IL-31

A Regulator of Skin Differentiation

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Abstract

The main function of human skin is forming of a barrier against physical, chemical, and biological stress. Many skin diseases are characterized by weakening of the skin barrier leading to transepidermal water loss and aggravation of inflammatory processes by enhanced penetration with allergens and pathogens. Atopic dermatitis (AD) is an inflammatory skin disease affecting 10-20% of children and 1-3% of adults in industrialized countries. Enhanced expression of Interleukin-31 (IL-31) is detected in skin samples of AD patients, but its physiological relevance is not known. IL-31 was recently discovered as a new member of the IL-6 family of cytokines. IL-31 signals through a heterodimeric receptor composed of the Oncostatin M receptor (OSMR) and the IL-31 receptor alpha (IL-31RA), a complex that stimulates the JAK-STAT, the RAS/ERK and the PI3K/AKT signal transduction pathways. The available data suggest that IL-31 is important for both innate and adaptive immunity in tissues that are in close contact with the environment, i.e. skin, airways and lung, and the lining of the intestine. Enhanced expression of IL-31 is not only associated with AD, but also with allergy and inflammatory bowel disease. In the affected tissues IL-31 coordinates the interaction of different immune cells, including T-cells, mast cells, and eosinophils, with epithelial cells.

I observed that IL-31 interferes with keratinocyte differentiation in 3D organotypic skin models. Gene expression analysis revealed a limited set of genes deregulated in response to IL-31, including *IL20* and *IL24*. In HaCaT keratinocytes with inducible IL-31RA, IL-31 inhibited proliferation upon induction of IL-31RA by inducing a cell cycle arrest. As in primary cells, IL-31 treated HaCaT cells elicited a differentiation defect in organotypic skin models, associated with reduced epidermal thickness, disturbed epidermal constitution, disturbed alignment of the *stratum basale* and poor development of the stratum granulosum. The differentiation defect was associated with a profound repression of terminal differentiation markers, including filaggrin, an essential factor for skin barrier formation. The highly induced pro-inflammatory cytokines IL-20 and IL-24 were responsible for part of the effect on *FLG* expression and thus for terminal differentiation.

Several other factors were identified as early IL-31 target genes that are involved in stress responses and p38/JNK signaling pathways including JUNB. JUNB is part of the AP-1 transcription factor family that controls cell cycle arrest in many different cell types. In keratinocytes it is also an essential factor in differentiation. JUN and JUNB antagonize each other in controlling the cell cycle by affecting the expression of different cell cycle regulators including Cyclin D1, p21, p16 and p53. All of them are also involved in regulating differentiation in keratinocytes. I identified changes in the expressional pattern of these factors upon IL-31 stimulation that would promote an early differentiation state of keratinocytes but inhibit late differentiation processes. These studies define IL-31 as an important regulator of keratinocyte differentiation and demonstrate a link between the presence of IL-31 in skin, as found in patients with AD, and weakening of the skin barrier function.

Zusammenfassung

Die Hauptfunktion humaner Haut ist die Bildung einer Barriere gegen physikalischen, chemischen und biologischen Stress. Viele Hautkrankheiten weisen eine Schwächung dieser Barrierefunktion auf, die zu einem transepidermalen Wasserverlust und einer erhöhten Penetration der Haut mit Allergenen und Pathogenen führt. Dies initiiert und verstärkt Entzündungsprozesse in der Haut. Atopische Dermatitis (AD) ist eine entzündliche Hauterkrankung die in 10-20% aller Kinder und 1-3% der erwachsenen Bevölkerung in den Industrieländern vorkommt. Eine erhöhte Expression von Interleukin-31 (IL-31) wurde in Hautbiopsien von AD Patienten festgestellt, die physiologische Funktion dieses Zytokin in AD ist bis heute aber unbekannt. IL-31 wurde kürzlich als neues Mitglied der IL-6 Zytokinfamilie entdeckt. Es bindet einen heterodimeren Komplex aus dem Oncostatin M Rezeptor (OSMR) und dem Interleukin 31 Rezeptor alpha (IL-31RA). Dieser Komplex aktiviert den JAK-STAT, den RAS/ERK und den PI3K/AKT Signaltransduktionsweg. Die vorhandenen Daten lassen vermuten, dass IL-31 eine wichtige Rolle in der angeborenen wie auch in der erworbenen Immunität in Geweben, welche in direktem Kontakt zur Umgebung stehen (wie z.B. die Haut, die Lunge oder der Verdauungstrakt), spielt. Erhöhte IL-31 Expression ist nicht nur messbar in AD, sondern auch in allergischen Erkrankungen sowie chronisch-entzündlichen Darmerkrankungen. In den betroffenen Organen koordiniert IL-31 die Interaktion zwischen verschiedenen Zellen des Immunsystems wie z.B. T-Zellen, Mast Zellen und Eosinophilen mit epithelialen Zellen.

In meiner Arbeit konnte ich feststellen, dass IL-31 in dreidimensionalen organotypischen Hautmodellen in die Keratinozytendifferenzierung störend eingreift. Genexpressionsanalysen zeigten, dass nur eine sehr limitierte Anzahl an Genen durch IL-31 dereguliert wurde. Zwei dieser Gene waren die Zytokine *IL20* und *IL24*. HaCaT Keratinozyten mit einem induzierbaren IL-31RA zeigten eine reduzierte Proliferationsrate nach IL-31 Stimulation durch die Induzierung eines Zellzyklusarrests. Wie primäre Zellen zeigten auch HaCaT Zellen einen Differenzierungsdefekt in organotypischen Modellen charakterisiert durch eine Reduzierung der epidermalen Dicke, einer Schwächung der epidermalen Konstitution, Störungen in der Formierung der basalen Zellschicht sowie ein schlecht entwickeltes

stratum granulosum. Der Differenzierungsdefekt war mit einer tiefgreifenden Reduktion in der Expression von terminalen Differenzierungsmarkern wie Filaggrin, einem essentiellen Faktor in der Formation der Hautbarriere, verbunden. Die induzierten Zytokine IL-20 und L-24 waren für die Reduktion der *FLG* Expression teilweise verantwortlich und trugen somit zu den Defekten in der terminalen Differenzierung bei.

Einige weitere Faktoren wurden als frühe IL-31 Zielgene identifiziert, welche alle in Stressantworten und p38/JNK Signaltransduktionswege involviert sind. Eines dieser Zielgene war JUNB. JUNB gehört zur AP-1 Familie von Transkriptionsfaktoren, die in vielen verschieden Zellen in die Kontrolle des Zellzyklus involviert sind. In Keratinozyten kontrollieren sie unter anderem auch viele Aspekte der Differenzierung. In der Kontrolle des Zellzyklus wirken JUN und JUNB entgegengesetzt durch die Expressionskontrolle verschiedener Zellzyklusregulatoren. Dazu gehören Cyclin D1, p21, p16 und p53. Diese Faktoren spielen ebenfalls eine Rolle in der Regulation der Keratinozytendifferenzierung. Ich stellte Veränderungen in der Expression dieser Faktoren nach IL-31 Stimulation fest, die eine frühes Differenzierungsstadium fördern, allerdings späte Differenzierungsprozesse inhibieren könnten. Diese Studien definieren IL-31 als einen wichtigen Faktor in der Regulation der Keratinozytendifferenzierung und schaffen eine Verbindung zwischen erhöhter IL-31 Expression in AD Patienten und der Schwächung der Hautbarriere in AD.

1 Introduction

1.1 The human skin

The human skin is the first barrier of the body to protect our organism from harmful influences from the "outside". It forms a barrier for physical, chemical and biological stress. The cornified envelope (CE) protects from physical stress and inhibits trans-epidermal water loss. The inner parts of the epidermis consist of lipids, acids (mainly fatty acids), hydrolytic enzymes, antimicrobial peptides and macrophages forming the chemical and biological barrier as part of the innate immunity. Other cellular components of the immune system reside in the skin to activate the adaptive immunity in case of penetration by pathogens. The epidermal barrier is mainly formed by differentiating keratinocytes and is divided into different cellular layers: the *stratum corneum* (SC) also called the CE, the *stratum granulosum* (SG), the *stratum spinosum* (SS) and the *stratum basale* (SB) (Fig. 1).

The basal layer of the epidermis maintains skin homeostasis by providing keratinocytes for terminal differentiation. For this purpose it harbors two types of cells; slow cycling epidermal stem cells (SCs), from which emenate all types of growing and differentiating keratinocytes and which posses an indefinite renewal potential, and transiently amplifying cells (TAC) that are committed to terminal differentiation but can still undergo a limited number of cell divisions (Watt, 2001). This cellular layer is attached to the basement membrane of the epidermis that is characterized by the presence of a small number of keratin filaments and matrix adhesion molecules including keratin (K) 5, 14, 15 (Fuchs and Green, 1980; Waseem et al., 1999) and α6-, β4-integrin (Margadant et al., 2010). SCs express high levels of β1-integrin that is suggested to prohibit differentiation of keratinocytes (Watt and Hogan, 2000). As soon as keratinocytes start to differentiate they loose the contact to the basement membrane by down-regulating the matrix adhesion molecules α6- and β4-integrin and migrate upwards into the SS (Watt, 2002). The expression of K5, K14 and K15 is down-regulated, they induce the expression of K1 and K10 and develop extensive cellular contacts in form of desmosomes (connecting the keratin-filament

cytoskeleton) and adherence junctions (connecting the actin cytoskeleton) (Fuchs, 1990; Waseem et al., 1999; Watt and Hogan, 2000). They transit from a mitotic phase into a post mitotic status (Koster et al., 2007). Before transition to the SG, cells in the upper part of the SS express involucrin, a late differentiation marker that becomes covalently cross-linked to each other as a component of the CE (Eckert et al., 1993).

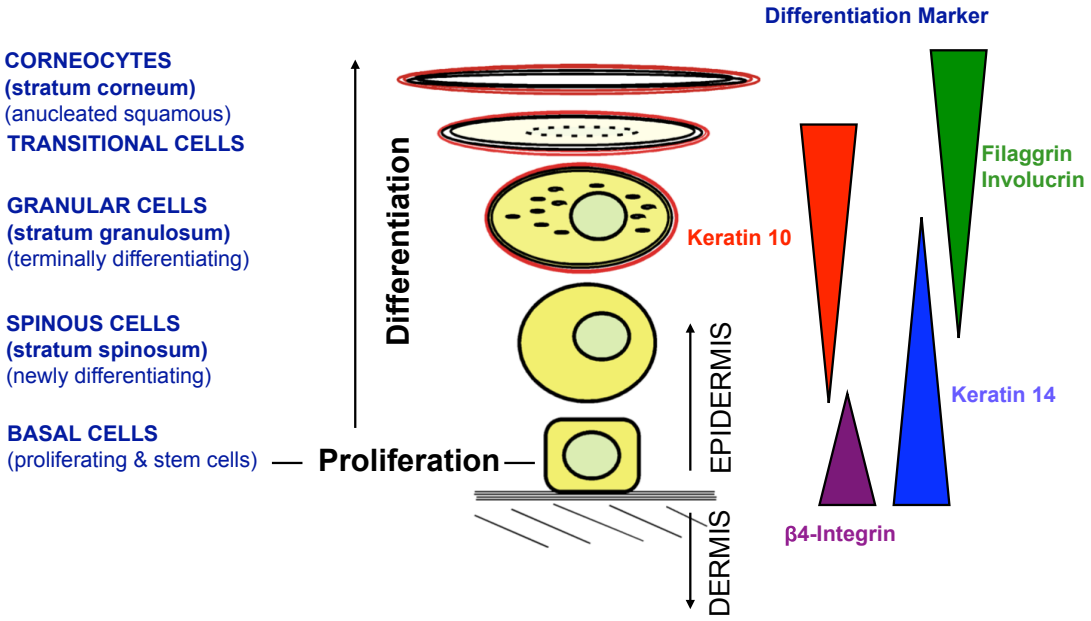


Figure 1. The human Epidermis.
The human epidermis is characterized by the presence of different cellular layers with cells in different stages of differentiation. Basal keratinocytes are attached to the basal membrane that divides the epidermis from the dermis. This basal layer comprises proliferating stem cells as well as transient amplifying cells. Detachment of the basal membrane induces early differentiation steps in the stratum spinosum. The stratum granulosum contains terminally differentiating cells characterized by lamellar and granular bodies containing lipids and proteins important for formation of the cornified and lipid envelope that forms the skin barrier in the stratum corneum. Cells that underwent terminal differentiation and apoptosis are called corneocytes. The different differentiation steps of keratinocytes are characterized by the expression of certain differentiation marker proteins. β4-intergin is exclusively present in hemidesmosomes at the basal cell – basal membrane interface. Keratin 14 is expressed in basal keratinocytes and downregulated in early differentiating cells. Keratin 10 is induced as soon as the cells leave the basal compartment and is repressed again in late terminal differentiation steps. Filaggrin and Involucrin are markers of the late *stratum granulosum* and *stratum corneum* and are major components of the cornified envelope.

Keratinocytes of the SG synthesize keratohyalin granules (Coulombe and Wong, 2004). The major component of these granules is profilaggrin; a protein consisting of 10-12 filaggrin repeats that is processed by phosphorylation and proteolysis to mature filaggrin monomers and an N-terminal filaggrin S100-protein fragment (Scott and Harding, 1981). This N-terminal proteolytic product is proposed to function as a transcription factor to stimulate further differentiation events (Pearton et al., 2002). The granules also include loricrin, an insoluble protein that becomes cross-linked in the cornified envelope (Steven et al., 1990).

1.1.1 The Cornified Envelope

Within the granular layer keratinocytes synthesize lipids in lamellar bodies. Keratins, loricrin, profilaggrin and other structural proteins are reorganized and bundled underneath the plasma membrane. Desmosomes undergo profound changes in their morphology and form a homogenous electron-dense plaque called corneodesmosome (Simon et al., 2001). In the next steps the lamellar bodies fuse with the membrane to secrete their content and form a lipid envelope outside of the cells. The cells undergo apoptosis, the plasma membrane gets disintegrated and the calcium influx activates transglutaminases and different proteolytic enzymes that irreversibly process and cross-link the proteins. Now the cells are referred to corneocytes and form the CE. Keratins mediate the mechanical resistance of the epidermis. Differences in the mechanical resistance are mediated by differential expression of different keratins. Other components of the CE mainly contribute to epidermal elasticity (Candi et al., 2005; Kalinin et al., 2002).

A large number of genes coding for structural proteins of the CE are located in the epidermal differentiation gene cluster (EDC) on chromosome 1q21 (Mischke et al., 1996). It includes profilaggrin, loricrin, involucrin and trichohyalin as well as several proteins of the late cornified envelope (LCE) and the small proline rich (SPR) and S100 calcium binding family.

During cornification, profilaggrin is processed to filaggrin monomers that aggregate keratin filaments into bundles, which leads to a flattened cell shape that is characteristic for corneocytes. In addition these bundles represent 80-90% of the whole epidermal protein mass and serve as a scaffold for further development of the CE. Filaggrin has a half-life of six hours and is degraded into free amino acids leading to a high concentration of up to 100nM hydrophilic amino acids within the CE. This contributes to the flexibility and the water retention abilities of the CE (Mack et al., 1993; Markova et al., 1993; Resing et al., 1993). During the formation of the keratin bundles other proteins are synthesized and localized to the plasma membrane including involucrin, loricrin, trichohyalin and SPRs. All these proteins become cross-linked by transglutaminases (TG) that are activated by changes in the calcium concentration e.g. after disintegration of the plasma membrane.

Involucrin is an early component of the CE that becomes cross-linked and serves as a further scaffold for proteins and ipids. It is attached to the plasma membrane and becomes replaced during further cornification (Nemes et al., 1999; Simon and Green, 1988).

Loricrin is a glycine-, serine- and cysteine-rich mainly unstructured protein that represents 70-85% of the CE protein mass (Hohl et al., 1991). The N- and C-terminal parts of the protein serve as substrates for TGs to cross-link loricrin monomers to multimeric structures. The inner part of the protein has no structure and thus mediates elasticity of the epidermis. In addition loricrin is cross-linked to other components of the CE, e.g. SPRs, to further increase CE stability (Candi et al., 1995; Steven et al., 1990).

SPRs are small unstructured proteins with a proline-rich flexible centre and glutamine and lysine-rich ends that are TG substrates. SPRs cross-link loricrin multimers to increase CE stability. The ratio of loricrin to SPRs correlates with the physical properties of the skin. For example the SPR to loricrin ratio in the thinner epidermis of the trunk is 1:100, while palm or sole epidermises exhibit a ratio of 1:10. The content of SPRs in the epidermis alters the physical properties of the CE and thus the epidermal structure and barrier function (Candi et al., 1999; Steinert et al., 1998; Tarcsa et al., 1998).

The cornified envelope is embedded in a lipid envelope that mainly contributes to the skin barrier function in preventing trans-epidermal water loss. This envelope contains ceramids, cholesterol, fatty acids and cholesterol esters that form lamellae between the corneocytes. These lipids are synthesized as precursors in the granular layer in lamellar granules and are extruded into the extracellular space during cornification and processed by hydrolases (Bouwstra et al., 2003; Freinkel and Traczyk, 1985; Madison, 2003). Although several models have been proposed, the structural composition of the lipid envelope and the physical interaction with the CE is not understood yet (Forslind, 1994; Norlen, 2001a, b).

1.1.2 Signals regulating skin differentiation

Whereas the changes in skin morphology during epidermal differentiation are well described, the molecular mechanisms and signaling events underlying these changes are poorly understood and most scientific findings were obtained from mouse models. The first event in epithelial differentiation is the transition of cells from the basal layer to the spinouse layer. Two factors controlling the transition are p63 and the Notch signaling pathway (Koster, 2010; Watt et al., 2008).

p63 is related to p53 (Yang et al., 1998) and primarily expressed in stratified epithelia like the epidermis. p63 deficient mice are born with severe abnormalities like a thin translucent skin and without any appendages like teeth or hair follicles and mammary glands (Mills et al., 1999; Yang et al., 1999). The reason for this is a complete failure in epidermal commitment during development (De Rosa et al., 2009; Koster et al., 2004).

p63 is expressed highest in the basal layer predominantly as it´s isoform ΔNp63α (Liefer et al., 2000; Yang et al., 1998). As described, the adult epidermis contains several different cell types including SCs and TACs that proliferate or already undergo differentiation. The role of p63 is not clear yet but it seems that p63 function depends on the cell type, in which it is expressed. p63 can maintain cell proliferation by inhibiting the expression of anti-proliferative cell cycle regulators like p21 and p16, promoting pro-proliferative factors like ADA and FASN (Lefkimmiatis et al., 2009; Su et al., 2009; Truong et al., 2006; Westfall et al., 2003). In contrast keratinocytes from mice with an inducible epidermis specific ΔNp63α knock out exhibit an increased proliferation rate upon ΔNp63α silencing (Koster et al., 2007), p63 induces the cyclin-dependent kinase inhibitor p57, which is induced during terminal differentiation of keratinocytes (Beretta et al., 2005), and it represses cyclin B2 and cdc2 (Testoni and Mantovani, 2006), important factors for cell cycle progression. This controversy can be explained by tight differential regulation of p63 and co-factors that might promote proliferation in early still cycling TACs and promote differentiation in late TACs.

ΔNp63α is rapidly down regulated to approximately 25% in differentiating suprabasal keratinocytes compared to basal layer keratinocytes (King et al., 2006) by microRNA-203 (Yi et al., 2008) and subjected to proteasomal degradation (Di Costanzo et al., 2009; Rossi et al., 2006; Vivo et al., 2009). The remaining ΔNp63α still functions as a transcription factor regulating further differentiation processes (King et al., 2006).

Notch cell surface receptors and their ligands play a crucial role in cell fate determination and epithelial differentiation (Artavanis-Tsakonas et al., 1999). Epidermal SCs express the Notch ligand Delta and are proposed to activate Notch signaling in neighboring cells and thus commit them to TACs (Lowell et al., 2000). After activation, the intracellular domain of Notch gets cleaved and translocates to the nucleus where it functions as the transcription factor NCID (Notch intracellular domain) (Bray, 2006). In the nucleus it forms a complex with the DNA binding protein RBP-Jk (recombination signal binding protein for Igκ J region) and turns it from a transcriptional repressor to an activator (Lai, 2002; Mumm and Kopan, 2000). Additional binding to co-activators of the mastermind family recruits chromatin-modifying enzymes like p300 to elevate RBP-Jk dependent gene activation (Ilagan and Kopan, 2007). Notch signaling induces cell cycle withdrawal by induction of p21 expression, activates the expression of the intermediate differentiation markers K1, K10 and involucrin and represses terminal differentiation markers (loricrin, filaggrin) as well as integrins β1 and β4 that are essential for basal proliferating cells (Rangarajan et al., 2001). In addition Notch activity directly inhibits p63 expression probably via activation of the NF-kB pathway (Nguyen et al., 2006). This indicates that p63 and Notch form a tightly balanced regulatory network that commits cells for proliferation or differentiation. Furthermore Notch influences the expression of C/EBPs, DNA binding proteins that, in combination with the AP-2 family of transcription factors, regulate the commitment to terminal differentiation (Wang et al., 2008).

An important trigger of terminal differentiation is the internal Ca^{2+} signaling pathway. An increasing extracellular and intracellular Ca^{2+} gradient is present from the spinous cell layer to the CE (Elias et al., 1998). Increased Ca^{2+} signaling activates kinases of the PKC protein family (Lee and Yuspa, 1991) that contribute to the transition of the spinouse layer to the granular layer by down-regulating K1 and K10 expression

(Dlugosz and Yuspa, 1993) and inducing the expression of loricrin, filaggrin and transglutaminases (Dlugosz and Yuspa, 1993, 1994). In addition to PKC several Ca^{2+} sensing proteins have been identified that might contribute to the barrier formation including Scarf 1 and 2 (Hwang et al., 2005; Hwang and Morasso, 2003), calmodulin like skin protein (CLSP) (Mehul et al., 2000), the calcium sensing receptor (Oda et al., 2000), and the calmodulin-like protein (Rogers et al., 2001). At least the calcium sensing receptor seems to be directly involved in the regulation of loricrin and filaggrin expression (Komuves et al., 2002). During differentiation several Ca^{2+} dependent proteases are expressed that process differentiation markers at the right step of differentiation as they get activated at a certain Ca^{2+} concentration (Candi et al., 2005; Ovaere et al., 2009).

Beside the processes of proliferation and differentiation, apoptosis signaling is a critical part of epidermal barrier formation although epidermal apoptosis is distinct from the common programmed cell death. Corneocytes are not phagocytosed, the cytoskeleton does get reorganized instead of broken down, the nuclear destruction is different compared to normal apoptosis and many apoptotic caspases are not activated during cornification (Lippens et al., 2005; Tschachler, 2005). This leads to the assumption that e.g. “apoptotic keratinocyte differentiation program” is a more appropriate term to describe the changes occurring in keratinocytes during differentiation. In the granular layer cells undergo apoptosis to differentiate to corneocytes but the “apoptotic keratinocyte differentiation program” is distributed through the whole epidermis. In the spinous layer apoptosis signal-regulating-kinase 1 (ASK-1) and dual leucine zipper-bearing kinase (DLK) are expressed and induce transglutaminase 1, loricrin and involucrin expression (Robitaille et al., 2005; Sayama et al., 2001). Basal keratinocytes express Bcl-2, protecting them from apoptosis (Hockenbery et al., 1991), while the expression of Bak, the pro-apoptotic inhibitor of Bcl-2, increases from the basal layer to the granular layer to promote cell death (Krajewski et al., 1996). Furthermore the expression of the p53 inhibitor MDM2 increases from the basal to the suprabasal cell layers (Ganguli et al., 2000), certain death receptors (TNF receptor 1, death receptor 4) and caspase 8 are expressed higher in the basal layers, while death receptor 5, Decoy R2, caspase-14 and Bcl-$_{xL}$ are more prevalent in the terminally differentiating parts of the epidermis (Nickoloff et al., 2002). This reflects a very tight regulation of apoptotic pathways, not only controlling

cell death in keratinocytes but also regulating differentiation processes. Although these factors are known to be important in skin differentiation, it remains to be determined how these different pro- and anti-apoptotic factors cooperate in regulating the apoptosis related pathways of differentiation.

Several agents have been described to enhance proliferation or differentiation of keratinocytes (Eckert et al., 1997). Cholera toxin, phorbol ester, retinoids, viral infection or transformation can enhance keratinocyte proliferation and/or reduce their differentiation. Vitamin D, Calcium, Hydrocortisone and phorbol ester are potent enhancers of differentiation.

Also growth factors and cytokines influence keratinocyte proliferation and differentiation. Insulin like growth factor (IGF) 1 and 2, epidermal growth factor (EGF) (Krane et al., 1991) and TGF-α (Barrandon and Green, 1987) enhance keratinocyte proliferation. Transforming growth factor (TGF)-β1 and 2 suppress keratinocyte proliferation and enhance their differentiation (Coffey et al., 1988; Massague and Gomis, 2006) as well as Interferon (IFN)-γ (Saunders and Jetten, 1994). Keratinocyte growth factor (KGF) stimulates both proliferation and differentiation (Marchese et al., 1990). Together many agents and cytokines control keratinocyte differentiation and formation of an epidermis that provides efficient protection. These agents and cytokines interact manifold, forming complex regulatory networks that are only poorly understood at present

Many skin diseases like atopic dermatitis are characterized by defects in skin differentiation and skin barrier formation. Although in many cases the molecular basis of these diseases is not understood it is likely that either the above mentioned regulatory molecules and/or essential components of the skin barrier are not functioning properly or are defect. In 2004 Interleukin 31 (IL-31) was discovered and evidence emerged that it might be involved in the pathogenesis of T_H2 cytokine mediated skin disorders. However the biological consequences of IL-31 signaling and its role in skin differentiation has not been determined yet.

1.2 Signaling by IL-31 and functional consequences

1.2.1 Introduction

Cytokines are secreted signaling molecules that enable cells to communicate with each other without requiring direct contact. They play important roles in a wide range of different cell types, tissues, and organs. Cytokines regulate innate and adaptive immunity, stimulate pro- or anti-inflammatory signaling processes, control cell differentiation, proliferation, survival and apoptosis. The large family of cytokines can be divided into different functional classes. These classifications are somewhat artificial because individual cytokines can possess more than one function and thus can be part of more than one functional class. Arguably the most simple classification is in pro- and anti-inflammatory cytokines with IL-1, IL-6, TNFα, IL-12, IL-18, MIF, IL-32, IL-33 being examples of the former and IL-10, IL-13, TGFβ, IL-22, IFNα/β of the latter. Relevant for the discussion in this review is that many cytokines can be classified dependent on the cell type in which they are expressed and synthesized. For example different T helper cell populations, i.e. T_H1, T_H2, T_H17, and T_H22 cells, synthesize different subsets of cytokines. T_H1 cells are characterized by the expression of IFNγ, IL-2, IL-12, and IL18; T_H2 cells by IL-4, IL-5, IL-13, IL-18, IL-25, IL-31, and IL-33; T_H17 cells by IL-17, IL-17F, IL-6 and TNFα; and T_H22 cells by IL-22 and IL-14 (Trifari et al., 2009; Wan, 2010; Weaver et al., 2006; Zhu and Paul, 2010). Moreover cytokines can be classified according to their functions in controlling lymphocyte proliferation, hematopoietic differentiation, neovascularization, and cellular migration to name just a few (Dinarello, 2007, 2010). In this review I summarize the available information on the molecular and biological functions of IL-31, a factor that belongs to the IL-6 cytokine family and that was originally described to be synthesized by T_H2 cells. More recent data provide evidence that additional cell types can produce IL-31. Importantly this cytokine has been implicated in allergic diseases, including atopic dermatitis. These findings suggest that IL-31 is a potentially interesting target for therapeutical approaches.

1.2.2 The IL-6 family of cytokines and their receptors

The family of IL-6/gp130 type cytokines includes IL-6, IL-11, IL-27, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin 1 and 2 (CT-1) and cardiotrophin-like cytokine (CLC) (Fig. 2) (Heinrich et al., 2003; Pflanz et al., 2002). The latest addition to this family is IL-31, which was discovered in 2004 (Dillon et al., 2004).

Although the members of the IL-6 type cytokine family share very low sequence homology, they are structurally highly related. These proteins form anti-parallel four-helix bundles with a characteristic "up-up-down-down" topology, a structure that is relevant for the binding of the cytokine to their respective receptor complexes (a model of IL-31 and its receptor is shown in Fig. 3) (Bazan, 1990a; Rozwarski et al., 1994). IL-6 type cytokines signal through type I cytokine receptors (Fig. 2). These share the cytokine binding domain (CBD) with conserved cysteine residues and a conserved WSxWS motif in the extracellular domain (indicated for the IL-31RA and OSMR in Fig. 3). The cysteines form disulfide bonds to stabilize the tertiary structure of the CBD. The WSxWS stabilizes the structure of the CBD and it contributes to the formation of the binding crevice. In addition they have a single transmembrane domain and an intracellular domain without intrinsic enzymatic activity (Bazan, 1990b). The receptors form heteromeric complexes that typically contain the glycoprotein 130 (gp130), which mediates the binding to members of the Janus tyrosine kinase (JAK) subfamily. Thus gp130 is important for activating downstream signaling pathways. In addition to gp130 more specific receptor subunits are used, including LIFR, OSMR and IL-27RA, which can also stimulate signaling cascades. In some instances additional subunits contribute to the binding of ligand exemplified by IL-6R, IL-11R or CNTFR. Other cytokines like CLC and IL-27 (or p28) bind soluble receptor subunits like CLF, soluble CNTFR or EBI3 prior to binding to their receptor complexes. In summary although different IL-6 family cytokines share some of the receptor subunits, the composition of the signaling-competent receptor complexes varies, providing specificity for individual cytokines. Thus the members of the IL-6/gp130 type cytokine family can be divided into different groups depending on how

the receptor complexes are organized (summarized in Fig. 2) (Elson et al., 2000; Heinrich et al., 2003; Pflanz et al., 2002; Plun-Favreau et al., 2001).

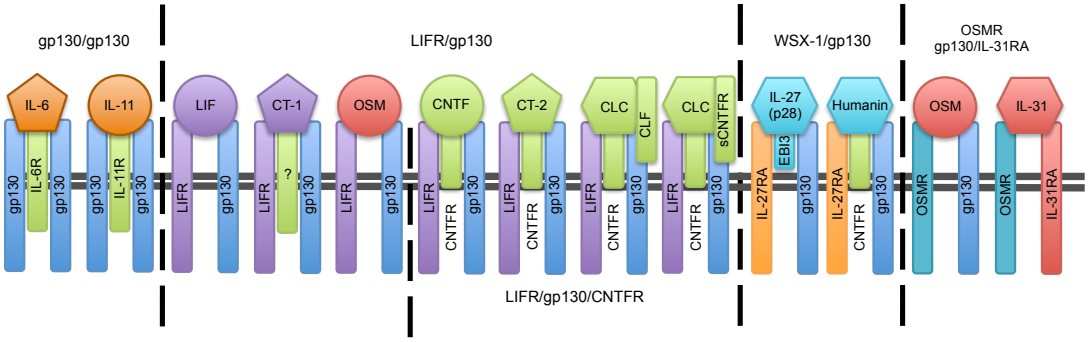


Figure 2. IL-6 type cytokines and their receptor complexes.
IL-6/gp130 type cytokines can be divided into different subgroups according to the receptor complex they interact with, i.e. heterotrimeric complexes with a glycoprotein 130 (gp130) dimer together with non-signaling receptors or accessory proteins that determine specificity (e.g. IL-6R) and heterodimers of gp130 with the leukemia inhibitory factor receptor alpha (LIFR), the interleukin 27 receptor alpha (IL-27RA), or the oncostatin M receptor (OSMR). The receptor for IL-31 is a heterodimer of the interleukin 31 receptor alpha (IL-31RA) and OSMR, without gp130, a set-up which is unique among these cytokine receptors. The LIFR/gp130 subgroup can be subdivided according to the presence or abscence of CNTFR within the receptor complex. An additional receptor component of CT-1 is proposed but not determined yet (displayed as "?" in the figure). IL-6 and IL-11 bind their specific receptor components (IL-6R; IL-11R) prior to binding to gp130. CLC and IL-27 (p28) bind their receptor complexes after dimerization with a soluble receptor component (CLF or sCNTFR and EBI3) (for details and references see text). Humanin is a 24 aa bioactive peptide with undetermined structure. It binds a WSX-1/CNTFR/gp130 trimeric complex (Hashimoto et al., 2009)

JAK kinases are associated constitutively with IL-6 family receptors. Upon ligand binding these kinases phosphorylate tyrosine (Tyr, Y) residues of the intracellular domain of IL-6/gp130 type receptors. These phosphorylated Tyr motifs serve as essential binding sites for proteins that relay receptor activation to downstream signaling cascades. One example are signal transducers and activators of transcription (STAT) proteins that not only bind to activated receptors but are also phosphorylated and activated by JAKs. This results in dimerization of STATs and leads to their translocation into the nucleus where they serve as DNA sequence-specific transcription factors. Phosphoinositide-3-kinase (PI3K) is another enzyme that can associate with specific phosphorylated Tyr residues. Again this results in the activation of the catalytic activity of PI3K and the stimulation of the PI3K/AKT signaling pathway. JAKs also mediate the binding of additional factors to the receptors, including Grb2, SOS, Shc and SHP-2 that allow activation of different MAPKinase signaling cascades (Bennett et al., 1994; Salcini et al., 1994). Thus the

receptors of IL-6 type cytokines control directly the activities of STAT, MAPKinase, and PI3K/AKT signaling pathways (Heinrich et al., 2003).

1.2.3 The IL-31 receptor alpha

Within the family of IL-6 type cytokines, IL-31 signals through a unique receptor complex. While all other members of this family use at least one gp130, as far as analyzed, IL-31 signaling does not depend on gp130. The heterodimeric IL-31 receptor is composed of the IL-31 receptor alpha (IL-31RA) subunit, originally described as GLM-R (for gp130-like monocyte receptor) or GPL (for gp130-like receptor) and OSMR (Fig. 3) (Diveu, 2003; Ghilardi, 2002). As the alternative names suggest, IL-31RA has similarities to gp130. Interestingly the *IL31RA* gene is located on chromosome 5q11.2, 24 kb telomeric to *IL6ST*, the gene that encodes gp130, with the two genes being organized in a head-to-head configuration. The coding regions share approximately 28% homology and a somewhat lower homology to the receptor of the granulocyte colony-stimulating factor (GCSF-R). These findings support the concept that IL-31RA replaces gp130 in the IL-31 receptor complex, the two being likely the product of a gene duplication.

The extracellular domain of IL-31RA consists of five fibronectin type III (FN III)-like domains (Fig. 3). The first two form the CBD with four conserved cysteins and the WSxWS motif. This structural organization is conserved between the IL-31RA and other receptors of the family but this structural homology is not represented by their primary amino acid sequences. These structural elements are so typical that they allow for the classification of type 1 cytokine receptors.

The IL-31RA has a single transmembrane domain. The intracellular domain possesses a proline rich motif (box1 motif) that mediates association with kinases of the JAK family and thus is essential for propagating the signal (Fig. 3). In addition it contains three Tyr residues (Y652, Y683, Y721) that are conserved between human and mouse (Diveu, 2003; Ghilardi, 2002). The phosphorylation of these Tyr residues allows for the interaction with proteins that promote downstream signaling or that are involved in feedback control. The most C-terminal Y721 of the IL-31RA recruits STAT3 to the receptor, whereas Y652 is required for STAT5 activation. All three Tyr residues

contribute to the activation of STAT1 (Dillon et al., 2004; Diveu, 2003; Dreuw et al., 2004). However the IL-31RA is unable to recruit SHP-2 or Shc and thus cannot activate the MAPKinase cascades, unlike the OSMR (see below) (Fig. 4).

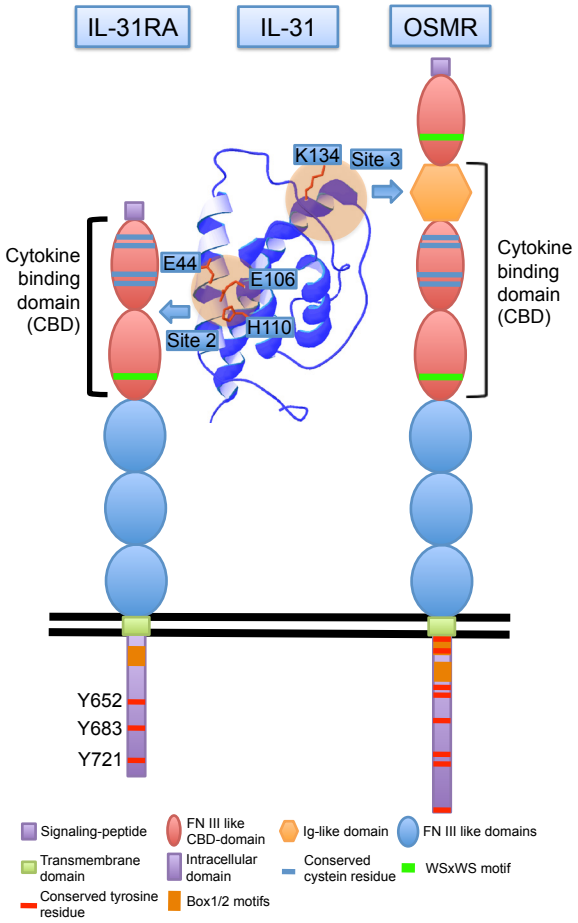


Figure 3. The IL-31/IL-31 receptor complex.
The IL-31 receptor is composed of the IL-31RA and the OSMR subunits. The domain structure of both receptor molecules is indicated. Both have a N-terminal signaling peptide that is cleaved off during translocation to the plasma membrane. IL-31 is displayed as a predicted 3-dimensional model of the 141 amino acid mature protein calculated by the web-based I-Tasser programm (Zhang, 2008). 4-helical bundle proteins bind their receptors through three binding sites numbered 1-3. IL-31 binds the IL-31RA through binding site two and the OSMR through binding site three indicated by circular surfaces. Binding site 1 mediates the binding to a third cytokine-specific receptor component like the IL-6RA, which is not present in the IL-31RA receptor complex and thus not relevant for the binding of this receptor. Amino acids demonstrated to be important for binding are indicated (in site 2: E44, E106, and H110; in site 3: K134, the numbers are according to the 164 amino acid immature protein). Binding of IL-31 to the two receptors appears to be sequential, with binding to IL-31RA being first, followed by binding to OSMR. The IL-31RA mediates, through recruitment of JAKs, phosphorylation of STAT proteins that depends on tyrosine residues in the intracellular domain of the receptors (Y652, Y683, Y721). The names of the different domains and structures are indicated in the figure.

Of note is the analysis of *IL31RA* cDNAs obtained from peripheral blood mononuclear cells (PBMC), U937 promyelocytes, and GO-G-UVM glioblastoma cells. These studies suggest that several splice variants of the *IL31RA* exist, potentially encoding several protein isoforms. Most of these alternative IL-31RA proteins are suggested to be membrane bound, but differ in the N-terminal signal sequence or in the cytosolic domain. Two protein variants consist only of parts of the extracellular domain and thus are predicted to be soluble and may therefore function potentially as antagonists (Dillon et al., 2004; Diveu, 2003). This is interesting in view of the trans-signaling concept defined for IL-6, suggesting that soluble IL-31RA may broaden the range of responsive cells and tissues. However it has to be noted that

it is presently unclear whether these different splice variants are indeed expressed and to what extent relative to the full-length protein has not been clarified.

The IL-31RA forms a heterodimer with OSMR (Fig. 3) (Dillon et al., 2004; Dreuw et al., 2004). The extracellular domain of the OSMR consists of 6 FN III-like domains and an immunoglobulin (Ig)-like domain (Fig. 3). The second and third FN III-like domain in combination with the Ig-like domain form the CBD on OSMR (Fig. 3). The intracellular domain binds JAK1 and JAK2 through its box1 and box2 motifs. Recruitment of JAKs results in the phosphorylation of Tyr residues within the intracellular domain. Subsequently through these phosphorylation sites STAT3 and STAT5 are recruited and phosphorylated by JAKs (Heinrich et al., 2003; Hintzen et al., 2008). In addition to STATs, PI3K is recruited, stimulating the PI3K/AKT pathway. In contrast to gp130, which binds SHP-2, the OSMR interacts with the adaptor protein Shc via Y861-P. Through Shc the RAS/RAF/MEK/ERK pathway is activated (Hermanns et al., 2000). The OSMR can also stimulate the p38 and JNK pathways (Murakami-Mori et al., 1999; Tong et al., 2004; Wang et al., 2000). These two appear to be activated also through Shc, an aspect that is not fully understood mechanistically because the p38 signaling seems to interfere with ERK1/2 activation (Böing et al., 2006). These findings were derived from studying the OSMR in a complex with gp130 and it remains to be analyzed whether the OSMR behaves identically when heterodimerized to the IL-31RA.

Together these findings demonstrate that the IL-31 receptor complex is composed of the IL-31RA and the OSMR (Fig. 4). This heterodimer recruits JAK1 and JAK2 and stimulates STATs, different MAPKinases and PI3K/AKT. These findings were obtained by studying modified and/or over-expressed receptor complexes. More recently these findings were substantiated in several reports demonstrating that IL-31 activates these pathways in different primary cells and cell lines through the endogenous receptor complex. IL-31 stimulates the ERK, JNK, and p38 MAPKinase pathways in human bronchial epithelial cells (Ip et al., 2007), activates STAT1, STAT3, ERK1/2 and AKT in colorectal cancer cell lines and in lung epithelial cells (Chattopadhyay et al., 2006; Dambacher et al., 2007; Jawa et al., 2008), and STAT3 in epidermal keratinocytes (Dillon et al., 2004; Heise et al., 2009; Kasraie et al., 2011). Because the IL-31RA does only activate STAT phosphorylation, the activation

of PI3K and MAPKinase pathways must be mediated by the OSMR within this complex (Fig. 3). Whether all pathways are activated in all cells that express the IL-31 receptor or whether tissue specific effects explain some of the differences implied by the findings described above remains to be determined by performing more systematic studies of the signaling capacity of the IL-31 receptor complex.

1.2.4 The cytokine IL-31

IL-31 was discovered in a Ba/F3 cell line stably expressing the IL-31RA and the OSMR. The proliferation of Ba/F3 cells is highly dependent on cytokine signaling and thus it was asked whether known cytokines can promote proliferation of these cells when IL-31RA, at that time an orphan receptor, was expressed. Since no known cytokine was able to stimulate cells with IL-31RA/OSMR, conditioned media and subsequently expression cloning resulted in the identification of IL-31 as a novel cytokine that fostered proliferation in Ba/F3-IL-31RA/OSMR cells but not in cells expressing IL-31RA together with other gp130 family members (Dillon et al., 2004). The human *IL31* gene is located on chromosome 12q24.31 and encodes a protein of 164 amino acids. While the human gene shares only 31% identity with the murine gene, the two proteins show 61% homology. The protein is processed through cleavage of the N-terminal signal peptide to a 141 amino acid mature protein (Dillon et al., 2004).

Even though the IL-31RA forms a complex with the OSMR, IL-31 binds first to the IL-31RA (Le Saux et al., 2010). However, the OSMR is essential for IL-31 signaling because in over-expression experiments only a co-expression of the IL-31RA and the OSMR led to IL-31-dependent induction of STAT phosphorylation (Diveu et al., 2004). In addition proliferation of Ba/F3-IL-31RA/OSMR cells was abrogated with an antagonistic OSMR antibody. Recent studies provided insight into how IL-31 binds to its receptor complex. Cytokines of the IL-6 family are composed of 4 α-helices (named A-D) with three different receptor-binding sites numbered 1-3 (Boulanger et al., 2003; Hammacher et al., 1998). IL-6 type cytokines belong to the family of long chain α-helical bundle cytokines with α-helices of 20-30 amino acids. In contrast cytokines like IL-4 or GM-CSF belong to the short chain cytokines with 20 amino acid α-helices and two small β-sheets. IL-31 possesses two long (A and D with 25-28 amino acids) and two short helices (B and C with 10-16 amino acids)

without any obvious β-sheets. Therefore IL-31 cannot be simply classified in any of these two groups but seems to be a compound of long and short chain cytokines. Site directed mutagenesis and co-immunoprecipitation experiments revealed that IL-31 binds first, through its binding site 2 (composed of residues of the A and C helices), to the IL-31RA and subsequently to the OSMR through site 3, which is located at the N-terminal end of helix D (Fig. 3). The initial binding of IL-31 to the IL-31RA is essential for binding to the OSMR, possibly because the binding to the IL-31RA induces some conformational change in IL-31, which is relevant for binding to OSMR. Glu44, Glu106 and His110 were identified as essential residues of binding site 2. Furthermore His131 and Lys134 are critical residues in binding site 3 for binding the OSMR, amino acids that are not needed for the binding of IL-31 to the IL-31RA (Fig. 3) (Le Saux et al., 2010). In this context binding site 1 is not relevant. In other cytokines site 1 mediates the initial binding to their non-signaling receptor subunits, e.g. to IL-6R or IL-11R in case of IL-6 and IL-11, respectively, a receptor component not present in the IL-31 receptor complex.

1.2.5 IL-31 sources and IL-31RA expressing tissues

First attempts towards understanding the biological functions of IL-31 and its receptor were to identify cells and tissues that express IL-31 and/or the IL-31RA. The first results indicated that IL-31 is synthesized by activated $CD4^+$ T-helper cells and in lower amounts also by activated $CD8^+$ T-cells. Conditions that skew T-cells towards a T_H1 or T_H2 phenotype revealed that T_H2 cells express preferentially *IL31* mRNA (Table 1). Low mRNA expression levels were detected in testis, bone marrow, skeletal muscle, kidney, colon, thymus, small intestine and trachea (Dillon et al., 2004). Recently it has been demonstrated that human mast cells are also a source of IL-31 (Ishii et al., 2009; Niyonsaba et al., 2010). Our own analyses revealed that in addition to T-cells, monocytes, macrophages, immature and especially mature monocyte-derived dendritic cells produce IL-31 in response to UV irradiation and H_2O_2 treatment. Moreover normal human epidermal keratinocytes and dermal fibroblast show enhanced *IL31* mRNA expression upon H_2O_2 stimulation (Table 1) (Cornelissen et al., 2011).

While the OSMR is ubiquitously expressed throughout human tissues and organs, the *IL31RA* mRNA is found in specific tissues, including trachea, skeletal muscle,

thymus, peripheral blood lymphocytes, placenta, bone marrow, thyroid, testis, brain and skin (Dillon et al., 2004). However it has not been clarified yet, whether all these tissues also express the IL-31RA protein and which of the postulated isoforms. Furthermore several cell lines and primary cells have been shown to express the *IL31RA* mRNA and/or the protein including primary keratinocytes, lung and prostate epithelium cells, monocytes and dendritic cells, and different tumor cell lines such as osteosarcomas, glioblastomas, melanomas, and myelomonocytic leukemias (Table 1) (Chattopadhyay et al., 2006; Dillon et al., 2004; Diveu et al., 2004; Ghilardi, 2002; Ip et al., 2007; Jawa et al., 2008). Moreover eosinophils (Cheung et al., 2010), colonic subepithelial myofibroblast (Yagi et al., 2007), and several cell lines derived from ileal and colon tissue (Dambacher et al., 2007) express the IL-31RA. In the mouse IL-31RA is expressed in a small subpopulation of neurons of the dorsal root ganglia and in small sized sensory neurons of trigeminal ganglia while other neuronal cells do not express the receptor. In addition the IL-31RA co-localizes with the OSMR in neuronal cells (Bando et al., 2006). The presence of IL-31RA in dorsal root ganglia was also measured in humans (Sonkoly et al., 2006). These findings suggest that quite a number of tissue and cell types are potentially responsive to IL-31 (summarized in Table 1). At present however it is largely unknown whether this is indeed the case.

Table 1: Sources of IL-31 and IL-31RA expressing cells

IL-31 SOURCES	REPORTED REGULATORY STIMULI	REPORTED BIOLOGICAL CONSEQUENCES	REPRESENTATIVE PUBLICATIONS
PBMCs	SEB, UVB, H_2O_2	Links IL-31 to AD associated penetration of lesional skin by S. aureus and to inflammatory processes induced by UV radiation.	Sonkoly et al., 2006 Cornelissen et al., 2011
T-cells ($CD4^+$, $CD8^+$, T_H1, T_H2, CLA^+)	inflammatory stimuli like UV radiation, allergens, pathogens, histamines	Enhancement of inflammatory processes by stimulation of e.g. eosinophils in various epithelial diseases.	Dillon et al., 2004 Bilsborough et al., 2006 Sonkoly et al., 2006 Gutzmer et al., 2009 Cornelissen et al., 2011
Monocytes, Macrophages, Dendritic cells	IFNγ, H_2O_2, UVB	Links IL-31to inflammatory processes in the skin induced by UV radiation.	Dillon et al., 2004 Cornelissen et al., 2011
Mast cellls	IgE mediated activation, human β-defensins, LL-37	Links IL-31 to the innate immunity. IL-31 stimulated MCs from MPN patiens secret elevated levels of histamines and leukotrienes and reduced levels of prostaglandinds - links IL-31 to puritic mediators	Ishii et al., 2009, Wang et al., 2009 Niyonsaba et al., 2010
Keratinocytes, Fibroblast	H_2O_2	Links IL-31 to inflammatory processes induced by UV radiation in the skin.	Cornelissen et al., 2011
HCT116	Induction of differentiation, TNFα, IL-1β, IFNγ, LPS		Dambacher et al., 2007

IL-31RA EXPRESSING CELLS			
Primary cells			
Monocytes	IFNγ		Ghilardi et al., 2002 Dillon et al., 2004 Diveu et al., 2004
Eosinophils		Potential enhancement of inflammatory processes by induced secretion of cytokines and chemokines	Cheung et al., 2010
Mast cells		Activation of the Fcε-RI leads to IL-31RA tyrosine phosphorylation indicating a potential role in allergic dissorders	Yamaoka et al., 2009
Dendritic cells	IL-1α, CD40L		Diveu et al., 2004
Keratinocytes	IFNγ, TLR-2	Enhancement of inflammatory processes by direct contact to immune cells in IL-31 stimulated tissues	Heise et al., 2009 Cheung et al., 2010 Kasraie et al., 2011
Human dermal microvascular endothelial cells	IFNγ		Feld et al., 2010
Primary cells of bronchial epithelia	TGFβ, Dexamethasone	Potential involvement in the pathology of allergic disorders	Chattopadhyay et al., 2006 Jawa et al., 2008
Pulmonary macrophages	IFNγ	Potential involvement in the pathology of allergic disorders	Jawa et al., 2008
Colonic subepithelial myofibroblasts		Secret cytokines, chemokines and MMPs after IL-31 stimulation. Potential cooperation of IL-21 and IL-17A in IBD	Yagi et al., 2007
Neurons of the dorsal root ganglia		Potential involvement in the sensation of disease ralated puritus	Bando et al., 2006 Sonkoly et al., 2006
Cell lines			
A549	TGFβ	IL-31 reduces cell proliferation by inducing cell cycle inhibitors	Chattopadhyay et al., 2006 Jawa et al., 2008
HBE	TGFβ, Dexamethasone		Jawa et al., 2008
BEAS-2B		IL-31 induces VEGF, EGF, CCL2 expression which are key regulators in bronchial inflammation. Co-culture with eosinophils enhances their IL-31 responsiveness	Ip et al., 2007
Cell lines derived from colon epithelia (HT-29, SW480, HCT116, CaCo-2)		HCT116, SW480 induce IL-8 upon IL-31 stimulation	Dambacher et al., 2007

HCT 116	Induction of differentiation, TNFα, IL-1β, IFNγ, LPS	IL-31 stimulation inhibits cell proliferation and induces migration	Dambacher et al., 2007
GO-G-UVM, U937, THP1, A172 CCF-S-TTG1. U87MG, A375, A549, DU145, MCF-7			Ghilardi et al., 2002 Diveu et al., 2004,

1.2.6 Regulation of IL-31 and IL-31RA

Although a number of studies have described tissues and cells that express IL-31 and/or its receptor, little is known about how either the ligand or the receptor are regulated. The few studies that have been published are summarized below. In general these are correlative studies and the molecular mechanisms underlying these observations have not been determined yet.

1.2.6.1 Regulation of the expression of IL-31

The superantigen staphylococcal enterotoxin B (SEB) of *S. aureus* is one of the stimuli identified, which induces *IL31* expression in PBMCs of atopic dermatitis (AD) patients (Sonkoly et al., 2006). SEB also induced *IL31* expression in *in vivo* patch tests in AD patients, while in the same experimental setup and in PBMCs of psoriasis patients no regulation of *IL31* was measurable. As pointed out above, IL-31 was originally identified as a T_H2 cytokine. Thus it is possible that these cells are responsible for the SEB effect in PBMCs. Also AD is a T_H2-linked skin disorder, whereas psoriasis is mainly characterized by the presence of T_H1 cells. These differences might explain the selective induction of *IL31* mRNA but further detailed investigations are needed for more definitive conclusions.

Skin biopsies of extrinsic atopic eczema (AE) patients display increased *IL31* mRNA expression compared to normal skin (Gambichler et al., 2008). A standard therapy for these patients is UVA irradiation. After 6 weeks of phototherapy patients show decreased *IL31* mRNA levels, indicating that IL-31 expression might correlate with the AE disease phenotype. Extrinsic AE is driven by an IgE sensitization of the skin, which prompts high levels of T_H2 cells in the skin. UVA irradiation promotes apoptosis of T-cells and this is thought to ameliorate the disease. The reduced number of T-cells in UVA irradiated skin could also explain the reduced *IL31* mRNA levels (Gambichler et al., 2008). Thus IL-31 is a candidate for influencing the severity of AE

because the decrease of *IL31* mRNA (and other T_H2 cytokines) after UVA therapy correlates with a decline of the symptoms.

Recently it was reported that $CD4^+$ T-cells express the histamine H_4 receptor, which is involved in allergic inflammation. This receptor responds to histamines secreted by mast cells and basophiles. The expression of the receptor can be up-regulated by stimulation of the cells with IL-4, a cytokine produced by T_H2 cells. Stimulation with the H_4 receptor agonist 4-methyl-histamine results in a significant up-regulation of *IL31* indicating a role of this cytokine in allergic inflammations (Gutzmer et al., 2009).

Furthermore antimicrobial peptides play an important role in activating mast cells. Human β-defensins (hBD) and cathelicidin LL-37 belong to these peptides. They can be secreted by monocytes in response to bacteria, LPS or IFNγ (Niyonsaba et al., 2006). Also keratinocytes express hBDs after stimulation with bacteria or inflammatory cytokines. These peptides are important components of innate immunity and it has been demonstrated that they also induce IL-31 expression and secretion in mast cells. IgE sensitized and subsequently activated mast cells also respond with an increase of IL-31 expression. The combined stimulation with hBDs or LL37 and anti-IgE results in a synergistic induction of IL-31 (Niyonsaba et al., 2010). The observation that IFNγ induces IL-31RA expression (see below) and that hBDs promote IL-31 production indicates a potential role of IL-31 in innate immunity (Duits et al., 2002; Fang et al., 2003).

1.2.6.2 Regulation of the expression of IL-31 receptor

The regulation of IL-31RA expression has been studied more extensively. It was observed that the receptor is induced in monocytes by the cytokine IFNγ that is expressed predominantly by T_H1 cells (Dillon et al., 2004; Diveu et al., 2004; Ghilardi et al., 2002). Moreover dendritic cells respond to IL-1α and CD40L by stimulating IL-31RA (Diveu et al., 2004). It was observed that proliferating and early-differentiating keratinocytes express the IL-31RA. The expression is rapidly down regulated during differentiation. Similar to the observation in monocytes, undifferentiated and early-differentiated keratinocytes also up-regulate the IL-31RA in response to IFNγ, while the pro-inflammatory cytokines IL-1α, IL-6 and TNFα have no effect in

keratinocytes. During differentiation keratinocytes down-regulate the IFNγ receptor, which might explain the lack of responsiveness to IFNγ in more differentiated cells. Taken together the results obtained in keratinocytes combined with the findings that IL-31 is high in certain skin diseases suggest that this cytokine might be involved in controlling keratinocyte differentiation (Heise et al., 2009).

Further support for a role of IL-31 in skin comes from recent studies demonstrating that Pam3Cys, a toll-like receptor 2 (TLR2) ligand, induces *IL31RA* expression in normal skin. Keratinocytes derived from AD patients did not show this effect, possibly explained by an impaired TLR2 expression in keratinocytes derived from such patients (Kasraie et al., 2011). Recently it has also been reported that human dermal microvascular endothelial cells express the *IL31RA* and its expression can be stimulated by IFNγ (Feld et al., 2010). Together these studies identify IFNγ as an important regulator of *IL31RA* expression and therefore IFNγ is most likely a critical factor to define the responsiveness of cells to IL-31. Whether all cells that express the IFNγ receptor respond by inducing IL-31RA is not known at present.

Human mast cells are also a source of IL-31. They express the high affinity IgE receptor Fcε-RI that plays an important role in allergic disorders (Kinet, 1999; Siraganian, 2003). Treatment of IgE-sensitized mast cells with anti-human IgE antibodies stimulates the receptor, which leads to its aggregation and activation of downstream signaling cascades. The comparison of the Tyr phosphoproteome between unstimulated and IgE-activated mast cells revealed that IL-31RA is highly Tyr phosphorylated (Yamaoka et al., 2009). It is possible that this is the consequence of IgE-mediated secretion of IL-31, which then stimulates its receptor in an autocrine feedforward loop. Alternatively IL-31RA may be the target of crosstalk signaling. Thus mechanistically it is unclear why IL-31RA is Tyr phosphorylated in these cells. Also it has not been resolved whether this results in the activation of IL-31RA downstream signaling pathways.

Finally the expression of IL-31RA was analyzed in several primary cell types of the respiratory system, which was indicated by the potential role of IL-31 in allergic reactions (Jawa et al., 2008). The expression of IL-31RA is induced in bronchial

epithelial cells and type II pneumocytes in response to TGFβ, whereas IFNγ had no effect. In contrast pulmonary macrophages respond to IFNγ but not to TGFβ and pulmonary fibroblast do not respond to either of the two cytokines when IL-31RA expression was measured. In A549, a transformed type II alveolar epithelial cell line, and in HBE, an immortalized human bronchial epithelial cell line, TGFβ induces IL-31RA, consistent with the findings in primary cells (Jawa et al., 2008).

Dexamethasone (Dex), a cortisol analog, is a potent anti-inflammatory drug. Dex can induce the expression of gp130 in different cell types. Dex also induces *IL31RA* mRNA expression in bronchial epithelial and HBE cells and to a lower extent in pulmonary cells. Although Dex did not significantly increase *IL31RA* expression in A549 cells, it markedly increased *IL31* signaling after Dex pre-treatment, indicating that the cells become IL-31 sensitive. Mechanistically it is not entirely clear how Dex and IL-31 cooperate in these cells. Taking together these findings suggest that IL-31RA expression in response to inflammatory mediators varies considerably between different cell types. At least in epithelial cells derived from the pulmonary system TGFβ replaces IFNγ as a potent IL-31RA regulating factor and induces the sensitivity of the cells to IL-31 (Jawa et al., 2008). Figure 4 briefly summarizes what is known about the regulation of IL-31 and its receptor and the IL-31 induced signaling pathways. Table 1 summarizes IL-31 sources and IL-31RA expressing cells including known regulatory stimuli and the biological relevance as far it was further analyzed.

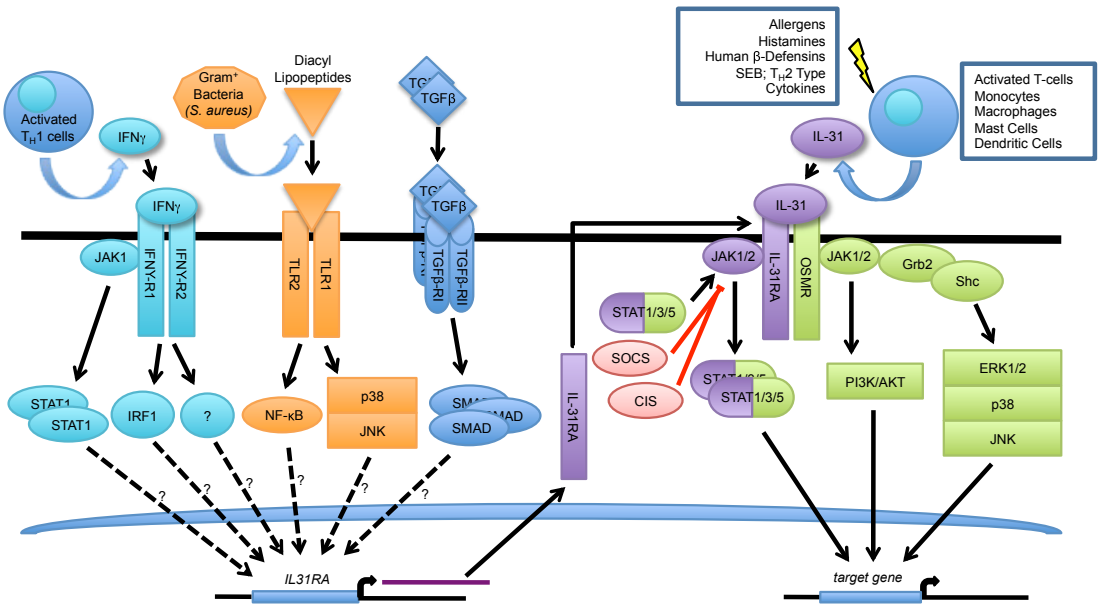


Figure 4. IL-31 signaling cascade.
IL-31 signals through a heterodimeric receptor composed of the IL-31RA and the OSMR. There are several stimuli described inducing IL-31RA expression, including IFNγ and TGFβ. IFNγ is mainly produced by activated T-cells and activates STAT1 and IRF1, which then translocate into the nucleus and regulate the expression of target genes. IFNγ also activates signaling pathways that control mRNA stability (not shown). TGFβ is expressed ubiquitously by a wide range of different cells and, when interacting with its receptors, induces the translocation of SMAD protein complexes into the nucleus. It is also described that toll-like receptor 1/2 (TLR1/2) activates the expression of *IL31RA* after binding of the synthetic ligand Pam3Cys. Under physiological conditions TLR1/2 is activated by components of gram$^+$ bacteria, e.g. derived from *S. aureus*, and induces NF-κB and p38 and JNK MAPKinase signaling. Which pathways directly influence the *IL31RA* expression has not been determined yet. Several cell types produce IL-31 and its expression can be induced by different stimuli as indicated. Upon binding to its receptor complex, IL-31 activates JAK kinases that subsequently phosphorylate and activate STAT1, STAT3, and STAT5. The co-localization with the OSMR enables the complex to activate also the PI3K/AKT and different MAPKinase signaling pathways (ERK, p38 and JNK). These control downstream effectors of IL-31 signaling and are most likely essential for controlling target gene expression.

1.2.7 The biological function of IL-31 in mouse models

1.2.7.1 IL-31 affects the immune response in the mouse skin

To address the biology of IL-31, transgenic mouse models over-expressing IL-31 were developed, in which the cytokine was either expressed under the control of a lymphocyte specific or a ubiquitously active promoter (Dillon 2004). In both models, the mice developed severe pruritus with alopecia and skin lesions that were characterized by hyperkeratosis, acanthosis, inflammatory cell infiltration and an increase in mast cells. Overall this phenotype shows similarities with the clinical picture of patients suffering from AD. No changes were observed in histological samples from lung, intestine, stomach, prostate or brain. A similar phenotype was seen in Rag1-deficient animals devoid of lymphocytes that were reconstituted with bone marrow of IL-31 transgenic mice (Dillon et al., 2004). This indicates that lymphocytes are an important source of IL-31 to develop an AD-like skin phenotype.

Moreover injection of BALB/c or C57BL/6 mice with mouse IL-31 via a subcutaneous mini-osmotic pump resulted in the development of severe pruritus with patchy regions of alopecia. This phenotype eased 6-9 days after the last IL-31 injection with a complete reduction of pruritus and alopecia after 40 days. These mice did not develop skin lesions indicating that under these experimental conditions pruritus was the main consequence of applying IL-31 systemically. Histological analysis of enlarged peripheral lymph nodes in IL-31 transgenic mice revealed an inverse ratio of B- and T-cells compared with wild type mice (36% B-cells to 63% T-

cells in IL-31 transgenic and 62% B-cells to 25% T-cells in wild type mice) and the portion of activated T-cells and memory T-cells within the T-cell fraction was up-regulated, specially in the $CD4^+$ fraction of T-cells. Serum levels of IgE and IgG were not altered, indicating that no change occurred in the development of cells of the myeloid or lymphoid lineage (Dillon et al., 2004). Thus the alterations observed in peripheral lymph nodes were probably a consequence of increased inflammatory processes triggered by infectious inflammation in the lesional skin. The composition of B- and T-cells was unaltered in other organs of the lymphoid system. These findings indicate that IL-31 induces pruritus and alopecia independent of IgE, which leads to the assumption that this model is more comparable to patients suffering from the non-IgE associated form of AD. Of note is that lymphocyte-deficient $Reg1^{-/-}$ mice treated with IL-31 injected via a subcutaneous mini-osmotic pump developed a severe pruritus with substantially delayed and reduced alopecia indicating that IL-31 mediated pruritus is lymphocyte-independent whereas the presence of lymphoid cells is necessary for the development of alopecia (Dillon et al., 2004). Subcutaneous local injection of IL-31 in BALB/c mice induces infiltration of inflammatory cells and epidermal thickening, which leads to the assumption that IL-31 has also chemotactic functions. Indeed stimulation by IL-31 enhances the expression of several chemokines in human keratinocytes including *GRO1α, TARC, MIP3β, MDC, MIP-3, MIP-1β* and *I-309* (Dillon et al., 2004). Together these findings demonstrate that IL-31 has a number of effects that point to a role in the regulation of immune responses in skin.

1.2.7.2 IL-31RA is required for IL-31-associated pruritus

IL-31RA-deficient mice have no obvious phenotype. There are no differences in the number of distinct immune cells in different lymphoid organs measureable. Injection of mouse IL-31 via subcutaneous mini-osmotic pump into IL-31RA-deficient animals did not result in pruritus or alopecia as seen in heterozygous littermate control mice or wild-type BALB/c mice, supporting the conclusion obtained from the findings with IL-31 transgenic mice that IL-31 is a strong inducer of pruritus and that IL-31RA is essential to mediate this effect (Dillon et al., 2004). Thus these findings suggest that IL-31 signals exclusively through the IL-31RA/OSMR heterodimeric complex.

1.2.7.3 The lack of IL-31 affects hematopoietic progenitor cells

Although no obvious phenotype is seen in IL-31RA$^{-/-}$ mice, these animals show alterations in hematopoietic progenitor cells (HPC) (Broxmeyer et al., 2007). But this does not result in any difference in the number of circulating red blood cells (RBC), platelets (PLT) or other blood cell types. However a significant reduction of CFU-GM (colony-forming unit granulocyte and macrophage), BFU-E (burst-forming unit erythroid), and CFU-GEEM (colony-forming unit erythroid, granulocyte, macrophage, and megakaryocyte) progenitor cells was measurable in bone marrow and spleen of IL-31RA$^{-/-}$ mice compared to control animals. Cells derived from the bone marrow of IL-31RA$^{-/-}$ mice are predominantly non-cycling whereas a significant fraction (40-50%) from wild type animals are cycling. Stimulation with granulocyte macrophage colony-stimulating factor (GM-CSF)/stem cell factor (SCF) (or alternatively to SCF fms-like tyrosine kinase ligand (FL)) or IL-3/SCF (or FL) induces maturation and proliferation of the "immature subset" of CFU-GMs. In contrast stimulation with GM-CSF, SCF or IL-31 alone supports maturation and proliferation of the "mature subset" of CFU-GMs (Shaheen and Broxmeyer, 2004). Although the total number of CFU-GM per femur that was able to differentiate was lower in IL-31RA$^{-/-}$ mice, the number of the "mature subset" of CFU-GMs, which proliferates in response to a single cytokine, was not different between wild type and IL-31RA$^{-/-}$ mice. In contrast the "immature subset", which proliferates with a combination of cytokines as mentioned above, was significantly under-represented in CFU-GM cells derived from IL-31RA$^{-/-}$ mice. This indicates that IL-31 might have a positive regulating effect on immature HPCs and that IL-31 influences the responsiveness to cytokine stimulation in immature CFU-GM. Although the absence of the IL-31RA seems to inhibit cytokine-induced proliferation of HPCs, the stimulation of wild type cells with recombinant IL-31 does not promote proliferation, neither alone nor in the presence of pro-proliferative cytokines. These findings are somewhat difficult to interpret with our current knowledge about IL-31. But it should also be remembered that the lack of IL-31RA may free OSMR that becomes available for other cytokines and associated signaling mechanisms that may explain some of the unexpected results.

Myeloid progenitors need cytokines for survival in cell culture. The combination of SDF-1 with CXCL12 is known to enhance survival of these cells. This is true for wild

type as well as IL-31RA$^{-/-}$ HPCs, indicating that the death rate of HPCs is not enhanced in the absence of IL-31RA. Surprisingly, however, the addition of IL-31 to the culture medium of wild type HPCs increases their survival, an effect that is abrogated in IL-31RA$^{-/-}$ HPCs (Broxmeyer et al., 2007). Thus the absence of IL-31RA leads to a significant reduction in the population of immature HPCs at a very early stage but does not affect myeloid differentiation under normal conditions. It leads to cell cycle arrest in the bone marrow while IL-31 stimulation of wild type cells promotes cell survival. These findings indicate that the lack of IL-31RA reduces the number of progenitors and enhance their sensitivity to apoptosis but the final number of mature cells is not affected. Obviously these observations are difficult to reconcile and are in need of further experimentation.

1.2.7.4 Mouse model for atopic dermatitis

Nc/Nga mice are a model for AD (Matsuda et al., 1997; Suto et al., 1999). These mice develop spontaneous skin lesions, have increased plasma IgE levels, show invasion of inflammatory cells into the skin and develop pruritus approximately 8 weeks after birth when kept under regular conditions. Under specific pathogen free (SPF) conditions they do not develop this phenotype, which is comparable to AD in humans. Skin lesions of these mice express higher levels of *IL31* and *IL4* (Takaoka et al., 2005). The developing skin lesions lead to an increased scratching behavior that correlates with increased *IL31* mRNA levels in injured skin compared to NC/Nga mice kept under SPF conditions. Treatment with 2,4,6-trinitrochlorbenzene (TNCB) induces lesions in the skin and serves as an additional model of allergic contact dermatitis. The treatment of NC/Nga mice kept under SPF conditions with TNCB induces skin lesions with high plasma IgE levels, infiltration of inflammatory cells, and increased IL-4 levels but no increase in the scratching behavior. Because no increase in *IL31* mRNA expression was observed compared to untreated animals, these findings indicated that IL-4 is an important mediator of the inflammatory process but does not contribute to pruritus. Instead it appears that IL-31 not only contributes to the scratching behavior, it might be the causative factor. It is accepted that pruritus is not only a symptom of AD, but it is also a cause for a more severe phenotype as it increases indirectly the injury of the skin and thus stimulates the inflammatory response as a result of the increased scratching behavior (Takaoka et al.,

2006). This hypothesis is supported by the observation that scratching behavior of NC/Nga mice increased before any signs of dermatitis were detectable. However application of anti-IL-31 antibodies in NC/Nga mice reduced scratching behavior but had no effect on the development of dermatitis. The treatment with IL-31 neutralizing antibodies was started several weeks after birth, thus at a time point when the mice had already developed an increased scratching behavior even though there were no signs of dermatitis (Grimstad et al., 2009). Whether IL-31 only contributes to pruritus under these circumstances or whether it interferes additionally with very early stages of dermatitis has to be further investigated.

As mentioned before it has been observed that the IL-31RA and the OSMR are co-expressed in a subset of neurons of the dorsal root ganglia (Bando et al., 2006), which represents the site where the soma of cutaneous sensory neurons are located while their sensory fibers protrude directly into the skin. These sensory neurons might be implicated in the sensing of itch and are possibly stimulated by IL-31. Another hypothesis suggests that IL-31 indirectly induces pruritus by stimulating keratinocytes (and/or other cells within the inflamed skin) that subsequently express not only inflammatory but also pruritic mediators (Fig. 5). Thus IL-31 may represent a new link between the immune and sensory nervous system (Steinhoff et al., 2006).

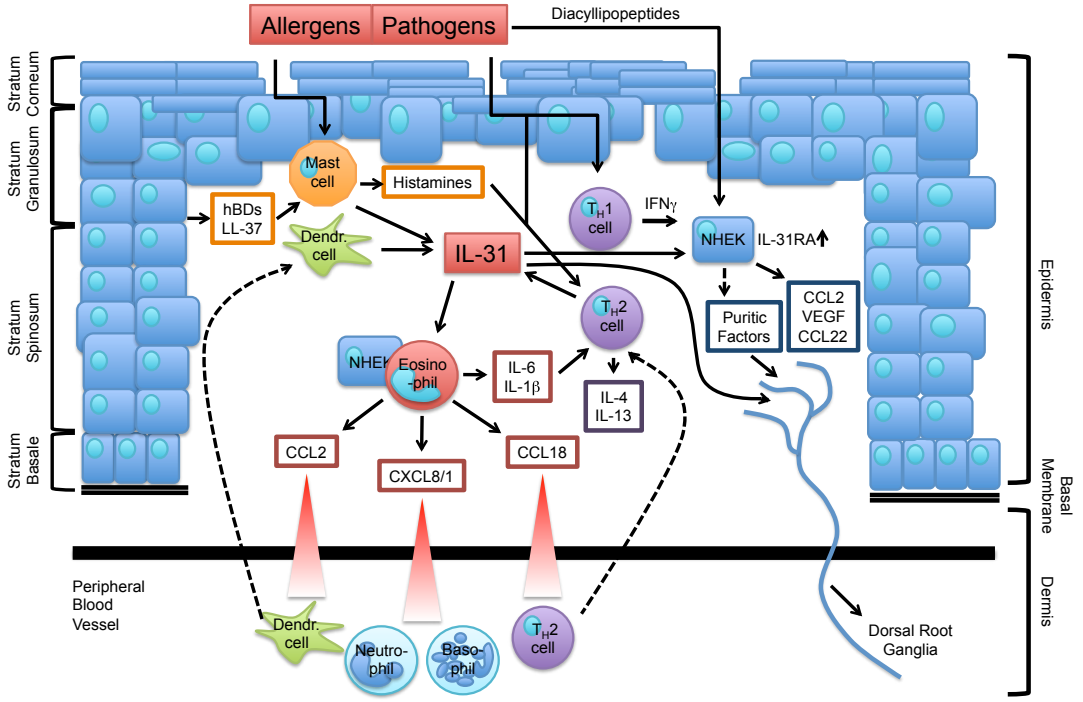


Figure 5. Center stage of IL-31 in skin disorders.
Disturbances in epidermal barrier formation (as e.g. in AD) allows increased penetration of the epidermis by allergens and pathogens. Mast cells and dendritic cells respond to these insults with an increased secretion of histamines and IL-31. The secretion of antimicrobial peptides (hBDs and LL-37) stimulates mast cells to secret IL-31. Furthermore certain antigens such as the *S. aureus* superantigen SEB stimulate IL-31 expression in T_H2 cells. IL-31 activates eosinophils to secret several cytokines and chemokines, an effect that is enhanced by direct interaction of eosinophils with keratinocytes. IL-6 and IL-1β activate T_H2 cells leading to the secretion of IL-4 and IL-13 and thus promoting the T_H2 immune response. CCL18 promotes the recruitment of T_H2 cells to the site of inflammation, whereas CCL2 attracts dendritic cells, and CXCL8/1 allure neutrophils and basophils. The recruitment of T_H2 cells and dendritic cells further increases the inflammatory response and thus enhances IL-31 levels at the site of inflammation. Although eosinophils secret CXCL8 and 1, it has not been reported yet whether basophils or neutrophils are important in IL-31 connected inflammations. NHEKs induce the expression of the IL-31RA after stimulation of the TLR1/2 receptor complex by components of $gram^+$ bacteria. Pathogens can stimulate T_H1 cells to secret IFNγ, which also induces IL-31RA expression on NHEKs and thus increasing IL-31 sensitivity. Moreover keratinocytes express cytokines and chemokines like CCL2, VEGF and CCL22 after IL-31 stimulation, also attracting inflammatory cells. In addition IL-31 might be involved in the development of pruritus in epidermal disorders. Dorsal root ganglia express the IL-31RA and their unmyelinated C fibers might be directly stimulated by IL-31 or IL-31 induces the expression of pruritic factors by NHEKs with subsequent activation of C fibers leading to the sensation of itch.

1.2.7.5 A role for IL-31 and its receptor in allergic diseases

Because IL-31 is mainly produced by T_H2 cells, a model for antigen-induced airway hyper-responsiveness was used that is characterized by the production of the T_H2 cytokines IL-4 and IL-13 (Tomkinson et al., 2001). The expression of *IL31RA* was investigated in an allergy mouse model, whereby ovalbumin (OVA) is applied intranasally to BALB/c or C57BL/6 mice and lung tissue and cellular infiltrates are collected by bronchoaveolar lavage. Both strains of mice had significantly increased

IL31RA mRNA levels in the lung tissue and the infiltrating lymphocytes upon OVA application compared to PBS treated mice (Dillon et al., 2004). BALB/c mice are known to be more susceptible for T_H2 cytokine associated diseases. This correlated with higher *IL31RA* mRNA levels in BALB/c compared to C57BL/6 mice. These data reveal a potential role of IL-31 in allergic disorders.

The involvement of IL-31/IL-31RA in inflammatory processes in the lung was also confirmed in IL-31RA deficient mice (Perrigoue et al., 2007). In the KO animals the composition of immune cells in thymus, spleen, and lymph nodes and in PBMCs was unchanged compared to wild-type animals. The intravenous injection of *Schistosoma mansoni* eggs, a model of type 2 inflammation in the lung, leads to the formation of IL-4- and IL-13-dependent granulomas in the small blood vessels of the lung (Warren et al., 1967; Wynn and Cheever, 1995). IL-31RA knockout mice develope more severe inflammation in the lung parenchym surrounding the eggs with increased granulocyte infiltration compared to wild type mice. Also KO cells located in the lymph nodes secreted more IL-4, IL-13, and IL-5 leading to higher IgE levels in the serum. *S.mansoni* infiltration in the lung is characterized by a T_H2 cytokine-dependent development of alternatively activated macrophages (AAMs) (Herbert et al., 2004). These AAMs were also present in a higher number compared to wild type mice. The presence of this macrophage subtype correlates with elevated collagen deposition and fibrosis, which is also observed in IL-31RA$^{-/-}$ mice compared to wild type mice. Taking this together these mice exhibit a deregulated type 2 inflammation in the lung. Furthermore activated naive CD4$^+$ T-cells of IL-31RA$^{-/-}$ mice have an increased proliferation rate and expression levels of IL-4 and IL-13 compared to wild type cells. In contrast CD4$^+$ T-cell derived T_H2 cells show no difference in proliferation or cytokine expression indicating that IL-31 can directly influence naïve CD4$^+$ T-cells but is not involved in proliferation or cytokine secretion processes in T_H2 cells (which are the major source of IL-31). This indicates that in this model system IL-31 might influence T_H2-mediated immune responses in a negative feedback mechanism rather than promoting T_H2-mediated inflammation (Perrigoue et al., 2007).

In contrast to the above-described association of the IL-31 system with T_H2-mediated inflammation, no interaction with T_H1-mediated immune response was seen. Injection of mice with the parasite *Leishmania major* elicits an immune response that is strictly

dependent on the production of IFNγ by T_H1 $CD4^+$ T-cells. The absence of the IL-31RA does neither influence the differentiation to T_H1 cells nor alter infection symptoms by *L. major* or interferes with IFNγ production in the lymph nodes indicating that IL-31 is not involved in IFNγ-dependent immune responses (Perrigoue et al., 2007).

1.2.7.6 The lack of IL-31RA affects OSM signaling

It has to be noted that the results obtained from IL-31 transgenic and IL-31RA knockout mice are somewhat contradictory. While the IL-31 over-expression induces T_H2-mediated immune responses with increased T_H2 cytokine levels, the IL-31RA knockout results in a more severe inflammatory response in a T_H2 cytokine-mediated inflammation model. The latter findings indicate that an intact IL-31 signaling cascade should inhibit T_H2 cytokine dependent inflammatory processes. If instead of genetically modified mice IL-31 neutralizing monoclonal antibodies are used to abrogate the IL-31 signal in antigen-induced immune models, no alterations in the expression of T_H2 cytokines by T-cells was observed (Bilsborough et al., 2010). Part of the observed differences may be due to changes in OSMR-dependent signals because the lack of IL-31RA increases the availability of OSMR to form complexes with gp130. Indeed the absence of IL-31RA in IL-$31RA^{-/-}$ mice in these studies led to an over-representation of gp130/OSMR complexes at the cell surface and an increased OSM responsiveness. This switch is most likely independent of IL-31. Consequently an elevated expression of IL-6, VEGF, and TIMP-1, whose genes are regulated by OSM, was measured in response to OSM in IL-$31RA^{-/-}$ mice. These observations lead to the hypothesis that rather enhanced OSM signaling than the absence of IL-31 signaling stimulated the T_H2 response in the lung of IL-$31RA^{-/-}$ mice. This may also relate to the increased expression of VEGF, which is known to induce remodeling of the lung epithelium, as seen also in the above mentioned airway models, and promote T_H2-mediated sensitization in the lung (Chapoval et al., 2009; Lee et al., 2004). The hypothesis, albeit attractive, that OSM or VEGF are responsible for the IL-$31RA^{-/-}$ phenotype requires further verification.

1.2.8 IL-31 in human diseases

1.2.8.1 Pruritic skin diseases

In order to evaluate the contribution of IL-31 signaling to different skin disease (Table 2), it is worth to briefly summarize key observations associated with some of these that will be relevant for the subsequent discussion. Atopic dermatitis (AD) is a chronic inflammatory skin disease that is characterized by xerosis, pruritus, defects in skin barrier formation with trans-epidermal water loss, erythematous lesions, the deregulation of inflammatory cytokines, and infiltration of inflammatory cells into the skin (Bieber, 2010; Leung and Bieber, 2003). It represents a systemic disease as a result of hypersensitivity of the immune system. As such it is a model of allergy and it is connected to food allergy, asthma bronchiale, and allergic rhinitis. The spectrum of severity of AD is very wide. Two sub-categories of atopic dermatitis can be classified (Bardana, 2004): (1) The immunoglobulin E (IgE)-mediated variant (referred to as extrinsic form or IgE-associated form of AD) is the most prevalent form of the disease (about 70–85% of AD patients). These patients have high serum IgE levels and positive skin prick test reactions to common environmental allergens such as food and aeroallergens. Target organ challenge with allergens has been shown to precipitate worsening of skin lesions, and the extent of IgE sensitization is directly associated with the disease severity. (2) The intrinsic form of AD (also referred to as non-IgE-associated form of AD) is not associated with sensitization to food or aeroallergens and total serum IgE is within normal limits. Nevertheless, these patients manifest identical skin lesions to the allergic form of the disease. AD is defined as a biphasic inflammatory disease with an initial induction of T_H2-type cytokines (IL-4, IL-5 and IL-13) in the acute phase, e.g. in response to skin penetrating allergens. In the chronic course of AD the cytokine expression pattern changes to predominantly T_H1-type cytokines, including IFNγ, IL-12, IL-5, and GM-CSF. In addition the maintenance of chronic AD is characterized by the production of T_H1-type cytokines like IL-12 and IL-18 as well as some remodeling-associated cytokines like IL-11, IL-17 and TGFβ (Bieber, 2010).

Allergic contact dermatitis (ACD) is a complex syndrome representing immunological responses to cutaneous exposure to protein-reactive chemicals, most frequently caused by low molecular weight electrophilic chemicals or metal ions.

ACD represents a common inflammatory skin disease, that is T cell-mediated, with a cumulative prevalence of 15–20% in the general population of western countries (Ott et al., 2010). Activation of DCs by contact allergens is a prerequisite for the induction of the pathogenic skin-specific T cell response. DC activation proceeds via a broad variety of innate immune receptors, including pattern recognition receptors, Toll-like receptors (TLRs), and NOD-like receptors (Weber et al., 2010). The onset of the disease is mostly due to the rapid recruitment of chemical-specific $CD8^+$ T cells, which induce apoptosis of keratinocytes. Additionally, $CD4^+$ T_H1 and T_H17 cells contribute to the extension of the inflammatory reaction by releasing pro-inflammatory cytokines that activate keratinocytes and other resident skin cells. ACD is a chronic disease, which lasts, in most cases, for the entire life of the affected individual (Cavani and De Luca, 2010).

Prurigo nodularis is a chronic condition characterized by a papulonodular pruriginous eruption of unknown aetiology. A variety of systemic conditions have been reported to be associated with prurigo nodularis but the underlying mechanisms are unknown. Nerve growth factor has been implicated in the pathogenesis of prurigo nodularis. Calcitonin gene-related peptide and Substance P immunoreactive nerves are markedly increased in prurigo nodularis when compared to normal skin. These neuropeptides may mediate the cutaneous neurogenic inflammation and pruritus in prurigo nodularis (Lee and Shumack, 2005).

The observations made in IL-31 transgenic mice indicated a potential role of IL-31 in AD. The expression of *IL4* and *IL13*, the major cytokines expressed by T_H2 cells in T_H2 mediated inflammation, and IFNγ, which is mainly expressed by T_H1 cells, in skin biopsies of 33 AD patients was analyzed. Serum IgE levels and IL-31 expression were measured in these patients. As expected the T_H2 cytokines *IL4* and *IL13* and serum IgE levels were high in AD patients compared to healthy donors. This was in contrast to IFNγ that is only expressed in chronic AD. Interestingly the expression of *IL31* mRNA was induced and correlated with the increased *IL4* and *IL13* expression. A comparison of the expression levels of these factors with the severity of AD of single patients revealed neither a correlation with *IL31*, *IL4* or *IL13* expression nor with serum IgE levels. Nevertheless these findings suggested that overall *IL31* expression correlated with an acute AD phenotype (Neis et al., 2006).

Biopsies of psoriasis patients, a T_H1 cytokine-mediated skin disease, did not show increased *IL4* and *IL13* levels but elevated *IFNγ* expression as expected. The *IL31* expression was even lower compared to normal skin. Also biopsies from atopic contact dermatitis (ACD) patients were analyzed, a disease that is proposed to be T_H1-mediated, but also shows aspects of T_H2-mediated diseases with a pathogenesis comparable to AD. Biopsies of 56 patients with acute ACD were analyzed and *IL31* mRNA expression was up regulated in 43%, *IL4* in 89% and IL-13 in 86% compared to healthy skin. Surprisingly *IFNγ* was induced only in a minority of patients (27%) (Neis et al., 2006). This indicates that also ACD, despite it being described as a T_H1 cytokine-mediated disease, probably has an acute phase with a T_H2 contribution including IL-31. This is consistent with the skin samples analyzed in this study that were taken during the acute phase of ACD. Evidence for an involvement of T_H2 cells and T_H2 cytokines in the development of contact hypersensitivity has been reported previously (Niiyama et al., 2010; Yokozeki et al., 2000).

These data were confirmed by another study comparing skin biopsies of healthy patients with biopsies of psoriasis, non-lesional AD, lesional AD and prurigo nodularis patients (Sonkoly et al., 2006). This study revealed that lesional AD and to a minor extend non-lesional AD as well as prurigo nodularis skin expresses increased *IL31* mRNA levels, while psoriasis samples were comparable to healthy skin. AD and prurigo nodularis (as well as ACD) are skin diseases often associated with severe pruritus while psoriasis rarely causes an itch sensation. This indicates a connection of IL-31 with pruritic skin diseases in humans and thus is similar to the observations made in the mouse. Genome-wide gene expression analysis in different human tissues revealed that *IL31RA* is highly expressed in dorsal root ganglia also in humans. Since the sensation of itch is mediated by unmyelinated C fibers of primary sensory neurons and the cell bodies of these neurons reside in the dorsal root ganglia, these observations led to the hypothesis that IL-31 is directly involved in modulating the signaling of sensory neurons in pruritic skin and may contribute to the itch sensation (Sonkoly et al., 2006). It is currently argued that IL-31 may induce pruritus through the induction of an as yet unknown keratinocyte-derived mediator, which subsequently activates unmyelinated C fibers in the skin. This suggests that IL-31 represents possibly a new link between the immune and the nervous system (Steinhoff et al., 2006). Alternatively IL-31RA may be present also in the C fibers and thus IL-

31 may directly stimulate these sensory neurons in the skin. Together these findings provide interesting models of how IL-31 may contribute to pruritus that can now be evaluated.

A recent clinical study compared the severity of AD in a group of 50 children with their IL-31 serum levels. The severity of AD was assessed according to different scoring systems and correlated to IL-31 levels (Ezzat et al., 2011). It was observed that serum IL-31 directly correlates with the severity of AD. The IL-31 levels decreased during quiescent phases of the disease but remained elevated if the previous burst of the disease was accompanied by high IL-31 serum levels. In contrast to IL-31, serum IgE levels, total LDH or absolute eosinophil counts in the blood, which were proposed to be indicators for the severity of AD, did not correlate (Ezzat et al., 2011).

Exposure of non-lesional skin of AD patients, who have a known house dust mite allergy, with house dust mite allergen induces skin lesions mimicking AD. In these lesions compared to non-treated biopsies, increased *IL31* mRNA expression was detected in some but not all patients. This supports the idea that IL-31 might be one important factor in allergen-induced AD. Skin of AD patients is often colonized with *S. aureus*, which is a known trigger factor for atopic skin inflammation. Exposure of skin of AD patients to *S. aureus* superantigen (SEB) in a patch test enhanced *IL31* mRNA expression and PBMCs derived from these patients show a significantly higher *IL31* mRNA expression compared to PBMCs of healthy donors after exposure to SEB. This indicates that AD patients respond to the exposure to allergens with increased IL-31 expression, while healthy individuals do not (Sonkoly et al., 2006).

Furthermore it has been demonstrated that circulating T-cells expressing the skin-homing receptor cutaneous lymphocyte antigen (CLA) induce *IL31* mRNA after activation by anti-CD3 and anti-CD28 antibodies in AD patients and healthy donors with a trend to higher expression by T-cells derived from AD patients. These T-cells accumulate at inflamed cutaneous sites, because CLA mediates binding to E-selectin (Teraki et al., 2000). These cells express also higher amounts of IL-4 and IL-13 in AD patients and respond to allergens more intensely. The number of circulating CLA positive T-cells is increased in AD patients. Thus these cells have been suggested to

be a major contributor to the pathology of AD (Bilsborough et al., 2006). Since they accumulate at inflammatory sites of AD patients (the majority of T-cells at these sites are CLA^+), this subtype of T-cells is most likely responsible for increased IL-31 expression in lesional skin of AD patients. In addition the *IL31RA* expression of keratinocytes in biopsies of lesional skin of AD patients is significantly higher compared to biopsies of healthy donors. Furthermore macrophages expressing the IL-31RA infiltrate inflammatory sites in AD patients. This suggests that IL-31 expressing T-cells accumulate at lesional skin sites of AD patients and interact with target cells with high IL-31RA. In contrast CLA^+ T-cells from psoriasis patients only express minimal *IL31* mRNA. These data provide further evidences for the involvement of IL-31 in pruritic skin disorders.

A common therapy for AD patients is UVA1 phototherapy at a wave length of 340-400nm. It has been demonstrated that *IL5*, *IL13* and *IL31* mRNA levels in UVA1 treated skin were significantly down-regulated compared to untreated skin. UVA1 stimulates apoptosis in T-cell. The reduction in mRNA levels supports the suggestion that T-cells are the major source of IL-31 in atopic skin (Gambichler et al., 2008).

One report suggested that the expression of IL-31 is affected by single nucleotide polymorphisms (Schulz et al., 2007). Homozygocity of one of the haplotypes identified correlates with increased *IL31* expression in activated PBMCs. Interestingly this haplotype is also strongly associated with the non-atopic form of dermatitis indicating that an altered IL-31 expression may be important in the pathogenesis of these patients. However, so far no additional evidence has been published to suggest that these three haplotypes differ in IL-31 expression.

Eosinophils are one type of inflammatory cells that infiltrate lesional skin of AD patients. Interestingly these cells express the IL-31RA (Cheung et al., 2010). In an *in vitro* co-culture system with keratinocytes, which also express the IL-31RA, it has been demonstrated that IL-31 delays the apoptosis of eosinophils and stimulates the secretion of pro-inflammatory cytokines and chemokines (IL-6, IL1β, CXCL1, CXCL8, CCL2, and CCL18) by eosinophils but not keratinocytes (Cheung et al., 2010). IL-6 and IL-1β are known to activate T_H cells and induce a T_H2-mediated immune response (Lacy and Moqbel, 2000). Thus IL-31 stimulation might contribute

to the inflammatory response by inducing the expression of these cytokines. The induction of the above mentioned chemokines might promote the infiltration of immune cells into lesional AD skin. CCL2 and CCL18 are AD associated chemokines (Homey et al., 2006). CCL18 is a highly expressed chemokine in AD skin and stimulates the recruitment of CLA^+ T-cells to the site of inflammation (Günther et al., 2005). CCL2 recruits dendritic cells while CXCL8 and CXCL1 induce infiltration of neutrophils and basophiles (Conti and DiGioacchino, 2001; McColl and Clark-Lewis, 1999; Rossi and Zlotnik, 2000). Taken together these results suggest that IL-31 might amplify the inflammatory responses in AD by initiating the expression of inflammatory cytokines and chemo-attracting chemokines. Although keratinocytes do not induce the expression of these above-mentioned inflammatory factors, they nevertheless might contribute to the secretion of these factors by eosinophils. The recruitment of inflammatory cells to the site of inflammation is a critical aspect of AD. Inflammatory cells are attracted by chemokines and maintained at the site of inflammation by adhesion molecules. Keratinocytes express the adhesion molecule ICAM-1, while eosinophils are positive for the integrin LFA-1, which is composed of CD11a/CD18. ICAM-1 interacts with LFA-1 and thus eosinophils are likely to be retained at the site of inflammation by direct interaction with keratinocytes (Larson and Springer, 1990; Nickoloff et al., 1990). In addition co-culturing of keratinocytes and eosinophils stimulates the expression of these adhesion molecules and thus enhances the possibility for efficient retention of eosinophils in the tissue.

Moreover the direct contact of these cells might contribute to the secretion of cytokines. Indeed the direct interaction of eosinophils and keratinocytes seems important for the production of cytokines and chemokines in response to IL-31. If the two cell types are separated in a trans-well chamber, the IL-31 effect was notedly reduced, indicating that the direct physical contact of these two cell types enhances the IL-31 stimulating effect on eosinophils (Cheung et al., 2010). The mechanistic base for this cooperative effect remains to be established.

An important aspect of AD is a defect in skin barrier formation and function, which results in the increased penetration of the skin with allergens and microbes. The skin produces a number of antimicrobial peptides and proteins as part of the innate immune system. Two of them are the human β-defensins (hBDs) and cathelicidin LL-

37 (Niyonsaba et al., 2006). Important cells mediating inflammatory and allergic responses in the skin are mast cells that are also recruited to the site of inflammation. Mast cells induce *IL31* mRNA expression upon treatment with hBDs and LL-37. Pre-sensitization of cells with IgE and co-stimulation of the cells with hBDs or LL-37 and anti-IgE antibodies synergistically increases the expression of *IL31* (Niyonsaba et al., 2010). This indicates an involvement of IL-31 in the innate immune response, an aspect that might be particularly relevant in AD patients, involving a feed-forward loop with innate immune cells and cytokines affecting the skin barrier further enhancing the effects of allergens. I suggest that IL-31 contributes to this vicious cycle.

As mentioned above, stimulation of lesional skin of AD patients with SEB induces IL-31 expression in various cell types. In addition keratinocytes seem to be able to enhance IL-31-mediated expression of inflammatory factors and therefore stimulate the inflammatory response in AD. Keratinocytes express the TLR-2, which is an important mediator of the innate immune response to gram-positive bacteria, including *S. aureus* (Takeuchi et al., 2001). Stimulation of human primary keratinocytes with the synthetic TLR-2/TLR-1 heterodimeric ligand Pam3Cys induces IL-31RA and OSMRβ expression, an effect that is even more potent for IL-31RA than the previously described T_H1-linked cytokine IFNγ (Morr et al., 2002). In addition the T_H2 cytokine IL-4 did not up-regulate IL-31RA in keratinocytes indicating a link of IL-31RA expression to T_H1 type cytokines in contrast to IL-31 that is linked with T_H2-type cytokines. After pre-stimulation of keratinocytes with Pam3Cys or IFNγ, the expression of CCL2, VEGF, and CCL22 was induced in keratinocytes in contrast to unstimulated cells (Kasraie et al., 2011). This demonstrates that keratinocytes can indeed directly contribute to changes in cytokine composition in AD skin, at least after pre-stimulation with IFNγ (i.e. as a consequence of activation of T_H1 cells) or activation of TLR-2 by *S. aureus* superantigens.

In addition chronic spontaneous urticaria (CU) is a skin disorder characterized by the development of highly pruritic relapses lasting for 24 hours up to periods of weeks or even years (Zuberbier et al., 2009). These patients show elevated serum IL-31 levels indicating a role of IL-31 in this pruritic disease (Raap et al., 2010).

Table 2: IL-31 related diseases

PUBLISHED IL-31 RELATED SKIN DISEASES	OBSERVATIONS INDICATING THE INVOLVEMENT OF IL-31 IN THESE DISEASES	REFERENCE
Pruritic skin diseases		
Atopic Dermatitis Non-atopic Dermatitis Atopic Contact Dermatitis	IL-31 mRNA expression correlates with IL-4 and IL-13 in biopsies of AD and ACD patients. IL-31 serum level correlate with AD severity in children. An IL-31 haplotype is associated with non-AD. IL-31 stimulates the secretion of inflammatory cytokines and chemokines in eosinophils that are in contact with keratinocytes.	Neis et al., 2006 Sonkoly et al., 2006 Ezzat et al., 2011 Bilsborough et al., 2006 Schulz et al., 2007 Cheung et al., 2010
Prurigo Nodularis	Skin biosies of patients show increased IL-31 level.	Sonkoly et al., 2006
Chronic Spontaneous Urticaria	Patiens have elevated IL-31 serum level.	Raap et al., 2010
Diseases of the repiratory system		
Allergic Asthma	Patiens show increased IL-31 expression in PBMCs. Lung epithelial cells and cell lines respond to IL-31 and secret inflammatory cytokines important in inflammatory deseases of the lung. Allergic mouse models exhibit an upregulation of IL-31 and IL-31RA in the lung tissue.	Lei et al., 2008 Chattopadhyay et al., 2006 Ip et al., 2007 Dillon et al., 2004 Perrigoue et al., 2007
Inflammatory Bowel Dieseases		
Crohn´s Disease Ulcerative Colitis	Upregulation of IL31RA, IL31 and OSMR mRNA in biopsies of inflammed tissues. Like IL-17, an important mediator in IBD, IL-31 induces the expression of cytokines, chemokines and MMPs in colonic subepithelial myofibroblasts.	Dambacher et al., 2007 Yagi et al., 2007
Philadelphia chromosome-negative myeloproliferative neoplasms	Mast cells derived from MPN patients show an incresed IL-31 secretion upon activation. Patients have elevated IL-31 plasma level.	Wang et al., 2009

1.2.8.2 IL-31 in the lung

IL-31 is not only important in the skin; it also seems to play a role in the lung epithelium (Table 2). Primary type II pneumocytes, primary bronchial epithelial cells, as well as A549 cells express the IL-31RA and respond to IL-31 by activating STAT3 and its target genes (Chattopadhyay et al., 2006). In addition ERK, JNK and AKT phosphorylation is induced in A549 cells. IL-31 stimulation efficiently suppresses proliferation in lung epithelial cells by altering the expression of cell cycle regulators. While the cyclin-dependent kinase (CDK) inhibitor $p27^{KIP1}$ is induced, cyclin B1, CDC2, and CDK6 are repressed (Chattopadhyay et al., 2006). Furthermore the *IL31RA* is expressed in a wide range of lung cells including primary bronchial epithelial cells, pulmonary macrophages and primary type II pneumocytes. Also HBE and A549 cells, cell lines derived from bronchial epithelial cells, express the *IL31RA* (Jawa et al., 2008). The broad expression spectrum of IL-31 and its receptor in lung epithelial cells supports the assumption that IL-31 is involved in inflammatory diseases associated with the airways, including allergic asthma.

Allergic asthma is a chronic inflammatory disease that is, similar to AD, characterized by the presence of T_H2 cytokines (IL-4 and IL-13), increased serum IgE levels, bronchial infiltration of immune cells, reversible bronchial obstruction, mucus hypersecretion and bronchial hyper reactivity (Chiappara et al., 2001). Thus some of these phenomenological alterations are comparable to those seen in the skin of AD patients. These findings resulted in the suggestion that IL-31 is also involved in inflammatory processes associated with allergic asthma. In BEAS-2B cells, a transformed bronchial epithelium cell line, CCL2, VEGF and EGF expression is induced upon IL-31 stimulation. In contrast the T_H2 cytokines IL-4 and IL-13 are unable to induce VEGF or EGF. The expression of the chemokine CCL2 is, however, induced by IL-4 and IL-13 and these two cytokines function cooperatively with IL-31. As described above eosinophils, which are prominent immune cells to infiltrate the bronchial tissue in allergic asthma, express EGF, CCL2, IL-6 and IL-8 after IL-31 stimulation, an effect that is significantly enhanced in co-cultures with bronchial epithelial cells. CCL2, VEGF and EGF are proposed to be key regulators of bronchial inflammation and remodeling of the lung tissue, frequently associated with lung fibrosis and loss of function, in bronchial asthma (Amishima et al., 1998; Ferrara and Davis-Smyth, 1997). Furthermore CCL2 and VEGF are highly involved in recruiting immune cells to inflammatory sites in the lung (Romagnani, 2002; Zittermann and Issekutz, 2006). In conclusion IL-31 might contribute to inflammation by inducing the expression of inflammatory cytokines in the lung and, similar to keratinocytes, enhance the inflammatory response of eosinophils at the site of inflammation (Ip et al., 2007).

In addition to the effects seen in lung tissue, serum levels of IL-31 and mRNA expression in PBMCs of allergic asthma patients are significantly up-regulated compared to non-allergic asthma patients and healthy individuals (Lei et al., 2008). These findings provide further support for a functional role of IL-31 in promoting and/or sustaining allergic asthma.

The assumption that IL-31 is important in allergic reactions in the skin and the respiratory system is further corroborated by the finding that $CD4^+$ T-cells, especially T_H2 cells, express the histamine H_4 receptor, which is up-regulated upon IL-4 treatment. Subsequent stimulation with a histamine agonist (see above) results in the activation of *IL31* mRNA expression in these T cells. This links IL-31 directly to

histamines that are secreted by mast cells and basophils in allergic reactions (Gutzmer et al., 2009). The finding that mast cells and eosinophils express IL-31 and IL-31RA, respectively, provides a connection of skin and lung epithelia with critical cells of the innate immune system through IL-31, indicating a direct involvement of IL-31 in innate immunity at sites of the body, where the initial confrontation with allergens and pathogens occurs.

1.2.8.3 IL-31 in inflammatory bowel disease

Since IL-31 is present in the epithelia of skin and lung, an obvious thought is that IL-31 is present also in other epithelia that are in close contact with the environment. Indeed, it has been reported that IL-31 is expressed in the small intestine and colon (Dillon et al., 2004). Inflammatory bowel disease (IBD) comprises a group of inflammatory disorders of the colon and the small intestine, with the major types being Crohn´s disease (CD) and ulcerative colitis (UC) (Mizoguchi and Mizoguchi, 2010). The etiology is largely unknown but a common view is that the mucosal immune system aberrantly responds to allergens and microbes in the gastrointestinal lumen, especially in patients with a genetic pre-disposition. For example mutations in the NOD2 gene, a cytosolic pattern recognition receptor that senses peptidoglycans from gram$^+$ and gram$^-$ bacteria, are associated with CD. It is suggested that an impaired NOD2 function promotes epithelial barrier dysfunctions with increased bacterial invasion and inflammation in the intestine (Lecat et al., 2010). This leads to infiltration of inflammatory cells including T-cells that express IL-17 and IL-22, two important mediators in IBD. The chronic inflammation results in mucosal damage by abnormal killing of bacteria of the normal microbial flora in the gut, disruption of the epithelial barrier, and the formation of epithelial ulcerations. Patients with IBD have an increased risk to develop colon cancer (Mizoguchi and Mizoguchi, 2008, 2010; Sartor and Muehlbauer, 2007).

It has been demonstrated that several human cell lines derived from the small intestine and the colon epithelia, including HT-29, SW480, HCT116 and CaCo-2, express IL-31RA and OSMR, and in low amounts *IL31* mRNA (Table 1) (Dambacher et al., 2007). Furthermore in HCT116 and SW480 the phosphorylation of STAT1, STAT3, ERK1/2 and AKT is induced in response to IL-31 stimulation, which results in the activation of downstream target genes, including *IL8*. IL-31 stimulation inhibits cell

proliferation of HCT116 cells, comparable to the effects in lung epithelial cells, and increases cell migration. Induction of differentiation in HCT116 cells, using sodium butyrate, increases the expression of *IL31RA*, *OSMR*, and *IL31*. The stimulation of HCT116 cells with the inflammatory cytokines TNFα, IL-1β, and IFNγ, and treatment with LPS induces the expression of all three genes (Dambacher et al., 2007). Whether this is relevant for tumor cell physiology remains to be addressed. Moreover in CD and UC patients *IL31RA*, *OSMR*, and *IL31* mRNA is upregulated in inflamed but not in unaffected skin biopsies (Dambacher et al., 2007). While CD is proposed to be a T_H1/T_H17 cytokine-mediated disorder, UC is more linked to T_H2 cytokines. The finding that IL-31 and IL-31RA are up-regulated in both forms of IBD suggests that although IL-31 is a T_H2 cytokine, it is not restricted to T_H2-mediated diseases.

Human colonic subepithelial myofibroblasts (SEMFs) respond to IL-31 stimulation by secretion of pro-inflammatory cytokines, chemokines and matrix metalloproteinase's (MMPs) that contribute to tissue destruction. SEMFs are located directly under the basement membrane of the intestinal mucosa and play a role in wound healing and inflammation. In microarray analyses, IL-31 was found to induce efficiently the chemokines *CXCL8 (IL-8), CXCL1 (GRO-α), CCL7 (MCP-3), CXCL3, CCL13* and *CCL15*, the pro-inflammatory cytokines *IL6, IL16* and *IL32*, as well as *MMP-1, MMP-3, MMP-25* and *MMP-7* in SEMFs. In addition the secretion of some of these proteins, including IL-6, IL-8, GRO-α, MCP-3, MMP-1 and MMP-3, was demonstrated. IL-17A has been identified as an important mediator for IBD also inducing IL-6, IL-8 and MMP-3 secretion (Andoh et al., 2008; Harrington et al., 2005). The effects of IL-17A were comparable to those seen with IL-31 and application of both cytokines resulted in additive effects on mRNA expression of these factors (Yagi et al., 2007). These results indicate that T_H2 T-cell secreted IL-31, together with IL-17, might contribute to the pathology of IBD by inducing the expression of the above-mentioned factors in SEMFs. It is tempting to speculate that IL-31 and IL-17 might cooperate in regulating inflammatory processes in the intestine, but possibly also in other tissues including skin.

1.2.8.4 Involvement of IL-31 in Philadelphia chromosome-negative myeloproliferative neoplasms (Ph^-MPN)

MPN are characterized by excessive proliferation of cells of one or more differentiated myeloid lineages. Polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) belong to the Ph^-MPN (Finazzi and Barbui, 2008; Mesa, 2002; Tefferi et al., 2009). An important aspect of Ph^-MPN is the development of pruritus in roughly 50% of all patients (Diehn and Tefferi, 2001; Gerlini et al., 2002). Mast cells appear to be important for Ph^-MPN-associated pruritus, possibly because they produce IL-31. It was observed that mast cells derived from SCF/IL-6-stimulated $CD34^+$ cells of MPN patients secrete significantly more pruritic factors than corresponding mast cells from healthy donors in response to IgE sensitization and anti-IgE activation (Wang et al., 2009). Moreover the secretion of histamines, prostaglandins and leukotrienes was significantly higher in patient-derived mast cells upon stimulation. Important for the discussion here, this was also true for IL-31 and, furthermore, IL-31 serum levels in MPN patients and secretion of IL-31 by $CD3^+$ T-cells from MPN patients after phytohemaglutinin activation were also elevated. The perception of pruritus in MPN patients correlates with the number of mast cells generated from bone marrow of these patients in culture, a decreased mast cell apoptosis rate and an increased IL-31 plasma level. In addition, as mentioned above the overall prostaglandin release of mast cells derived from MPN patients is higher compared to control cells, but mast cells derived from MPN patients that suffer from pruritus release significantly less prostaglandins than patients without pruritus (Ishii et al., 2009). This further increases the itch sensation as prostaglandins are proposed to play a role in inhibiting pruritus (Arai et al., 2004; Sugimoto et al., 2005; Sugimoto et al., 2007). In summary activated mast cells secrete IL-31 in combination with other pruritic factors in MPN. Furthermore mast cells derived from MPN patients proliferate faster and show a lower apoptosis rate. Importantly they accumulate in the skin of PV patients with pruritus. In combination with the proposed role of IL-31 in pruritic skin diseases, it is attractive to suggest that this cytokine contributes to MPN associated pruritus.

1.2.9 Conclusions

Although the literature on IL-31 function is not very extensive yet, the available findings suggest that this cytokine regulates aspects of innate as well as adaptive immunity in tissues that are exposed to the environment. In this setting IL-31 is associated with a number of diseases, including atopic dermatitis, inflammatory bowl diseases, and allergic asthma (summarized in Table 2). Common to these diseases is the deregulation of cells of the innate immune system, including mast cells and eosinophils, and of the adaptive immune system. For the latter T_H2 helper T cells appear to be particularly relevant but contributions by other T cell subpopulations, e.g. T_H1 and T_H17 cells, may also turn out to be relevant. These cells together with the epithelial cells in the skin, intestine, and lung form a network of interactions, both through cytokines and direct cell-cell contacts, in which IL-31 appears to play an important role. Clearly furthermore detailed molecular studies will be required to firmly establish the role of IL-31 in the normal physiology of these organs and in different disease states.

The molecular studies have established that IL-31 signals through a heterodimeric receptor composed of the IL-31RA and OSMR. This complex can stimulate different signaling pathways through receptor-associated JAK kinases. This results in the activation of STATs, MAPKinases, and PI3K/AKT, factors that control many aspects of cell physiology. Although these signaling pathways are well studied, their relative contributions to different IL-31 phenotypes have not been established yet.

One of the conclusions that can be drawn from the work in mice is that IL-31 plays a key function in pruritus. This is supported by work using IL-31 transgenic and IL-$31RA^{-/-}$ mouse models and by experiments, in which IL-31 was subcutaneously injected into the skin. In all these situations T_H2 cells are activated and secrete a cocktail of cytokines that includes IL-31. Moreover the stimulation of mast cells through antigens and allergens results in the release of IL-31 in combination with other factors. It appears that IL-31 then stimulates other immune cells, which results in further cytokine production and provokes the release of pruritic factors from keratinocytes. It is thought that the latter somehow affects sensory neurons and thus induces itch sensation. Although these experiments cannot be directly compared to the

human situation, several lines of evidence suggest that IL-31 is also a pro-pruritic factor in humans. This is of interest because pruritus is a severe symptom of many skin diseases. Thus interfering with IL-31-mediated itch sensation could be therapeutically relevant and substantially improve substantially the condition of patients with AD or some forms of MPN.

IL-31 has additional consequences in skin, lung, and intestinal epithelia. In this respect an important finding is that keratinocytes express the IL-31 receptor and therefore are responsive to this cytokine. From the interaction of different immune cells with keratinocytes in the skin, it seems likely that IL-31 contributes to skin diseases (Fig. 5). In this tissue IL-31 may control not only the presence and activity of different immune cells but also affect the proliferation and differentiation of keratinocytes and by this contribute to the function of the skin as a protective organ. For example IL-31 might affect skin barrier function, thereby controlling the exposure to antigens and allergens but also microorganisms. This again provides opportunities for specific molecular intervention, e.g. in AD.

Because several cell types in lung and intestine carry the IL-31 receptor, it is likely that also in these tissues IL-31 has specific effects. However the molecular consequences are less well studied. Nevertheless, also in these tissues barrier function might be controlled by IL-31, in analogy to the skin.

In conclusion the information available suggests that human IL-31 is an important factor in innate immunity and inflammation that enhances immune responses in epithelial tissues. How important IL-31 in epithelial inflammations is remains an open question. But the evidence that is being accumulated indicates that IL-31 contributes to the severity of T_H2-mediated diseases, including AD, allergic asthma and inflammatory bowel diseases. Most likely IL-31 is able to amplify the expression of pro-inflammatory cytokines and chemokines and thus increases the recruitment of inflammatory cells to the site of inflammation. Although IL-31 is probably not directly involved in T_H1-mediated diseases, the fact that IL-31RA can be regulated by cytokines of T_H1 cells provides support for the hypothesis that IL-31 links T_H2- and T_H1-mediated immune responses. Preliminary evidence also suggests that IL-31 controls the proliferation and differentiation of cells of non-hematopoietic origin,

lending support to the concept that this cytokine plays an integrative role in the proper formation of epithelia.

2 Results

2.1 The biological effect of IL-31 treatment: IL-31 interferes with keratinocyte differentiation in an organotypic skin model

To understand the role of IL-31 for keratinocyte proliferation and differentiation, recombinant human (rh) IL-31 was applied to organotypic human 3D models of skin differentiation. These reconstructs were generated from primary normal human epidermal keratinocytes (NHEK) and primary dermal fibroblasts (see Materials and Methods). IL-31 treatment resulted in a disturbed epidermal differentiation, characterized by a thin epidermal layer with hypogranulosis and an abolished alignment of keratinocytes in the stratum basale (Fig. 6A). The proliferation of keratinocytes was impaired as measured by the reduced staining for Ki67. In addition reduced expression of epidermal differentiation markers, including filaggrin and keratin (K) 10 was observed in the upper epidermal layer. These findings were confirmed by measuring the *FLG*, *KRT10, INV* and *ITGB4* mRNA levels of the 3D models (Fig. 6B). *FLG* and *KRT10* expression was considerably delayed and reduced during differentiation in response to rhIL-31. The expression of involucrin was also reduced (Fig. 6A), but this was only apparent at day 2 of the model at the mRNA level (Fig. 6B). Moreover the expression of β4-integrin and K14 was disorganized (Fig. 6A). β4-integrin expression seems not to be restricted to the basal cell layer, but rather is distributed throughout the whole model and at least at day 2 a 25-fold increased *ITGB4* mRNA expression compared to non-treated cells was observed (Fig. 6B).

These results implicated that IL-31 modulates fundamental aspects of keratinocyte proliferation and differentiation. Therefore NHEKs were stimulated for 1h with rhIL-31 and gene expression was measured using Affymetrix gene arrays. One could observe changes in the expression of a rather limited set of genes (Fig. 6C). The most highly regulated gene was *SOCS3*, a direct target of the JAK-STAT3 pathway activated by the IL-31RA/OSMR receptor complex as described above. In addition the expression of several cytokines and transcriptional regulators was enhanced. Of

particular interest were the genes that encode IL-20 and IL-24. They are located in the *IL10* gene cluster containing two further cytokine genes, *IL10* and *IL19*, flanked by genes encoding the mitogenic activated protein kinase activated kinase 2 (*MAPKAPK2*), an upstream kinase of the MAP-kinase signaling pathway (Zu et al., 1994), the fas apoptotic inhibitory molecule 3 (*FAIM3*), an Fc receptor for immunoglobulin (Ig) M (Shima et al., 2010), and the (*PIGR*), an Fc receptor that facilitates the secretion of IgA and IgM (Kaetzel, 2005) (summarized in Fig. 6D).

We observed that *IL20* and *IL24* gene expression was robustly activated within one hour of rhIL-31 application (Fig. 6E). In contrast the neighboring genes were either not expressed (*IL10, IL19, FAIM3* and *PIGR*) or their expression was not affected by rhIL-31 treatment (*MAPKAPK2*). Similarly the expression of *IL31RA* and *OSMR* was not regulated by rhIL-31. These findings validate the IL-31-dependent regulation of *IL20* and *IL24* and suggest that these two cytokines are involved in the downstream effects of IL-31. Indeed both IL-20 and IL-24 have been suggested to affect keratinocyte differentiation, although some of the reports are controversial (Sabat, 2010).

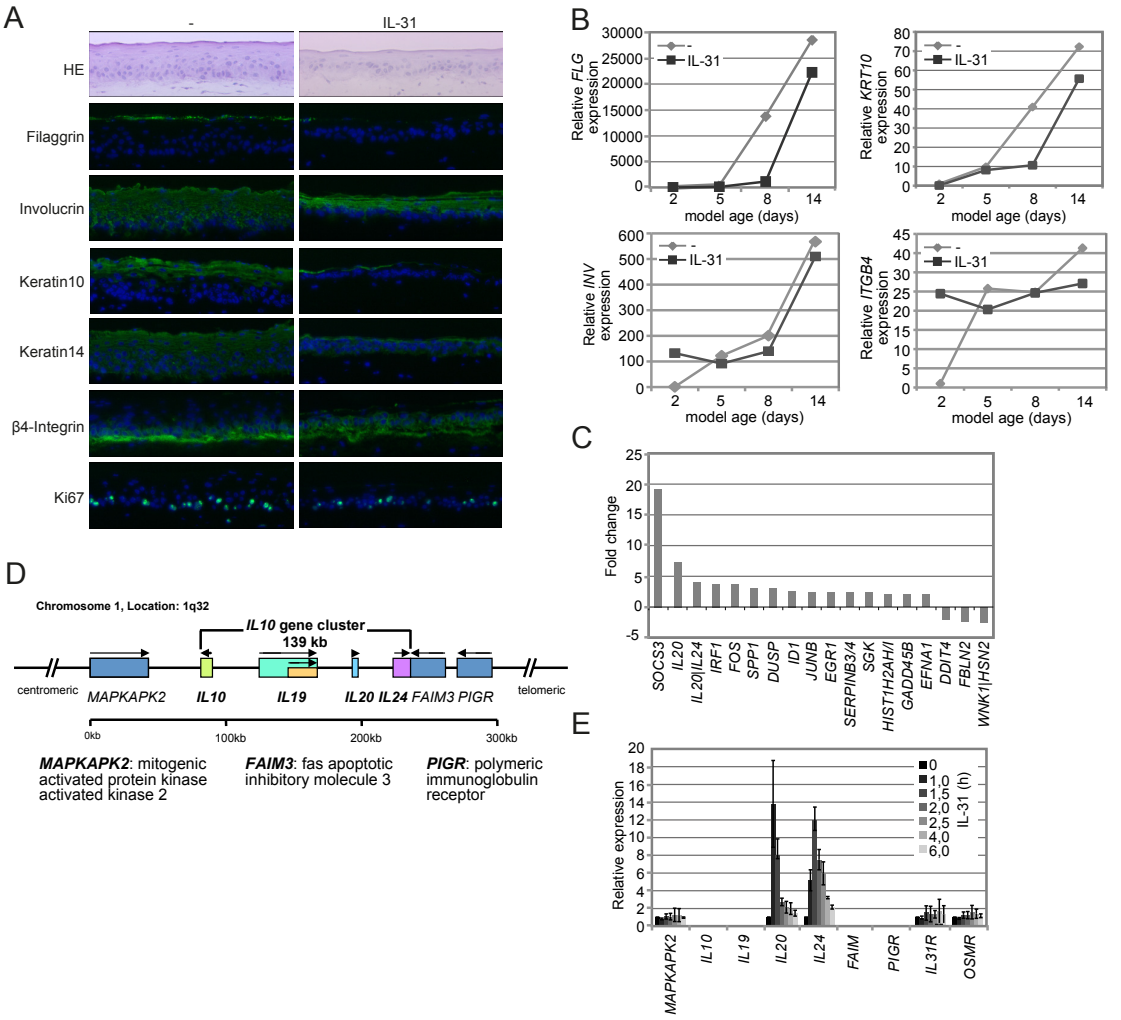
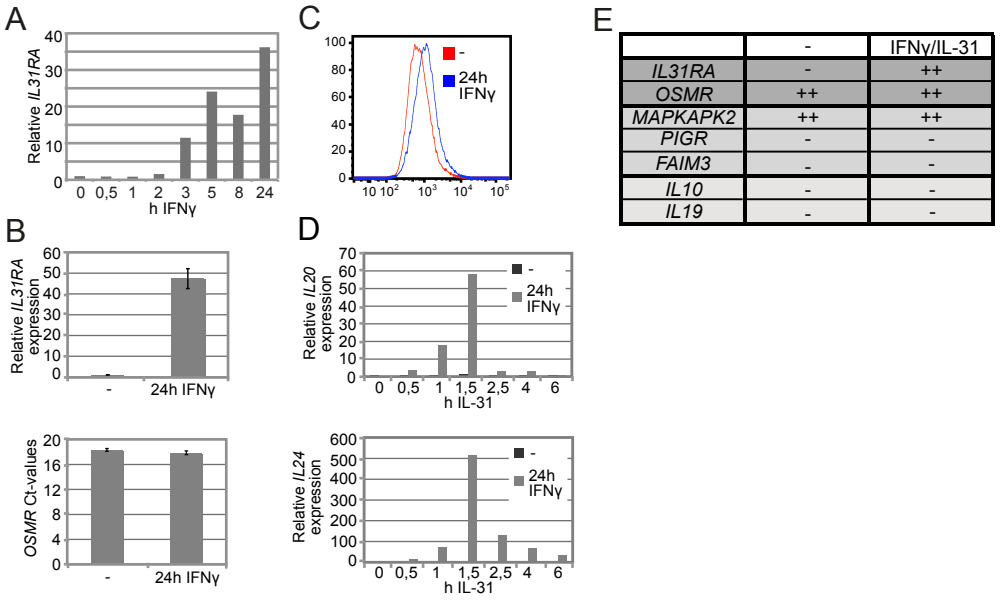


Figure 6. IL-31 disturbs differentiation of primary NHEKs in organotypic skin equivalents.
A. NHEKs and fibroblasts were grown as 3D organotypic skin equivalents and stimulated with or without rhIL-31 (100ng/ml; every second day) as indicated after lifting the cells to the air – liquid interphase. The skin equivalents were harvested and histological sections were stained for the indicated markers at day 8 (1 of 6 models).
B. Organotypic skin equivalents grown in parallel to the one in panel A (the same primary cells were used) were harvested at day 2, 5, 8, and 14 and the expression of *FLG, KRT10, INV* and *ITGB4* determined by RT-qPCR. Displayed are relative expression levels compared to the non-stimulated day 2 sample (normalized to *HPRT* expression). One representative experiment of 3 is shown.
C. NHEKs were stimulated with rhIL-31 for 1 h; mRNA was isolated and analyzed on Affymetrix Exon arrays. Displayed are genes that are regulated more than two fold compared to a non-stimulated control in two independent experiments.
D. Schematic representation of the *IL10* gene cluster located on chromosome 1 that spans a region of about 140 kb. The *IL19* gene is transcribed from two different promoters. The indicated genes flank the cluster and the chromosomal orientation is marked.
E. NHEKs from three different donors were isolated and stimulated with rhIL-31 (100ng/ml) for the indicated time periods (h). The expression of *IL20* and *IL24* was analyzed in comparison to other genes in the *IL10* gene cluster, flanking genes, and *IL31RA* and *OSMR* by RT-qPCR.

2.2 Development of model systems:

2.2.1 HaCaT cells treated with IFN-γ

Primary keratinocytes often show donor dependent variability, which was noted by studying the response to rhIL-31 in many different batches of primary NHEKs. Therefore I wanted to establish a donor-independent reproducible cellular system for subsequent studies. HaCaT cells are spontaneously immortalized primary keratinocytes that are still capable to differentiate in organotypic skin models (Boukamp et al., 1988). HaCaT keratinocytes constitutively express the OSMR (Fig. 7B) but not the IL-31RA receptor chain and are therefore not capable of responding to IL-31. The expression of the IL-31RA in HaCaT cells can be induced by IFN-γ (Heise et al., 2009) with expression first measureable by 2-3 hours (Fig. 7A, B and C), which enables the cells to respond to rhIL-31 and to induce target genes, including *IL20* and *IL24* (Fig. 7D). Similar to NHEKs *IL10, IL19, FAIM3* and *PIGR* are not expressed and *MAPKAPK2* and *OSMR* are not affected by rhIL-31 stimulation (summarized in Fig. 7E). IFN-γ controls many cellular processes and induces cell cycle arrest, making it difficult to investigate IL-31 specific functions during differentiation. To overcome this limitation I generated stable cell clones expressing IL-31RA under a tetracycline controllable promoter.



	-	IFNγ/IL-31
IL31RA	-	++
OSMR	++	++
MAPKAPK2	++	++
PIGR	-	-
FAIM3	-	-
IL10	-	-
IL19	-	-

Figure 7. HaCaT cells express IL-31RA in response to IFNγ and subsequently respond to IL-31
A. HaCaT cells were stimulated with IFNγ (20ng/ml) for the indicated time points (h). Then *IL31RA* expression was analyzed by RT-qPCR. Displayed are relative expression levels compared to non-stimulated cells, normalized to *HPRT* expression of one representative experiment.

B. HaCaT cells were stimulated with IFNγ (20ng/ml) for 24 h. Then *IL31RA* and *OSMR* expression was analyzed by RT-qPCR. Displayed are relative expression levels (*IL31RA*) and Ct-values (*OSMR*) comparing non-stimulated with stimulated cells, normalized to *HPRT* expression (mean values and standard deviations of three biological replicates).
C. The expression of IL-31RA at the cell surface was evaluated in cells treated as described in panel A using FACS.
D. HaCaT cells either pre-treated or not with IFNγ for 24h, were stimulated with rhIL-31 (100ng/ml) for the indicated time periods (h). The expression of *IL20* and *IL24* was analyzed by RT-qPCR. Displayed are relative expression levels compared to non-stimulated cells, normalized to *HPRT* expression of one representative experiment (of a total of 3).
E. Summary of relative expression values of the two IL-31RA receptor components *IL31RA* and *OSMR*, the three *IL10*-cluster flanking genes *MAPKAPK2*, *PIGR* and *FAIM3* and the *IL10*-cluster members *IL10* and *IL19* in untreated (-) and 24h IFNγ/1,5h IL-31 treated HaCaT cells. The displayed genes are either highly (++) or not (-) expressed.

2.2.2 HaCaT-pRTS IL31RA cell lines with inducible *IL31RA* expression

The human *IL31RA* cDNA was obtained from Imagenes cloned into the pENTR221 Gateway® (Invitrogen) entry vector, which allows easy sub-cloning into gateway compatible vectors via recombination. For expression of the IL-31RA in HaCaT cells I chose the pRTS vector (Bornkamm et al., 2005) (relevant features summarized in Fig. 8A). This vector persists as a replication-competent episomal vector within the nucleus of the target cell. It harbors an ampicillin resistance for prokaryotic and a hygromycin resistance for eukaryotic selection. The bi-directional tetracycline controllable promoter (P_{tet}bi-1) can drive the expression of a gene of interest in one direction and the expression of another protein or shRNA in the opposite direction. For the stimulated expression of the gene of interest, doxycycline (dox), a semi-synthetic derivate of tetracycline, is used. The bi-directional promoter is blocked by the binding of a repressor (rtTA-M2) in dox-free conditions. Addition of dox leads to the release of the repressor from the promoter and binding of a dox dependent activator protein (tTSKRAB). Both repressor and activator are optimized for the binding of dox and binding to the tetracycline responsive element (TRE) within the tetracycline controllable promoter to obtain high specificity. This combination of activator and repressor is meant to mediate a very tight regulation of expression. The repressor and activator are expressed in a single mRNA connected by an internal ribosomal entry site (IRES) and its expression is controlled by the mouse heavy chain intron enhancer (Eμ) in combination with the chicken β-actin promoter (Fig. 8B).

The cDNA of pENTR221-IL31RA was fully sequenced before amplification of the *IL31RA* cDNA via PCR and subsequent insertion of the amplicon into an EcoRV

digested pSFIexpress vector generating pSFI-IL31RA-HA. pSFI serves as a sub-cloning vector adding a haemaglutinin (HA)-tag to the C-terminus of the protein and embedding the HA-tagged cDNA into SfiI restriction sites for cloning it into pRTS. The IL31RA-HA cDNA was fully sequenced and pSFI-IL31RA-HA and pRTS were SfiI digested. The resulting IL31RA-HA fragment was ligated into the linearized pRTS generating pRTS-IL31RA-HA. pRTS harbors a gene expressing the green fluorescence protein (GFP) at the opposite side of the bi-directional promoter which was removed by digestion with BglII and SwaI. After converting the resulting sticky DNA ends to blunt ends via incubation with the Klenow fragment of polymerase II, the plasmid was religated to generate pRTS-IL31RA-HAΔGFP. This step was performed to avoid side effects on cellular behavior and experimental procedures by the expression of GFP that includes the detection of other fluorescently labeled proteins.

The dox-dependent expression of *IL31RA* mRNA was evaluated by RT-qPCR in transiently transfected HEK293 cells (Fig. 8B). The expression of the protein was confirmed in lysates of transfected and dox induced HEK293 cells using different combinations of primary and secondary antibodies to ensure a specific detection of IL-31RA (Fig. 8C).

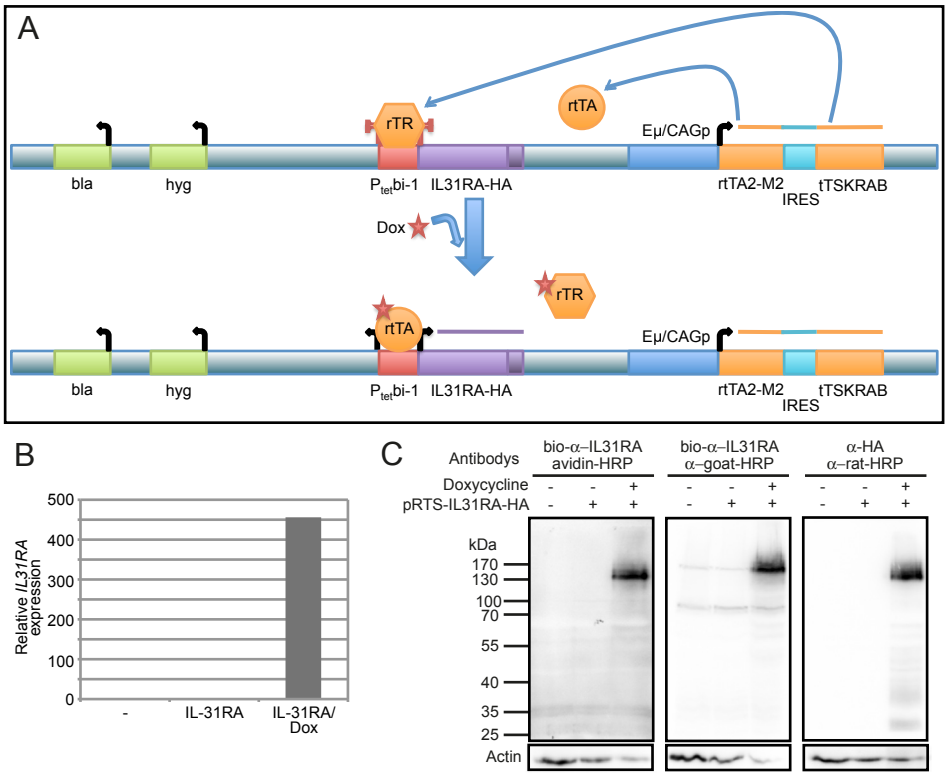


Figure 8. pRTS-IL-31RA-HAΔGFP induces IL-31RA expression after doxycycline induction in HEK293 cells.
A. Scheme of relevant pRTS IL-31RA-HAΔGFP features. The vector contains genes providing ampicilline resistance (bla) as bacterial selection marker and hygromycine (hyg) resistance for eukaryotic selection. The *IL31RA* gene, with a C-terminal added HA-tag (IL31RA-HA), is under the control of a bi-directional tetracycline dependent promoter (Ptetbi-1). The tet-activator (rtTA2-M2) and tet-repressor (tTSKRAB) are expresses from a single mRNA with an internal ribosomal entry site (IRES) and constitutively expressed under the control of the mouse heavy chain intron enhancer (Eμ) and the chicken ß-actin promoter (CAGp). The vector exists as an episomal replication-competent vector in the target cells. Under doxycycline free conditions the tet-repressor binds to the bi-directional promoter to inhibit expression. Addition of dox leads to the replacement of the repressor by the activator and subsequent expression of *IL31RA*.
B. HEK293 cells were mock or pRTS IL-31RA-HAΔGFP transfected and stimulated with dox (1μg/ml) for 16h. The *IL31RA* expression was analyzed by RT-qPCR. Displayed are relative expression levels (*IL31RA*) comparing mock transfected with non-stimulated and dox stimulated cells normalized to *HPRT* expression (one representative experiments of three).
C. HEK293 cells were treated as described in B. The IL-31RA-HA expression was analyzed in western blot using different combinations of primary (either a goat derived biotinylated IL-31RA specific antibody or a rat derived anti-HA antibody) and secondary antibodies coupled to horseradish peroxidase (HRP) as indicated.

To generate a stable pRTS-IL31RA containing cell line, HaCaT cells were seeded in 10cm dishes and transfected with Superfect (Qiagen) according to the manufacturers recommendations. Transfected cells were selected by addition of 400ng/ml hygromycine and seeded at low density to select individual cell clones that were called HaCaT-pRTS-IL31RA. Well-growing individual clones were stimulated with dox for 16h and *IL31RA* expression was measured by RT-qPCR (Fig. 9A). All selected clones induced *IL31RA* expression, which was also confirmed at the protein level (Fig. 9B). For further characterization clone K2 and K15 were selected. Clone K15 induced IL-31RA protein expression in a concentration-dependent manner (Fig. 9C). After 16h of dox pre-treatment and 30min rhIL-31 (100ng/ml) stimulation, STAT3 phosphorylation could be detected, indicating that the receptor is active (Fig. 9D). Furthermore the receptor was expressed at the cell surface as evaluated by fluorescence microscopy and indirect confocal immunofluorescence microscopy of paraformaldehyde fixed but not permeabilized cells to prevent the binding of the primary antibodies to intracellular IL-31RA (Fig. 9E). Clone K15 induced *IL31RA* expression upon dox treatment (Fig. 9F) and subsequent stimulation with IL-31 for 1 hour led to the expression of the IL-31 target genes *IL20* and *IL24* (Fig. 9G). Similar results were obtained with clone K2 (data not shown).

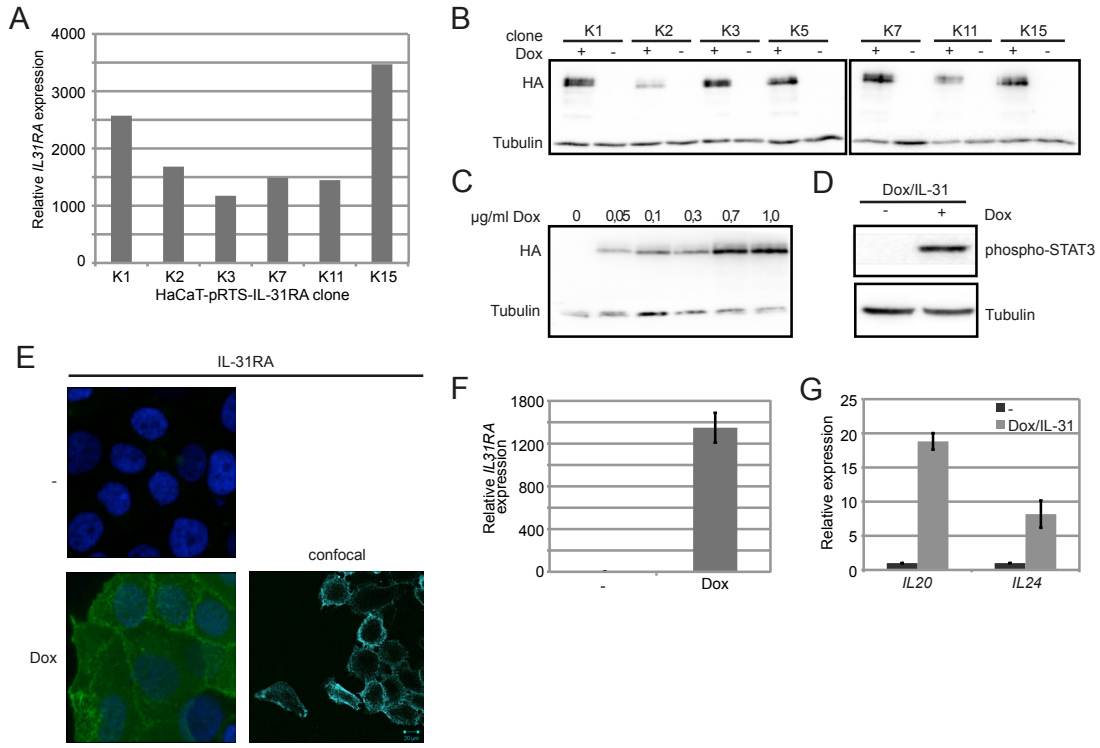


Figure 9. HaCaT-pRTS-IL-31RA cells induce *IL31RA* expression and respond to IL-31.
A. HaCaT cells were transfected with pRTS IL-31RA-HAΔGFP and individual clones selected with hygromycine. The clones were stimulated with or without dox (1µg/ml) for 16h and the expression of *IL31RA* mRNA was analyzed by RT-qPCR compared to the non-stimulated control and normalized to *HPRT* expression.
B. IL-31RA protein expression was analyzed in lysates of cells treated with or without dox (1µg/ml, 16 hrs) by western blotting. Tubuline served as loading control.
C. Clone K15 was stimulated with the indicated concentrations of dox for 16h and IL-31RA protein expression was analyzed by western blotting. Tubuline served as loading control.
D. The phosphorylation of STAT3 was analyzed by western blotting in lysates of clone K15 that were 16h stimulated with dox and 30min with rhIL-31 (100ng/ml).
E. IL-31RA protein expression was evaluated in clone K15 cells treated with or without dox for 16h and visualized using fluorescence microscopy (panels on the left) and indirect confocal immunofluoresence microscopy (panels on the right). Comparable findings were obtained with clone K2.
F and G. Clone K15 cells were pre-treated with dox for 16h and stimulated with rhIL-31 for 1h. *IL31RA* (F) and *IL20*/*IL24* (G) expression was analyzed by RT-qPCR. Displayed are the mean values and standard deviation of three biological replicates using *HPRT* for normalization.

2.2.3 IL-31 disturbs proliferation of HaCaT-pRTS-IL31RA cells in monolayer cultures and differentiation in organotypic skin equivalents

As IL-31 affected the formation of the epidermis in organotypic skin equivalents of NHEKs, now the question was addressed whether this is also true for HaCaT-pRTS-IL31RA cells. In a monolayer culture dox and rhIL-31 co-stimulation significantly reduced the proliferation rate of clone K2 and clone K15 (Fig. 10A), which is consistent with the reduced Ki67 staining observed in the NHEK model (Fig. 6A). In

addition the induction of IL-31RA by dox alone was already sufficient to reduce the proliferation rate of these two clones by 50% compared to the dox/rhIL-31 co-stimulation. Furthermore rhIL-31 treatment alone slightly reduced the proliferation rate of the cells (Fig. 10A). This indicates that HaCaT-pRTS-IL31RA cells probably exhibit some basal IL-31RA expression without dox induction and that the high overexpression of IL-31RA after dox pre-treatment may cause auto-activation of the receptor. Nevertheless in organotypic skin equivalents dox/rhIL-31 stimulation resulted in similar defects in formation of the epidermal structure in both clone K2 and clone K15 compared to primary cells (Fig. 10B). The haematoxylin-eosin staining revealed no (clone K15) or only minor differences (clone K2) in the dox or rhIL-31 alone treated model, compared to the non-stimulated control. In addition the clone K15 model was also stained for phosphorylated STAT3 to visualize IL-31 dependent signaling activation (Fig. 10B; red staining). The dox/rhIL-31 treated model shows increased STAT3 activation compared to the controls demonstrating the functionality of the induced IL-31RA in organotypic skin equivalents.

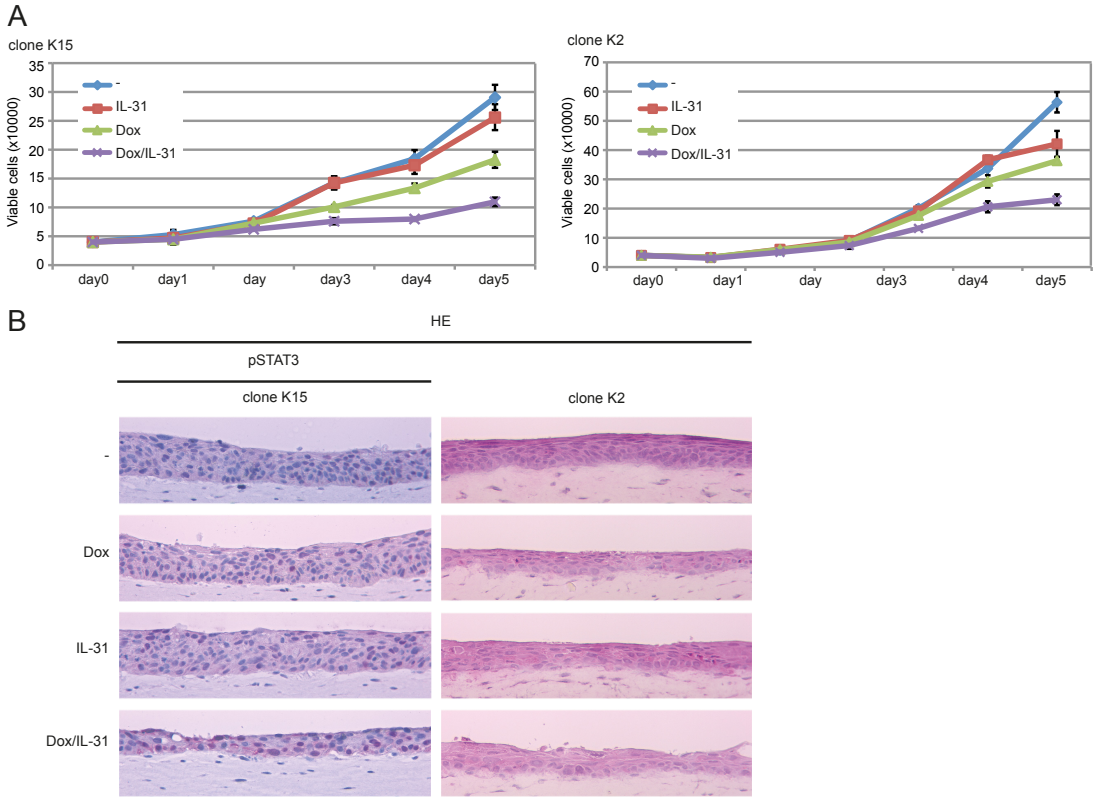


Figure 10. IL-31 disturbs proliferation and differentiation of HaCaT-pRTS-IL-31RA in organotypic skin equivalents.
A. The proliferation rate of HaCaT-pRTS-IL-31RA clone K15 or clone K2 was measured by counting cells incubated in the presence or absence of dox and rhIL-31. The cultures were re-stimulated with dox and rhIL-31 daily. At the indicated time points the cell number of the differently treated cultures was determined. Displayed are mean values with standard deviation of three biological replicates.

B. HaCaT-pRTS-IL-31RA clone K15 or clone K2 and fibroblasts were grown as 3D organotypic skin equivalents and stimulated with or without dox (1µg/ml) and/or rhIL-31 (100ng/ml) every second day as indicated after lifting the cells to the air – liquid interphase. The skin equivalents were harvested and histological sections were haematoxylin-eosin stained (HE) (clone K2) and stained for phosphorylated STAT3 as indicated (clone K15) at day 7.

2.2.4 Individual HaCaT-pRTS-IL31RA clones are not stable

As demonstrated in Fig. 10A, rhIL-31 or dox treatment alone is sufficient to cause a reduced HaCaT-pRTS-IL31RA proliferation rate. To evaluate if also the expression of IL-31 target genes can be induced without induction of *IL31RA*, individual clones were stimulated with either dox or rhIL31 or with a combination of both followed by measuring *IL20* and *IL24* expression using qRT-PCR. All tested HaCaT-pRTS-IL31RA clones induced *IL20* and *IL24* expression after rhIL-31 stimulation without initial induction of the IL-31RA by dox pre-treatment (Fig. 11A). In contrast to the proliferation rate measured in Fig. 10B, dox treatment alone was not enough to induce *IL20* or *IL24* expression. This indicates that HaCaT-pRTS-IL31RA cells exhibit a basal *IL31RA* expression without the presence of dox that is sufficient to induce IL-31 signaling, leading to target gene expression. However pre-treatment of dox with subsequent IL-31 stimulation further increased the induction of *IL20* and *IL24* expression in all clones (Fig. 11A). To evaluate whether HaCaT-pRTS-IL31RA cells in later passages still express the IL-31RA and induce IL-31 signaling to a similar degree, clone K15 cells in passage 24 (after isolation) were pretreated with dox for 16h and stimulated with rhIL-31 for 30min. The expression of the IL-31RA and the induction of STAT3 phosphorylation were visualized by fluorescence microscopy (Fig. 11B).

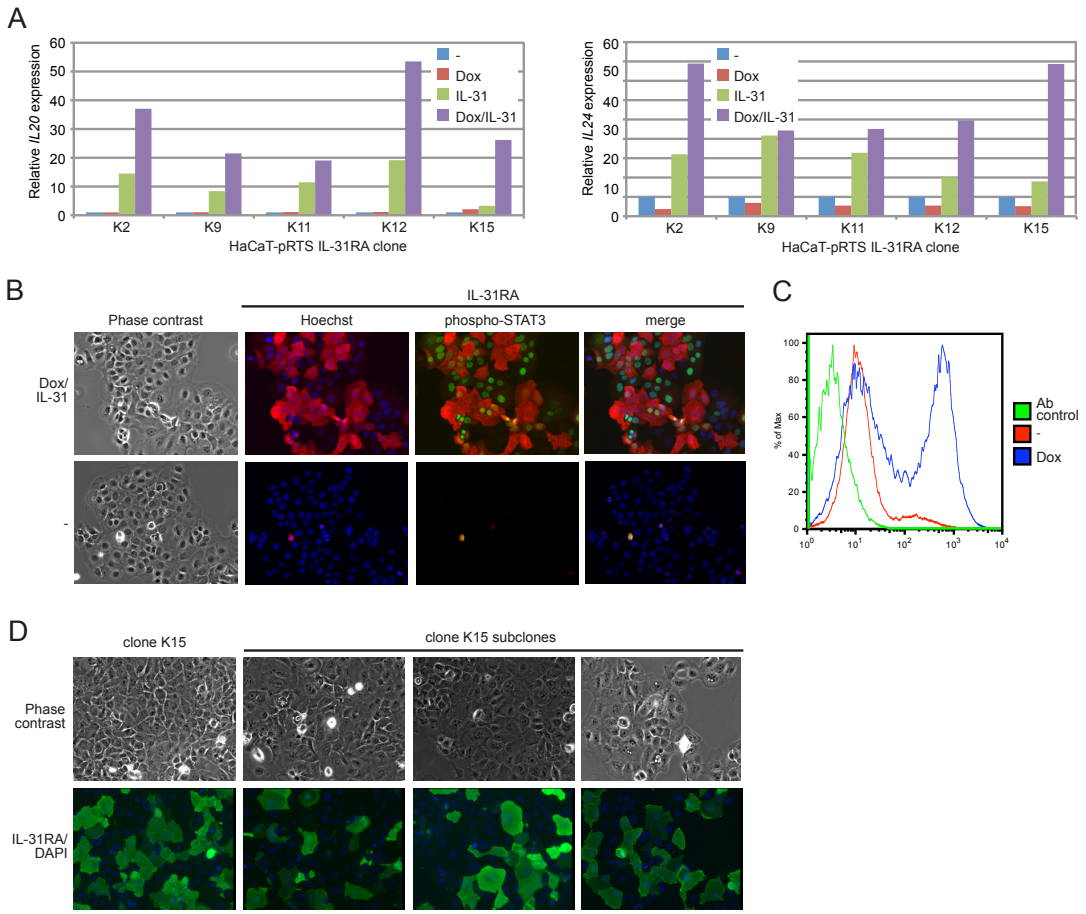


Figure 11. Individual HaCaT-pRTS IL-31RA clones are not stable
A. The expression of *IL20* and *IL24* was analyzed by RT-qPCR in response to dox (1µg/ml for 16h), rhIL-31 (100ng/ml for 1h) or a combination of the two. Displayed are relative expression values compared to non-treated cells *HPRT* expression was used for normalization.
B. IL-31RA protein expression and STAT3 phosphorylation was evaluated in clone K15 cells treated with or without dox (1µg/ml, 16h)/rhIL-31 (100ng/ml), 30min) and visualized using fluorescence microscopy. The DNA was stained with Hoechst 33258.
C. L-31RA protein expression was evaluated in clone K15 cells treated with or without dox for 16h and visualized using FACS.
D. Clone K15 cells were seeded in very low density and individual sub clones were selected and analyzed for IL-31RA protein expression after dox stimulation for 16h visualized using fluorescence microscopy.

The immunofluorescence images showed a very heterogeneous distribution of IL-31RA with no, low or highly expressing cells. In addition the induction of STAT3 phosphorylation did not correlate with the IL-31RA expression (Fig. 11B). Furthermore FACS analysis of the IL-31RA expression revealed that after dox treatment of K15 cells two populations existed; one expressing low to medium the other high amounts of IL-31RA (Fig. 11C). To solve this problem clone K15 cells were seeded at very low density to isolate single cell subclones. Several subclones were tested for IL-31RA expression after dox induction by immunofluorescence

microscopy. All subclones still demonstrated a heterogeneous expression of the IL-31RA (Fig. 11D).

2.3 HaCaT-pSLIK-IL31RA cell lines with inducible IL31RA expression

Because HaCaT-pRTS-IL31RA seem to silence parts of the pRTS vector over time, leading to differences in their dox responsiveness and expression of *IL31RA,* it was decided to clone a new monoclonal dox inducible cell line, using the pSLIK vector system (Shin et al., 2006). This vector is delivered into the cells by lentiviral infection and its dox controllable expression cassette integrates into the target cell genome via its long terminal repeats (LTR). The IL-31RA expression is controlled by a tetracycline responsive element (TRE) that binds the TRE- and dox-binding optimized activator rtTA3. The *rtTA3* gene is expressed from one mRNA together with a neomycine (Neo) selection marker connected by an internal ribosomal entry site (IRES). This mRNA is constitutively expressed under the control of an ubiquitin-C-promoter. Binding of dox to the rtTA3 protein leads to association with the TRE and activation of *IL31RA* expression. The rtTA3-IRES-Neo expression cassette is directly located behind the *IL31RA* thus transactivation by rtTA3 also enhances the expression of the transactivator and the selection marker. In addition the vector has an HIV derived DNA-Flap region supporting the integration into the host genome with its 3 dimensional structure, and the 3´ SIN (self-inactivating) LTR lacks elements necessary for reverse transcription and thus inhibits viral replication. Furthermore a WRE (woodchuck regulatory element) between the neomycin resistance gene and the 3´SIN LTR promotes mRNA export and protein expression. Fig. 12A summarizes the pSLIK-IL31RA cassette that integrates into the target cell genome.

The vector was cloned using pSFI-IL31RA-HA. To keep the HA-tag, the IL-31RA cDNA including the HA-tag was PCR-amplified and sub-cloned into the pSLIK entry vector pEN_TGmirc3 (Shin et al., 2006) into Klenow-fragment blunted NcoI/XbaI restriction sites generating pEN-IL31RA-HA. The integrated PCR fragment was fully sequenced. Sub-cloning into pEN_TGmirc3 adds the TRE in front of the *IL31RA-HA* and embeds the gene within attL sites for recombination. Using the Gateway® LR

Clonase® enzyme mix (Invitrogen) according to the manufactures recommendations, the TRE-IL31RA-HA cassette was integrated into the pSLIKNeo vector via *in vitro* recombination between attL and attR sites creating pSLIK-IL31RA-HA.

Lentiviral pSLIK-IL31RA-HA particles were generated by transient transfection of HEK293T cells together with the appropriate helper plasmids (Materials and Methods). HaCaT cells were infected and selected by adding 800ng/ml Geneticin (G418), whose activity is also inhibited by the Neo selection marker, and seeded in low density to select single cell clones that were called HaCaT-pSLIK-IL31RA.

The resulting clones expressed *IL31RA* mRNA (Fig. 12B) and protein (Fig. 12C) upon addition of dox except clone 7 that was G418 resistant but did not induce *IL31RA* expression, which was not further investigated. IL-31RA was expressed at the cell surface (Fig. 12F) and was functional because stimulation with rhIL-31 resulted in the phosphorylation of STAT3 (Fig. 12G), an immediate downstream target of receptor-bound JAK kinases. The HaCaT-pSLIK-IL31RA clones responded also with induction of *IL20* and *IL24* mRNA expression (Fig 12D). Clones K12 and K14 were selected for the subsequent experiments. In these two clones the induction of the *IL20* and *IL24* genes was observed exclusively when the IL-31RA was induced and the cells were stimulated by rhIL-31. In contrast to HaCaT-pRTS-IL31RA cells, only the combination of the two treatments induced *IL20* and *IL24,* while neither of the two treatments alone was sufficient (Fig. 12E).

2.3.1 IL-31 inhibits proliferation in HaCaT-pSLIK-IL31RA cells

Now the question was addressed how the established HaCaT-pSLIK-IL31RA clones reacted to rhIL-31 stimulation. Treatment of these cells with rhIL-31 resulted in a reduced proliferation, whereas the induction of the IL-31RA or the treatment with IL-31 alone had no effect (Fig. 13A). FACS analyses revealed that within 48h the number of cells in S/G2 phase of the cell cycle was significantly reduced with a parallel increase in G1 phase cells (Fig. 13B). Since no signs of apoptosis were observed by staining the cells for Annexin V (Fig. 13C), by evaluating the cells morphologically, and by measuring sub-G1 cells (data not shown), the proliferation effect is most likely due to reduced cell cycle progression, consistent with the reduced

Ki67 staining observed in the 3D NHEK model (Fig. 6A) and the proliferation rate observed in HaCaT-pRTS-IL31RA cells (Fig 10B).

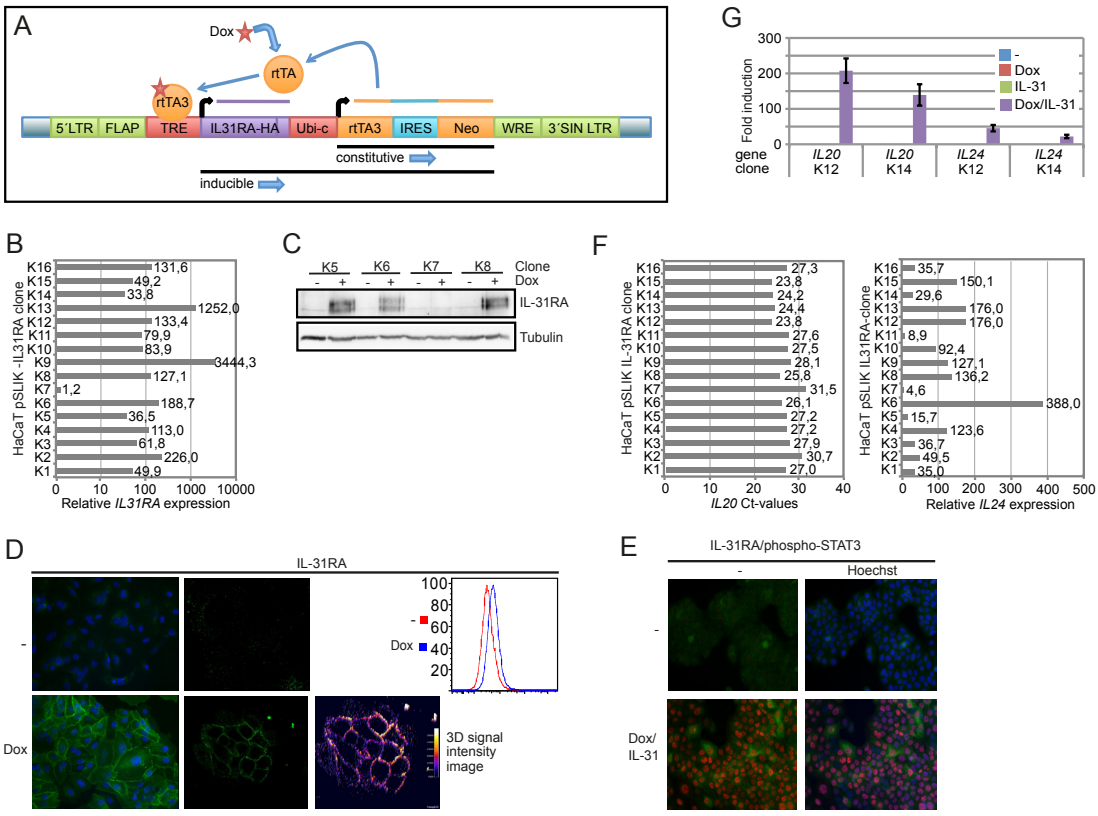


Figure 12. HaCaT-pSLIK-IL-31RA cells induce *IL31RA* expression and respond to IL-31.
A. Scheme of the pSLIK-IL31RA-HA vector that integrates into the genome of the target cell via its long terminal repeats (5´LTR and 3´SIN LTR) after lentiviral infection. The 3´SIN (self inactivating) LTR furthermore inhibits viral replication because it lacks important enhancer elements. The FLAP region promotes the import of the viral genome into the nucleus. The IL-31RA-HA gene is under the control of a tet-responsive element (TRE) that induces target gene expression after binding of dox to the dox and TRE binding optimized tet-transactivator (rtTa3). The genes encoding the tet-transactivator and the neomycine resistance (neo) are located on one mRNA connected by an internal ribosomal entry site (IRES). Their expression is controlled by an ubiquitin-C promoter (Ubi-c). The WRE (woodchuck regulatory element) promotes mRNA export into the cytosol and enhances protein expression. Transactivation by the rtTA also enhances the expression of the rtTa3-IRES-Neo cassette.
B. HaCaT cells were infected with pSLIK-IL31RA-HA and individual clones were selected with G418 (800ng/ml). The clones were stimulated with or without dox (1µg/ml) for 16h, the expression of *IL31RA* mRNA was analyzed by RT-qPCR and normalized to *HPRT* expression.
C. IL-31RA protein expression was analyzed in lysates of cells treated with or without dox (1µg/ml, 16h) by western blotting. Tubulin served as loading control.
D. IL-31RA protein expression was evaluated in clone K14 cells treated with or without dox (1µg/ml) for 16h and visualized using fluorescence microscopy (panels on the left) indirect confocal immunofluoresence microscopy (panels in the middle) and FACS (panel on the right). Comparable findings were obtained with at least 5 clones.
E. Clone K14 cells were treated with or without dox (1µg/ml, 16h), stimulated with rhIL-31 for 45min, fixed and subsequently stained for IL-31RA (Alex-Fluor488, green) and phospho-STAT3 (Alexa-Fluor555, red). The DNA was stained with Hoechst 33258. Comparable findings were obtained with at least 5 clones.
F. The expression of *IL20* and *IL24* was analyzed by RT-qPCR in response to 16h dox/1h rhIL-31 stimulation. The *IL20* expression in individual clones is displayed as Ct-values because the basal expression values for *IL20* in the different clones was either not detectable or very low. The *IL24* expression is shown as relative expression compared to cells without dox and rhIL-31 treatment. *HPRT* expression was used for normalization.

G. The expression of *IL20* and *IL24* was analyzed by RT-qPCR in two individual clones in response to dox (16 hrs), rhIL-31 (1h) or co-treatment. Displayed are the mean values and standard deviation of three biological replicates using *HPRT* for normalization.

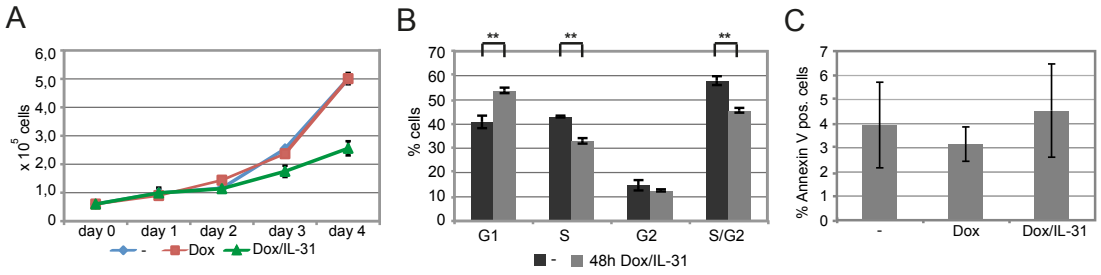


Figure 13. IL-31 inhibits proliferation in HaCaT-pSLIK-IL31RA cells.
A. The proliferation rate of HaCaT-pSLIK-IL31RA clone K14 cells was measured by counting cells incubated in the presence or absence of dox (1µg/ml) and rhIL-31 (100ng/ml). The cultures were re-stimulated with dox and rhIL-31 daily. At the indicated time points (days) the cell number of the differently treated cultures was determined. Displayed are mean values with standard deviation of three biological replicates. Similar findings were obtained with clone K12.
B. Cell cycle distribution of HaCaT-pSLIK-IL31RA clone K14 cells was determined in response to dox/rhIL-31 treatment after 48h. The percentage of cells in G1-, S- and G2-phase was determined by FACS. Displayed are mean values with standard deviation of three biological replicates. Statistical significance was determined by the two-sided Student's t-test (**p<0,01). Comparable results were obtained with clone K12 cells.
C. HaCaT-pSLIK-IL31RA cells were stimulated with or without dox (1µg/ml) and rhIL-31 (100ng/ml) as indicated. The cells were then stained for AnnexinV and analyzed by FACS. Displayed are mean values with standard deviation of three experiments. The differences were not statistically significant.

2.3.2 IL-31 interferes with HaCaT-pSLIK-IL31RA cell differentiation in the organotypic 3D skin model

To evaluate whether IL-31 compromised differentiation of HaCaT cells, these clones were utilized in the organotypic 3D skin model and the consequences of IL-31 signaling were determined. Representative micrographs depict the effect of continuous rhIL-31 treatment (at day 7 and day 14) on 3D reconstructs of HaCaT-pSLIK-IL31RA cell clones (Fig. 14A). Similar to the findings with primary cells, IL31-treament significantly disturbed epidermal differentiation. This is specific for IL-31 signaling, because neither dox nor rhIL-31 alone resulted in changes in the differentiation compared to the control. This disturbance became evident by a thin and disordered epidermal layer and is further supported by immunohistology, which showed abolished expression of epidermal differentiation markers in the upper epidermal layer including filaggrin, involucrin, and K10 as well as an increased distribution of β4-integrin through the whole epidermal layer (Fig. 14B).

The IL-31 effects were equivalent to the ones seen in the organotypic 3D skin model using NHEK cells. Therefore the HaCaT-pSLIK-IL31RA cell clones were used as a model system to further study the effects of IL-31 exposure in a defined cellular genetic background.

2.4 Quantitative and morphological evaluation of the IL-31 effect on differentiation

For a semi-quantitative estimation of IL-31-induced disordered differentiation, an expert dematopathologist (PD Dr. med Jörg Wenzel, Klinik und Poliklinik für Dermatologie und Allergologie, Universitätsklinikum Bonn) was asked to examine blinded the different haematoxylin-eosin-stained slides from three independent experiments. He defined a scoring method for semi-quantitative analysis of the epidermal constitution (Wenzel et al., 2005).

The epidermal thickness of each reconstruct was measured (Fig. 14C) and the specimens were scored according to the epidermal constitution, alignment of the stratum basale, and development of the stratum granulosum on a scale from 0 (completely disturbed) to 3 (normally differentiated, i.e. equivalent to healthy human skin) as described previously (Fig. 14D) (Wenzel et al., 2005).

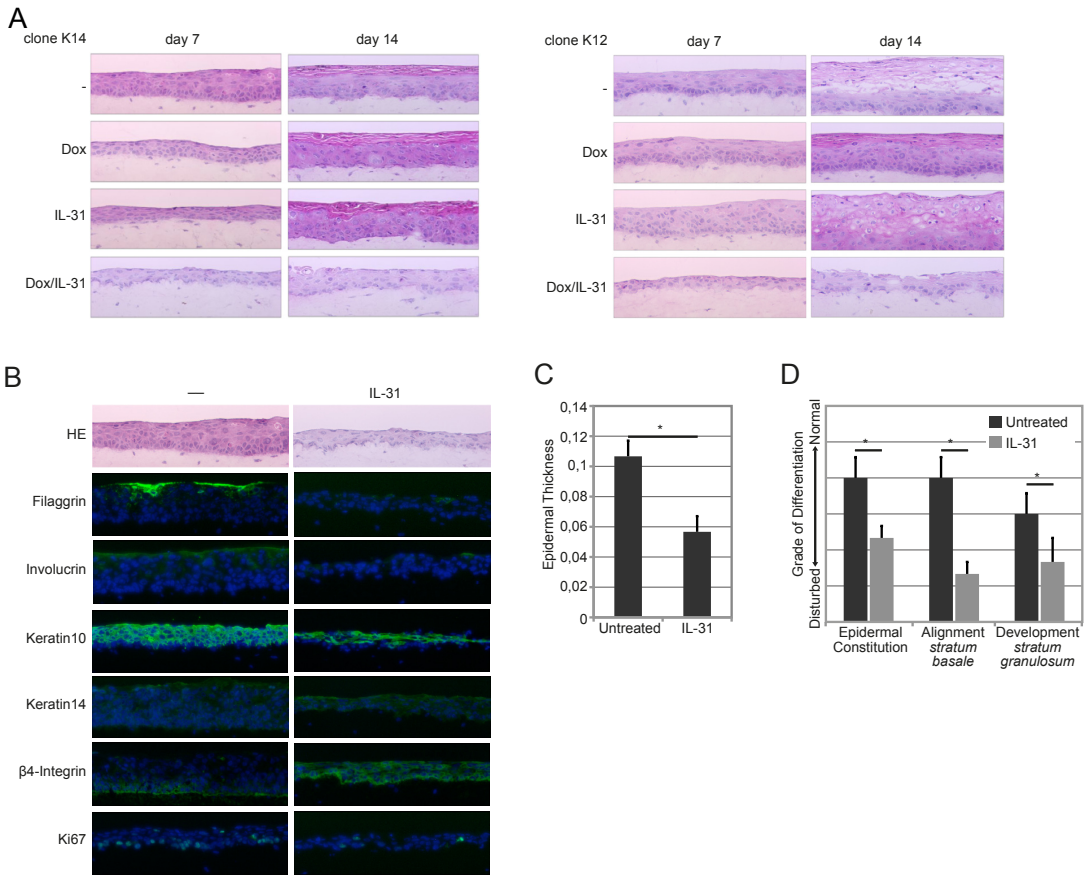


Figure 14. IL-31 interferes with HaCaT-pSLIK-IL31RA cell differentiation.
A. Clone K14 (panel on the left) and clone K12 (panel on the right) cells and primary dermal fibroblasts were grown as organotypic skin equivalents in the presence or absence of dox (1µg/ml) and/or rhIL-31 (100ng/ml). Dox and rhIL-31 were added every second day. The skin equivalents were harvested after 7 and 14 days and histological sections were stained with haematoxylin-eosin.
B. Clone K14 cells and primary dermal fibroblasts were grown as organotypic skin equivalents in the presence or absence of dox and rhIL-31 (100ng/ml). Dox and rhIL-31 were added every second day. The skin equivalents were harvested after 7 days and analyzed histologically (HE, haematoxylin-eosin) or stained for the indicated marker proteins. These results are from the same experiment as shown in panel A. C. and D. Haematoxylin-eosin-stained histological sections were examined blinded. The epidermal thickness of each epidermal reconstruct was measured (C) and the specimens were scored semi-quantitatively on a scale from 0 (completely disturbed) to 3 ("normally differentiated" = similar to healthy human skin) regarding the epidermal constitution, the alignment of the stratum basale, and the development of the stratum granulosum (D) as described in a previous study (Wenzel et al., 2005). Displayed are mean values of three biological replicates. Statistical significance was determined by the Mann-Withney U test (*p<0,05).

In addition most striking was the reduction of both proliferation (Ki67) and the expression of late differentiation markers (filaggrin, involucrin, and keratin 10), which are strong indications for inappropriate differentiation. Most prominent was the effect on filaggrin expression, as seen also in the primary cell model (Fig. 6A). The *FLG, INV* and *KRT10* expression was strongly reduced when the 3D model was analyzed upon permanent rhIL-31 treatment at day 7 (Fig. 15B). The expression of β4-integrin was also disorganized and no longer confined to the basal layer although

changes on mRNA levels were not observed (Fig. 15B). rhIL-31 pulse experiments were also performed and it was evident that 2h were not sufficient to observe a phenotype in the 3D model (data not shown) but 8h and 24h treatment resulted in a partial phenotype after developing the model for 7 days (Fig. 15A), suggesting that an initial, relatively short IL-31 stimulus is sufficient to deregulate late differentiation processes. *FLG* was a particularly sensitive marker because a reduction of expression already after an IL-31 pulse of 2 hrs could be observed, while *KRT10* expression was less sensitive and *INV* expression even showed a slight increase rather than a decrease after IL-31 pulse stimulation (Fig. 15B). Furthermore *ITGB4* expression was also not altered after IL-31 pulse stimulation. This supports the hypothesis that IL-31 induces a series of molecular events that culminate in defective differentiation.

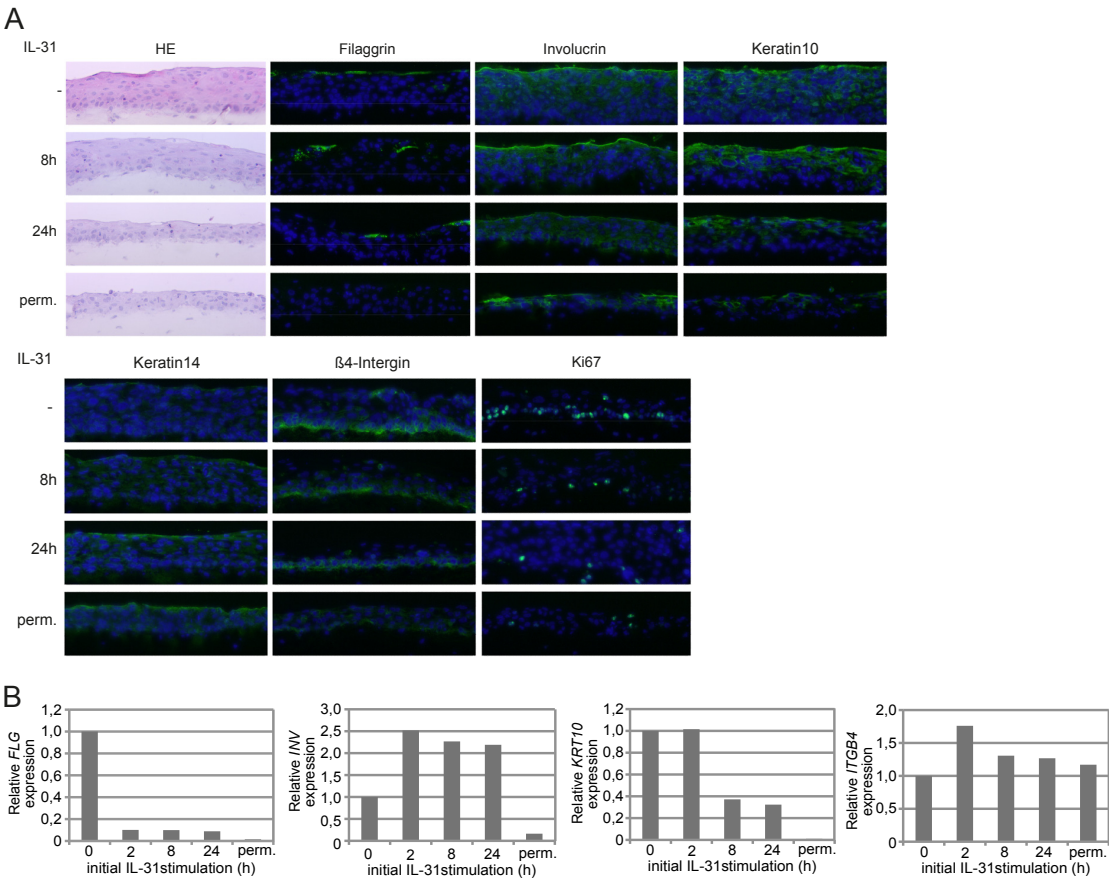


Figure 15. IL-31 interferes with HaCaT-pSLIK-IL31RA cell differentiation.
A. Clone K14 and clone K12 (data not shown) cells and primary dermal fibroblasts were grown as organotypic skin equivalents in the presence or absence of dox. rhIL-31 (100ng/ml) was added for the indicated times and the skin equivalents were harvested after 7 days and processed as described in Fig. 14
B. Parts of the organotypic skin equivalents displayed in panel A were used for total mRNA isolation and the expression of *FLG*, *KRT10*, *INV* and *ITGB4* was determined by RT-qPCR. Displayed are relative expression levels compared to the non-stimulated sample (normalized to *HPRT* expression).

2.5 Evaluation of signaling pathways downstream of IL-31

2.5.1 IL-20 and IL-24 mediate part of the IL-31 effect on keratinocyte differentiation

Candidates for mediating the above discussed IL-31 effects are IL-20 and IL-24, which are induced rapidly in response to IL-31 (Fig. 16A). In addition an enhanced expression of both genes was also measured upon prolonged stimulation of HaCaT-pSLIK-IL31RA cells with IL-31 (Fig. 16B). IL-31 pulse stimulation was sufficient to cause a slight increase in *IL20* and *IL24* expression also in 7 day old organotypic skin equivalents and that is significantly further increased upon permanent IL-31 stimulation (Fig. 16C). The specificity of induction of these two genes was further elucidated by using kinase inhibitors. The inhibition of JAK kinases completely blocked the expression of *IL20* and *IL24*, consistent with a key role of these kinases in IL-31RA/OSMR-dependent signal transduction (Fig. 16D). In addition the p38 and ERK kinases but neither JNK nor PI3K kinases were critical to induce the two cytokine genes (Fig. 16D). Thus HaCaT cells stably expressing an inducible IL-31RA receptor subunit and NHEK cells respond comparably to IL-31 signaling when *IL20* and *IL24* expression is measured requiring the activation of distinct signaling pathways. To evaluate if the receptor heterodimers of these two cytokines, i.e. IL-20Rα/IL-20Rβ and IL-22Rα/IL-20Rβ that are both recognized by IL-20 and IL-24 (Parrish-Novak et al., 2002), are expressed, their surface expression was determined using specific antibodies and FACS. All three receptor subunits were found at the cell surface of HaCaT-pSLIK-IL31RA cells (Fig. 16E). Therefore I tested whether rhIL-20 and rhIL-24 affected keratinocyte differentiation. While rhIL-20 and rhIL-24 alone gave variable and small effects, the combination of the two cytokines resulted in reduced differentiation (Fig. 16F). This was associated with reduced *FLG* and, to a lesser extent, *INV* expression while *KRT10* and *ITGB4* were not affected in the model displayed in Fig. 16F (Fig. 16G). By analyzing the expression of *FLG* and *KRT10* in two different *IL20/IL24* stimulated models from clone K12 and clone K14 the reduction in expression of these two differentiation markers was rather small (Fig. 16H). Moreover reduced *FLG* and *KRT10* were seen early during differentiation of 3D equivalents of primary NHEK (Fig. 16H), while at later time points the expression of the two genes recovered (data not shown). The treatment with rhIL-20 and rhIL-24

was clearly less efficient than rhIL-31, suggesting that other genes regulated by IL-31 are involved additionally in controlling keratinocyte proliferation and differentiation.

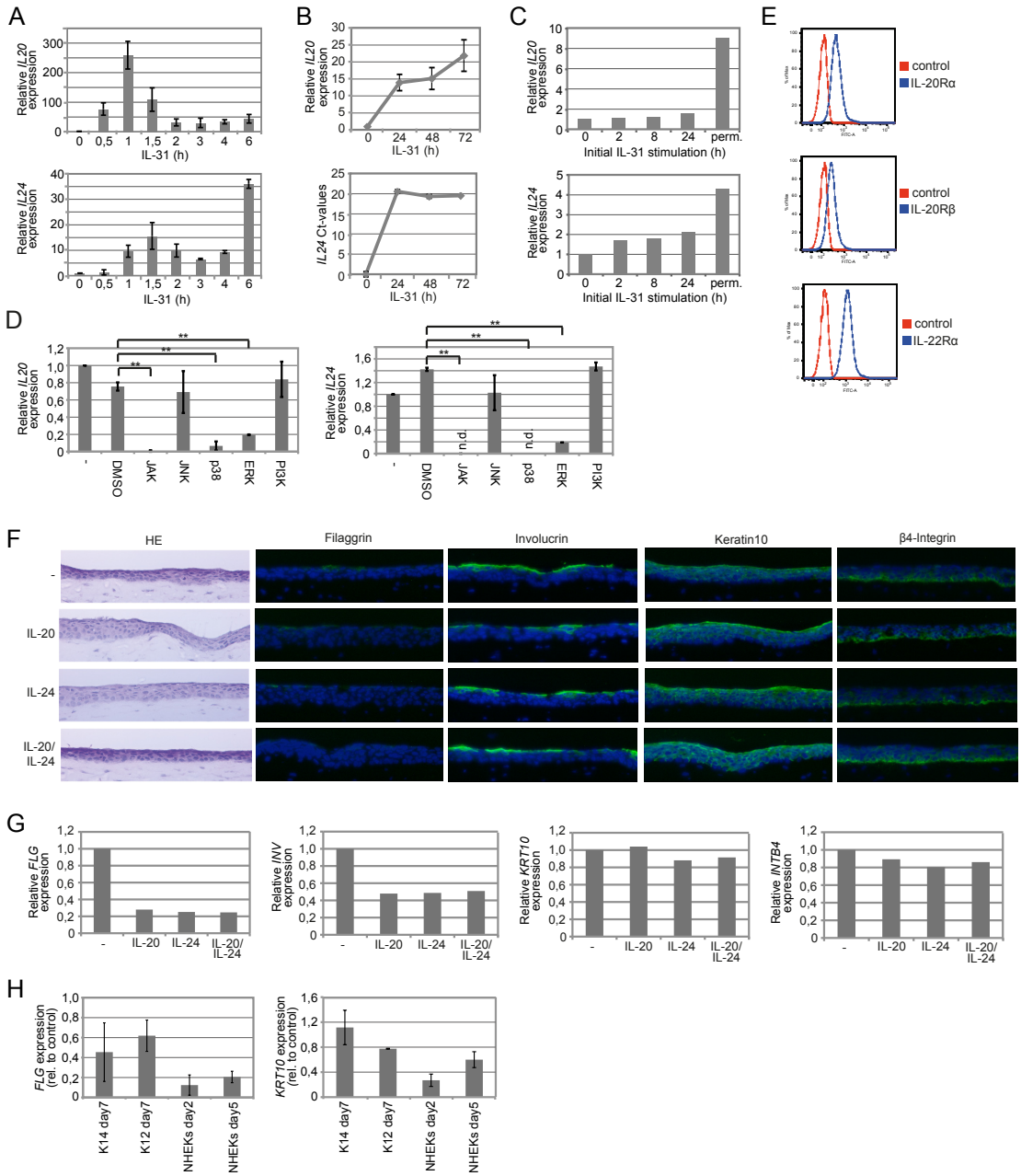


Figure 16. IL-20 and IL-24 impair differentiation of HaCaT-pSLIK-IL31RA cells in organotypic 3D co-cultures

A. and B. HaCaT-pSLIK-IL31RA clone K14 cells were 16h pre-treated with dox (1µg/ml) and rhIL-31 (100ng/ml) stimulated for the indicated time periods and the expression of *IL20* and *IL24* was analyzed by RT-qPCR. Displayed are relative expression levels comparing non-stimulated to stimulated cells, normalized to *HPRT* expression (*IL20*). For *IL24* Ct-values are given, because no signals were detectable from unstimulated controls (mean values and standard deviations of three biological replicates).

C. Parts of the organotypic skin equivalents displayed in Fig. 15A were used for total mRNA isolation and the expression of *IL20* and *IL24* was determined by RT-qPCR. Displayed are relative expression levels compared to the non-stimulated sample (normalized to *HPRT* expression).
D. The expression of *IL20* and *IL24* was analyzed by RT-qPCR in clone K14 cells in response to dox (16h), rhIL-31 (1h) or co-treatment. In addition the indicated kinase inhibitors (JAK inh. I, 100nM; JNK inh. II, 20μM; SB202190, selective for p38 MAP kinases (20μM); U0126, selective for ERK kinases (20μM); Wortmannin, selective for PI3-Kinases (500nM)) were added 1h prior to stimulation with rhIL-31. Mean values and standard deviations of 3 independent experiments are displayed. Statistical significance was evaluated using the two-sided Student's t-test (**p< 0,01; n.d.: not detectable).
E. HaCaT-pSLIK-IL31RA clone K14 cells were fixed with 3,8% paraformaldehyde and incubated with antibodies specific for IL-20Rα, IL-20Rβ and IL-22Rα and a secondary antibodies coupled to Alexa-Fluor 488. Cells incubated with the secondary antibodies alone served as a control. The cell surface expression was analyzed by flow cytometry. Displayed is one representative experiments of three.
F. HaCaT-pSLIK-IL31RA clone K14 cells and fibroblasts were grown in an organotypic skin equivalent in the presence or absence of rhIL-20 (20ng/ml) and rhIL-24 (20ng/ml). The equivalents were harvested at day 7 and stained for differentiation markers as indicated. One representative experiment of three is shown.
G and H. Total RNA was isolated from the organotypic skin equivalents displayed in panel F (G. and H.) and from comparable samples of clone K12 and of 3D equivalents of primary NHEKs that were grown in the presence or absence of rhIL-20 (20ng/ml) and rhIL-24 (20ng/ml) (H). The expression of *FLG, INV, ITGB4* and *KRT10* was analyzed by RT-qPCR at the time points indicated. Displayed are relative expression levels compared to the non-stimulated control (normalized to *HPRT* expression). Displayed are the findings from two experiments with triplicate measurements.

2.5.2 Additional targets of IL-31

As demonstrated in Fig. 6C, beside *IL20* and *IL24* several other factors that are involved in stress response, cell cycle progression and p38/JNK signaling pathways are induced upon IL-31 stimulation in NHEKs. *IRF1, DUSP1, JUNB, EGR1* and *GADD45β* were further verified as IL-31 target genes. All genes were rapidly induced after IL-31 stimulation in HaCaT-pSLIK-IL31RA clone K12 and K14 cells (Fig. 17A and 18A). Both clones showed similar expression patterns with slight differences in the expression peaks and later during the time course in silencing of gene expression. The specificity of induction was further elucidated by using kinase inhibitors. The inhibition of JAK kinases completely blocked the expression of all investigated genes, which is consistent with the observations made for *IL20* and *IL24* expression (Fig. 17C and 18C). In addition MAPKinase pathway specific inhibitors show differences in abrogating the expression of certain IL-31 target genes. The induction of *IRF1* expression, a negative regulator of cell proliferation and a classical target of STAT1 signaling (Romeo et al., 2002), was significantly repressed only by the inhibitor of JAK kinases indicating that the most critical *IRF1* activating pathway was indeed STAT signaling in HaCaT-pSLIK-IL31RA cells. The same is true for *GADD45β*, a factor involved in stress response especially in myeloid cells (Hoffman and Liebermann, 2009). *DUSP1*, a p38-specific phosphatase that gets activated by p38

signaling (Owens and Keyse, 2007), was significantly repressed by p38 and ERK pathway specific inhibitors. EGR1, a transcription factor that is involved in the regulation of several cellular processes including the suppression of transformation, cell growth and apoptosis (Baron et al., 2006; Khachigian and Collins, 1998; Liu et al., 1998), was specifically repressed by the ERK specific inhibitor and at least in clone K12 cells also by the p38 specific inhibitor (Fig. 17C). Finally *JUNB* was repressed in both clones by JAK inhibition and at least clone 12 showed a significant decrease in JUNB expression after inhibition of the ERK and p38 pathways (Fig. 17C and 13C). Taking these results together JAK/STAT, p38 and ERK signaling play a critical role in early activation of the investigated IL-31 target genes while JNK and PI3K signaling do not seem to be relevant.

2.5.3 JUNB target gene expression is affected by IL-31 signaling

Prolonged stimulation of IL-31 revealed that especially *JUNB* expression is constantly increased during IL-31 stimulation in both clonesat the mRNA (Fig. 17B and 18B) and the protein level (Fig. 19C and 20B). JUNB belongs to the AP-1 family of transcription factors that forms heterodimers with other JUN or FOS proteins (Shaulian and Karin, 2001, 2002). Another transcription factor of the AP-1 family is JUN whose function is antagonized by JUNB (Shaulian and Karin, 2001). In a large variety of cells JUNB and JUN are involved in the control of cell cycle progression by directly or indirectly altering the expression of cell cycle regulators (summarized in Fig. 19A right side of the panel). JUNB directly induces p16 expression, an inhibitor of cyclin D1/CDK complexes, and inhibits cyclin D1 expression, an essential cyclin for G1-S transition (Bakiri et al., 2000; Shaulian and Karin, 2001). In addition it inhibits JUN function probably by forming heterodimers with JUN or by competing for JUN binding sites or heterodimerization partners for JUN like FOS (Shaulian and Karin, 2002). In contrast to JUNB, JUN inhibits p16 expression and induces cyclin D1 expression and thus promotes cell cycle progression (Shaulian and Karin, 2001). In addition JUN is directly involved in the expressional control of p21, a cyclin dependent kinase inhibitor important to repress cell cycle progression (Dotto, 2000), and p53, a transcription factor that promotes cell cycle exit e.g. by activating p21 expression and furthermore can induce apoptosis (Vogelstein et al., 2000; Vousden, 2000). By antagonizing JUN function JUNB influences probably also the expression of p21 and p53. In differentiating keratinocytes JUNB and JUN act as regulators of

differentiation (summarized in Fig 19A left side of the panel). JUNB promotes early differentiation and inhibits terminal differentiation while JUN seems to promote terminal differentiation processes (Angel et al., 2001) but the mechanisms underlying these effects are not fully understood. p21 is a JUN target gene that is probably involved in controlling early differentiation but needs to be repressed to enable the cells to enter terminal differentiation processes (Di Cunto et al., 1998). Cyclin D1 needs to be repressed in differentiation for the cells to be able to exit the cell cycle in early differentiation and p53 (among other proteins involved in apoptosis pathways like Bak, MDM2, and Bcl-2) is important for controlling apoptotic pathways that are involved in terminal differentiation as described above.

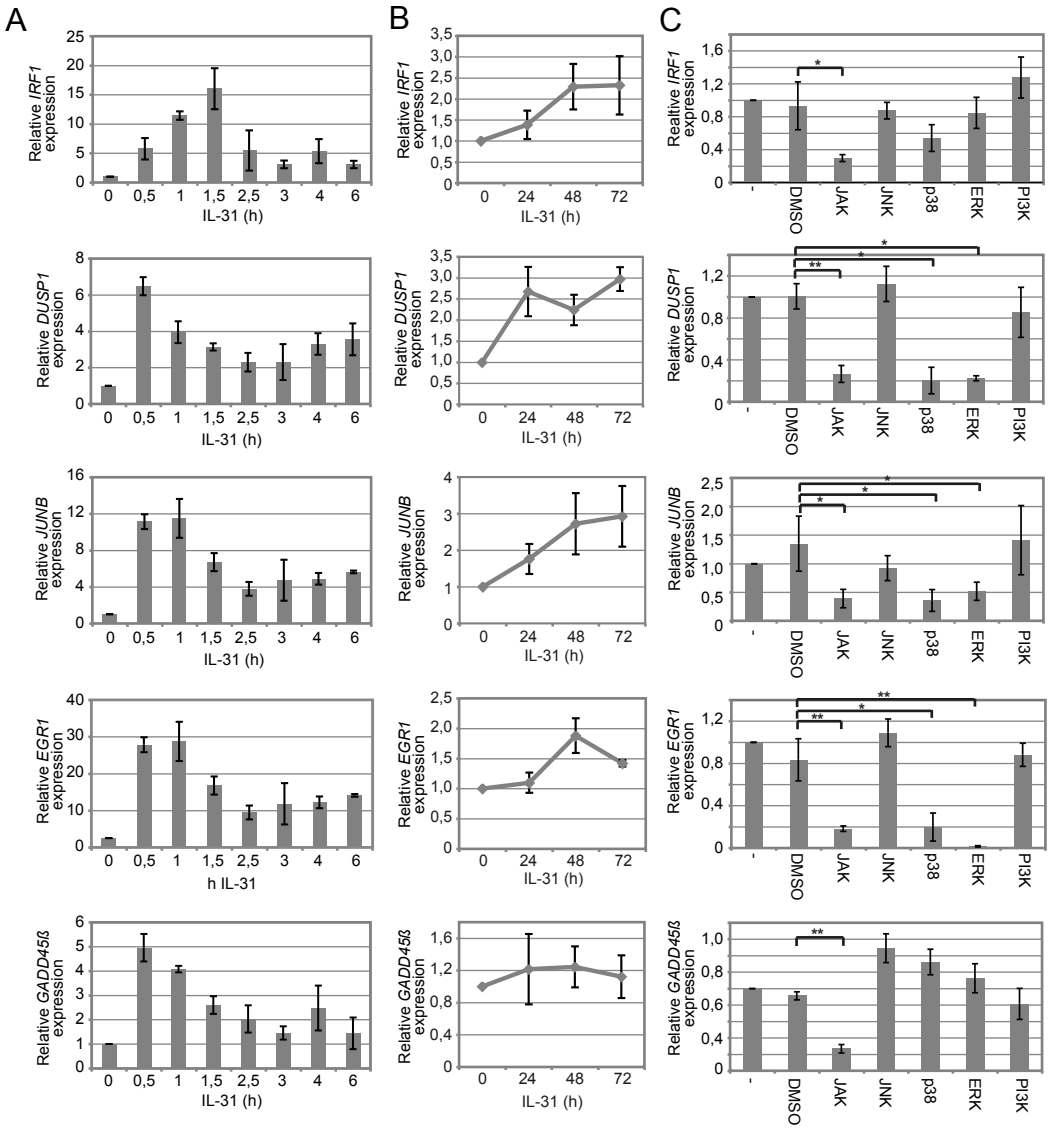


Figure 17. *IRF1, DUSP1, JUNB, EGR1* and *GADD45β* are targets of IL-31 in HaCaT-pSLIK-IL31RA clone K12 cells

A. and B. HaCaT pSLIK-IL31RA clone K12 cells were pre-treated with dox (1µg/ml) for 16h and stimulated with rhIL-31 (100ng/ml) for the indicated time periods. The expression of *IRF1*, *DUSP1, JUNB, EGR1* and *GADD45β* was analyzed by RT-qPCR. Displayed are relative expression levels comparing non-stimulated to stimulated cells, normalized to *HPRT* expression (mean values and standard deviations of three biological replicates).
C. The expression of *IRF1*, *DUSP1*, *JUNB*, *EGR1* and *GADD45β* was analyzed by RT-qPCR in clone K12 cells in response to dox (16h), rhIL-31 (1h) or both together. In addition the indicated kinase inhibitors (JAK inh. I; 100nM; JNK inh. II, 20µM; SB202190, selective for p38 MAP kinases (20µM); U0126, selective for ERK kinases (20µM); Wortmannin, selective for PI3-Kinases (500nM)) were added 1h prior to stimulation with rhIL-31. Mean values and standard deviations of 3 independent experiments are displayed. Statistical significance was evaluated using the two-sided Student's t-test. (**p< 0,01; n.d.: not detectable)

We analyzed the expression of the JUN/JUNB target genes *CDKN1A* (p21), *CDKN2A* (p16), *CCND1* (cyclin D1) and *TP53* (p53) in HaCaT-pSLIK-IL31RA clone K12 and K14 cells stimulated with rhIL-31 for up to 72h. The expression of *CDKN1A* was increasing constantly on both the mRNA (Fig. 19B and 20A) and protein level (Fig. 19C and 20B). *TP53* and *CCND1* expression was repressed by IL-31 at the mRNA level (Fig. 19B and 20A), which was also confirmed at the protein level for p53 (Fig. 19C and 20B). *CDKN2A* expression was reduced by IL-31 signaling (Fig. 19B and 20A), an observation that could not be confirmed when the protein was analyzed (Fig. 19C and 20B). In addition the mRNA obtained from organotypic skin equivalents of NHEKs, displayed in Fig. 6A and 6B, was analyzed for the expression of JUN/JUNB target genes. While 2, 5, 8 and 14 day old models showed no significant differences in *CDKN2A* and *TP53* expression in the presence or absence of rhIL-31, *JUNB* and *CDKN1A* expression was significantly stimulated at day 2 (Fig. 19D). At later time points JUNB expression constantly increased but did not show differences between the IL-31 stimulated and non-stimulated control. In contrast the *CDKN1A* expression levels stayed significantly higher at day 8 and day 14 of the model compared to the non-stimulated control. Taking together these results suggest that IL-31 induces *JUNB* expression, which subsequently alters the expression of p21, p53, cyclin D1 and p16 in HaCaT-pSLIK-IL31RA cells. These proteins, probably together with additional factors, appear to promote an early-differentiated state of the cells and moreover seem to inhibit terminal differentiation. In organotypic models of NHEKs the early induction of *JUNB* expression might cause a constant increase of p21 expression during the whole differentiation process. It is possible that this deregulated p21 expression contributes to the IL-31 phenotype, consistent with the findings that pulse stimulation with IL-31 is sufficient to cause a differentiation phenotype (Fig. 15).

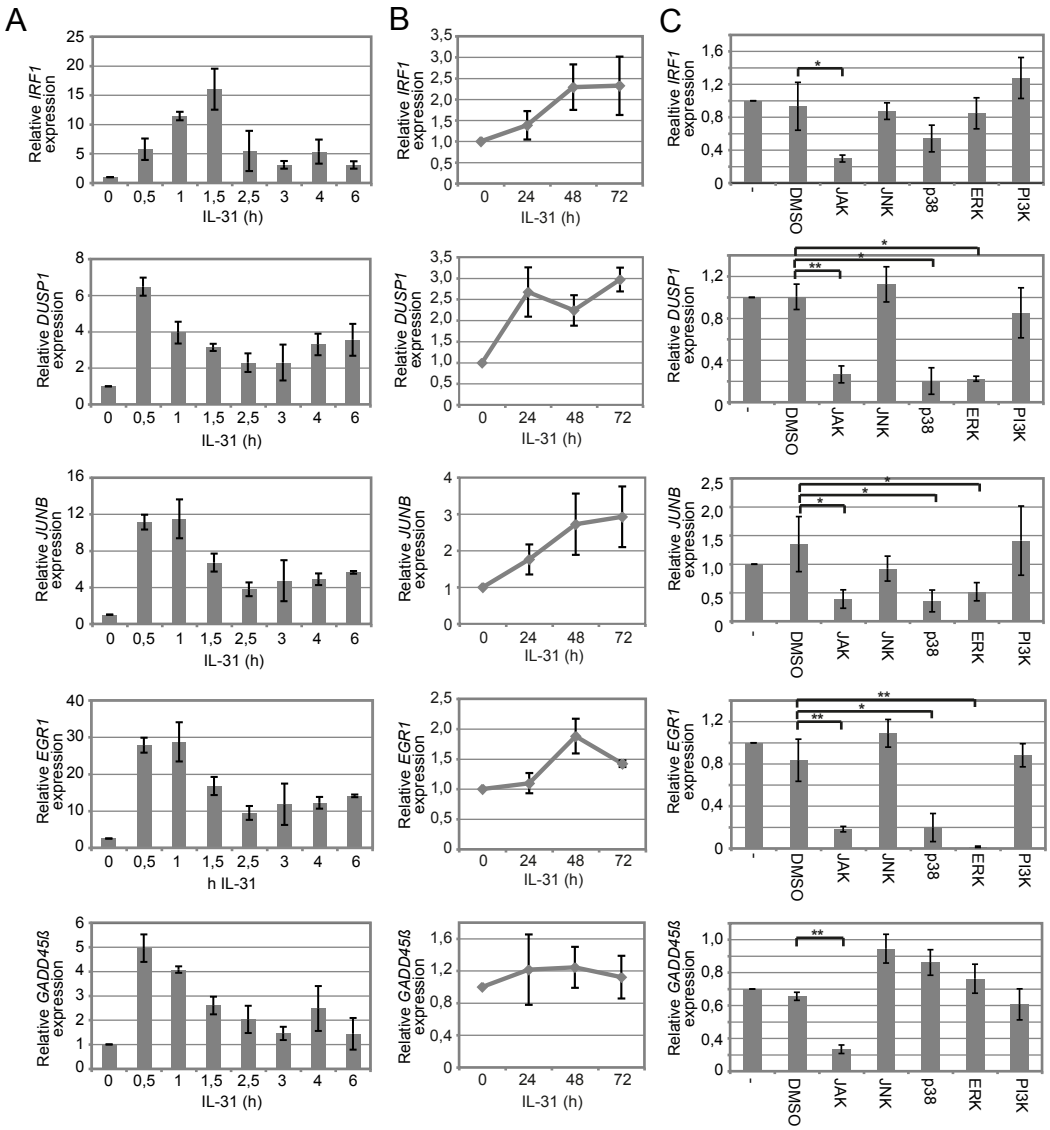


Figure 18. *IRF1, DUSP1, JUNB, EGR1* and *GADD45β* are targets of IL-31 in HaCaT-pSLIK-IL31RA clone K14 cells

A. and B. HaCaT pSLIK-IL31RA clone K14 cells were pre-treated with dox (1µg/ml) for 16h and stimulated with rhIL-31 (100ng/ml) for the indicated time periods. The expression of *IRF1*, *DUSP1, JUNB, EGR1* and *GADD45β* was analyzed by RT-qPCR. Displayed are relative expression levels comparing non-stimulated to stimulated cells, normalized to *HPRT* expression (mean values and standard deviations of three biological replicates).

C. The expression of *IRF1*, *DUSP1*, *JUNB*, *EGR1* and *GADD45β* was analyzed by RT-qPCR in clone K14 cells in response to dox (16h), rhIL-31 (1h) or both together. In addition the indicated kinase inhibitors (JAK inh. I; 100nM; JNK inh. II, 20µM; SB202190, selective for p38 MAP kinases (20µM); U0126, selective for ERK kinases (20µM); Wortmannin, selective for PI3-Kinases (500nM)) were added 1h prior to stimulation with rhIL-31. Mean values and standard deviations of 3 independent experiments are displayed. Statistical significance was evaluated using the two-sided Student's t-test. (**p< 0,01; n.d.: not detectable)

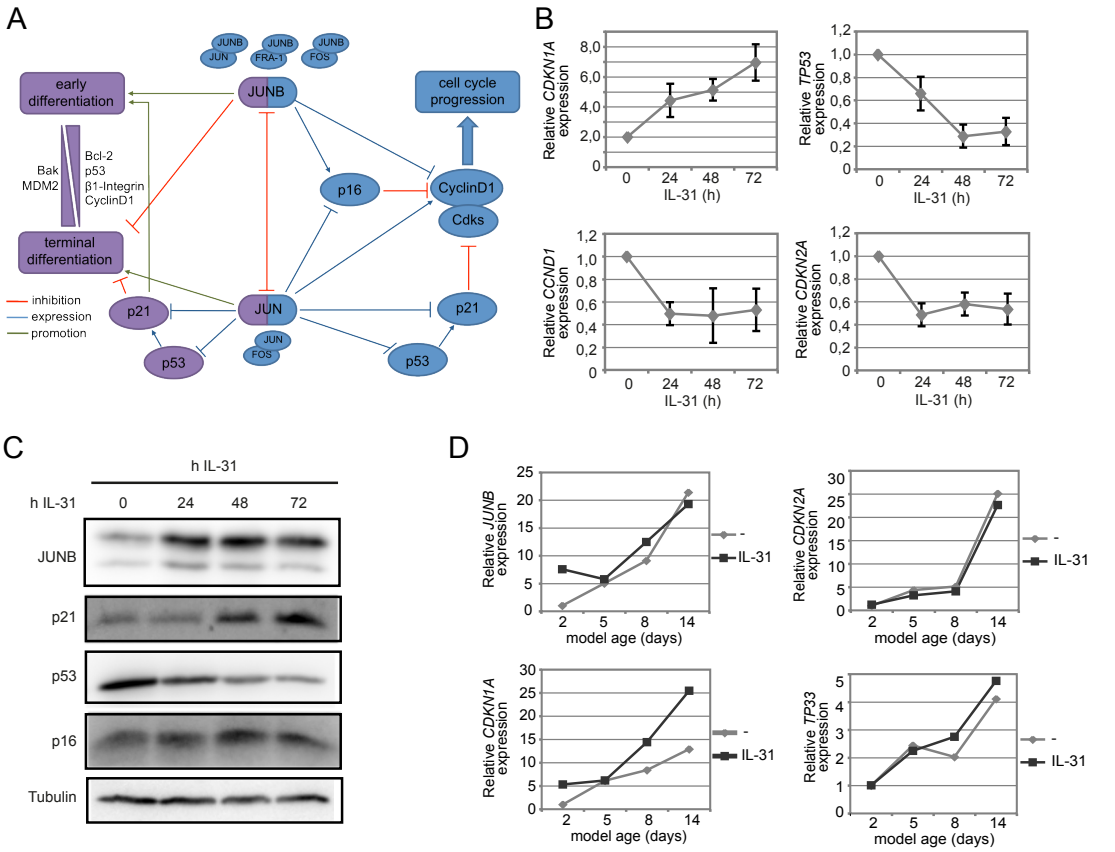


Figure 19. JUNB target gene expression is affected by IL-31 signaling in HaCaT pSLIK-IL31RA clone K14 cells

A. Scheme of the JUN-JUNB network controlling cell cycle progression (blue site) and skin differentiation (purple site). JUN and JUNB antagonize each other in their function in cell cycle progression leading to alterations in p16, CyclinD1, p21 and p53 expression. JUN and JUNB belong to the AP-1 family of transcription factors that form heterodimers and bind DNA. JUN can form dimers with FOS, which then promote cell cycle progression. JUNB forms dimers with FOS, FRA-1 and also with JUN, which then antagonizes the pro-proliferative functions of JUN. In skin differentiation both proteins have additional functions that are not well characterized. While JUNB promotes early differentiation and inhibits late differentiation, JUN is described as a promoter of late differentiation by direct activation of differentiation related genes or indirectly by controlling p21 and p53 expression. In addition apoptosis related factors like MDM2, Bak and Bcl-2 or matrix proteins like β1-integrin are important mediators in the transition from early to late differentiation, that might be controlled by JUN and JUNB.

B. HaCaT-pSLIK-IL31RA clone K14 cells were pre-treated with dox (1µg/ml) for 16h and stimulated with rhIL-31 (100ng/ml) for the indicated time periods. The expression of *CDKN1A*, *CDKN2A*, *TP53* and *CCND1* was analyzed by RT-qPCR. Displayed are relative expression levels comparing non-stimulated to stimulated cells, normalized to *HPRT* expression (mean values and standard deviations of three biological replicates).

C. JUNB, p21, p53 and p16 protein expression was analyzed in lysates of cells treated with dox (1µg/ml) and rhIL-31 (100ng/ml) for 0, 24, 48 or 72h by western blotting. Tubulin served as loading control.

D. Total mRNA obtained from organotypic skin equivalents described in Fig. 6B was analyzed for *JUNB, CDKN1A, CDKN2A* and *TP53* expression by RT-qPCR. Displayed are relative expression levels compared to the non-stimulated day2 sample normalized to *HPRT* expression.

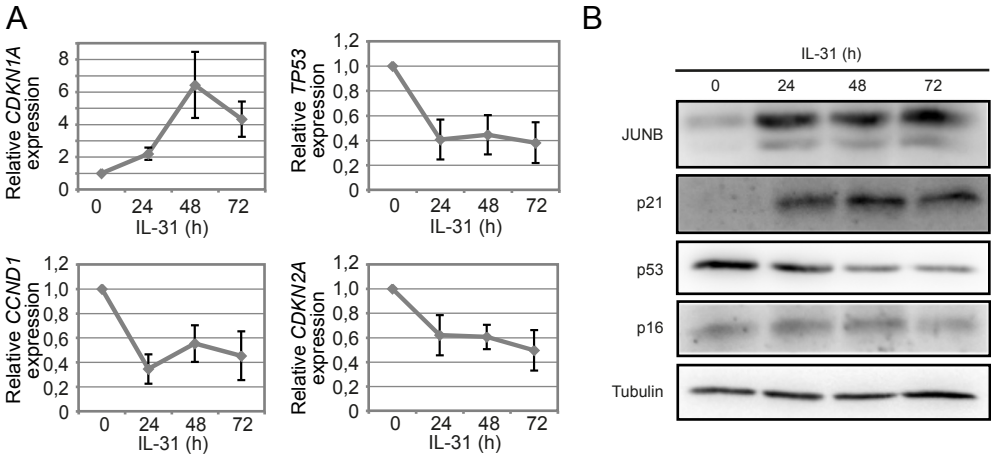


Figure 20. JUNB target gene expression is affected by IL-31 signaling in HaCaT-pSLIK-IL31RA clone K12 cells

A. HaCaT-pSLIK-IL31RA clone K12 cells were pre-treated with dox (1µg/ml) for 16h and stimulated with IL-31 (100ng/ml) for the indicated time periods. The expression of *CDKN1A*, *CDKN2A, TP53* and *CCND1* was analyzed by RT-qPCR. Displayed are relative expression levels comparing non-stimulated to stimulated cells, normalized to *HPRT* expression (mean values and standard deviations of three biological replicates).

B. JUNB, p21, p53 and p16 protein expression was analyzed in lysates of clone K12 cells treated with dox (1µg/ml) and rhIL-31 (100ng/ml) for 0, 24, 48 or 72h by western blotting. Tubulin served as loading control.

3 Discussion

3.1 Involvement of IL-31 in the inflammatory processes of atopic dermatitis

AD is characterized by the deregulation of a number of cytokines that are postulated to participate in the disease progression (Carmi-Levy et al., 2011) One of the T_H2 effector cytokines implicated in AD is IL-31, in addition to a number of other cytokines including IL-4 and IL-13. IL-31 is also produced by monocytes, dendritic cells and mast cells (Ishii et al., 2009; Niyonsaba et al., 2010). UV and reactive oxygen species strongly enhance the expression of IL-31 in at least some of these cells that have the capacity to infiltrate human skin or are resident skin cells (Cornelissen et al., 2011). From these observations an important role of IL-31 in AD pathology has been postulated. While several cell types in the skin are capable to synthesize IL-31, as described in the introduction, the target cells and the physiological consequences on these cells are less well described. Keratinocytes express the IL-31RA and OSMR receptor subunits, the expression of the former can be further stimulated with pro-inflammatory cytokines including IFNγ (Fig. 7A, B and C) (Heise et al., 2009) suggesting that keratinocytes are responsive to IL-31. Another relevant cell type found in lesions of AD patients are eosinophils, which express the IL-31 receptor (Cheung et al., 2010). IL-31 stimulates the secretion of a number of different pro-inflammatory cytokines and chemokines by eosinophils. Together these findings suggest that IL-31 has broad pro-inflammatory effects in the skin and thus IL-31 is a valid candidate to contribute to the development of AD pathology.

Human mast cells play an important role in allergic disorders and are an additional source of IL-31 (Abraham and St John, 2010; Yamaoka et al., 2009). Mast cells are localized in tissues that are in contact with the environment such as the skin, where mast cells play an important role in pathogen surveillance and innate immunity. Recent findings suggest that anti-IgE treatment and stimulation with antimicrobial peptides such as human β-defensins (hBD) and cathelicidin LL-37 induce IL-31 expression and secretion in mast cells (Niyonsaba et al., 2010). These peptides,

important components of innate immunity, are secreted from monocytes and keratinocytes upon stimulation by bacteria and pro-inflammatory cytokines (Abraham and St John, 2010). Moreover $CD4^+$ T-cells, especially T_H2 cells, express the histamine H_4 receptor, which is upregulated upon IL-4 treatment. Stimulation with a histamine agonist results in the activation of *IL31* mRNA expression in these T cells (Gutzmer et al., 2009). Together these findings further support a role of IL-31 in AD. A possible scenario is that the reduced skin barrier formation observed in AD patients allows the activation of monocytes and mast cells during infection and by allergens stimulating IL-31 production. Increasing amounts of this cytokine will then further impair proper differentiation of keratinocytes, thereby worsening the skin lesions. In addition the findings summarized above provide a direct link of histamine secretion and IL-31 function in allergic reactions.

Another important aspect of AD is the development of pruritus. Enhanced expression of IL-31RA was discovered in cells of the human and murine dorsal root ganglia and in murine primary afferent fibers of the spinal cord and dermis that are proposed to be involved in the sensation of itch (Bando et al., 2006; Sonkoly et al., 2006). These cells, comparable to most cells, express the OSMR. A putative functional role of IL-31 in induction of pruritus in patients with AD was suggested. These findings are supported by studies in an atopic dermatitis-like murine model (NC/Nga mice) revealing an association between high IL-31 mRNA expression and itch-associated scratching behavior (Takaoka et al., 2006). Although a direct correlation of itch sensation and IL-31 in AD patients is missing, Ishii et al. demonstrated a correlation of pruritus with IL-31 serum level and the number of mast cells in patients suffering from Philadelphia chromosome-negative myeloproliferative neoplasms (Ishii et al., 2009). Moreover significantly enhanced expression of IL-31 was detected in skin and serum samples of patients with AD (Neis et al., 2006) correlating with the severity of AD (Ezzat et al., 2011).

Finally it is worth noting that genetic predispositions associated with the *IL31* locus appear to influence the pathogenesis of AD. Three different haplotypes of single nucleotide polymorphisms (SNP) in the *IL31* gene and promoter were identified (Schulz et al., 2007). One of these is significantly associated with non-IgE-mediated AD. PBMCs of healthy individuals of this risk-haplotype responded with a stronger

induction of *IL31* expression in response to CD3/CD28-mediated stimulation compared to the other haplotypes. This study provides additional evidence for a determining role of IL-31 in AD.

A critical role of IL-31 in skin was also seen in a mouse model, in which IL-31 was overexpressed. These mice developed severe pruritus with alopecia and skin lesions that were characterized by hyperkeratosis, acanthosis, inflammatory cell infiltration and an increase in mast cells (Dillon et al., 2004). These studies also highlight the role of infiltrating monocytes and mast cells, consistent with the findings in human cells.

3.2 IL-31 regulates epidermal differentiation

3.2.1 The anti-proliferative function of IL-31

Beside its potential role in inflammatory processes it was observed in the studies presented here that IL-31 inhibited proliferation and deregulated differentiation in both primary NHEKs and in two different HaCaT cell clones conditionally expressing the IL-31RA receptor subunit (Figs. 6, 10, 13, 14 and 15). The effect of IL-31 on proliferation are consistent with the observations that this cytokine interferes with the proliferation of lung epithelial cells and of the colon carcinoma cell line HCT116 (Chattopadhyay et al., 2007; Dambacher et al., 2007). In lung epithelial cells IL-31 represses different cell cycle regulators, including cyclin B1 and CDK1, and stimulates the expression of p27, a negative regulator of cyclin/CDK complexes relevant for the transition from G1 into S phase and progression through S phase of cells. These findings are somewhat unexpected since IL-31 stimulates in different cell types several signaling pathways, i.e. the RAS/ERK, the PI3K/AKT, and the JAK/STAT pathway, all of which are rather pro-proliferative (Dillon et al., 2004; Diveu et al., 2004; Dreuw et al., 2004; Ip et al., 2007; Kasraie et al., 2011). At present it is not clear whether the combination of pathways or as yet not described signaling pathways are key to understand the anti-proliferative phenotype of IL-31. Furthermore an IL-31 effect on cell cycle progression is only reported for a very limited number of cell types indicating a cell type specific effect of IL-31. It is also

not excluded that downstream effectors of IL-31 signaling function as inhibitors of proliferation. In fact my studies revealed an effect of IL-31 on the expression of JUNB, Cyclin D1, p21 and p53 that have all been described to affect cell proliferation. The impact of IL-31 on these genes and their involvement in keratinocyte proliferation and differentiation is discussed further below.

3.2.2 IL-31 stabilizes β4-integrin in keratinocyte differentiation

My findings suggest that IL-31 interferes with the proper differentiation of keratinocytes. Marker proteins for various differentiation steps of the epidermis (described in the introduction) were investigated. IL-31 interferes with the expression of the late differentiation markers filaggrin and to a lesser extent involucrin. Also the marker of the spinouse layer, K10 is reduced and β4-integrin, which needs to be down-regulated during differentiation to enable the cells to detach from the basal membrane, seems to be distributed throughout the whole cellular layer. Differences in β4-integrin mRNA level could only be observed early in the NHEK 3D models (Fig. 6B, day2) but no differences were present at day 7 of the HaCaT-pSLIK-IL31RA models (Fig. 15B). The missing differences on mRNA expression in the 3D models are probably due to the presence of cells in many different stages of differentiation within the models and/or the result of translational or post-translational regulation of β4-integrin expression by IL-31. Nevertheless the difference in β4-integrin expression is clearly visible in the immunofluorescence stainings of the 3D models (Figs. 6A, 14B and 15A).

β4-integrin is located in the basal cellular layer and forms a heterodimeric complex with α6-integrin. It is essential for the attachment of cells in the basal layer to the basal membrane because mutations or depletion of β4-integrin leads to severe blistering of the skin in mice and humans associated with a high mortality rate in both (Ashton et al., 2001; Murgia et al., 1998; Nakano, 2004). It is exclusively located at hemidesmosomes where it binds laminin-5 (also called laminin-332) at the cell surface and hereby connecting the extracellular matrix to the intracellular cytoskeleton and stabilizing the interaction of the cells with the basal membrane. It connects the intermediate filaments in the cytosol through the cytoskeleton linker protein plectin (Margadant et al., 2010). The lack of β4-integrin also destabilizes α6-

integrin and leads to a failure in forming hemidesmosomes (Dowling et al., 1996; van der Neut et al., 1996). Beside its essential role in keratinocyte adhesion other functions of β4-integrin, including the regulation of the cell cycle and later differentiation steps are rather controversial because most mouse models with β4- or α6-integrin depletion or expression of modified proteins demonstrate no or only minor differences in epidermal differentiation (Margadant et al., 2010).

Nevertheless an extensive crosstalk of β4-integrin with growth factor dependent signaling pathways has been reported in tumors and cancer cell lines. The current opinion is that in normal epithelia β4-integrin, located in hemidesmosomes of cells in the basal layer, does not come in contact with proteins exhibiting signaling capacity. Detachment of cells from the basal membrane leads to disappearance of the hemidesmosomes and thus re-distribution of β4-integrin over the whole cell surface if it is not down-regulated. This leads to the association with receptor tyrosine kinases, which frequently seems to happen in tumor development also with other types of integrins (Guo and Giancotti, 2004).

The cytoplasmic tail of β4-integrin is phosphorylated following stimulation with hepatocyte growth factor (HGF), macrophage-stimulating protein (MSP) or epidermal growth factor (EGF) in different carcinoma models and might form a complex with the corresponding receptors to amplify and/or alter their signaling capacity (Margadant et al., 2010; Wilhelmsen et al., 2006). The biological consequences of β4-integrin, not associated with hemidesmosomes, range from tumor promotion to inhibition and seem to depend on the tumor type and genetic background of the tumor. For example in the presence of functional p53 α6β4-integrin stimulates apoptosis by stimulating caspase-3-dependent AKT/PKB kinase cleavage, while it promotes survival AKT/PKB signaling dependent on a p53 negative background (Bachelder et al., 1999; Clarke et al., 1995). Human keratinocytes lacking α6β4-integrin are resistant to transformation mediated by oncogenic RAS or blocking of NF-κB signaling (Dajee et al., 2003), indicating its influence on both signaling pathways. Furthermore tumor growth in isolated p53 and Smad4 deficient mouse tumor-initiating cells (mTICs) is inhibited by α6β4-integrin, which depends on its ability to bind intracellular plectin (Raymond et al., 2007), a linker-protein to the cytoskeleton that is able to activate signaling pathways, like PKC and RHO family

GTPases. In contrast addition of oncogenic RAS into these cells changes the tumor inhibiting function of α6β4-integrin to a tumor promoting function, which is probably due to further enhanced RAS signaling (Raymond et al., 2007). One central molecule in β4-integrin mediated signaling seems to be the PI3-Kinase. In breast carcinoma cell lines α6β4-integrin cooperates with ErbB2, a receptor of the EGFR family, in activating PI3-K signaling and enhancing tumor progression (Mercurio et al., 2001). These and more examples demonstrate that β4-integrin can have broad effects on internal signaling pathways (Oldak et al., 2006).

Hemidesmosome free β4-integrin could significantly change signaling pathways activated by growth factors that are essential for a proper keratinocyte differentiation. In organotypic cultures the presence of fibroblasts as feeder cells is needed. They secret e.g. EGF that promotes the proliferative state of the basal epidermal layer, which is essential for the development of organotypic cultures and the epidermis in vivo (Kern et al., 2011; Pastore et al., 2008). Broad effects of β4-integrin on different cell types are reported but the role of IL-31 induced stabilization of β4-integrin in the examined organotypic co-cultures remains an open question. In addition a physiological role for β4-integrin in non-transformed cells, tissues and organs in vivo, beside its essential function in attaching cells to a basal membrane, has not been reported. If IL-31 mediated stabilization of β4-integrin has physiological consequences has to be clarified in the future.

3.2.3 Inhibition of terminal differentiation markers by IL-31

The studies presented here demonstrate that K10, involucrin and filaggrin expression is negatively affected by IL-31 signaling. The morphological changes in keratinocyte differentiation and the expressional changes of differentiation markers are well described in the literature (as summarized in the introduction). Although the molecular changes in signaling events occurring during differentiation are less well understood, several secreted factors and signaling pathways are identified, e.g. leading to the activation of important transcription factors, that positively or negatively influence the differentiation process. However missing are direct links of signaling events to the molecular changes at e.g. promoters of differentiation markers leading to changes in gene transcription. A large number of terminal differentiation markers

including involucrin and filaggrin are located in an approximately 2 MB large gene cluster (termed the Epidermal Differentiation Cluster (EDC)) on chromosome 1q21 (Hoffjan and Stemmler, 2007). This cluster includes more than 50 genes all being regulated exclusively during epidermal differentiation. A similar genomic organization is true for keratins. Type 1 keratins are located in two clusters on chromosome 17 (17p11-12 and 17q11-13), type II keratins including K10 are located in a single cluster on chromosome 12 (12q11-13) (Bowden, 2005). These observations allow the hypothesis that epigenetic remodeling of chromatin might be the most important factor and that IL-31 might interfere with epigenetic mechanisms that normally lead to the expression of K10, involucrin and filaggrin during differentiation. But this hypothesis has not been verified yet.

Nevertheless IL-31 is able to activate signaling pathways and transcription factors that might influence differentiation marker expression after chromatin remodeling including MAPkinases, JAK/STAT signaling and the PI3-K/AKT pathway.

There is very little information available about the direct transcriptional regulation of K10 beside the observation that keratin 10 expression seems to be regulated by a p63/Notch-dependent mechanism. At the transition from the basal cell layer to the spinous layer the expression of the basal keratins K5 and K14 is switched off and K1/K10 expression is induced. This switch is regulated by p63/Notch. p63 promotes K5/K14 expression and inhibits the induction of differentiation, which is counteracted by Notch signaling that promotes K1/K10 expression (Koster, 2010; Lefort and Dotto, 2004; Watt et al., 2008). If or how IL-31 might influence p63/Notch signaling has not been determined but is one central future question since my results indicate that IL-31 might interfere with early differentiation steps that are mainly controlled by p63/Notch and their downstream signaling cascades.

The regulation of involucrin is described a bit more extensively. The involucrin promoter harbors a distal- (DRR) and a proximal-regulatory region (PRR) that are essential for optimal promoter activity. Both elements have well defined AP-1 binding sites, recruiting JUNB, JUND and Fra-1 in a keratinocyte specific manner (Welter et al., 1995), which is essential for promoter activation (Crish et al., 2002). Separated from these AP-1 sites, an additional SP-1 site is present in the DRR that contributes to

promoter activity (Banks et al., 1998). Addition of extracellular calcium activates the *INV* promoter dependent on the action of PKC (Deucher et al., 2002; Ng et al., 2000). The PRR contains an additional C/EBP binding site that also contributes to promoter activation while the intensity of activation seems to depend on the composition of C/EBP proteins in the cell (Agarwal et al., 1999). p38 is the main MAPkinase that controls involucrin expression by regulating AP-1, SP-1 and C/EBP factors (Agarwal et al., 1999; Banks et al., 1998; Efimova et al., 1998). In addition efficient activation of involucrin expression requires repression of ERK1/2 (Efimova et al., 2003; Efimova et al., 2002). How IL-31 might directly affect involucrin expression or eventually indirectly by activating the expression of certain transcription factors like JUNB, remains to be determined.

Filaggrin is one of the most important proteins in formation of the skin barrier. Loss-of-function mutations in *FLG* cause ichthyosis vulgaris (Sandilands et al., 2007; Weidinger et al., 2007). Filaggrin is essential for the formation of the stratum corneum and for skin barrier function (Mildner et al., 2010; Sandilands et al., 2009). Considering that *FLG* mutations are frequent in the general Western population, the enhanced expression of IL-31 with the here-described consequence on filaggrin suggests that IL-31 has the capacity to aggravate AD pathology and to further weaken skin barrier function. The IL-31 pulse experiments that was performed suggest that *FLG* is not a direct target of IL-31-induced signaling pathways (Fig. 15). Although the *FLG* promoter has not been studied in great detail, the AP-1 family of transcriptional regulators, POU-domain transcription factors, and p63 have been implicated in controlling *FLG* expression (Candi et al., 2006; Jang et al., 2000; Jang et al., 1996). Moreover AP-1 factors have been demonstrated to regulate different aspects of skin differentiation (Angel et al., 2001). Recently several signaling pathways have been proposed to be implicated in *FLG* regulation. Ca^{2+} signaling in the transition from the spinous to the granular cell layer induces filaggrin expression via PKC signaling, the Ca^{2+} sensing receptor and Dlx3 (Koster and Roop, 2007). A recent publication demonstrated that TNF-α expression in psoriasis patients correlates with reduced filaggrin and loricrin expression and an activation of the JUN N-terminal kinase-dependent pathway (Kim et al., 2011). Another recent publication revealed a potential role of NFκ-B signaling on filaggrin expression in HaCaT keratinocytes. Overexpression of S100A8 and A9 (both parts of the EDC) increases

NFκ-B activation leading to increased expression of involucrin and filaggrin (Voss et al., 2011). NFκ-B and components of the NFκ-B system have been demonstrated to be important mediators in epidermal homeostasis especially in the regulation of the "apoptotic keratinocyte differentiation program" (Lippens et al., 2005). IL-31 can activate the expression of AP-1 transcription factors (JUNB and FOS) but implications of IL-31 in other pathways that have been demonstrated to regulate filaggrin expression have not been published. Since IL-31 activates typical receptor tyrosine kinase pathways and *FLG* is not an early target of IL-31 stimulation and only expressed in late differentiation it is most likely an indirect IL-31 target gene.

Taking together these results suggests that IL-31 stimulation promotes early differentiation steps but inhibits the transition of cells towards terminal differentiation. This leads to weakening of the skin barrier, a typical symptom of AD, giving IL-31 the potential to aggravate AD pathology. This hypothesis is supported by the findings that the basal differentiation marker β4-integrin is not restricted to the basal cellular layer and the expression of the early differentiation marker of the stratum spinosum, keratin 10 as well as the expression of the terminal markers involucrin and filaggrin are all affected by IL-31. In addition basal cells exit the cell cycle and accumulate in G1 phase indicating an induction of differentiation. Furthermore the IL-31 induced phenotype is most likely indirect but IL-31 induces a persistent IL-31 specific differentiation process already after short stimulation times demonstrated by IL-31 pulse stimulation. The IL-31 phenotype in organotypic models of NHEKs is present predominantly in early stages of the models (day 2 and day 5). Older models seem to recover since no or only minor differences were present. This is probably due to the down-regulation of the IL-31RA rapidly after initiation of differentiation as demonstrated by Heise et al. 2009. The HaCaT cell clones stably express the IL-31RA after dox induction and thus do not loose their IL-31 responsiveness.

3.3 IL-20 and IL-24 in IL-31 mediated defects in skin differentiation

In order to identify factors that are involved in the IL-31 phenotype the potential of IL-20 and IL-24 to influence keratinocyte differentiation was investigated. Both are

early IL-31 target genes (Figs. 6C, 6E and 16A) and their mRNA expression constantly increases over time in IL-31 stimulated HaCaT-IL-31RA clones (Fig. 16B). In 3D models *IL20* and *IL24* expression is already affected after an IL-31 pulse stimulation (Fig. 16C). IL-20 and IL-24 do affect the expression of the late differentiation markers filaggrin and involucrin in NHEK and HaCaT 3D models while keratin 10 was not affected (Fig. 16F-H).

IL-20 has been discussed to be one of the key players by the pathology of psoriasis. Overexpression of IL-20 in transgenic mice is neonatal lethal caused by a psoriasis-like skin phenotype including hyperkeratosis, thickened epidermis and aberrant epidermal differentiation. This includes the overexpression of basal epidermal layer markers in suprabasal keratinocytes (K5 and K14) and the weakening of the barrier formation although the expression of filaggrin and loricrin are not altered. Taken together this leads to the assumption that IL-20 mainly affects the differentiation of early-differentiated cell layers (Blumberg et al., 2001). Keratinocytes from psoriatic lesions demonstrate an increased expression of IL-20 and IL-20 receptor components accompanied by an elevated STAT3 activation, a major signaling pathway activated by IL-20 (Blumberg et al., 2001; Wei et al., 2005). Moreover easing psoriasis symptoms by successful therapy correlates with a reduced cutaneous IL-20 level (Ogilvie et al., 2006; Otkjaer et al., 2005). But in contrast to AD, psoriasis is thought to be a disease mediated by T_H1 type cytokines with increased expression of IL-2, IL-6, IL-8, IL-12, INF-γ and TNF-α (Asadullah et al., 1999) and thus until today IL-20 was not proposed to play a role in T_H2 mediated skin diseases. This is supported by the findings that other T_H1 mediated diseases like rheumatoid arthritis (Hsu et al., 2006) and atherosclerosis (Hsieh et al., 2006) also exhibit elevated IL-20 levels. Main sources of IL-20 are monocytes, keratinocytes and endothelial cells while other cells of the immune system do not express IL-20 (Chen et al., 2006; Kunz et al., 2006; Wolk et al., 2002). The mechanisms how IL-20 might influence skin differentiation in psoriasis are largely unknown. It has been demonstrated that mice expressing a constitutively active STAT3-variant develop a psoriasis-like phenotype (Sano et al., 2005) but if this is also the main signaling pathway activated by IL-20 leading to this phenotype remains open. Neither resting nor activated immune cell populations express the IL-20 receptor chains and thus are not able to respond to IL-20 (Kunz et al., 2006; Wolk et al., 2002; Wolk et al., 2004; Wolk et al., 2005). The current opinion

is that the pro-inflammatory potential of IL-20 is mainly directed against keratinocytes and thus it was hypothesized that IL-31-induced IL-20 expression contributes to the IL-31 phenotype, although the pathology of psoriasis is clearly distinct from the AD like-phenotype that was observed in my studies. Recent studies demonstrated that maturated dendritic cells, derived by TNF-α/IL-1β stimulation of monocytes, induce IL-20 expression giving a potential link to innate immunity (Wolk et al., 2008). In addition IL-20 stimulated keratinocytes increased the sensitivity of the cells to Toll-like receptor 2 and 3 ligands leading to increased IL-8 expression. Furthermore IL-20 induced human β defensin-2 expression supporting a role for IL-20 in innate immunity. These observations are in agreement with the potential role of IL-31 in innate immunity as described in the introduction.

How IL-31 exactly regulates *IL20* expression remains to be determined. IL-1β induced IL-20 expression in keratinocytes depends on the p38 MAPkinase pathway and NFκ-B signaling (Otkjaer et al., 2007). The authors proposed a model indicating a transactivation of NFκ-B signaling by p38 activated MSK-1 leading to *IL20* expression. Figure 11D demonstrates the importance of p38 MAPkinase signaling for IL-31 stimulated *IL20* expression. However the activation of NFκ-B by IL-31 has not been tested but will be an aspect for future studies.

IL-24 was originally identified as a tumor suppressor (melanoma differentiation associated gene 7: MDA-7). Adenoviral overexpression in various tumor cells leads to the inhibition of tumor growth (Dash et al., 2010; Jiang et al., 1995). Surprisingly the physiological und patho-physiological function of IL-24 is largely unknown. Recent studies describe very similar functions in psoriasis and psoriatic skin compared to IL-20. An IL-24 transgenic mouse develops a similar psoriasis-like skin (He and Liang, 2010), psoriasis lesions (Kunz et al., 2006; Romer et al., 2003) and rheumatoid arthritis, comparable to the phenotype seen in humans (Kragstrup et al., 2008), attended by elevated IL-24 levels. Main sources of IL-24 are monocytes, T-cells (Wolk et al., 2002) and keratinocytes (Kunz et al., 2006; Tohyama et al., 2009). Furthermore IL-4 is able to induce IL-24 expression in keratinocytes (Kunz et al., 2006) and IL-24 signals through the same receptor complexes as IL-20 (Sabat, 2010), supporting the assumption of similar target cells and functions. As mentioned, besides its role in skin diseases, IL-24 (together with its family members IL-19 and IL-20) is

also thought to be involved in immune-mediated diseases like rheumatoid arthritis. It may induce the production of IL-6 and some other chemokines and aggravate the inflammatory process by attracting neutrophilic granulocytes (Hsu et al., 2006; Sakurai et al., 2008). Although little is known about IL-24 function the available information indicates a similar role as IL-20 in inflammatory skin diseases and thus IL-31 induced IL-24 expression might cooperate with IL-31 and IL-20 in altering keratinocyte differentiation. Indeed IL-20 and IL-24 have a similar influence on keratinocyte differentiation like IL-31 but to a much weaker extent (Figs. 11F and G). One report comparing the expression of IL-20 and IL-24 in psoriatic and AD lesions demonstrates that both skin diseases have similarly increased cytokine levels (Kunz et al., 2006) although both diseases have a quite different pathology concerning the defects in the skin differentiation and the involved inflammatory cytokines. This indicates that probably the inflammatory environment independently of the disease itself leads to induction of IL-20 and IL-24 in the skin and thus stimulates functions in the inflammatory process that are distinct from their role in epidermal differentiation. Since IL-20 and IL-24 expression has only minor effects on keratinocyte differentiation compared to IL-31, the IL-31 mediated expression of IL-20 and IL-24 might have another function distinct from epidermal differentiation and thus the IL-31 induced defects in skin barrier formation are directly mediated most likely by IL-31 induced signaling pathways and expressional changes of IL-31 target genes that are not secreted.

While IL-20 and IL-24, as direct targets of IL-31, contribute to the IL-31 differentiation phenotype, the array data indicate that different AP-1 family members, i.e. *FOS* and *JUNB*, as well as genes that code for components of MAPKinase signaling pathway are also targets of IL-31. This suggests that these genes will also contribute to altered differentiation of keratinocytes and disregulated filaggrin expression and differentiation.

3.4 JUNB in keratinocyte differentiation and involvement in the IL-31 phenotype

3.4.1 The JUN-JUNB network in cell cycle arrest and epidermal differentiation

We identified *JUNB* as an early target gene of IL-31 signaling in keratinocytes that is induced on the mRNA (Fig. 6C, Fig. 17 and Fig. 18) and protein level (Fig. 19C, Fig. 20B). JUNB belongs to the group of JUN proteins (like JUN and JUND) that are part of the AP-1 family of transcription factors. Further members of this family are FOS (FOS, FOSB, FRA-1, FRA-2), ATF (ATFa, ATF2, ATF3), JDF (JDF-1 and JDF-2) and MAF (c-MAF, MAFB, MAFA, MAFG/F/K, NRL) proteins (Shaulian, 2010). All AP-1 proteins form homo- or heterodimers and function as transcription factors by binding to DNA via their basic region-leucine zipper motif (Angel and Karin, 1991; Karin, 1995). JUN proteins, dimerized with FOS proteins, bind DNA elements known as TREs (phorpol 12-O-tetradeconate-13-acetate (TPA) response element) (Angel et al., 1987) to regulate gene expression. ATF homodimers or JUN/ATF heterodimers bind cAMP responsive elements (CREs) (Ryseck and Bravo, 1991). AP-1 transcription factors regulate various cellular processes including cell growth, cell cycle progression, apoptosis and transformation. JUN gets activated by growth factors, pro-inflammatory cytokines and UV irradiation (Karin, 1995). Growth factors induce the activation of the ERK MAPkinase pathway leading to FOS gene expression and heterodimerization with JUN. This leads to a further increase in JUN transcription (Angel et al., 1988). Cytokine and UV mediated activation of AP-1 factors mainly depends on the activation of the JNK and p38 MAP kinase pathways (Karin, 1995). A large number of reports demonstrate that JUN proteins are involved in cell cycle progression and transformation in a wide range of different cell types (Hess et al., 2004; Shaulian, 2010; Shaulian and Karin, 2001, 2002).

JUN is the most extensively studied member of the AP-1 family and in general promotes cell cycle progression especially in situations of cellular stress. In this context one target gene of JUN is Cyclin D1 (*CCND1*) that binds CDK4 and promotes G1-S phase transition (Sherr, 1996). This is consistent with the findings that JUN is essential for G1-S transition (Schreiber et al., 1999; Smith and Prochownik, 1992).

But Cyclin D1 contributes only partially to the cell cycle-promoting effects of JUN (Wisdom et al., 1999). Activated JUN directly represses p53 expression (Schreiber et al., 1999; Shaulian et al., 2000), a negative regulator of cell proliferation (Vogelstein et al., 2000; Vousden, 2000), which subsequently also represses the expression of the p53 target gene p21. p21 belongs to the family of cyclin-dependent kinase inhibitors that inhibit the activity of Cyclin/CDK complexes (Dotto, 2000) and thus results in cell cycle arrest. In fact in UV dependent cell cycle arrest the repression of p21 seems to be the most important function of JUN induction to allow reentering into the cell cycle (Shaulian et al., 2000). In addition p16, another member of the CDK inhibitor family, is repressed by JUN at least in transient transfection experiments (Passegue and Wagner, 2000).

JUNB directly antagonizes JUN functions (Passegué et al., 2002). It represses Cyclin D1 expression (Bakiri et al., 2000) and induces p16 expression (Passegue and Wagner, 2000), while its absence leads to down-regulation of p16 and increased JUN levels (Passegue et al., 2001). Notably replacement of JUN in JUN deficient MEFs by a JUNB transgene abrogates anti-proliferative effects of JUNB and rescues a deregulated Cyclin D1, p53 and p21 expression (Passegué et al., 2002). This indicates that anti-proliferative effects of JUNB depend on the presence of JUN probably by forming a growth inhibitory JUN/JUNB heterodimer. Nevertheless JUNB exhibits a much weaker dimerization affinity with other AP-1 family members and a much weaker DNA binding activity due to changes in the amino acid sequence within the bZIP region compared to JUN (Deng and Karin, 1993; Mechta-Grigoriou et al., 2001). These findings allow to postulate that JUN and JUNB differ considerably in the target genes they regulate and in their capacity to control expression. In fact JUNB activates efficiently only those target genes which harbor multiple AP-1 binding sites but can block effectively the expression of JUN target genes with only one AP-1 binding site by either competing with JUN for binding sites or maybe forming repressive JUN/JUNB heterodimers (Zenz and Wagner, 2006).

Beside the general role of JUN proteins in cell cycle progression, they play an important role in keratinocyte differentiation and wound healing. Studies in mouse and human epidermis revealed a differential expression of AP-1 proteins throughout differentiated skin (Angel et al., 2001). While JUNB is highly expressed in the

basement membrane and the early to mid-differentiated keratinocytes of the stratum spinosum and the stratum granulosum, JUN is hardly detectable in basal and early differentiating keratinocytes while it is highly expressed in the stratum granulosum (Angel et al., 2001). In addition also other members of the AP-1 family are expressed dependent on the differentiation state indicating that certain sets of AP-1 factors are associated with the differentiation status of keratinocytes (Angel et al., 2001; Eckert and Welter, 1996). The differing expression pattern of JUN family members indicates that the JUN-JUNB antagonizing function on cell proliferation might be less important in differentiating keratinocytes and that JUN proteins contribute also directly to keratinocyte differentiation. Several AP-1 target genes encoding critical markers in epidermal differentiation have been identified. A large number of keratins (including K5, K6, K8, K14, K18 and K19) and transglutaminases are potential AP-1 target genes (Eckert and Welter, 1996; Hu and Gudas, 1994; Navarro et al., 1995). K14 and K18 expression depend on their AP-1 binding sites (Rhodes and Oshima, 1998; Sinha et al., 2000), high basal profilaggrin expression in late differentiated keratinocytes depends on JUN/FOS dimers (Jang et al., 1996), involucrin transcription is regulated by AP-1 and SP-1 (Lopez-Bayghen et al., 1996) and JUND, Fra-1 and Fra-2, which are regulated by intracellular calcium concentration, bind calcium response elements in the involucrin promoter (Ng et al., 2000). Furthermore JUNB, JUND and Fra-1 seem to be responsible for involucrin regulation by phorbol ester (Welter et al., 1995). Furthermore co-transfection experiments indicate that JUNB/FOS dimers are involved in the differentiation-specific expression of loricrin (DiSepio et al., 1999) and AP-1 and ETS proteins regulate K14 expression (Sinha et al., 2000). Recently it was reported that JUNB/Fra-1 dimers bind the ß4-integrin promoter and activate its transcription (Oldak et al., 2010). Finally several differentiation-induced pathways including the MAP kinase and PKC pathway are involved in modulating AP-1 activity (Angel et al., 2001).

3.4.2 JUN-JUNB target genes in IL-31 stimulation affected epidermal differentiation

AP-1 dimers are directly involved in the transcription of differentiation-related genes like filaggrin or involucrin but they also regulate the expression of other genes that are

involved in regulating differentiation. Initiation of keratinocyte differentiation depends on a cell cycle arrest in the G1 phase (Hauser et al., 2004; Martinez et al., 1999). Downregulation of Cyclin D1 is associated with a G1 arrest in the cell cycle. Keratinocyte cell lines undergoing differentiation by detachment from the surface depend on the down-regulation of Cyclin D1 for permanent cell cycle exit (Nishi et al., 2009). Non-melanoma skin cancers (like keratoacanthomas or squamous cell carcinomas) are associated with Cyclin D1 overexpression preventing a cell cycle exit (Burnworth et al., 2006). In the epidermis Cyclin D1 is preferentially expressed in the basal proliferating cell layer (Belso et al., 2008) and down-regulated by expression of late differentiation markers like K10 (Paramio et al., 1999). Primary human oral squamous epithelial keratinocytes are immortalized by overexpression of Cyclin D1 and p53 inactivation (Opitz et al., 2001). These studies demonstrate that Cyclin D1 is critical to control cell proliferation in keratinocytes.

Moreover p21, a direct p53 target, is essential for the initiation of differentiation in keratinocytes and contributes to the differentiation-induced cell cycle arrest (Missero et al., 1996; Topley et al., 1999). On the other hand at later stages of differentiation p21 expression needs to be reduced to enable the cells to further proceed in differentiation (Di Cunto et al., 1998) indicating a negative feedback loop of p21 expression during differentiation. This influence of p21 on differentiation is not limited to keratinocytes. For example differentiating osteoblasts show a similar expression pattern of p21 during differentiation with inhibitory effects of overexpressed p21 during later stages of differentiation (Bellosta et al., 2003). However during differentiation p21 seems to play an important role that is distinct from cell cycle control since p21 overexpression specifically increases MAPkinase signaling in keratinocytes and suppresses keratinocyte differentiation independently of the cell cycle (Devgan et al., 2006). In this case MAPkinase activation is mediated by the induction of IGF-1 expression by p21 leading to disturbed differentiation. This is consistent with the finding that overexpression of p21 in promyelocytic leukemia cells does not affect cell cycle progression but clearly differentiation processes (Casini and Pelicci, 1999). Furthermore deletion of the N-terminus of p21 that mediates the binding to Cyclin/CDK complexes and thus cell cycle exit, does not abrogate the inhibitory effects on cell differentiation (Di Cunto et al., 1998; Dotto, 2000). To summarize this: in cell differentiation p21 has functions that can either

promote or inhibit differentiation and are distinct from its ability to control cell cycle progression.

In addition p21 is an important factor in committing stem cell fate in keratinocytes. As mentioned in the introduction two populations of proliferating cells exist in the epidermis: self-renewing stem cells and transient amplifying cells that are committed to differentiation. In the absence of p21 the pool of keratinocytes with stem cell potential is markedly increased with an enhanced possibility of carcinogenesis in mice (Philipp et al., 1999; Topley et al., 1999; Weinberg et al., 1999) indicating that p21 restricts the self-renewal potential of keratinocyte stem cells and promotes commitment for differentiation. In normal keratinocyte differentiation p21 activity is mainly controlled by Notch and p63 signaling in a p53 independent manner (Dotto, 2008; Mack et al., 2005).

Although the cell cycle inhibitory function of p16 is well described, information about its role in keratinocyte differentiation are rather limited. p16 deficiency in mice has no epidermal phenotype (like p21) but p16/p21 deficient mice show increased keratinocyte proliferation and defects in adult differentiation processes. Both seem to depend on alterations in Rb and p53 signaling (Paramio et al., 2001). Furthermore the limited proliferation potential and the exit from the cell cycle in keratinocytes (in contrast to several other cell types) potentially depends on p16, because immortalization of keratinocytes by hTERT fails as long as they express p16 (Dickson et al., 2000) and aging of keratinocytes in culture comes along with increased p16 levels independent of telomere length (Darbro et al., 2005). In addition keratinocyte-like cells derived from human embryonic stem cells undergo senescence associated with an increase of p16 expression (Dabelsteen et al., 2009) and normal human oral keratinocytes exhibit an extended life span when they are stimulated with retinoid acid which comes along with decreased p16 expression (You et al., 2000). p16 (like p21 and p27) expression is induced in keratinocytes that undergo differentiation by culturing them in suspension culture, accompanied by the induction of differentiation markers. In addition overexpression of p16 effects cell cycle progression but does not induce the expression of differentiation markers indicating that it is involved in differentiation-induced cell cycle exit but possibly does not play a role in other differentiation processes (Harvat et al., 1998).

p21 is a well-described p53 target with important functions in differentiation as described above. Although p53 is a JUN target and itself activates p21 transcription its function in keratinocyte differentiation is only poorly described. In fact p63, which belongs to the p53 protein family, is a very important factor in skin differentiation (as described in the introduction) while p53 seems to play only a minor role. Furthermore p21 regulation in differentiation mainly seems to be p53 independent. Nevertheless loss of p63 expression in keratinocytes by siRNA up-regulates p21 expression and results in cell cycle arrest (Truong et al., 2006). Simultaneous knock-out of p53 in this p63-/- background rescues the cell cycle arrest completely but a p21 knock-out only partially rescues this effect (Truong et al., 2006). This suggests that p21 is not the main mediator of p53 induced cell cycle arrest in keratinocytes. p63 is required for maintaining the proliferation potential of basal epidermal cells which seems to be depended on the inhibitory function of p63 on p53 expression (Truong and Khavari, 2007) probably by competing for the same DNA binding sites (Truong and Khavari, 2007; Yang et al., 1998). Thus the general opinion is that p63 and p53 counteract each another, one by maintaining cell cycle progression, the other inducing cell cycle arrest by inhibiting or promoting the expression of cell cycle regulators like p21, 14-3-3$^{\sigma}$ or IGFBP-3 (Truong and Khavari, 2007). At this point it is important to mention that p63/p53-dependent regulation of cell cycle progression in keratinocytes does not affect keratinocyte differentiation. Rescue of the p63 knock-out phenotype by a simultaneous p53 knock-out does not restore a p63 knock-out dependent loss of the differentiation potential of keratinocytes (Truong et al., 2006). This implies an only minor role for p53 in the differentiation program although it is important in the control of the cell cycle. In fact p63 dependent regulation of differentiation is only poorly understood but probably relies on a differential expression of p63 isoforms during differentiation and not on the counteracting functions of p63 and p53 (Wu et al., 2003).

The studies presented in this thesis revealed a significant impact of IL-31 on the expression of major regulators in keratinocyte cell cycle arrest and differentiation, most notably JUNB and p21, in HaCaT-pSLIK-IL31RA monolayer culture and NHEK organotypic skin equivalents (Figs. 17-20). JUNB and p21 are induced after IL-31 stimulation while Cyclin D1, p53 and p16 expression is repressed. In case of p16 this repression on the mRNA level was not apparent on the protein level. These

data give first hints how IL-31 might be involved in regulating keratinocyte differentiation.

The JUN/JUNB network in cell cycle control might be less relevant in keratinocyte differentiation since these proteins are differentially expressed in the epidermis suggesting that JUN and JUNB have distinct unique functions in differentiation that might not be affected directly by each other. The mRNA expression in IL-31 stimulated HaCaT cells supports this hypothesis since JUNB up-regulation in many other cells would lead to p16 induction and Cyclin D1 repression. JUNB dependent inhibition of JUN induces p53 and subsequently p21 expression. In IL-31 stimulated HaCaT cells I observe a different pattern as it would be expected in a JUNB dependent regulation of the cell cycle and thus might be IL-31 regulated differentiation dependent.

Repression of Cyclin D1 is in agreement with the observation that IL-31 stimulated HaCaT keratinocytes have a reduced proliferation rate in monolayer culture and organotypic skin equivalents and accumulate in G1 phase of the cell cycle (Fig. 10B, Fig. 13A and B, Fig. 14B). Also NHEKs arrest in the cell cycle, which was demonstrated by a reduced Ki67 nuclear staining in organotypic skin equivalents (Fig. 6A). As described above down-regulation of Cyclin D1 is essential for the progression of keratinocyte differentiation at very early stages of the process and in intact epidermis it is preferentially expressed in the basal proliferating cell layer. Down-regulation of Cyclin D1 might be a direct consequence of IL-31-dependent induction of JUNB expression and is in common with the JUN/JUNB dependent cell cycle control network.

IL-31 induced repression of p16 expression could only be observed in monolayer cultures of keratinocytes on the mRNA level and is probably not JUNB dependent since JUNB induces p16 in other cellular systems. It is most likely that p16 does not contribute to the IL-31 dependent phenotype because a p16 repression is connected in general with hyper-proliferation of cells and would maintain the proliferating state of keratinocytes which is not present in the observed differentiation models. In agreement with this hypothesis is the missing reduction on the protein level and the

missing regulation in the NHEK organotypic skin equivalents that might explain these missing known p16 effects.

Down-regulation of p53 during differentiation has been observed before (Dazard et al., 2000) and seems to be connected to the transition from transient amplifying cells to terminal differentiating cells and is connected with an induction of the p53 regulator MDM2. IL-31 stimulation also induces p53 repression in monolayer culture but could not be observed at the mRNA level in NHEK organotypic skin equivalents. Finally p21 induced expression is present in monolayer cultures of keratinocytes and NHEK organotypic skin equivalents. Since no direct regulation of p21 by JUNB has been reported yet and JUN negatively regulates p21 only indirectly by inhibition of p53, it is difficult to suggest how IL-31 or JUNB regulate p21. Nevertheless the constant overexpression of JUNB and p21 after IL-31 stimulation and the down-regulation of p53 and Cyclin D1 connected to normal differentiation in monolayer keratinocyte cultures further supports the hypothesis that IL-31 might impair the transition of keratinocytes from an early differentiated stage to terminal differentiating cells. JUNB and p21 are candidates for mediating this phenotype. JUNB down-regulation is essential in late differentiating cells. Furthermore JUNB/Fra-1 heterodimers can induce β4-intergrin expression, which is in agreement with the increased distribution of β4-intergrin in the observed organotypic skin equivalents described above. But β4-integrin silencing is an essential step in the transition of cells from the basal layer to the stratum spinosum (see above). Also the down-regulation of p21 after a high expression in early differentiated steps is needed for proper differentiation and a constantly high p21 level contributes most likely to the induction of differentiation by committing amplifying cells for differentiation but impairs the final formation of the skin barrier and keeps the cells in an early differentiated state.

If and how JUNB, Cyclin D1, p53, p21 and p16 contribute to the IL-31 phenotype is not proven yet. The described hypothesizes are based on the simple observation that these factors are induced after IL-31 stimulation in monolayer cultures and fully satisfying results from organotypic skin equivalents that also proof this regulation pattern in differentiating keratinocytes could not be observed. Monolayer cultures have the advantage of a consistent cellular state but in normal cell culture HaCaT keratinocytes do not differentiate. In contrast organotypic skin equivalents contain

feeder fibroblasts in combination with keratinocytes in different differentiation steps and thus with a potentially very different transcriptome. As displayed in Fig. 18D differences in JUNB expression could only be observed at day 2 after IL-31 stimulation of a NHEK organotypic skin equivalent. p21 was constantly higher compared to the non-IL-31 stimulated model (except at day 5) and for p53 and p16 no differences between IL-31 stimulated and non- stimulated models were observed. In HaCaT derived skin equivalents that were all harvested after 7 or 14 days of IL-31 stimulation, no differences in the mRNA expression were measured (data not shown). This is probably due to IL-31 induced transcriptional changes only in a minor subset of cells in a certain differentiation step that also affects later differentiation processes, which would concur with the observation that an IL-31 pulse stimulation of 24 hours is already sufficient to partially induce the IL-31 phenotype. The large number of cells with different transcriptomes does not represent this minor subset of IL-31 responsive cells that are present perhaps only in the basal layer and early spinous layer and thus IL-31 dependent changes are no longer measurable in 7 or 14 day old skin equivalents. To clarify this question, monolayer differentiation models (e.g. NHEK differentiation in monolayers can be induced by the increase of the Ca^{2+} concentration in the culture medium; HaCaT differentiation can be induced by prolonged detachment of the cells from a surface) are needed or the opportunity to investigate the transcriptome of certain cellular layers within an organotypic skin equivalent e.g. by single cell PCR and/or micro-dissection.

3.5 Conclusions

In conclusion several publications report that IL-31 is involved in the inflammatory processes in human skin diseases like AD and might have the capacity to enhance the inflammatory response by stimulating indirectly the recruitment of inflammatory cells. This thesis demonstrates for the first time that IL-31 can directly interfere with proper differentiation and skin barrier formation of keratinocytes. Impaired skin barrier formation is the most important symptom of AD as an increased penetration of the skin by allergens and pathogens leads to an aggravation of the inflammatory response. Filaggrin mutations are frequently associated with AD but the severity of AD does not correlate with the type of mutation, especially in heterozygouse

mutations. IL-31 has the ability to further weaken the skin barrier formation by inhibiting filaggrin expression. Thus IL-31 might be another important factor especially in patients suffering from a filaggrin mutation associated AD.

Furthermore IL-20 and IL-24 are induced by IL-31 stimulation, contributing to the IL-31 phenotype. They also might contribute to inflammatory processes in the skin since they have been shown to be tightly associated to psoriasis.

IL-31 induces a phenotype that is closely related to the earliest steps of differentiation but seems to inhibit further progression in the differentiation process indicated by the exit of the cell cycle and the missing down-regulation of β4-integrin associated with the inhibition of terminal differentiation markers. This finding is supported by expressional changes in cell cycle and differention regulators associated with the JUN/JUNB network that would promote early differentiation but inhibit late differentiation.

Thus I can conclude that IL-31 is an important regulator in skin differentiation and might highly contribute to inflammatory processes and differentiation in AD pathology.

4 Materials and Methods

Materials and Methods are described according to standard protocols used in the Institute of Biochemistry and Molecular Biology, RWTH Aachen University, and modified regarding individual differences in experimental procedures.

4.1 Consumables and reagents

4.1.1 Consumables

Consumables were purchased from: Amersham Biosciences, Ansell, Becton Dickinson, Biometra, Bio-Rad, Biosan, Biotron, Biozym, Brand, Braun, Cellstar, Costar, Eppendorf, eBioscience, Falcon, Fisher Scientific, Fuji, Gibco BRL, Heidolph, Hirschmann, Integra Biosciences, Komberley-Clark, Kojair, Millipore, Nalgene, Nerbe Plus, Omnilab, Rainin, Roth, Sanyo, Sarstedt, Sartorius, Schott Duran, Semper med, Siemens, TPP, VWR.

4.1.2 Reagents

Reagents met at least the criteria for the purity standard *p.a* and were purchased from: Amersham Pharmacia Biotech, AppliChem, Bayer, BD Biosciences, Bode, Biometra, Bio-Rad, Calbiochem, Chroma, Clontech, Eurogentec, Fermentas, Finnzymes, Fluka BioChemika, Gibco, Invitrogen, Invivogen, Merck, New England Biolabs, PAA, Pierce, Qiagen, Roche, Santa Cruz Biotechnology, Seromed, Serva, Sigma, Zymo Research.

4.2 Oligonucleotides

The following nucleotides were purchased from Eurofins-MWG-Operon, Sigma-Aldrich, Qiagen or Applied Biosystems.

Sequencing primers		
Primer name	**Sequence (5´-3´)**	**Function**
M13 (-21) forward	TGTAAAACGACGGCCAGT	Sequencing of pEN221 inserts
M13 reverse	CAGGAAACAGCTATGACC	Sequencing of pEN221 inserts
IL-31RAORFseq_fw	AAGTTCTGGAGTGACTGGAGCCAA	Sequencing of IL31RA cDNA
IL-31RAORFseq_rev	TTTCCTGACCCGTTCTGGCTTCAT	Sequencing of IL31RA cDNA

EGFP-N1_forward	CAACGGGACTTTCCAAAATG	Sequencing of pSFI express inserts
EGFP-N1_rev	CGGACACGCTGAACTTGTG	Sequencing of pSFI express inserts
pTRE_for	TAAGCAGAGCTCGTTTAGTG	Sequencing of pEN_TGmirc3 inserts
SeqL-B	Designed by Seqlab laboratories	Sequencing of pEN_TGmirc3 inserts

PCR primers		
Primer name	**Sequence (5´-3´)**	**Function**
IL31RAclon_fw	ATGTGCATCAGGCAACTCAAG	amplification of *IL31RA* cDNA from pEN-IL31RA
IL31RAclon_rev	TTAGACTTCTCCCTTGGTGTG	amplification of *IL31RA* cDNA from pEN-IL31RA
IL-31RA-pSLIK_fw	ATGATGTGGACCTGGGCACTG	amplification of *IL31RA-HA* cDNA from pSFI-IL31RA-HA
IL-31RA-pSLIK-HA_rev	TTAGGCATAATCTGGCACATCATA	amplification of *IL31RA-HA* cDNA from pSFI-IL31RA-HA
qRT-PCR primers and TaqMan® primer/probe sets		
Target	**Sequence/cataloge number**	**Company/reference**
OSMR_rev	TCTGTGCTAATGACTGTGCTTGTGGT	Savarese et al., 2002)
OSMR_fw	GTGTGGGTGCTTCTCCTGCTTCTGTA	(Savarese et al., 2002)
FAIM3	QT00011221	Qiagen
PIGR	QT00084651	Qiagen
MAPKAPK2	QT01012508	Qiagen
IL10	QT00041658	Qiagen
IL24	QT00059059	Qiagen
IL31RA	QT00010794	Qiagen
IL20	QT00044905	Qiagen
IL19	QT00022015	Qiagen
CCND1	QT00495285	Qiagen
CDKN2A	QT00089964	Qiagen
TP53	QT00060235	Qiagen
CDKN1A	QT00062090	Qiagen
IRF1	QT00494536	Qiagen
JUNB	QT00201341	Qiagen
GADD45B	QT00018480	Qiagen
DUSP1	QT00036638	Qiagen
EGR1	QT00999964	Qiagen
FLG	Hs00418578_m1 TaqMan®	Applied Biosystems
INV	Hs00902520_m1 TaqMan®	Applied Biosystems
KRT10	Hs00166289_m1 TaqMan®	Applied Biosystems

ITGB4	Hs00236216_m1 TaqMan®	Applied Biosystems
HPRT_fw	TGACACTGGCAAAACAATGCA	Lüscher Lab Pool
HPRT_rev	GGTCCTTTTCACCAGCAAGCT	Lüscher Lab Pool
HPRT	Hs99999909_m1 TaqMan®	Applied Biosystems

4.3 Plasmids

pENTR221-IL31RA

The full open reading frame of IL-31RA was cloned into pENTR221. The clone was obtained from Imagenes (IOH34840, #ReqNo: 9801604). pENTR221 is an ORF expression vector expressing the gene of interest under the control of a T7 promoter. *IL31RA* was cloned in between attL sites for easy recombination within the Gateway® recombination system (Invitrogen). The vector harbors a kanamycin resistance gene for bacterial selection.

pSFIexpress

Sub-cloning vector for cloning into pRTS. Cloning of a gene of interest into an EcoRV restriction site adds the sequence for a C-terminal HA-tag to the cDNA and embeds the cDNA into SfiI restriction sites (Hölzel et al., 2007).

pSFI-IL31RA-HA

The cDNA of IL-31RA was cloned into pSFIexpress after PCR amplification from pENTR221-IL31RA.

pRTS

Episomal doxycycline controllable expression vector for the creation of stable cell clones (Hölzel et al., 2007). This vector is extensively described in the results section.

pRTS-IL31RA-HA

The IL31RA-HA cDNA was cloned via SfiI restriction sites from pSFI-IL31RA-HA into pRTS.

pRTS-IL31RA-HAΔGFP

pRTS-IL31RA-HA has a bi-directional tetracycline-controllable promoter with the IL-31RA cDNA on the one side and a GFP cDNA on the other side. The GFP cDNA was removed by digestion of pRTS-IL31RA-HA with BglII and SwaI.

pEN_TGmirc3

Sub-cloning vector for insertion of a gene of interest into pSLIKneo via recombination (Shin et al., 2006). Cloning into pEN_TGmirc3 adds a tetracycline responsive element (TRE) in front of a cDNA and embeds this combination into attL sites for recombination.

pEN-IL31RA-HA

The IL-31RA-HA cDNA was PCR amplified from pSFI-IL31RA-HA and cloned into pEN_TGmirc3.

pSLIKneo

Tetracycline controllable expression vector that integrates into the target cell genome via lentiviral infection (Shin et al., 2006). The vector is extensively described in the results section

pSLIK-IL31RA-HA

The IL-31RA.HA cDNA was cloned into pSLIKneo via recombination with pEN-IL31RA-HA

4.4 Antibodies

Target	Company	Catnr.	Species	Dilution
primary antibodies				
Filaggrin	Santa Cruz	sc-66192	mouse	1:100
Involucrin	Sigma	I-9018	mouse	1:2000
Keratin 10	Dako	M7002	mouse	1:500
Keratin14	Chemicon	CBL197	mouse	1:200
β4-Integrin	Millipore	MAB2059	mouse	1:200
Ki67	Dako	M7240	mouse	1:100
IL-31RA (biotinylated)	R&D	BAF2769	goat	1:200
Hämagglutinin (HA)-tag	E.Krämer (München)	3F10 (clone)	mouse	1:1000

α-Tubulin	Sigma	T-5168	mouse	1:2500
Actin	MP Biomedicals	69100	mouse	1:1000
STAT3	Santa Cruz	SC-482	rabbit	1:1000
phospho-STAT3 (Tyr705)	Cell Signaling	#9131	rabbit	1:1000
IL-22R	R&D	MAB2770	mouse	1:200
IL-20Rβ	Santa Cruz	sc-47058	goat	1:200
IL-20Rα	R&D	AF1176	goat	1:200
JUNB	Santa Cruz	sc-73	rabbit	1:1000
p53	Santa Cruz	sc-126	mouse	1:1000
p21	BD PharMingen	556431	mouse	1:1000
p16	Santa Cruz	SC-1661	mouse	1:1000
secondary antibodies				
mouse IgG & IgM (H+L) HRP coupled	Jackson Immuno Research	115-036-068	goat	1:5000
rabbit IgG & IgM (H+L) HRP coupled	Jackson Immuno Research	111-035-144	goat	1:5000
rat IgG & IgM (H+L) HRP coupled	Jackson Immuno Research	112-035-068	goat	1:5000
goat IgG &IgM (H+L) HRP coupled	Santa Cruz	sc-2768	rabbit	1:5000
goat IgG (H+L)-Alexa Fluor 488	Invitrogen	A11055	donkey	1:2000
rabbit IgG (H+L)-Alexa Fluor 555	Invitrogen	A21428	goat	1:2000
rabbit IgG (H+L)-Alexa Fluor 488	Invitrogen	A11034	goat	1:2000
mouse IgG (H+L)-Alexa Fluor 488	Invitrogen	A11029	goat	1:2000
mouse IgG (H+L)-Alexa Fluor 555	Invitrogen	A21422	goat	1:2000

4.5 Inhibitors and cytokines

Inhibitors				
Name	**Target**	**Cataloge number**	**Company**	**final concentration**
JAK Inh. I	JAK kinases	420099	Calbiochem	100nM
SB202190	p38	559388	Calbiochem	20µM
JNK Inh. II	JNK kinases	420119	Calbiochem	20µM
U0126	MEK1 (ERK)	662005	Calbiochem	20µM
Wortmannin	PI3-kinase	691675	Calbiochem	500nM

recombinant human (rh) Cytokines			
Name	**Catalog number**	**Company**	**final concentration**
rhIL-31	200-31	Peprotech	100ng/ml
rhIFNγ	AF-300-02	Peprotech	20ng/ml
rhIL-20	1102IL	R&D	20ng/ml
rhIL-24	1965IL	R&D	20ng/ml

4.6 Work with nucleic acids

4.6.1 DNA preparation

Plasmid DNA from prokaryotic cultures was extracted using the Nucleobond Xtra Maxi plus kit (Macherey-Nagel) for large scale preparations and the Plasmid Miniprep Kit (Zymo Research) for small-scale preparations, according to the manufactures recommendations.

4.6.2 Enzymatic Manipulation of Plasmid DNA

For digestion, modification and ligation of Plasmid DNA restriction enzymes, calf intestine alkaline phosphatase, Klenow fragment and T4 DNA Ligase were purchased from Fermentas, Germany. Plasmid DNA digestions and double digestions were performed in a 20-50µl Volume for 1h to o/n with the appropriate buffers and temperatures according to the restriction enzyme specific recommendations. De-phosphorylation of DNA 5´-ends was achieved by incubation with calf intestine alkaline phosphatase for 1h at 37°C. Refill of sticky-ends to blunt-ends was achieved by addition of deoxynucleotides and incubation with Klenow Fragment at 37°C for 1h. Sticky end ligations were carried out for 1-2h at 37°C with T4 DNA ligase. For Blunt-end ligations 5% PEG2000 was added to the reaction and the time was extended to 2h-o/n.

4.6.3 Agarose gel electrophoresis

Gel Electrophoresis was performed in 0,8-3,0% low EEO agarose (Sigma-Aldrich) as described before (Sambrook, 1989).

4.6.4 Gel extraction of DNA

DNA from agarose gels was recovered using the Zippy Gel DNA Recovery Kit (Zymo Research) according to the manufactures recommendations.

4.6.5 Polymerase Chain Reaction (PCR)

All PCR reactions were performed using the Phusion High Fidelity Polymerase Kit (Finnzymes) according to the manufactures recommendations.

For a 50µl reaction volume:

plasmid DNA	10ng
10x Phusion HF buffer	10µl
Primer	0,5µM
dNTPs	200µM
Phusion DNA polymerase	0,02 U/µl
H_2O PCR grade	added to 50 µl final reaction volume

PCR program:

All PCR reactions were performed using the peqSTAR 96 Universal Gradient Thermocycler (Peqlab).

Initial denaturation		98°C	30 sec
35 cycles:	Denaturation	98°C	10 sec
	Annealing	60°C	20 sec
	Elongation	72°C	20 sec
Final extension		72°C	5 min

4.7 Work with prokaryotic cells

4.7.1 Bacterial strains:

E.coli XL10-Gold
(Stratagene)
Tetr *D(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacIqZDM15 Tn10* (Tetr) Amy Camr]

E.coli DH5α
(Invitrogen)
F- 80d*lacZ* M15 (*lacZYA-arg*F) U169 *recA1 endA1 hsdR17*(rk-mk+) *phoA supE44 - thi-1 gyrA96 relA1*

4.7.2 Materials

LB-Medium:	1% (w/v) Tryptone (AppliChem) 0,5% (w/v) Yeast extract (AppliChem) 1% (w/v) NaCl pH 7,0
Agar-Plates:	LB-Medium 1,5% (w/v) Bacto-Agar (Difco)
Antibiotics:	100µg/ml Ampicillin or 50µg/ml Kanamycin

4.7.3 Bacterial Transformation and cultures for plasmid DNA preparation

100µl chemically competent bacteria were thawed in a 1,5ml reaction tube and mixed with 100- 500ng plasmid DNA. The mixture was incubated on ice for 10-30min and heat-shocked for 45sec at 42°C and immediately placed back on ice. 1ml 37°C LB

medium was added to the bacteria and they were incubated at 37°C for 1h. The bacteria were pelleted by centrifugation, re-suspended in 100µl LB Medium and plated on LB agar plates containing the appropriate antibiotic for selection. The cells were incubated at 37°C until bacterial colonies could be selected. Single cell colonies were selected to seed o/n cultures for plasmid DNA preparation. For small-scale preparations 3ml LB-Medium and for large-scale preparations 300ml LB Medium, substituted with the plasmid specific selection antibiotic, were incubated in a shaker at 37°C and 160rpm.

4.8 Work with eukaryotic cells

4.8.1 Primary cells

HDFs

Human dermal fibroblasts

NHEKs

Normal human epidermal keratinocytes

Isolation of HDFs and NHEKs

Normal human keratinocytes were isolated from skin biopsies of adult skin and foreskin (obtained from healthy volunteers after coutaneous surgery), rinsed with 70% ethanol and washed 3 times in PBS with antibiotics/ antimycotics, and cut into pieces of 1cm^2. The epidermis was separated by incubation in 50 Caseinolytic units/ml Dispase (BD Bioscience) for 2h at 37ºC. After incubation in 0,25mg/ml trypsin/EDTA (Lonza) for 30minutes at 37ºC a single cell suspension of keratinocytes was released by gentle pipetting. After inactivation of the trypsin with Trypsin Neutralisation Solution TNS (Lonza) cells were cultured in KGM (Lonza) in 100x20mm cell culture dishes (Sarstedt) at 37ºC in a humidified atmosphere at 5%

CO_2.

Keratinocytes were used at their first to second passage for organotypic skin equivalents or monolayer cultures.

Normal human fibroblasts were isolated from the remaining dermis after incubation of skin in Dispase. The Dermis was digested in Collagenase 1A 100U/ml (Sigma) and cultivated in DMEM (Invitrogen) substituted with 10% Fetal Calf Serum (Biochrom) and penicillin (50U/ml)/streptomycin (50µg/ml)

The Isolation of HDFs and NHEKs was performed by Yvonne Marquard (Dermatology Department, Medical School, RWTH Aachen University) with informed consent by the donor.

4.8.2 Eukaryotic cell lines

HaCaT

HaCaT cells are a spontaneously transformed cell line derived from keratinocytes of histological normal skin of a 63 years old male (Boukamp et al., 1988) and were a generous gift of N. Fusenig.

HEK 293

Human epidermal kidney (HEK) cell line (ATCC CRL-1573) transformed with Adenovirus 5 DNA, integrated into chromosome 19q13.2.Obtained from ATCC.

HEK 293T

A cell line derived from HEK293 cells containing the large T-antigen from the Simian Virus 40 (ATCC CRL-11268). Obtained from ATCC.

HaCaT pRTS-IL31RA

HaCaT derived cell line containing a stably integrated episomal vector that leads to the expression of a HA-tagged IL-31RA upon doxycycline addition to the cell culture medium. The cell line was generated by Superfect (Qiagen) transfection of wt HaCaT cells with the vector pRTS-IL31RA-HAΔGFP.

HaCaT pSLIK-IL31RA

HaCaT derived cell line containing a stably integrated cassette that leads to the expression of a HA-tagged IL-31RA upon doxycycline addition to the cell culture medium. The cell line was generated by lentiviral infection of wt HaCaT cells with the vector pSLIK-IL31RA-HA.

4.8.3 Cell culture media, supplements, buffers and plastic materials

DMEM (Gibco) with 4,5g/l Glycose and GlutaMax

KGM® Keratinocyte Growth Medium (Lonza)

Supplements (Lonza):	Bovine pituitary extract (BPE)
	hEGF
	Insulin
	Hydrocortisone

PBS	140mM NaCl
	2,6mM KCl
	2mM Na_2HPO_4
	1,45mM KH_2PO_4

Trypsin/EDTA (Seromed)	0,5/0,02% (w/v) in PBS
Trypsin/EDTA (Lonza)	0,25/0,01% (W/V) in PBS
Trypsin Neutralizing Solution (Lonza)	
Hygromycin B solution (Roth)	50mg/ml
G418-Solution (Roth)	50mg/ml
Puromycine (Sigma-Aldrich)	1mg/ml
Doxycycline (Sigma-Aldrich)	1mg/ml
Penicillin/Streptomycin (Seromed)	10.000 Units/ml / 10.000 µg/ml
FCS (Fetal calf serum, Gibco)	
Cell culture dishes (Sarstedt)	Ø 6cm, 10cm, 15cm
Cell culture plates (TPP)	6-well, 12-well, 24-well, 48-well, 96-well
1ml cryotubes (Nalgene)	

4.8.4 Culture conditions

All cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37°C. NHEKs are cultured in KGM supplemented with BPM, hEGF, Insulin and Hydrocortisone. HDFs are cultured in DMEM supplemented with 10% FCS and 1% Penicillin/Streptomycin until they are seeded in co-culture with NHEKs. HaCaT, HEK293 and HEK293T cells were cultured in DMEM supplemented with 10% FCS and 1% Penicillin/Streptomycin. HaCaT pRTS-IL31RA cells were cultured by addition of 400µg/ml Hygromycin B to the normal HaCaT culture medium. To the culture medium of HaCaT pSLIK-IL31RA 800µg/ml G418 was added.

4.8.5 Protocols for work with prokaryotic cells

4.8.5.1 Cryoconservation

For cryconservation 5-8x10^6 cells were pelleted by centrifugation and re-suspended in 1ml 90%FCS/10%DMSO (v/v) and transferred to a cryotube. After incubation on ice for 30min the cells were wrapped in several layers of tissue paper and within 16-48h cooled down to -80°C in an isolated box. For long term storage the cryotubes were transferred to -150°C.

4.8.5.2 Thawing of cells

Cryotubes with frozen cells were thawed fast in a water bath at 37°C until all ice crystals were melted. The cells were immediately re-suspended in 37°C fresh culture medium, pelleted by centrifugation, taken up in 10ml culture medium and transferred to an appropriate culture dish.

4.8.5.3 Seeding and passaging of cells

To detach the cells from their surface, the culture medium was aspirated and the cells were washed once with PBS to remove bivalent ions that would inactivate trypsin. HaCaT cell lines were washed with PBS containing 10mg/ml EDTA to remove bivalent ions more efficiently. All cell lines were incubated in Trypsin/ EDTA (0,5%/0,02%, NHEKs: 0,25%/0,01%) until they detached from the plastic surface. By

addition of culture medium the trypsin was inactivated. The cell number/ml was determined by using the Casy Cell Counter (Innovatis) and the cells were seeded in the appropriate dilutions in new cell culture plates.

4.8.5.4 Transient transfections

Calcium-phosphate method

2x Hebs buffer:	274mM NaCl
	42mM HEPES
	9,6mM KCl
	1,4mM Na_2HPO_4
	pH 7,1
HEPES buffer:	142mM NaCl
	10mM HEPES
	6,7mM KCl
	pH 7,3

250mM $CaCl_2$

This transient transfection method is based on the fact that calcium-phosphate-DNA complexes form precipitates that can be taken up by adherent cells through endocytosis (Bousset et al., 1994; Chen and Okayama, 1988). This method was used to transfect HEK293 and HEK293T cells.

The day before transfection approximately 10^6 cells (10cm dishes), $4x10^5$ (6cm dishes) or 10^5 cells (6-well plates) were seeded in cell culture dishes. 20µg (10cm), 7µg (6cm) or 3µg (6-Well) of total DNA was diluted in 1/20 culture medium volume of 250mM $CaCl_2$. The same volume 2x HEBS was added drop wise under continuous mixing by agitation to the DNA-$CaCl_2$ solution. The solution was incubated for 15min at room temperature and afterwards added drop wise by pipetting to the cell monolayers. The cells were incubated approximately for 6 hours and residual precipitate was removed by washing the cells with HEPES buffer followed by addition of fresh medium.

Superfect (Qiagen) transfection of HaCaT cells

SuperFect Reagent consists of activated-dendrimer molecules with a defined spherical architecture. Branches radiate from a central core and terminate at charged amino groups which can then interact with negatively charged phosphate groups of nucleic acids. SuperFect Reagent assembles DNA into compact structures that bind to the cell surface and are taken into the cell by nonspecific endocytosis. The reagent buffers the pH of the endosome, leading to pH inhibition of endosomal nucleases, which ensures stability of SuperFect–DNA complexes. Due to highly controlled chemical synthesis the activated-dendrimer molecules in SuperFect Reagent have a precise size and a defined shape. This ensures consistent transfection-complex formation and reproducible transfection results (from www.qiagen.com) (Tang et al., 1996)

According to the manufactures recommendations the day before transfection $2x10^6$ cells were seeded in 10cm dishes. At the day of transfection 10µg pRTS-IL31RA-HAΔGFP were diluted in 300µl serum- and antibiotic-free DMEM medium and mixed with 50µl Superfect reagent. The mixture was incubated at RT for 10 min. The cells were washed four times with PBS, 2,5 ml normal growth medium was added to the plasmid-Superfect mixture and applied to the cells. After 3 h of incubation under normal cell culture conditions the transfection medium was aspirated, the cells were washed once with PBS and normal growth medium was added.

4.8.5.5 Lentiviral infection

Lentiviruses belong to the family of Retroviruses. They harbor a single stranded RNA which is reverse transcribed into a cDNA and integrated into the genome via long terminal repeats (LTR). The most prominent member of this virus family is HIV. The system how HIV integrates its genomic information into the host genome can be used to deliver transgenes into the genome of target cells. In a first step HEK293T cells are used to produce virus particles that harbor a lentiviral expression vector that is capable to deliver the genetic information into the cells via infection. To enable HEK293T cells to produce viral particles, the cells are transfected with so called helper or packaging plasmids. These plasmids encode essential proteins needed for virus formation. The plasmid pLP1 codes for the viral genes *gag/pol* (core and structural proteins, protease and integrase), pLP2 encodes the reverse transcriptase *rev* and pLP/VSV-G encodes lentiviral envelope proteins needed for binding and

infection of the target cell. The HEK293T cells express all proteins and the virus particles are formed automatically and are released into the supernatant. For biosafety reasons the vectors for the gene of interest include only an expression cassette and no additional genes needed for viral reproduction to ensure that any infected cell cannot produce new viral particles.

The day before transfection $2{,}4x10^6$ HEK293T (passage ≤ 20) cells were seeded on 10cm cell culture dishes. The cells were transfected with the calcium-phosphate-method with a total DNA amount of 20µg (5µg of each plasmid pLP1, pLP2, pLP/VSV-G and the lentiviral expression vector). At day 3 the medium was replaced by 8ml fresh medium. The target cells were seeded in 10cm dishes in a density that reaches 10-50% confluency on the next day. At day 4 the virus particle containing supernatant of the HEK293T cells was sterile filtered with a 0,45µm PVDF membrane filter. In some instances the medium of the HEK293T cells was replaced by fresh medium for a second harvest of viral particles at day 5.Half of the target cell medium was replaced by the virus containing supernatant and 8µg/ml polybrene was added, which promotes the viral infection.

After 6-9h of infection the medium of the target cells was replaced. At day 6 successfully infected cells were selected by addition of the appropriate selection marker to the culture medium.

4.9 Three dimensional organotypic skin equivalents

4.9.1 NHEK skin equivalents:

Keratinocytes and fibroblasts were prepared from sterile human skin samples and cultivated under regular cell culture conditions. Organotypic skin equivalents were constructed as described previously and cultured over a period of up to 14 days at the air liquid interphase (Neis et al., 2010). Skin equivalents were lifted at the air – liquid interphase and treated with the different cytokines as indicated in the figure legends. The reconstructs were harvested at the days indicated, cut into pieces and either fixed according to a standardized protocol for routine histology or embedded in Tissue Tec

(O.C.T.) (Sakura Finetek) compound for cryosectioning. Parts of the cultures were stored in RNA Later (Roche) for RNA isolation.

4.9.2 HaCaT pRTS-IL31RA/ HaCaT pSLIK-IL31RA skin equvalents:

For 3D organotypic cultures, primary dermal fibroblasts were isolated, cultured and seeded into collagen gels at a density of $5x10^5$/ml. These dermal equivalents were covered with DMEM containing 10% FCS. The following day, HaCaT-IL31RA cells ($8x10^5$/cm2) were plated onto the fibroblast-collagen matrix. After two days of culturing, the skin equivalents were lifted to the air - liquid interphase. The day before lifting the models to the air liquid interphase, doxycyclin (1µg/ml) was added to cultures to induce IL-31RA expression. Once lifted to the air - liquid interphase, interleukins were added as described in the figure legends. Untreated models were maintained as negative controls. For the pulse experiments, rhIL-31 (100ng/ml) was added to the culture for 2h, 8h and 24h. Models were carefully washed and further cultured with medium at the air – liquid interphase. Organotypc skin equivalents were prepared by Yvonne Marquard (Dermatology Department, Medical School, RWTH Aachen University).

4.9.3 Haematoxilin-eosin staining of three dimensional skin equivalents:

4µm thin slices were cut from cryo-conserved parts of three dimensional skin equivalents and transferred onto microscope slips. Acidic structures (that are concentrated in the nucleus and ER) are stained by 5min incubation of the slides in haemalaun with subsequent 10min incubation in water that increases the pH of the samples leading to a blue staining. Basic structures (e.g. most cytoplasmic proteins) are stained by 3min incubation of the samples in 0,1% eosin in ddH_2O. Eosin is removed by 2x 3 min washing in water. Water within the samples is removed by 2x 3min washing in 70% ethanol followed by 2x 3min in 90% ethanol and 2x 3min in 100% ethanol. Subsequently the slides are 3x 3min washed in Xylol and embedded under a cover slip with Vitro Clud® (Langenbrinck).

4.9.4 Immunostainings of three dimensional skin equivalents:

4µm thin slices were cut from cryo-conserved parts of three dimensional skin equivalents and transferred onto microscope slides. They were fixed for 10min in 4°C

aceton, air dried and washed with Ca^{2+} and Mg^{2+} free PBS. Afterwards they were incubated for 1hr with the appropriate dilution of the primary antibody in antibody diluent (Dako). The slides were washed 3 times with PBS and incubated 1 hr with the appropriate dilution of the secondary Alexa Fluor 488 labeled antibody in antibody diluent (Dako). After three PBS washing steps the slides were incubated 5min with a 1:5000 dilution of Hoechst 33258 (10mg/ml), washed three times with PBS and embedded under a cover slip with fluorescent mounting medium (Dako). Immunostainings of organotypic skin equivalents were performed by Yvonne Marquard (Dermatology Department, Medical School, RWTH Aachen University).

4.10 Work with proteins

4.10.1 Preparation of cell lysates

RIPA extracts

RIPA-buffer:	10mM TrisCl, pH 7,4
	0,15M NaCl
	1% NP-40
	0,1% SDS
	1% Deoxycholate (DOC)
100x ProteoBlock™ Protease:	100mM AEBSF HCl
Inhibitor Cocktail (Fermentas)	80µM Aproptinin
	5mM Bestatin
	1,5mM E64
	2mM Leupeptin
	1mM Pepstatin A
	0,5mM EDTA
Phosphatase inhibitors:	20mM β- Glycerophosphat
	1mM EGTA

0,1mM Vanadate
50mM NaF
50mM Okadaic acid

All steps were performed on ice. 250µl (150µl) RIPA-buffer, freshly substituted with the protease inhibitor cocktail (1:100) and phosphatase Inhibitors, was added to 6cm (6-well) cell culture dishes and incubated for 5min. The lysates were scraped off the plates and transferred to a 1,5ml reaction tube. The lysates were sonified in the Bioraptor (Diagenode) sonifier for 15min, 30sec cycles, max intensity, 4°C. Un-dissolvable cell fragments were pelleted by centrifugation (13200rpm, 20min, 4°C) The supernatants were transferred to a new tube and stored at -20°C.

4.10.2 Denaturing discontinues polyacrylamide gel electrophoresis (SDS-PAGE)

2x sample buffer:	160mM Tris-HCl, pH 6,8
	20% (v/v) glycerol
	10% (w/v) SDS
	0,25% (w/v) BPB
	100mM β-Mercaptoethanol
Laemmli-buffer pH 6,8:	25mM Tris base
	250mM glycine
	0,1% (w/v) methanol
Protein ladder:	Page Ruler™ Prestained Protein Ladder
	10-180kDa (Fermentas)

The denaturing SDS-PAGE separates complex protein mixtures according to the molecular size of the proteins. SDS quantitatively binds denatured proteins and leads to a large negative net charge that makes the natural protein charge, caused by the different amino acids, negligible. This ensures a separation towards the anode only influenced by the protein size. Discontinuous gels are composed of a large-pored

stacking gel and a small-pored separation gel. The large-pored gel leads to accumulation of the proteins at the small-pored border because of a change in pore size and the change in the pH of the gel (6,8 in the accumulation gel to 8,8 in the separation gel).

The discontinuous SDS-PAGE was performed according to Laemmli (Laemmli, 1970). RIPA-lysates were diluted 1:1 with 2x sample buffer, incubated 5 min at 95°C to denature the proteins, and loaded on a gel composed of a 5% stacking gel and a 10/12/15 % separation gel (dependent on the size of the protein of interest). The proteins were separated at 200V using a Mini-PROTEAN electrophoresis system (BIO-RAD).

4.10.3 Western Blot

10x Semi-Dry-buffer:	250mM Tris-base
	1,92M Glycin
	pH 8,3-8,8
1x Semi-Dry-buffer:	10% 10x Semi-Dry-buffer
	20% Methanol
Wash buffer :	PBS
	0,05% (v/v) Tween-20
Blocking buffer:	Wash buffer
	5% (w/v) dry milk powder

In a Western Blot proteins in a polyacryamide gel matrix are transferred vertically onto a nitrocellulose membrane via electrophoresis.

In the semi-dry transfer of proteins, the gel is covered by a nitrocellulose membrane (Anode site). Gel and membrane are surrounded by Whatman papers soaked in semi-dry buffer. This “sandwich” is placed in a blotting chamber between electrode plates. It is important to ensure that the size of each “sandwich” component is the same to ensure an optimal electrical flow through the “sandwich”. It is also important to remove every air bubble within the “sandwich”. The proteins are transferred onto the membrane by 1,2mA/cm^2 of the membrane size for 1-1,5h. After blotting the membrane is washed 1-2 times in H_2O.

4.10.4 Immunodetection of proteins

PBS-T: PBS
0,05% (v/v) Tween-20

Blocking buffer: PBS-T
5% (w/v) dry milk powder
0,2% (w/v) Ponceau S in 1% (v/v) acetic acid

ECL-Kit (Pierce)

Femto ECL-Kit (Pierce)

After Western blotting the membrane was stained 5 min in Ponceau S solution and washed with H_2O to remove the background staining of the membrane to make the protein bands visible. This proves proper blotting of the proteins and facilitates the cutting of the Western blot in pieces according to the size of the protein of interest. Afterwards the membrane was blocked in blocking buffer for 30 min at room temperature to saturate all free protein binding sites at the nitrocellulose membrane. The membrane was washed twice with PBS-T. The primary antibody targeting the protein of interest was diluted in an appropriate amount of PBS-T and incubated with the membrane for at least 2h at room temperature or o/n at 4°C. The membrane was washed 3 times for at least 10 min in PBS-T to remove unbound primary antibody and incubated afterwards with an appropriate dilution of a horse radish peroxidase (HRP) coupled secondary antibody in PBS-T for 1-2h at room temperature. The secondary antibody is directed against the invariable part of the primary antibody. After washing the membranes at least 3 times for 10 min in PBS-T the detection was performed using the ECL-Kit or the Femto ECL-Kit according to the manufactures recommendations with a computer assisted camera (Las-3000, Fuji). The ECL-Kit contains a chemieluminescent substrate for the HRP enzyme. After being processed by HRP the substrate emits light which can be detected.

4.11 Analysis of gene expression using the Affymetrix GeneChip® Human Exon 1.0 ST array

Probe preparation and GeneChip hybridization were performed using the GeneChip®Whole Transcript (WT) Sense Target Labelling Assay (Affymetrix) according to the manufactures recommendations using a protocol for 2µg total RNA. RNA and probe quality was analyzed using the Nanodrop ND-1000 spectrometer (Peqlab) and the Agilent 2100 Bioanalyzer (Agilent). Gene ChiP scanning was performed using the Affymetrix 428 scanner. Probe preparation was performed by Yvonne Marquard (Dermatology Department, Medical School, RWTH Aachen University). GeneChip hybridization and scanning were performed in the Chip core facility of the IZKF (Interdisziplinäres Zentrum für Klinische Forschung) Aachen. GeneChip data analysis was performed by Jens-Malte Baron (Dermatology Department, Medical School, RWTH Aachen University). The array for IL-31 stimulated and the non-stimulated control was performed twice.

4.12 Quantitative reverse transcriptase PCR (qRT-PCR)

4.12.1 Total RNA preparation

For preparation total RNA the RNeasy Mini Kit (Qiagen) was used according to the manufactures recommendations. The cell lysates were homogenized using the QIAShredder (Qiagen) columns. Remaining genomic DNA was digested on column using the RNase free DNase Set (Qiagen) for 15min at RT. The RNA concentration was determined using the Nanodrop 100 UV/VIS Spectrophotometer (Peqlab/Thermo Scientific). RNA was stored at -80°C and thawed on ice.

4.12.2 cDNA synthesis

Complementary (c)DNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufactures recommendations. 500ng or 1µg RNA was used for cDNA synthesis depending on the total RNA concentration. 2µl genomic DNA wipe out buffer was added to 1µg total RNA and filled up to total 12µl reaction

volume with RNase free water (PCR grade) and incubated for 2min at 42°C. A mixture of 4µl RT-buffer, 1µl random hexamer/oligo-dT primer and 1µl Reverese Transcriptase was added and incubated for another 15 min at 42°C. The reaction was stopped after 3 min incubation at 96°C. The cDNA was stored at -20°C.

4.12.3 Real-time PCR

In the real-time PCR a fluorescent dye is added to the PCR reaction that emits light at a certain wavelength after excitation only when it is incorporated into double stranded DNA. The total fluorescence of the reaction is measured after every elongation step within a PCR cycle. By setting a certain threshold for a positive signal the measured Ct-value (cycle threshold) directly correlates with the amount of cDNA in the reaction. This allows conclusions about the initial amount of transcript (e.g. mRNA of a certain gene) in the samples and gives the opportunity for e.g. comparable gene expression analyses. Since PCR is an exponentially growing reaction tight controls are needed. The amount of total RNA used in the cDNA synthesis reaction is set to the same amount in every sample (500ng or 1µg) and the amount of cDNA used in every real-time PCR reaction has to be strictly the same (in this case 2µl of a 1:10 dilution of a 1µg cDNA synthesis reactions, or a 1:5 dilution for 500ng cDNA synthesis reactions respectively, of a 20µl cDNA sample). To normalize for pipetting variances the Ct-value of the gene of interest (GOI) is normalized to the Ct-value of a so called house-keeping gene. The expression of this gene is not allowed to change under the performed treatments within the experiment and thus is the same in every sample. Since PCR is an exponential growing reaction the primer performance is another important aspect. Before performing a qRT-PCR experiment the primer specificity was verified by performing a melt curve and agarose gel electrophoresis to ensure the amplification of a single PCR product at the expected size. Finally the primer efficiency was determined by performing a standard curve with dilutions of a characterized template.

All real-time PCR experiments were performed using the Rotor-Gene 6000 (Corbett/Qiagen).

Used PCR Mastermixes: Universal Mastermix (Diagenode)
SensiMix™ *Plus* SYBR Kit (Quantace)

Both mixes use SYBR green as fluorescent dye

Reaction: 5 µl Mastermix
2 µl template cDNA (1:10 or 1:5 diluted in RNase free water)
1 µl primer mix of forward and reverse primers:
QuantiTect primer assays 1:4 diluted in TE pH 8,0,
individually ordered oligonucleotides, 100nM, 1:40 diluted
2 µl RNase free water

PCR programs:

Universal Mastermix: 10 min 95°C
40 cycles 10 sec 95°C
60 sec 60°C
(fluorescence measurement)
Melt curve: 60°C-95°C in 0,5°C steps, 5sec each step, fluorescence measurement after every step

Sensimix: 10 min 95°C
40 cycles 15 sec 95°C
15 sec 60°C
30 sec 72°C
(fluorescence measurement)
Melt curve: 60°C-95°C in 0,5°C steps, 5 sec each step, fluorescence measurement after every step

4.13 Flow Cytometry

4.13.1 Analysis of the cell cycle

Propidium iodide (PI) is a fluorescent dye that incorporates into a DNA double strand. It has an absorption peak at 488 nm and an emission peak at 562-588 nm. DNA of fixed and permeabilized cells can be stained with PI and the emission of light can be measured in a FACS (fluorescence activated cell sorting) machine. The emitted light

directly correlates with the amount of DNA in the cell. The different cell cycle phases (G0/G1, S and G2/M phase) differ in the amount of DNA. From G0/G1 with a single set of chromosomes the amount of DNA increases in S phase until in G2/M phase a doubled set of chromosomes and thus a doubled fluorescence intensity is present. This can be used to analyze the stage in the cell cycle of single cells and allow conclusions about the influence of cell treatments on the cell cycle.

Materials:
- FACSCanto II (BD)
- 1-3 x 10^6 cells per sample
- PBS/EDTA
- Trypsin/EDTA
- Culture Medium containing 10% FCS
- PBS
- 100% Methanol (-20°C)
- 20mg/ml RNase A
- PI solution (1mg/ml, Roth)

HaCaT cells were washed with PBS/EDTA and detached from the surface by adding Trypsin/EDTA. Trypsin was inactivated by adding normal culture medium containing 10% FCS. The cells were pelleted by centrifugation at 1500 rpm for 3 min and washed twice with PBS. The supernatant was aspirated and under continuous vortexing 3ml Methanol (-20°C) was added to fix and permeabilize the cells. The cells were incubated at -20°C for 20min. After pelleting the cells by centrifugation and 3 washing steps with PBS, 200µl 20µg/ml RNase A in PBS was added and incubated for 5min at RT. Subsequently 400µl of 1:20 PI solution diluted in PBS was added and the cells were incubated in the dark at RT for at least 30min. The cellular fluorescence was analyzed with the FACSCanto II system. Analysis of the data was performed with the FLOWJO software (Tree Star).

4.13.2 Analysis of the presents of cell surface proteins

Flow cytometry can also be used to detect cellular surface markers. For this purpose the cells were fixed in paraformaldehyde to keep the cell surface intact and prevent antibody penetration to the inner parts of the cell. Subsequently the cells are incubated

with a primary antibody directed against the extracellular domain of cell surface markers. A species specific secondary antibody linked to a fluorescent dye stains the primary antibody and allows the detection in Flow Cytometry.

Materials:
- $1x10^6$ cells per sample
- PBS/EDTA
- Trypsin/EDTA
- Culture medium with 10% FCS
- 3,7 % Paraformaldehyde (PFA) in PBS
- PBS
- PBS/1 % bovine serum albumin (BSA) 4°C
- Primary antibody
- Secondary Alexa Fluor coupled antibody

HaCaT cells were washed with PBS/EDTA and detached from the surface by adding Trypsin/EDTA. Trypsin was inactivated by adding normal culture medium containing 10% FCS. The cells were pelleted by centrifugation at 1500 rpm for 3min and washed twice with PBS. Under continuous vortexing 1ml 3,7% PFA was added the cells and they were incubated at RT for 20min. The cells were pelleted and washed 3 times with PBS/1% BSA and blocked 30min in PBS/1% BSA. The primary antibody was diluted in 200µl per sample PBS/1% BSA in the appropriate dilution, added to the cells and incubated for 1h at RT. The cells were washed twice with PBS/1% BSA and incubated with a 1:2000 dilution of the secondary antibody in PBS/1% BSA for 20min in the dark. The cells were washed 3 times with PBS/1% BSA, resuspended in 500µl PBS/1% BSA and analyzed for fluorescence in the FACSCanto II system. Analysis of the data was performed with the FLOWJO software (Tree Star).

4.13.3 Analysis of apoptotic cells

One of the first changes that occur when cells undergo apoptosis is the translocation of Annexin V from the cytosolic side of the plasma membrane to the cell surface. Annexin V is a surface marker that can be detected like described in the above section. In addition in late apoptosis the plasma membrane disintegrates. PI cannot pass an intact plasma membrane but it passes a disintegrated membrane and thus

binds to DNA in the nucleus. Apoptotic cells can be analyzed by a double staining of the cells with PI and for Annexin V on the cell surface to detect apoptotic cells and distinguish between early and late apoptotic cells. Early apoptotic cells are Annexin V positive and PI negative while late apoptotic cells are always PI and Annexin V positive because anti-Annexin V antibodies also pass a disintegrated membrane and bind to Annexin V at the cytosolic side of the plasma membrane.

Materials: see above
Annexin V-eFluor 450 Apoptosis detection kit (eBioscience)

The cells were treated as described in the above section. The PFA fixed and PBS/1% BSA washed/blocked cells were washed once in 100µl Annexin V binding buffer and resuspended in 100µl Annexin V binding buffer. 5µl FITC labeled anti-Annexin V antibody was added and incubated 15min at RT in the dark. The cells were washed twice in PBS/1% BSA, resuspended in 500µl PBS/1% BSA and 5µl PI solution was added. The cellular fluorescence was analyzed with the FACSCanto II system. Analysis of the data was performed with the FLOWJO software (Tree Star).

4.14 Immunocytochemistry

Immunocytochemistry is used to fluorescently label cellular proteins for fluorescence microscopy or confocal laser microscopy to determine the expression and localization of these factors. Surface proteins can be stained by non-permeabilizing fixation of the cells. For cytosolic proteins or proteins in other cell compartments the cells can be permeabilized additional by adding a detergent.

Materials: Glass coverslips
PBS
3,7 % PFA in PBS
0,1 % Triton-X100 in PBS
PBS/1 % BSA
Hoechst 33258 10mg/ml

Mowiol 4-88
Primary antibody
Secondary AlexaFluor labeled antibody

Cells grown on cover slips were washed twice in PBS, fixed for 20min with 3,7% PFA and permeabilized for another 15min with 0,1% Triton-X100 as needed. Blocking of unspecific binding sites was achieved by washing and incubation of the cells with PBS/1% BSA. The primary antibody was diluted in PBS/1% BSA and incubated for 60min at RT. The cover slips were washed twice with PBS/1% BSA and incubated with a 1:2000 dilution of the secondary antibody in PBS/1% BSA for another 30-60min at RT in the dark. The cells were washed twice with PBS/1%BSA, once with PBS and incubated for 5min at RT with a 1:5000 dilution of Hoechst stain in ddH_2O. After additional two washing with ddH_2O the cover slips were embedded in Mowiol 4-88 and left at 4°C over night in the dark. The next day stained cells were evaluated by fluorescence microscopy on an Olympus IX50 microskope or by confocal laser microscopy on a Zeiss Axiovert LSM510. The images were processed with the ImageJ programm (National Institute of Health, USA).

4.15 Proliferation assay

To determine the proliferation rate of cells, 60000 cells were seeded in 6 well plates in triplicates and treated as indicated in the figure legends. For a period of 4 days, every day one triplicate sample was washed in PBS/EDTA and detached from the surface by adding exactly 400µl Trypsin/EDTA. To inactivate trypsin 600µl normal growth medium substituted with 10% FCS was added. The total cell number was determined in the Casy Cell counting system (Innovatis).

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6 Appendix

6.1 Abbreviations

°C	degree Celsius
µ	micro
3D	three dimensional
A	ampere
A. dest	destilled water
aa	amino acid
AAM	alternatively activated macrophage
Ab	antibody
ACD	allergic contact dermatitis
AD	atopic dermatitis
ADA	adenosine deaminase
AE	atopic eczema
AKT	v-akt murine thymoma viral oncogene homolog 1
Amp	ampicillin
AP-1	activator protein 1
Arf	p16, cyclin- dependent kinase inhibitor 2A
ASK-1	apoptosis signal-regulating-kinase 1
ATCC	American Type Culture Collection
Bak	Bcl2-antagonist/killer
Bcl	B-cell CCL/lymphoma2
BFU-E	burst-forming unit erythroid
bio	biotin
bla	β-lactamase
bp	base pair
BSA	bovine serum albumin
C/EBP	CCAAT enhancer binding protein
ca.	circa
CBD	cytokine binding domain
CCL	CC chemokine receptor ligand
CD	cluster of differentiation
CD	Crohn´s disease
Cdc2	CDK1, cyclin-dependent kinase 1
CDK	cyclin dependent kinase
CDK	cyclin-dependent kinase
cDNA	copy DNA
CE	cornified envelope
CFU-GEEM	colony-forming unit erythroid, granulocyte, macrophage, and megakaryocyte
CFU-GM	colony-forming unit granulocyte and macrophage
CLA	cutaneous lymphocyte antigen
CLC	cardiotrophin-like cytokine
CLSP	calmodulin like skin protein
CNTF	ciliary neurotrophic factor
CRE	cAMP responsive elements
CT	cardiotrophin

Ct	cycle threshold#
CU	spontaneous urticarial
CXCL	CXC chemokine receptor ligand
Da	Dalton
DC	dendritic cell
Dex	dexamethasone
DLK	dual leucine zipper-bearing kinase
Dlx3	distal-less homeobox 3
DMSO	dimethyl sulfoxide
DNA	desoxyribo-nucleotide-acid
dNTPs	desoxynucleotide-tri-phosphate
dox	doxycycline
DRR	distal regulatory region
DTT	dithiothreitol
DUSP	dual specific phosphatase
E.coli	*Escherichia coli*
EBI3	Epstein-Barr virus induced 3
ECL	enhanced chemoluminescence
EDC	epidermal differentiation cluster
EDTA	Ethylenediaminetetraacetic acid
EGF	epithelial growth factor
EGR1	early growth response 1
ErbB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
ET	essential thrombocythemia
et.al.	*et alii*
FACS	fluorescence activated cell sorting
FAIM3	fas apoptotic inhibitory molecule 3
FASN	fatty acid synthase
FCS	fetal calf serum
FL	fms-like tyrosine kinase ligand
FLG	filaggrin
FN III	fibronectin type III
FOS	FBJ osteosarcoma oncogene
FRA-1	FOS-like antigen 1
g	gramm
GADD45β	growth arrest and DNA-damage-inducible, beta
GCSF	granulocyte colony-stimulating factor
GFP	green fluorescence protein
GLMR	gp130-like monocyte receptor
GM-CSF	granulocyte macrophage colony-stimulating factor
gp130	glycoprotein 130
GPL	gp130-like receptor
Grb2	growth factor receptor-bound protein 2
GRO1	CXCL1, CXC chemokine ligand 1
GTP	guanosinetriphosphate
HA	haemaglutinin
hBD	human β-defensins
HBE	human bronchial epithelial (cell line)
HE	haematoxylin-eosin
HEK	human embryonic kidney

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
HPC	hematopoietic progenitor cell
HPRT	hypoxanthine guanine phosphoribosyl transferase
hr(s)	hour(s)
HRP	horse radish peroxidase
hyg	hygromycine
i.e.	that is
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IF	immunofluorescence
IFN	interferon
Ig	immune globulin
IGF	insulin like growth factor
IL	Interleukin
IL-31RA	IL-31 receptor alpha
Ink4a	p16, cyclin-dependent kinase inhibitor 2A
INV	Involucrin
IRES	internal ribosomal entry site
IRF	interferon regulatory factor
ITGB4	β4-integrin
JAK	janus tyrosine kinase
JNK	Jun N-terminal kinase
JUN	c-JUN, jun proto-oncogene
JUNB	Jun-B oncogene
K	keratin
k	kilo
KGF	keratinocyte growth factor
Ki67	antigen identified by monoclonal antibody Ki 67
KO	knock out
l	liter
LB	luria broth
LCE	late cornified envelope
LDH	lactate dehydrogenáse
LFA-1	ITGB2, integrin β2
LIF	leukemia inhibitory factor
LIFR	leukemia inhibitory factor receptor
LPS	lipopolysaccharide
LTR	long terminal repeat
m	milli
M	molar (Mol/l)
MAPK	mitogen activated protein kinase
MAPKAPK2	mitogenic activated protein kinase activated kinase 2
max	maximum
MB	mega bases
MC	mast cell
MCS	multiple cloning site
MDA7	melanoma differentiation associated gene 7
MDC	CCL22, CC chemokine ligand 22
MDM2	Mdm2 p53 binding protein homolog (mouse)
MEK	MAP2K, mitogen-activated protein kinase kinase
MIF	macrophage migration inhibitory factor

min	minimum
min	minutes
MIP	major intrinsic protein of lens fiber
MMP	matrix metallo- proteases
MPN	myeloproliferative neoplasms
mRNA	messenger RNA
MSP	macrophage-stimulating protein
mTICs	mouse tumor-initiating cells
n	nano
NCID	notch intracellular domain
Neo	neomycin
NFκ-B	nuclear factor kappa B
NHEK	normal human epidermal keratinocyte
NOD	ATN1, atrophin1
NOD2	nucleotide binding oligomerization domain
NP-40	Nonidet P-40
ORF	open reading frame
OSM	oncostatin M
OSMR	oncostatin M receptor
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	poly-ethylene glycol
Ph$^-$MPN	chromosome-negative myeloproliferative neoplasms
PI3-K	Phosphoinositide-3-kinase
PIGR	polymeric immunoglobulin receptor
PKB/C	protein kinase B/C
PKC	protein kinase C
PLT	platelets
PMF	primary myelofibrosis
PRR	proximal regulator region
PV	polycythemia vera
RAS	rat sarcoma viral oncogene
RBC	red blood cell
RBP-Jk	recombination signal binding protein for Igκ J region
Reg1	regenerating islet-derived 1
rh	recombinant human
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RT-qPCR	quantitative reverse transcriptase polymerase chain reaction
SB	stratum basale
SC	stem cells
SC	stratum corneum
Scarf	Calm4, calmodulin 4
SCF	stem cell factor
SDF	CXCR4, CXC chemokine receptor 4
SDS	sodium dodecyl sulfate
SEB	superantigen staphylococcal enterotoxin B
SEMF	Human colonic subepithelial myofibroblast

SG	stratum granulosum
Shc	Src homology 2 domain containing transforming protein
SHP-2	PTPN11, protein tyrosine phosphatase, non-receptor type 11
shRNA	small hairpin RNA
SIN	self-inactivating
SMAD	mothers against decapentaplegic homolog or SMAD family member
SNP	single nucleotide polymorphisms
SOCS	suppressor of cytokine signaling
SOS	son of sevenless
SP-1	specificity protein 1
SPF	specific pathogen free
SPR	small proline rich
SS	stratum spinosum
STAT	signal transducers and activators of transcription
TAC	transient amplifying cells
TARC	CCL17, CC chemokine ligand 17
TE	Tris-EDTA
TG	transglutaminase
TGF	transforming growth factor
TIMP-1	TIMP metallopeptidase inhibitor 1
TLR	toll like receptor
TNCB	2,4,6-trinitrochlorbenzene
TNF	tumor necrosis factor
TRE	tetracycline responsive element
Tyr, Y	tyrosine
U	unit
Ubi-C	ubiquitin C
UC	ulcerative colitis
UV	ultra violet light
v/v	volume per volume
VEGF	vascular epithelial growth factor
w/v	weight per volume
WB	western blot
WRE	woodchuck regulatory element
WSX-1	IL27RA, IL-27 receptor alpha
WT	wild type
x	times/-fold
β-ME	β-mercaptoethanol
λ	wavelength of light

6.2 Curriculum vitae

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6.3 Publications

ten Haaf, A., Franken, L., Heymann, C., von Serenyi, S., **Cornelissen, C.**, de Hoon, J.P., Veeck, J., Luscher, B., Knuchel, R., and Dahl, E. (2011). Paradox of sonic hedgehog (SHH) transcriptional regulation: Alternative transcription initiation overrides the effect of downstream promoter DNA methylation. Epigenetics *6*, 465-477.

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6.4 Eidesstattliche Erklärung

Ich erkläre eidesstattlich, dass ich die vorliegende Dissertation selbstständig verfasst und alle in Anspruch genommenen Hilfen in der Dissertation angegeben habe. Des Weiteren erkläre ich, dass die vorliegende Dissertation nicht bereits als Diplomarbeit oder vergleichbare Prüfungsarbeit verwendet wurde.

Aachen, den 21.11.2011 Christian Cornelissen

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