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Maria do Nascimento Lopes Primo

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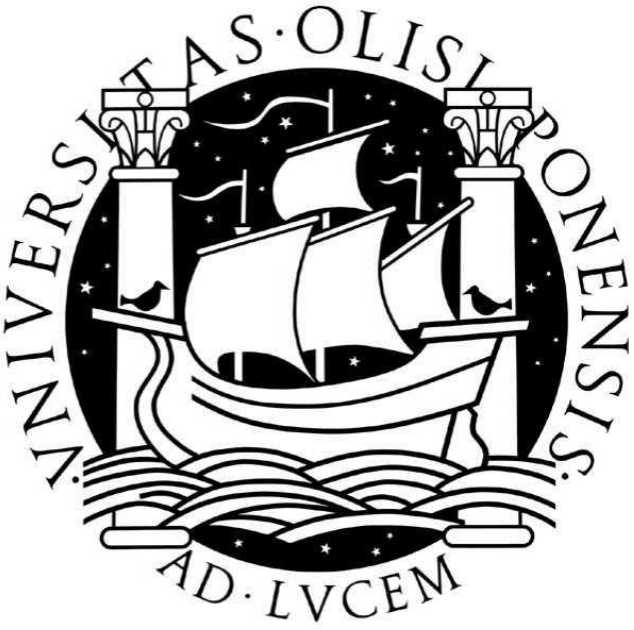

CHARACTERIZATION OF IMMUNE-MODULATORY MIRNAS AND THEIR ROLE IN INFLAMMATORY SKIN DISEASE

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“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”

Marie Curie (French Physicist, 1867-1934)

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To all you I dedicate this paper.

Resumo

A *psoríase vulgares* é uma doença cutânea que ocorre aproximadamente em 3% da população mundial adulta, afectando negativamente a qualidade de vida dos doentes. É uma doença crónica, resultante da activação do sistema imunitário e semelhante a outras doenças auto-imunes como a artrite reumatóide ou a esclerose múltipla. Histologicamente, a psoríase é caracterizada por um aumento da espessura da epiderme devido à hiperproliferação de queratinócitos, resultando no aparecimento de escamas e placas com diferentes tamanhos. Outra particularidade desta doença é o aumento da vascularização da derme, assim como a presença de um elevado número de células imunitárias, tais como linfócitos T e células dendríticas. Ao nível molecular, pensa-se que as manifestações clínicas e laboratoriais descritas são resultantes da desregulação da complexa rede de citocinas devido à acção combinada das células imunitárias que infiltraram a derme e dos queratinócitos presentes na epiderme.

Durante a última década foram identificadas inúmeras espécies de pequenos RNA denominados *small non-coding RNAs*, que contribuem para a intrincada rede de vias que regulam os genes humanos. Entre estes, os microRNA (miRNA) emergiram como reguladores relevantes da tradução proteica, e actualmente acredita-se que os miRNA participam na regulação da maioria dos processos celulares e tecidulares. A pele não é excepção, e de momento os miRNA são intensamente estudados para determinar a sua relevância na regulação da homeostase da pele e no aparecimento de doenças cutâneas.

O possível envolvimento de miRNA na patologia da psoríase foi identificado pela primeira vez em 2007, quando a equipa liderada por Sonkoly comparou os perfis de expressão de miRNA em pele psoriática e em pele normal. Entre os miRNA identificados, potencialmente associados à psoríase, o miRNA-203 foi identificado predominantemente em queratinócitos e sobre-expressado em psoríase, quando comparado com a pele normal. Recentemente, a mesma equipa científica demonstrou também que o miRNA-203 e o transcrito de um componente regulador da sinalização da resposta imunitária, o SOCS3 (*supressor of cytokine signalling 3*), apresentam níveis de expressão inversamente relacionados. Sabe-se ainda que a proteína SOCS3 regula negativamente a activação de um factor de transcrição – *STAT3* (*signal transducer and activator of transcription 3*) – que está sobre-expressado em psoríase e implicado na rede de citocinas. Assim, a sobre-expressão do miRNA-203 em psoríase pode ter importantes implicações na patologia da doença uma vez que impossibilita a expressão de SOCS3, proteína que regula negativamente a rede de citocinas.

O objectivo principal deste projecto foi descrever a interacção entre miRNA e a expressão de citocinas em pele humana. Com especial interesse no miRNA-203, o projecto procurou elucidar a função específica dos miRNA moduladores da resposta imunitária na complexa rede de interacções moleculares, levando ao desenvolvimento da doença inflamatória na pele. No começo deste projecto, procurou-se estabelecer um sistema *in vitro* que permitisse a identificação e análise de potenciais genes-alvo de miRNA associados à psoríase. Assim, através da utilização da proteína *Renilla luciferase* (*R-luc*), foi desenvolvido um ensaio repórter para a detecção de interacções directas entre o miRNA-203 e diversos elementos da resposta imunitária, através da medição de bioluminescência. Os DNA complementares (cDNA) de seis diferentes citocinas (IL12B, IL15, IL17, IL20, IL24 e TNFα) assim como os cDNA de proteínas supressoras da sinalização das citocinas (SOCS3 e SOCS6) foram fundidos ao gene codificante da *R-luc*, e as medidas de bioluminescência foram avaliadas quando os vectores codificantes da *R-luc* foram co-transfectados com um vector expressando o miRNA-203 em células HEK293 (*Human kidney embryo cells*). Dos ensaios desenvolvidos, identificaram-se três potenciais genes-alvo do miRNA-203: *IL24*, *SOCS6* e *TNFα*.

Para a confirmação da interacção directa entre o miRNA-203 e os genes-alvo mencionados foi desenvolvido um estudo bioinformático para a identificação de sequências nucleotídicas na região 3'UTR dos genes-alvo complementares à sequência nucleotídica do miRNA-203. Uma vez identificadas as possíveis sequências nucleotídicas que permitem o estabelecimento de interacções entre o miRNA-203 e os RNA mensageiro (mRNA) dos genes-alvo, as sequências identificadas nas regiões 3'UTR foram mutadas de forma a inibir as interacções miRNA-mRNA. Os transcritos 3'UTR mutantes dos genes *IL24, SOCS6* e *TNFα* foram fundidos ao gene codificante da *R-luc* e novos ensaios repórteres foram elaborados. Em paralelo, procedeu-se também ao desenvolvimento de uma linha celular de queratinócitos (denominada HaCaT-203), através da utilização do sistema transposão de DNA "*sleeping beauty*", para expressar constitutivamente o miRNA-203. Esta nova linha celular de queratinócitos foi especialmente desenvolvida para a confirmação de potenciais genes-alvo do miRNA-203, através da quantificação dos níveis de expressão dos genes-alvo por qRT-PCR. A quantificação e a análise dos valores de bioluminiscência obtidos a partir da expressão do gene da *R-luc* fundido aos transcritos dos três 3'UTR mutantes de IL24, SOCS6 e TNFα identificaram a interacção directa entre os três mRNA e o miRNA-203. Resultados semelhantes foram obtidos através da quantificação dos níveis endógenos dos mRNA de IL24, SOCS6 e TNFα em queratinócitos, confirmando a especificidade da interacção entre o miRNA-203 e os genes-alvo mencionados. A identificação destes novos genes-alvo veio contribuir para a compreensão e caracterização das implicações do miRNA-203 na expressão de citocinas em pele humana. Não menos importante, também foi o facto de que os resultados obtidos

vieram consolidar a hipótese de que o miRNA-203 poderá ter implicações na patologia da psoríase uma vez que o miRNA-203 foi identificado como um modulador activo na complexa regulação da rede de citocinas em queratinócitos.

A equipa com a qual foi desenvolvido este projecto documentou anteriormente a aplicabilidade terapêutica da utilização de lentivirus para a inibição dos mRNA de TNFα e IL12B através da "entrega" (*delivery*) de RNA efectores anti-TNFα e anti-IL12B, respectivamente, em pele psoriática humana xenotransplantada em ratos imunodeficientes (*xenografted psoriatic skin*). No projecto actual, foi desenvolvido um novo vector lentiviral codificando um inibidor de miRNA específico para o miRNA-203 (denominado *antagomiR-203*). Os RNA efectores anti-miRNA (*antagomirs*) são caracterizados por sequências oligonucleotídicas complementares aos miRNA de interesse. Assim, os antagomir estabelecem interacções específicas com o miRNA-alvo, conduzindo à inibição funcional dos mesmos. Através da utilização do vector lentiviral codificando o antagomiR-203, vários ensaios *in vitro* foram desenvolvidos para testar a funcionalidade e a potência do antagomiR-203. Com base nas avaliações funcionais, identificou-se uma nítida sobre-expressão do gene da *R-luc* quando fundido com uma sequência oligonucleotídica complementar ao miRNA-203, quando co-expresso com o vector lentiviral codificando o antagomiR-203. Foi também identificada uma redução dos níveis de expressão do miRNA-203, quando as linhas celulares de queratinócitos foram infectadas com partículas lentivirais expressando o antagomiR-203. Os resultados obtidos confirmaram assim a funcionalidade e especificidade do antagomiR-203 relativamente ao miRNA-203, permitindo o estabelecimento de uma nova plataforma para a regulação de miRNA através da utilização de lentivirus como veículo de transporte de anti-miRNA.

Com base na tecnologia de expressão de antagomir, este projecto procurou ainda abrir caminho para os estudos de gene-alvos endógenos, permitindo experimentalmente estudar a função de miRNA em queratinócitos em pele normal e pele psoriática. Como objectivo final deste projecto, testou-se a aplicabilidade terapêutica da utilização de lentivirus para a "entrega" de RNA efectores anti-miRNA-203 em pele psoriática humana xenotransplantada em ratos imunodeficientes. A administração intradérmica de uma única dose de partículas lentivirais em pele psoriática humana resultou num aumento dos níveis de expressão do miRNA-203 e na não alteração da espessura da epiderme em enxertos de pele tratados com o antagomiR-203. Em conformidade com os resultados obtidos, a avaliação clínica do fenótipo psoriático dos enxertos de pele não identificou nenhuma melhoria clínica e histológica do fenótipo psoriático no ensaio desenvolvido.

O potencial para a concepção de medicamentos moleculares baseados na modulação de miRNA endógenos é muito ambicionado, mas actualmente está ainda muito inexplorado. Os

estudos desenvolvidos ao longo deste projecto demonstraram que o aumento da expressão do miRNA-203 na patologia da psoríase é complexo, claramente evidenciando a necessidade de estudos futuros para uma melhor compreensão das funções desempenhadas pelos miRNA na regulação da resposta imunitária. O projecto realizado procurou também explorar a aplicabilidade terapêutica de fármacos dirigidos aos miRNA, tendo-se identificado algumas dificuldades relacionadas com a funcionalidade dos antagomirs *in vivo*. No entanto, é de salientar que a abordagem utilizada neste estudo foi única, na medida em que se procurou explorar uma nova metodologia de administração de antagomirs, permitindo a entrega de material genético em tecidos específicos, de modo a aumentar a segurança na utilização de terapia genética em humanos.

Palavras-chave: miRNA-203, psoríase, rede de citocinas, vectores lentivirais, antagomirs

Observação: Resumo escrito de acordo com a antiga ortografia.

Abstract

Psoriasis vulgaris is a chronic inflammatory skin disease which is characterized by an excessive growth of skin epithelial cells, increased dermal angiogenesis and infiltration of immune cells into the skin. The past decade has unveiled a plethora of small RNA species that contribute to the intricate network of pathways regulating our genes. Among these, microRNAs have emerged as key regulators of translation and are believed to play a role in almost any cellular process and tissue. One of the most upregulated microRNAs in psoriasis skin is miRNA-203. The aim of this study was to describe the interplay between microRNAs, with focus on miRNA-203, and cytokine-encoding mRNAs in human skin. Based on the v-antagomir expression technology, the project may pave the way for studies of endogenous targets, allowing us to experimentally address microRNA function in keratinocytes and skin inflammation.

Three components of the cytokine circuit, interleukin-24, suppressor of cytokine signaling-6 and tumor necrosis factor-α, were identified as direct targets for suppression by miRNA-203 by luciferase reporter assay. In the present study, we also used lentiviral vectors as potent carriers of antagomir-encoding gene cassettes. Lentiviral vectors are attractive gene vehicles primarily due to their ability to establish persistent expression owning to genomic integration of the vector DNA reverse-transcribed from virally delivered single-stranded RNA. Potent and persistent knockdown of miRNA-203 expression following transduction of lentiviral vectors encoding antagomiR-203 in keratinocytes was reported. In contrast to the data collected *in vitro*, miRNA-203 knockdown was not identified *in vivo* after a three week treatment of xenografted psoriatic skin with lentivirus-encoded antagomiR-203.

Our studies consolidate the properties of lentiviral vectors as a tool in experimental dermatology with particular significance for cutaneous RNA managing and *in vivo* genetic intervention. However, the therapeutic potential of targeting miRNA-203 in psoriasis is here questioned.

Key words: miRNA-203, psoriasis, cytokine network, lentiviral vectors, antagomirs

List of manuscripts

This thesis is based on the following papers, which are referred to by their Roman numbers in the text:

I. **Primo MN***, Bak RO, Mikkelsen JG. Lentiviral vectors for cutaneous RNA managing. *Review article submitted to Experimental Dermatology on 22nd of September 2011.*

II. **Primo MN***, Bak RO, Mikkelsen JG. Managing miR-203 in keratinocytes demonstrates roles in cytokine regulation. *Manuscript in preparation.*

* Corresponding author

Abbreviations

293T cells	Variant of Human kidney embryo cells
Ago	Argonaute
Bp	Basepair
cDNA	Complementary DNA
CMV	Cytomegalovirus
DGCR8	DiGeorge syndrome critical region gene 8
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
FGFR2	Fibroblast growth factor receptor 2
G418	Geneticin (aminoglycoside antibiotic)
GW182	TNRC6A trinucleotide repeat containing 6A
HaCaT cells	Human keratinocyte cells
HEK293 cells	Human kidney embryo cells
HeLa cells	Human cervical adenocarcinoma cells
HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
LV	Lentiviral vector
miRNA	MicroRNA
MLV	Moleney murine leukemia virus
MOI	Multiplicity of infection
mRNA	Messenger RNA
mSB	Mutant sleeping beauty transposase
Neg	Negative Control
Neo	Neomycin
NF	Nuclear factor
PACT	Protein activator of PKR
PCR	Polymerase chain reaction
pri-miRNA	Primary microRNA
R-luc	Renilla luciferase
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RPLP0	Ribosomal protein, large P0
qRT-PCR	Real time reverse transcriptase polymerase chain reaction
RU48	Small nucleolar RNA, C/D box 48
SB	Sleeping beauty transposase
SCID	Severe immunodeficient mice
SEM	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activation of transcription
SV40	Simian virus 40
TLR	Toll-like receptor
TNF	Tumor suppressor factor
TRBP	Tar RNA binding protein
UNG	Uracil N-glycosylase
UTR	Untranslated region
XPO5	Exportin 5

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Introduction

Psoriasis vulgaris is a common inflammatory skin disease which is characterized by excessive growth of skin epithelial cells, increased dermal angiogenesis and infiltration of immune cells into the skin leading to focal formation of inflamed, scaly skin lesions. Recent evidence proposes that miRNAs are involved in immune system regulation and that miRNA levels are regulated upon stress induction. One of the most upregulated miRNAs in psoriatic skin is miRNA-203. The aim of this study was to describe the interplay between miRNAs, with focus on miRNA-203, and cytokine-encoding mRNAs in human skin.

Psoriasis vulgaris, a disease-model in skin inflammation

Pathogenesis of psoriasis

Psoriasis vulgaris is one of the most common chronic inflammatory skin disorders affecting approximately 3% of the population in Europe and North America. It is an organ-specific autoimmune disease that is triggered by an activated cellular immune system [1; 2]. The histological changes observed within lesional skin are striking, and include (1) a thickened epidermis from rapid keratinocyte proliferation, (2) a reduced or absent granular layer, (3) marked dilatation of blood vessels in the papillary dermis, and (4) dense clusters of infiltrated mononuclear leukocytes (T-cells and dendritic cells) into the dermis (Figure 1) [3; 4]. Cytokine interactions in psoriasis have previously been illustrated as 'type-1 pathway', which assumes a linear relationship between proximal inducers (IL23 and IL12), production of IFNγ and TNFα by type-1 T-cells, and downstream activation of numerous IFN-responsive genes through STAT1 [4].

However, it is now known that this model accounts for only a small fraction of the inflammatory circuitry in psoriasis. Figure 1 represents an alternative view of the cytokine interactions in psoriasis, which is more of a network or interactive model [5]. Following a stimulus, such as infection or stress in genetically predisposed individuals, resident T-cells become activated through interaction with resident dendritic cells and macrophages, leading to the production of numerous cytokines, such as TNFα and IL23. Depending on the exposure to different cytokines, naïve T-cells may develop into one of the at least five different CD4+ T-cell lineages. T-cell activation leads to activation of STAT1, STAT3 and NF-κB transcription factors which will be involved in amplifying inflammation process, resulting in upregulation of several cytokines as TNFs, IL1, IL6, IL12, IL17, IL20, IL22, IL24 and IFNs [6; 7]. Keratinocytes are then involved in the cytokine-mediated inflammation by responding to and producing cytokines, which have several functions including promotion of angiogenesis, amplification of immune cell trafficking and immune cell adhesion to endothelial cells [8]. Both events will contribute to the start of a vicious cycle of inflammation within lesional skin which results in epidermal re-

modeling with altered proliferation and differentiation of keratinocytes, culminating in the formation of psoriatic plaques.

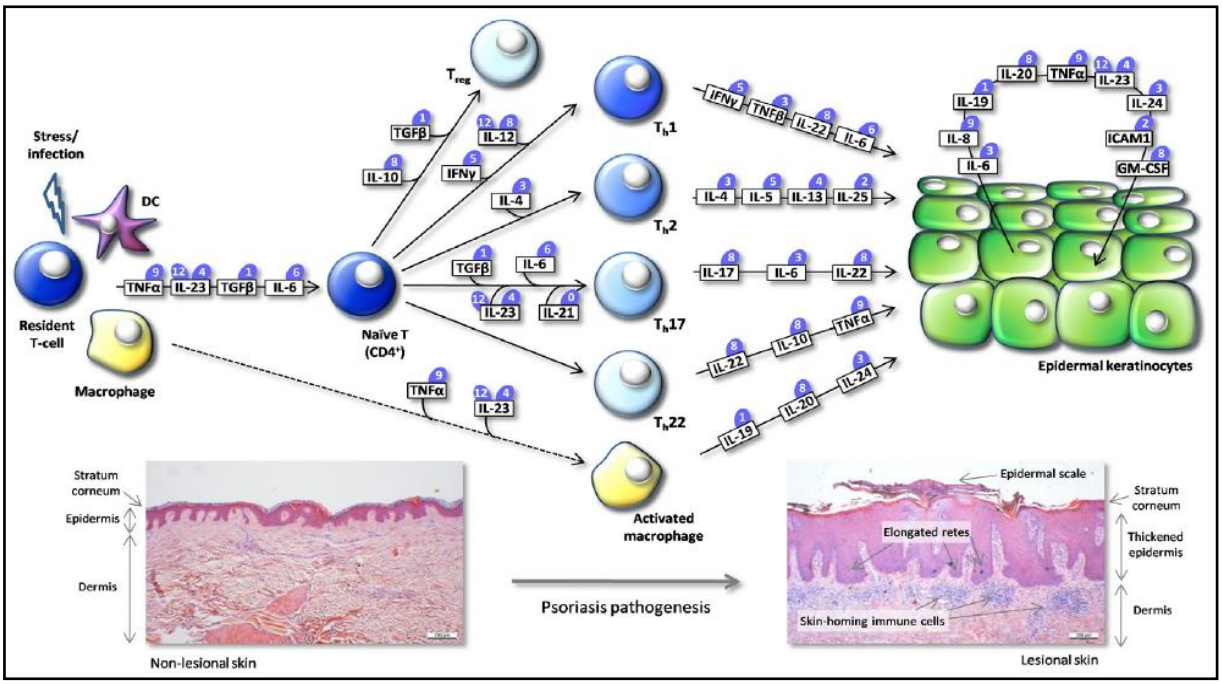

Figure 1. Schematic representation of the cytokine network driving development of psoriasis. Cytokines mediate intercellular communication between skin-infiltrating activated immune cells and epidermal keratinocytes. Following a stimulus, resident T-cells and antigen-presenting cells are activated leading to production of cytokines. Naïve $CD4^+$ T-cells undergo differentiation upon exposure to different cytokine milieus, which produce a subset of cytokines that stimulate epidermal keratinocytes. This leads to production of an additional set of cytokines triggering epidermal remodeling through altered keratinocyte growth and differentiation as well as angiogenesis. *Adapted from Bak et al, 2010.*

Non-coding RNAs: Biogenesis pathways and function

miRNAs comprise a large family of non-coding and single stranded RNAs of approximately 22 nucleotides, which are estimated to regulate up to one-third of human genes via either translational repression or mRNA degradation [9; 10; 11]. miRNAs regulate expression of their target mRNAs through RISC, which is composed of Ago family proteins as the core component [12; 13; 14]. Functional miRNA binding sequences are frequently located in the 3' UTR of the target mRNA, but can also exist within the 5' UTR or coding region. The 5' end of the miRNA (nucleotide 2-7), termed 'seed region', is partially important for mRNA repression by miRNA [15; 16]. When only seed region binds to the mRNA target sequence, two core components of the RISC, namely Ago (Ago-1 to Ago-4) and GW182, facilitate gene suppression mainly by recruiting mRNA destabilizing factors or by repressing translation [10; 17; 18; 19; 20]. However, full or near-full sequence complementarity between the miRNA and the mRNA target sequences may occur, resulting in mRNA cleavage facilitated by Ago-2 [14]. miRNAs are encoded by genomic DNA and are transcribed by RNA polymerase II into pri-miRNAs transcripts, which are usually several kilobases long [21; 22; 23] (Figure 2).

Some miRNA-containing primary transcripts produce a single miRNA, whereas other miRNAs are grouped in clusters on a single unprocessed transcript and are expressed together as polycistronic primary transcripts. The pri-miRNAs fold into hairpins and, next, the stem-loop structure is endonucleolytically cleaved by the nuclear microprocessor complex formed by the RNase III Drosha and its cofactor, DGCR8 [24; 25; 26]. The two RNase domains of Drosha cleave the 5' and 3' arms of the pri-miRNA hairpin, whereas DGCR8 functions as a molecular ruler to determine the precise cleavage site, resulting in the release of ~70-nucleotide pre-miRNAs, which fold into imperfect stem-loop structures [24; 25; 27; 28; 29]. Recently, it has been identified in mammals that Drosha-mediated processing of pri-miRNAs into pre-miRNAs is not obligatory [30] (Figure 2). These miRNAs have been denominated 'mirtrons', as they are introns-derived miRNAs which are released from their host transcripts after splicing [31; 32]. If the introns resulting from the action of the splicing machinery have the appropriate size to form a hairpin structure resembling a pre-miRNA, it bypasses Drosha cleavage and

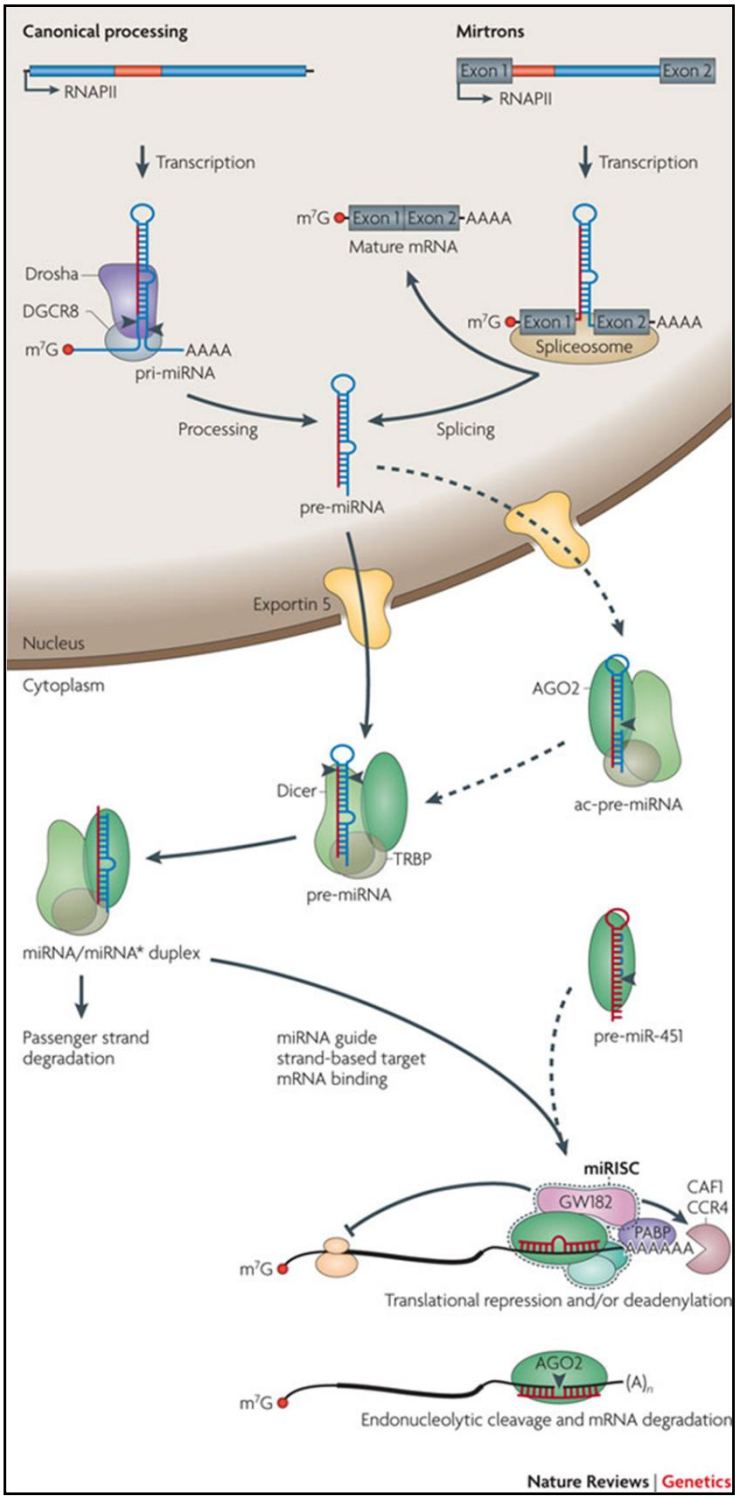

Figure 2. Schematic representation of miRNA biogenesis. The miRNA processing pathway has been long viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of pri-miRNA transcripts by RNA polymerase II and cleavage of pri-miRNAs by the microprocessor complex Drosha-DGCR8 in the nucleus. The resulting precursor hairpin is exported from the nucleus by Exportin-5-Ran-GTP. In the cytoplasm, the RNase Dicer in complex with dsRNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Ago proteins into the RISC, where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation. *Adapted from Krol et al, 2010.*

is further processed in the cytoplasm. After nuclear processing, pre-miRNAs are exported into the cytoplasm through nuclear pore complexes, by a RanGTP-dependent double stranded DNA-binding protein, the XPO5 [33; 34; 35; 36].

On reaching the cytoplasm, the cytoplasmic RNase III Dicer cleaves off the loop of the pre-miRNAs, generating mature ~22-nucleotide miRNA duplexes with two nucleotides protruding as overhangs at each 3'end [37; 38; 39]. Dicer-mediated cleavage of the pre-miRNA is facilitated by the double-stranded RNA-binding domain proteins TRBP and PACT, which in association with Ago2 compose the multi-protein RISC loading complex [40; 41; 42; 43]. After Dicer-mediated cleavage, Dicer and its interactors, TRBP and PACT, dissociate from the miRNA duplex. To form the active RISC that performs gene silencing, the double-stranded RNA duplex needs to be separated into (i) the functional guide strand, which is complementary to the mRNA target and guides RISC to silence target mRNAs and (ii) the passive strand, which is subsequently degraded. Studies on small siRNA duplexes indicate that the relative thermodynamic stability of the basepairs at the two ends of the duplex determines which strand is loaded into RISC - the miRNA strand which is less stable at the 5' end is loaded into the RISC [44].

In 1998, Fire and Mello discovered the RNA interference pathway which showed that exogenously derived dsRNA could give rise to sequence-specific degradation of RNA transcripts with complementary sequence, with resemblance to miRNA-mediated RNA regulation [45]. Since then, it has been firmly established that components of the cellular RNAi machinery are shared by both pathways. Exogenous dsRNA or stem-loop structured RNAs, either artificially introduced into cells or originating from viral dsRNA, are recognized by Dicer as pre-miRNAs and cleaved into mature ~22 nucleotides siRNAs from which one of the strands is incorporated into RISC. siRNA duplexes are usually asymmetric according to their structures and thermodynamic stability, in order to favor only one specific strand of the small RNA duplex assembled into RISC components, ultimately giving rise to sequence-specific cleavage of target RNA with complementary sequence [46]. Approaches to induce miRNA loss of function also represent a powerful functional genomic tool. *In vitro* chemically modified miRNA inhibitors, such as 'antagomirs' and miRNA sponges in mammalian cells have recently proved to be effective in blocking functions of specific miRNA families [47; 48]. Based on antisense strategy, oligonucleotides complementary to miRNAs act as competitive inhibitors of endogenous mRNAs to bind to miRNAs, which lead to suppression of miRNA functions. The addition of these unique technologies to the molecular toolbox has allowed researchers to study small RNAs, individual gene functions, functional genomics, and various biological questions in both plants and animals.

Involvement of miRNAs in psoriasis

The first study regarding miRNA expression in psoriasis was reported in 2007 by Sonkoly and colleagues. In this study they have identified a specific miRNA expression profile in psoriasis-affected skin, when compared with healthy skin [49]. Among the psoriasis-specific miRNAs, they have identified upregulation of miR-21, miR-146a and miR-203 expression patterns, in contrast to miR-125b expression pattern that was shown to be downregulated. Moreover, they have shown that miR-21 and miR-125b were expressed by structural and inflammatory cells, whereas miR-146a was preferentially expressed by immune cells and miR-203 showed a keratinocyte-specific expression pattern.

miR-146a was one of the first miRNAs identified to be involved in the regulation of immune functions [50]. Taganov and co-workers have shown that miR-146a expression is induced after Toll-like receptor activation in an NF-κB-dependent way. Induction of miR-146a was identified as a negative regulator of innate immune responses since it was observed that miR-146a potentially targeted tumor necrosis factor receptor-associated family-6 and interleukin-1 receptor-associated kinase, which are both regulators of the NF-κB signalling pathway. Besides miR-146a, miR-21 is also a central player in many inflammatory pathways, including in Toll-like receptor signalling. In a recent report by Sheedy and colleagues, it was found that the control of the tumor suppressor PDCD4 expression is crucial in the negative regulation of the inflammatory response to lipopolysaccharide, acting as a molecular switch between the pro-inflammatory (NF-κB) and anti-inflammatory (IL10) response [51]. This switch was identified as being controlled by the activation of miR-21, resulting in a decrease in PDCD4 protein abundance. This process positively influences IL10 production, leading to inhibition of the NF-κB signaling pathway. Together with the fact that both miR-146a and miR-21 are induced by resolving D1, an anti-inflammatory and pro-resolving lipid molecule, these data lend further support to their roles as negative regulators of the inflammatory response [52].

miR-125b has been shown to be expressed in the majority of human organs, in which this particular miRNA may play several roles in pathological and physiological processes [53], in some cases with potential implications for carcinogenesis [54; 55]. Concerning psoriasis, a recent publication by Sonkoly *et al.* suggests that miR-125b may play a role in the regulation of keratinocyte proliferation and differentiation, partially through regulation of *FGFR2* [56]. According to collected data, reduced levels of miR-125b in primary keratinocytes result in increased FGFR2 expression, which partially contributes to hyperproliferation and aberrant differentiation of keratinocytes in psoriasis.

Since miR-203 was identified as being a skin-specific miRNA, numerous studies have been developed for identification and characterization of miR-203 targets. In 2008, Yi and col-

leagues looked at miRNA frequencies in epidermal miRNA libraries and identified increased expression of miR-203 during skin development, promoting epidermal stratification and differentiation [57]. Further studies have recently demonstrated that increased expression of miR-203 is required for human keratinocyte differentiation, supporting the crucial role of miR-203 during the entire process of epidermal development [58; 59]. In agreement with these observations, miR-203 was identified to negatively regulate the transcription factor p63, which is involved in signaling pathways for stem-cell maintenance and epidermal stratification [60; 61]. Besides, miR-203 has recently been identified as a possible player in the regulation of the cytokine network in keratinocytes. miR-203 and SOCS3, which is a negative regulator of STAT3 pathway, were shown to have reciprocal expression patterns in psoriatic skin [59; 62]. As mentioned before, the STAT3 pathway is activated by inflammatory cytokines and has important functions in the regulation of both innate and adaptive immunity. In a supporting study, it was observed that constitutive activation of this pathway led to a psoriasis-like phenotype in mice and that inhibition of STAT3 ameliorated the psoriasis-associated pathological conditions [63]. Therefore, it appears reasonable to hypothesize that overexpression of miR-203 may contribute to maintenance of skin inflammation in response to T-cell-derived cytokines due to an aberrant negative feedback in cytokine signaling in keratinocytes.

Over the last year, other miRNAs such as miR-19a, miR-99a and miR-424 have been associated to pathogenesis of psoriasis, either through comparison of miRNA expression in normal skin versus psoriatic involved and uninvolved skin [64; 65] or through examination of miRNA levels in serum samples from psoriasis patients [66; 67]. In the years to follow, it is critical to focus on the cytokine network and its relevance in disease pathogenesis as it is still unclear whether deregulation of miRNAs in psoriasis is a result of unbalanced cytokine signaling or a causative reason for disease development.

Cutaneous gene delivery

As the most accessible of body organs, the skin is an attractive tissue for direct gene delivery and gene therapy applications, which can give rise to restoration, correction, augmentation, or inhibition of gene function. In addition to easy administration, the skin is an easy organ for clinical observation following gene transfer, with uncomplicated removal of genetically engineered tissue if necessary, thereby enhancing its applicability in gene therapy [68].

As the skin provides a primary barrier to microbial invasion and desiccation, cutaneous tissue poses substantial obstacles to effective insertion of foreign DNA. The two basic approaches for gene delivery in skin are based on *ex vivo* and *in vivo* gene delivery (Figure 3).

For *ex vivo* gene delivery, target cells are isolated from skin biopsies and treated with gene vehicles ('vectors') in culture prior to re-engraftment of the tissue into the patient. In contrast, for *in vivo* approaches, which are often considered more straight-forward, the gene-carrying vector is directly administered to skin. Direct administration has been undertaken using a variety of approaches with both nonviral and viral vectors, including topical application, direct injection, application to wounded skin surfaces, electroporation and bioplastic particle insertion [68].

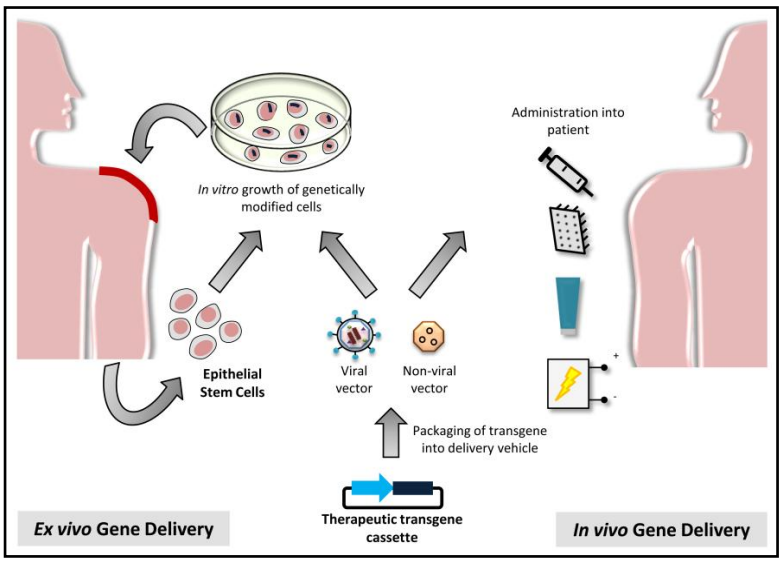

Figure 3. Approaches for cutaneous gene delivery. The two basic approaches to therapeutic gene delivery in skin involve *ex vivo* and *in vivo* gene delivery. *Ex vivo* delivery is a relative complex procedure, which can be divided into three major steps. In the first step, cells from the patient are isolated and propagated in the laboratory. Then, the therapeutic transgene cassette is packaged into lentiviral expression delivery vectors and the patient cells are transduced with lentiviral particles. Finally, the genetically-modified cells are grown in culture followed by re-grafting to the patient. In contrast to *ex vivo* cutaneous gene delivery, *in vivo* gene transfer delivers genetic material directly to the patient skin tissue and is thus generally simpler. Direct administration has been undertaken using a variety of approaches with lentiviral vectors, including direct injection, topical application and microneedle electroporation. *Adapted from manuscript Primo et al, 2011.*

Lentiviral gene delivery to skin

The transfer of genetic material to skin is facing the same challenges as conventional drug types and therefore requires penetration-enhancing carriers or physical methods to overcome the barriers of the skin. Viruses have through evolution developed and refined the capacity to carry and transfer genetic material between cells and have in recent years been extensively explored as vehicles for nucleic acids. Retroviral vectors are attractive tools for human gene therapy as they have evolved specialized molecular mechanisms to stably integrate into the chromosomes of their targets, a likely requisite for long-term expression. However, the use of retroviruses such as the MLV-derived retroviral vector involves the requirement of the breakdown of the nuclear membrane, which makes them capable of transducing only proliferating cells [69]. Lentiviruses, another subclass of retroviruses, have recently been adapted as gene delivery vehicles. Opposed to MLV-derived vectors, lentiviral vectors are capable of transducing both dividing and non-dividing cells due to interactions with the nuclear import machinery of the target cells. Illustrating these properties, vectors derived from HIV-1 allow for the efficient delivery, integration, and stable expression of transgenes into cells such as neurons, hepatocytes, and myocytes [70; 71; 72; 73; 74; 75; 76].

The extensive studies of HIV-1 in relation to the global HIV pandemic have created a detailed knowledge of lentiviral replication and biology and have made HIV-1 the preferred parental virus for development of LVs. Since the first pioneering reports on HIV-1-based vectors back in the mid-1990s, several increasingly safer generations of the vector system have appeared. State-of-the-art vectors are today self-inactivating replication incompetent vectors that harbor multiple modifications for optimized safety. These engineered vectors do not contain viral protein-encoding information required for viral replication and are restricted, therefore to a single infection cycle. Instead, genes encoding the viral structural and enzymatic proteins are provided on three separate plasmid constructs (packaging vectors) that are simultaneously supplied to cells that will subsequently produce virus particles. Moreover, removal of the parental viral promoter from the vector construct ensures that transgene expression is driven from an internal promoter and that potential adverse effects on neighboring genomic DNA are limited.

In 2001, Baek and co-workers developed one of the first studies using HIV-derived vectors for gene delivery in human skin [77]. They showed that after a single intracutaneous injection into full-thickness human skin grafts on immunodeficient mice, LVs encoding human erythropoietin produced dose-dependent increases of human erythropoietin levels in serum, which remained stable subsequently. Additionally, it was shown that removal of the skin graft led to rapid and total loss of human erythropoietin, confirming that the injected virus was exclusively targeting the cells in the skin graft and elegantly demonstrating a reversible approach for gene-based delivery of therapeutic proteins to the bloodstream. Since then, different studies using lentiviruses as gene delivery vehicles have been successfully developed in monogenic skin diseases with an autosomal recessive mode of inheritance, such as the tumor-prone Xeroderma Pigmentosum and the inherited Epidermolysis Bullosa [78; 79; 80; 81].

An extensive number of *in vivo* studies using LVs to express shRNAs have been performed to evaluate the effectiveness of RNAi in the treatment of cancer, cardiac disease, retinal disease, neurodegenerative disease, virus infections and other diseases. The properties of LVs as facilitators of persistent transgene expression in skin have prompted the idea of utilizing viral vectors as vehicles of DNA-encoded small RNA effectors. The first *in vitro* and *in vivo* studies for possible future cutaneous siRNA treatments using LVs for transgene delivery started in 2006 concerning a possible treatment for melanoma [82]. Since then, other *in vivo* studies have been developed for cutaneous siRNA treatments including two studies from Mikkelsen research group, which have aimed at targeting central cytokines for amelioration of psoriasis [83; 84]. Potent and persistent transgene expression following a single intradermal injection of LVs in xenografted human skin was reported in both studies, consolidating the properties of lentiviral vectors as a valuable tool for cutaneous gene delivery.

Aim of the present work

Inflammation involves a well-coordinated response, which includes the activation of several hundred genes including multiple cytokines, chemokines, matrix remodeling proteases, reactive oxygen, nitrogen species and others. The observation that miRNAs are likely regulators of cytokine mRNAs suggests that they might be involved in diseases related to abnormal immune responses including certain inflammatory disorders [85]. Psoriatic skin is characterized by a specific miRNA expression profile that is different from healthy skin and skin affected by other chronic inflammatory diseases, such as atopic eczema. The keratinocyte-specific miR-203 may play a role in the unbalanced cytokine signaling network in psoriasis as it is known to be upregulated in psoriatic plaques, leading to a concurrent downregulation of SOCS3 protein translation.

The main goal of this project was to demonstrate the roles played by miRNAs in the complex network of regulatory pathways controlling the production of cytokines in human skin. With focus on miR-203, this work combined three major objectives:

- Establishment of lentiviral vector-encoded antagomir production for specific miR-203 inhibition in human cell lines and *in vivo* studies;
- Analysis of expression of miRNAs with a putative role in cytokine regulation;
- Identification and analysis of miR-203 target genes in human skin.

With a deeper understanding of RNAi and continued progress in designing more safer and effective RNAi effectors, lentiviral vector-mediated RNAi has the potential to change the way that numerous cutaneous diseases are studied and treated. As a result, the outlined studies tried to explore the applicability of miRNA-directed drugs and to address some of the main hurdles related to specificity of miRNA inhibitor specificity and the capacity of miRNAs to regulate numerous genes.

Materials and Methods

Plasmid construction

Cloning of H1-antagomir expression cassettes

pCCL-PGK-Puro-H1-MCS (designated pLV/vehicle; see Appendix II, Figure AI) was constructed as reported by Bak *et al.* [83]. Oligonucleotide designed was performed as described in Scherr *et al.* [86] . For each of the antagomir expression constructs, pCCL-PGK-Puro-H1-AntagomiR-203 and –Negative control (neg), complementary sense and antisense oligonucleotides (see Appendix I, Table 1) were annealed by incubation at 100°C for 5 minutes followed by room temperature cooling. The annealed oligonucleotides were designed to leave overhangs for cloning into the *AvrII/AscI*-digested pCCL-PGK-Puro-H1-MCS vector.

Cloning of miR-203 targets into psiCHECK2

To generate psiCHECK2-gene targets (see Appendix I, Table 2), mRNA was isolated from HaCaT cells stimulated with anisomycin (500µg/mL) using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer's protocol. The mRNA was reverse-transcribed into cDNA using iScript™ cDNA Synthesis Kit (BIO-RAD, Hercules, CA) according to manufacturer's protocol. All target genes were amplified by PCR using gene-specific primers (see Appendix I, Table 3), which were made with restriction site containing linkers (*XhoI/NotI*). The generated fragments were digested with *XhoI/NotI* and inserted into *XhoI/NotI*-digested psiCHECK2 (Promega, Madison, WI).

To generate miR-203-perfect target and miR-125b-perfect target constructs (psiCHECK2-miR-203 and psiCHECK2-miR-125b, respectively), complementary sense and antisense oligonucleotides (see Appendix I, Table 4) were annealed by incubation at 100°C for 5 minutes followed by room temperature cooling. The annealed oligonucleotides were designed to leave overhangs for cloning into the *XhoI/NotI*-digested psiCHECK2 (Promega, Madison, WI).

Generation of 3'UTR mutated sequences and cloning into psiCHECK2

To generate IL24, SOCS6 and TNFα 3'UTR mutants, self-complementary DNA oligonucleotides encompassing the sequences of IL24-, SOCS6- and TNFα-mut 3'UTRs were chemically synthesized with 5' *XhoI* and a 3' *NotI* restriction sites (GenScript, Piscataway, NJ, USA). The sequences are shown in Appendix III-V. The generated fragments were digested with *XhoI/NotI* and inserted into *XhoI/NotI*-digested psiCHECK2 (Promega, Madison, WI).

Generation of pri-miR-203 and pri-miR-125b-expressing constructs

The vector encoding pri-miR-203 was created by amplification of pri-miR-203 cDNA from genomic DNA extracted from HaCaT cells with primers 5' GCGTCTAAGGCGTCCGGTAC 3' and 5' GTCGCCGGCGCACCCCT 3'. The two primers were made with restriction site containing linkers (*NotI*). eGFP was excised using *NotI* from a biscitronic DNA transposon vector, pT2/CMV-eGFP(s).SV40-neo. The pri-miR-203 PCR amplicon was inserted into the vector in the sense orientation using the *NotI* sites (see Appendix II, Figure AI). Similar approach was employed for generation of pri-miR-125b-expressing construct, by amplifying pri-miR-125b cDNA from genomic DNA extracted from HaCaT cells with primers 5' CATCTTAGTTATGAACCTCGAACAG 3' and 5' AAATTGTCTTTAGGTCCTCGACGG 3'.

Cell lines

HaCaT, HeLa, HEK293 and 293T cells were cultured at 37°C in 5% (v/v) CO_2 and maintained in Dulbeccoo's modified Eagle's medium (Cambrex, Verviers, Belgium) supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and L-glutamine (265 mg/L).

Generation of miR-203-expressing HaCaT cell line, HaCaT-203

For generation of miR-203-expressing HaCaT cell line, an approach based on the Sleeping Beauty DNA transposon system was employed (see Appendix II, Figure AI). Transposition in the HaCaT cell line was performed in 6-well plates into which $5x10^4$ HaCaT cells were seeded one day prior to transfection. Co-transfections were performed with 1.82 µg of pT2/CMV-pri-miR-203.SV40-neo and 0.18 µg of either transposase-encoding vector (SB100X) or mutated transposase (mSB) using FuGene-6 (Roche, Basel, Switzerland) according to manufacturer's protocol. One day after transfection cells were trypsinized and re-seeded in appropriate dilutions. The cells were selected for positive transposition by G418-supplemented medium (700 µg/mL) for fourteen days. From the wells with the lowest colony-count, singles clones were isolated to separate dishes and expanded for one week with standard medium, followed by total RNA extraction using Tri Reagent (Sigma, St Louis, MO). miR-203 expression analysis was assessed by qRT-PCR employing the TaqMan® Universal Master Mix II, No UNG (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. miR-203 expression was determined using miR-203 primers and probes (Assay ID 000507). A single miR-203 HaCaT clone was chosen for subsequent experiments.

Dual-Luciferase Reporter Assay

For co-transfection experiments, HEK293 cells were seeded in 96-well plates (3 x 10^3 cells/well) one day before transfection. Co-transfections were performed with a total of 60 ng (54 ng of pT2/CMV-pri-miR-203.SV40-neo, pT2/CMV-pri-miR-125b.SV40-neo or pUC19 stuffer DNA and 6 ng psiCHECK2-gene targets-encoding vectors) using FuGene-6 (Roche, Basel, Switzerland) according to manufacturer's protocol. Forty-eight hours post-transfection, Renilla and Firefly luciferase activities were analyzed by the use of the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Reactions were carried out in 96-well plates and luminescence readings were performed in a multisample platereading luminometer (Berthold, Bad Wild-bad, Germany). Renilla luciferase activity was normalized to Firefly luciferase and presented relative to the negative control (pUC19).

To test the functionality of antagomir-encoding lentiviral vectors, HeLa, HaCaT and HaCaT-203 cells were seeded in 24-well plates (1,9x10^4 cells/ well) one day before transfection. Co-transfections were performed with a total of 400 ng (40 ng of psiCHECK2-miR-203-perfect target and 0,180, 270 or 360 ng of pCCL-PGK-Puro-H1-antagomiR-203) using FuGene-6 (Roche, Basel, Switzerland) according to manufacturer's protocol. Additionally, 90,180 or 360 ng of pUC19 was included as stuffer DNA to ensure the equal amounts of DNA used in each transfection. Luciferase activities were measured forty-eight hours post-transfection as described above.

In transduction studies of antagomir-encoding lentiviral vectors, HaCaT cells were seeded in 24-well plates (1,9x10^4 cells/ well) one day before they were transduced at an MOI of 120. The viral supernatant was supplemented with polybrene (8 µg/mL; Sigma-Aldrich, Milwaukee, WI). One day post-transduction, co-transfections with 40 ng of psiCHECK2-miR-203-perfect target and 360 ng of pUC19 using FuGene-6 (Roche, Basel, Switzerland) according to manufacturer's protocol and luciferase activities were measured forty-eight hours post-transfection as described above.

All the dual luciferase assay experiments were performed at least in triplicates.

Lentiviral vector production

For production of lentiviral vectors, 293T cells were seeded at a density of 4 x 10^6 cells/well in 10-cm dishes one day before transfection. Cells were transfected with calcium phosphate treatment with 3.75 µg pMD.2G (envelope plasmid), 3 µg pRSV-Rev (Rev-expressing plasmid), 13 µg pMDGP-Lg/RRE (packaging plasmid) and 13 µg lentiviral transfer vector. Forty-

eight hours after transfection the viral supernatant was harvested and passed through 0.45 µm filters to remove cellular debris (Sarstedt, Nümbrecht, Germany). The resulting lentiviral vectors were designated LV/vehicle, LV/antagomiR-neg and LV/antagomiR-203. Colony-forming titer assays were performed on HaCaT cells seeded in 6-well plates (5 x 10^6 cells/well) one day before transduction. Lentiviral supernatants were serially diluted and supplemented with polybrene (8 µg/mL; Sigma-Aldrich, Milwaukee, WI) before addition to the cells. Transduced cells were grown in 1 µg/mL puromycin-containing medium (Sigma-Aldrich, Milwaukee, WI) for ten days after which the number of colonies was counted in the wells.

For *in vivo* transductions of xenografted human skin, the lentiviral supernatants were ultracentrifuged for two hours (4°C at 25000 r.p.m.) in a SW28 rotor (Beckman Coulter, Fullerton, CA). Virus pellets were resuspended overnight in $PBS^{-/-}$ at 4°C in a volume of 1/300 of the original volume. The lentiviral vector yield was determined by measuring the amount of p24 Gag protein using HIV-1 p24 Antigen ELISA Kit (ZeptoMetrix, Buffalo, NY) according to manufacturer's protocol.

Human xenograft transplantation model

Six psoriatic plaque skin biopsies were obtained from donors with moderate to severe plaque psoriasis. The psoriasis of the participants was untreated for at least one month prior to the time of biopsy. Informed consent was obtained and the study was approved by the Central Ethical Committee and conducted according to the Declaration of Helsinki protocols. Animal studies were carried out with permission from the Danish Experimental Animal Inspectorate. Each keratome skin biopsy, containing both epidermis and dermis, was split into several grafts (each 1.5 x 1.5 x 0.05 cm) and transplanted onto C.B-17 severe combined immunodeficient (SCID) mice, 6-8 weeks old (Taconic M & B, Silkeborg, Denmark), as described [87]. Shortly, the mice were anesthetized prior to surgery by a subcutaneous injection of Ketaminol (ketamine, 100 mg/kg; Intervet, Skovlunde, Denmark) and Narcoxyl (xylazine, 10 mg/kg; Intervet, Skovlunde, Denmark). The back was shaved and part of the exposed skin removed. The grafts were sutured with absorbable 6-0 suture (Caprosyn, Tyco, Copenhagen, Denmark) and covered with Xeroform dressings (Sherwood Medical Company; Markham, Ontario, Canada) for one week. The mice were kept under pathogen-free conditions throughout the study. The grafts healed for ten days before the mice were randomized and subjected to treatment as indicated.

In vivo administration of antagomir-encoding lentiviral vectors

LV/antagomiR-neg and LV/antagomiR-203 were administered intradermally into psoriatic skin grafts (at a dose of 65 µg p24 Gag/mL in 150 µL) as a single treatment.

Xenograft evaluation following treatment

The severity of psoriatic lesions in the grafts was assessed blinded twice weekly and scored semi-quantitatively according to the average of the following clinical signs: scaliness, induration, and erythema. The parameters were scored using a four-point scale: 0, complete lack of cutaneous involvement; 1, slight involvement; 2, moderate involvement; 3, sever involvement. On a scale from 0 to 3 a maximal score of 3 represents severe scale, induration and erythema of the xenografted psoriatic skin. After three weeks of treatment, biopsies from the center of the graft were obtained, fixed and embedded in paraffin. The remaining grafted skin was snap-frozen in liquid nitrogen and stored at -80°C for further analysis. Employing standard methods, sections were stained histochemically with hematoxylin and eosin. Epidermal thickness was measured at least ten random places from stratum corneum to the deepest part of the rete pegs on three equally distantly cut sections. All sections were blinded prior to evaluation and evaluated randomly. Mean epidermal thickness values for each graft in each treatment group were calculated, and the data summarized as mean ± SEM.

RNA isolation and quantitative RT-PCR

Skin biopsies from *in vivo* transduced xenografted psoriatic and normal skin were incubated in RNAlater-ICE (Ambion, Austin, TX) and stored at -20°C for twenty-four hours prior to RNA isolation. From both *in vitro* transduced cells and skin biopsies, total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer's protocol. In the lysis buffer the biopsies were homogenized 2 x 2 minutes at 25 Hz using a TissueLyser (Qiagen, Valencia, California, USA). Isolated RNA was dissolved in RNase-/DNase-free water and stored until further use at -150°C. First strand cDNA synthesis was performed using the Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, St. Leon-Rot, Germany) according to manufacturer's protocol. SOCS6, TNFα, IL12B, IL20 and IL24 mRNA levels were assessed by qRT-PCR employing the TaqMan® Universal Master Mix II, No UNG (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. SOCS6, TNFα, IL12B, IL20 and IL24 mRNA expression was determined using SOCS6, TNFα, IL12B, IL20 and IL24 primers and probes (FAM-labeled MGB-probes Hs00926356_g1, Hs00174128_m1, Hs01011518_m1, Hs00218888_m1 and Hs01114274_m1, respectively, Applied Biosystems, Foster City, CA). Expression of each

gene was analyzed at least in duplicates using a LightCycler 480 (Roche, Basel, Switzerland). SOCS6, TNFα, IL12B, IL20 and IL24 mRNA levels were normalized to the expression of the reference gene ribosomal protein, large P0 (RPLP0) using RPLP0 specific primers (FAM-labeled MGB-probes Hs99999902_m1, Applied Biosystems, Foster City, CA).

Quantification of miRNAs by TaqMan® microRNA Assays (Applied Biosystems, Foster City, CA) was carried out according to manufacturer's protocol. RU48, miR-16, miR-125b and miR-203 levels were assessed by qRT-PCR employing the TaqMan® Universal Master Mix II, No UNG (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. RU48, miR-16, miR-125b and miR-203 expressions were determined using RU48, miR-16, miR-125b and miR-203 primers and probes (AB Assay ID 001006, 000391, 000449 and 000507, respectively). Expression of each gene was analyzed at least in duplicates using a LightCycler 480 (Roche, Basel, Switzerland). miR-203 levels were normalized to the expression of the reference small nucleolar RNA, C/D box 48 (RU48) using RU48 specific primers (AB Assay ID 001006, Applied Biosystems, Foster City, CA).

For all qPCR experiments, relative RNA levels were determined using the relative standard curve method. Briefly, a standard curve for each gene was made from serial dilutions of the cDNA. The standard curve was then used to calculate relative amounts of target RNA in the samples. Mean RNA values were calculated and the data summarized as mean + SEM.

Bioinformatics

miR-203 target sites for IL24, SOCS6 and TNFα 3'UTR sequences were predicted by PicTar 5, RNAhybrid and TargetScan 5.2 softwares available at http://pictar.mdc-berlin.de/, http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/ and http://www.targetscan.org/, respectively.

Statistical Analyses

All p-values were calculated by a two-tailed Student's T-test to test the null hypothesis of no difference between two compared groups. The assumption of equal variances was tested by the F-test. In all statistical analyses, p-values < 0.05 were considered significant.

Results

Towards identification and analyses of miR-203 target genes

To study the regulatory properties of psoriasis-related miRNAs with a role in cytokine production, we decided to focus on two prominent candidates, miR-203 and miR-125b. Therefore, we started by establishing expression vectors encoding miR-203 and miR-125b by PCR-amplifying a human genomic DNA segment containing part of the pri-miRNA sequences. For amplification of pri-miR-203 and pri-miR-125b sequences we used primer sets flanking the pre-miRNA sequences of both miRNAs (with approximately 50 nucleotides extra margin), as we were trying to develop cDNA sequences mimicking the endogenous pri-miRNAs (see Appendix II, Figure AI). To examine miRNA efficiency, we transiently transfected HEK293 cells with plasmid DNA expressing each of the pri-miRNAs from a CMV promoter together with miR-203- and miR-125b-perfect target constructs. By using a dual luciferase assay in which oligonucleotide sequences complementary to miR-203 and miR-125b were transiently expressed as a fusion RNA with the coding sequence of the *R-luc* gene, we measured the ability of each miRNA to downregulate expression of R-luc reporter. Transfections with both pri-miRNAs constructs reduced R-luc expression in >75% compared to negative control (data not shown).

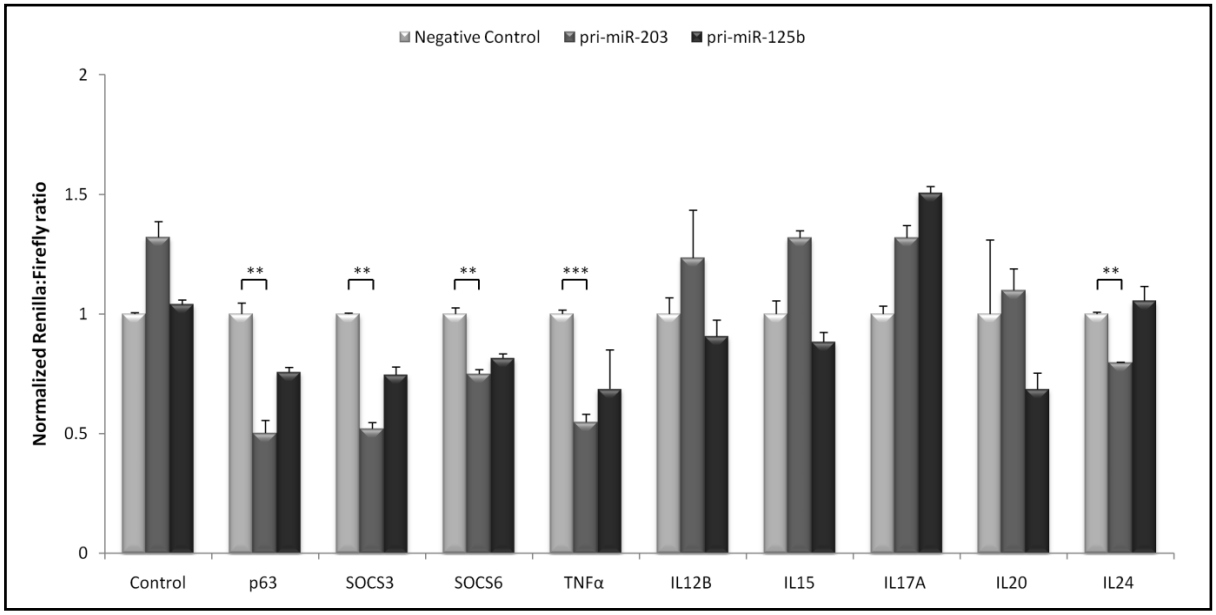

Figure 4. Functional screening of potential microRNA-203 and microRNA-125b targets in psoriasis. Screening of miR-203 targeting six different psoriasis-related cytokine mRNAs using the dual luciferase assay. HEK293 cells were co-transfected either with pUC19 (Negative control), pri-miR-203- or pri-miR-125b-encoding SB transposon vectors and nine psiCHECK2-gene target vectors and psiCHECK2 vector (control), all encoding Firefly luciferase for transfection normalization and a fusion mRNA consisting of Renilla luciferase and potential miR-203 targets. Luciferase activities were measured forty-eight hours post-transfection and Renilla luciferase activity was normalized to Firefly luciferase activity and depicted relative to each transfection with the negative control, pUC19 vector, not encoding a miRNA. *P*-values for the comparisons indicated by brackets were as follows: $^{**}P < 0.01$; $^{***}P < 0.001$. All experiments were performed in triplicates and data are depicted as mean + SEM. *Adapted from manuscript Primo et al, 2011.*

Inflammation is characterized by deregulated expression of a wealth of cytokines, many of which are candidates for miRNA-directed expression control. Towards identification of miR-203 targets in psoriasis, nine psiCHECK2 constructs were generated encoding different mRNA transcripts associated with the cytokine network in psoriasis - six of them encoding psoriasis-relevant cytokines. In addition to cytokines, we included *p63* and *SOCS3* genes in miR-203 target screen, as they are both miR-203 validated targets. At last, we also included SOCS6 gene in miR-203 target screen as we have found by computational predictions that mRNA encoding SOCS6 contains target sites for several miRNAs, including miR-203. Based on the initial miRNA efficiency assay, miR-203 potential targets were verified by co-transfecting these plasmid vectors into HEK293 cells with plasmid DNA transiently expressing pri-miRNAs. As shown in Figure 4, transient expression of pri-miR-203 from the transposon vector resulted in at least 20% reduction of p63, SOCS3, SOCS6, TNFα and IL24 fused protein expressions compared to the level of the respective fused proteins in the negative control ($p < 0.01$). As mRNAs encoding p63 and SOCS3 were already validated miR-203 targets, this analysis proposed that mRNA transcripts encoding SOCS6, TNFα and IL24 could be new possible targets for miR-203.

Human miRNAs mainly target the 3'UTR of mRNAs, predominantly by complete sequence complementarity within the 'seed region'. Thus, to confirm whether mRNAs encoding IL24, SOCS6 and TNFα were miR-203 targets, we performed bioinformatic studies using PicTar 5, RNAhybrid and TargetScan 5.2 softwares for identification of possible miR-203 binding sites in the 3'UTRs of the mentioned mRNAs. As a criterion, we decided to restrict the 3'UTR-miRNA interactions within the seed region, although additional basepairing is known to increase miRNA functionality. As a result, we identified four potential miR-203 binding sites within 3'UTRs of *IL24* and *SOCS6* and two within 3'UTR of *TNFα* (Figure 5a and see Appendix III-V, Figures AII-AIV).

Subsequently, we agreed on developing a mutant miR-203 binding site within 3'UTRs of *IL24*, *SOCS6* and *TNFα*, based on informatic studies. As a criterion, we decided to create a mutant miR-203 binding site that would not present full complementarity to any known seed region of human miRNAs in order to inhibit as many as possible mRNA-miRNA interactions. Consequently, 3'UTR cDNA mutants were generated for the three genes, in which the identified miR-203 binding sites were modified with the following sequence 'CACAAAA' (Figure 5a). Once the 3'UTR mutants were designed, psiCHECK2 constructs encoding IL24, SOCS6 and TNFα 3'UTR mRNA mutant transcripts were generated. When the 3'UTR mutant vectors were co-transfected into HEK293 cells with plasmid DNA transiently expressing pri-miR-203, miR-203-mediated repression was abolished and luciferase activity was restored, as shown

in Figure 5b. Together, these results indicate that the effect of miR-203 on IL24, SOCS6 and TNFα mRNA transcripts is direct and mediated by specific 3'UTR target sites.

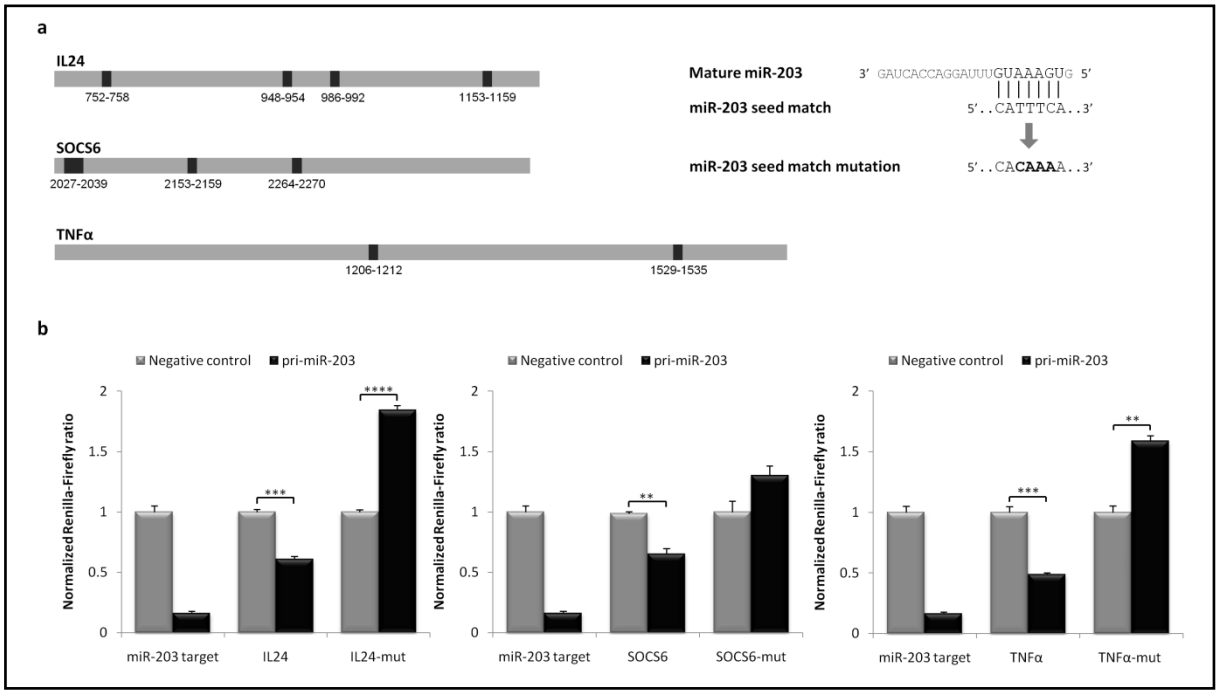

Figure 5. Development of 3'UTR mutants for confirmation of IL24, SOCS6 and TNFα mRNA transcripts as direct targets of microRNA-203. (**a**) Schematic representation of the 3'UTR sequence of IL24, SOCS6 and TNFα transcripts (grey bar) with the predicted target sites for miR-203 (black lines). The numbers underlying the black lines represent the first and the last basepair position within the NCBI reference for each represented transcript. Schematic representation of the miR-203 seed match mutation. Nucleotides written in bold represent the mutated nucleotides. (**b**) Confirmation of IL24, SOCS6 and TNFα 3'UTR transcripts as miR-203 targets. Comparison between HEK293 cells co-transfected with Firefly luciferase constructs containing the wild-type or mutant 3'UTR and pUC19 (Negative control) or pri-miR-203-encoding SB DNA transposon vector. Luciferase activities were measured forty-eight hours post-transfection. Renilla luciferase activity was normalized to Firefly luciferase activity and depicted relative to each transfection with the negative control, pUC19 vector, not encoding a miRNA. *P*-values for the comparisons indicated by brackets were as follows: ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. All experiments were performed in triplicates and data are depicted as mean + SEM. *Adapted from manuscript Primo et al, 2011.*

Downregulation of miR-203 targets following stable expression of miR-203

To confirm that IL24, SOCS6 and TNFα mRNA transcripts were indeed genuine targets of miR-203, a pri-miR-203 expression cassette was inserted into a HaCaT cell line by means of the Sleeping Beauty DNA transposon system [88]. Plasmid DNA, containing the pri-miR-203 expression cassette and neomycin resistance gene driven by a SV40 promoter within the context of a SB DNA transposon, was co-transfected with helper plasmid encoding either the SB100X transposase or a mutated, inactive variant. The transposition efficiency of the pri-miR-203 transposon vector was evaluated by transfecting HaCaT cells with pT2/CMV-pri-miR-203.SV40-neo together with one of both transposase-encoding plasmids. Numbers of G418-resistant colonies (see Appendix VI, Figure AVa) indicated that *neo* expression cas-

sette was inserted markedly more frequently in the presence of active transposase relative to the inactive variant, indicating that genomic integration of the pri-miR-203 vector was efficiently accomplished by a transposase-directed mechanism. We next isolated and expanded three individual clones containing the pri-miR-203 expression cassette inserted by the SB100X transposase, and miR-203 expression was confirmed by qRT-PCR (see Appendix VI, Figure AVb). A single HaCaT-203 clone (clone C) was chosen for subsequent experiments.

To evaluate knockdown of endogenous IL24, SOCS6 and TNFα mRNA transcripts due to miR-203 stable overexpression, RNA samples were collected and evaluated by quantitative RT-PCR on naïve HaCaT and HaCaT-203 cells. Endogenous expressions of IL24, SOCS6 and TNFα mRNA transcripts were efficiently targeted by miR-203 expression, as shown in Figure 6a. We detected a knockdown ranging from 60% to 95% of SOCS6 and TNFα mRNA transcripts, respectively (Figure 6a). These findings demonstrated the potential specificity of the interaction between miR-203 and the mentioned targets.

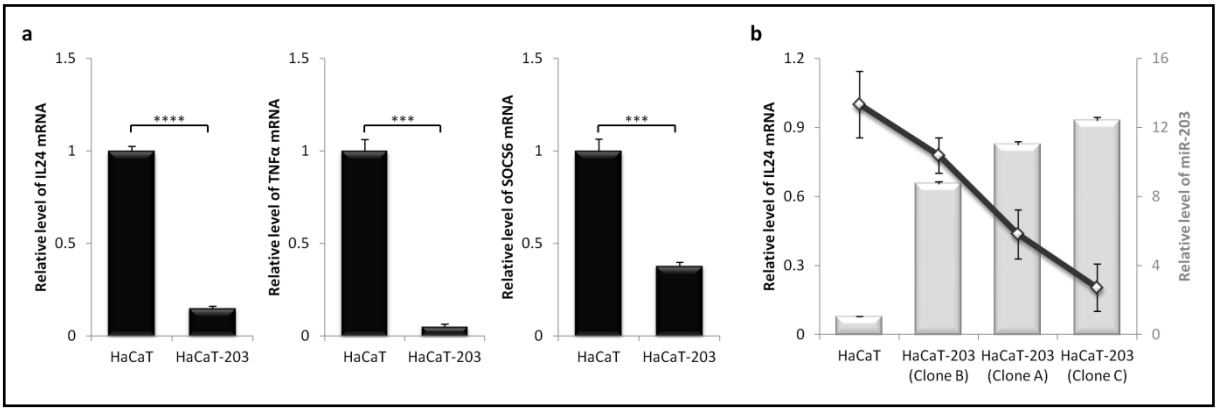

Figure 6. Endogenous knockdown of IL24, SOCS6 and TNFα mRNA transcripts by stable overexpression of microRNA-203. (**a**) IL24, SOCS6 and TNFα mRNA levels were evaluated by qRT-PCR in a naïve HaCaT cell line and in a miR-203 expressing HaCaT cell line. (**b**) IL24 mRNA levels and miR-203 levels were evaluated by qRT-PCR in a naïve HaCaT cell line and in three different HaCaT clones stably overexpressing miR-203, generated by means of the Sleeping Beauty DNA transposon system. IL24, SOCS6 and TNFα mRNA levels were normalized to the expression of the reference gene ribosomal protein, large P0 (RPLP0) and miR-203 levels were normalized to the expression of the reference small nucleolar RNA, C/D box 48 (RU48). *P*-values for the comparisons indicated by brackets were as follows: ****P* < 0.001; *****P* < 0.0001. All experiments were performed in triplicates and data are depicted as mean + SEM. *Adapted from manuscript Primo et al, 2011.*

To certify that endogenous knockdown of the target genes was the result of increased expression of miR-203 in the HaCaT cell line and not due to an unknown particularity of HaCaT-203 cell line - clone C, we decided to analyze the endogenous mRNA expression of one of miR-203-targets in the other two HaCaT-203 clones. The knockdown potency was evaluated by quantitative RT-PCR analysis, and endogenously expressed IL24 was efficiently targeted by miR-203 in all different HaCaT-203 clones. As shown in Figure 6b, miR-203 and IL24 mRNA levels were inversely related in the different HaCaT populations, indicating that repression of IL24 mRNA transcript was only dependent of miR-203 expression levels. Tak-

en together, these results demonstrated the specificity of miRNA-mRNA interactions between miR-203 and the IL24, SOCS6 and TNFα mRNA transcripts.

Establishment of miR-203 inhibition by vector-encoded 'antagomirs'

Jacob Mikkelsen research group has previously provided evidence of potent and sustained RNAi-mediated *in vivo* knockdown of IL12B and TNFα mRNAs in human skin by the use of lentiviral vectors [83; 84]. Hence, we decided to explore the same lentiviral backbone (see Appendix II, Figure AI) for design of vectors expressing antisense RNAs complementary to immune-modulatory miRNAs (v-antagomirs), as a method for persistently antagonizing miRNA function in cultured cells and in mice. Therefore, two antagomirs were designed in this study – the first one encoding an antisense RNA complementary to human miR-203 (antagomiR-203) and the second one encoding an antisense RNA not complementary to any known human miRNA, named antagomiR-Negative control (antagomiR-neg).

Before we monitored the efficiency of antagomirs against endogenous miR-203, we decided to characterize the expression of miRNAs with a putative role in cytokine regulation in different cell lines. This assay was developed to identify the cell line with the highest miR-203 expression for subsequent investigations of antagomir functionality. In our pilot study we evaluated by quantitative RT-PCR analyses the RNA expression levels of RU48, miR-203, miR-125b and miR-16 (the latter has been implicated in posttranscriptional regulation of TNFα) in three different cell lines and in an extra RNA sample collected from psoriasis lesional skin (see Appendix VI, Figure AVI). Since miR-203 expression levels were shown to be very reduced in HEK293 cells and as we did not identify a statistical significant difference between miR-203 expression levels in HaCaT cells with and without stress stimulation, we decided to proceed with HeLa and HaCaT cell lines without stress stimulation for examination of miR-203 inhibition by antagomirs.

To monitor the potency of miRNA inhibition triggered by antagomirs, antagomir-encoding lentiviral vectors were analyzed for their ability to target endogenous miR-203 by using a dual luciferase expression assay. HeLa and HaCaT cells were co-transfected with psiCHECK2-miR-203-perfect target together with different amounts of antagomiR-203-encoding lentiviral vector. Luciferase activities were measured forty-eight hours post-transfection, and antagomiR-203 potencies were determined by normalizing to the expression levels obtained in the absence of antagomiR-203-expressing lentiviral vector. As shown in Figure 7a, a dose-response relationship was obtained in all cell lines, in which increased amounts of lentiviral vector expressing antagomiR-203 resulted in increased expression of *R-luc,* due to inhibition of endogenous miR-203 activity. We also observed that the highest miR-203 knockdown was

achieved in HaCaT-203 cells as those cells were the ones that presented the highest miR-203 expression levels within the same setup conditions.

To certify that the antagomir-encoding gene cassette was efficiently transferred by the lentiviral vector system, we first measured the transductional efficiency of antagomir-expressing vectors in experiments that included the LV/vehicle vector as a control. High transduction titers of antagomir-expressing lentiviral vectors were confirmed (ranging from 3.40 x 10^6 to 4.05 x 10^6 colony forming units per mL [cfu/mL] on HaCaT cells), although the titers on average were reduced relative to the control vector, which did not express an antagomir (data not shown).

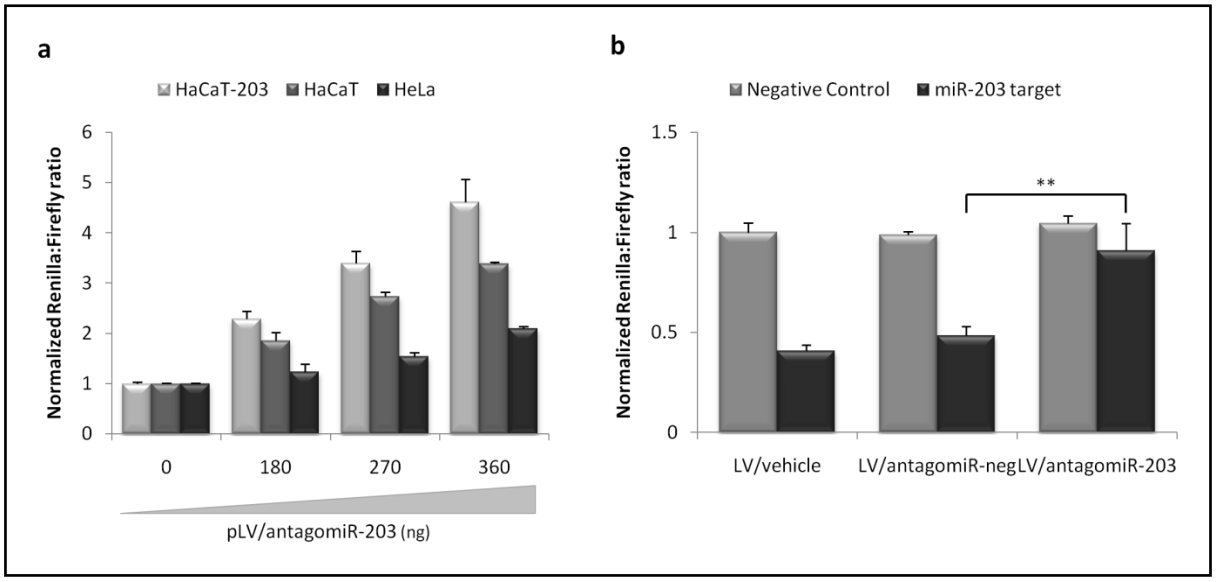

Figure 7. Functional evaluation of microRNA-203 targeting by antagomiR-203 and confirmation of antagomir potency after lentiviral transduction. (**a**) Upregulation of transiently expressed miR-203-perfect target by transfection with lentiviral construct encoding antagomiR-203. miR-203-perfect match sequence was fused to the Renilla luciferase gene in the psiCHECK2 vector and the plasmid was co-transfected together with pLV/antagomiR-203 into HaCaT-203 cells, HaCaT cells and HeLa cells. Luciferase activities were measured forty-eight hours post-transfection. Renilla luciferase activity was normalized to Firefly luciferase activity and depicted relative to non-transfected cells with pLV/antagomiR-203. (**b**) Antagomir potency evaluation after transduction with antagomir-encoding lentiviral vectors. HaCaT cells were transduced at an MOI of 120. Cells were transfected with either psiCHECK2 vector (Negative control) or miR-203-perfect target construct one day post-transduction and luciferase activity was measured forty-eight hours post-transfection. Renilla luciferase activity was normalized to Firefly luciferase activity and depicted relative to transduction with lentiviral vector encoding no antagomir (LV/vehicle). *P*-values for the comparisons indicated by brackets were as follows: ***P* < 0.01. All experiments were performed at least in triplicates and data are depicted as mean + SEM. *Adapted from manuscript Primo et al, 2011.*

To evaluate antagomir potency when delivered by lentiviral vectors, HEK293 cells were transduced at a multiplicity of infection (MOI) of 120 with three lentiviral vectors (LV/antagomiR-203, LV/antagomiR-neg and LV/vehicle, as a control), one day prior to transfection. On the following day, cells were transfected either with the psiCHECK2-miR-203-perfect target or pUC19 stuffer DNA, and luciferase activities were measured forty-eight hours post-transfection. As shown in Figure 7b, transient expression of antagomiR-203, when delivered by lentiviral vectors at an MOI of 120, resulted in abolishment of miR-203-mediated repression. We detected a 50% knockdown of miR-203 activity in cells transduced

with LV/antagomiR-203 ($p < 0.01$), an effect that was not identified in cells transduced with either LV/vehicle or LV/antagomiR-neg. Taken together, these results demonstrated a high potential of lentiviral delivery of antagomiR-203 for efficient targeting of aberrantly expressed miR-203 for RNAi-mediated knockdown.

Efficient and persistent upregulation of IL24 following lentiviral transduction

To investigate the effects of miR-203 knockdown by delivery of antagomiR-203-expressing lentiviral vector into HaCaT cells, we evaluated by qRT-PCR the expression levels of miR-203 and IL24 mRNA transcripts. Therefore, two different HaCaT cell lines (naïve HaCaT cells and HaCaT-203 cells) were transduced at an MOI of 50 and at an MOI of 5 (followed by a 14-day puromycin selection) to test whether a few inserted copies of the antagomiR-203-expressing vector would have a persistent effect on miR-203 knockdown.

As shown in Figure 8, endogenously miR-203 activity was efficiently targeted and abolished

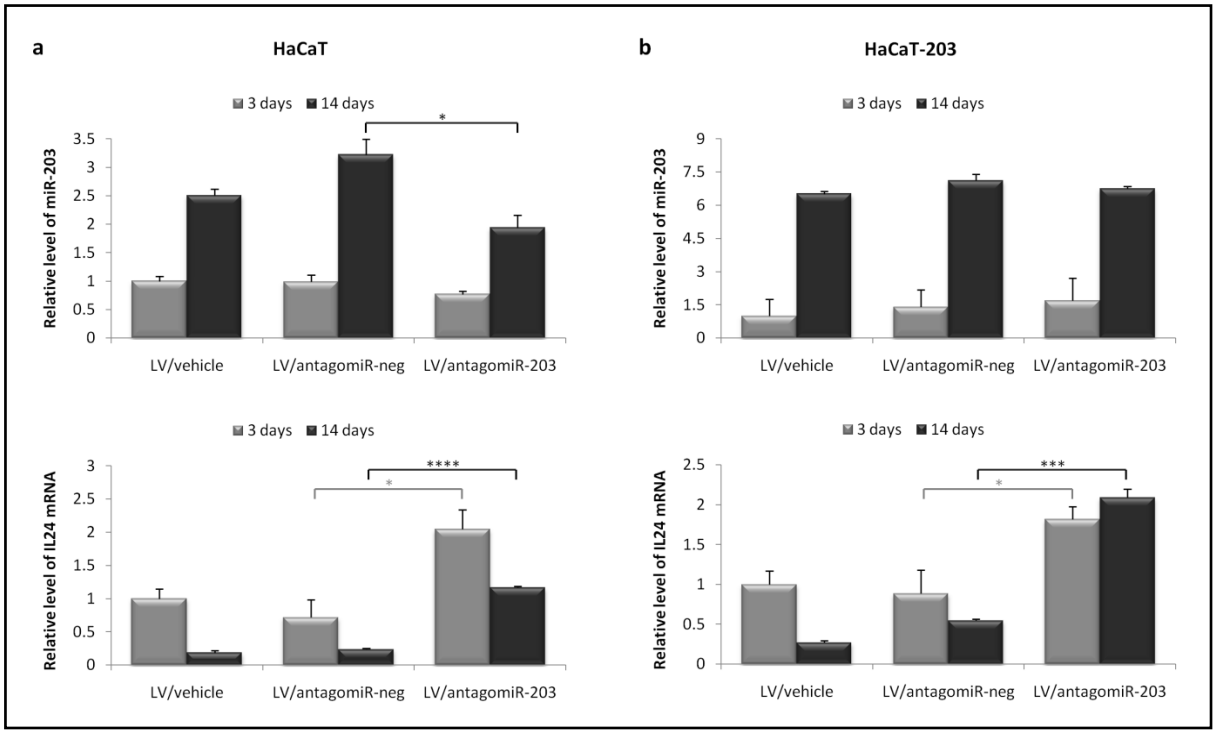

Figure 8. ***In vitro*** **knockdown of microRNA-203 after transduction with antagomir-encoding lentiviral vectors and upregulation of IL24 mRNA expression.** (**a, b**) Knockdown of miR-203 expression following transduction with lentiviral vector-encoded antagomiR-203. (**a**) Naïve HaCaT cells and (**b**) HaCaT-203 cells were transduced with LV/vehicle, LV/antagomiR-neg or LV/antagomiR-203, as indicated below each column. Both naïve HaCaT and HaCaT-203 cells were transduced at an MOI of 50 (grey bars) or 5 (black bars) followed by puromycin selection for fourteen days to ensure that cells harbored only few lentiviral insertions. miR-203 and IL24 mRNA levels were evaluated by qRT-PCR after three days and fourteen days post-transduction in (**a**) naïve HaCaT cells and (**b**) HaCaT-203 cells. IL24 mRNA levels were normalized to the expression of the reference gene ribosomal protein, large P0 (RPLP0) and miR-203 levels were normalized to the expression of the reference small nucleolar RNA, C/D box 48 (RU48). *P*-values for the comparisons indicated by brackets were as follows: **P* < 0.05; ****P* < 0.001; *****P* < 0.0001. All experiments were performed in triplicates and data are depicted as mean + SEM. *Adapted from manuscript Primo et al. 2011.*

by lentiviral delivery of antagomiR-203, in both naïve and HaCaT-203 cells, as we detected a 2-fold increase of IL24 mRNA transcripts three days after transduction ($p < 0.05$). When analyzing IL24 mRNA transcripts fourteen days after lentiviral transduction, we also identified an increase of IL24 mRNA expression levels ($p < 0.001$), providing persistent regulatory effect of lentivirally encoded antagomiR-203 effectors. By comparison of IL24 mRNA expression levels within both cell lines, we observed that the highest levels of IL24 mRNA transcripts were quantified fourteen days after transduction in the naïve HaCaT cell line (Figure 8a). In agreement with this observation was the fact that the highest miR-203 knockdown was identified fourteen days after transduction in the same HaCaT cell line (Figure 8b). Taken together, these observations indicated that miR-203 and IL24 mRNA levels were inversely expressed in HaCaT cells, clearly demonstrating the direct occurrence of a specific miRNA-mRNA interaction.

According to the proposed function of the antagomirs, this type of inhibitor effectively competes with miRNA targets by a stronger binding to their specific miRNA target on the RISC complex, resulting in inhibition of miRNA activities. Our observations regarding IL24 mRNA transcripts after delivery of antagomiR-203-expressing lentiviral vector in HaCaT cells were in agreement with the previous statement, demonstrating that antagomiR-203 was a potent inhibitor of miR-203. Antagomirs are also known to induce the degradation of the targeted miRNAs with as of yet unknown mechanisms [47; 89]. Regarding induced-miR-203 degradation by antagomiR-203 expression, no change in miR-203 expression levels was observed in both HaCaT cell lines three days after lentiviral transduction (Figure 8a and 8b). RNA samples collected fourteen days after cell transduction shown that miR-203 expression levels were markedly diminished (60% knockdown) in naïve HaCaT cells transduced with antagomiR-203-expressing lentiviral vector, when compared to cells transduced with antagomiR-neg-expressing lentiviral vector (Figure 8a). A similar result was not identified in HaCaT cells stably expressing miR-203. In this HaCaT-203 cell line, miR-203 expression levels did not differ within HaCaT cells transduced with antagomiR-203- and antagomiR-neg-expressing lentiviral vectors (Figure 8b). The results collectively demonstrated that induced miR-203 knockdown by antagomiR-203 expression presented a dose-dependent response as antagomiR-203 knockdown efficiency was inversely correlated to miR-203 endogenous expression.

In vivo lentiviral delivery of antagomiR-203 in xenografted psoriatic skin

In the previous experiments we demonstrated that miR-203 could mediate post-transcriptional suppression through *IL24* 3'UTR. These proof-of-concept studies confirmed that keratinocyte-specific miR-203 plays a role in cytokine expression in keratinocytes. Since

psoriatic skin is characterized by a specific miR-203 expression profile that is different in healthy skin, we decided to extend the exploration of antagomir-mediated miRNA knockdown in psoriatic human skin. To investigate the effects of lentivirally delivered antagomiR-203 in human psoriatic skin *in vivo*, we used the psoriasis xenograft transplantation model. Upon transplantation of psoriatic keratome skin biopsies, the mice were divided into two groups. The first group (n = 3) was treated with a single intradermal dose LV/antagomiR-203 (65 µg p24 Gag/mL in 150 µl), whereas the second group (n = 3) was treated with a single intradermal dose of LV/antagomiR-neg (same dose as group one) as a negative control (Figure 9a). The mice were assigned a baseline semiquantitative clinical psoriasis score based on the average erythema, thickness and scaliness of the psoriatic plaques. The effects of treatment were evaluated in a blinded fashion by semiquantitative clinical scores given twice weekly for three weeks until killing. The outlined study tried to explore the applicability of miRNA-directed drugs and address some of the main hurdles related to antagomir specificity and the capacity of miRNAs to regulate numerous genes.

The final endpoint in evaluating the effect on psoriasis was determined histologically by measuring epidermal thickness and by quantification of miR-203 expression levels and several cytokine mRNA transcripts in the biopsies obtained from psoriatic skin grafts. As evident from Figures 9b, skin grafts treated with antagomiR-203-encoding lentiviral vectors did not seem to have an effect on the clinical phenotype of psoriatic skin, when compared to the negative control. Histological assessment of skin grafts did not reveal amelioration of psoriasis phenotype by the injected antagomiR-203-encoding lentiviral vectors as a characteristic psoriatic phenotype with elongated rete pegs was retained (Figure 9c). Average epidermal thicknesses, as shown in Figure 9d, gave the indication that grafts injected with antagomiR-203-encoding lentiviral vectors exhibited a slight reduction in average epidermal thickness compared to the negative control, although this reduction did not appear to be significant. Additionally, we investigated whether miR-203 was downregulated in psoriasis skin grafts treated with LV/antagomiR-203 and, therefore, performed quantitative RT-PCR on RNA extracted from treated skin grafts. Unexpectedly, miR-203 expression levels in the grafts treated with lentiviral vectors encoding antagomiR-203 were increased 60% relative to grafts isolated from mice in the negative control group (Figure 9e). Lastly, we carried out quantitative RT-PCR analysis of IL24 mRNA transcripts and other two inflammatory-related cytokines, such as IL12B and IL20. As evident from Figure 9f, treatment with LV/antagomiR-203 elicited a 33% reduction of IL24 mRNA transcripts in the skin grafts in average ($p < 0.01$). We also identified a 4-fold increase of IL12B and IL20 mRNA transcripts in the skin grafts transduced with antagomiR-203-encoding lentiviral vectors. Taken together, our observations suggested

that intradermally administered antagomiR-203 impacts the levels of miR-203, resulting in altered expression of cytokines expression.

It remains a matter of speculation why treatment with the antagomir triggered an increased in the detected level of miR-203. Overall, these preliminary findings could indicate that the psoriatic phenotype was not affected by targeting miR-203 in the psoriasis xenograft transplantation model.

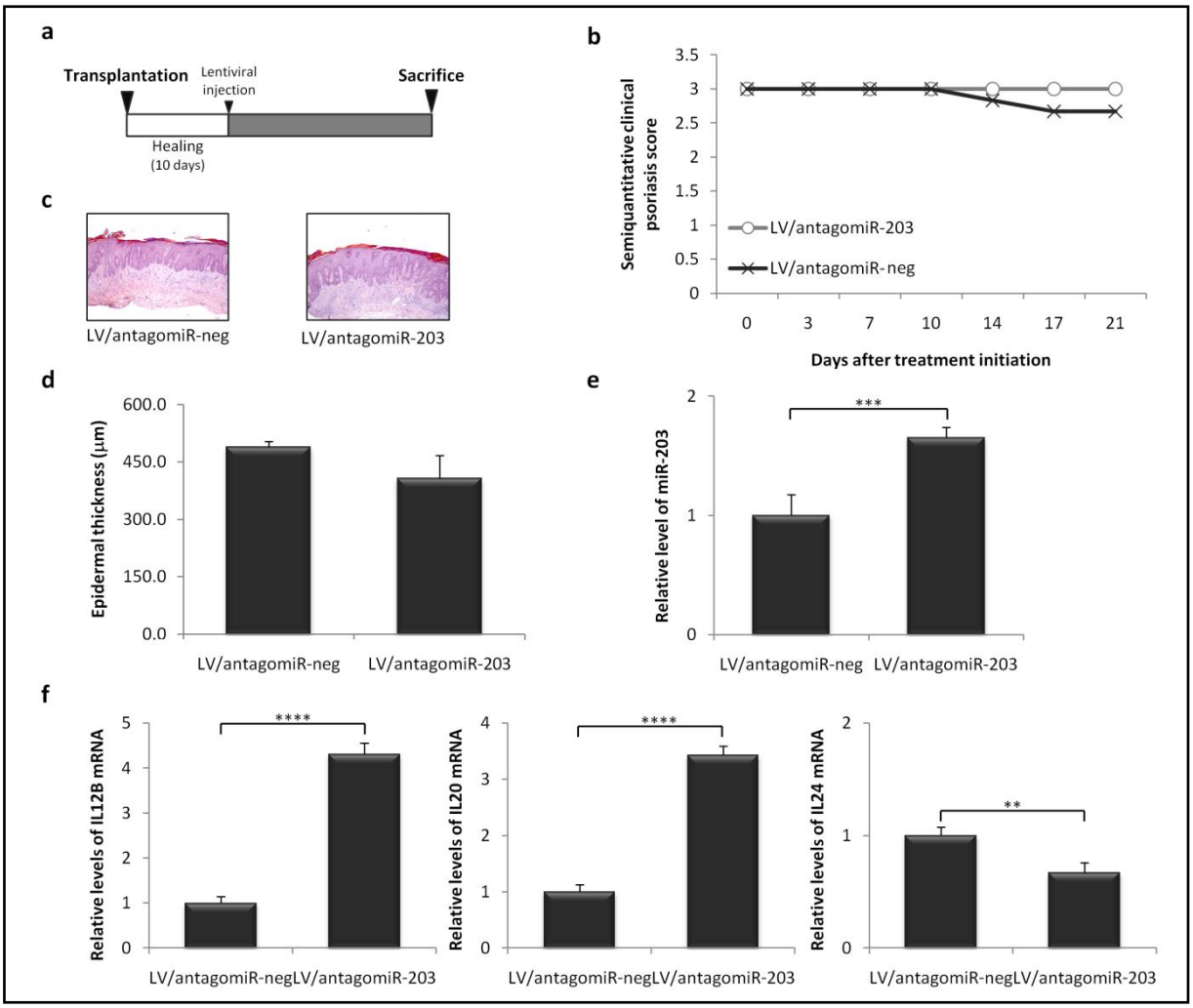

Figure 9. ***In vivo*** **knockdown of microRNA-203 in xenografted psoriatic skin by lentiviral delivery of antagomiR-203.** (**a**) Schematic schedule of treatment with antagomir-encoding lentiviral vectors. The skin grafts were treated by a single intradermal injection of lentiviral vectors encoding either antagomiR-203 or an irrelevant antagomiR (LV/antagomiR-neg), and the mice were sacrificed three weeks after treatment. (**b**) Semiquantitative clinical psoriasis scores were given twice weekly for three weeks to mice treated with LV/antagomiR-203 (*n* = 3) and LV/antagomiR-neg (*n* = 3) (**c**) Histological assessment of grafted skin samples. Representative tissue samples of each treatment group are shown. (**d**) At treatment endpoint, three weeks post-transduction of the skin samples, mice were sacrificed and biopsies from the skin grafts were fixed, paraffin-embedded, H&E-stained, and epidermal thickness was measured in each graft. (**e, f**) Biopsies from the xenografted psoriatic skin injected with antagomir-encoding lentiviral vectors were acquired at treatment endpoint (three weeks post-transduction) and evaluated for (**e**) miR-203 expression levels and for (**f**) IL12B, IL20 and IL24 mRNA expression by qRT-PCR. IL12B, IL20 and 24 mRNA levels were normalized to the expression of the reference gene ribosomal protein, large P0 (RPLP0) and miR-203 levels were normalized to the expression of the reference small nucleolar RNA, C/D box 48 (RU48). *P*-values for the comparisons indicated by brackets were as follows: ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. Data are presented as mean + SEM.

Discussion

Jacob Mikkelsen research group has previously provided evidence of potent and persistent *in vivo* knockdown of TNFα and IL12B mRNA transcripts following a single intradermal injection of lentiviral vectors into xenografted human skin [83; 84]. These proof-of-concept studies confirmed the therapeutic applicability of shRNA expression in skin and documented the potential use of RNAi in the treatment of psoriasis. In the present study, we explored the cytokine-directed shRNA-encoding lentiviral vector for design of vectors expressing antagomirs. As a focus of these studies, we chose miR-203, which was recently identified as a miRNA significantly overexpressed in psoriasis and which is exclusively expressed in keratinocytes [49]. Whether miR-203 may contribute or not to increased skin inflammation in response to T-cell derived-cytokines is still unknown, as miR-203 has only been shown to target SOCS3. Therefore, an initial screening of a panel of psoriasis related-cytokines was necessary to identify possible new miR-203 targets. Indeed, in the initial luciferase-based miR-203 screen targeting six different cytokines and two protein suppressors of the cytokine signaling, two cytokines (*IL24* and *TNFα*) and *SOCS6* were identified as potential miR-203 targets. Importantly, the mRNAs encoding the three different targets contained potential miR-203-binding sites in their 3'UTRs. To determine whether the regulation of the luciferase reporter gene was mediated by the predicted target sites in 3'UTR of the potential targets, we mutated the seven nucleotides within the seed-matching sequences of the predicted target sites. Mutation of the seed-matching sequences in each potential target gene led to complete restoration of luciferase activity, confirming the direct interaction between the target genes and miR-203. Nevertheless, variations in target accessibility in the fusion mRNA context could have influenced RNAi activity and thus provide a false identification of a potential miRNA target. This emphasized the need to validate miR-203 knockdown on native endogenously expressed targets. Moving the experimental setup to endogenously expressed targets, we showed that miR-203 activity toward the mentioned targets was maintained clearly demonstrating that miR-203 directly regulates IL24, SOCS6 and TNFα mRNA transcripts in human keratinocytes.

On receptor-expressing keratinocyte cells, IL24 is known to activate STAT pathway, by phosphorylation of STAT3 [90; 91]. In response to IL24, keratinocytes proliferate and express pro-inflammatory genes including TNF-α, which leads to activation of NF-κB. Accordingly to the mentioned studies, the data collected propose that miR-203 may play a role in the regulation of NF-κB activity in human keratinocytes. As miR-203 targets IL24 and TNFα mRNA transcripts in keratinocytes, IL24 and TNFα protein expressions will diminish, leading to inhibition of NF-κB signaling pathway. Conversely, miR-203 also targets SOCS3 mRNA tran-

script, which is a negative regulator of STAT3 pathway, resulting in STAT3 activation and, consequently, activation of NF-κB signaling pathway. According to the data collected here and to our understanding of miR-203 targets, it is reasonable to assume that miR-203 may have a meaning in NF-κB pathway in human keratinocytes, although further studies are still relevant to confirm and clarify miR-203 significance on NF-κB pathway tight regulation.

Moreover, we generated two antagomir-expressing lentiviral constructs (antagomiR-neg and antagomiR-203). Based on functional evaluations, we identified a solid upregulation of Renilla luciferase gene fused to a full miR-203 complementary sequence when co-expressed with either antagomiR-203-encoding plasmid or lentiviral vector. miR-203 knockdown was confirmed when the antagomiR-203 was expressed from very few lentiviral integrations (MOI of 5) and after extrachromosomal non-integrated lentiviral vectors have been lost. The mechanism of oligonucleotide-mediated miRNA silencing is still unknown, although previous data from other groups suggests that this process involves degradation of the miRNA *in vitro* and *in vivo* [47; 89]. Our collected data is partially in agreement with the mentioned hypothesis, as we identified a clearly reduction of miR-203 expression levels in HaCaT naïve cells following transduction of antagomiR-203-expressing lentiviral vector. Nonetheless, the same experiment was developed in a HaCaT cell line stably overexpressing miR-203, and no reduction of miR-203 expressing levels was detected, despite the fact that a release of miR-203 repression on IL24 mRNA target was detected in this particular cell line. In this study, it is important to take into account that antagomiR-203 sequence did not present any chemical modifications, such as phosphorothioate modification or 2'-O-methyl sugar modification which protect oligonucleotides against exonuclease or endonuclease activity, respectively. This may indicate that, when expressed endogenously, antagomirs and miRNAs may share similar degradation pathways. Therefore, our results suggest that miRNA-target cell expression levels and antagomir chemical modifications are relevant parameters to take into account when designing and evaluating antagomir potency.

The psoriasis xenograft transplantation model currently appears to be the best tool to screen anti-psoriatic therapeutic strategies in psoriasis before introducing them into the clinic [92]. Using the psoriasis xenograft transplantation model, we aimed to evaluate if lentiviral delivery of antagomiR-203 could decrease miR-203 levels and if this would have a beneficial effect on the disease phenotype. Following a single, intradermally administered dose of lentiviral vector in human skin, we detected a 60% increase of miR-203 in antagomiR-203-treated skin grafts compared to the negative control. Recent *in vitro* and *in vivo* investigations indicate that miR-203 impacts epidermal proliferation predominantly by restricting the proliferative potential of basal-cell precursors as they migrate from the basal layer to the suprabasal layers [57; 60]. It is hypothesized that miR-203 might be absent or only expressed at low levels in

multipotent proliferating cells to enable p63-dependent epidermal commitment, whereas its expression increases as the multipotent epidermal cells exit the proliferating basal layer and terminally differentiate. These data indicate that it was rational to expect an increased epidermal thickness following intradermal administration of antagomiR-203-expressing lentiviral vectors in skin. Conversely, we did not identify any clinical and histological improvement of psoriasis phenotype, as epidermal thickness and semiquantitative clinical psoriasis score remained unchanged during the three-week observations. As we had tested antagomiR-203 functionality in HaCaT keratinocyte cell line before developing miR-203 knockdown study *in vivo*, miR-203 upregulation in antagomiR-203-treated skin grafts was to a great extent an unexpected result. For that reason, we decided to analyze the expression levels of IL24 mRNA transcripts, as we have previously demonstrated that miR-203 targets IL24 mRNA transcript. In agreement with our previous results, miR-203 expression levels and IL24 mRNA transcripts were inversely related in antagomiR-203-treated skin grafts compared to the negative control. This observation validated the result obtained from miR-203 quantification in the skin grafts.

According to the data collected and to our understanding of miR-203 function, we might hypothesize that miR-203 upregulation in antagomiR-203-treated skin samples was the outcome of a negative feedback for regulation of miR-203 expression levels in cytokine-activated keratinocytes. As antagomiR-203 potentially facilitates inhibition of miR-203 function in keratinocytes, p63 expression levels increase which lead to increased processing efficiency of growth promoting pre-miRNAs, resulting in increased number of ‘transiently amplifying-keratinocyte’ cells. Since these proliferative keratinocytes are generated within an inflammatory microenvironment loaded of keratinocyte-released cytokines, it is reasonable to assume that these new progenitor cells will develop into cytokine-activated keratinocytes, re-establishing a psoriatic phenotype by acting on the crosstalk between keratinocytes and infiltrating cells. This theory would, therefore, explain the fact that upregulation of miR-203, IL12B and IL20 expression levels were detected in the skin grafts tested with antagomiR-203, features characteristic from psoriatic lesions, which may have lead to no amelioration of the psoriatic phenotype.

Concluding remarks and future perspectives

In this study, we identified *IL24, SOCS6* and *TNFα* as experimentally verified target genes for miR-203 in human keratinocytes. Importantly, mRNAs encoded by *IL24*, *SOCS6* and *TNFα* have been associated with cytokine network as well as *TNFα* has previously been established as a player in psoriasis pathogenesis, clearly demonstrating that miR-203 plays a role in cytokine signaling network. Here, stable production of RNA antagomirs (v-antagomirs) encoded by lentiviral vectors was also established as a method for persistently antagonizing miRNA function in cultured cells and in mice. It is relevant to mention that this approach was unique in exploring a vector-driven route of antagomir administration that allowed non-systemic and tissue-specific antagomir delivery to increase safety. Nonetheless, attempted knockdown of miR-203 in psoriasis xenograft transplantation model resulted in no amelioration of psoriasis phenotype, as evaluated by semi-quantitative clinical scoring. This study clearly demonstrates that the role of increased miR-203 expression in psoriasis is complex and further studies are still needed to comprehend miRNA functions in the cytokine network as well as to establish whether targeting of miRNAs by RNAi-mediated degradation is or not therapeutically relevant in psoriasis.

For the future, it is relevant to develop a similar setup as the one described above, in order to (1) confirm potent and persistent antagomir expression following a single intradermal injection of lentiviral vectors in xenografted human skin and (2) confirm that miR-203 knockdown occurs in psoriasis skin grafts treated with an antagomiR-203-encoding lentiviral vector. In case the results demonstrated here are reproducible, it is pertinent to carry out immunohistochemical staining for cell proliferation markers as well as for skin-homing T-cells in order to understand in more detail the crosstalk between keratinocytes and infiltrating cells. As an additional point, it has been recently validated that miR-125b targets *STAT3* [93]. As miR-203 and miR-125b expression levels are deregulated in psoriasis, specifically in keratinocytes, and both miRNAs are associated with STAT3 regulation pathway, I assume it would be pertinent to try to design a lentiviral construct allowing simultaneous expression of miR-125b and knockdown of miR-203, for following experimentation in psoriasis xenograft transplantation model. This study would be significant to understand how relevant miRNAs expressed in keratinocytes are in the regulation of the cytokine network and in the pathogenesis of psoriasis, since STAT3 is involved in amplifying the inflammation process in psoriatic keratinocytes, resulting in upregulation of several cytokines.

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APPENDIX

Appendix I. Oligonucleotide sequences of the cloned transgenes.

Table 1. Oligonucleotide sequences and predicted transcripts for antagomiR-203 and antagomiR-Negative control.

Name	Sequence (5' - 3')
FO-antagomiR-203	CTAGCCC CTAGTGGTCCTAAACATTTCAC TTTTTGGAAA
RO-antagomiR-203	CGCGTTTCCAAAAA GTGAAATGTTTAGGACCACTAG GGG
antagomiR-203	CUAGUGGUCCUAAACAUUUCAC UU
FO-antagomiR-neg	CTAGCCC GTGTAACACGTCTATACGCCCA TTTTTGGAAA
RO-antagomiR-neg	CGCGTTTCCAAAAA TGGGCGTATAGACGTGTTACAC GGG
antagomiR-neg*	GUGUAACACGUCUAUACGCCCA UU

* Sequence from Exiqon's miRCURY LNA™ Knockdown Control Probe.

Table 2. Sequence information about the target genes cloned into psiCHECK2 vector.

Gene	NCBI Reference	First Position (bp)	Last Position(bp)	Length (bp)	Characteristics
IL12B	NM_002187.2	12	2347	2336	~ Complete transcript
IL15	NM_000585.4	864	1448	585	3' UTR
IL17A	NM_002190.2	511	1774	1264	3' UTR
IL20	NM_018724.3	576	1210	635	3' UTR
IL24	NM_006850.3	695	1202	508	3' UTR
p63	NM_003722.4	3854	4372	519	3' UTR
SOCS3	NM_003955.3	2253	2720	468	3' UTR
SOCS6	NM_004232.3	2010	2504	495	3' UTR
TNFα	NM_000594.2	89	1600	1512	~ Complete transcript

Table 3. Primer sequences of the target genes cloned into psiCHECK2 vector.

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
IL12B	CCATTGGACTCTCCGTCCTG	CTAATGTTTCTTCTCAAAAA
IL15	TTGCAATTGATTCTTTTTAA	GTATTGTCAACGAAATATGT
IL17A	TAAGAGCTCTGGGGAGCCCA	GAGTATGAATTTCAAGCAAG
IL20	GAGGAAAGTGATGCTGCTGC	CATCTTTATGTACGACCGGC
IL24	GGCCAACAACTTTGTTCTCA	CGGAAGTACATTGAATGTCA
p63	GTGGATTGCCTCTGAAAAGT	CCGATCACGGTTTAGGTACT
SOCS3	CTGGAGGTCATTGGAGAGGC	TATCACGAGAAATAATATTT
SOCS6	CTTTTGCTGCCATAACTATT	TTCTATACAACGTCTAGAGG
TNFα	CCCTGACAAGCTGCCAGGCA	CTCGTCTCCGAGTCGTTACT

Table 4. Oligonucleotide sequences and predicted transcripts for psoriasis related-miRNA-perfect targets.

Name	Sequence (5' - 3')
FO-miRNA-125b target	TCGAGTAA TCACAAGTTAGGGTCTCAGGGA AGG
RO-miRNA-125b target	GGCCCCT TCCCTGAGACCCTAACTTGTGA TTAC
miRNA-125b target	UAAUCACAAGUUAGGGUCUCAGGGAAGG
FO-miRNA-203 target	TCGAGGGT CTAGTGGTCCTAAACATTTCAC AAT
RO-miRNA-203 target	GGCCATT GTGAAATGTTTAGGACCACTAG ACCC
miRNA-203 target	GGUCUAGUGGUCCUAAACAUUUCACAAU

Appendix II. Schematic representation of the different vectors.

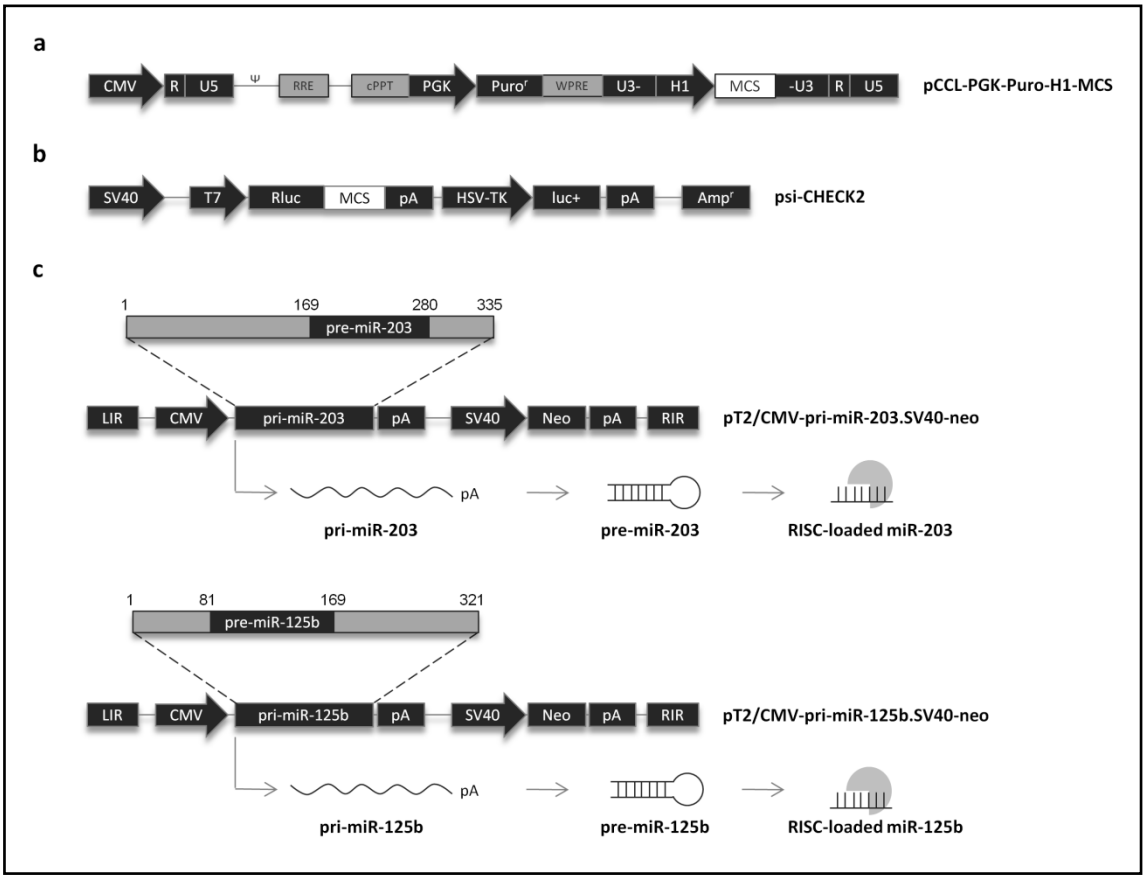

Figure AI. Schematic overview of the different vectors used in this project. (**a**) Schematic overview of the lentiviral vector, pCCL-PGK-Puro-H1-MCS, for an easy one-step antagomir cloning. Antagomirs with compatible overhangs can be cloned into the multiple cloning site (MCS) from which antagomir expression will be driven by the H1 promoter. (**b**) Schematic overview of the dual-reporter vector, psiCHECK2, for development of functional studies. 3'UTRs with compatible overhangs can be cloned into the MCS from which expression will be driven by T7 promoter. (**c**) Schematic overview of the Sleeping Beauty (SB) DNA transposon-based sensor, pT2/CMV-MCS.SV40-neo, for expression of pri-miR-203 and pri-miR-125b. Modules of the sensor are flanked by the left and right inverted repeat (LIR and RIR, respectively) of the SB transposon. pri-miRNA gene cassettes with compatible overhangs can be cloned into the MCS from which pri-miRNA expression will be driven by the CMV promoter and enter the miRNA processing pathway. *Adapted from manuscript Primo et al, 2011.*

Appendix III. Potential miR-203-binding sites in IL24 3'UTR sequence.

IL24 Sequence Modification

IL24 3'UTR Sequence

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1     GGCCAACAACTTTGTTCTCATCGTGTCACAACTGCAACCCAGTCAAGAAA
51    ATGAGATGTTTTTCCATCAGAGACAGTGCACACAGGCGGTTTCTGCTATTC
101   CGGAGAGCATTCAAACAGTTGGACGTAGAAGCAGCTCTGACCAAAGCCCT
151   TGGGGAAGTGGACATTCTTCTGACCTGGATGCAGAAATTCTACAAGCTCT
201   GAATGTCTAGACCAGGACCTCCCTCCCCCTGGCACTGGTTTGTTCCCTGT
251   GTCATTTCAAACAGTCTCCCTTCCTATGCTGTTCACTGGACACTTCACGC
301   CCTTGGCCATGGGTCCCATTCTTGGCCCAGGATTATTGTCAAAGAAGTCA
351   TTCTTTAAGCAGCGCCAGTGACAGTCAGGGAAGGTGCCTCTGGATGCTGT
401   GAAGAGTCTACAGAGAAGATTCTTGTATTTATTACAACTCTATTTAATTA
451   ATGTCAGTATTTCAACTGAAGTTCTATTTATTTGTGAGACTGTAAGTTAC
501   ATGAAGGC
```

IL24-mut 3'UTR Sequence

```
1     GGCCAACAACTTTGTTCTCATCGTGTCACAACTGCAACCCAGTCAAGAAA
51    ATGAGACACAAAACATCAGAGACAGTGCACACAGGCGGTTTCTGCTATTC
101   CGGAGAGCATTCAAACAGTTGGACGTAGAAGCAGCTCTGACCAAAGCCCT
151   TGGGGAAGTGGACATTCTTCTGACCTGGATGCAGAAATTCTACAAGCTCT
201   GAATGTCTAGACCAGGACCTCCCTCCCCCTGGCACTGGTTTGTTCCCTGT
251   GTCACAAAAAACAGTCTCCCTTCCTATGCTGTTCACTGGACACAAAACGC
301   CCTTGGCCATGGGTCCCATTCTTGGCCCAGGATTATTGTCAAAGAAGTCA
351   TTCTTTAAGCAGCGCCAGTGACAGTCAGGGAAGGTGCCTCTGGATGCTGT
401   GAAGAGTCTACAGAGAAGATTCTTGTATTTATTACAACTCTATTTAATTA
451   ATGTCAGCACAAAAACTGAAGTTCTATTTATTTGTGAGACTGTAAGTTAC
501   ATGAAGGC
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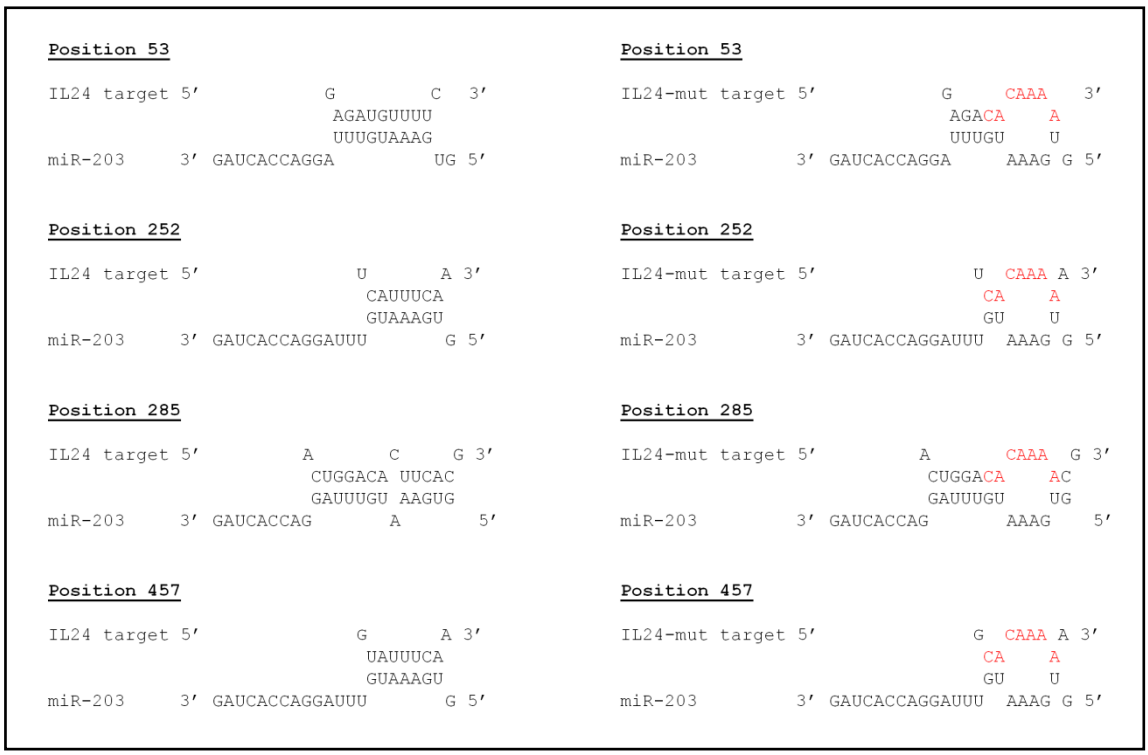

Figure AII. Schematic representation of the potential miRNA-203 binding sites in IL24 3'UTR sequence. Nucleotide resolution of the predicted target sites in IL24 naïve (IL24 target) and IL24-mutant (IL24-mut target) 3'UTR sequences by miR-203. Mutated seed match sequence in red letters.

Appendix IV. Potential miR-203-binding sites in SOCS6 3'UTR sequence.

SOCS6 Sequence Modification

SOCS6 3'UTR Sequence

```
1     CTTTTGCTGCCATAAC TATTTCAGTTTTA TGTGTAAAAGAGTCATCAGTT
51    TGTTTAGGGGTGGGGAAGTGTCAGCAAGGTGTCTTGGGTTTATTTTGGTT
101   CTTTAAAAAAGGGAAGTCTTGAGGTTTTAGAGGTGTGAATTA TGTTTCA T
151   CAATGTGCAGAATAATCACAATGTGAATTATCAAATTCTCCTCAATGCCC
201   CCCCCGCCCAGTCCTTTGCTGCTATCCACTGTGATTTTTATGCATTAAAA
251   GCA CATTTCA TGTGTATTCAACCCTAAGTAAAGTTGAATGAAACTTAACA
301   GAATGGAAATTGCTATGTCTTTTTAAATGGTCCATTTTCAAAAGACAGTG
351   TTGAATAAACATACCTGTGTGATAAAACACAGAATTTACATATACACTGA
401   AGATGAGTTTTTAATCTCTTACTTTAAAAAGATTTATTTAGAATCGTGAA
451   TTGACATAATCTTGGGTAATGGAACGGAGATCTGCAACATATCTT
```

SOCS6-mut 3'UTR Sequence

```
1     CTTTTGCTGCCATAAC CACAAAACACAAA TGTGTAAAAGAGTCATCAGTT
51    TGTTTAGGGGTGGGGAAGTGTCAGCAAGGTGTCTTGGGTTTATTTTGGTT
101   CTTTAAAAAAGGGAAGTCTTGAGGTTTTAGAGGTGTGAATTA CACAAAA T
151   CAATGTGCAGAATAATCACAATGTGAATTATCAAATTCTCCTCAATGCCC
201   CCCCCGCCCAGTCCTTTGCTGCTATCCACTGTGATTTTTATGCATTAAAA
251   GCA CACAAAA TGTGTATTCAACCCTAAGTAAAGTTGAATGAAACTTAACA
301   GAATGGAAATTGCTATGTCTTTTTAAATGGTCCATTTTCAAAAGACAGTG
351   TTGAATAAACATACCTGTGTGATAAAACACAGAATTTACATATACACTGA
401   AGATGAGTTTTTAATCTCTTACTTTAAAAAGATTTATTTAGAATCGTGAA
451   TTGACATAATCTTGGGTAATGGAACGGAGATCTGCAACATATCTT
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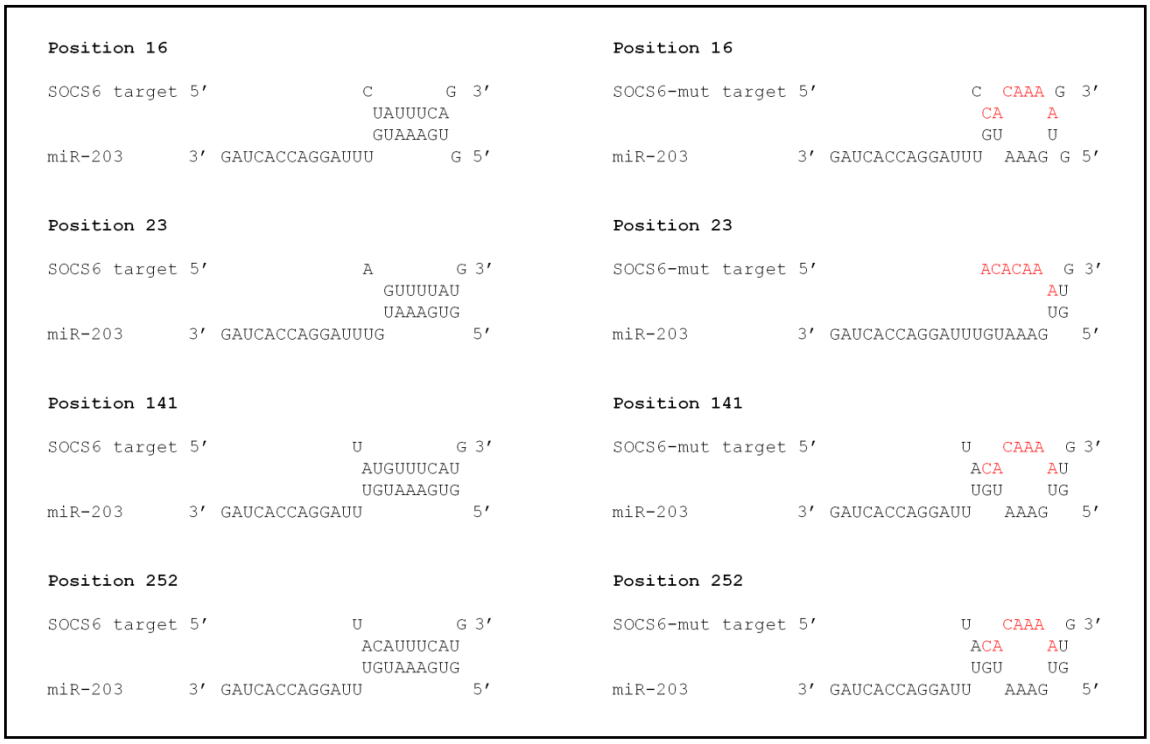

Figure AIII. Schematic representation of the potential miRNA-203 binding sites in SOCS6 3'UTR sequence. Nucleotide resolution of the predicted target sites in SOCS6 naïve (SOCS6 target) and SOCS6-mutant (SOCS6-mut target) 3'UTR sequences by miR-203. Mutated seed match sequence in red letters.

Appendix V. Potential miR-203-binding sites in TNFα 3'UTR sequence.

TNFα Sequence Modification

TNFα 3'UTR Sequence

```
1     GGAGGACGAACATCCAACCTTCCCAAACGCCTCCCCTGCCCCAATCCCTT
51    TATTACCCCCTCCTTCAGACACCCTCAACCTCTTCTGGCTCAAAAAGAGA
101   ATTGGGGGCTTAGGGTCGGAACCCAAGCTTAGAACTTTAAGCAACAAGAC
151   CACCACTTCGAAACCTGGGATTCAGGAATGTGTGGCCTGCACAGTGAAGT
201   GCTGGCAACCACTAAGAATTCAAACTGGGGCCTCCAGAACTCACTGGGGC
251   CTACAGCTTTGATCCCTGACATCTGGAATCTGGAGACCAGGGAGCCTTTG
301   GTTCTGGCCAGAATGCTGCAGGACTTGAGAAGACCTCACCTAGAAATTGA
351   CACAAGTGGACCTTAGGCCTTCCTCTCTCCAGATGTTTCCAGACTTCCTT
401   GAGACACGGAGCCCAGCCCTCCCCATGGAGCCAGCTCCCTCTATTTATGT
451   TTGCACTTGTGATTATTTATTATTTATTTATTATTTATTTATTTACAGAT
501   GAATGTATTTATTTGGGAGACCGGGGTATCCTGGGGGACCCAATGTAGGA
551   GCTGCCTTGGCTCAGACATGTTTTCCGTGAAAACGGAGCTGAACAATAGG
601   CTGTTCCCATGTAGCCCCCTGGCCTCTGTGCCTTCTTTTGATTATGTTTT
651   TTAAAATATTTATCTGATTAAGTTGTCTAAACAATGCTGATTTGGTGACC
701   AACTGTCACTCATTGCTGAGCCTCTGCTCCCCAGGGGAGTTGTGTCTGTA
751   ATCGCCCTACTATTCAGTGGC
```

TNFα-mut 3'UTR Sequence

```
1     GGAGGACGAACATCCAACCTTCCCAAACGCCTCCCCTGCCCCAATCCCTT
51    TATTACCCCCTCCTTCAGACACCCTCAACCTCTTCTGGCTCAAAAAGAGA
101   ATTGGGGGCTTAGGGTCGGAACCCAAGCTTAGAACTTTAAGCAACAAGAC
151   CACCACTTCGAAACCTGGGATTCAGGAATGTGTGGCCTGCACAGTGAAGT
201   GCTGGCAACCACTAAGAATTCAAACTGGGGCCTCCAGAACTCACTGGGGC
251   CTACAGCTTTGATCCCTGACATCTGGAATCTGGAGACCAGGGAGCCTTTG
301   GTTCTGGCCAGAATGCTGCAGGACTTGAGAAGACCTCACCTAGAAATTGA
351   CACAAGTGGACCTTAGGCCTTCCTCTCTCCAGACACAAAAAGACTTCCTT
401   GAGACACGGAGCCCAGCCCTCCCCATGGAGCCAGCTCCCTCTATTTATGT
451   TTGCACTTGTGATTATTTATTATTTATTTATTATTTATTTATTTACAGAT
501   GAATGTATTTATTTGGGAGACCGGGGTATCCTGGGGGACCCAATGTAGGA
551   GCTGCCTTGGCTCAGACATGTTTTCCGTGAAAACGGAGCTGAACAATAGG
601   CTGTTCCCATGTAGCCCCCTGGCCTCTGTGCCTTCTTTTGATTATGTTTT
651   TTAAAACACAAAACTGATTAAGTTGTCTAAACAATGCTGATTTGGTGACC
701   AACTGTCACTCATTGCTGAGCCTCTGCTCCCCAGGGGAGTTGTGTCTGTA
751   ATCGCCCTACTATTCAGTGGC
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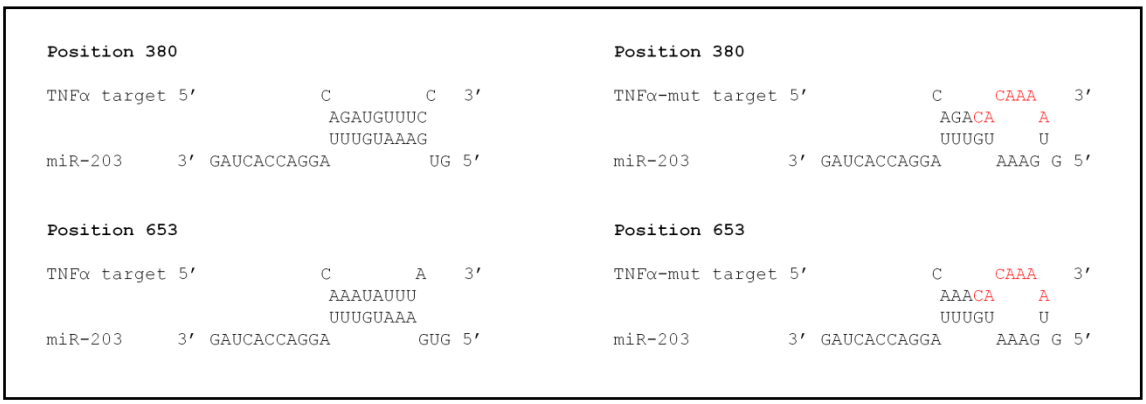

Figure AIV. Schematic representation of the potential miRNA-203 binding sites in TNFα 3'UTR sequence. Nucleotide resolution of the predicted target sites in TNFα naïve (TNFα target) and TNFα-mutant (TNFα-mut target) 3'UTR sequences by miR-203. Mutated seed match sequence in red letters.

Appendix VI. Supplementary data.

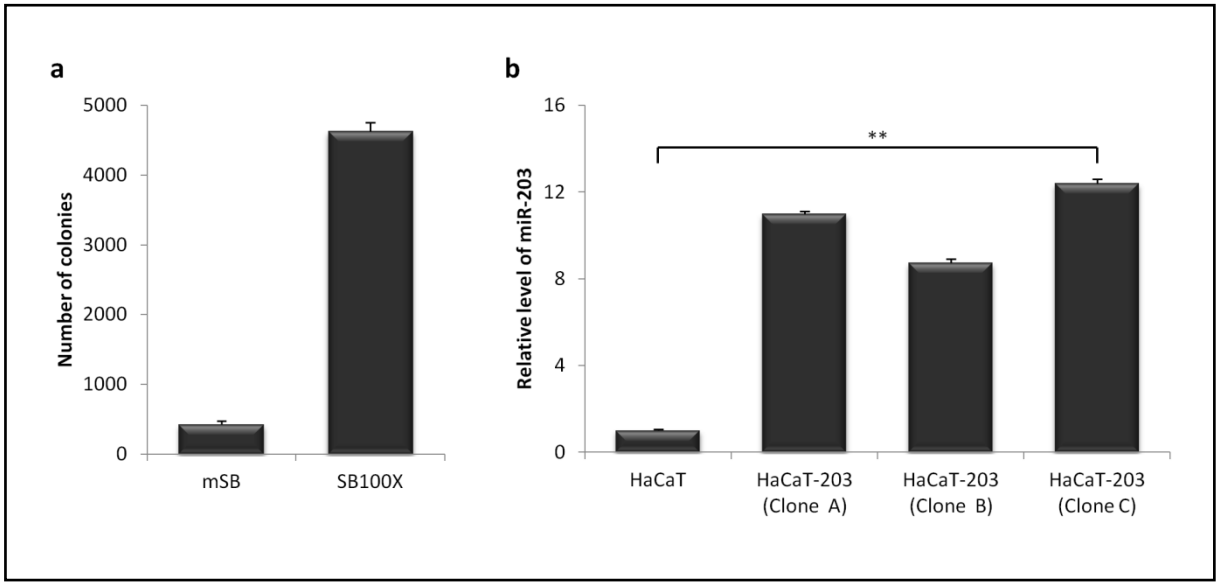

Figure AV. Generation of stable HaCaT cell lines expressing miRNA-203 by transposition assay. (**a**) The transposition assay was carried out with two transposases, the mutated, inactive variant (mSB) and the SB100X transposase (SB100X). HaCaT cells were transfected with 1.82 µg pT2/CMV-pri-miR-203.SV40-neo and 180 ng plasmid encoding mSB or SB100X. Transposition activity was measured by counting the number of G418-resistant colonies 14 days post-transfection. (**b**) miR-203 levels were evaluated by qRT-PCR in naïve HaCaT cells and in three individual clones expressing miR-203 (HaCaT-203). miR-203 levels were normalized to the expression of the reference small nucleolar RNA, C/D box 48 (RU48).*P*-values for the comparisons indicated by brackets were as follows: ***P* < 0.01. All assays were performed in triplicates and are the data are depicted as mean + SEM. *Adapted from manuscript Primo et al, 2011.*

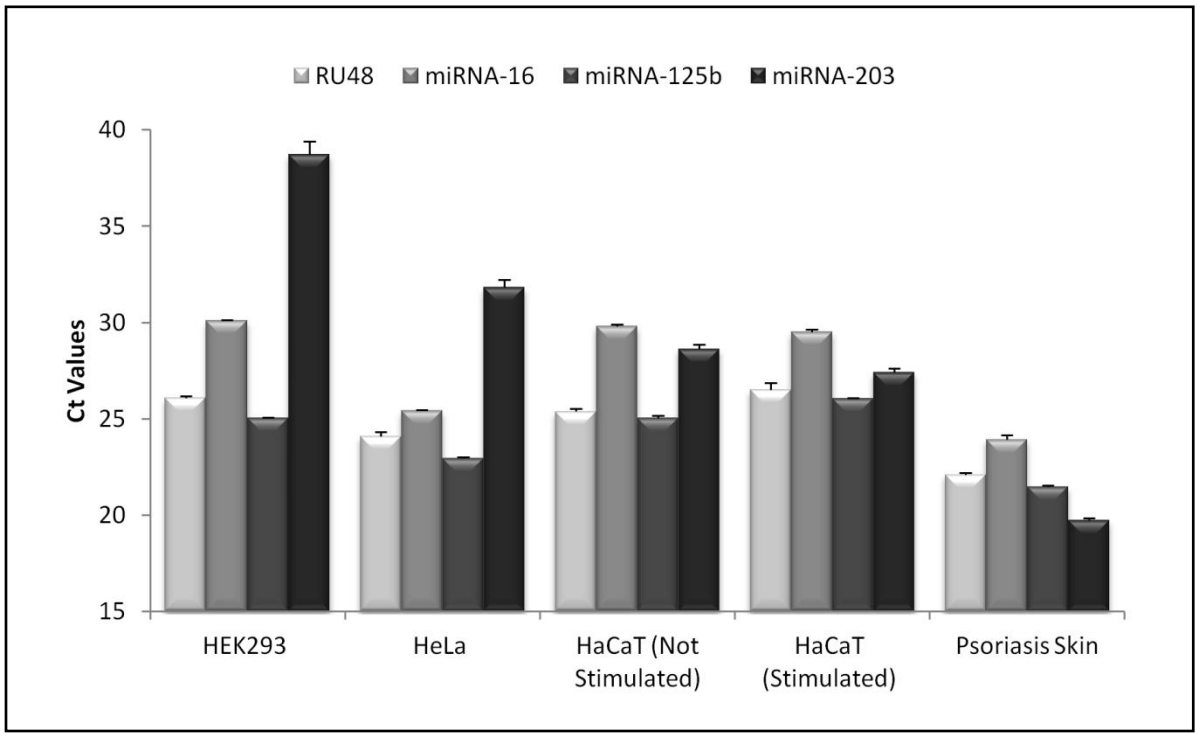

Figure AVI. Quantification of RNA Ct values in human cell lines. RU48, miRNA-16, miRNA-125b and miRNA-203 Ct values were evaluated by qRT-PCR in different cell lines: HEK293, HeLa, HaCaT (with and without stress stimulation) and RNA extracted from psoriatic skin. All assays were performed in triplicates and are the data are depicted as mean + SEM.