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Ubiquitin-specific peptidase 2 as a potential link between microRNA-125b and psoriasis

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Summary

Background The extensive involvement of microRNA (miRNA) in the pathophysiology of psoriasis is well documented. However, in order for this information to be useful in therapeutic manipulation of miRNA levels, it is essential that detailed functional mechanisms are elucidated. miR-125b has previously been shown to be strongly associated with psoriasis, and presents as an obvious candidate for further investigation.

Objectives To elucidate the specific pathway and mechanism of interest in this association.

Methods A three-step bioinformatical hypothesis-generation pipeline was performed to identify genes of interest. This pipeline was based on miR-125b binding, expression in psoriatic lesions and genome-wide association study-based evidence of involvement. The identified candidate gene was then carefully evaluated using luciferase binding assays, in vitro overexpression, small interfering RNA knock-down and downstream gene readouts.

Results Based on our bioinformatical pipeline, ubiquitin-specific peptidase 2 was selected as a likely candidate for a mechanistic explanation for psoriasis association. After establishing a definite connection to miR-125b, we proceeded to show that modulation of nuclear factor kappa B-mediated inflammation is the likely mechanism through which this miRNA gene pair functioned.

Conclusions Shedding further light on the multifactorial causes of psoriasis is essential, if the goal is to progress towards finer control of therapeutic tools in disease management. Findings, such as the ones presented herein, are therefore necessary in order to achieve the future of personalized medicine.

What’s already known about this topic?

- MicroRNA (MiR)-125b has previously been shown to be strongly associated with psoriasis.

What does this study add?

- This study adds a likely molecular mechanism to the association between miR-125b and psoriasis. Additionally, it provides a possible novel pathway for therapeutic treatment.

What is the translational message?

- MiR-125b has previously been shown to be strongly associated with psoriasis.
- To benefit clinically from this knowledge it is important to understand the underlying mechanism.
Psoriasis is a common chronic inflammatory skin disease that affects approximately 2–3% of the population. The cause of psoriasis is still unclear and a complete cure is lacking. Nonetheless, significant progress has been made in understanding the cellular immunology and biology of psoriasis. This includes the importance of interleukin (IL)-12 and IL-23 and the T helper (Th)1 and Th17 inflammatory pathways, as is already well established through the success of ustekinumab in treatment of moderate-to-severe psoriasis. Likewise, it is unsurprising that more unspecific modulation of the immune system is beneficial in psoriasis [corticosteroids, methotrexate and tumour necrosis factor (TNF) inhibitors]. However, a multifactorial disease like psoriasis is likely to originate in a complex interplay among many different effector molecules and further studies into the specific pathophysiological pathways are warranted.

One particularly interesting aspect of psoriasis-specific pathophysiological pathways is the extensive study of micro-RNAs (miRNAs), which have been extensively discussed as important regulators in psoriasis.1,2 Direct pharmacological targeting of miRNAs has even been suggested.3 Undoubtedly, the broad regulatory activity of miRNA makes it very interesting from a biological point of view, but it should be noted that it also creates the one weakness of miRNA targeting, which is that it can be problematic to modulate pharmacologically molecules with such broad consequences. It is therefore of interest to further elucidate the related molecular pathways for the purpose of gaining increased pathophysiological understanding.

In this study we set out to characterize the targets of psoriasis-associated miRNA, miR-125b, which have previously been shown to be downregulated in psoriatic lesions, playing an important role in modulating keratinocyte function.4,5 As with all miRNAs, a number of different targets have been proposed and characterized for each miRNA, which is not surprising given the functionality of miRNA. Therefore, the first steps of our study involved the search for a useful gene for further characterization as target molecule. We required such a gene to be (i) a direct target of a known psoriasis-associated miRNA; (ii) to be overexpressed in psoriatic lesions; and (iii) to have evidence of causal involvement in disease formation.

The first and second requirements give a direct link to psoriasis and previous discoveries. The third tries to overcome the problem that a gene upregulated in diseased tissue may be the cause of the disease or simply a consequence of the disease. Short of large-scale clinical drug trials and knockout animals, it is difficult to overcome that fact. One manageable method is the use of human genetics to investigate if natural variation in the expression level of a gene coincides with variation in risk of disease.6 Taken together, these methods constitute the foundation for our selection of ubiquitin-specific protease 2 (USP2) as a potential target of interest.

Having established this, we set out to characterize the interplay between USP2 and miR-125b and its effects on the three hallmarks of psoriasis: differentiation, proliferation and inflammation. The overall aim of these experiments was to provide new knowledge about this aspect of psoriatic pathogenesis signalling.

Materials and methods

Selection of target gene USP2

For the miRNA target gene list, Targetscan release 6.2 was used, with the default settings, using lsa-mir-125b as input in the mammalian database, retrieving all genes with a probability of conserved targeting score ($P_o$) of $>0.9$.7 Gene expression data from psoriatic skin lesions and healthy controls were downloaded from the Gene Expression Omnibus entry (accession number GSE13355), and renormalized using robust multiarray average and log2 transformation.8,9 The validation measurements shown in Figure 1b were performed on an independent cohort from the Karolinska Institute, using real-time polymerase chain reaction (PCR)s analysed according to the $2^{\Delta \Delta Ct}$ methodology. Comparisons between groups were calculated using a heteroscedastic Student’s t-test. Patients with psoriasis had not received systemic treatments for at least 1 month, and topical therapy for at least 2 weeks before skin biopsy. The study was approved by the Stockholm Regional Ethics Committee, and conducted according to the principles of the Declaration of Helsinki.

For the investigation of genetic disease association, genome-wide association data were downloaded from the dbGap project (accession number phs000019.v1.p1) and P-values and all odds ratios were used as reported. Genetics of gene expression data were downloaded from array expression accession number E-MTAB-2232, and gene expression data were used as provided. USP2 was measured using the ILMN_1755502 microarray probe in the 24-h LPS-stimulated dataset. Association with genotype was calculated using an additive linear model, in which the TT was coded as 0, the CT as 1 and the CC genotype as 2.

Plasmid mutagenesis and 3’ untranslated region luciferase-binding assays

Firefly luciferase reporter plasmids containing the 3’-untranslated region (UTR) of USP2 and empty luciferase vector were
obtained from Promega (Madison, WI, U.S.A.). The nucleotides of USP2 3’-UTR containing the miR-125b binding site were cloned into psiCHECK-2 (Promega) using the restriction sites XhoI/NotI. The 3’UTR of human USP2 was amplified by PCR using the following primers: forward 5’–GGCTCGTTGTGCATTGCAGT–3’; reverse 5’–GGCAGGACTGGAGACC–3’.

To delete the seed sequence of miR-125b on the USP2 3’UTR, we used a Quick-Change Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, U.S.A.) and the following primers: forward 5’–TGTCGGAGCCAGTACCTC–3’; reverse 5’–GGCTCAGTTGGGCAGAAACTCGG–3’.

For the reporter luciferase assay human embryonic kidney-293 cells were co-transfected in a 12-well plate with 1 µg psiCheck2-USP2 and/or psiCheck2-Mut and 100 nM of miR-125b or scrambled-sequence miRNA control (Thermo Scientific, Waltham, MA, U.S.A.). A Dual Luciferase Assay (Promega) was performed 24 h after transfection, according to the manufacturer’s instructions.

**Immunostaining**

USP2 protein expression was analysed in frozen skin sections using rabbit antihuman USP2 antibody (ab66556 diluted 1:100; Abcam, Cambridge, U.K.) and antirabbit conjugated with horseradish peroxidase (P 0448 diluted 1:200; DAKO, Glostrup, Denmark). Sections were mounted using Glycergel/C226 Mounting Medium, Aqueous (DAKO). The omission of the primary antibody in the staining procedure was used as a negative control.

**Cell culture and transfection**

Human adult skin epidermal keratinocytes (Cascade Biologics, Portland, OR, U.S.A.) were cultured in EpiLife serum-free keratinocyte growth medium, including human keratinocyte growth supplement at a final Ca\(^{2+}\) concentration of 0.06 mmol L\(^{-1}\) (Cascade Biologics). Third-passage keratinocytes were used at 50–70% confluence for all experiments.

Keratinocyte transfection was carried out using Lipofectamine\textsuperscript{®} RNAiMAX (Invitrogen, Carlsbad, CA, U.S.A.), following the manufacturer’s instructions. In total, 20 nmol L\(^{-1}\) miRCURY\textsuperscript{™} LNA Inhibitor (Exiqon, Vedbaek, Denmark) was used for inhibition of miR-125b. In total, 50 nmol L\(^{-1}\) small interfering RNA (siRNA) was used for the specific knockdown of USP2 (Santa Cruz Biotechnology, Dallas, TX, U.S.A.). Silencer select negative control #1 (Ambion, Austin, TX, U.S.A.).

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U.S.A.) was used as the negative control. Keratinocytes were treated with TNF-α (50 ng mL⁻¹; R&D system, Minneapolis, MN, U.S.A.) at the indicated time points. Transfection efficiency for pre-miR-125b was 51.4 ± 13.9-fold. For anti-miR-125b experiments all measurements were below the detectable range of 40 cycle thresholds.

Proliferation assay

5-Ethynyl-2'-deoxyuridine (EdU) was added at a final concentration of 10 μM L⁻¹ to the transfected cells 2 h before harvesting. A Click-it® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Invitrogen) was used according to the manufacturer’s instructions and analysed by flow cytometry (Beckman-Coulter, Fullerton, CA, U.S.A.) to determine EdU-positive cells.

Quantitative real-time polymerase chain reaction

For the quantification of USP2, involucrin, K10, IL-8, chemokine (C-X-C motif) ligand (CXCL)1 and CXCL5, 20 ng cDNA per reaction was amplified in the presence of TaqMan® Universal Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) and TaqMan® Gene Expression Assay: USP2 (Hs00899199-g1), involucrin (Hs 00846307-s1), K10 (Hs01043114-g1), IL-8 (Hs00174103-m1), CXCL1 (Hs00605382-gH), CXCL5 (Hs01099660-g1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [Hs02758991-g1, Hs00605382-gH]. Target gene expression was normalized based on the values of the expression of GAPDH (Applied Biosystems).

Results

Investigation of microRNA-125b targets that are overexpressed in psoriasis

A list of target genes of miR-125b was obtained using the TargetScan software. This TargetScan algorithm searches for binding motifs for specific miRNAs, typically returning hundreds of potential and real target genes. For miR-125b, a total of 202 genes with an aggregate binding score > 0.9 were identified.

To further narrow down the list of potential candidate genes we investigated a publicly available gene expression microarray dataset of psoriatic skin lesions and healthy skin biopsies. We omitted those of the 202 genes that were not upregulated in psoriatic lesions with a minimum significance of P < 0.001 and 2.5-fold upregulation (corresponding to a Bonferroni-corrected P-value of 0.05 for testing 202 genes). This left 57 genes of potential interest, quite likely all having some measure of importance in the interplay of miR-125b and psoriasis. However, for the purpose of this study we chose to continue with USP2. This choice was not based on any further filtering strategies, but rather motivated by its known function as a requirement for TNF-α-induced nuclear factor kappa B (NFkB) signalling. Overexpression of USP2 in microarray data is shown in Figure 1a. To validate the finding of overexpression, we measured USP2 expression using real-time PCR in an independent cohort with psoriatic lesions (n = 25), nonlesional skin from patients with psoriasis (n = 10) and skin from healthy volunteers (n = 22) (Fig. 1b). In accordance with the microarray data, quantitative real-time PCR results showed a significantly increased USP2 level in psoriatic skin when compared with nonlesional psoriatic skin and healthy skin (P < 0.001; Fig. 1b). In addition, we confirmed by immunohistochemistry the presence of USP2 protein in the corresponding frozen samples. Stronger expression of USP2 was observed in the suprabasal layer of psoriasis lesion but not in healthy skin (Fig. 1c).

Investigation of human genetics resources for involvement of USP2 in psoriasis

Using a previously published genome-wide association study of 1446 patients with psoriasis and 1432 healthy controls, we investigated if there were any single-nucleotide polymorphisms (SNPs) associated with psoriasis found near to USP2. The rs10892465 SNP was found to have an association with psoriasis (P = 0.002; Fig. 1d). This was significant at a false-discovery rate of 5%, calculated over the 142 measured SNPs found within a 500-kilobase pair (kb) range of the gene. Additionally, this SNP was found to affect the expression level of USP2 in monocytes. This was done in a set of lipopolysaccharide (LPS) and interferon-stimulated monocytes for which both gene expression and genotypes were available. From the dataset of LPS-stimulated monocytes we observed that the C allele of the SNP for risk of psoriasis caused a slight increase in USP2 expression (P = 0.027; Fig. 1c). On their own, the confidences of these two findings are not sufficiently strong to support USP2 as a candidate psoriasis gene, but taken together and viewed in light of the miR-125b hypothesis, they strengthen interest in further pursuing detailed characterization of USP2 in the pathogenesis of psoriasis.

In vitro confirmation of the link between miR-125b and USP2

To confirm the TargetScan observation linking miR-125b and USP2, we generated a site-specific mutation in the miR-125b binding site. Luciferase reporter assay revealed up to 60% direct inhibition of USP2 by miR-125b. When the predicted miR-125b binding site in the 3’UTR of USP2 mRNA was mutated, luciferase activity was restored (Fig. 2b). We further performed an anti-miR knock-down experiment and a pre-miR overexpression experiment, which showed the expected results: that pre-miR-125b reduced USP2 expression and that anti-miR-125b increased USP2 expression (Fig. 2c, d). Taken together, these experiments solidified the link...
Effects of USP2 and miR-125 on keratinocyte differentiation and proliferation

A hallmark of psoriasis is the increased proliferation and altered differentiation of keratinocytes. Accordingly, we investigated the influence of USP2 on these two aspects. Firstly, the differentiation markers keratin 10 (K10) (Fig. 3b) and involucrin (Fig. 3a) were measured after transfection with siUSP2 at the mRNA level. Knock-down of USP2 significantly increased mRNA expression of both differentiation markers. The induction of early differentiation marker K10 was also shown at the protein level. Only a slight increase was detected in the protein expression of the late differentiation marker involucrin (data not shown).

Secondly, the effect of USP2 knock-down on keratinocyte proliferation was measured. We analysed the cell proliferation rate by an EdU incorporation assay using flow cytometry and gating on live keratinocytes (Fig. 4). This showed that siUSP2 strongly reduced keratinocyte proliferation, particularly at the 48-h time point. As reduced differentiation and increased proliferation is the important cellular state in psoriasis, these two findings are consistent with a hypothesis that USP2 modulation could have a beneficial effect in vivo.

Exploration of USP2 and miR-125b effects on the NFκB signalling pathway

Previously published observations that USP2 works through the NFκB signalling pathway prompted us to investigate the effect of miR-125b and USP2 modulation on characteristic NFκB signalling consequences, namely regulation of IL8, CXCL1 and CXCL5.10 The purpose of this investigation was to gain a better understanding of the direct mechanism through which USP2 exerted any psoriasis-related function. As expected miR-125b knock-down increases expression of these genes, whereas USP2 knock-down decreases expression. miR-125 knock-down is shown in Figure 5a; IL8 expression was

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**Fig 2.** USP2 is the direct target of microRNA miR-125b. (a) Targeting mapping and mutation. DNA sequence alignment of the evolutionary conserved miR-125b binding site in the USP2 3’-untranslated region (UTR) of mammals. The miR-125b seed-match region is highlighted in red uppercase letters. (b) The relative expression obtained when human embryonic kidney 293 cells were transfected with reporter constructs containing wild-type or mutant USP2 3’-UTR or empty luciferase expression vector (vector) together with miR-125b or miRNA control (miR-ctrl). The experiments were repeated three times and a representative result is shown. Each bar represents the mean ± SD for three wells. ***p < 0.001. (c) knock-down and (d) overexpression of miR-125b at different time points in human keratinocytes, followed by real-time polymerase chain reaction measurement of USP2 expression. The experiment was repeated three times and a representative result is shown. Each bar represents the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. (d) Hypothesis on function of miR-125B and USP connection, and planning for subsequent experiments. RU, relative units; NFκB, nuclear factor kappa B.

between miR-125b and USP2, further suggesting the involvement of USP2 in psoriasis.1,5 It has been shown that overexpression of miR-125b prohibits keratinocyte proliferation and promotes keratinocyte differentiation.4 As a target of miR-125b and a pivotal gene in TNF-α-induced NFκB signalling, we therefore hypothesize that knock-down of USP2 has effects on keratinocyte proliferation/differentiation and NFκB signalling (Fig. 2c).
Fig 3. USP2 inhibits keratinocyte differentiation. Human keratinocytes were transfected with small interfering (si)USP2 and siRNA control (con). The cells were harvested for RNA extraction 24 h and 48 h after transfection, followed by real-time polymerase chain reaction measurement of (a) involucrin and (b) keratin 10 (K10). The experiment was repeated three times and a representative result is shown. Each bar represents the mean ± SD. *P < 0.05, **P < 0.01. RU, relative units.

Fig 4. Ubiquitin-specific protease 2 (USP2) enhances keratinocyte proliferation. (a) Human keratinocytes were transfected with small interfering (si)USP2 and siRNA control. The percentage of dividing cells was assessed by anti-5-ethynyl-2'-deoxyuridine (anti-EdU) Alexa Fluor 488 staining 24- and 48-h post-transfection. The representative flow cytometry histograms gated on live propidium iodide (PI)-negative cells are shown and the percentages of EdU-positive cells are indicated. (b) The data from one representative experiment performed in triplicate are shown. The experiment was repeated three times. **P < 0.01. con, control.
Fig 5. USP2 promotes nuclear factor kappa B (NFκB) signalling activation. (a) Expression of IL8, CXCL5 and CXCL1 after knock-down of microRNA (miR)-125b. (b) Expression of IL8, CXCL5 and CXCL1 after knock-down of USP2. The 48-h [tumour necrosis factor (TNF)] group was additionally treated with 50 ng mL⁻¹ TNF-α, to obtain a sufficient basal level of NFκB signalling. (c) Effect of combined knock-down of miR-125b and USP2 in the expression of IL8 and CXCL1 with or without TNF-α stimulation. Human keratinocytes were co-transfected with anti-miR-125b and small interfering (si)USP2 for 24 h, followed by 4 h of TNF-α stimulation. The cells were harvested for real-time polymerase chain reaction detection. All experiments were repeated three times and a representative result is shown. Each bar represents the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 con, control; RU, relative units.
increased 4–8-fold compared with antimiR-con at 24 h post-transfection. CXCL5 and CXCL1 were upregulated threefold and 1–4-fold, respectively. Correspondingly, the downregulation seen with siUSP2 treatment was 3–6-, 1.5- and 3.3-fold for IL8, CXCL5 and CXCL1, respectively (Fig. 5b). Addition of 50-fold miR-125b to keratinocytes also affected levels of IL-8 and CXCL1, although in an upward direction (Fig. 5i; see Supporting Information). The USP2 knock-down experiment was also conducted after TNF stimulation to obtain a sufficient active level of NFkB signalling. The same suppressive effects on NFkB signalling were shown.

Additionally, we performed a combined miR-125b and USP2 double-knock-down experiment to confirm the interaction between the effects of USP2 and miR-125b on NFkB signalling. siUSP2 alone diminished the expression of two NFkB downstream genes to the lowest level. In combination with miR-125b knock-down, expression of IL-8 and CXCL1 was recovered by 21% and 43%, respectively (Fig. 5c). The increase in NFkB signalling activity by anti-miR125b was only observed without TNF stimulation.

Discussion

Starting with the aim of elucidating miRNA-related pathways leading to psoriasis, we investigated potential targets of the known psoriasis-associated miRNA, miR-125b. From a list of 57 mir-125b target genes we selected USP2, fully acknowledging that this was not the only possible candidate. However, according to the notation that solid functional characterization of molecular pathways is nonetheless required, we proceeded to further elucidate the connection between miR-125b and USP2 in psoriasis. Firstly, we firmly substantiated the relationship between miRNA and the gene, using both a luciferase assay and knock-down/overexpression assays. Secondly, we investigated whether modulation of the gene had any direct effect on the cellular events of psoriasis, namely reduced keratinocyte differentiation and increased proliferation. Prompted by siRNA screen findings that USP2 is a required factor for TNF-α-induced NFkB signalling,10 we proceeded to analyse the relationship between USP2, miR-125b and established NFkB-induced genes. Finally, we tested whether miR-125 rescues the decrease in NFkB signalling otherwise caused by USP2 knock-down, which it did. Therefore, our overall conclusion is that one effect of miR-125b is through USP2, then NFkB signalling and, ultimately, towards a psoriasis phenotype.

A central question is how to conclude on findings for separate target molecules for miR-125b, for example fibroblast growth factor receptor and matrix metalloproteinase 13.4,5 Likewise, it is puzzling how both miR-125b knock-down and overexpression can cause increased expression of IL8/CXCL1 [Fig. 5a and Fig. S1 (see Supporting Information)]. It is generally established that each miRNA has many targets.13 Thus, the miRNA-regulated output of a pathway can depend on interaction with different genes with different threshold levels, and resulting complex patterns. It is therefore important to underscore that these results only shed light on the miR-125b-USP2 interaction, and further on the downstream effects of USP2. We are, of course, unable to conclude on all the effects of miR-125b. However, ultimately, understanding the many specific arms of their regulation will lead to increased knowledge about the pathogenesis of diseases.

An additional aspect of our interest in USP2 is the genetic findings showing a slight, but significant, naturally occurring variation of USP2 dependent on a single SNP (rs10892465). Having the high-expression USP2 variant also gives individuals a slightly higher lifetime risk of psoriasis. Expression-level changes from natural genetic variations are much smaller than expression-level changes seen when comparing healthy and diseased tissue. However, a central advantage of these genetic studies is that, unlike disease gene-expression studies, a causality link can be established, following a theory for genetic drug candidate selection that is discussed elsewhere.5,14 The modest strength of the genetic findings is one property of this study that could be improved. However, although larger publicly funded genome-wide association studies have been described, they are not accessible by other researchers.15 Likewise, it would be of interest to investigate if the SNP in question has any influence on the effect of miR-125b on USP2. It is possible that miR-125b-mediated downregulation will be decreased in patients carrying the C allele. Other limitations worth discussing are the purely in vitro set-ups for experimentation. Ideally, a conditional knockout animal model would be created for USP2. However, such undertakings were outside the scope of our resources. However, ultimately, a main interest of all these investigations is the identification of novel drug targets for psoriasis. The current setting is one of unspecific immune system modulators as the first line of treatment, followed by more specific targeting of drugs such as ustekinumab. In this setting, it is beneficial to expand our knowledge towards as many other pathways into disease as possible. This is because a broader range of highly specific drugs is likely to go well together with a future setting in which personalized medicine is much more established. We hope that increased knowledge about the USP2-dependent NFkB signalling pathway will contribute a bit towards that goal.

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References


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Fig S1. Transfection of human keratinocytes.