratinocytes

protein epsilon (YWHAE), and a gene associated with melanocyte differentiation, SRY (sex determining region Y)-Box 10 (SOX10), were included in the analysis. Of IFN-γ-inducible genes, we analysed the effect of miR-155 on interferon-induced transmembrane protein 1 (IFITM1), interferon regulatory factor 1 (IRF1) (21) and previously described miR-155 direct target SOCS1. Fig. 3B and C demonstrate that the overexpression of miR-155 suppressed the expression of SOX10 (p < 0.05), TYRP1 (p < 0.01) and YWHAE (p < 0.01) in unstimulated melanocytes. The mRNA level of SDCBP was inhibited both in unstimulated and IFN-γ-stimulated melanocytes with p < 0.01 and p < 0.05, respectively. Of interferon-inducible genes, SOCS1 was suppressed by overexpression of miR-155, both in unstimulated (p < 0.01) and stimulated (p < 0.05) melanocytes. IRF1 was decreased (p < 0.001) and IFITM1 was increased (p < 0.05) in unstimulated melanocytes transfected with miR-155 compared with the control transfection. IFITM1 and IRF1 were strongly induced in response to IFN-γ, and IFITM1 was further increased (p < 0.01) upon overexpression of miR-155. For IRF1, no difference between miR-155 and control-transfected cells was observed when the cells were stimulated by IFN-γ (Fig. 3D).

In the similar overexpression experiment in keratinocytes, miR-155 suppressed the expression of YWHAE (p < 0.05) in unstimulated conditions (Fig. 3E). Among interferon-inducible genes, SOCS1 and IRF1 were suppressed by overexpression of miR-155, both in unstimulated (p < 0.05 and p < 0.01, respectively) and stimulated (both with p < 0.05) keratinocytes. Similarly to the effect in melanocytes, IFITM1 was increased in unstimulated (p < 0.001) and IFN-γ-stimulated (p < 0.001) keratinocytes transfected with miR-155 compared with the control transfections (Fig. 3F). In conclusion, these results demonstrate that miR-155 has the capacity to impact melanogenesis and inflammatory responses in vitiligo directly through its effect in melanocytes and keratinocytes.

DISCUSSION

Little is known about the functions of miRNA in the pathogenesis of vitiligo. This study revealed that miR-99b, miR-155, miR-199a-3p, miR-125b and miR-145 are dysregulated in the skin of patients with vitiligo, of which miR-99b, miR-125b, miR-155 and miR-199a-3p were found to have putative targets associated with melanocyte differentiation and melanogenesis. Among the dysregulated miRNAs, we demonstrated increased expression of miR-155 in the epidermis of patients with vitiligo. Thus, miR-155 was induced by vitiligo-associated cytokines TNF-α, IFN-α, IFN-γ and IL-1β in human primary melanocytes and keratinocytes. When
overexpressed, miR-155 inhibited the expression of genes known to affect melanocyte differentiation and melanogenesis, such as TYRP1, YWHAE, SDCBP and SOX10 in melanocytes, and YWHAE in keratinocytes. In addition, miR-155 overexpression altered the levels of interferon-regulated genes SOCS1, IRF1 and IFITM1 in melanocytes and keratinocytes. Our results suggest that miR-155 and other miRNAs contribute to the pathogenesis of vitiligo.

Previously, miRNA expression profiles of peripheral blood mononuclear cells (14), serum (13) and skin (12) of patients with vitiligo have been studied. We report here for the first time the aberrant expression of miR-125b, miR-155 and miR-199a-3p and confirm the altered expression of miR-99b, in the skin of patients with vitiligo. In addition, miR-146b was significantly upregulated in vitiligo lesional skin compared with non-lesional skin. An increased expression level of miR-145 and miR-10a in the non-lesional skin from patients with vitiligo has been reported previously (12). The results of our study did not confirm these differences, as miR-145 was downregulated in vitiligo lesional skin and miR-10a showed no differences between the studied groups. This might be due to differences in participant characteristics or technical approach. For the other studied miRNAs, miR-125a, miR-203, miR-223 and miR-511, we did not detect any differences in the expression levels in the skin of control subjects and patients with vitiligo. Previously, the expression of miR-10a and miR-125b was observed to be downregulated and miR-223 upregulated in serum from patients with vitiligo (13).

Consistent with its overexpression in the epidermis, miR-155 was induced in response to TNF-α, IFN-α, IFN-γ and IL-1β, the proinflammatory cytokines that have been previously reported to be associated with vitiligo pathogenesis (see also Appendix SI1, Table SIII1) in cultured melanocytes and keratinocytes. miR-155 was overexpressed in the skin from patients with atopic dermatitis, mainly due to the presence of immune cells (22). In our study, in line with stimulation experiments in melanocytes and keratinocytes, miR-155 was observed to be increased in the epidermis of lesional vitiligo skin, when analysed by in situ hybridization. This suggests that the presence of inflammatory cytokines in the skin of patients with vitiligo modulates inflammatory responses and activates the expression of miR-155 in melanocytes and keratinocytes.

miR-155 is known as a proinflammatory miRNA, which, among other targets, inhibits suppressor of cytokine signalling 1 (SOCS1). This results in the activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway and, consequently, of types I and II interferon signalling (23). Here, we found that miR-155 modulates several interferon-inducible genes, such as SOCS1, IFITM1 and IRF1, as well as melanogenesis-associated genes, TYRP1, YWHAE, SDCBP and SOX10, in melanocytes and keratinocytes, which suggests that miR-155 contributes to the pathogenesis of vitiligo, both through targeting of melanogenesis-associated targets and via modulation of interferon signalling (see also Appendix SI1, Table SIV1).

In addition to miR-155, other dysregulated miRNAs might contribute to the development of vitiligo. For example, miR-125b is downregulated after the induction of pigmentation in melanocytes (18) and miR-125b mimics have been shown to inhibit expression of pigmentation-related genes (24). Similar to miR-125b, the expression of miR-145 has been shown to be reduced in cultured pigment cells after induction of pigmentation (18). The pathway analysis in our study detected possible direct targets for miR-145 among the MAPK, JNK and TGF-β pathways, which can interfere with the viability and functionality of melanocytes (25). Although no significant overlap was revealed between miR-155 targets and genes associated with pigmentation, we detected the presence of binding sites for miR-145 in the mRNAs of melanogenesis-associated genes ras-related protein rab-27A (RAB27A), SRY (sex determining region Y)-Box9 (SOX9) and fascin actin-bundling protein 1 (FCSNI) using TargetsCan (data not shown). This is in line with previous results demonstrating the influence of miR-145 on genes involved in the pigmentation process in miR-145-transfected cells (18).

In conclusion, this study demonstrates that miRNAs miR-99b, miR-125b, miR-145, miR-155 and miR-199a-3p are dysregulated in the skin of patients with vitiligo, and shows that miR-155 has the capacity to modulate melanogenesis-associated and interferon-inducible genes in melanocytes and keratinocytes. Further studies are needed to clarify whether miR-155 and other dysregulated miRNAs described in the present study are suitable diagnostic markers and/or targets for the treatment of vitiligo.

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The authors declare no conflicts of interest.

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