THE ROLE OF MIR-203 IN KERATINOCYTE BIOLOGY AND PSORIASIS

Tianling Wei
韦天玲
M.D.

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Supervisors:

Dr. Andor Pivarcsi
Department of Medicine
Unit of Dermatology and Venereology
Karolinska Institutet, Stockholm, Sweden

Professor Mona Ståhle
Department of Medicine
Unit of Dermatology and Venereology
Karolinska Institutet, Stockholm, Sweden

Dr. Enikő Pivarcsi Sonkoly
Department of Medicine
Unit of Dermatology and Venereology
Karolinska Institutet, Stockholm, Sweden

Faculty opponent:

Professor Lone Skov
Department of Dermato-Allergology
Gentofte University Hospital, Copenhagen, Denmark

Thesis committee:

Professor Anna Wedell
Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

Professor Dan Grandér
Department of Onkology and Pathology
Cancer Center Karolinska (CCK)
Karolinska Institutet, Stockholm, Sweden

Professor Anders Vahlquist
Department of Medical Sciences
Unit of Dermatology and Venereology
Uppsala University, Uppsala, Sweden

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Cover image was painted by Lasse Folkersen. It illustrates the skin structure.
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I may not have gone where I intended to go, but I think I have ended up where I needed to be.

......*The Hitchhiker’s Guide to the Galaxy*

*To my family and friends*
Psoriasis is one of the most common chronic inflammatory skin diseases. It is a life long suffering disease affecting approximately 2 to 3% of the population in Scandinavia. Significant progress has been made to understand the cellular immunology and biology of psoriasis. However, the cause is still unclear and we still lack a cure for this common and enigmatic disease. MicroRNAs (miRNAs) are 18-22 nucleotides non-coding RNAs that regulate gene expression at the post-transcriptional level by targeting mRNAs for translational suppression or degradation. Extensive studies in the last few years showed that miRNAs play important roles in physiological processes and diseases. Before our investigation, no connection had been established between miRNAs and psoriasis.

The general aim of this thesis was to investigate the involvement of miRNAs in the pathogenesis of psoriasis and skin/keratinocyte biology.

In paper I, we showed for the first time that psoriasis-affected skin has a specific microRNA expression profile compared with healthy human skin or with another chronic inflammatory skin disease, atopic eczema. Among the psoriasis-associated microRNAs, we identified one skin and keratinocyte-specific microRNA, miR-203. The up-regulation of miR-203 in psoriatic plaques was concurrent with the down-regulation of one of its evolutionary conserved target, suppressor of cytokine signaling 3 (SOCS-3), which is involved in inflammatory responses and keratinocyte functions. This paper unveiled the involvement of microRNAs in inflammatory skin diseases.

In paper II, we particularly examined the role of miR-203 in keratinocyte differentiation. Out of 365 miRNAs tested, miR-203 was the most upregulated miRNA during keratinocyte differentiation. Furthermore, we found that upregulation of miR-203 is required for keratinocyte differentiation and is dependent on the activation of the PKC/AP-1 pathway.

In paper III, we explored the expression of miR-203 during human skin morphogenesis. MiR-203 is barely detectable at 14 weeks of estimated gestation age (EGA). Its expression became prominent from week 17 and was most pronounced in the suprabasal layers of the epidermis. The direct targets of miR-203, p63 and SOCS-3, were preferentially expressed in the basal layer. Our results suggest miR-203 is involved in the regulation of human foetal skin development and provide a basis for further studies to investigate the role of miR-203 in this process.

In paper IV, we studied the role of miR-203 in NF-κB signaling in keratinocytes. We found that overexpression of miR-203 in human primary keratinocytes suppressed NF-κB activity by 1) suppressing downstream genes in NF-κB pathway; 2) preventing the nuclear translocation of p65 and repressing NF-κB-driven promoter luciferase activity. The results suggested that miR-203 plays a potential role in keeping skin homeostasis and controlling inflammation by modulating NF-κB signaling.

In conclusion, our data in the thesis suggest that miR-203 plays a role as ‘safety guard’ in skin by regulating inflammation-, differentiation-, proliferation- and morphogenesis-associated processes.
LIST OF PUBLICATIONS

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


\textsuperscript{*} Corresponding author
OTHER RELATED PAPERS


   *Equal contribution

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<tr>
<td>AP 1</td>
<td>Activating Protein 1</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>CE</td>
<td>Cornified Envelop</td>
</tr>
<tr>
<td>EGA</td>
<td>Estimated Gestational Age</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HKGS</td>
<td>Human Keratinocyte Growth Supplement</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>KGF</td>
<td>Keratinocyte Growth Factor</td>
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<tr>
<td>LNA</td>
<td>Locked Nucleic Acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MDDC</td>
<td>Monocyte-Derived Dendritic Cells</td>
</tr>
<tr>
<td>miRISC</td>
<td>miRNA-containing RNA-induced silencing complex</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>miRNP</td>
<td>miRNA-containing ribonucleoprotein</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>premiRNA</td>
<td>Precursor of miRNA</td>
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<tr>
<td>PUVA</td>
<td>Psoralen + UVA treatment</td>
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<tr>
<td>RT</td>
<td>Reverse Transcription</td>
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<td>SAM</td>
<td>Significance Analysis of Microarrays</td>
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<td>SHIP 2</td>
<td>SH2 domain-containing inositol phosphatase 2</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SNP</td>
<td>Single-Nucleotide Polymorphism</td>
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<td>STAT 3</td>
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<tr>
<td>TLDA</td>
<td>TaqMan® Low Density Array</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-Tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>UVR</td>
<td>UltraViolet Radiation</td>
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1 INTRODUCTION

Psoriasis is a chronic inflammatory skin disease of multifactorial origin, affecting approximately 2-3% of the population in Scandinavia. Both genetic and environmental factors contribute to the development of the disease. It is a lifelong disease with spontaneous remissions and exacerbations, and with wide clinical variations ranging from mild almost cosmetic to severe with extensive lesions covering most of the body. Comorbidities are an important feature of the disease, up to 30% of patients develop psoriatic arthritis and severe psoriasis is associated with a significant risk for cardiovascular disease. Thus, psoriasis can have a major impact on the patients’ life quality and constitutes an important health problem in society. Much of the clinical disease phenotype is caused by hyperproliferation and altered differentiation of epidermal keratinocytes, and increased growth of dermal blood vessels. The immune response in psoriasis is markedly polarized towards a Th1 cell response, with the presence of T cells, Th17 cells, dendritic cells and a broad range of cytokines and chemokines in the inflammatory lesions. Although significant progress has been made in the understanding of the underlying immunology and biology of psoriasis, the cause remains unknown and we still lack a cure for this common and enigmatic disease.

MicroRNAs (miRNAs) are ~22 nucleotides (nt) non-coding RNAs that regulate gene expression at the post-transcriptional level by targeting mRNAs for translational suppression or degradation. MiRNAs mediate their regulatory action through imperfect binding to the 3’ untranslated region (3’UTR) of target miRNAs carrying complementary sites. Base-pairing at position 2-8 nt relative to the 5’ end of small RNA, termed as the ‘seed’ region, appears to be the most important for target recognition. Typically, miRNAs regulate biological functions through suppressing hundreds of target miRNAs simultaneously. Many miRNAs are highly conserved, suggesting strong evolutionary pressure and participation in essential biological processes such as development, differentiation, apoptosis, fat metabolism, viral infection, and cancer. Before our investigation, no connection had been established between miRNAs and psoriasis.

The work presented in this thesis focuses on exploring the role of miRNAs in pathogenesis of psoriasis and keratinocyte biology.
1.1 **SKIN**

The skin is the largest organ in the body, making up 16% of body weight, with a surface area of approximately 1.8m². It is the boundary between ourselves and the world around us. Its primary role is that of a barrier, preventing the entry of noxious chemicals and infectious organisms, and preventing the exit of water and other chemicals. It is a sort of ‘space suit’, nicely evolved to house all the other organs and chemicals in our bodies (Goldsmith, 1990; Norlen, 2003; Tobin, 2006).

Except for being a barrier, skin has other roles too. It is an important sensory organ, and controls body temperature. It can sweat, grow hair, erect its hairs, change colour, smell, grow nails and secrete sebum. When confronted with insults from outside, it usually adapts easily and returns to a normal state, but sometimes it fails to do so and a skin disorder appears.

The skin is organized into an elaborate layered structure consisting mainly of the outermost epidermis and the underlying dermis. A subcutaneous adipose-storing hypodermis layer (subcutis) and various appendages such as hair follicles, sweat glands, sebaceous glands, nerves, lymphatics, and blood vessels are also present in the skin.

![Skin Structure Diagram](source: Pearson Scott Foresman publishing)

**Figure 1.** Skin structure (source: Pearson Scott Foresman publishing).

1.1.1 **Epidermis**

Epidermis is the outmost layer of the skin, serving as the physical and chemical barrier between the interior body and exterior environment. The thickness of the epidermis varies in different types of skin. It is thinnest on the eyelids at 0.05 mm and thickest on the palms and
soles at 1.5 mm. The epidermis contains four histological distinct layers which, from the inside to the outside, are the stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The bottom layer, the stratum basale, has cells that are shaped like columns. In this layer the cells divide and push already formed cells into higher layers. As the cells move into the higher layers, they flatten and eventually die. The top layer of the epidermis, the stratum corneum, is made of dead, flat skin cells that shed about every 2 weeks.

There are three major cell types in epidermis: keratinocytes, melanocytes and Langerhans cells. Keratinocytes are the major cellular constituent, constituting 95% of the epidermis. Melanocytes are found in the stratum basale. These cells produce pigment called melanin, which is responsible for different skin colour. Langerhans cells are immune cells found in the epidermis, and are responsible for helping the body learn and later recognise new ‘antigens’ (foreign material to the body). Langerhans cells break the foreign material into smaller pieces then migrate from the epidermis into the dermis. They find their way to lymphatics and blood vessels before eventually reaching the lymph nodes. Here they present the antigens to immune cells called lymphocytes. Once the antigen is successfully presented, the lymphocytes initiate a sequence of events to (1) initiate an immune reaction to destroy the foreign material, and (2) stimulate proliferation of more lymphocytes that recognise and remember the foreign material in the future. Merkel cells are the fourth cells found in the basal layer of the epidermis. Their exact role and function is not well understood.

1.1.1.1 Morphogenesis of human epidermis
During embryonic development, the epidermis derives from a single-layered epithelium, the surface ectoderm. The ectoderm proliferates in the 4th week of estimated gestational age (EGA), and produces two layers of cells (Breathnach and Wyllie, 1965; Holbrook and Odland, 1975). The inner layer of cells is the basal layer while the outer layer is called the periderm, and proliferation takes place in both cell layers (Holbrook and Odland, 1975). In the 11th week of EGA, a new intermediate cell layer is produced between the basal and the periderm layer. It marks the beginning of stratification of the epidermis. At 12 weeks of EGA, appendages begin to form from cells of the basal cell layer. Groups of cells develop into hair follicles and associated granular keratinocytes. The periderm cells stop dividing, become larger and elevated, and exhibit rounded blebs on their outer surfaces (Holbrook and Odland, 1975). The nondividing periderm cells then form a cornified cell envelope. By 21–24 weeks
of EGA, the intermediate cell layer has apparently become the three layers of the outer epidermis: the spinous, the granular, and the cornified cell layers. As the stratification proceeds, the periderm layer is gradually shed into the amniotic fluid by 24-25 weeks of EGA (Holbrook and Odland, 1980). The periderm cells show characteristics consistent with apoptosis before being sloughed off (Polakowska et al., 1994). Cornified cell envelope is formed in the upper cell layers of epidermis after the shedding of the periderm cells (Akiyama et al., 2000; Akiyama et al., 1999).

![Figure 2. The structure of epidermis.](image)

### 1.1.2 Dermis

The dermis is mainly composed of collagen and elastin fibres, giving the skin flexibility and strength. The dermis also contains nerve endings, sweat glands and sebaceous glands, hair follicles, and blood vessels (Spellberg, 2000).

The nerve endings provide the body with the information from outside world. The sweat glands produce sweat in response to heat and stress. As sweat evaporates off the skin, it helps cool the body temperature. The sebaceous glands secrete sebum into hair follicles. Sebum is oil that keeps the skin moist and soft and acts as part of barrier against foreign substances. The hair follicles produce the assorted types of hair throughout the body. Hair not only contributes to a person's appearance but also has few important physical roles including
regulating body temperature, providing protection from injury, and enhancing sensation. A part of the follicle contains stem cells capable of repairing damaged epidermis. The blood vessels of the dermis provide nutrients to the skin and help regulate body temperature. Heat enlarges the blood vessels, allowing certain amounts of blood to circulate near the skin surface, where the heat can be released. Cold constricts the blood vessels, retaining the body's heat.

1.1.3 Subcutis
The subcutis is the fat layer below the dermis and epidermis. It is also called subcutaneous tissue, hypodermis or panniculus. The subcutis mainly consists of fat cells (adipocytes), nerves and blood vessels. Fat cells are organised into lobules, which are separated by structures called septae. The septae contain nerves, larger blood vessels, fibrous tissue and fibroblasts. Fibrous septae may form dimples in the skin (so-called cellulite).

1.2 Keratinocytes
The human epidermis provides a first-line defence barrier for the host. Keratinocytes are the major cell type in epidermis, contributing significantly to form the barrier against various physical and immunological insults and stimuli (Kim J, 2003; Stingl G, 2003). Keratinocytes are generated from two sources in the epidermis: the interfollicular epidermal stem cells in the basal layer and stem cells in the bulge region of hair follicles. Interfollicular stem cells differentiate into epidermal keratinocytes and other skin appendix cells. Hair follicle stem cells differentiate into epidermal keratinocytes, skin appendix cells as well as neuronal cells and mesenchymal cells (Cotsarelis, 2006; Kaur, 2006; Yan and Owens, 2008).

When cultured in vitro, keratinocytes have a limited lifespan. They gradually become slow in growth, and eventually stop dividing. This process is known as cellular senescence. It is thought to limit the vulnerability of aging cells to disease. Human keratinocytes are priceless for the study of skin biology and the pathogenesis of skin-related diseases, but their short lifespan in culture is a limitation.
1.2.1 Keratinocyte differentiation

In the epidermis, keratinocytes produce major structural components of the epidermal barrier through a programmed and multistep process. The progress of keratinocyte differentiation begins in the basal layer of the epidermis, which contains undifferentiated keratinocytes with high proliferative potential (stratum basale). One daughter cell starts migrating upwards in the epidermis and undergoes differentiation, which is characterized with profound changes in gene expression. As cells move upwards to the epidermal surface, the cells cease division and eventually shed away from the skin surface. During the transit from stratum basale to the skin surface, the cells undergo morphological changes to form stratum spinosum, stratum granulosum and stratum corneum. Cells in stratum spinosum are characterized by the presence of extensive intercellular desmosomal connections, whereas cells in stratum granulosum are distinguished by the presence of granules that contain the products of keratinocyte differentiation. Continued differentiation of the granular layer cells results in formation of the transition zone which separates the dead from living epidermal layers. It is in this zone that the cellular constituents are extensively remodelled. The formation of stratum corneum results from this remodelling (Green, 1980). During this process, keratinocytes initiate the synthesis of important differentiation-dependent structural and catalytic proteins including loricrin (Yoneda et al., 1992), involucrin (Rice and Green, 1977, 1979), the keratins (Green et al., 1982), filaggrin (Dale et al., 1985), and transglutaminase (Thacher and Rice, 1985). Each keratinocyte in stratum corneum, which is called corneocyte, is surrounded by a protein shell called a cell cornified envelope (CE). The CE composes primarily of two proteins, loricin and involucrin. These proteins make CE the most insoluble structure (Candi et al., 2005).

The principal markers for keratinocyte differentiation are particular keratin pairs. More than 30 keratins are currently known. The majority of these are associated with the skin and its appendages. Broadly speaking, proliferative keratinocytes in the basal cell layer of the epidermis express K5 and K14, while keratinocytes in the early stages of differentiating switch to K1 and K10. Moreover, K6 and K16 are expressed in epithelial cells of the outer root sheath of hair follicles, nail beds, and epithelium of oral mucosa, and the expression of K6 and K16 in supra-basal layers can occur when keratinocytes proliferate in a non-physiological manner such as in psoriasis and wound healing (Lane and McLean, 2004; Tobin, 2006).
Another important keratinocyte differentiation marker is involucrin. Involucrin expression initiates in the early spinous layer and is maintained in the granular layer. In the transition zone, involucrin is incorporated, via the action of transglutaminase, as a component of the cornified envelope (Eckert et al., 1993; Yaffe et al., 1992).

There are several methods established to mimic keratinocyte differentiation in vitro, using monolayer culture of primary human keratinocytes. Keratinocyte differentiation can be induced by increasing calcium concentration in the medium, treatment with TPA or vitamin D. Contact inhibition can also induce keratinocyte differentiation. Thus keratinocyte differentiation can be induced by culturing cells at high confluence.

Figure 3. Keratinocyte monolayer culture in different confluence.

1.3 PSORIASIS

Psoriasis was originally thought of as a disorder primarily of epidermal keratinocytes, but is now recognized as one of the commonest immune-mediated disorders (Griffiths and Barker, 2007). Psoriasis is most frequent in the Caucasian population, and it is estimated to affect only 0.3% of the general population in China. The prevalence of psoriasis is between 1.5% to 3% of the general Scandinavian population, with men and women equally affected. The incidence in white population is estimated to be 60 cases per 100000 individuals of population per year (Griffiths CEM, 2005)

1.3.1 Clinical features of psoriasis

The most common type of psoriasis, accounting for 90% of all cases, is psoriasis vulgaris, in which papulosquamous plaques are well-delineated from surrounding normal skin. The plaques are red or salmon pink in color, covered by white or silvery scales, and may be thick, thin, large or small. Plaques are usually distributed symmetrically, and occur most commonly
on the extensor aspects of elbows and knees; scalp, lumbosacral region, and umbilicus (Griffiths CEM, 2005).

Figure 4. Psoriasis plague.

Children and adolescents can develop an acute form of psoriasis known as guttate psoriasis, in which papules less than 1 cm in diameter erupt on the trunk about 2 weeks after a β-haemolytic streptococcal infection such as tonsillitis or pharyngitis, or a viral infection. Guttate psoriasis is self-limiting, resolving within 3–4 months of onset, although its long-term prognosis is unknown. One study indicated that only a third of individuals with guttate psoriasis develop classic plaque disease (Martin et al., 1996).

About 50% of patients with psoriasis have distinctive nail changes related to the disease: the commonest is pitting, which is best seen under oblique lighting conditions; onycholysis (nail plate separation); oil spots (orange-yellow sub-ungual discolouration), and dystrophy psoriatic nail disease occurs most commonly in patients with psoriatic arthritis (Farber and Nall, 1992; Jiaravuthisan et al., 2007).

Psoriatic arthritis is a seronegative inflammatory arthritis that occurs in the presence of psoriasis (Moll and Wright, 1973). Five types of psoriatic arthritis have been proposed: distal interphalangeal joint only; asymmetrical oligoarthritis; polyarthritis; spondylitis; and arthritis mutilans. Classic psoriatic arthritis consists of oligoarthritis, distal interphalangeal joint involvement, dactylitis, and calcaneal enthesitis (Helliwell and Taylor, 2005). Recently
presented data indicate that its prevalence has been greatly underestimated, and may be as high as 25% in people with psoriasis. In about 10% of people with psoriatic arthritis, the arthritis appears before skin manifestations of psoriasis (Zachariae, 2003).

![Healthy skin vs. Psoriasis skin](image)

**Figure 5.** Histological features of psoriasis vs. healthy skin.

### 1.3.2 Histological features of psoriasis

Psoriasis has three principal histological features: abnormal keratinocyte differentiation and epidermal hyperplasia; dilated, increased growth of blood vessels in the dermis; and infiltrate of inflammatory cells, predominantly into the dermis. Histology of uninvolved psoriasis skin is normal (Braverman and Yen, 1974; Krueger and Bowcock, 2005).

#### 1.3.2.1 Abnormal keratinocyte differentiation

Several keratinocyte differentiation markers have found to be abnormal, and all have implications in the pathogenesis of the disease. These include aberrations of keratinocyte transglutaminase type I (TGase K), involucrin, filaggrin and keratin expression.

**TGase K**, which catalyzes a critical step of CE formation, is upregulated in psoriasis (Schroeder *et al.*, 1992). The overexpression of this enzyme causes the excessive cornification seen in psoriatic lesions, which directly contributes to the hard lesions manifested on the skin in psoriatic patients (Candi *et al.*, 2005).
**Involucrin**, which helps to stabilize the CE, is also upregulated in psoriasis vulgaris. In normal skin, this protein is a major constituent of the CE only during its early stages of assembly. However, in psoriatic skin, CE formation seems to be initiated prematurely, and involucrin remains the major constituent of the CE during maturation (Ishida-Yamamoto and Iizuka, 1995).

**Keratin** expression is also disrupted in psoriasis. K6 and K16, markers of abnormal hyperproliferative conditions, are upregulated in psoriatic epidermis, whereas K1 and K10, markers of terminal differentiation, are downregulated (Thewes *et al.*, 1991). This build-up of cells then accumulates on the skin surface in the form of the psoriatic plaque.

**Filaggrin**, which is normally found in the granular layer of the skin, is absent in psoriatic lesions (Bernard *et al.*, 1988; Gerritsen *et al.*, 1997). The loss of the granular layer through scaling in psoriasis accounts for the absence of filaggrin.

### 1.3.2.2 Keratinocyte hyperproliferation

Several possible biochemical causes for the overproduction of the keratinocytes have been implicated in psoriatic skin: epidermal growth factor (EGF), bone morphogenetic protein-6 (BMP-6), activating protein 1 (AP1) and mitogen-activated protein kinase (MAPK).

**EGF**, Stimulation of growth and differentiation of human epidermis by EGF is mediated by its binding to specific receptors. Nanney *et al.* found that there were no differences in EGF receptor expression between normal and psoriatic stratum basale. In the upper layers of the epidermis, a 2-fold increase in EGF binding capacity was observed in psoriatic skin as compared with normal thin or thick skin (Nanney *et al.*, 1986). This increase in binding contributes to the growth of the keratinocytes, causing hyperproliferation.

**BMP-6**, another growth factor, is present in newborns, but normally disappears by adulthood, except in psoriatic patients. Blessing *et al.* were able to induce psoriasis-like skin lesion in mice overexpressing BMP-6 in suprabasal layers of interfollicular epidermis, which makes it a prime candidate for a growth factor sponsoring the formation of psoriatic lesions in humans (Blessing *et al.*, 1996).
**AP1**, a complex of the oncoproteins *Jun* and *Fos*, stimulates the expression of many genes that are important in cell proliferation and inflammation (Nagpal *et al.*, 1995). These factors were shown to have an altered expression pattern in plaque psoriasis, and so they have also been implicated in the pathogenesis of psoriasis (Basset-Seguin *et al.*, 1991; Zenz *et al.*, 2005).

**MAPK**, helps to regulate cellular proliferation (Dimon-Gadal *et al.*, 1998). Numerous growth factors and cytokines modulate MAPK activity, which is higher in psoriatic fibroblasts (Dimon-Gadal *et al.*, 1998). Determination of the exact mechanism by which elevated MAPK causes hyperproliferation of the keratinocyte could add another valuable piece to the puzzle of the pathogenesis of psoriasis.

### 1.3.2.3 Inflammatory elements in psoriasis

Psoriasis is a chronic inflammatory cutaneous disorder (Krueger and Bowcock, 2005). The inflammatory aspect of psoriasis is physically evident by the redness of psoriatic lesion. Recent data indicate that T cells and cytokines are of major importance in the pathophysiology of psoriasis (Asadullah *et al.*, 1999). The cutaneous and systemic overexpression of several proinflammatory cytokines, particularly type-1 cytokines such as IL-2, IL-6, IL-8, IL-12, IL-23, IFN-gamma and tumor necrosis factor (TNF)-alpha, has been demonstrated, supporting psoriasis as a ‘Th1 disease’ for years (Duncan *et al.*, 1991; Ettehadi *et al.*, 1994; Grossman *et al.*, 1989; Lee *et al.*, 2004a; Schulz *et al.*, 1993; Szabo *et al.*, 1998; Yawalkar *et al.*, 1998). In 2007, a new population of IL-17-producing T helper cells, Th17 cells, has been involved with psoriasis. IL-23 stimulates Th17 cells to produce sets of cytokines such as IL-17A, IL-17F, TNF-alpha, IL-21, and IL-22, espousing psoriasis as a ‘Th17 disease’ (Fitch *et al.*, 2007; Kastelein *et al.*, 2007). The overexpression of these proinflammatory cytokines is considered to be responsible for initiation, maintenance and recurrence of skin lesions. The cellular composition of the inflammatory infiltrate within the plaques as well as the keratinocyte hyperproliferation appears to be directed by cytokines as well. Does psoriasis represent a ‘Th1 diseases’ or a ‘Th17 disease’ or some combination of both? The most recent data suggest that IL-23/Th17 axis is key mediator of disease pathogenesis (Fitch *et al.*, 2007; Lowes *et al.*, 2007; Nickoloff, 2007).

Studies have also shown that overexpression of the chemoattractant IL-8 contributes to the accumulation of granulocytes, a characteristic finding in psoriatic lesions. In contrast to the
overexpression of proinflammatory cytokines, a relatively low level of expression of the anti-inflammatory cytokines IL-1RA and IL-10 has been found, suggesting an insufficient counter-regulatory capacity in psoriasis which might have a genetic background (Asadullah et al., 1998; Cooper et al., 1990). New understanding of psoriasis pathophysiology offers the opportunity for well-targeted therapeutic interventions which should be more effective and better tolerated than the approaches used thus far.

1.3.3 Genetic basis in psoriasis
Epidemiology studies have shown that there is strong genetic basis behind psoriasis. The incidence of psoriasis vulgaris is greater in first and second degree relatives of patients than in the general population. About 30% of individuals with psoriasis vulgaris have an affected first degree relative. If both parents and a sibling are affected, a further child has a 50% chance of developing psoriasis vulgaris; if the sibling is affected but not the parents, the risk drops to 8%. The risk of psoriasis vulgaris is two to three times greater in monozygotic than in dizygotic twins (Krueger and Bowcock, 2005).

In recent years, genetic analyses of multiply affected families or cases have identified several susceptibility variants for psoriasis. At least nine chromosomal loci (PSORS1–9) have been identified for which statistically significant evidence for linkage to psoriasis has been observed. By far the major genetic determinant of psoriasis is PSORS1, which probably accounts for 35–50% of the heritability of the disease. HLA-Cw6 as the susceptibility factor at PSORS1 is one of the most compelling susceptibility factors for psoriasis. It was found in, 10% of healthy northern European controls and, 50% of psoriasis patients but in, 20% of patients with psoriatic arthritis. Other susceptibility loci for psoriasis reside on chromosomes 1q21, 3q21, 4q, 7p, 8, 11, 16q, 17q, and 20p, and many others have been proposed following genome-wide linkage scans of primarily nuclear families with two affected individuals (Roberson and Bowcock, 2010; Valdimarsson, 2007).

1.3.4 Current and future management of psoriasis
Three therapeutic modalities can be used singly or in combination: topical agents (steroidal and non-steroidal), phototherapy (appropriate wavelengths of ultraviolet radiation (UVR)), and systemic medications. Topical agents used predominantly for mild disease and for ‘stubborn’ lesions in more severe disease; phototherapy for moderate disease; and systemic
agents including photochemotherapy, oral agents, and newer injectable biological agents targeting TNF-α and IL-12/23, which have revolutionised the management of severe psoriasis. Other innovative treatments are undergoing clinical studies, with the aim of maintaining safe, long-term control of the condition (Menter and Griffiths, 2007).

Psoriasis is an incurable disease that affects individuals differently. In the long term, individualized therapeutic strategies based on distinguished set of biomarkers and patients’ psychosocial status are encouraged.

1.4 MicroRNAs

MicroRNA is defined as a single-strand RNA of ~22 nucleotides in length, which is generated by the RNase-III-type enzyme Dicer from an endogenous transcript that contains a local hairpin structure (Ambros et al., 2003; Kim, 2005).

The first miRNAs were characterized in the early 1990s, but miRNAs were not recognized as a distinct class of biologic regulators with conserved functions until the early 2000s. Until Sep. 2010, there were 1048 miRNA registered in human in miRBAse 16.0.

1.4.1 The discovery of microRNAs
Victor Ambros and colleagues, Rosalind Lee and Rhonda Feinbaum, discovered that lin-4, a gene known to control the timing of C. elegans larval development does not code for a protein but instead produces a pair of small RNAs (Lee et al., 1993; Wightman et al., 1991; Wightman et al., 1993). One RNA is approximately 22nt in length, and the other is approximately 61nt which folds into a stem loop proposed to be the precursor of the shorter one (Lee et al., 1993). Furthermore, Ambros et al. noticed that these lin-4 RNAs had antisense effect to multiple sites in the 3’UTR of the lin-14 gene. Ruvkun et al. continued to investigate the importance of these complementarity sites for regulation of lin-14 by lin-4, showing that this regulation substantially reduces the amount of lin-14 protein without noticeable change in levels of lin-14 mRNA (Wightman et al., 1993).

The shorter small RNAs derives from lin-4 gene are now recognised as the founding member of miRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).
1.4.2 MicroRNA biogenesis

Most of miRNAs are encoded in intergenic regions quite distance from previously annotated genes, suggesting that they are from independent transcription units (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Rodriguez et al., 2004). However, a minority of miRNAs was found in the intronic regions of known genes. These miRNAs are preferentially in the same orientation as the predicted mRNAs, implying that most of these miRNAs are not transcribed from their own promoters, but are processed from the introns (Altuvia et al., 2005; Ambros, 2003; Aravin et al., 2003; Lai et al., 2003; Lim et al., 2003). Another interesting observation was that around 50% of known miRNAs are found in close proximity to other miRNAs, raising the possibility that these clustered miRNAs might be transcribed from a single polycistronic transcription unit (Lagos-Quintana et al., 2001; Lau et al., 2001; Mourelatos et al., 2002).

Transcription of miRNAs is mediated by RNA polymerase II (pol II) (Cai et al., 2004; Lee et al., 2004b). The primary transcript is several kilobases long, capped and polyadenylated pri-miRNA containing a local hairpin structure. The stem-loop structure is cleaved by nuclear RNase III Drosha to release the precursor of miRNA (pre-miRNA) (Cai et al., 2004; Lee et al., 2003; Lee et al., 2004b). The remaining fragments from the cleaved pri-miRNAs are thought to be degraded in the nucleus (Lee et al., 2003).

Following the nuclear processing by Drosha, the hairpin-like pre-miRNAs are exported to cytoplasm. Once there, the hairpin loop is removed by Dicer (another RNase III enzyme) to form ~22-nucleotide miRNA duplex (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). The cleavage products do not persist in the cell for long. Usually, one strand of this short-lived duplex becomes the mature miRNA and the other strand is discarded at the final step of miRNA maturation (Gregory et al., 2005; Hutvagner et al., 2001). Studies on siRNA duplexes indicate that the relative thermodynamic stability of the two ends of the duplex determines which strand is to be selected to be mature miRNA. The strand with relatively unstable base pairs at the 5’ end typically remains (Khvorova et al., 2003; Schwarz et al., 2003). The same rule is also thought to be applicable to generate miRNA.

The mature miRNAs are incorporated into a member of the Argonaute (Ago) family of proteins, representing the core component of the effector complexes that are known as
‘miRNP’ (miRNA-containing ribonucleoprotein complex), or ‘miRISC’ (miRNA-containing RNA-induced silencing complex), to regulate the gene expression (Hammond et al., 2001; Liu et al., 2004; Meister et al., 2004).

An alternative nuclear pathway for miRNA biogenesis was recently reported (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). Short introns mimicking the structural features of pre-miRNAs, termed mirtrons (premiRNA/introns), enter the miRNA-processing pathway by passing Drosha-mediated cleavage. Debranched mirtrons access the canonical miRNA pathway during nuclear export, and are then cleaved by Dicer and incorporated into silencing complexes.

Figure 6. MicroRNA biogenesis.
1.4.3 The regulatory function of microRNAs in gene expression

In general, miRNAs can regulate gene expression by pairing the miRISC to the mRNAs 3’UTR by either of two posttranscriptional mechanisms: mRNA cleavage or translational repression. The miRNA will specify cleavage if the mRNA has sufficient complementarity to the miRNA, or it will repress productive translation if the mRNA does not have sufficient complementarity to be cleaved but does have a suitable constellation of miRNA complementary sites (Doench et al., 2003; Hutvagner and Zamore, 2002; Zeng et al., 2002).

2-8 nucleotides relative to the 5’ end of miRNAs is called seed region. It is important for target recognition that ~7 nt sites in mRNA match the seed region of the miRNA. Messenger RNA downregulated after introducing miRNA are most associated with four types of sites. These include one 6mer, two 7mers, and one 8mer. The 6mer is the perfect 6 nt match to the miRNA seed region (Lewis et al., 2005). The best 7mer site, the 7mer-m8 site, contains the seed match augmented by a match to miRNA nucleotide 8 (Brennecke et al., 2005; Krek et al., 2005; Lewis et al., 2005; Lewis et al., 2003). Another 7mer, the 7mer-A1 site, which contains the seed match augmented by an A at target position 1 (Lewis et al., 2005). The 8mer site comprises the seed match flanked by both the match at position 8 and the A at position 1 (Lewis et al., 2005). The pairing to the seed region is not only important for recognition but also that in some cases appear sufficient to gene repression (Brennecke et al., 2005; Doench and Sharp, 2004).

![Figure 7. Types of miRNA complementary sites.](image_url)
A central goal for understanding miRNAs functions has been to understand how they recognize their target messages (Bartel, 2004). Studies have suggested five general features of site context that boost site efficacy: (1) AU-rich nucleotide composition near the site; (2) Proximity to sites for coexpressed miRNAs (which leads to cooperative action); (3) Proximity to residues pairing to miRNA nucleotides 13–16; (4) Positioning within the 3’UTR at least 15 nt from the stop codon; (5) Positioning away from the center of long UTRs (Grimson et al., 2007). To predict the target genes, several bioinformatics methods can be used: TargetScan (http://www.targetscan.org) (Lewis et al., 2005), PicTar (http://pictar.mdc-berlin.de) (Krek et al., 2005), miRWalk (http://mirwalk.uni-hd.de) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) (Rehmsmeier et al., 2004).

1.4.4 MicroRNAs in diseases and development
Gene expression is tightly controlled in many levels. The discovery of miRNAs has fundamentally altered our understanding of gene regulation and added a new dimension to already complex gene regulatory networks (Bartel, 2004). Since the early 2000, studies have revealed that miRNAs are involved in different physiological and pathophysiological processes such as development, differentiation, apoptosis, fat metabolism, viral infection, and cancer (Miska, 2005). In 2003, Wienholds et al reported that defective in miRNA processing arrest Zebrafish development (Wienholds et al., 2003). Moreover, miRNAs were shown to be dispensable for cell fate determination, axis formation, and cell differentiation but are required for brain morphogenesis in zebrafish embryos (Giraldez et al., 2005). Taken together, miRNAs can play essential roles in development. In cancers, microRNAs can act as oncogenes or tumor-suppressors. ‘Oncogenic’ miRNAs (i.e. miR-155, miR-21 and miR-17) are frequently overexpressed in tumors, while ‘tumor-suppressor’ miRNAs (i.e. let-7 and miR-16) are consistently lost in most cancers (Lu et al., 2005; Volinia et al., 2006). In fat metabolism, up regulation of miR-143 has been associated with obesity in adipose and inhibition of miR-143 effectively suppressed adipocyte differentiation (Takanabe et al., 2008; Weiler et al., 2006). Interestingly, recent study showed that cellular miRNA can act synergistically with a human cytomegalovirus (HCMV) miRNA to escape immune elimination during HCMV infection (Nachmani et al., 2010), suggesting miRNAs as potential targets for therapeutic purposes.
1.4.5 MicroRNAs in skin
In skin, Yi and Fuchs et al observed that epidermis-specific removal of miRNA activity from mice by conditionally targeting Dicer1 gene in embryonic skin progenitors leads to disturbed epidermal morphogenesis, highlighted the important role of microRNAs in skin biology (Yi et al., 2006). However, at that time, the expression and function of miRNAs in human skin was largely unknown and there were no any studies about the involvement of miRNAs in skin inflammatory diseases. We set out this study to investigate the role of microRNAs in human skin inflammation, especially in psoriasis (Sonkoly et al., 2007).
2 AIMS OF THE THESIS

The overall aim of the study is to investigate the function of microRNAs in pathogenesis of psoriasis and skin biology. The specific aims for individual papers are:

- **Paper I**: to examine the potential involvement of microRNAs in the pathogenesis of psoriasis and skin inflammation and to identify psoriasis-associated microRNAs.

- **Paper II**: to investigate the involvement of microRNAs in keratinocyte differentiation and to further explore the signaling pathways that regulate the expression of miR-203 during keratinocyte differentiation.

- **Paper III**: to explore the role of miR-203 in human skin morphogenesis.

- **Paper IV**: to explore the signaling pathways regulated by miR-203 and to further investigate the role of miR-203 involved in NF-κB signaling.
3 MATERIALS AND METHODS

3.1 Patient material (paper I, III)

All in vivo studies are carried out according to the Declaration of Helsinki Principles, and were approved by the Stockholm Regional Ethics Committee (Dnr. 2007/20-31/2 for paper I, Dnr. 2009/550-323 for paper III). In paper I, both psoriasis patients and healthy controls were between 18–65 years old. Patients had not received systemic immunosuppressive treatment or PUVA/solarium/UV, for at least 1 month, and topical therapy for at least 2 weeks before skin biopsy was taken. After obtaining informed consent, 4mm punch biopsies were taken and snap frozen, from lesional skin of patients with moderate or severe chronic plaque psoriasis (n= 25), lesional skin of patients with moderate to severe chronic atopic eczema (n =20), and from non-inflamed, non-irritated skin of healthy individuals (n= 26). In paper III, human foetal skin biopsies from 14, 17, 20 and 22 weeks of EGA were obtained from electively aborted foetuses. Adult skin biopsies were obtained from healthy donors.

3.2 Cells (paper I, II, III and IV)

In paper I, primary human keratinocytes, dermal fibroblasts and melanocytes were isolated from healthy skin using standard protocols and cultured as described (Homey et al., 2000; Pivarcsi et al., 2003). Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) from healthy blood donors (Karolinska University Hospital Blood Bank, Stockholm, Sweden) using MACS separation. Immature monocyte-derived dendritic cells (MDDCs) were generated by culturing separated monocytes in the presence of GM-CSF (550 IU/ml), and IL-4 (800 IU/ml) (Biosource International, Camarillo, CA, USA) for 6 days. CD4⁺, CD8⁺, CD4⁺CD25high, CD56⁺, CD19⁺, and CD69⁺ cells were isolated from PBMCs from healthy blood donors by FACS sorting using a Becton Dickinson (BD) FACSArria cell sorting system and BD FACSDiva software v 4.1.2 (BD Biosciences, Franklin Lakes, NJ, USA). Granulocytes and eosinophils were FACS sorted from whole blood following RBC lysis with ACK lysis buffer.

In paper II and IV, human adult skin epidermal keratinocytes (obtained from Cascade Biologics, Inc., Portland, OR) were cultured in EpiLife® serum-free keratinocyte growth
medium including Human Keratinocyte Growth Supplement (HKGS) at a final Ca2+-concentration of 0.06mM (Cascade Biologics, Inc.).

In paper III and IV, HeLa and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum, 100 U/ml penicillin/streptomycin (Invitrogen, Grand Island, NY).

All the cells are cultured at 37°C in 5% CO2.

3.3 Keratinocyte differentiation models (paper II)

To investigate the effects of keratinocyte differentiation inducers in regulating miRNAs expression, keratinocytes were subcultured into six-well plates at a density of 24,000 cells per well and exposed to 1.5mM CaCl2 (Cascade Biologics), 50 ng/ml TPA, and 100 nM vitamin D3 (1,25(OH)2D3) (both from Sigma, St Louis, MO). Medium was used as a control for CaCl2, and DMSO was used as a control for TPA and for vitamin D3. Plates to be used for RNA preparation, real-time PCR, and LDA profiling (Applied Biosystems) were washed twice with phosphate-buffered saline (PBS) at 1, 4, 24, and 48 hours after calcium and TPA treatment, and at 3, 6, 12, 24, and 48 hours after vitamin D3 treatment. To avoid effects of changes in keratinocyte physiology during culture, each time point includes treated and corresponding untreated, control samples. To examine whether confluent status of keratinocytes influences miR-203 expression, RNA were collected from the cells at 25, 50, and 100% confluence and at 4 days after confluence.

3.4 Biopsies homogenization&RNA isolation (paper I, II, III and IV)

In paper I, skin biopsies were homogenized with a Mikro-Dismembrator S laboratory ball mill (MTI corporation, Richmond, CA, USA), and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according manufacture’s instruction.

Total RNA from cultured cells was prepared using Trizol (Invotrogen).
3.5 miRCURY LNA™ miRNA microarray and data analysis (paper I)

Locked nucleic acid (LNA™) nucleosides are a class of nucleic acid analogues in which the ribose ring is ‘locked’ by a methylene bridge connecting the 2’-O atom with the 4’-C atom. LNA™ nucleosides contain the six common nucleobases (T, C, G, A, U and mC) that appear in DNA and RNA and are able to form base pairs according to standard Watson-Crick base pairing rules. Moreover, the locking is constrained in the ideal conformation for Watson-Crick binding. (Kloosterman et al., 2006).

Figure 8. LNA vs. DNA.

In paper I, total RNA from lesional skin of psoriasis patients (n= 3) and atopic eczema patients (n= 3) and skin of healthy individuals (n= 4) was isolated using TRIzol reagent (Invitrogen). Two μg of total RNA from each sample were labeled using the miRCURY™ Hy3™/Hy5™ labelling kit and hybridized on the miRCURY™ LNA Array (v.8.0) (Exiqon, Vedbøk, Denmark). Signal intensities were normalized using the global Lowess regression algorithm. For subsequent analysis, we used the log2 of the background-subtracted, normalized median spot intensities of ratios from the two channels (Hy3/Hy5). To find consistently differentially expressed genes, the data were subjected to SAM analysis as described previously (Tusher et al., 2001). For visualization of differentially expressed miRNAs, a heat map was generated using TreeView (http://jtreeview.sourceforge.net). All microarray data reported in the manuscript is described in accordance with MIAME guidelines and have been deposited at EMBL-EBI (accession number: E-MEXP-1123).

3.6 MiRNA-specific real-time PCR (paper I, II)
In paper I, total RNA of skin biopsies and cells was extracted using TRIzol reagent. RNA from 20 different normal human organs was obtained from Ambion (FirstChoiceH Human Total RNA Survey Panel). Quantification of miRNAs by TaqMan® Real-Time PCR was carried out as described by the manufacturer (Applied Biosystems, Foster City, CA). Briefly, 10 ng of template RNA was reverse transcribed using the TaqMan® MiRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems). 1.5 μl RT product was introduced into the 20 μl PCR reactions which were incubated in 384-well plates on the ABI 7900HT thermocycler (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Target gene expression was normalized between different samples based on the values of U48 RNA expression.

In paper II, 80 ng of template RNA was reverse transcribed using the TaqMan MiRNA Reverse Transcription Kit and the multiplex RT primer pools containing miRNA-specific stem-loop primers (Applied Biosystems). Diluted RT product (1.5 μl) was introduced into the 20 μl PCR reactions, which were incubated in 384-well plates on the ABI 7900HT thermocycler (Applied Biosystems) at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Target gene expression was normalized between different samples based on the values of U48 small RNA expression.

3.7 miRCURY LNA™ miRNAs in situ hybridization (paper I, III)

miRCURY LNA™ miRNA Detection Probes for in situ hybridization bind to their target with high affinity and enables specific and sensitive detection of miRNAs. In paper I, in situ transcriptional levels of miR-203 were determined on frozen sections (10 μm) of skin biopsy specimens from six psoriasis patients and six healthy individuals according to the manufacturer’s instructions (Exiqon). Briefly, sections were hybridized overnight with digoxygenin-labeled miRCURY LNA™ probes (Exiqon) and incubated with antidigoxygenin antibody conjugated with alkaline phosphatase (Roche, Basel, Swissland) for 1 h. Sections were visualized by using BM purple substrate (Roche) together with 2 mM levamisole (Roche). The color reaction was performed overnight. We followed the protocol recommended by the manufacturer (Exiqon). The stained sections were reviewed with a Zeiss microscope.
In paper II, the global miRNA profiling for 365 human miRNAs was carried out using the TaqMan LDA Human miRNA Panel v1.0 (MicroFluidic card, Applied Biosystems). Relative levels of miRNAs expression are determined ABI7900 HT analyzer with TLDA upgrade and analyzed with RQ Manager Software provided by Applied Biosystems.

**Figure 9.** TLDA flow chart.

Total RNA from cultured keratinocytes was prepared using Trizol (Invotrogen). MiRNAs were reverse transcribed by Megaplex™ RT Primers&TaqMan MiRNA Reverse Transcription Kit and amplified (PCR) using the multiplex reverse transcription (RT) TaqMan MiRNA Low Density Array (TLDA) (Applied Biosystems). An amount of 640 ng of starting total RNA (80 ng for each of the eight RT-PCR) was used for each sample. All the quality control tests were validated: blanks and reproducibility (standard deviation of cycle threshold (C_T) <1) of the two small nucleolar housekeeping RNAs RNU48 (SNORD48) and RNU44 (SNORD44). The amount of RNA from each sample was calibrated to the more stable (between the different arrays) small nucleolar housekeeping RNA, RNU48. To find consistently differentially expressed genes, the data were subjected to SAM analysis as described (Sonkoly et al., 2007). Genes showing at least 1.5-fold regulation and a q-value <20% were considered to be differentially expressed.
3.9 Quantitative real-time PCR (paper II, IV)

For the quantification of IL-8 and hCAP18, 20 ng of cDNA were amplified per reaction in the presence of TaqManR universal master mix (Applied Biosystems) and the following specific primers and probes: IL-8-F: 5’-CCA CAC TGC GCC AAC A-3’, IL-8-R: 5’-GCA TCT TCA CTG ATT CTT GGA T-3’, IL-8-PR: 5’- CTG GTG GCA GAG GGT TGT GG-3’. (Stage 1: 50°C for 2 minutes, stage 2: 95°C for 5 min, stage 3: 95°C for 15 s, 57°C for 45 s, repeated 42 times); hCAP18-F: 5’-GTCACCAGAGGATTGTGACTTCAA-3’, hCAP18-R: 5’-TTTAGGGTCACTGTCCCCATA-3’, hCAP18-PR: 5’-CCGCTTCACCAGCCCGTCTTCT-3’. (Stage 1: 50°C for 2 minutes; stage 2: 95°C for 10 minutes; and stage 3: 95°C for 15 seconds, 60°C for 1 minute, repeated 40 times).

For the quantification of involucrin, 20 ng of cDNA was amplified per reaction in the presence of SYBR green master mix, (Applied Biosystems) and the following specific primers: involucrin—F: 5’-ACCCATCAGGAGCAAATGAAA-3’ and R: 5’-GCTCGACAGGCACCTTCTGGCA-3’ (Stage 1: 95°C for 5 minutes, stage 2: 95°C for 15 seconds, 60°C for 1 minute, repeated 40 times, stage 3: 95°C for 1 minute, stage 4: 55°C for 1 minute; stage 5: 55°C for 10 seconds, increasing the set point temperature after cycle 2 by 0.5°C, repeated 80 times).

For the quantification of IL-1α, 20ng of cDNA were amplified per reaction in presence of TaqMan® universal master mix (Applied Biosystems) and TaqMan® Gene Expression Assay (Hs00899848_m1) (Applied Biosystems) (stage 1, 50°C for 2 min, stage 2, 95°C for 10 min and stage 3, 95°C for 15 s, 60°C for 1 min, repeated 40 times). Gene-specific PCR products were measured by means of an ABI PRISM R 7000 Sequence Detection Systems (Applied Biosystems). Target gene expression was normalized based on the values of the expression of 18S RNA (18S-F: 5’-CGG CTA CCA CAT CCA AGG AA-3’, 18S-R: 5’-GCT GGA ATT ACC GCG GCT-3’, 18S TaqMan probe: 5’-FAM/ TGC TGG CAC CAG ACT TGC CCT C -3’).

3.10 Specific up- and down-regulation of miR-203 activity (paper II and IV)
The activity of miR-203 was up-regulated by transfection of keratinocytes at 50 to 60% confluence with miR-203 miRNA precursor (pre-miR-203) (Ambion, Foster City, CA) which is a small, double stranded, chemically modified nucleic acid that mimic miR-203 precursor molecules; or miRNA precursors negative control #1 (pre-miR-CON) (Ambion) which is a double stranded RNA oligonucleotide designed to serve as a negative control for experiments involving Pre-miR miRNA Precursors;

For the specific down-regulation of miR-203, we transfected keratinocytes with LNA™miR-203 inhibitors (anti-miR-203) (Santaris Pharma, Hørsholm, Denmark) or universal LNA-based negative control (anti-miR-CON) (Santaris Pharma).

### 3.11 RT² Profiler™ PCR Array (paper IV)

In paper IV, to investigate the signal pathways regulated by miR-203, RT² Profiler™ PCR Array was applied (SuperArray Bioscience Corp, Frederick, USA). Human keratinocytes were transfected with pre-miR-203 or scramble control (pre-miR-CON) for 72 hours. We used the RT² Profiler PCR Array System to compare the expression of 84 genes, selected activation markers of 18 signal transduction pathways, in keratinocytes overexpressing miR-203 or scramble control. Real-time PCR detection was carried out by the manufacturer's instructions. The experimental cocktail was prepared by adding 1275μL of the SuperArray RT² qPCR master mix and 1173μL ddH₂O to 102 μL of the diluted cDNA mixture. For real-time PCR detection, 25μL of this cocktail was added to each well of the 96-well PCR array. The array was then cycled on a real-time thermal cycler through the following program: 1 cycle of 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and of 1 minute at 60°C. SYBR Green fluorescence was detected from each well during the annealing step. Values were exported to a template Excel file for analysis. Analyses of the raw data were done through the Superarray Data Analysis Web Portal (SuperArray Bioscience Corp).

### 3.12 p65 Translocation assay (paper IV)

The NF-κB family of transcription factors consists of five members in mammalian cells: RELA (also known as p65), REL (also known as c-REL), RELB, p50 and p52, which can
form homodimers or heterodimers, p65/p50 is most abundant dimer. Following cell stimulation, NF-κB dimers translocate from the cytoplasm to the nucleus, and it binds to the response elements of downstream genes (Bonizzi and Karin, 2004; Sen and Baltimore, 1986a, b).

In paper IV, to examine the effect of miR-203 in p65 translocation, keratinocytes were cultured in 60mm Petri dishes at a density of 100 000 cells/well and transfected with 10nM pre-miR-203 or pre-miR-CON (Ambion) when reaching 50% confluence. 48h after transfection, keratinocytes were exposed to 50ng/ml recombinant human TNF-α (R&D system) for 15 min, 30 min and 45 min or left untreated. The nuclear fraction was extracted by nuclear extract kit (Active motif, CA, USA). The protein expression was analyzed by Western blotting with purified mouse anti-human NF-κB p65 (1:1000) (BD Transduction Laboritories™, Franklin Lakes, New Jersey, USA). The protein levels were visualized by ECL (GE Healthcare, Niskayuna, NY) using horseradish peroxidise (HPR)-conjugated anti-mouse antibody (1:2000) (DakoCytomation, Glostrup, Denmark). As loading controls, the blots were re-probed using a rabbit anti-human TATA binding protein (Abcam, Cambridge, UK) and secondary HPR–conjugated anti-rabbit antibody (1:2000) (DakoCytomation).

3.13 NF-κB luciferase reporter assay (paper IV)

Upon proper stimuli, the dimers of NF-κB family translocate into the cell nucleus and bind to the response elements located in the promoter regions of its target genes and active their transcription (Sen and Baltimore, 1986a, b).

In paper IV, to investigate whether miR-203 can affect NF-κB signaling, it is necessary to examine if miR-203 can influence the binding between the dimers of NF-κB family and the NF-κB response elements. To this end, luciferase reporter plasmid (PGL4.32) containing five copies of an NF-κB response element were purchased from Promega. Keratinocytes were plated in 24-well tissue-culture dishes and transfected at 50% confluence with PGL4.32 (200ng per well) together with Pre-miR-203 (50nM per well) or pre-miR-CON (50nM per well). 24h after transfection, the cells were treated with 20ng/ml TNF-α or medium for 5h. All transfections were carried out in triplicate with Fugene6 (Roche, Basel, Switzerland) following
the manufacturer’s protocol. Luciferase activity was determined using Dual Luciferase® Reporter Assay System (Promega, Madison, USA).

### 3.14 AP-1 transcription factor assay (paper II)

In paper II, to examine the involvement of the AP1 transcription factor complex in the regulation of miR-203, keratinocytes were cultured in 60mm Petri dishes at a density of 50,000 cells per well and cultured in EpiLife serum-free keratinocyte growth medium excluding Human Keratinocyte Growth Supplement when reaching to 70% confluence. One day after starvation, 100 nM Ro31-8220 (Calbiochem) or 10 mM GF109203X (Calbiochem) were added to keratinocytes, followed by 50 ng/ml TPA stimulation. The nuclear fraction was extracted by nuclear extract kit (Active Motif, Carlsbad, CA), and activation of JunB and c-Jun were measured using the TransAM AP-1 Family Transcription Factor Assay Kit (Active Motif) according to the manufacturer’s instruction.

### 3.15 Firefly luciferase assay (paper III and IV)

To date, several methods have been established to show miRNAs regulating their putative target genes. Most commonly used are luciferase reporter constructs containing the target 3′ UTR with the putative binding site downstream of the reporter coding region. These constructs are used to transfect cells expressing the relevant miRNA, along with vectors carrying mutant versions of binding sites. Evidence for miRNA activity can be established when wild-type reporters have less activity than their respective mutants (Krutzfeldt et al., 2006).

In paper III, to verify if SOCS3 is the direct target of miR-203, the SOCS3 3’UTR fragment encompassing the putative target site or the mutated fragment was introduced into luciferase reporter vector (200 ng per well) (pMIR-REPORTTM miRNA Expression Reporter Vector System, Ambion, Austin, TX, USA). Luciferase activity was measured 24 h after cotransfection of the luciferase reporter gene and exogenous precursor miR-203 (Ambion) into HeLa cells.
In paper IV, to confirm that IL-8 is the direct target of miR-203, firefly luciferase reporter plasmids containing 3'UTR of the IL-8 gene and empty luciferase vector were obtained from SwitchGear Genomics (Menlo Park, CA). HEK293 cells were plated in 24-well tissue-culture dishes 24 hours prior to transfection at a density of 80,000 cells per well. Cells were transfected with the firefly luciferase reporters (50 ng per well), pRL-TK renilla luciferase vector (10 ng per well) (Promega, Madison, USA) together with Pre-miR-203 (10nM per well) or pre-miR-CON (10nM per well).

### 3.16 Enzyme-linked immunosorbent assay (ELISA) (paper IV)

In paper IV, to investigate if miR-203 suppressed the protein expression of IL-8 and IL-1α, two well-known downstream genes of NF-κB pathway. We performed ELISA to detect IL-8 and IL-1α protein expression in culture supernatants from keratinocytes.

Keratinocytes were cultured in 12-well plates and transfected with 10nM pre-miR-203 or 10nM pre-miR-CON (Ambion), 10nM anti-miR-203 (Santaris Pharma) or 10nM anti-miR-CON (Santaris) (Ambion) when reaching 50% confluence. To investigate the effect of miR-203 in IL-8 and IL-1α protein expression upon activated NF-κB signaling, keratinocytes were treated with 50ng/ml recombinant human TNF-α (R&D system, Minneapolis, MN, USA) 24 hours after transfection. 6 and 24 hours after TNF-α treatment, culture supernatants were collected respectively. To explore whether miR-203 modulates IL-8 and IL-1α protein expression upon non-activated NF-κB signaling, culture supernatants were harvested 24, 48, 72 and 96 hours after transfection. Culture supernatants were analysed for IL-8 and IL-1α expression by ELISA (R&D system), according to the manufacture’s protocol.

### 3.17 Western blotting (paper I, II and IV)

Western blotting allows the identification of proteins of interest through probing with specific antibodies.

In paper I, to examine SOCS3 protein expression in lesion skin of psoriasis patients and in healthy skin, a mouse anti-human SOCS-3 antibody (1:2500) (Alexis Biochemical, Lausen, Switzerland) were used. In paper II, to investigate if miR-203 can regulate keratinocytes...
differentiation, keratinocytes were transfected 10nM pre-miR-203 or 10nM pre-miR-CON; 5nM anti-miR-203 or 10nM anti-miR-CON for 72h. Keratinocytes lysates were analyzed for protein expression by western blotting with a mouse anti-human involucrin antibody at a concentration of 1:1000 (Sigma Aldrich, St Louis, MO). In paper IV, to explore if miR-203 modulates p65 translocation, a mouse anti-human NF-κB p65 (1:1000) (BD Transduction Laboratories™, Franklin Lakes, New Jersey, USA) was used with keratinocytes nuclear fraction. The protein levels were visualized by ECL (GE Healthcare, Niskayuna, NY) using horseradish peroxidise (HPR)-conjugated anti-mouse antibody (1:2000) (DakoCytomation, Glostrup, Denmark). As loading controls, the blot from nuclear fraction was re-probed using a rabbit anti-human TATA binding protein (1:4000) (Abcam, Cambridge, UK) and secondary HPR–conjugated anti-rabbit antibody (1:2000) (DakoCytomation). The blots from whole cell lysates was re-probed using a mouse anti-human β-actin (1:50,000) (Sigma).

3.18 Immunohistochemistry (paper I and III)

In paper I, SOCS-3 protein expression was analyzed in skin from 9 psoriasis patients and 8 healthy control individuals. Cryostat sections (7 mm) from skin biopsies were stained with the ABC-ELITE (Vector Laboratories) immunohistochemical staining method, using rabbit anti-human SOCS-3 (1:400) (Santa Cruz, CA, USA), following the manufacturer’s instructions. In paper III, the expression of p63 (1:200), SOCS3 (1:400) (Santa Cruz), involucrin (1:200) (Visionbiosystems, Newcastle, UK) and filaggrin (1:400) (Abcam, Cambridge, UK) was analysed in foetal skin biopsies from 14, 17, 20 and 22 weeks of EGA and adult skin sections by ABC-ELITE immunohistochemical staining.

3.19 Immunofluorescence (paper II)

In paper II, to investigate the effects of miR-203 on keratinocyte differentiation, keratinocytes were cultured in cover slips precoated with collagen IV and were transfected with 10nM pre-miR-203 or 10nM pre-miR-CON; 5nM anti-miR-203 or 5nM anti-miR-CON using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. In the inhibitor experiments, the cells were exposed to 1.5mM CaCl2 (Cascade Biologics) after 16 hours to induce endogenous miR-203. 72h after transfection, the cells were washed in PBS, followed by 10 minutes fixation in 4% formaldehyde and 5 minutes blocking in serum-free protein
block (Dako, Denmark) at room temperature. The primary antibody (anti-human involucrin antibody, Sigma Aldrich) diluted at 1:200 in 1%BSA/PBS was added and incubated overnight. The secondary antibody, 1 mg/ml Alexa Fluor 594 donkey anti-mouse IgG (Invitrogen), was diluted in 1%BSA/PBS and incubated with the cells for 17 minutes at room temperature without exposing to direct light. Sections were mounted using 40-6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories, Burlington, CA).
4 RESULTS AND DISCUSSION

4.1 Paper I – MicroRNAs: Novel Regulators Involved in the Pathogenesis of Psoriasis?

4.1.1 A characteristic miRNA signature identified in psoriasis skin
To examine the potential involvement of miRNAs in the pathogenesis of psoriasis, we performed microRNA profiling for 342 miRNAs in skin lesions of patients with psoriasis (n=3) and healthy skin (n=4). To take a broader view of skin inflammation, we also included 3 lesional skin samples from atopic eczema, another common skin inflammatory disease. We showed for the first time that psoriasis-affected skin has a specific microRNA expression profile compared with healthy human skin or atopic eczema. 29 miRNAs were consistently differentially expressed between psoriasis and healthy skin. Among the 29 identified miRNAs, there were (I) miRNAs specifically up-regulated in psoriasis, (II) miRNAs with increased expression in both psoriasis and atopic eczema, (III) miRNAs specifically down-regulated in psoriasis, and (IV) miRNAs uniformly down-regulated in both skin diseases. 17 miRNAs were found to be specifically up-regulated or down-regulated in psoriasis as compared with healthy skin.

To confirm the result of the miRNA profiling, we analysed the expression of miR-203, miR-146a, miR-21 and miR-125b in larger cohort, psoriasis (n= 25), healthy skin (n= 26) or atopic eczema lesions (n =20) using microRNA specific-quantitative real time PCR. In line with the result from microarray study, quantitative real-time PCR results showed that miR-203 (p<0.001) and miR-146a (p<0.001) were specifically overexpressed in psoriasis skin. Unlike miR-203 and miR-146a, miR-21 was significantly (p<0.001) up-regulated both in psoriasis and atopic eczema, suggesting that this miRNA is involved in inflammation in general and not specifically in psoriasis. MiR-146a upregulation has been associated with rheumatoid arthritis (RA) (Li et al., 2010; Nakasa et al., 2008). Overexpression of miR-146a suppresses T cell apoptosis indicate a role of miR-146a in RA pathogenesis and provide potential novel therapeutic targets (Li et al., 2010). MiR-125b showed the opposite expression pattern to miR-21: the level of this miRNA significantly (p<0.001) decreased both in psoriasis and atopic eczema. Taken together, the distinct microRNAs expression pattern distinguished psoriasis from healthy skin and atopic eczema lesion.
4.1.2 miR-203 showed skin- and keratinocyte-specific expression pattern

To get further insight into the function of those four miRNAs, we systematically analysed the expression of miR-203, miR-146a, miR-21 and miR-125b in a panel of 21 different human organs and tissues. Interestingly, miR-203, a miRNA specifically upregulated in psoriasis, was expressed more than 100-fold higher in skin compared with most other studied organs. In addition to skin, miR-203 was expressed at lower levels in organs, esophagus and cervix, that also contain squamous epithelium, suggesting that a specific function for this miRNA in the formation or function of squamous epithelia. In contrast to miR-203, the mature forms of miR-146a, miR-21 and miR-125b were detected in all studied organs with different expression pattern.

In psoriasis, there is evidence for the pathogenic relevance of several different cell types that normally occur in skin: keratinocytes (Sano et al., 2005), fibroblasts (Dimon-Gadal et al., 2000), monocyte-derived immunocytes (Lowes et al., 2007; Nestle et al., 2005), T cells (Nickoloff and Wrone-Smith, 1999), and mast cells (Fischer et al., 2006). Therefore it is likely that this disease is the outcome of aberrantly activated mechanisms that involve a variety of different cell populations (Lowes et al., 2007; Stratis et al., 2006). To identify the cell types expressing those four miRNAs, we systematically analysed the expression of miR-203, miR-146a, miR-21 and miR-125b in the panel of cells present in healthy and/or inflamed skin including both resident cells (keratinocytes, dermal fibroblasts and melanocytes) and leukocyte/immune cell subsets (CD4+, CD8+ and CD4+ CD25^{high} T cell subsets, NK cells, granulocytes, B cells, dendritic cells and mast cells). MiR-203, the miRNA specifically upregulated in psoriasis and was specifically expressed in skin, showed keratinocyte-specific expression pattern, suggesting a strong link between miR-203 and keratinocytes and hinting a potential involvement of miR-203 in pathogenesis of psoriasis.

4.1.3 A plausible link between miR-203 and keratinocyte dysfunction in psoriasis through the regulation of SOCS-3 signaling

Epidermal keratinocytes are active participants in the formation of psoriasis plaques. Psoriatic keratinocytes show abnormal differentiation and proliferation, have aberrant cell signaling and produce mediators that contribute to the recruitment and activation of immune cells (Sano et al., 2005). The specific expression of miR-203 in skin and in keratinocytes as well as its
specific up-regulation in psoriasis implied that this miRNA plays a role in the regulation of keratinocyte functions.

MiRNAs exert their regulatory function by binding to the 3’UTRs of their target genes for translational repression and potentially mRNA degradation (Bartel, 2004). To investigate the functions of miR-203 in keratinocytes and the pathogenesis of psoriasis, we predicted target genes that can be regulated by miR-203 using bioinformatic tools. One of the identified evolutionarily conserved target genes was Suppressor Of Cytokine Signaling 3 (SOCS-3). SOCS-3 is part of a negative feedback loop in cytokine signaling inhibiting the activation of STAT3, a transcription factor whose activation in keratinocytes is essential for the development of psoriatic plaques (Lowes et al., 2007; Sano et al., 2005). It has been shown that SOCS-3 is upregulated in atopic eczema (Ekelund et al., 2006). However, before our study, SOCS-3 was not known to be associated with psoriasis. We analysed the expression pattern of miR-203 and SOCS-3 in the lesional skin of psoriasis patients and healthy skin by immunohistochemistry and LNA-modified nucleotide probe in situ hybridization, respectively. In comparison to healthy skin, the expression of miR-203 was increased in psoriatic lesional skin in all epidermal layers, consistent with the real-time PCR results. Analysis of SOCS-3 protein expression pattern showed a complementary pattern with the miR-203 expression. SOCS-3 was strongly expressed by the basal layer of keratinocytes in healthy skin, while it was suppressed in the epidermis of psoriasis lesions. Down-regulation of SOCS-3 expression in psoriatic lesional skin was further confirmed by Western blot analysis. However, quantitative real time PCR analysis showed no significant difference in SOCS-3 mRNA expression between psoriatic and healthy skin (data not shown), suggesting that the down-regulation of SOCS-3 in psoriasis occurs at the posttranscriptional level.

One study has shown that SOCS-3 deficiency results in prolonged activation of signal transducer and activator 3 (STAT3) after IL-6 stimulation (Croker et al., 2003), a cytokine contributing to the pathogenesis of psoriasis (Grossman et al., 1989; Lowes et al., 2007). This suggests that the suppression of SOCS-3 by miR-203 in psoriatic lesions would in turn lead to constant activation of STAT3. The psoriatic hyperplastic epidermis shows increased STAT3 activation and constitutively active STAT3 in keratinocytes leads to the spontaneous development of psoriasis in transgenic mice (Sano et al., 2005). Accordingly, the up-regulation of miR-203 may have important implications for psoriasis pathogenesis by preventing the up-regulation of SOCS-3 in response to cytokines. It can be speculated that
suppression of SOCS-3 in psoriatic keratinocytes leads to sustained activation of the STAT3 pathway, leading to the infiltration of leukocytes and the development of psoriatic plaques. In addition to the modulation of the inflammatory responses, SOCS-3 has also been implicated in the regulation of keratinocyte proliferation and differentiation. It has been shown that overexpression of SOCS-3 in keratinocytes leads to final differentiation and inhibits serum-stimulated proliferation (Goren et al., 2006). Therefore, miRNA-mediated suppression of SOCS-3 expression in keratinocytes may not only modulate cytokine signaling but also contribute to keratinocyte hyperproliferation and alteration in keratinocyte differentiation in psoriatic plaques.

Taken together, we identified SOCS-3 as a target of miR-203 and showed its down-regulation in psoriasis. Studies have reported that one miRNA can affect the cellular processes by regulating the expression of multiple genes (Bartel, 2004; Hutvagner and Zamore, 2002; McDaneld, 2009). Therefore it is not likely that miR-203 functions in psoriasis are mediated solely through the suppression of SOCS-3. Instead, the function of miR-203 can be interpreted as a function of the sum of all of its target proteins and the consequences of their interactions. Thus, researches in the forthcoming years will be needed to understand fully the consequence of the deregulation of miR-203 and other miRNAs in psoriasis. In 2009, Skov et al published another paper regarding miRNAs and potential target interactions in psoriasis. They further confirmed the significant upregulation of miR-203 and miR-21 in psoriasis lesion compared to healthy skin. More importantly, they extended our finding to investigate the global miRNA–mRNA target interactions in psoriatic lesion by associating the miRNA expression with potential targets in the mRNA expression profile in psoriasis on the basis of previously experimentally validated target interactions from the literature. Based on the miRNA and mRNA array, they found the following potential mRNA targets: PDCD4, TPM1, P57, C-KIT, RTN4, SHIP2, TIMP3, RECK and NFIB. The identified target mRNAs were likely to be involved in cellular growth, proliferation, apoptosis and degradation of the extracellular matrix, indicating the novel roles of miRNAs in pathogenesis of psoriasis (Zibert et al., 2010).
4.2 Paper II – Protein Kinase C-Dependent Upregulation of miR-203 Induces the Differentiation of Human Keratinocytes

4.2.1 Calcium-induced differentiation alters miRNA expression in keratinocytes

Keratinocyte differentiation is a multistep process requiring coordination of many genes (Eckert and Rorke, 1989). To investigate the involvement of miRNAs in this process, we carried out a comprehensive analysis of miRNA expression in primary proliferating (cultured in low-calcium (0.06mM) media) or differentiated (cultured in high-calcium (1.5mM) media) keratinocytes. Using the TaqMan Low Density Array (TLDA) platform, the expression of the biologically active form of 365 human miRNAs was examined in keratinocytes 48 hours after calcium treatment.

Interestingly, the most abundant miRNAs in human keratinocytes (either proliferating or differentiated keratinocytes) could be classified into several gene families sharing 5’ seed sequences. The miR-17 family (miR-20a, miR-19a and miR-19b, miR-92, and miR-93), miR-221 family (miR-221 and miR-222), miR-26 family (miR-26a and miR-26b), miR-99 family

Figure 10. A proposed interaction between miR-203 and SOCS-3 contributing the pathogenesis of psoriasis.
(miR-99a and miR-100), the miR-125 family (miR-125a and miR-125b), and the miR-15 family (miR-15b and miR-16). Expressing multiple miRNAs with shared seed sequences may enable the suppression of miRNA targets more effectively and rapidly. Moreover, as the thermodynamics of RNA–RNA binding plays an essential role in miRNA interaction with target mRNA, it is expected that sequence variations such as SNPs or mutations at the miRNAs seed sequence or at miRNA-binding sites may affect the expression of miRNA targets (Brennecke et al., 2005). Thus the cluster expression of miRNAs in keratinocytes may ensure that mutation or SNPs at one miRNA gene or miRNA-binding sites will not have detrimental effect on cells as other family members may compensate for the loss of function.

In addition to these miRNA families, miR-205, miR-200c, miR-31, miR-24, and miR-21 were identified as particularly abundant miRNAs in human keratinocytes. To date, several studies have been carried out to investigate the function of those miRNAs in keratinocyte biology (Mardaryev et al., 2010; Yu et al., 2010; Yu et al., 2008). It was shown that miR-184 can antagonize miR-205 to maintain SH2 domain-containing inositol phosphatase 2 (SHIP2) levels in epithelia (Yu et al., 2008) and upregulation of miR-205 promoted keratinocyte migration by targeting the lipid phosphatase SHIP2 (Yu et al., 2010). MiR-31 has shown to ablate the expression of the hypoxia inducible factor (HIF) regulatory factor FIH to activate the HIF pathway in head and neck carcinoma (Liu et al., 2010). Moreover, administration of miR-31 inhibitor into mouse skin during the early- and midanagen phases of the hair cycle results in accelerated anagen development, and altered differentiation of hair matrix keratinocytes and hair shaft formation (Mardaryev et al., 2010). MiR-200c is a member of the miR-200 family and loss of its expression can result in epithelial to mesenchymal transition (Gregory et al., 2008). MiR-24 is upregulated in oral squamous cell carcinoma (OSCC). Experiments blocking miR-24 and using exogenous miR-24 expression indicated that miR-24 contributes to the growth of OSCC cells and that miR-24 may target p57 (Lin et al., 2010).

### 4.2.2 miR-203 is upregulated during keratinocyte differentiation

Comparing the miRNA expression profiles of proliferating and differentiated keratinocytes using the significance analysis of microarrays (SAM), we found that miR-203 was the most upregulated (8.3-fold change) miRNA in differentiated keratinocytes in comparison with proliferating controls and showed the highest upregulation among all the miRNAs. In 2010, Hildebrand J et al extended the miRNA profiling during keratinocyte differentiation to several
time points in vitro as well as in vivo. They confirmed that miR-203 was the most stringently
upregulated miRNA under high calcium conditions (Hildebrand et al.). We therefore focused
on characterizing the regulation of miR-203 by agents that are known to induce keratinocyte
differentiation. Calcium, TPA and vitamin D were chosen. After 24–48 hours of treatment
with TPA or calcium, keratinocytes showed morphological signs of terminal differentiation.
MiR-203 expression was significantly (P<0.05) increased 24 hours after exposure of
keratinocytes to high calcium. We observed further upregulation of miR-203 48 hours after
calcium treatment. Stimulation with TPA resulted in a detectable and significant (P<0.01)
upregulation of miR-203 expression already after 4 hours, and this effect was even stronger
24 and 48 hours post treatment. Quantitative real-time PCR results showed significantly
increased miR-203 expression after 24 (P<0.001) and 48 hours after vitamin D3 (P<0.001)
treatment. These results showed that the upregulation of miR-203 expression in differentiating
keratinocytes is not specific for calcium-induced differentiation but can also be achieved by
other inducers of keratinocyte differentiation.

To further investigate the upregulation of miR-203 expression during keratinocyte
differentiation, we set out to measure miR-203 levels in monolayer keratinocyte cultures at
different cell densities. This is the natural keratinocyte differentiation model. As cultures
grow to confluence, cells become tightly packed, maintain epithelial morphology, and close
association to one another, eventually establishing close contacts as a large continuous
monolayer sheet of cells. After confluence, cells become stratified and few mitoses are
observed (Eckert and Rorke, 1989; Green, 1980). We observed more than 10-fold increase in
miR-203 expression in cells that were kept in confluence for 4 days, compared with sub
confluent keratinocytes. The induction of miR-203 during keratinocyte differentiation is in
line with the expression pattern previously reported by us, where miR-203 was mainly
expressed by keratinocytes in the suprabasal layers in the epidermis of healthy human skin
(Sonkoly et al., 2007), implying the tight links between miR-203 expression and the
differentiation programme in keratinocytes.

4.2.3 miR-203 expression is repressed when keratinocyte differentiation
is prevented
We have shown that miR-203 was upregulated during keratinocyte differentiation. The next
question was what would happen if we prevented keratinocytes from differentiation. Would
miR-203 expression be suppressed? To answer this question, primary human keratinocytes
were treated with recombinant human EGF or keratinocyte growth factor (KGF) or medium for 24 and 48 hours and miR-203 expression was analyzed. EGF and KGF are well known keratinocyte growth factors. Suitable concentration of EGF and KGF can prevent keratinocytes from differentiation and promote proliferation (Chen et al., 1993; Gniadecki, 1998). Quantitative real-time PCR results showed a significant decrease in miR-203 expression 24 hours after treatment with KGF (P<0.001) and EGF (P<0.05). The suppressive effect of both EGF and KGF on miR-203 expression in keratinocytes was more pronounced 48 hours after treatment. These results show that KGF and EGF suppress miR-203 expression in keratinocytes.

4.2.4 Upregulation of miR-203 during keratinocyte differentiation is PKC-dependent

Next, we asked what the signaling pathways involved in the regulation of miR-203 expression during keratinocyte differentiation were. It has been known from the literature that TPA-induced terminal differentiation of keratinocytes is mediated by the activation of protein kinase C (PKC) (Yuspa et al., 1982). In vitro, PKC inhibitors effectively block the terminal differentiation program induced by calcium or TPA in keratinocytes (Dlugosz and Yuspa, 1993). To examine if the upregulation of miR-203 is PKC-dependent, primary human keratinocytes were treated with specific PKC inhibitors, GF109203X and Ro31-8220 (Cuenda and Alessi, 2000; Toullec et al., 1991), together with TPA or DMSO alone. 24 hours after TPA treatment, quantitative real time PCR was applied to analyse miR-203 and involucrin expression. The results showed a significant upregulation of involucrin expression by TPA stimulation, which was equally reversed by both PKC inhibitors. Similarly, TPA treatment induced a five fold, significant (P<0.001) increase in the expression of miR-203 in comparison with DMSO-treated cells 24 hours after treatment. Treatment of keratinocytes with the specific PKC inhibitors not only significantly (P<0.001) blocked TPA-induced miR-203 expression but also suppressed it to below the basal level. The results suggested that activation of PKC is required for differentiation-induced upregulation of miR-203 in keratinocytes. The two specific PKC inhibitors suppressed miR-203 expression below the basal level, suggesting that endogenous PKC activation is required for homeostatic miR-203 expression.
We then aimed to further investigate how PKC activation regulates the expression of miR-203. Analysis of the putative promoter region of the miR-203 gene showed the presence of an AP-1 transcription factor-binding site (unpublished observation), suggesting that activator protein-1 (AP-1) is directly involved in the transcriptional regulation of miR-203. AP-1 is a well-known downstream target of PKC action in keratinocytes, which is a transcription factor consisting of homodimers or heterodimers of the Jun and Fos families of nuclear protein (Rutberg et al., 1996; Shaulian and Karin, 2001). AP-1 proteins have essential roles in the regulation of keratinocyte growth and differentiation. Different members of the AP-1 family can have antagonistic effects. While c-Jun is primarily a positive regulator of proliferation, JunB suppresses proliferation and promotes differentiation (Ikebe et al., 2007; Shaulian and Karin, 2001; Welter and Eckert, 1995; Zenz and Wagner, 2006). To explore the regulation of Jun family members by TPA in primary keratinocytes, we measured JunB and c-Jun activity in TPA-treated keratinocytes using transcription factor assays. TPA treatment led to significantly (P<0.05) increased JunB activation, which was prevented by the PKC inhibitors GF109203X (P<0.001) and Ro31-8220 (P<0.01). In contrast to JunB, the activation of c-Jun was decreased in TPA-treated keratinocytes (P<0.01), and this decrease was prevented by GF109203X (P<0.05) but not by Ro31-8220.

Furthermore, we set out to investigate if dysregulation of Jun family members could affect the expression of miR-203. To this end, we analyzed miR-203 expression in HaCaT keratinocytes stably overexpressing c-Jun, or a dominant-negative mutant of JunB, JunBΔN (Ikebe et al., 2007). The results showed that miR-203 expression was suppressed in both HaCaT-c-Jun and HaCaT- JunBΔN cell lines, implying that JunB is a positive regulator and c-Jun is a negative regulator of miR-203 expression which is in line with their antagonistic roles in keratinocyte functions (Welter and Eckert, 1995; Zenz and Wagner, 2006). Hence, reduced JunB or increased c-Jun activity led to decreased miR-203 expression in HaCaT keratinocytes, indicating the involvement of AP-1 in the regulation of miR-203. In conclusion, our results suggest that differentiation-induced miR-203 upregulation is dependent on the activation of PKC and an altered balance between AP-1 family members.

4.2.5 The effect of miR-203 dysregulation in keratinocyte differentiation
Next we aimed to investigate the role of miR-203 in keratinocyte differentiation. To answer this question, we used two parallel approached: 1) Transient overexpression of miR-203 in
undifferentiated keratinocytes using synthetic precursor of miR-203 (pre-miR-203) and 2) Inhibition of endogenous miR-203 during calcium-induced differentiation of keratinocytes using LNA™ antagomirs specific for miR-203 (anti-miR-203).

First, we transiently overexpressed miR-203 in primary keratinocytes by transfection of pre-miR-203, and analyzed the expression of involucrin in the transfected cells. Immunofluorescence as well as western blot showed the upregulation of involucrin in miR-203-overexpressing keratinocytes compared with those transfected with a scrambled control (pre-miR-CON), indicating that overexpression of miR-203 induces differentiation of keratinocytes also in the absence of calcium or TPA.

Next, we investigated the effect of miR-203 inhibition in keratinocyte differentiation. In monolayer keratinocyte culture system at subconfluence, keratinocytes are proliferating. As a consequence, there is low expression of miR-203. To enhance and magnify the effect of miR-203 inhibition in keratinocyte differentiation, we transfected keratinocytes with anti-miR-203 or nonspecific scrambled control LNA antagomir (anti-miR-CON) and subsequently treated the cells with calcium. Both immunofluorescence and western blot showed that inhibition of miR-203 resulted in decreased involucrin expression after calcium treatment in comparison with controls.

In paper I, we identified miR-203 to be significantly overexpressed in psoriasis in comparison with healthy skin and atopic eczema (Sonkoly et al., 2007). Psoriasis is characterized by altered differentiation and hyperproliferation (Bernard et al., 1985; Griffiths and Barker, 2007). Because of the hyperproliferation, we initially expected miR-203 expression to be decreased during differentiation. This is clearly not the case. Psoriasis keratinocytes do not show an undifferentiated phenotype but undergo an alternative differentiation program and early terminal differentiation markers such as involucrin are indeed overexpressed (Bernard et al., 1985; Ishida-Yamamoto and Iizuka, 1995).

In conclusion, miR-203 upregulation is required for complete keratinocyte differentiation. Because (1) miRNAs have negative effects on protein output; (2) there are no predicted binding sites for miR-203 in the 3’UTR of involucrin mRNA (unpublished observation), the effect of miR-203 in involucrin expression is likely to be indirect. We hypothesize that miR-203-induced involucrin expression is mediated by miR-203 targets that have negative effects
on differentiation. Overexpression of miR-203 would eventually lead to the post-
transcriptional suppression of these genes, contributing to keratinocyte differentiation. To date, SOCS-3, p63, c-abl oncogene 1 (ABL 1) have been identified as direct targets of miR-203 (Bueno et al., 2008; Lena et al., 2008; Sonkoly et al., 2007; Yi et al., 2008). However, additional target genes that may serve as a link between miR-203 and differentiation still wait to be discovered.

In 2008, Yi et al. found that transgenic mice overexpressing miR-203 driven by the keratin 14 promoter had a thinner epidermis with lower p63 expression in comparison with wild-type mice. Moreover, transgenic mouse keratinocytes overexpressing miR-203 had a reduced proliferation rate and reduced colony-forming capacity in comparison with wild-type keratinocytes (Yi et al., 2008). However, epidermal differentiation markers were not induced by overexpression of miR-203 as judged by the mRNA expression of K10, Filaggrin, and Loricrin mRNAs (Yi et al., 2008). In contrast, we found that ectopic overexpression of miR-203 led to increased keratinocyte differentiation judged by involucrin protein level. Moreover, inhibition of endogenous miR-203 impaired calcium-induced increase of involucrin protein. We hypothesize that the following conditions would contribute to the discrepancy: (1) the measurement of gene expression at the protein or mRNA level; (2) the use of different differentiation markers; (3) the use of synthetic miRNA precursors instead of viral vectors; (4) the expression of a different set of targets in human and mouse, although miR-203 is highly conserved between mice and humans, this does not necessarily imply that all of their targets are conserved as it is indicated by the obvious differences between the human and the mouse epidermis. Further investigation and probably the identification of additional miR-203 targets will be necessary to determine the reasons causing the discrepancy.
Figure 11. A proposed model for the regulation of miR-203 in keratinocyte differentiation.

4.3 Paper III – The expression of microRNA-203 during human skin morphogenesis

In 2006, Yi et al. found that when miRNAs are globally ablated in mice skin epithelium by targeting the gene that encodes the miRNA-processing enzyme Dicer1, hair follicles fail to invaginate. This distorts epidermal morphology, underscoring the functional importance of miRNAs in skin development (Yi et al., 2006). In 2008, it was shown that transgenic overexpression of miR-203 in mice, driven by keratin 14 promoter resulted in the formation of thinner epidermis and decreased proliferation, demonstrating critical roles for miR-203 in epidermal morphogenesis (Yi et al., 2008). Furthermore, Yi and Lena et al. found that miR-203 induces cell-cycle exit and represses “stemness” in epidermal progenitors by targeting p63, an essential regulator of stem-cell maintenance in stratified epithelial tissues (Lena et al., 2008; Yi et al., 2008). We wondered if miR-203 has a similar role in human skin morphogenesis. In this paper we aimed to investigate the expression of miR-203 and the interaction between miR-203 and p63, SOCS3 during human skin morphogenesis.
To this end, *in situ* hybridization for miR-203 using LNA-modified nucleotide probe was performed on frozen sections of human fetal skin from 14 weeks, 17 weeks, 20 weeks and 22 weeks of EGA. The expression of p63 and SOCS-3, two conserved targets of miR-203, was analyzed in corresponding fetal skin sections by ABC-ELITE immunohistochemical staining. To confirm whether SOCS-3 is the direct miR-203 target, luciferase assay was used. SOCS-3 3’UTR fragment encompassing the putative target site or the mutated fragment was introduced into a luciferase reporter vector. Luciferase activity was measured 24h after cotransfection of the luciferase reporter gene and exogenous precursor miR-203 into HeLa cells.

*In situ* hybridization revealed that miR-203 expression was first detectable at 17 weeks of EGA. Both p63 and SOCS-3 were expressed in the epidermis already at 14 weeks. Expression of miR-203 was most pronounced in the suprabasal layers of the epidermis in both foetal and adult skin. As one may expect it with miRNA:target pairs, p63 and SOCS-3 as its targets were preferentially expressed in the basal epidermal layer where miR-203 was absent. When SOCS-3 3’UTR fragment was cloned into the luciferase reporter vector and cotransfected with pre-miR-203 into HeLa cells, we observed significant (P<0.05) repression in luciferase activity compared to those transfected with pre-miR-CON. The repression was alleviated when the predicted target sites were mutated (P<0.01), suggesting that SOCS-3 is the direct target of miR-203. In accordance with the role of miR-203 in promoting differentiation and stratification during skin morphogenesis (Yi et al., 2008) as well as differentiation of adult human keratinocytes (Sonkoly et al., 2010), we found that miR-203 was expressed in the suprabasal epidermal layers in both foetal and adult skin. MiR-203 was first detectable in human foetal skin at 17 weeks of EGA. During mouse skin development, miR-203 becomes one of the most abundant epidermal miRNAs from mouse embryonic day 15.5 (Yi et al., 2008), which equates to approximately 8 weeks’ human gestation (O'Rahilly, 1979). Hence, human miR-203 appears later during skin development compared to its murine orthologue.

p63 is critical for the development of epidermis and other stratified epithelia, and it acts as a molecular switch between basal cell with proliferating capacity and suprabasal cells (Candi et al., 2008; Koster et al., 2004; Mills et al., 1999; Yang et al., 1999). p63 has been shown to be directly targeted by miR-203 (Lena et al., 2008; Yi et al., 2008). In concordance, we found that p63 and miR-203 showed mutually exclusive expression pattern within the epidermis.
during human skin development, further supporting a role for miR-203 as a switch between proliferating basal cells and differentiating suprabasal cells.

Previously, we identified SOCS-3 as another potential target for miR-203 (Sonkoly et al., 2007). Here, we demonstrate that SOCS-3 is directly targeted by miR-203 and show that SOCS-3 is expressed in the basal epidermal layer of foetal and adult skin. SOCS-3 is a negative regulator of STAT3 (Kimura et al., 2004; Mori et al., 2004), a transcription factor involved in inflammation, skin remodelling and wound healing (Bromberg, 2002; Levy and Darnell, 2002). MiR-203-mediated suppression represents an additional layer of the complex regulation of SOCS-3, which may have important implications for keratinocyte functions in the developing and adult skin.

4.4 Paper IV – miR-203 suppresses NF-κB signaling in human keratinocytes

4.4.1 miR-203 is involved in the regulation of NF-κB pathway
From paper I, II and III, we proved that there is strong link between miR-203 and keratinocyte biology. To get further insight into the role of miR-203 in keratinocyte biology, we set out to explore the signaling pathways regulated by miR-203. To this end, human keratinocytes were transfected with pre-miR-203 or scramble control (pre-miR-CON) for 72 hours. RT2 Profiler PCR Array System was used to compare the expression of 84 genes, selected activation markers of 18 signal transduction pathways, in transfected keratinocytes.

Analysis of results identified 22 genes, which were regulated by overexpression of miR-203. Interestingly, we found that the miR-203-regulated genes were not randomly distributed among all pathways represented on the array but genes in NF-κB pathway were markedly overrepresented among the downregulated genes indicating a pivotal role for miR-203 in the regulation of this pathway. Out of the 10 down-regulated genes, 7 were targets of the NF-κB pathway. Among the 14 NF-κB genes represented on the array, 9 were expressed in primary human keratinocytes. Out of them 7 (IL-8, CCL20, IL-1α, TNF-α, TANK, ICAM1 and PTGS2/COX2) were down-regulated, 2 (NFκB and IKBKB) were unchanged. The high proportion of NF-κB target genes regulated by miR-203 prompted us to further investigate the potential involvement of miR-203 in the regulation of NF-κB signal pathway in keratinocytes.
NF-κB signal pathway in skin is crucial for morphogenesis and homeostasis. Abnormal NF-κB activity has been linked to developmental skin defects, inflammatory skin disease, and skin cancer (Bell et al., 2003; Sur et al., 2008). The NF-κB signal pathway has been implicated in the pathogenesis of psoriasis (Abdou and Hanout, 2008; Tsuruta, 2009). The expression of TNF-α, a proinflammatory cytokine playing key roles in psoriasis, is regulated by NF-κB activation (Pahl, 1999; Pasparakis, 2009). In recent years, anti-TNF antibodies have been used successfully in the treatment of psoriasis, indicating a crucial role for the NF-κB/TNF-α pathway in psoriasis (Kircik and Del Rosso, 2009). To date, there is nothing known about the role of miR-203 in NF-κB signal pathway in keratinocytes.

### 4.4.2 miR-203 suppresses IL-8 and IL-1α expression in keratinocytes

To validate the array results from the RT2 Profiler PCR Array, we measured the expression of two miR-203-regulated NF-κB target genes, IL-8 and IL-1α, in human keratinocytes in time-course experiments. It was previously shown that IL-8 and IL-1α play important roles in skin inflammation (Hoffmann et al., 2002; Lee et al., 1994). We measured the mRNA and protein expression of these genes 24, 48, 72 and 96 hours after transfection with pre-miR-203 or pre-miR-CON by quantitative real-time PCR and ELISA. Overexpression of miR-203 significantly repressed the mRNA expression of IL-8 (P<0.05) and IL-1α (P<0.01) already 24h posttransfection. The suppressive effect of miR-203 overexpression in IL-8 and IL-1α mRNA expression was sustained through all time points analyzed. Overexpression of miR-203 significantly repressed IL-8 (P<0.05) and IL-1α (P<0.001) protein expression 48 hours after transfection. The repression was enhanced at 72 and 96 hours posttransfection, suggesting that miR-203 may suppress the baseline expression of these NF-κB genes in keratinocytes.

Under physiological conditions, cultured keratinocytes express IL-8 and IL-1α at a relatively low level. We wondered whether miR-203 could regulate IL-8 and IL-1α expression also in TNF-α-stimulated cells. To examine the regulation of miR-203 in IL-8 and IL-1α mRNA expression after TNF-α stimulation, human keratinocytes were transfected with pre-miR-203 or pre-miR-CON for 24 hours, followed by TNF-α stimulation for 3 and 6 hours. The expression of IL-8 and IL-1α was examined by quantitative real-time PCR. IL-8 and IL-1α were induced in TNF-α stimulated keratinocytes (P<0.001). At both 3 and 6 hours post TNF-α stimulation, the induction of IL-8 and IL-1α mRNA expression was significantly suppressed.
by miR-203. Next, we set out to investigate the regulation of miR-203 in IL-8 and IL-1α protein expression after TNF-α stimulation. Keratinocytes were transfected with pre-miR-203 or pre-miR-CON for 24 hours, followed by TNF-α stimulation for 6 and 24 hours. ELISA was used to analyze IL-8 protein expression in culture supernatant. IL-8 protein expression was increased 3.8 times after 6 hours of TNF-α treatment as compared with the control cells transfected only with pre-miR-CON. MiR-203 suppressed the induction by 30% (P<0.05). 6 hours after TNF-α treatment, IL-1α protein expression was not increased as compared with the control cells only transfected with pre-miR-CON. IL-1α protein expression was increased 1.8 times after 24 hours of TNF-α treatment as compared with control cells. MiR-203 suppressed the induction by 27% (P<0.001).

Next, we asked whether the inhibition of miR-203 could rescue the suppressed expression of IL-8 and IL-1α. To answer this question, keratinocytes were transfected with pre-miR-203 or pre-miR-CON, anti-miR-203 or anti-miR-CON respectively, or cotransfected with two combinations: i) pre-miR-203 and anti-miR-203, ii) pre-miR-203 and anti-miR-CON. The transfected cells were harvested 48 hours after transfection and we performed quantitative real-time PCR and ELISA to analyse the expression of IL-8 and IL-1α expression. In accordance with our earlier results, IL-8 mRNA (P<0.01) and protein (P<0.001) expression were significantly suppressed by overexpression of miR-203. By contrast, treatment of cells with anti-miR-203 significantly (P<0.05) increased IL-8 protein level compared with the scramble control. Next, we aimed to determine whether pre-treatment of keratinocytes with anti-miR-203 could antagonize the effect of pre-miR-203. We observed increased IL-8 protein in cells transfected with anti-miR-203 and pre-miR-203 in comparison with cells transfected with anti-miR-CON and pre-miR-203 (P<0.05). Hence, inhibition of miR-203 rescued IL-8 protein expression. IL-1α protein expression was suppressed by overexpression of miR-203. However, the de-repression by miR-203 inhibitor was not observed (data not shown) suggesting that there are differences in the regulation of IL-8 and IL-1α by miR-203 or in the biogenesis of these proteins.

4.4.3 miR-203 suppresses the nuclear translocation of NF-κB and NF-κB-dependent promoter activity

NF-κB is a family of transcription factors consisting of five members in mammalian cells: RELA (also known as p65), REL (also known as c-REL), RELB, p50 and p52, which can form homodimers or heterodimers, p65/p50 is most abundant dimmer. In resting cells, NF-κB dimers are normally kept in an inactive state by association with proteins of the inhibitor of
NF-κB (IκB) family. Following cell stimulation, NF-κB dimers translocate from the cytoplasm to the nucleus, and it binds to the response elements in the promoter region of NF-κB target genes. (Bonizzi and Karin, 2004; Sen and Baltimore, 1986b).

To examine whether miR-203 can modulate NF-κB translocation, keratinocytes were transfected with pre-miR-203 or pre-miR-CON, and 48 hours after transfection, keratinocytes were exposed to TNF-α for 15 min, 30 min and 45 min or left untreated. We performed western blotting to detect total p65 protein expression in the nuclear extract from the transfected cells. Overexpression of miR-203 delayed p65 translocation in comparison to cells transfected with pre-miR-CON, suggesting that miR-203 interferes with the nuclear translocation of p65.

To investigate the effect of miR-203 on NF-κB-driven promoter activity, we transfected keratinocytes with an NF-κB luciferase reporter vector containing NF-κB response elements together with pre-miR-203 or pre-miR-CON. 24 hours after transfection with the miRNA mimics, keratinocytes were treated with TNF-α or medium for 5 hours. As expected, treatment of keratinocytes with TNF-α increased luciferase activity 3.6 times to that of controls treated with medium only. Overexpression of miR-203 significantly (P < 0.01) suppressed the TNF-α-induced luciferase activity, suggesting that miR-203 can affect NF-κB signaling activity by modulating NF-κB-driven promoter luciferase activity.

4.4.4 Potential targets of miR-203 in NF-κB pathway
To further add to the complexity of miRNA-mediated gene regulation, bioinformatics analysis revealed that several genes of the NF-κB pathway and several target genes of NF-κB are putative miR-203 targets. Interestingly, IL-8 and IL-1α are among the predicted miR-203 targets in the NF-κB pathway. To verify whether IL-8 is indeed a direct target gene for miR-203, we performed luciferase reporter assays. To this end, the entire 3'UTR of IL-8 mRNA was cloned into a luciferase reporter plasmid and subsequently transfected into HEK293 cells together with pre-miR-203 or pre-miR-CON. Luciferase activity was analyzed 24 hours after transfection. Overexpression of miR-203 significantly repressed luciferase activity by 38% as compared to the scramble control with pre-miR-CON (P<0.01) indicating that IL-8 is a direct target of miR-203 for posttranscriptional suppression. The target validation for IL-1α is ongoing.

48
Table 1. MiR-203 target genes involved in NF-κB signaling pathway from TargetScanHuman 5.1

<table>
<thead>
<tr>
<th>gene name</th>
<th>numbers of predicted binding sites</th>
<th>type of binding sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8 mer</td>
</tr>
<tr>
<td>TLR4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>MyD88</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>IRAK2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>NOD2</td>
<td>2</td>
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</tr>
<tr>
<td>RIPK2</td>
<td>1</td>
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</tr>
<tr>
<td>IKK2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IL-8</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>IL-1α</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TNFRSF19</td>
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</tr>
<tr>
<td>TNFRSF8</td>
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<td>0</td>
</tr>
<tr>
<td>TNFRSF9</td>
<td>1</td>
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</tr>
</tbody>
</table>

We found that overexpression of miR-203 suppressed IL-8 both at the mRNA and protein level while the inhibition of miR-203 rescued the suppressed IL-8 in keratinocytes. Moreover, our results suggest that IL-8 is also a direct target of the miR-203 for posttranscriptional suppression. IL-8 is a member of the CXC chemokine family and was initially identified as a neutrophil chemotactic and activating factor (Baggiolini et al., 1994; Hoffmann et al., 2002). The IL-8 core promoter contains several binding sites for NF-κB and AP-1 transcript factors families. Unlike the NF-κB site, the AP-1 site is not essential for induction but is required for maximal gene expression (Kunsch et al., 1994; Lee et al., 1997). Based on our findings, one would anticipate the downregulation of IL-8 expression in psoriasis lesion due to the overexpression of miR-203. However, increased expression of epidermal IL-8 and IL-8 receptor has been shown in psoriasis (Schroder et al., 1992; Schulz et al., 1993). Of note, IL-8 is produced not only by keratinocytes but also by several types of infiltrating cells in the inflammatory skin (Koch et al., 1992; Samanta et al., 1990), while, miR-203 is expressed only in keratinocytes (Sonkoly et al., 2007). Therefore, the production of miR-203 in psoriasis lesions may not be enough to suppress IL-8 in the tissue.

Similar to IL-8, we found that miR-203 suppresses IL-1α expression both at the mRNA and at the protein level. IL-1α is one of the most common initiators of keratinocyte activation and can be produced when keratinocytes respond to injury (Freedberg et al., 2001). IL-1α can act as both target gene and activator of NF-κB signaling (Kupper and Groves, 1995; Steude et al.,...
A previous study showed that IL-1α expression was decreased in psoriasis epidermis (Cooper et al., 1990). Thus upregulation of miR-203 in psoriasis may partially be responsible for the downregulation of IL-1α expression. Moreover, among the predicted targets of miR-203 shown in the table, we found IL-1α, MyD88 and IRAK2. Muzio et al., discovered that IRAK-2 and MyD88 as the proximal mediators of IL-1 signaling (Muzio et al., 1997). IL-1α, MyD88 and IRAK2 compose an axis to exert their function within NF-κB signaling. Taken together, miR-203 suppresses the IL-1α induction by directly binding to 3′UTRs of IL-1α axis genes, leading to maximal gene repression.

![Figure 12. A proposed model for the involvement of miR-203 in NF-κB signaling pathway.](image)

### 4.4.5 Why does overexpression of miR-203 not lead to suppressed NF-κB activity in psoriasis?

Our results showed that miR-203 suppresses NF-κB activity in primary human keratinocytes as indicated by 1) the delayed nuclear translocation of p65 upon TNF-α-treatment; 2) the suppressed NF-κB-driven promoter luciferase activity; 3) the suppressed expression of NF-κB target genes in keratinocytes overexpressing miR-203. NF-κB is crucial for morphogenesis and homeostasis in the skin (Pasparakis, 2009). Surprisingly, both the
constitutive activation and the inhibition of NF-κB signalling leads to skin inflammation (Klement et al., 1996; Omori et al., 2006; Pasparakis et al., 2002; Sur et al., 2008), illustrating how complex effects this pathway have on skin homeostasis. NF-κB signaling is known to be activated in psoriasis lesion as many of its targets have an increased level in the inflamed skin (Abdou and Hanout, 2008; Tsuruta, 2009). Our result suggested that miR-203 overexpression in psoriasis may serve as a negative feedback that aims to suppress NF-κB activation. Of note, psoriasis is a chronically relapsing disease, with spontaneous remissions and exacerbations during the whole life (Krueger and Bowcock, 2005). Suppression of NF-κB signaling by miR-203 may have a role in spontaneous remissions. This hypothesis would predict that the level of miR-203 varies with the phases of psoriasis with highest level before the beginning of the remission phase.

SNPs in miRNAs and in miRNA binding sites are commonly found to be associated with diseases (Mishra et al., 2008; Nicoloso et al.). Epidemiological and family studies have clearly demonstrated a strong genetic component to the risk to psoriasis ((Duffin et al., 2009; Lee et al., 2008; Li et al., 2009; Zhang et al., 2007). It is conceivable that SNPs in the miR-203 binding sites of some of its target genes diminish or prevent miR-203 effects (i.e. the suppression of inflammation) in psoriatic individuals. This hypothesis predicts that miR-203 may have different effect on keratinocytes obtained from psoriasis individuals and those from healthy donors.
5 CONCLUDING REMARKS

• We show for the first time that psoriasis-affected skin has a specific microRNA expression profile when compared with healthy human skin or with another chronic inflammatory skin disease, atopic eczema. Among the psoriasis-specific microRNAs, we identified one keratinocyte-derived and skin-specific microRNA, miR-203. The up-regulation of miR-203 in psoriatic plaques was concurrent with the down-regulation of an evolutionary conserved target of miR-203, SOCS-3, which is involved in inflammatory responses and keratinocyte functions. Our results suggest that microRNA deregulation is involved in the pathogenesis of psoriasis and contributes to the dysfunction of the cross talk between resident and infiltrating cells. Taken together, a new layer of regulatory mechanisms is involved in the pathogenesis of chronic inflammatory skin diseases.

• Out of 365 miRNAs tested, miR-203 is the most upregulated miRNA during keratinocyte differentiation. Differentiation-induced upregulation of miR-203 expression was blocked by treatment with specific inhibitors of protein kinase C (PKC), GF109203X, and Ro31-8220. Moreover, our results showed that the activator protein-1 (AP-1) proteins c-Jun and JunB regulate miR-203 expression in keratinocytes. In contrast to inducers of keratinocyte differentiation, epidermal growth factor and keratinocyte growth factor suppressed miR-203 expression in keratinocytes below the basal level. Overexpression of miR-203 in keratinocytes resulted in enhanced differentiation, whereas inhibition of miR-203 suppressed calcium-induced terminal differentiation as judged by involucrin expression. These results suggest that upregulation of miR-203 in human keratinocytes is required for their differentiation and is dependent on the activation of the PKC/AP-1 pathway.

• During human skin morphogenesis, miR-203 is barely detectable at 14 weeks. Its expression became prominent from week 17 and was most pronounced in the suprabasal layers of the epidermis. The direct targets of miR-203, p63 and SOCS-3, were preferentially expressed in the basal layer. Differentiation markers such as involucrin and filaggrin were expressed mainly in the suprabasal layers of epidermis, similar to miR-203. Our results suggest miR-203 is involved in the regulation of
human foetal skin development and provide a basis for further studies to investigate
the role of miR-203 in this process.

- Overexpression of miR-203 in human primary keratinocytes suppressed NF-κB
  activity as indicated by the expression of 8 NF-κB target genes. MiR-203 suppressed
  IL-8 and IL-1α, two well-known downstream genes in NF-κB pathway, both at the
  mRNA and protein level. Inhibition of either endogenous or extraneous miR-203
  rescued suppressed IL-8 protein expression. Moreover, we found that overexpression
  of miR-203 prevented the nuclear translocation of p65 and repressed NF-κB-driven
  promoter luciferase activity. Taken together, our finding showed the modulation of
  NF-κB by miR-203 in human keratinocytes, suggesting a potential role for miR-203 in
  keeping skin homeostasis and controlling inflammation.

In conclusion, our data in the thesis suggest the potential involvement of microRNAs in skin
biology and psoriasis. Since miRNAs exert their regulatory function for cellular processes
through the regulation of several proteins, miRNA-based therapies may be more effective
than drugs targeting single protein. The disease-specific miRNAs identified in our study
represent the potential therapeutic targets in the treatment of chronic skin inflammation. The
focus of this thesis is miR-203. Our data showed that miR-203 plays a role as ‘safety guard’
in the skin by regulating inflammation-, differentiation-, proliferation- and morphogenesis-
associated processes. In the future, introducing miR-203 mimic in the diseased tissue could
achieve favourable therapeutic responses in the treatment of psoriasis.
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**Lasse,** my love, seems that I have been expecting too much from you. Since I knew the time for my dissertation in October, I have demanded you to 1) make a painting for my thesis FrontPage; 2) organize my dissertation party; 3) do my thesis prove-reading…… something I can’t even remember☺. I know stress can be the trigger for developing psoriasis. But I didn’t know stress can cause temporary memory loss until you came to me one day to ask what my name was (100% true story, Said and Tatjana can be the witnesses). I am soooooo sorry that I have been careless and putting so much pressure to you in the past few month. But I am really amazed that you have successfully/excellently completed all the tasks: drew the splendid FrontPage; did three times proof-reading for my thesis; organized/ing my dissertation party…… Thank you for being so caring☺. **Che Guevara,** thank you for letting me know that life is so fragile, for making me be more appreciated and cherish what I have now. **Civilization 5,** thank you for entertaining Lasse while I am busy with writing thesis☺.
REFERENCES


