Doctoral Thesis

Paracrine regulation of skin homeostasis and repair by fibroblast growth factors

Author(s):
Müller, Anna-Katharina

Publication Date:
2012

Permanent Link:
https://doi.org/10.3929/ethz-a-007587433

Rights / License:
In Copyright - Non-Commercial Use Permitted
Paracrine regulation of skin homeostasis and repair by fibroblast growth factors

A dissertation submitted to
ETH ZURICH
for the degree of
Doctor of Sciences

presented by

ANNA-KATHARINA MÜLLER

Dipl. Natw. ETH Zurich, Switzerland
Born July 17th, 1981
Citizen of Zihlschlacht-Sitterdorf (TG), Switzerland

accepted on the recommendation of

Prof. Dr. Sabine Werner, examiner
Prof. Dr. Cornelia Halin Winter, co-examiner

2012
Table of contents

Zusammenfassung I
Summary III
1. Introduction 1

1.1. The skin 1

1.1.1. Functions of the skin 1
1.1.2. The epidermis 2
1.1.3. The immune cells of the epidermis 4
1.1.4. The dermis 5
1.1.5. The immune cells of the dermis 6
1.1.6. The hypodermis 7

1.2. Cutaneous wound healing 8

1.2.1. The inflammatory phase 10
1.2.2. The phase of new tissue formation 10
   1.2.2.1. Migrating keratinocytes 11
   1.2.2.2. Dermal repair 14
1.2.3. The remodeling phase 14

1.3. Fibroblast growth factors (FGFs) and their receptors (FGFRs) 15

1.3.1. The FGFs 15
1.3.2. The FGFRs 16
1.3.3. Functional FGF – FGFR complex 17
1.3.4. Expression of FGF7 subfamily members and their receptors in the skin 18

1.4. Aim of this PhD thesis 20

References 21
## 2. Materials and Methods

### 2.1. Materials

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.1. Chemicals and other consumables</td>
<td>25</td>
</tr>
<tr>
<td>2.1.2. Enzymes</td>
<td>28</td>
</tr>
<tr>
<td>2.1.3. Nucleotids</td>
<td>29</td>
</tr>
<tr>
<td>2.1.4. Oligonucleotids</td>
<td>29</td>
</tr>
<tr>
<td>2.1.4.1. Primers for genotyping</td>
<td>29</td>
</tr>
<tr>
<td>2.1.4.2. Primers for qRT-PCR</td>
<td>29</td>
</tr>
<tr>
<td>2.1.5. Antibodies</td>
<td>30</td>
</tr>
<tr>
<td>2.1.5.1. Antibodies used for immunostaining</td>
<td>30</td>
</tr>
<tr>
<td>2.1.5.2. Antibodies used for Western blots</td>
<td>31</td>
</tr>
<tr>
<td>2.1.5.3. Fluorescent dyes</td>
<td>32</td>
</tr>
<tr>
<td>2.1.5.4. Antibodies used for FACS analysis</td>
<td>32</td>
</tr>
<tr>
<td>2.1.6. Kits</td>
<td>32</td>
</tr>
<tr>
<td>2.1.7. Cell culture reagents</td>
<td>32</td>
</tr>
<tr>
<td>2.1.8. Standard buffers and solutions</td>
<td>33</td>
</tr>
</tbody>
</table>

### 2.2. Methods

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1. DNA methods</td>
<td>34</td>
</tr>
<tr>
<td>2.2.1.1. Genomic DNA isolation</td>
<td>34</td>
</tr>
<tr>
<td>2.2.1.2. Polymerase chain reaction</td>
<td>34</td>
</tr>
<tr>
<td>2.2.2. RNA methods</td>
<td>35</td>
</tr>
<tr>
<td>2.2.2.1. RNA extraction from large tissue samples</td>
<td>35</td>
</tr>
<tr>
<td>2.2.2.2. RNA extraction with kit (for small tissue samples)</td>
<td>35</td>
</tr>
<tr>
<td>2.2.2.3. RNA extraction from cells</td>
<td>36</td>
</tr>
<tr>
<td>2.2.2.4. Nucleic acid concentration measurement</td>
<td>36</td>
</tr>
<tr>
<td>2.2.2.5. Agarose gel electrophoresis</td>
<td>36</td>
</tr>
<tr>
<td>2.2.2.6. qRT-PCR</td>
<td>37</td>
</tr>
<tr>
<td>2.2.2.7. RNase Protection Assay</td>
<td>37</td>
</tr>
<tr>
<td>2.2.3. Protein methods</td>
<td>38</td>
</tr>
<tr>
<td>2.2.3.1. Preparation of protein lysates from mouse skin</td>
<td>38</td>
</tr>
<tr>
<td>2.2.3.2. Preparation of protein lysates from keratinocytes</td>
<td>39</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>2.2.3.3</td>
<td>Determination of protein concentration</td>
</tr>
<tr>
<td>2.2.3.4</td>
<td>Tris/glycine SDS-polyacrylamide gel electrophoresis (SDS-PAGE)</td>
</tr>
<tr>
<td>2.2.3.5</td>
<td>Western blotting</td>
</tr>
<tr>
<td>2.2.3.6</td>
<td>Stripping of Western blot nitrocellulose membrane</td>
</tr>
<tr>
<td>2.2.3.7</td>
<td>Zymography</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Preparation of single cell suspensions from mouse tissues and fluorescence-activated cell sorting (FACS) analysis</td>
</tr>
<tr>
<td>2.2.4.1</td>
<td>Preparation of single cell suspensions from mouse spleen</td>
</tr>
<tr>
<td>2.2.4.2</td>
<td>Preparation of single cell suspensions from mouse skin</td>
</tr>
<tr>
<td>2.2.4.3</td>
<td>Fluorescence-Activated Cell Sorting (FACS)</td>
</tr>
<tr>
<td>2.2.5</td>
<td>Cell culture</td>
</tr>
<tr>
<td>2.2.5.1</td>
<td>Isolation of primary keratinocytes</td>
</tr>
<tr>
<td>2.2.5.2</td>
<td>Transwell/Boyden chamber assay</td>
</tr>
<tr>
<td>2.2.5.3</td>
<td>Adhesion assay</td>
</tr>
<tr>
<td>2.2.5.4</td>
<td>Rac1 pull-down assay</td>
</tr>
<tr>
<td>2.2.5.5</td>
<td>Spreading/adhesion assay</td>
</tr>
<tr>
<td>2.2.5.6</td>
<td>Scratch assay</td>
</tr>
<tr>
<td>2.2.5.7</td>
<td>BrdU assay for cells</td>
</tr>
<tr>
<td>2.2.5.8</td>
<td>Treatment of cultured cells with FGF7</td>
</tr>
<tr>
<td>2.2.5.9</td>
<td>IL-1F8 treatment of fibroblasts</td>
</tr>
<tr>
<td>2.2.5.10</td>
<td>Analysis of transepithelial electrical resistance</td>
</tr>
<tr>
<td>2.2.5.11</td>
<td>Live cell imaging and analysis of scratch assay movies</td>
</tr>
<tr>
<td>2.2.5.12</td>
<td>Cell ruffle analysis</td>
</tr>
<tr>
<td>2.2.6</td>
<td>Histology</td>
</tr>
<tr>
<td>2.2.6.1</td>
<td>Preparation of paraffin sections</td>
</tr>
<tr>
<td>2.2.6.2</td>
<td>Preparation of cryosections</td>
</tr>
<tr>
<td>2.2.6.3</td>
<td>Deyparaffinization</td>
</tr>
<tr>
<td>2.2.6.4</td>
<td>Haematoxylin/Eosin staining</td>
</tr>
<tr>
<td>2.2.6.5</td>
<td>Masson Trichrome staining</td>
</tr>
<tr>
<td>2.2.6.6</td>
<td>Mast cell staining</td>
</tr>
<tr>
<td>2.2.6.7</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>2.2.6.8</td>
<td>Immunofluorescence</td>
</tr>
</tbody>
</table>
2.2.6.9. Immunofluorescence staining of cells 55
2.2.6.10. BrdU staining using immunohistochemistry 56
2.2.6.11. BrdU staining using immunofluorescence 56
2.2.6.12. Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay 56

2.2.7. Microscopy 57
2.2.7.1. Confocal microscopy 57

2.2.8. Animal experiments 57
2.2.8.1. Animal experiments (general) 57
2.2.8.2. Wounding and preparation of wound tissue 57
2.2.8.3. BrdU incorporation assay 58
2.2.8.4. Measurement of transepidermal water loss (TEWL) 58
2.2.8.5. Tissue collection and follow-up preparation 58
2.2.8.6. Cream treatment of mouse skin 59
2.2.8.7. Acetone treatment of mouse skin 59
2.2.8.8. IL-1F8 injection 59

2.2.9. Statistics 60
2.2.9.1. Statistical analysis 60

References 61

3. Results 62
3.1. FGF receptors 1 and 2 in keratinocytes control the epidermal barrier and cutaneous homeostasis 62
3.2. The role of chronic inflammation in cutaneous fibrosis: FGFR deficiency in keratinocytes as an example 101
3.3. FGF receptors 1 and 2 are key regulators of keratinocyte migration in vitro and in wounded skin 114
3.4. The roles of receptor tyrosine kinases and their ligands in the wound repair process 144

4. General discussion 169

References 177
<table>
<thead>
<tr>
<th>Acknowledgements</th>
<th>179</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publications</td>
<td>180</td>
</tr>
</tbody>
</table>
Zusammenfassung


Nach Verwundung der Doppel-Knockoutmäuse zeigten diese eine Verzögerung im Heilungsprozess. Dies war auf eine reduzierte Kontraktion sowie auf Defekte

Summary

The skin protects our body from harmful environmental insults, such as pathogens and UV irradiation, and from excessive water loss. Due to the essential functions of the skin, injuries, which disrupt the skin’s barrier function, need to be rapidly and efficiently repaired. Wound healing disorders are a major problem in aged individuals as well as in patients with certain diseases, such as diabetes. The development of strategies to improve the wound healing process is therefore essential, and this requires a thorough understanding of the mechanisms underlying normal and impaired wound repair.

Previous studies performed in our laboratory revealed the importance of the FGF signaling pathway in wound healing, since mice expressing a dominant-negative FGFR2-IIIb mutant in keratinocytes showed a severe delay in reepithelialization. However, this approach did not allow to identify the ligands or receptors that are responsible for the delay in wound healing. For this reason we generated mice lacking FGFR1 and FGFR2 in keratinocytes. Amongst other phenotypic abnormalities, these double knockout mice loose their hair and they show severe defects in reproduction. Surprisingly, a progressive acanthosis was observed in the epidermis. This resulted from reduced expression of major tight junction components in the absence of FGFR1 and FGFR2 in keratinocytes. The junctional defect caused severe skin dryness, resulting in activation of epidermal γδ T cells, and production of IL-1F8 by these cells. This cytokine combined with keratinocyte-derived S100A8 and S100A9 caused keratinocyte hyperproliferation through induction of a double paracrine loop involving stromal cells of the dermis. In support of a role of skin dryness in the phenotype, topical treatment with moisturizing cream reduced the inflammatory infiltrate observed in the double knockout mice.

Upon full-thickness skin wounding, the double knockout mice showed a delay in the wound repair process. This was due to impairments in contraction as well as in reepithelialization. The contraction defect is a secondary effect that arises from alterations in the connective tissue composition in the dermis. The delay in
reepithelialization, however, is a direct effect of the FGFR1/2 knockout in keratinocytes of these animals. Keratinocytes from double knockout mice had a reduced migratory speed in vitro and their directional migration was impaired. Furthermore, attachment of these cells to the extracellular matrix was reduced and delayed. As the underlying mechanism we identified reduced expression of paxillin and focal adhesion kinase, two focal adhesion proteins, in FGFR1/2-deficient keratinocytes.

In summary, we identified new targets of the FGF signaling pathway in murine skin, including the tight junction proteins claudin 1, claudin 3 and occludin as well as the focal adhesion proteins paxillin and focal adhesion kinase. The impaired expression of tight junction components resulted in the development of a phenotype resembling atopic dermatitis, whereas the reduced expression of focal adhesion components caused wound healing abnormalities with similarities to chronic human skin ulcers. Therefore, expression and activity of FGFRs and the levels of their ligands should be determined in the skin of patients with atopic dermatitis or chronic ulcers. The similarities of the abnormalities of our mice with those seen in major human skin diseases suggest that the FGFR1/2 double knockout mice could serve as an interesting model system to study at least certain aspects of the pathogenesis of these disorders and for the development of novel treatment strategies.
1. Introduction

1.1 The skin

1.1.1 Functions of the skin

The skin of mammals is the largest organ of the body and consists of the skin itself and its appendages - the hair follicles, sebaceous glands and arrector pili muscles (pilosebaceous unit), the sweat glands, and the nails. It serves as an anatomical barrier and protects the body against invading pathogens and irritants with the help of specific structures that serve as a permeability barrier and of the innate (non-specific) and adaptive (specific) immune systems. Furthermore, it prevents desiccation of the body as it regulates the extent of water loss. By means of sweat glands and blood vessels the skin helps to regulate the temperature. While evaporation of water and increased perfusion help to reduce the body temperature, constricted blood vessels limit cutaneous blood flow and thus prevent heat loss to the environment.

As the sun reaches the skin and its appendages, cells in this tissue have to be protected from the deleterious effects of UV light. Melanin, a pigment produced by melanocytes that is transported to keratinocytes, efficiently absorbs dangerous UV-radiation. In addition, various intracellular defense mechanisms, such as low molecular weight antioxidants, antioxidant proteins and DNA repair enzymes protect cells of the skin from UV-induced damage. However, sunlight also has beneficial effects in the skin: it helps to convert 7-dehydrocholesterol to vitamin D, an important regulator of calcium homeostasis with anti-inflammatory functions (Benson et al., 2012).

The skin is also a sensory organ containing a variety of nerve endings, which sense pain, temperature and mechanical stimuli such as pressure, stretch and touch.

Normal skin is stretchable and compressible. It consists of three layers, namely the hypodermis (subcutis), the dermis and the epidermis. The hypodermis is composed mostly of fat (50% of the body fat) that absorbs shocks, and the
dermis is a loose connective tissue that provides elasticity. The composition and functions of the different skin layers are described below (Sundberg et al., 2012).

**Figure 1.1:** Schematic representation of a cross-section of mouse skin (from Wagner et al., 2010).

### 1.1.2 The epidermis

The epidermis originates from the ectoderm of the embryo and is a stratified squamous epithelium consisting of four layers: the *stratum basale* (basal layer), the *stratum spinosum* (spinous layer), the *stratum granulosum* (granular layer), and the *stratum corneum* (cornified layer). Keratinocytes at different stages of differentiation comprise 95% of all cells in the epidermis. They start as non-differentiated, proliferation-competent cells and end as dead, terminally differentiated cells (Fuchs and Raghavan, 2002). The differentiation process is in part controlled by a calcium gradient, which is highest in the outermost layer of the skin. There are two types of keratinocytes in the basal layer - a small population of pluripotent (stem) cells as well as a larger population of transit amplifying cells, which undergo various rounds of proliferation until they initiate the differentiation process. Upon induction of differentiation, keratinocytes dissociate their hemidesmosomal attachments to the basement membrane and
move upwards. Keratinocytes in the basal layer express keratins 5 and 14 (K5 and K14), which serve as marker proteins for this layer (Fig. 1.2). A stable population of pigment (melanin) producing cells, the melanocytes, is also located in the basal layer. The extensions of the melanocytes reach into the upper layers of the skin to distribute the melanin to all nucleated keratinocytes. Other cell types with dendrites are located in the basal layer of the epidermis of mouse skin, namely the epidermal γδ T cells (also called dendritic epidermal T cells; DETCs). Merkel cells, which are located in this layer as well, have neurosensory functions. In the spinous layer, the keratinocytes have lost their proliferative capacity under normal conditions and become flattened and partially differentiated. They are called “prickle cells” due to the appearance of tight desmosomal connections to adjacent cells. This part of the epidermis consists of more than one layer of cells (in human skin) in contrast to the basal monolayer. It includes the Langerhans cells, which are components of the immune system. The keratinocytes in the spinous layer express K1 and K10 and start to synthesize lamellar bodies, which are secretory organelles that contain lipids, hydrolytic enzymes and proteins. Keratinocytes in the following layer, the granular layer, secrete the content of the lamellar bodies into the extracellular space. These lipids help to build up an impermeable barrier, while the hydrolytic enzymes are needed for cell shedding in the outermost layer. The tight junctions, which hold neighboring keratinocytes together, seal the layer towards the outside and contribute to the barrier function of the skin. Different claudins and occludin are the major components of tight junctions. The relative amount of these proteins needs to be strictly controlled, since an appropriate composition of claudins is important for the formation of functional tight junctions and thus for maintaining an efficient barrier function (Furuse et al., 2002). On the way to the next layer keratinocytes terminally differentiate and thereby dehydrate and flatten further. In addition, their cell nuclei as well as most other organelles are degraded. This outermost layer of the skin is the cornified layer. Keratinocytes of this layer are called corneocytes - they lack nuclei and organelles and have lost their viability. The major function of this layer is to build a permeability barrier to protect the underlying tissue. It does so by forming a continuous sheet of protein-enriched corneocytes held together by corneodesmosomes and
surrounded by an intercellular matrix. This matrix is rich in non-polar lipids like ceramides, free fatty acids and cholesterol. The corneocytes have a flattened shape, because filaggrin aggregates keratin filaments to a tight bundle, thereby causing the cells to collapse. Characteristic for the corneocytes is their cornified envelope were proteins like involucrin, loricrin and trichohyalin are cross-linked by transglutaminase with small proline-rich proteins. On the outside of the cornified envelope a covalently bound lipid layer replaces the plasma membrane (Eckert et al., 1997; Proksch et al., 2008).

Figure 1.2: Representation of the different layers of the mammalian epidermis together with marker proteins, which are specifically expressed in keratinocytes of these layers (from Radtke and Raj, 2003).

1.1.3 The immune cells of the epidermis

The epidermis is populated with two major types of immune cells, Langerhans cells and T cells. Of particular importance for this work are epidermal $\gamma\delta$ T cells (DETCs), which are therefore described in more detail. DETCs or $\gamma\delta$ T cells expressing the $V\gamma3/V\delta1$ T cell receptor are unique in mice and do not exist in humans. In humans, the epidermal $\gamma\delta$ T cells are called $V\gamma9/V\delta2$ T cells, named after their V gamma locus in the genome. $\gamma\delta$ T cells are generally less abundant in human than in mouse epidermis. The $\gamma\delta$ T cells not only recognize stress antigens exposed on keratinocytes, but they also exchange signals with the keratinocytes under steady state conditions. Through this physiological interaction of $\gamma\delta$ T cells with keratinocytes the T cells are pushed into a pre-
active state. Upon activation of γδ T cells they either produce mitogens and survival factors for keratinocytes as seen for example during wound healing (Jameson et al., 2002) or they secrete granzymes and other proteins that lead to elimination of mutated keratinocytes, thereby preventing cancer development (Hayday and Tigelaar, 2003). The ligands of the γδ T cell receptor that are exposed by keratinocytes under these different stress conditions or even under physiological conditions remain to be identified (Chodaczek et al., 2012).

In contrast to the mouse epidermis, which includes almost exclusively γδ T cells under normal conditions, different types of αβ T cells are present in normal human skin (Spetz et al., 1996). Additional immune cells present in the epidermis are Langerhans cells (LCs), which are located in the suprabasal layer between the keratinocytes. Langerhans cells are the first sentinel cells that get in contact with pathogens from the outside. LCs phagocytose these pathogens and migrate to a skin-draining lymph node. In their lysosomes, they process certain pathogen-derived proteins and subsequently present them on their surface on MHC class II molecules to naïve T cells in order to activate them (Nestle et al., 2009). Finally, different types of αβ T cells as well as neutrophils and macrophages can infiltrate into the epidermis under inflammatory conditions (Cooper et al., 1993).

1.1.4 The dermis

The dermis originates from the mesoderm of the embryo and is separated from the epidermis by the basement membrane. It is a loose connective tissue and responsible for the nutrition and waste disposal of the whole skin. Capillary loops close to the basal layer of the epidermis exchange nutrients and deliver oxygen, and the lymphatic vessels are responsible for draining interstitial fluid back into the blood stream and for fighting infections with the help of lymphocytes, which are part of the lymphatic fluid. All the skin appendages as well as the nerve endings protrude into the dermis. The major connective tissue forming cells are the fibroblasts, which produce various components of the extracellular matrix, including collagens, glycosaminoglycans, and elastin. These components are responsible for the softness and flexibility of the skin. Different
types of immune cells are located in or are able to infiltrate into the dermis. In the case of injury these cells help to defend the body against pathogens coming from the outside and support the closure of skin wounds by secreting growth factors and cytokines acting on resident cells (Sundberg et al., 2012; Nestle et al., 2009; Schafer and Werner, 2008).

1.1.5 The immune cells of the dermis

Dermal dendritic cells (DDC), αβ T cells, γδ T cells, natural killer (NK) cells, mast cells, monocytes/macrophages and neutrophils are immune cells residing in or attracted to the dermis upon local release of cytokines or chemokines (Fig. 1.3). The DDCs are situated right below the epidermal-dermal junction and also deeper in the dermis. Interferon-α released from LCs in the epidermis stimulates DDCs in the dermis. DDCs secrete cytokines and chemokines and rapidly dislocate to the lymph nodes where they present antigens to T cells. Dendritic cells are also important in building up tolerance (Nestle et al., 2009). Regulatory T cells (Tregs) are the second cell type playing a crucial role in peripheral tolerance. They abort unnecessary immune reactions against harmless bacteria or pathogens in the skin and they control the magnitude of the immune response. If tolerance is not established properly, autoimmune diseases may develop (Di Meglio et al., 2011).

The dermal γδ T cells are located just beneath the epidermis as well as around capillaries and next to appendages. They proliferate in situ and orchestrate an immunosurveillance program by attracting neutrophils and triggering the expansion of CD4+ T cells (Di Meglio et al., 2011; Nestle et al., 2009). B cells are normally not present in the skin. However, they may become activated, resulting in the production of antibodies against foreign and endogenous antigens, which may be deposited in the skin (de Visser et al., 2006). Another important component of the adaptive immune system of the skin are mast cells. They are located in the upper dermis and are essentially involved in protecting the skin from infections as well as from severe venom effects after insect stings or snake bites. Additionally, mast cells are involved in wound healing (Weller et al., 2006). Finally, these cells play a crucial role in allergies.
Upon contact with foreign antigens, B cells produce large amounts of IgEs against these antigens. These IgEs subsequently bind with high affinity to the surface of mast cells via their $F_C$ region. Upon re-exposure to the original antigen, the latter binds to the IgEs on mast cells, resulting in their activation and degranulation. This results in the release of various cytokines, chemokines, peptidases and of histamine. This low molecular weight mediator dilates blood vessels and irritates nerve endings (Alenius et al., 2002; Harvima and Nilsson, 2011).

Figure 1.3: Immune cells that reside in the skin (from Feldmeyer et al., 2010).

1.1.6 The hypodermis

The hypodermis or subcutis is the lowermost layer of the integumentary system in vertebrates and originates from the mesoderm. It is a loose connective tissue with lobules of fat. Fibroblasts, adipose cells and several immune cells, in particular macrophages, are found in the hypodermis. Fibrous bands anchor the skin to the deep fascia, and energy is stored in the form of fat (Sundberg et al., 2012).
1.2 Cutaneous wound healing

Injuries of the skin need to be healed as fast as possible to re-establish the barrier function. Impaired wound healing can have various reasons, e.g. chemotherapy treatment, old age, treatment with anti-inflammatory steroids, or diabetes. These conditions frequently lead to the formation of chronic, non-healing ulcers. On the other hand, wound healing can also be excessive, resulting in the formation of hypertrophic scars and keloids (Gurtner et al., 2008). To find efficient treatments for these medical problems a good understanding of the normal wound healing process is required (Schafer and Werner, 2008).

The wound healing process is highly complex and involves a large number of different cell types and signaling pathways. Closure of wounds is achieved through a combination of reepithelialization (coverage of the wound with a neo-epidermis) and wound contraction. In parallel, a new tissue is built to replace the damaged dermis. This new tissue is initially called the granulation tissue, and it subsequently develops into a scar. Wound healing takes place in three partially overlapping phases namely the a) inflammatory phase b) the phase of new tissue formation and c) the remodeling phase (Fig. 1.4). At the end of wound healing these processes need to be shut down properly, and the tissue needs to return to homeostasis. It is still unclear if contact inhibition between cells, epigenetic control, or other processes are responsible for the termination of the wound healing program. A scar, an imperfectly healed piece of skin, remains at the site of injury in adult mammals. Prior to the beginning of the third trimester of development, however, mammals are able to heal a wound without scar formation (Schafer and Werner, 2008). The different phases of wound healing in adult mammals are described below.
Figure 1.4: The three stages of cutaneous wound healing. A) Inflammatory phase, b) phase of new tissue formation and c) remodeling phase (from Gurtner et al., 2008).
1.2.1 The inflammatory phase

Immediately after skin injury, fluid loss from the body must be prevented, dead and dying cells need to be removed, and an infection must be avoided. The disruption of blood vessels and the injury of cells caused by wounding stimulate the tissue to start the wound healing process. Platelets are activated and aggregate at the site of wounding, thereby forming a blood clot, which temporarily closes the wound (Szpaderska et al., 2003). The clot consists of cross-linked fibrin, fibronectin, vitronectin, thrombospondin and several less abundant components, and serves both as a scaffold for infiltrating cells and as a growth factor reservoir (Clark, 1993). Resident mast cells secrete histamine upon injury. This leads to vessel dilation and thus facilitation of immune cell extravasation and their infiltration into the wound area (Trautmann et al., 2000). Within a few minutes after wounding neutrophils are recruited to the site of injury by complement activation, degranulation of platelets, and products of bacterial degradation. Neutrophils mainly attack invading microorganisms by release of reactive oxygen species (ROS) and proteases and by engulfing and degrading microorganisms (Weiss, 1989). They also phagocytose cell debris and secrete growth factors and cytokines that initiate the next phase of wound healing (Hubner et al., 1996). Two to three days after injury monocytes infiltrate the wound and mature into macrophages. They coordinate many of the further events in the wound healing process (Leibovich and Ross, 1975).

1.2.2 The phase of new tissue formation

The phase of new tissue formation or the proliferative phase starts approximately two days after injury and lasts for several days depending on the size of the wound. This phase is mainly characterized by proliferation and migration of cells (Martin, 1997). Stimulated by the growth factors and cytokines released from immune cells and resident cells, keratinocytes of the epidermis at the wound edge and of injured hair follicles start to migrate. This is followed by a wave of proliferation to replace the cells that had been lost upon injury. Since keratinocyte migration is such an essential event for wound healing and severely
impaired in the FGFR1/2-deficient mice used in this thesis, the process of migration is described in more detail.

1.2.2.1 Keratinocyte migration

Upon wounding, keratinocytes from the epidermis at the wound edges as well as from injured skin appendages start to migrate towards the middle of the wound. This requires their transformation from stationary keratinocytes to agile migrating cells. Keratinocytes change their shape into flat and elongated cells, which extend lamellipodia. The migrating keratinocytes loose the hemidesmosomes, which connect the cell to the extracellular matrix (ECM), as well as their desmosomes, which link two cells together. Keratin filaments retract from the periphery, and the actin cytoskeleton gets rearranged into lamelli- and filopodia. Keratinocytes at the tip of the epithelial tongue of skin wounds as well as activated inflammatory cells produce different types of matrix metalloproteinases (MMPs) and other proteolytic enzymes. MMPs are initially produced in a latent (inactive or pro-) form. Upon secretion, they get activated by other types of proteases or by other MMP family members through cleavage of a short part of the N-terminus. Active MMPs degrade various extracellular matrix proteins as well other bioactive molecules, thereby facilitating the migratory process. Upon wounding, keratinocytes initially migrate along the injured dermis and subsequently use the provisional matrix of the granulation tissue as a substrate for adhesion and migration. This requires the upregulation of novel types of integrins (Raja et al., 2007).

The migration process of keratinocytes can be mechanistically studied in culture. Upon addition of a pro-motogenic factor, cultured keratinocytes polarize – they rearrange their organelles and cytoskeleton, which results in an asymmetrical layout of the cell. At the front end lamellipodia and filopodia are assembled and establish the first adhesions to the ECM called focal complexes (FX). These are dot-like adhesions located at the cell periphery that mature into focal adhesions (FA). FAs are stronger adhesions than FX due to their connection of the ECM through stress fibers. The last step of maturation leads to even stronger adhesions, called fibrillar adhesions (FBA). All of the described structures need
to be assembled and disassembled in order for the cell to migrate (Dubash et al., 2009).

Focal adhesions are multiprotein complexes that include transmembrane proteins that bind to the ECM (integrins and syndecan-4), proteins that link the transmembrane proteins to the actin filaments (tensin, talin, vinculin, α-actinin, zyxin, paxillin), and proteins with enzymatic activity (focal adhesion kinase (FAK), Src). Integrins are composed of α- and β-subunits, which assemble into heterodimers to form functional receptors. They have a large extracellular domain to bind to the ECM and a small cytoplasmic domain without enzymatic activity. Integrin engagement leads to the phosphorylation of the Src tyrosine kinase, which in turn activates the small GTPases Cdc42 and Rac1 (Fig. 1.5). Additionally, a negative regulator of RhoA is activated, resulting in RhoA inhibition. Active Rac1 and Cdc42 induce the formation of lamellipodia and filopodia and initiate the first weak and punctuated adhesions, the focal complexes. At a later stage, RhoA gets activated by a guanylate exchange factor and triggers the formation of the stronger focal adhesions and concomitantly of stress fibers.

**Figure 1.5: Integrin clustering recruits focal adhesion proteins** (from Avraamides et al., 2008).
Clustering and activation of integrins triggers the recruitment of various other proteins. One of them, paxillin, serves as a scaffold protein with docking sites for many other proteins and is phosphorylated by focal adhesion kinase (FAK), which is recruited to FAs as well and binds to talin. The binding of FAK to the FA induces a conformational change and auto-phosphorylation at tyrosine 397. This leads to recruitment of Src, and the complex of Src and FAK further phosphorylates FAK, thereby completing its activation. The complex also phosphorylates and activates p190RhoGAP, leading to a decreased activity of RhoA. Phosphorylation of p120Cas by the complex on the other hand leads to the activation of Rac1 and Cdc42 (Tomar and Schlaepfer, 2009). All of these processes need to be induced via an external signal. This can be an integrin ligand or a growth factor that binds to a receptor tyrosine kinase (Sieg et al., 2000; Fig. 1. 6).

Previous work using receptor knockout mice identified hepatocyte growth factor and the ligands of the epidermal growth factor receptor as well as FGFRs as important regulators of keratinocyte migration in wounded skin (Chmielowiec et al., 2007; chapter 3.3 of this thesis). However, additional factors are likely to be involved in this process as demonstrated in this thesis.

![Diagram](image_url)

**Figure 1.6:** Focal adhesion kinase serves as a signal integrator for integrins and growth factor receptors (from McLean et al., 2005).
1.2.2.2 Dermal repair

In addition to keratinocytes, fibroblasts at the wound edge in the dermis start to migrate and to proliferate upon stimulation by growth factors released from hematopoietic cells and resident cells. Wound fibroblasts initially deposit predominantly collagen III. PDGF and TGF-beta that are released at the wound site together with mechanical forces trigger the differentiation of fibroblasts into myofibroblasts, which are smooth muscle-like cells. The myofibroblasts contract the wound and secrete extracellular matrix (ECM) components like fibronectin, collagens I and III, proteoglycans, glycosaminoglycans as well as proteases (Hinz, 2007). The newly formed tissue needs to be supplied with oxygen and nutrients. Neoangiogenesis fulfills this need, and many new capillaries are formed within the wound area. The granular appearance of these new capillaries gives the wound tissue its name - granulation tissue. Lymphangiogenesis as well as nerve sprouting also take place during this phase (Werner and Grose, 2003).

1.2.3 The remodeling phase

Two to three weeks after wounding the remodeling phase is initiated, which can last for several weeks or even much longer. The existing granulation tissue that mainly consists of collagen III is now remodeled. Collagen I is synthesized by fibroblasts, and collagen III is degraded by MMPs. Immature collagen fibers are rearranged into higher order structures, forming cross-linked bundles that align parallel along a tensile line. This collagen structure differs from the one found in non-injured skin where the collagen fibers are arranged in even higher order structures. This results in reduced tensile strength with the scar tissue only having 70 to 80 percent of tensile strength of non-injured skin. In addition, the scar tissue lacks elastic fibers and therefore has a reduced elasticity. Skin appendages like hair follicles, sebaceous glands and sweat glands are absent in the scar, since they cannot regenerate (Hinz et al., 2007; Ulrich et al., 2007).

Most macrophages and myofibroblasts undergo apoptosis after the second stage of wound healing is concluded (Haslett, 1992). Surplus blood vessels are eliminated by apoptosis as well. The scar tissue hence has only few cells, but
extracellular matrix proteins are abundant. Keratinocytes that proliferated and migrated in the previous phases reassume their normal differentiation program (Gurtner et al., 2008).

In the remodeling phase all non-homeostatic processes are stopped and the cells return to their homeostatic programs. This is different to cancer where this arrest does not take place. However, various other cellular and molecular mechanisms involved in wound healing are also hallmarks of cancer (Schafer and Werner, 2008), and cancer has therefore been designated as “wounds that do not heal” (Dvorak, 1986). Studying and understanding wound healing could therefore shed light on processes that also occur in cancer and thus provide the basis for the development of strategies to treat impaired wound healing and also malignant disease.

The wound healing process is controlled by a wide variety of growth factors and cytokines (Werner and Grose, 2003; chapter 3.3 of this thesis). Of particular importance for this thesis are the FGFs and their receptors, which are therefore described in more detail.

1.3 Fibroblast growth factors (FGFs) and their receptors (FGFRs)

1.3.1 The FGFs

The FGF family comprises 22 members, of which 4 members (FGF11, FGF12, FGF13 and FGF14) do not function as ligands for the FGF receptors (FGFRs), but act intracellularly and are referred to as FGF homologues factors. FGF15 and FGF19 are orthologues, and FGF15 only exists in mice and FGF19 only in humans. At the amino acid level the different members of the FGF family share an internal core region. Twenty-eight amino acids are highly conserved, and 6 of them are invariant. Beta-sheets make up the core region with binding sites for the FGFRs and heparan sulfate proteoglycans. The molecular weight of FGFs ranges from 17 to 34 kDa in vertebrates. FGFs can be classified into subgroups corresponding to their structure and biochemical properties. Of particular interest for the work described in this thesis is the subfamily, which includes FGF3, FGF7, FGF10 (keratinocyte growth factor 1 and 2) and FGF22. FGF7,
FGF10 and FGF22 are expressed in the skin and control proliferation, migration, differentiation and survival of keratinocytes (Steiling and Werner, 2003). With the exception of the FGF homologous factors, FGFs exert their biological functions through binding to the FGFRs (Ornitz and Itoh, 2001).

**Figure 1.7:** The seven subfamilies of FGFs depicted in a phylogenetic tree (from Itoh and Ornitz, 2011).

### 1.3.2 The FGFRs

The family of FGFRs consists of 4 members (FGFR1–4) and one related receptor (FGFR-like 1) that can bind FGFs, but lacks a tyrosine kinase domain. The receptors comprise three extracellular immunoglobulin (Ig)-like domains, a hydrophobic transmembrane domain and an intracellular protein tyrosine kinase domain. Alternative splicing in the third Ig-like domain leads to different variants of FGFR1, FGFR2 and FGFR3 (Fig. 1.8). The variants are called IIIb (exon 8) or IIIc (exon 9) and they regulate the specificity of these receptors for FGFs. They are expressed in a cell type- and tissue-specific manner, allowing directional signaling across epithelial-mesenchymal borders. The IIIb variants are predominantly expressed in epithelial lineages (e.g. in keratinocytes),
whereas the IIIc variants tend to be expressed in mesenchymal lineages (e.g. in fibroblasts) as well as in immune cells. FGFRs 1, 2 and 3 – in particular or even exclusively the IIib variants - are expressed in keratinocytes of the epidermis. Whereas FGFR1-IIib and FGFR2-IIib have an overlapping ligand binding specificity and are activated by members of the FGF7 subfamily (FGF3, FGF7, FGF10, and FGF22), FGFR3-IIib binds another subgroup of FGFs (FGF1, 2, 9 and 18) (Ornitz et al., 1996; Zhang et al., 2006).

Figure 1.8: The splice variants of the FGFRs (from Powers et al., 2000).

1.3.3 Functional FGF-FGFR complex

The basic structure of the ligand-receptor complex includes two receptor molecules, two FGF molecules and one heparan sulfate proteoglycan chain (HSPG). HSPGs serve as a local depot of ligands in the extracellular space and protect the FGFs from degradation. Upon dimerization of homo- or heterodimers of receptor-ligand complexes the receptors transphosphorylate each other at tyrosine residues in the intracellular domain, thereby creating new binding sites for signaling proteins. The major FGF signaling protein is FGF receptor substrate (FRS)2α, which is fairly specific for FGFRs, binds them on phosphorylated sites with its phosphotyrosine-binding (PTB) domain and is phosphorylated by the activated receptors. Four key downstream pathways are turned on through the FGF signal: the MAPK pathway, the PI3K-AKT pathway, the STAT pathway and the phospholipase Cγ (PLCγ) pathway (Fig. 1.9). Their activation affects
proliferation, differentiation, migration, and survival of cells. To modulate these signals, receptors on the one hand get internalized after binding of ligands and on the other hand get inhibited by a variety of negative regulators, like FGFR-like1 or Sef. Additional intracellular inhibitors of FGF signaling are sprouty1-4 and MAPK phosphatase 1 (Bottcher and Niehrs, 2005; Murphy et al., 2010; Ornitz, 2000).

![Diagram of FGF signaling pathways](image)

**Figure 1.9:** The signaling network of FGFs binding to FGFRs (from Turner and Grose, 2010).

### 1.3.4 Expression of FGF7 subfamily members and their receptors in the skin

Fibroblasts, epidermal γδ T cells and dermal papilla cells of the skin secrete FGF7 and FGF10, which act in a paracrine manner on keratinocytes via FGFR1-IIIb and FGFR2-IIIb. FGF22 is produced by keratinocytes and acts in an autocrine manner on the same receptors (Fig. 1.10). FGF7, FGF10 and FGF22 have a high affinity for
FGFR2-IIIb. FGF10 and FGF22 also bind to FGFR1-IIIb, but with lower affinity. Previous work demonstrated that upon wounding FGF7 is highly expressed in the wound tissue (Werner et al., 1992). Mice lacking FGF7 or FGF22, however, did not show a wound healing phenotype, presumably because of compensation by FGF10 (Jarocz et al., 2012; Guo et al., 1996). The consequences of the loss of FGF10 for wound repair have as yet not been determined, since mice lacking FGF10 in all cells die immediately after birth (Min et al., 1998). The importance of FGFR signaling in the skin has been demonstrated by the phenotype of transgenic mice expressing a dominant-negative FGFR2-IIIb mutant in keratinocytes. These mice had a thinner epidermis, a fibrotic dermis, hair follicle abnormalities and a strong wound healing defect (Werner et al., 1994). However, the dominant-negative FGFR2-IIIb mutant blocks the action of other FGFRs in response to the same ligands and therefore, the importance of individual FGFs and FGFRs for skin morphogenesis and wound healing could not be determined. Furthermore, the mechanisms of FGF action in keratinocytes of normal and wounded skin had remained unclear.

Figure 1.10: FGFs expressed in the skin and their function in cutaneous wound healing (from Steiling and Werner, 2003).
1.4. Aim of this PhD thesis

Based on the above-mentioned findings and open questions, mice were generated in our laboratory, which lack both FGFR1 and FGFR2 in keratinocytes of the epidermis and the appendages. The first aim of this thesis was to characterize the non-injured skin of these mice at different stages of postnatal development using histology and immunohistochemistry and to unravel the cellular and molecular mechanisms underlying the skin phenotype. The second aim was to study the wound healing process in these mice with particular emphasis on reepithelialization as well as the migration phenotype of FGFR-deficient keratinocytes.

The results presented in this thesis revealed novel functions of FGFs and FGFRs in epidermal barrier function, skin homeostasis, keratinocyte migration and wound healing and point to a role of impaired FGFR signaling in the pathogenesis of impaired wound healing and inflammatory skin disease.
References


I. Introduction

1. Background

2. Significance

II. Fibroblast Growth Factors

1. Description

2. Functions

3. Regulation

III. Adhesion and Signaling

1. Molecular Interactions

2. Cellular Receptors

IV. Role in Development

1. Embryonic Development

2. Postnatal Development

V. Role in Disease

1. Tumor Development

2. Wound Healing

VI. Conclusion

References


2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and other consumables

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Acetone</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Acrylamide/bisacrylamide</td>
<td>Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Agarose, SeaKem ME</td>
<td>Biozym, FMC, Rockland, USA</td>
</tr>
<tr>
<td>4-(2-aminoethyl)benzene-sulfonylfluoride (AEBSF)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Ammonium thiocyanate</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Boric acid</td>
<td>Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>5-Bromo-2’-deoxyuridine (BrdU)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂)</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Casein</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Coomassie Brilliant Blue R-250</td>
<td>SERVA, Heidelberg, Germany</td>
</tr>
<tr>
<td>3,3-Diaminobenzidine (DAB)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>D,L-Dithiothreitol (DDT)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Diethylpyrocarbonate (DEPC)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Di-sodium hydrogenphosphate-Dihydrate (Na₂HPO₄x 2H₂O)</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Dispase</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>DNA marker</td>
<td>MBI Fermentas, Nunningen, Switzerland</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>Supplier</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>dNTP</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Dulbecco's Modified Medium (DMEM)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Eosin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Ethanol (EtOH)</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Ethidium bromide (EtBr)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Ethylenediamine tetra-acetic acid (EDTA)</td>
<td>Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Eukitt Mounting Medium</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Excipial Protect cream</td>
<td>Spirig Pharma AG, Egerkingen, Switzerland</td>
</tr>
<tr>
<td>Ferric chloride (FeCl₃)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Formamide</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Schweizerhall Chemie AG, Basel, Switzerland</td>
</tr>
<tr>
<td>Glycine</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Guanidinium thiocyanate (GSCN)</td>
<td>Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Haematoxylin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Hoechst</td>
<td>Molecular Probes, Basel, Switzerland</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Hydrogen peroxide (H₂O₂)</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Ketamine</td>
<td>Veterinaria, Zurich, Switzerland</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Magnesium sulfate (MgSO₄)</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Methanol (MeOH)</td>
<td>Schweizerhall Chemie AG, Basel, Switzerland</td>
</tr>
<tr>
<td>Meyer's haematoxylin solution</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Mowiol</td>
<td>Hoechst, Frankfurt, Germany</td>
</tr>
<tr>
<td>Item</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>NPAG</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>N,N,N',N'-Tetramethylene-diamine (TEMED)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Nitrocellulose transfer membrane (Protran)</td>
<td>Schleicher&amp;Schuell GmbH, Dassel, Germany</td>
</tr>
<tr>
<td>NP-40 (Igepal CA 630)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Paraffin S</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Pentobarbital (Esnarkon)</td>
<td>Streuli Pharma AG, Uznach, Switzerland</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Phenol</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Picric acid</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Ponceau S</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (KH$_2$PO$_4$)</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Prestained Protein Marker (broad range)</td>
<td>MBI Fermentas, Nunningen, Switzerland</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Skimmed milk powder</td>
<td>Migros, Zurich, Switzerland</td>
</tr>
<tr>
<td>Slides Superfrost</td>
<td>Shandon, Frankfurt, Germany</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sodium azide (NaN$_3$)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Sodium bicarbonate (NaHCO$_3$)</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>Fluka Chemie AG, Buchs, Switzerland</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate monohydrate (NaH$_2$PO$_4$·H$_2$O)</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
</tbody>
</table>
2.1.2 Enzymes

Collagenase II  Worthington, Lakewood, NJ
Collagenase IV  Invitrogen, Paisley, UK
DNase I  Sigma, Buchs, Switzerland
DNase RQ1  Promega, Madison, USA
Hyaluronidase  BioConcept, Allschwil, Switzerland
Proteinase K  Roche, Rotkreuz, Switzerland
RNA Polymerase (T3, T7)  Roche, Rotkreuz, Switzerland
RNase A  Roche, Rotkreuz, Switzerland
RNase T1  Roche, Rotkreuz, Switzerland
RNasin ribonuclease inhibitor  Promega, Madison, USA
Taq DNA Polymerase  New England BioLabs, Ipswich, USA

2.1.3 Nucleotides

dNTPs  Invitrogen, Paisley, UK

\( ^{32}P \text{-UTP} \)  Amersham, Braunschweig, Germany

(800Ci/mmol, 20mCi/ml)

2.1.4 Oligonucleotides

All primers were synthetized by Microsynth (Balgach, Switzerland).

2.1.4.1 Primers for genotyping

\textbf{mCre}:

5'Cre  5'-AAC ATG CTT CAT CGT CGG-3'
3'Cre  5'-TTC GGA TCA TCA GCT ACA CC-3'

\textbf{mFgfr1}:

5'FGFR1  5'-CGA ATG GAC AAG CCC AGT AAC-3'
3'FGFR1  5'-CTC CTG CTT CCT TCA GAG C-3'

\textbf{mFgfr2}

5'FGFR2  5'-TGC AAG AAG CGA CCA GTC AG-3'
3'FGFR2  5'-ATA GGA GCA ACA GGC GG-3'

2.1.4.2 Primers for qRT-PCR

The Primer-BLAST (NCBI, Bethesda, MD) or the Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA) programs were used to design primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin 1</td>
<td>CTTCTCTGGGATGGATCG</td>
<td>GGGTTGCCTGCAAAGTACTGT</td>
</tr>
</tbody>
</table>
2.1.5 Antibodies

2.1.5.1 Antibodies used for immunostaining

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Host</th>
<th>Catalog Nr.</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-BrdU IgG-POD</td>
<td>mouse</td>
<td>BMG-6H8</td>
<td>Roche, Rotkreuz, Switzerland</td>
</tr>
<tr>
<td>anti-CD3 IgG</td>
<td>rabbit</td>
<td>A0452</td>
<td>DAKO, Glostrup, Denmark</td>
</tr>
<tr>
<td>anti-Claudin3 IgG</td>
<td>rabbit</td>
<td>#34-1700</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>anti-F4/80 IgG</td>
<td>rat</td>
<td>T-2006</td>
<td>BMA Biomedicals, Augst, Switzerland</td>
</tr>
<tr>
<td>anti-Giantin IgG</td>
<td>rabbit</td>
<td>PRB-114C</td>
<td>Covance, Princeton, NY</td>
</tr>
<tr>
<td>anti-Keratin 6 IgG</td>
<td>rabbit</td>
<td>PRB-169P</td>
<td>Covance</td>
</tr>
<tr>
<td>anti-Keratin 10 IgG</td>
<td>mouse</td>
<td>M7002</td>
<td>DAKO</td>
</tr>
<tr>
<td>anti-Keratin 14 IgG</td>
<td>rabbit</td>
<td>PRB-155P</td>
<td>Covance</td>
</tr>
<tr>
<td>anti-Ly6G IgG</td>
<td>rat</td>
<td>01211A</td>
<td>BD Bioscience, Allschwil, Switzerland</td>
</tr>
<tr>
<td>anti-Paxillin IgG</td>
<td>mouse</td>
<td>610054</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>anti-phospho-Paxillin</td>
<td>rabbit</td>
<td>#2541S</td>
<td>Cell Signaling, Danvers, MA</td>
</tr>
<tr>
<td>anti-PCNA IgG</td>
<td>rabbit</td>
<td>Sc-7070</td>
<td>Santa Cruz, Santa Cruz, CA</td>
</tr>
<tr>
<td>anti-α-SMA IgG-FITC</td>
<td>mouse</td>
<td>F3777</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
</tbody>
</table>
### 2.1.5.2 Antibodies used for Western blots

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Host</th>
<th>Catalog Nr.</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-β-Actin IgG</td>
<td>mouse</td>
<td>A5441</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-Cdc42 IgG</td>
<td>rabbit</td>
<td>#2466</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-Claudin1 IgG</td>
<td>mouse</td>
<td>37-4900</td>
<td>Zymed Laboratories, Inc, South San Francisco, CA</td>
</tr>
<tr>
<td>anti-Claudin3 IgG</td>
<td>rabbit</td>
<td>34-1700</td>
<td>Zymed Laboratories</td>
</tr>
<tr>
<td>anti-Erk1/2 IgG</td>
<td>goat</td>
<td>Sc-94</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-phospho-Erk1/2 IgG</td>
<td>rabbit</td>
<td>#9102</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-FAK IgG</td>
<td>mouse</td>
<td>610088</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>anti-phospho-FAK (Y397)</td>
<td>rabbit</td>
<td>44624G</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>anti-Filaggrin IgG</td>
<td>rabbit</td>
<td>PRB-417P</td>
<td>Covance</td>
</tr>
<tr>
<td>anti-phospho-FRS2α IgG</td>
<td>rabbit</td>
<td>#3864</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-Gapdh IgG</td>
<td>mouse</td>
<td>5G4</td>
<td>HyTest, Turku, Finland</td>
</tr>
<tr>
<td>anti-LaminA IgG</td>
<td>goat</td>
<td>Sc-6214</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-Loricrin IgG</td>
<td>rabbit</td>
<td>PRB-145P</td>
<td>Covance</td>
</tr>
<tr>
<td>anti-NF-κB IgG</td>
<td>rabbit</td>
<td>Sc-372</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-phospho-NF-κB IgG</td>
<td>rabbit</td>
<td>#3033</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-Occludin IgG</td>
<td>rabbit</td>
<td>71-1500</td>
<td>Zymed Laboratories</td>
</tr>
<tr>
<td>anti-phospho-p38 IgG</td>
<td>rabbit</td>
<td>#9211</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-Paxillin IgG</td>
<td>mouse</td>
<td>610054</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-phospho-Paxillin (Y118)</td>
<td>rabbit</td>
<td>#2541S</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-rac1 IgG</td>
<td>mouse</td>
<td>05-389</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>anti-Stat3 IgG</td>
<td>rabbit</td>
<td>#9132</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-phospho-Stat3 IgG</td>
<td>rabbit</td>
<td>#9131</td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Host</th>
<th>Catalog Nr.</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-mouse-Cy3 IgG</td>
<td>goat</td>
<td>#115-166-062</td>
<td>Jackson, West Grove, PA</td>
</tr>
<tr>
<td>anti-mouse IgG-HRP</td>
<td>goat</td>
<td>W4021</td>
<td>Promega</td>
</tr>
<tr>
<td>anti-rabbit-Cy2 IgG</td>
<td>goat</td>
<td>#111-225-003</td>
<td>Jackson</td>
</tr>
<tr>
<td>anti-rabbit-Cy3 IgG</td>
<td>goat</td>
<td>#111-166-006</td>
<td>Jackson</td>
</tr>
<tr>
<td>anti-rabbit IgG-HRP</td>
<td>goat</td>
<td>W4011</td>
<td>Promega, Madison, WI</td>
</tr>
<tr>
<td>anti-rat-Cy3 IgG</td>
<td>donkey</td>
<td>#712-165-150</td>
<td>Jackson</td>
</tr>
</tbody>
</table>
### 2.1.5.3 Fluorescent dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Catalog Nr.</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoechst</td>
<td>B2261</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rhodamine phalloidine</td>
<td>R415</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Propidium Iodide</td>
<td>81845</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

### 2.1.5.4 Antibodies used for FACS analysis

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Catalog Nr.</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-A/1-E</td>
<td>558593</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>anti-CD3</td>
<td>553066</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>anti-CD11b</td>
<td>12-0112</td>
<td>eBioscience, San Diego, CA</td>
</tr>
<tr>
<td>anti-CD32</td>
<td>#553142</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>anti-CD45</td>
<td>#17-0451</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>anti-F4/80</td>
<td>MF48004</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>anti-Ly6G</td>
<td>11-5931</td>
<td>eBioscience</td>
</tr>
<tr>
<td>anti-αβ TCR</td>
<td>12-5961</td>
<td>eBioscience</td>
</tr>
<tr>
<td>anti-γδ TCR</td>
<td>#553177</td>
<td>BD Bioscience</td>
</tr>
</tbody>
</table>

### 2.1.6 Kits

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEC Substrate Kit for Peroxidase</td>
<td>Vector Laboratories, Burlingame, CA</td>
</tr>
<tr>
<td>BCA Protein Assay</td>
<td>Pierce, Rockford, IL</td>
</tr>
<tr>
<td>In Situ Cell Death Detection kit</td>
<td>Roche, Rotkreuz, Switzerland</td>
</tr>
<tr>
<td>iScript cDNA Synthesis kit</td>
<td>Bio-Rad Laboratories, Hercules, CA</td>
</tr>
<tr>
<td>Lumi-Light Western blotting Substrate</td>
<td>Roche, Rotkreuz, Switzerland</td>
</tr>
<tr>
<td>Qiagen RNeasy Fibrous Tissue Mini kit</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Qiagen RNeasy MinElute Cleanup kit</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>RNase-Free DNase Set</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>T-Per, tissue protein extraction reagent</td>
<td>Pierce, Rockford, IL</td>
</tr>
</tbody>
</table>

### 2.1.7 Cell culture reagents

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-amino-actinomycin (7-AAD)</td>
<td>BD Pharmingenen, Allschwil, Switzerland</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Cell culture plastic material</td>
<td>Nunc, Roskilde, Denmark</td>
</tr>
<tr>
<td>Cholera Toxin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Costar 12mm Snapwell Insert with 3.0µm Pore Polycarbonate Membrane</td>
<td>Corning, Lowell, MA</td>
</tr>
<tr>
<td>Defined keratinocyte serum free medium</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Item</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>DMEM</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>DMSO</td>
<td>VWR, Dietikon, Switzerland</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Fetal calf serum (FCS)</td>
<td>Amimed, BioConcept, Allschwil, Switzerland</td>
</tr>
<tr>
<td>Fibroblast growth factor (FGF10)</td>
<td>R&amp;D Systems, Minneapolis, MN</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Fungizone</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>IL-1F8</td>
<td>R&amp;D Systems, Minneapolis, MN</td>
</tr>
<tr>
<td>Insulin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Calbiochem, San Diego, CA</td>
</tr>
<tr>
<td>Keratinocyte growth factor (KGF/FGF7)</td>
<td>Amgen, Thousands Oaks, CA</td>
</tr>
<tr>
<td>Minimal Essential Medium Eagle</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Nylon cell strainer (70µm)</td>
<td>BC, Allschwil, Switzerland</td>
</tr>
<tr>
<td>Penicillin/streptomycin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Pharm Lyse lysis buffer</td>
<td>BD Pharmingen, Ort</td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Swabs</td>
<td>Paul Hartmann AG, Heidenheim · Germany</td>
</tr>
<tr>
<td>Triiodo-L-Thyronine (T3)</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Trypsin (1:250)</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Trypsin/EDTA solution</td>
<td>Sigma, Buchs, Switzerland</td>
</tr>
<tr>
<td>Vitrogen Collagen In Solution</td>
<td>Cohesion Technologies Inc., Palo Alto, CA</td>
</tr>
</tbody>
</table>

### 2.1.8 Standard buffers and solutions

Standard solutions were prepared as described previously in “Current Protocols in Molecular Biology” (Ausubel, 2001).
2.2 Methods

2.2.1 DNA methods

2.2.1.1 Genomic DNA isolation

Small parts (0.2 cm) of tail tips from mice at the age of 18 to 21 days were clipped to extract genomic DNA. Tails were digested in 250 µl of Lysis buffer together with 10 µl of proteinase K (10 mg/ml). The samples were incubated overnight at 55°C and thereafter heated to 95°C for 5 min. Before using 1 µl of the supernatants for genotyping PCR, samples were vortexed and centrifuged at 13000 rpm for 5 min in a microcentrifuge to remove cell debris.

*Lysis buffer (in water):*

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (pH 8.0)</td>
<td>50 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>50 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>NP-40</td>
<td>0.45%</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.45%</td>
</tr>
</tbody>
</table>

2.2.1.2 Polymerase Chain Reaction

The genotyping PCR was carried out in 20 µl reaction volume. Water samples served as negative controls.

*Reaction mix:*

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Extracted tail) DNA</td>
<td>1 µl</td>
<td>1.94°C</td>
<td>4 min</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>2.5 µl</td>
<td>2.94°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>0.45 µl</td>
<td>3.60°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.15 µl</td>
<td>4.72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Primer mix (10 mM each)</td>
<td>1 µl</td>
<td>5.72°C</td>
<td>10 min</td>
</tr>
<tr>
<td>dH₂O</td>
<td>14.9 µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genotyping PCR with FGFR1IIIb primers was conducted at 58°C instead of 60°C as annealing temperature.
2.2.2 RNA methods

2.2.2.1 RNA extraction from large tissue samples

Collection of tissue samples was performed as described in 2.2.8.5. RNA extraction was performed using a modified protocol originally described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). In brief: Snap frozen samples were homogenized using an Ultra Turrax (Janke & Kunkel, Staufen, Germany) in Trizol buffer on ice. Samples were centrifuged thereafter at 4000 rpm for 20 min at 4°C in a microcentrifuge. The supernatant was transferred to a new tube and 0.2x volume of chloroform was added to the sample, mixed well, and the mixture was centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and 0.5x volume of chloroform was added, mixed well, and the mixture was centrifuged for 10 min. This step was repeated once. 0.5x volume of 100% isopropanol and 0.5x volume of 0.8M sodium citrate/1.2M NaCl was subsequently added to the supernatant, mixed well, and the samples were incubated overnight at -20°C. Samples were then centrifuged at 4000 rpm for 1h at 4°C and the pellets were washed once with 75% ethanol, centrifuged for 5 min, and all the liquid was removed afterwards. Samples were air-dried prior to dissolving the pellets in DEPC-H₂O. RNAs were frozen in liquid nitrogen and stored at -80°C.

*Trizol buffer (in DEPC-water):*

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol in saturated buffer</td>
<td>38%</td>
</tr>
<tr>
<td>Guanidinium thiocyanate</td>
<td>0.8M</td>
</tr>
<tr>
<td>Ammonium thiocyanate</td>
<td>0.4M</td>
</tr>
<tr>
<td>Sodium acetate (pH 5.0)</td>
<td>0.1M</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5%</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>1%</td>
</tr>
</tbody>
</table>

2.2.2.2 RNA extraction with kit (for small tissue samples)

To isolate the RNA from small tissue samples the RNeasy (Fibrous Tissue) Mini kit (Qiagen, Hilden, Germany) was used following the manufacturer’s
recommendations. Either on-column digestion of potentially contaminating DNA with the RNase-Free DNase Set (Qiagen) or treatment of purified RNA with RQ1 DNase (Promega, Madison, WI) was performed.

2.2.2.3 RNA extraction from cells

Ice-cold PBS was used to wash cultured cells twice before adding Trizol buffer to the cells.

The cells were scraped off the dish and were then treated following the protocol described in 2.2.2.1.

2.2.2.4 Determination of nucleic acid concentrations

The absorption at 260nm was used to measure nucleic acid concentration. A Nanodrop Spectrophotometer ND-1000 (NanoDrop products, Wilmington, DE) was used for the determination of RNA and DNA concentration. The following approximations were used:

\[ \text{OD}_{260} = 1 \approx 50 \mu g \text{ DNA/ml} \]
\[ \text{OD}_{260} = 1 \approx 40 \mu g \text{ RNA/ml} \]

The relation of nucleic acid absorbance at 260nm to protein absorbance at 280nm was taken as an index of purity of the measured nucleic acid solution. Ideal values are 2.0 for RNA and 1.8 for DNA.

2.2.2.5 Agarose gel electrophoresis

Separation of RNA and DNA fragments was performed using 1% agarose gels in TBE for RNA and 2% agarose gels for DNA containing 1 \( \mu g/ml \) ethidium bromide. Loading dye was added to RNA and DNA samples and separation was performed at 120V until single bands were distinguishable. Gels were photographed thereafter in a Molecular Imager Gel Doc WR System (Bio-Rad Laboratories, Hercules, CA) with UV light (265nm).
**TBE buffer (in water):**

Tris-HCl 38.5 mM  
Boric acid 50 mM  
Na₂EDTA 1 mM  

**Loading buffer (in water):**

Saccharose 40%  
Bromophenol blue 0.25%  
Xylene Cyanol 0.25%  

2.2.2.6 qRT-PCR

A Roche LightCycler 480 SYBR Green system (Roche, Rotkreuz, Switzerland) was used following the manufacturer’s instructions to determine relative expression levels of genes. 20 µl reaction volumes were used and the different samples were run in duplicates. Enzyme reaction mix supplied by the manufacturer was applied and a standard amplification program was applied (95°C, 10 sec; 60°C, 20 sec; 72°C, 30 sec). Negative control samples (“minus RT”) should detect genomic DNA contaminations and verify the specificity of the self-designed primers.

2.2.2.7 RNase Protection Assay

The detection of the expression level of certain mRNAs was performed as described previously (Ma et al., 1996). In brief: Template DNA in a pBluescript II KS(+/−) vector was digested using the appropriate restriction enzyme. The linearized template DNA then was extracted, precipitated and washed and thereafter incubated with a reaction mix for 1h at 37°C to obtain the radioactively labeled RNA-probe. The probe was next separated by SDS-PAGE from the template, excised from the gel and eluted with elution buffer. The probe’s activity was measured in a scintillation counter (Tri-Carb 1500, Liquid Scintillation Analyzer, Canberra Packard, Schwadorf, Austria) and 0.5 to 1x10⁵ cpm were used for hybridization of the probe with the sample RNA. Probe and sample RNA were precipitated, and the pellet was resuspended in FAB solution.
For hybridization the samples were denatured and incubated in a 42°C water bath overnight. Single-stranded RNA was digested with an RNase cocktail to get rid of non-probe-bound RNA. Pellets were resuspended in FLB solution, the hybridized RNAs denatured and separated by SDS-PAGE. Labeled probes were visualized by exposing the dried gel to an X-ray film (Phosphor Imager Storm 820, GE Healthcare, Glattbrugg, Switzerland) overnight.

**Reaction mix:**

- DEPC-H₂O: 3.5 µl
- 10x nucleotide mix: 1.0 µl
  (5mM ATP, CTP, GTP)
- 10x transcription buffer: 1.0 µl
- RNasin (40 µg/µl): 0.5 µl
- RNA polymerase: 0.5 µl
- ³²P-UTP: 2.5 µl
- Linearized template (1 µg/µl): 1.0 µl

**FLB solution:**

- Deionized formamide: 100%
- Bromophenol blue: 0.05%
- Xylene cyanol: 0.05%

**RNase cocktail:**

- RNase buffer: 297 µl
- RNase A (10 µg/µl): 1 µl
- RNase T1 (100 U/µl): 2 µl

### 2.2.3 Protein methods

#### 2.2.3.1 Preparation of protein lysates from mouse skin

An Ultra Turax homogenizer (Janke & Kunkel, Staufen, Germany) was used to homogenize snap frozen tissue samples in tissue protein extraction reagent (T-Per, Pierce, Rockford, USA) loaded with phosphatase and protease inhibitors. On
ice the lysed samples were sonicated and centrifuged (1h, 4°C, 4000rpm) thereafter. Three rounds of transferring supernatant to a new tube spilling over fat and centrifuging were performed. (dieser Satz ist unverständlich). The samples were then snap frozen in liquid nitrogen and stored at -80°C.

**T-Per plus:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>10 µg/µl</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>50 µg/µl</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>100 µg/µl</td>
</tr>
<tr>
<td>AEBSF</td>
<td>0.525 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>Na₄VO₃</td>
<td>1 mM</td>
</tr>
<tr>
<td>PAO</td>
<td>20 µM</td>
</tr>
<tr>
<td>NaF</td>
<td>50 mM</td>
</tr>
<tr>
<td>Na₄P₂O₇</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

2.2.3.2 Preparation of protein lysates from keratinocytes

Cells were immediately put on ice, medium was aspirated, keratinocytes were washed once with PBS, and T-Per buffer supplemented with protease and phosphatase inhibitors was added. Lysates were then centrifuged for 15 min at 13000 rpm (4°C) in a microcentrifuge, and the supernatant was transferred to a fresh tube, snap frozen and stored at -80°C.

2.2.3.3 Determination of protein concentrations

The BCA Protein Assay kit (Pierce, Rockford, IL) was used for the measurement of protein concentration in lysates following the manufacturer`s instructions. For this purpose, 25 µl of sample was mixed with 200 µl of BCA working reagent, incubated for 30 min at 37°C and measured spectrophotometrically at 562 nm using a Molecular Devices SpectraMax 190 (Bucher Biotec AG, Basel, Switzerland).
2.2.3.4 Tris/glycine SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Stacking and separating gel were prepared using the appropriate percentage of acrylamide/biscrylamide. Protein lysates were denatured by adding Laemmli buffer (2.5x) and subsequent incubation for 5 min at 95°C. They were then loaded onto the polymerized gel. Electrophoresis was performed at 20 mA per gel in SDS-PAGE running buffer.

**Separating gel:**

<table>
<thead>
<tr>
<th></th>
<th>8%</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>4.7 ml</td>
<td>3.3 ml</td>
<td>2.35 ml</td>
</tr>
<tr>
<td>30% Acrylamide/</td>
<td>2.6 ml</td>
<td>4.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>0.8% bisacrylamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris (1.5M, pH 8.8)</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004 ml</td>
<td>0.004 ml</td>
<td>0.004 ml</td>
</tr>
</tbody>
</table>

**Stacking gel:**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>4.1 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide/</td>
<td>1.0 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8% bisacrylamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris (1M, pH 6.8)</td>
<td>0.75 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.06 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% APS</td>
<td>0.06 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>0.006 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**SDS-PAGE running buffer (in water):**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>25 mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>192 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

**Laemmli buffer (5x, in water):**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 6.8)</td>
<td>312.5 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>10%</td>
</tr>
</tbody>
</table>
Glycerol 50%
2-Mercaptoethanol 12.5%
Bromophenol blue 0.5%

2.2.3.5 Western blotting

A Criterion Wet Blotter (Bio-Rad, Reinach, Switzerland) was used to transfer proteins from the gel to a nitrocellulose membrane. The gel was placed on top of the membrane in a transfer buffer bath. Three Whatman 3 MM papers, soaked in transfer buffer, were placed on both sides of this sandwich. The whole stack was placed into the blotter so that the gel was next to the cathode and the membrane next to the anode. The transfer was performed for 1.5 h at 100 V and 4°C using Towbin buffer as transfer buffer. Membranes were washed once with water after blotting and were stained with Ponceau S to rapidly and reversibly detect the proteins on the membrane. The staining solution was washed off with TBST.

Proteins were blocked with 5% skimmed milk and incubated overnight with primary antibody in 5% BSA/TBST solution at 4°C. The membrane was washed with TBST for 20 min 3 times at RT and thereafter incubated with the corresponding HRP-coupled secondary antibody in 5% milk for 1 h at RT. After washing 3 times with TBST for 20 min, Lumi-Light Western Blotting Substrate (Roche, Rotkreuz, Switzerland) was used according to the manufacturer’s recommendation to detect the protein of interest.

* * *

**Towbin buffer (in water):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>25 mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>192 mM</td>
</tr>
<tr>
<td>Methanol</td>
<td>20%</td>
</tr>
</tbody>
</table>

2.2.3.6 Stripping of Western blot nitrocellulose membranes

To remove primary and secondary antibodies from the membrane and to investigate the expression of other proteins, the membrane was shrink-wrapped and 10 ml of stripping buffer was added. The membrane was incubated like this
in a 55°C water bath for 30 min. It was rinsed with water several times and
washed 4 times for 15 min with TBST. Now the membrane was ready for
blocking with skimmed milk and antibody incubation as described above.

**Stripping buffer (in water):**

- Tris-HCl, pH 6.8: 62.5 mM
- SDS (w/v): 2%
- 2-Mercaptoethanol: 0.65%

2.2.3.7 Zymography

Immortalized keratinocytes from control and K5-R1/R2 mice were cultured for 3
days at 100% confluence. Supernatant of the cells was then harvested and
centrifuged to remove dead cells and cell debris. Centrifugal filter units (Amicon,
Millipore, Billerica, MA) were used to concentrate the proteins in the
supernatant. Equal amounts of native protein were analyzed on a 10%
polyacrylamide gel either containing 1 mg/ml casein or 1.5 mg/ml gelatin by gel
electrophoresis performed at 4°C and 120V for 2h. The gels were subsequently
incubated in a 2.5% Triton X-100 solution three times for 15min. After
thoroughly washing of the gels with water they were incubated for 24h at 37°C
in incubation buffer. Gels were stained for 30min with Coomassie blue and
destained thereafter.

**Triton solution (in water):**

- Triton X-100: 2.5%

**Incubation buffer (in water):**

- Tris-HCl, pH 7.5: 50mM
- CaCl₂: 10mM
- NaN₃: 0.02%

**Staining solution (in water):**

- Methanol: 50%
- Acetic acid: 10%
- Coomassie: 2.5mg/ml
Brilliant Blue R-250

*Destaining solution (in water)*:

Methanol 40%

Acetic acid 10%

2.2.4 Preparation of single cell suspensions from mouse tissues and fluorescence-activated cell sorting (FACS)

2.2.4.1 Preparation of single cell suspensions from mouse spleen

Spleens were harvested from mice and passed through a 70 μm nylon cell strainer (BD Falcon), which was washed with DMEM medium. The solution was then centrifuged for 10 min at 4°C and 1200 rpm. The pelleted cells were resuspended in Pharm Lyse lysis buffer (BD Pharmingen) and incubated for 5 min to rupture the erythrocytes. After centrifugation, the cell suspension was washed with FACS buffer.

*FACS buffer (in PBS)*:

PBS 1 mM

BSA 0.5 mM

EDTA 10 μg/ml

2.2.4.2 Preparation of single cell suspensions from mouse skin

Single cell suspensions from mouse skin were prepared as described previously (Sullivan et al., 1985). In brief: Skin was collected from mice, and dermis and epidermis were separated by incubating the total skin in 0.2% trypsin in DMEM at 37°C for 2h. The separated layers were thereafter incubated for another 10 min in 0.2% trypsin in DMEM containing 0.1 mg/ml DNase I. 10% FCS was added to stop the trypsin activity. The dermis was then cut in small fragments and incubated in Dermis buffer under continuous slow shaking for 1h at 37°C. Before dermis and epidermis cell suspensions were washed with FACS buffer they were filtered through 70 μm nylon cell strainers (BD Falcon).
**Dermis buffer (in DMEM):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase II</td>
<td>2.5 mg/ml</td>
</tr>
<tr>
<td>Collagenase IV</td>
<td>2.5 mg/ml</td>
</tr>
<tr>
<td>DNase</td>
<td>0.5 mg/ml</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>1 mg/ml</td>
</tr>
</tbody>
</table>

### 2.2.4.3 Fluorescence-activated cell sorting (FACS)

Cell suspensions were blocked with rat anti-mouse CD16/CD32 at 4°C for 10 min prior to washing the cells with FACS buffer once. Fluorochrome-coupled primary antibodies were incubated with the cell suspension at 4°C for 20 min and cells were washed thereafter once with FACS buffer. For the identification of dead cells 7-AAD was added to the cell suspensions. The following data acquisition was done using FACSCalibur together with CellQuestPro software (BD Bioscience, San Jose, CA). The FlowJo software (Tree Star Inc., Ashland, OR) was used to perform analysis of the collected data.

### 2.2.5 Cell culture

#### 2.2.5.1 Isolation of primary keratinocytes

Isolation of primary keratinocytes from mouse skin was performed as previously described (Braun et al., 2002) with some changes. In short, epidermis of 3-6 day-old or 21-23 day-old mice was separated as described in 2.2.8.5. The epidermis next was incubated in DMEM (Invitrogen, Basel, Switzerland) supplemented with 10% FCS, 0.25 mg/ml DNaseI, 200 µg/ml streptomycin, 200 U/ml penicillin at RT for 1h under continuous shaking. After filtering the cell suspension through a 70 µm cell strainer (BD Falcon), samples were centrifuged 5 min at 1200 rpm. Cells were resuspended in fresh defined keratinocyte serum-free medium (Invitrogen, Basel, Switzerland) containing 10 ng/ml EGF and seeded in collagen IV (2.5 µg/cm² (Sigma, Buchs, Switzerland)) coated dishes at a density of 5x 10⁴ cells/ cm². After one day of attachment, non-adherent cells were washed away.
and fresh medium was added. Freshly isolated cells were cultured at 37°C with 5% CO₂.

2.2.5.2 Transwell/Boyden chamber assay

The lower side of a transwell filter (Costar #3422) was coated with 200 µl of coating medium for 2h at 37°C. The filter was then rinsed with PBS. Keratinocytes were trypsinized, centrifuged for 5 min at 1200 rpm, resuspended in pure (no supplements and no EGF) defined Keratinocyte-Serum Free Medium (df) and counted. 4x10⁴ cells were seeded on each filter in 200 µl of pure df medium. To the bottom part of the well 600 µl of pure df medium was added containing FCS, EGF, FGF7 or FGF10. Df medium without additives served as a control. Keratinocytes migrated for 24h, and filters were then transferred to PBS to wash them. Thereafter, cells on the upper side of the filter that had not migrated were wiped off with a cotton swab. Cells on the lower side of the filter were fixed for 30min in 4% PFA. To visualize the cells, Haematoxylin/Eosin staining was performed as described in 2.2.6.4, although haematoxylin-staining time was prolonged to 1h. To determine the number of migrated cells 4 independent microscopic fields (200x magnification) per filter were counted.

*Coating medium:*

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>25 ml</td>
</tr>
<tr>
<td>BSA (1 mg/ml)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Hepes (1M)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Vitrogen 100 (3 mg/ml)</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>Fibronectin (1 mg/ml)</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>CaCl₂ (100 mM)</td>
<td>0.29 ml</td>
</tr>
</tbody>
</table>

*Growth factor concentrations:*

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>10%</td>
</tr>
<tr>
<td>EGF</td>
<td>20 ng/ml</td>
</tr>
<tr>
<td>FGF7</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>FGF10</td>
<td>10 ng/ml</td>
</tr>
</tbody>
</table>
2.2.5.3 Adhesion assay

A 48-well plate was coated at 4°C overnight with different extracellular matrix proteins. The coated wells were subsequently washed with PBS once and blocked with 1% BSA/PBS for 3h at RT. Immortalized keratinocytes were seeded into the wells at a density of 4x10^4 cells per well. Keratinocytes were allowed to attach for 45 min at 37°C. Floating cells were removed by washing the wells 3 times with PBS. Substrate buffer was then added and the cells were incubated overnight at 37°C. Before absorbance was measured at 405 nm in a plate reader (Molecular Devices, Spectra Max190), stop buffer was added to the cells for visualization.

Coating proteins (in PBS):
BSA/PBS 1%
Collagen I 5 µg/ml
Collagen IV 5 µg/ml
Fibronectin 5 µg/ml
Laminin I 5 µg/ml

Substrate buffer (in water):
Sodium citrate pH 5.0 0.1 M
NPAG 7.5 mM
Triton X-100 0.5%

Stop buffer (in water):
Glycine 3.75 g/l
EDTA (0.5 M) 1%

2.2.5.4 Rac1 pull-down assay

45 µl glutathione-coupled beads were added to GST-fused-PAK1 protein and incubated at 4°C for 30 min under continuous rotation. Beads were next washed 3 times with Bacterial Lysis buffer and once with FISH buffer. Another 200 µl of glutathione-coupled beads were washed 4 times with FISH buffer without adding
GST-fused-PAK1 protein. The two bead suspensions were subsequently pooled and split into 3 fractions. As a negative control one aliquot was not incubated with cell suspension. 80% confluent keratinocytes of control and double-knockout mice were lysed, centrifuged, and the lysates were added to the washed beads. 10% of the cell lysate was saved as input. Beads were incubated with cell suspension at 4°C for 1h under continuous rotation. After washing the beads 3 times with FISH buffer they were resuspended in 5 µl FISH buffer and 15 µl 5x Laemmlli buffer. Samples were heated at 95°C for 10 min and subsequently filtered through a Mobitec column (MoBiTec, Goettingen, Germany). The samples were then stored at -80°C.

2.2.5.5 Spreading/adhesion assay

Keratinocytes of both genotypes were seeded into 4-chamber-slides. 20 or 90 min after seeding cells were fixed and stained as described under 2.2.6.9. with antibodies against Paxillin and phospho-Paxillin.

2.2.5.6 Scratch assay

Keratinocytes were seeded either in 4-chamber slides or in 12-well plates. When they reached 100% confluence, the medium was changed to a 1:3 mix of keratinocyte growth medium (Chrostek et al., 2006) / defined medium. 4h after changing the medium one or several scratches were made into the cell layer with a 200 µl pipette tip. For live cell imaging cells were immediately transferred to the microscope. When keratinocytes were stained after scratch wounding they were fixed 6h after scratching the cell layer and stained as described in 2.2.6.8.

2.2.5.7 BrdU assay for cells

To determine the in vitro proliferation rate, cells were seeded in defined medium with additives into 4-chamber slides or 6-well plates such that they were 50% confluent the next morning. 24h after seeding the medium was replaced by medium containing 100µM BrdU for 2h. Thereafter, cells were washed twice with PBS, fixed in 4% PFA for 30 min at RT and treated with 2M HCl/0.1% Triton
X-100 for 30 min to denature the DNA and to permeabilize the cells. The acid solution was buffered by incubating the cells with 0.1 M sodium borate (pH 8.5) for 5 min. Cells were subsequently incubated with blocking solution for 30 min to block unspecific binding sites, followed by incubation with FITC-conjugated anti-BrdU antibody and propidium iodide overnight at 4°C. After washing the cells 3 times with PBS they were mounted with Mowiol.

2.2.5.8 Treatment of cultured cells with FGF7

Cells were seeded into 12-well plates at a density of 5x10^5 cells per well. When the cells reached confluency they were starved in pure defined medium (no addition of supplements and EGF) for 24h. Thereafter, 10 ng/ml FGF7 was added to the cells and they were harvested at different time points. Cells cultured without FGF7 served as negative controls and cells cultured with 20 ng/ml EGF served as positive controls. Harvested cells were then used for RNA and protein analysis and processed accordingly.

2.2.5.9 IL-1F8 treatment of fibroblasts

Embryonic immortalized fibroblasts were seeded in 12-well plates and cultured in DMEM supplemented with 10% FCS. They were starved overnight (DMEM containing 0.1% FCS) and treated thereafter with 500 ng/ml IL-1F8 for 15 min or 3h. Non-treated fibroblasts served as negative control. RNA was isolated from the cells and used for qRT-PCR analysis.

2.2.5.10 Analysis of transepithelial electrical resistance

Three different immortalized cell lines of control and K5-R1/R2 mice were seeded on a permeable support (Costar Snapwell, Sigma, Buchs, Switzerland) containing a 0.4 μm pore polycarbonate filter membrane and grown to confluence in defined medium. Medium was then changed to differentiation medium and TER was measured daily for 7 – 14 days with an epithelial tissue volt ohmmeter (World Precision Instrument, Sarasota, FL) as described in Balda et al., 1996.
**Differentiation medium (in DME/F-12, 1:1):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>10%</td>
</tr>
<tr>
<td>T3</td>
<td>2 nM</td>
</tr>
<tr>
<td>Transferrin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.4 µg/ml</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>Insulin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Penicillin/streptomycin</td>
<td>1%</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>0.1 nM</td>
</tr>
<tr>
<td>EGF</td>
<td>10 ng/ml</td>
</tr>
</tbody>
</table>

**2.2.5.11 Live cell imaging and analysis of scratch assay movies**

A 12-well plate with scratched cell layers of keratinocytes of control and K5-R1/R2 mice was placed into the incubator box of a Zeiss live cell station and pictures were acquired for 13h (Zeiss 200, Zeiss, Feldbach, Switzerland) by means of Metamorph 7.53 (Molecular Devices, Sunnyvale, CA). 8 cells of each genotype from 5 different movies were analyzed with regard to velocity, displacement (linear distance from starting to end point), persistence coefficient (as described in (Hartwig et al., 2007) ) and perpendicular movement into the scratch.

**2.2.5.12 Cell ruffle analysis**

Single pictures taken at 4 different time points of the live cell imaging experiment were used to count cells with ruffles in each control and K5-R1/R2 cell line. For every time point at least 5 pictures were analyzed.
2.2.6 Histology

2.2.6.1 Preparation of paraffin sections

Mice were sacrificed and the back skin was excised and placed on a nylon membrane (Millipore, type HPLV 0.45 µm). Pieces of the back skin were fixed in 4% PFA in PBS or in AcOH (acidic ethanol: 1% acetic acid, 95% ethanol) at 4°C overnight and processed in a Microm STP 120 Spin Tissue Processor (ThermoScientific, Walldorf, Germany). The following programs were used:

**PFA-fixed samples:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>30 min</td>
</tr>
<tr>
<td>0.9% NaCl</td>
<td>30 min</td>
</tr>
<tr>
<td>30% EtOH/0.9% NaCl</td>
<td>30 min</td>
</tr>
<tr>
<td>50% EtOH/0.9% NaCl</td>
<td>30 min</td>
</tr>
<tr>
<td>70% EtOH/0.9% NaCl</td>
<td>30 min</td>
</tr>
<tr>
<td>90% EtOH/0.9% NaCl</td>
<td>30 min</td>
</tr>
<tr>
<td>96% EtOH/0.9% NaCl</td>
<td>30 min</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>60 min</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>30 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>30 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>30 min</td>
</tr>
<tr>
<td>Paraffin (60°C)</td>
<td>2.5h</td>
</tr>
<tr>
<td>Paraffin (60°C)</td>
<td>≥ 3h</td>
</tr>
</tbody>
</table>

**Acetic ethanol-fixed samples:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% EtOH</td>
<td>60 min</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>30 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>30 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>30 min</td>
</tr>
<tr>
<td>Paraffin (60°C)</td>
<td>2.5h</td>
</tr>
<tr>
<td>Paraffin (60°C)</td>
<td>≥ 3h</td>
</tr>
</tbody>
</table>
Using a Leica EG 1150H (Leica, Wetzlar, Germany) tissue embedding machine the skin samples were embedded in paraffin after processing and preserved at 4°C.

Paraffin embedded skin was cut in 7µm thick slices using a HM 355S microtome with Cool Cut and Section-Transfer-System STS (Microm AG, Walldorf, Germany). After heating the sections in a 40°C water bath for better spreading, sections were placed onto glass slides. Thereafter, sections were dried overnight at 37°C and stored at 4°C.

2.2.6.2 Preparation of cryosections

Back skin was harvested from mice and placed on a nylon membrane. The tissue was embedded in tissue freezing medium (Jung, Nussloch, Germany), slowly frozen on dry ice and stored at -80°C. Using a Microm cryostat (Walldorf, Germany) 7µm thick sections were prepared, dried at room temperature and stored at -80°C.

2.2.6.3 Deparaffinization

Prior to staining skin sections they were deparaffinized according to the following protocol:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene I</td>
<td>10 min</td>
</tr>
<tr>
<td>Xylene II</td>
<td>10 min</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>96% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>90% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>80% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>50% Ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>dH₂O</td>
<td>1 min</td>
</tr>
</tbody>
</table>
2.2.6.4 Haematoxylin/Eosin staining

Sections were deparaffinized and stained as follows:

- Haematoxylin: 1 min
- ddH$_2$O: 3 x 10 sec
- Scott-water: 30 sec
- 70% EtOH: 10 sec
- Eosin/90% EtOH: 1 min
- 80% EtOH: 2 x 10 sec
- 95% EtOH: 2 x 10 sec
- 100% EtOH: 2 x 10 sec
- Xylene I: 10 min
- Xylene II: 10 min

Thereafter, sections were mounted with Eukitt permanent mounting medium and dried overnight at room temperature.

2.2.6.5 Masson Trichrome staining

To visualize collagen in skin sections Masson Trichrome staining was performed with deparaffinized PFA-fixed sections using the following protocol:

- Bouin's solution: 1h 60°C
- dH$_2$O: 10 sec 60°C
- dH$_2$O: 2 x 10 sec RT
- Weigert's Haematoxylin: 10min RT
- Tap H$_2$O: 2 x 10 min RT
- dH$_2$O: 10 sec RT
- BSAF: 10 min RT
- dH$_2$O: 2 x 10 sec RT
- PMPT: 5 min RT
- Aniline Blue: 5 min RT
Sections were then mounted with Eukitt permanent mounting medium and dried overnight at RT.

*Bouin’s solution:*

Saturated picric acid 375 ml
37% formaldehyde 125 ml
Acetic acid 25 ml

*Weigert’s haematoxylin solution A:*

Haematoxylin 5 g
95% EtOH 500 ml

*Weigert’s haematoxylin solution B:*

29% FeCl₃ 20 ml
dH₂O 475 ml
HCl 5 ml

*Weigert’s Haematoxylin:*

Weigert’s A + Weigert’s B 1:1

*BSAF:*

1% Biebrich Scarlett 180 ml
1% Acid Fuchsin 20 ml
Acetic acid 2 ml

*PMA:*

Phosphomolybdic acid 15 g
**dH₂O**: 300 ml

**PTA**:  
Phosphotungstic acid 15 g  
dH₂O 300 ml

**PMPT**:  
PMA + PTA 1:1

**Aniline Blue**:  
Aniline Blue 5 g  
dH₂O 200 ml  
Acetic acid 4 ml

### 2.2.6.6 Mast cell staining

Deparaffinized PFA- or AcOH-fixed sections were incubated with 0.5% Toluidine Blue in 0.5N HCl for 30 min to stain mast cells. Sections were counterstained with haematoxylin.

### 2.2.6.7 Immunohistochemistry

Deparaffinized back skin sections were treated with 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity. The sections were then washed in PBS for 10 minutes and boiled in a pressure cooker (micro MED TT Mega) in 10mM citrate pH 6.0 for 15 minutes. After cooling, sections were rinsed 2 to 3 times with PBS and blocked with 3% BSA, 0.1%NP-40, 0.02% sodium azide for 1h at room temperature. Primary antibody diluted in blocking solution was added overnight at 4°C. Sections were then washed once in PBST for 10 minutes and additionally 3 times in PBS for 5 minutes. Secondary antibody conjugated with biotin was added diluted in blocking solution for 1h at room temperature. Sections then were washed 3 times in PBST for 10 minutes and incubated for 30 minutes at room temperature with Vectastain ABC (avidin-biotin-complex) solution. The solution contains preformed avidin/biotinylated enzyme (horseradish peroxidase) complex. After washing the sections again for
3 times with PBST the complex was visualized by adding the substrate for peroxidase, namely 3-amino-9-ethylcarbazole (AEC) solution. After rinsing the sections with water to stop the color reaction sections were counterstained with haematoxylin.

2.2.6.8 Immunofluorescence

Cryosections were air-dried for 15 min and fixed in ice-cold methanol or acetone at -20°C for 10 min and subsequently washed in PBST. Acetic ethanol-fixed and PFA-fixed sections were deparaffinized as described in 2.2.6.3. and washed with PBST prior to PBS. The antigen retrieval procedure was generally applied to PFA-fixed sections. This procedure included an incubation for 15 min at 100°C in a pressure cooker in 0.01M citrate buffer (pH 6.0). Sections were then blocked with 3% BSA, 0.1%NP-40, 0.02% sodium azide for 1h at room temperature. Primary antibody diluted in blocking solution was applied over night at 4°C. Sections were washed once in PBST and twice in PBS for 10 min and incubated with fluorochrome-conjugated secondary antibody for 1h at room temperature. For nuclear staining Hoechst or propidium iodide were added. After washing 3 times with PBS for 10 min sections were mounted with Mowiol.

2.2.6.9 Immunofluorescence staining of cells

Cells were washed twice with PBS for 5 min after aspirating the medium. They were then fixed in 4% PFA for 30 min and subsequently washed 3 times for 5 min in PBS at RT. To permeabilize the cells they were treated with 0.1% Triton X-100 in PBS for 30 min at RT and washed thereafter 3 times for 5 min with PBS. Before adding primary antibody solution overnight at 4°C cells unspecific binding sites on the cells were blocked for 1h with blocking solution at RT. Cells were washed thereafter first once with PBST for 5 min and then twice with PBS for 5 min and incubated with secondary antibody and Hoechst for nuclei staining for 1h at RT. After washing the cells once with PBST for 5 min and twice with PBS for 5 min cells were mounted with Mowiol and air-dried.
**2.2.6.10 BrdU staining using immunohistochemistry**

Acetic ethanol-fixed skin sections were deparaffinized as described in 2.2.6.3. and treated with 3% hydrogen peroxide methanol for 40 minutes at room temperature and washed thereafter twice for 10 minutes in PBS. To denature the DNA, sections were incubated with 2N HCl for 30 minutes and subsequently washed twice with 70% ethanol/0.1 M Tris pH 7.5, twice with 70% ethanol and twice with PBS for 10 minutes. Primary anti-BrdU-POD antibody diluted in 3% BSA, 0.1%NP-40, 0.02% sodium azide was applied overnight. After washing the sections twice with PBS for 10 minutes they were developed with DAB solution (0.05% DAB in 1mM Tris/HCl pH 7.5, 0.005% H₂O₂) in PBS. To stop the reaction, sections were rinsed with tap water for 5 minutes. Sections were counterstained with haematoxylin afterwards.

**2.2.6.11 BrdU staining using immunofluorescence**

Acetic ethanol-fixed sections were deparaffinized as described in 2.2.6.3. and then immersed in cold methanol for 10 min at 4°C. DNA was denatured by incubation of the sections in 2N HCl for 1h at 37°C and thereafter sections were incubated with 0.1M borate buffer (pH 8.5) 3 times for 10 min to neutralize the acid solution. Subsequently, sections were rinsed with PBS and incubated with blocking solution for 1h at RT. FITC-coupled anti-BrdU antibody diluted in blocking solution was added for overnight incubation at 4°C. This solution contained propidium iodide for staining of nuclei. Sections were washed 3 times with PBS for 10 min and mounted with Mowiol.

**2.2.6.12 Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay**

PFA-fixed sections were dewaxed and heated in 200ml of 0.1M NaCitrate buffer (pH 6.0) in a microwave (750W) for 1 min. Sections were cooled immediately by adding 80 ml of ddH₂O, and blocking solution (0.1M Tris/HCl pH 7.0, 3% BSA, 20% BSA) was added and incubated for 30 min. After 2 washes with PBS the sections were incubated in 50 µl of TUNEL reaction solution mixture (In situ Cell
Death Detection Kit, Roche) for 120 min at RT in the dark. They were then again washed 3 times for 5 min in PBS and mounted with Mowiol.

2.2.7 Microscopy

2.2.7.1 Confocal microscopy

Pictures of single, stained cells were taken at a confocal microscope (Leica TCS NT SP1, Leica, Heerbrugg, Switzerland) equipped with the Leica LCS software at a magnification of 6300x.

2.2.8 Animal experiments

2.2.8.1 Animal experiments (general)

Mice were kept under optimal hygienic conditions in accordance with the federal guidelines. Mice received water and food *ad libitum*. The local veterinary authorities of Zurich, Switzerland (Kantonales Veterinäramt der Stadt Zürich) had approved all mouse experiments.

2.2.8.2 Wounding and preparation of wound tissue

Mice at the age of 6 weeks, 3 or 5 months were anesthetized using ketamine (75 mg/kg)/xylazine (5mg/kg) in a single intraperitoneal injection. On both lateral sides of the back of the mice two full-thickness excisional wounds were made with a diameter of 5mm. Following the description of Thorey et al., 2001 the skin and rodent-specific subcutaneous muscle *panniculus carnosus* were excised. To track the process of wound healing mice were sacrificed at different time points after wounding and the wounds were harvested for histological analysis as well as RNA and protein expression level investigations. The histological specimens were processed thereafter according to 2.2.6.1. and stained sections from bisected wounds were analyzed for the different parameters as previously described (Kumin et al., 2007).
2.2.8.3 BrdU incorporation assay

Mice were injected intraperitoneally with a 5-bromo-2'-deoxyuridine (BrdU) solution (16.7 mg/ml in isotonic saline). Per 25 g of mouse body weight 5 mg of BrdU was used (equivalent to 300 µl of above-mentioned solution). 2h after injection mice were sacrificed and the tissue collected and fixed in acidic ethanol (95% ethanol, 1% acetic acid). Staining of BrdU incorporated cells was performed using immunohistochemistry 2.2.6.7. or immunofluorescence 2.2.6.8. Stained cells in four different microscopic fields (200x magnification) were counted for each section from mouse wounds to determine the percentage of BrdU positive cells.

2.2.8.4 Measurement of transepidermal water loss (TEWL)

30 min after shaving, transepidermal water loss of mice was measured using a Tewameter (Courage and Khazaka Electronic GmbH, Cologne, Germany). As following the manufacturer’s instructions, the probe was placed on the back skin of the mice and 4x20 sequential measurements were performed.

2.2.8.5 Tissue collection and follow-up preparation

Mice were euthanized by carbon dioxide inhalation, shaved at the back and dipped in an iodine solution prior to washing in 70% ethanol and PBS solutions. The skin was excised and subcutaneous fat was scraped off to increase protease efficiency. Skin fragments were then incubated twice for 5 min in PBS solutions supplemented with 50 µg/ml gentamycin. Subsequently, skin pieces were incubated in 0.25% dispase or 0.8% trypsin solution for 2h at 37°C, washed in PBS, and epidermis and dermis were separated using forceps. Dermis and epidermis were cut into small pieces, frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation or preparation of protein lysates. Longitudinal or cross sections of total skin were fixed as described in 2.2.6.1. Alternatively, they were directly frozen in tissue freezing medium for histological analysis.
For blood analysis mice were injected with a lethal dose of pentobarbital and the heart of the animals was punctuated. Blood was kept 30 min at RT to allow coagulation and subsequently centrifuged at 3000 rpm for 15 min. Plasma was collected, snap frozen in liquid nitrogen and stored at -80°C.

2.2.8.6 Cream treatment of mouse skin

Mice at the age of 10 days or 12 months were treated on their back and tail skin with moisturizing cream (Excipial Protect, SPIRIG PHARMA AG, Switzerland) containing aluminum-hydroxychloride and glycerine. Non-creamed mice served as negative controls. Creaming was either performed on the entire back or only on one flank of the back. In the latter case the other flank was used as not-treated control. Mice were treated daily for two months.

2.2.8.7 Acetone treatment of mouse skin

Seven adult female control mice were shaved at the back and the skin was cleaned with a water-soaked tissue. One flank of the back of the mouse was wiped ten times with a delipidized swab that had been soaked in acetone. The other flank was used as negative control and therefore treated in the same way with water. Mice were treated daily for 2 weeks, injected with BrdU thereafter and sacrificed 2h later for histological analysis.

2.2.8.8 IL-1F8 injection

Adult wild-type mice were injected intradermally at the back with 50 µl of 1 µg/ml IL-1F8 (R&D Systems) in 0.1% BSA in PBS. Vehicle injections served as negative control. 24h after injection the skin was isolated and proliferating cells were analyzed after an additional injection of BrdU 2h before sacrificing the mice.
2.2.9 Statistics

2.2.9.1 Statistical analysis

PRISM software (Graph Pad Software Inc., La Jolla, CA) was used for statistical analysis. For experiments examining differences between groups the Mann-Whitney U test was applied. *P ≤ 0.05, **P ≤ 0.005, ***P ≤ 0.001.
References


3. Results

3.1 FGF receptors 1 and 2 in keratinocytes control the epidermal barrier and cutaneous homeostasis

Jingxuan Yang1*, Michael Meyer1*, Anna-Katharina Müller1, Friederike Böhmd, Richard Grose2, Tina Dauwalder3, Francois Verrey3, Manfred Kopf4, Juha Partanen5, Wilhelm Bloch6, David M. Ornitz7, and Sabine Werner1

1Department of Biology, Institute of Cell Biology, ETH Zurich, Switzerland
2Centre for Tumour Biology, Institute of Cancer, Bart’s and The London, School of Medicine & Dentistry, Queen Mary University of London, UK
3Institute of Physiology and Center for Integrative Human Physiology, University of Zurich, Switzerland
4Department of Environmental Sciences, Institute of Integrative Biology, ETH Zurich, Switzerland
5Institute of Biotechnology, Viikki Biocenter, Helsinki, Finland
6Department of Molecular and Cellular Sport Medicine, German Sport University Cologne, Cologne, Germany
7Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO, USA
*Equal contribution

Condensed title: FGFs in skin homeostasis and barrier function

Key words: FGF, keratinocyte, tight junction, epidermal barrier, inflammation
Manuscript JCB 200910126
Abstract

Fibroblast growth factors (FGFs) are master regulators of organogenesis and tissue homeostasis. In this study we used different combinations of FGF receptor (FGFR)-deficient mice to unravel their functions in the skin. Loss of the IIIb splice variants of FGFR1 and FGFR2 in keratinocytes caused progressive loss of skin appendages, cutaneous inflammation, keratinocyte hyperproliferation and acanthosis. We identified loss of FGF-induced expression of tight junction components with subsequent deficits in epidermal barrier function as the mechanism underlying the progressive inflammatory skin disease. The defective barrier causes activation of keratinocytes and epidermal γδ T cells, which produce interleukin-1 family member 8 and S100A8/A9 proteins. These cytokines initiate an inflammatory response and induce a double paracrine loop through production of keratinocyte mitogens by dermal cells. Our results identify essential roles for FGFs in the regulation of the epidermal barrier and in the prevention of cutaneous inflammation and highlight the importance of stromal-epithelial interactions in skin homeostasis and disease.
List of non-standard abbreviations

FGFR: Fibroblast growth factor receptor
FRS: Fibroblast growth factor receptor substrate
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
G-CSF: Granulocyte colony stimulating factor
GM-CSF: Granulocyte macrophage colony stimulating factor
H/E: Hematoxylin/eosin
HGF: Hepatocyte growth factor
ICAM-1: Inter-cellular adhesion molecule 1
IL: Interleukin
PCNA: Proliferating cell nuclear antigen
RPS29: Ribosomal protein S29
TCR: T cell receptor
TER: Transepithelial electrical resistance
TEWL: Transepidermal water loss
Introduction

Fibroblast growth factors (FGFs) comprise a family of 22 polypeptides, which regulate migration, proliferation, differentiation and survival of different cell types. They exert these functions through activation of four transmembrane tyrosine kinase receptors, designated FGFR1-4 (Beenken and Mohammadi, 2009; Ornitz and Itoh, 2001). Further complexity is achieved by alternative splicing in the FGFR genes. Of particular importance is alternative splicing in the third immunoglobulin-like domain of FGFR1-3, which generates IIIb and IIIc variants of these receptors that are characterized by different ligand binding specificities (Ornitz and Itoh, 2001). For example, the IIIb splice variant of FGFR2 (FGFR2IIIb) is a high affinity receptor for FGF7, FGF10, and FGF22, whereas the IIIc variant (FGFR2IIIc) binds a variety of other FGF ligands (Zhang et al., 2006). Previous studies revealed important roles of FGFs in development, homeostasis and repair of the skin (Steiling and Werner, 2003). Several FGFs are expressed in this tissue, and most of them are upregulated upon injury (Komi-Kuramochi et al., 2005; Werner et al., 1992; Werner et al., 1993). Of particular interest are ligands of FGFR2IIIb, since transgenic mice expressing a dominant-negative mutant of this receptor in keratinocytes showed epidermal atrophy, hair follicle abnormalities, and impaired wound reepithelialization (Werner et al., 1994). However, the responsible receptor(s) remain(s) to be identified, since the dominant-negative mutant blocks the action of all FGF receptors in response to common FGF ligands (Ueno et al., 1992). The abnormalities seen in these animals were not observed in FGF7 knockout mice (Guo et al., 1996), suggesting functional redundancy among different FGFs and possibly FGF receptors. Indeed, expression studies revealed that FGF10 and FGF22 are also expressed in normal and wounded skin (Beer et al., 1997; Beyer et al., 2003; Nakatake et al., 2001). Together with FGF7 they can activate the “b” splice variants of FGFRs 1 and 2 (Zhang et al., 2006) that are expressed in keratinocytes (Beer et al., 2000; Zhang et al., 2004). In case of FGF7 and FGF10, the activation occurs in a paracrine manner, since both ligands are produced by fibroblasts in the dermal papilla and in the interfollicular dermis as well as by epidermal γδ T cells (Rosenquist and
Martin, 1996; Suzuki et al., 2000; Werner et al., 1993). By contrast, FGF22 is mainly expressed in the inner root sheath of the hair follicle (FGF22) (Nakatake et al., 2001) and most likely acts in an autocrine manner (Fig.1A).

To determine the function of these FGFs and their receptors in the skin, we generated mice lacking FGFR1, FGFR2 or both receptors in keratinocytes. Our results revealed that these receptors cooperate to maintain the epidermal barrier and cutaneous homeostasis.
Fig.1: Expression and activation of FGFR1IIIb and FGFR2IIIb in the skin of control and K5-R1/R2 mice. A: The expression pattern of FGF7, FGF10 and FGF22 in the skin is shown schematically. FGF7 and FGF10 are expressed by fibroblasts of the dermis and the dermal papilla (DP) of the hair follicles and by epidermal γδ T cells. FGF22 is expressed by keratinocytes. These FGFs activate FGFR1IIIb and FGFR2IIIb on keratinocytes. Bar: 50 µm. B: RNA from P0, P12 and P18 back skin epidermis of control and K5-R1/R2 mice was analyzed by Real-Time RT-PCR for the levels of Fgfr1 and Fgfr2 mRNAs. Error bars indicate mean ±SD. N=3 K5-R1/R2 mice and N=2 control mice at P0, N=5 mice per genotype for P12 and P18. Gapdh mRNA was used for normalization. Data are indicated as % of control. C: RNA was isolated from the epidermis of adult K5-R1/R2 mice and age-matched control mice. RNA from mouse liver was used as a positive control for FGFR4. Samples of 20 µg RNA were analyzed by RNase protection assay for expression of FGFR3, FGFR4 or GAPDH. D: Primary keratinocytes from control and K5-R1/R2 mice were grown to confluency, serum-starved and treated for 10 min with FGF7, FGF10, EGF or medium without growth factors (medium). Lysates were analyzed by western blotting using antibodies against total and phosphorylated signaling proteins or lamin A (loading control).

Results

Generation of mice lacking FGFR1, FGFR2 or both receptors in keratinocytes
Mice with floxed Fgfr1 (Pirvola et al., 2002) and Fgfr2 alleles (Yu et al., 2003) were mated with transgenic mice expressing Cre recombinase under the control of the keratin 5 (K5) promoter. This promoter allows excision of floxed alleles in basal cells of stratified epithelia after embryonic day 15.5 (Ramirez et al., 2004). The progeny of our breeding included mice lacking FGFR1, FGFR2, or both receptors in keratinocytes (designated K5-R1, K5-R2 and K5-R1/R2 mice). Mice with floxed Fgfr alleles but lacking the Cre transgene were used as controls. Mice heterozygous for the floxed alleles that express Cre were used as an additional
control in some experiments, and they never revealed phenotypic abnormalities (data not shown). Real-Time RT-PCR using RNA from isolated epidermis of control and mutant mice demonstrated a strong reduction of *Fgfr1* and *Fgfr2* expression (Fig.1B) in newborn single and double knockout mice, which further declined until P18 (Fig.1B and data not shown). There was no compensatory upregulation of *Fgfr3* expression, and *Fgfr4* mRNA could not be detected in mice of all genotypes using RNase protection assay (Fig.1C).

When primary keratinocytes from P3 mice were stimulated with FGF7 or FGF10, efficient phosphorylation of FGFR substrate 2α (FRS2α), Erk1/2, and p38 was observed in cells from control but not from K5-R1/R2 mice, demonstrating efficient inhibition of FGFR signalling in the latter. Epidermal growth factor (EGF) activated these signalling pathways in cells of both genotypes (Fig.1D).

**Overlapping functions of FGFR1 and FGFR2 in the skin**

Macroscopically, no obvious abnormalities were observed in K5-R1 mice at any stage of postnatal development (Fig.2B). By contrast, K5-R2 mice revealed a phenotype (Fig.2C), which fully matches the abnormalities seen in mice lacking FGFR2IIb in keratinocytes (K5-R2IIb mice) (Grose et al., 2007). The latter show hair abnormalities, a reduction in the number of hairs and loss of sebaceous glands. The phenotype was much more severe in the double knockout mice (Fig.2D), whereas the loss of only one *Fgfr2* allele in addition to both *Fgfr1* alleles did not cause obvious phenotypic abnormalities (data not shown). All females and approximately 60% of the males were infertile, and both were much smaller than control littermates. They also progressively lost their hair and were hairless by the age of 2-4 months (Fig.2E). However, we did not observe an increased mortality rate, and we were able to maintain the animals for up to two years.
Fig. 2: Macroscopic and histological abnormalities in FGFR mutant mice. A-F: Pictures were taken from control (A), K5-R1 (B), K5-R2 (C), K5-R1/R2 (D,E) and K5-R2/R1IIIb (F) mice at the age of 1 month (1M; A-D) or 5 months (5M; E,F).

FGFR1IIIb cooperates with FGFR2IIIb in the regulation of epidermal homeostasis

To identify the splice variant that cooperates with FGFR2 in keratinocytes, we generated mice lacking FGFR1IIIb in all cells and FGFR2 in keratinocytes (K5-R2/R1IIIb mice). FGFR1IIIb deficient mice are phenotypically normal and do not display an obvious skin phenotype, since only the IIIb exon of the Fgfr1 gene was deleted in these mice, whereas all other FGFR1 splice variants are normally expressed (Zhang et al., 2004). The phenotype of K5-R2/R1-IIIb mice was identical to the phenotype observed in K5-R1/R2 mice at the macroscopic (Fig.2E,F) and histological level (Fig.3E and data not shown). Therefore, we
conclude that FGFR1IIIb and FGFR2IIIb cooperate in the regulation of epidermal homeostasis.

Loss of skin appendages and progressive acanthosis in K5-R1/R2 mice

We next focused on the phenotype of the double mutant mice. At P5 (first anagen), a mild hypotrophy of the epidermis was observed, but the dermis and appendages appeared normal (Fig.3A). Although smaller and abnormally shaped, hair follicles were still present at P18 (first telogen) and their number was similar to control mice (24 follicles / mm in control mice versus 25 follicles / mm in K5-R1/R2 mice, N = 6 control and 5 K5-R1/R2 mice). At this stage the epidermis had a normal thickness, and no dermal abnormalities were observed (Fig.3B). By P30, control mice had entered the second anagen. However, most follicles from K5-R1/R2 mice were in telogen, although their number was still similar to the number in control mice (approximately 5 follicles / mm in mice of both genotypes; N=4 control and 3 K5-R1/R2 mice) (Fig.3C). Hair follicles and sebaceous glands were virtually absent in the back skin of older mice and only a few cysts were present in the dermis (data not shown), demonstrating progressive loss of appendages. Concomitantly, fibrosis developed in the dermis (Fig.3D). These abnormalities were reminiscent of the phenotype seen in K5-R2IIIb mice (Grose et al., 2007), but they were more severe in double knockout mice. In contrast to all single knockout mice, however, K5-R1/R2 as well as K5-R2/R1IIIb mice developed epidermal hyperthickening (acanthosis) combined with disorganization of the keratinocytes at the age of 2-3 months, and this phenotype further progressed upon aging (Fig.3D,E). Acanthosis was seen in all areas of the skin and was particular severe in the tail skin (Fig.3E). This phenotype was unexpected, since FGFs are potent mitogens for keratinocytes (Steiling and Werner, 2003). Therefore, we next focused on the mechanisms underlying the epidermal abnormalities and the progressive skin disease that developed in these mice.
Fig.3: Progressive loss of skin appendages in K5-R1/R2 mice. A-D: Longitudinal paraffin sections from back skin of K5-R1/R2 female mice and control (ctrl.) female littermates at P5, P18, 1 month (1M), or 6 months (6M) were stained with H/E. Bar: 100 μm. (E): Paraffin sections from the tail skin of control, K5-R1/R2 and K5-R2/FGFR1IIIb animals were stained with H/E. D: Dermis, E: Epidermis, HF: hair follicles. Bar: 22 μm.
Loss of FGFR1 and FGFR2 in keratinocytes causes hyperproliferation in vivo but not in vitro

Acanthosis may result from reduced apoptosis, impaired differentiation or enhanced proliferation of keratinocytes, and these possibilities were explored. Apoptotic cells were extremely rare in the epidermis of control or K5-R1/R2 mice (data not shown). Immunofluorescence analysis of epidermal differentiation markers revealed appropriate expression of keratin 14 (K14) in the basal layer. K10 expression started in the first suprabasal layer in mice of both genotypes, but the number of K10 positive layers was increased in K5-R1/R2 mice. Loricrin was expressed in the granular and cornified layers in mice of all genotypes, although the loricrin-positive part of the epidermis was thicker in K5-R1/R2 animals. K6, which is restricted to hair follicle keratinocytes in normal skin, was abnormally expressed in the interfollicular epidermis of adult K5-R1/R2 mice (Suppl. Fig.S1). Interfollicular expression of this keratin is characteristic for hyperplastic and hyperproliferative skin. Overexpression of K6 was already seen at the RNA level at P18, but only a weak immunoreactivity was observed in the interfollicular epidermis at this time point (data not shown).

Cell proliferation was assessed by in vivo labelling with 5-bromo-2’deoxyuridine (BrdU). At P18, keratinocyte proliferation was only mildly increased in K5-R1/R2 mice (Fig.4B, left panel). At the age of three months, however, keratinocyte proliferation was strongly increased in the back and tail skin of K5-R1/R2 mice but not of K5-R1 or K5-R2 mice (Fig.4A and B, right panel and data not shown). The increase in keratinocyte proliferation upon aging suggests that the hyperproliferation is not a cell autonomous effect but results from the progressive inflammation. This hypothesis is supported by the normal in vitro proliferation rate of primary keratinocytes isolated from K5-R1/R2 mice at P3 (Fig.4C, left panel). Proliferation was even reduced in cells from P23 K5-R1/R2 mice compared to cells from control mice of the same age (Fig.4C, right panel).
Supplemental Fig. S1: Keratinocyte differentiation is only mildly altered in K5-R1/R2 mice. Paraffin sections from tail skin of control and K5-R1, K5-R2 and K5-R1/R2 mice (5 months of age) were stained with hematoxylin/eosin (upper panel) or analyzed by immunofluorescence with antibodies against keratin 10 (green) and keratin 14 (red) (second panel), keratin 6 (red; third panel) or loricrin (green; lower panel). Nuclei were counterstained with Hoechst (blue) in the two lower panels. E: Epidermis, D: Dermis, HF: Hair follicles. Bars: 100 µm.
Fig. 4: Enhanced keratinocyte proliferation in aged K5-R1/R2 mice in vivo but not in vitro. K5-R1/R2 mice and control littermates were injected with BrdU at P18 or 3 months of age. Tail skin sections were stained with a peroxidase-conjugated antibody against BrdU. A: Representative sections from 3 months-old mice are shown. Bar: 100 μm. B: The number of BrdU positive cells/mm basement membrane was counted using at least 10 sections/mouse. Error bars indicate mean ±SD. P18: N=8 control and 6 K5-R1/R2 mice; 3 months (3M): N=4 control and 6 K5-R1/R2 mice. C: Primary keratinocytes of control and K5-R1/R2 mice were isolated at P3 or P23, seeded at equal density and labeled with BrdU. The percentage of BrdU positive cells was determined. Error bars
indicate mean ±SD; N=4 per genotype for P3 and N=3 for P23. At least two microscopic areas were counted per dish.

**Progressive skin inflammation in K5-R1/R2 mice**

To test the possible role of inflammation in the hyperthickening of the epidermis, we analyzed the immune cell infiltrate. The most obvious difference that we observed by immunofluorescence was the strong (60%) increase in epidermal γδ T cells (Fig.5A). This was verified by FACS analysis of cells from isolated epidermis (data not shown). Interestingly, the number of γδ T cells was already significantly increased at P18, whereas no difference was observed at P12 (Suppl. Fig.S2A). The number of Langerhans cells was similar between control and K5-R1/R2 mice (data not shown). Toluidine blue staining revealed significantly more mast cells in the dermis of K5-R1/R2 mice at the age of 6 months and P18, but no differences at P12 (Fig.5A and Suppl. Fig.S2B).

FACS analysis of dermal cells showed a significant increase in the number of CD45 positive immune cells, in particular of αβ and γδ T cells, in adult mice (Fig.5B and Suppl. Fig.S3A). This was confirmed by immunofluorescence, and an increased number of CD45 positive cells was already seen at P36 (Suppl. Fig.S2C). By contrast, no significant difference in the number of macrophages and neutrophils was detected using antibodies against CD11b and F4/80 or Ly-6G, respectively (Fig.5B and Suppl. Fig.S3A). The lack of a macrophage or neutrophil infiltrate was also confirmed by immunohistochemistry (data not shown).
Fig. 5: Immune cell infiltrate in the skin of K5-R1/R2 mice. A: Back skin sections from 6 months-old (6M) control and K5-R1/R2 mice were stained with...
antibodies against the $\gamma\delta$ T cell receptor. The white dotted line indicates the basement membrane. In addition, sections were stained with toluidine blue to identify mast cells. Bar: 33 $\mu$m. The number of $\gamma\delta$ T cells and mast cells per mm basement membrane was counted using at least 5 sections/mouse. N=5 mice per genotype for $\gamma\delta$ T cells and 7 mice per genotype for mast cells. B: Cells from the dermis of 12-months old mice were analyzed by FACS using antibodies against different inflammatory cell markers. N=4 mice per genotype. The frequencies of the individual inflammatory cells are shown. Original FACS data are shown in Suppl. Fig.S3A. C: Dermal protein lysates (N = 3 control and 4 K5-R1/R2 mice) or serum (N = 3 per genotype) of mice at the age of 12 months were analyzed for the levels of IgG1, IgG2a and IgE by ELISA. Error bars indicate mean ± SD.

Supplemental Fig.S2: Quantification of mast cells, $\gamma\delta$ T cells and CD45 positive cells in control and K5-R1/R2 mice.
(A): γδ T cells were identified by immunofluorescence with an antibody against the γδ T cell receptor. Their number in the back skin epidermis was quantified in 7 sections per mouse at 200x magnification. Error bars indicate mean ±SD, N= 3 control mice and 6 K5-R1/R2 mice at P12 and 7 mice per genotype at P18.

(B): Mast cells were stained with toluidine blue. Their number in the dermis of back skin was quantified in 8 serial images at 200x magnification. Error bars indicate mean ±SD.N= 4 control mice and 6 K5-R1/R2 mice at P12 and 6 mice per genotype at P18.

(C): CD45 positive cells were identified by immunofluorescence in P36 back skin from control and K5-R1/R2 mice. Error bars indicate mean ±SD N=4 mice per genotype. *P≤0.05, **P≤0.005, ***P≤0.001.

B cells do not accumulate in the skin, but their activation in adult K5-R1/R2 mice was demonstrated by the presence of enhanced levels of immunoglobulins (Ig)G1, G2a and E in the dermis and of IgE in the serum (Fig.5C). These results demonstrate that the loss of FGFR1 and FGFR2 in keratinocytes initiates an inflammatory response. This is also reflected by enhanced levels of phosphorylated (activated) and also of total NF-κB in the epidermis of aged mutant mice. Levels of total and phosphorylated STAT3 were also higher compared to controls, whereas expression and activation of p38 were not affected (Fig.6A).

Real-Time RT-PCR analysis of RNAs from dermis and epidermis of aged mice revealed enhanced expression of the pro-inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and of IL-1 family member 8 (IL-1F8), a new member of the IL-1 family (Barksby et al., 2007) (Fig.6B). We also found upregulation of S100A8 and S100A9, which are expressed by activated keratinocytes in hyperproliferative epidermis of psoriatic patients and in epidermal skin cancers and which act as chemoattractants for inflammatory cells (Gebhardt et al., 2006). In addition, intercellular adhesion molecule 1 (ICAM-1) was strongly expressed in the dermis (Fig.6B). This reflects the progressive skin inflammation in K5-R1/R2 mice.
Fig.6: Progressive inflammation in the skin of K5-R1/R2 mice through production of cytokines by activated keratinocytes and γδ T cells. A: Epidermal lysates from control and K5-R1/R2 mice at the age of P18 and 5 months (5M) were analyzed by western blotting for the levels of phosphorylated and total NF-κB (p65), Stat3, Erk1/2, and p38. B: RNAs from isolated dermis and epidermis of K5-R1/R2 mice and littermate controls (8M) were analyzed for expression of inflammatory markers using Real-Time RT-PCR. Gapdh mRNA was used for normalization. Results are shown in a table: -: not detectable; +: weak expression, ++: moderate expression, +++: strong expression. C: RNAs from the epidermis of K5-R1/R2 mice and littermate controls at P18 were analyzed for expression of inflammatory markers using Real-Time RT-PCR. Gapdh mRNA was used for normalization. Error bars represent mean +/- SD. N=3 per genotype. D: Keratinocytes and γδ T cells were purified from epidermal sheets by preparative FACS. Original FACS data are shown in Suppl. Fig.S3A and B. RNAs from the
purified cell populations were analyzed by RT-PCR for the expression of S100A8, S100A9 and IL-1F8. Ribosomal protein S29 (RPS29) mRNA was used for normalization; expression of keratin 14 and of the γδ T cell receptor was analyzed to verify the enrichment of the two cell populations.

Supplemental Fig. S3: Analysis of immune cells in the dermis and epidermis of adult control and K5-R1/R2 mice by FACS. (A): Cells from isolated dermis of 12-months old mice were analyzed by FACS using antibodies against different
inflammatory cell markers. N=4 mice per genotype. Original FACS data are shown. Results are summarized in Fig.5B. (B,C): Cells from isolated epidermis of adult control (B) and K5-R1/R2 (C) mice were sorted by FACS using antibodies against CD3 and the γδ T cell receptor to separate γδ T cells from the remaining epidermal cells (mainly keratinocytes). Original FACS data are shown. The sorted cells were used for RNA isolation and subsequent RT-PCR analysis (shown in Fig.6D).

**Role of keratinocyte- and γδ T cell-derived cytokines in the initiation of the inflammatory response**

To identify the factors that initiate the inflammation in K5-R1/R2 mice, we determined the expression of the above-mentioned cytokines at P18 using RNA from isolated epidermis. The efficient separation of epidermis from dermis was verified by RT-PCR for K14 (epidermal marker) and vimentin (dermal marker) (data not shown). At P18 the loss of FGFR expression was almost complete (Fig.1B), but the epidermis was not yet hyperthickened (Fig.3B). We found a strongly increased expression of the genes encoding S100A8, S100A9, and IL-1F8 in the epidermis of K5-R1/R2 mice, whereas TNF-α and IL-1β expression was unaltered (Fig.6C and data not shown). However, S100A8, S100A9 and IL-1F8 were not upregulated in cultured keratinocytes from K5-R1/R2 mice – neither in exponentially growing cells nor in quiescent cells that had undergone in vitro differentiation (data not shown). This finding suggests that these cytokines are not directly regulated by the loss of FGFR1 and FGFR2, but rather through a pro-inflammatory stimulus that is only present in vivo. Alternatively, they may be produced by other cell types in the epidermis, e.g. by γδ T cells. To distinguish between these possibilities, we separated γδ T cells from other epidermal cells (predominantly keratinocytes) by FACS using dissociated cells from epidermal sheets (Suppl. Fig.S3B and C). The efficient enrichment of keratinocytes and γδ T cells was verified by semi-quantitative RT-PCR analysis of mRNAs encoding K14 or the γδ T cell receptor, respectively (Fig.6D). Using RNAs from the purified cell populations we found that S100A8/A9 are mainly produced by keratinocytes and upregulated in this cell type in K5-R1/R2 mice,
whereas IL-1F8 was predominantly expressed by γδ T cells - in particular in the knockout mice (Fig.6D). Therefore, both keratinocytes and γδ T cells appear to contribute to the inflammatory phenotype.

**IL-1F8 stimulates keratinocyte proliferation and production of keratinocyte mitogens by stromal cells**

IL-1 released from keratinocytes is a potent inducer of keratinocyte mitogens in fibroblasts, resulting in keratinocyte proliferation through a double paracrine loop (Szabowski et al., 2000). To determine if IL-1F8 plays a similar role, we injected IL-1F8 or BSA as control intradermally into the skin of wild-type mice. 24h later, increased keratinocyte proliferation was observed in IL-1F8-injected mice as determined by BrdU-labeling as well as by staining of skin sections with an antibody against proliferating cell nuclear antigen (PCNA) (Fig.7A). To determine if this is a direct effect of IL-1F8 or mediated via stromal cells we treated murine keratinocytes with recombinant IL-1F8 at concentrations used in previous studies (Magne et al., 2006) and found a mild pro-mitogenic effect of this cytokine (60% increase; Fig.7B). Treatment of serum-starved murine fibroblasts with IL-1F8 induced the expression of IL-6 and IL-8 as previously reported (Magne et al., 2006) (Fig.7C and data not shown). In addition, increased mRNA levels of transforming growth factor α (TGF-α), hepatocyte growth factor (HGF) and FGF7 were observed in response to IL-1F8 treatment (Fig.7C). Most importantly, these mitogens were also overexpressed in the dermal compartment of K5-R1/R2 skin, in particular in aged mice, together with granulocyte colony stimulating factor (G-CSF) (Fig.7D). Although the FGFR1/2-deficient keratinocytes can no longer respond to FGF7, the other growth factors are likely to contribute strongly to the hyperproliferative phenotype.
Fig. 7: IL-1F8 stimulates keratinocyte proliferation and production of keratinocyte mitogens by fibroblasts. A: IL-1F8 or BSA were intradermally injected into wild-type mice. 24h later proliferating cells were identified by BrdU labeling or by immunostaining with an antibody against PCNA. Bar: 33 μm. B: Immortalized keratinocytes from wild-type mice were treated with 500 ng/ml IL-1F8 for 2h and labeled with BrdU. The percentage of BrdU positive cells was determined. Error bars indicate mean ±SD; N=3. C: Immortalized embryonic fibroblasts were starved overnight in medium with 0.1% FCS and subsequently treated for 15 min or 3 h with 500 ng/ml IL-1F8. RNA was isolated from these cells before and after IL-1F8 treatment and analyzed by Real-Time RT-PCR for the mRNA levels of different keratinocyte mitogens as indicated. Gapdh mRNA was used for normalization. Bars represent mean from duplicate determinations. The IL-1F8 induced expression of keratinocyte mitogens was reproduced with an independent fibroblast cell line. D: RNA from the dermis of 8 months old (8M) control and K5-R1/R2 mice was analyzed by Real-Time RT-PCR for the mRNA levels of different keratinocyte mitogens as indicated. RPS29 mRNA was used for normalization. Error bars represent mean +/- SD. N=3 per genotype (5 for TGF-α).
**Epidermal barrier function is disturbed in K5-R1/R2 mice**

Since cutaneous inflammation frequently results from a defect in epidermal barrier function (Segre, 2006), we analyzed the trans-epidermal water loss (TEWL) that reflects the status of the permeability barrier (Fluhr et al., 2006). At P18, TEWL was slightly increased in K5-R1/R2 mice. This phenotype strongly increased with age, and the TEWL was significantly higher in the double knockout mice compared to control animals at the age of 6 months (Fig.8A). This is consistent with their high consumption of drinking water (data not shown), as well as with the dry and fragile appearance of the skin.

Epidermal barrier function is conferred by the cornified envelope (Segre, 2006) and by tight junctions (Furuse et al., 2002) (Pummi et al., 2001) (Brandner et al., 2002) (Langbein et al., 2002). Expression of loricrin was enhanced in the knockout mice (Suppl. Fig.S1), and the mRNA levels of SPRR2A, another component of the cornified layer, were more than 30-fold upregulated (data not shown). By contrast, expression of tight junction components was strongly reduced. The mRNA levels of claudin 3, claudin 8 and occludin were much lower in K5-R1/R2 mice as determined by Real-Time RT-PCR analysis of epidermal RNAs (Fig.8B). A strong down-regulation was already seen at P12 (2-18% of control). Westernblot analysis of epidermal lysates confirmed the down-regulation of claudin 3 and occludin at the protein level and also revealed reduced claudin-1 expression in K5-R1/R2 mice (Fig.8C). Importantly, the down-regulation of tight junction gene expression preceded the onset of inflammation and hair loss, strongly suggesting that this is a direct consequence of the loss of FGFR1 and 2.

As a consequence of the down-regulation of tight junction gene expression, only a rudimentary development of tight junctions was observed in the epidermis of K5-R1/R2 mice using transmission electron microscopy (Fig.8E), whereas tight junctions were well developed in control mice (Fig.8D). In most of the sections from adult K5-R1/R2 animals we also found bubble-like intercellular clefts between the keratinocytes of the *stratum granulosum*, which most likely result from water-filled cavities (Fig.8F). Enlargement of the intercellular gaps was already seen occasionally in mutant mice at P18, and the phenotype progressed upon aging (data not shown).
Fig. 8: Impaired barrier function in K5-R1/R2 mice. A: TEWL was determined in control and K5-R1/R2 mice at the age of P18 or 5 months (5M). Error bars indicate mean ±SD; N=6 control mice P18, 9 K5-R1/R2 mice P18; N=4 mice per genotype 5M. B: RNA from isolated epidermis of mice at P12, P18 or 8 months was analyzed by Real-Time RT-PCR for expression of different claudins and occludin. RPS29 mRNA was used for normalization. N=3-5 per time point and genotype. Expression in control mice was arbitrarily set as 100%. Error bars represent mean +/- SD. (*) indicates a P value between 0.05 and 0.06. C: Epidermal lysates of control and K5-R1/R2 mice (P18 or 8 months old (8M)) were analyzed by western blotting for the levels of claudin 1, claudin 3, occludin, and total ERK (loading control). D-F: High magnification ultrastructure revealed a typical desmosome – tight junction (arrow) complex between keratinocytes of the stratum granulosum in a 4-months old control mouse (D), while only a
rudimentary cell-cell contact is formed adjacent to a desmosome of a 4-month old K5-R1/R2 mouse (E). In these animals, bubble-like clefts (asterisk) were frequently seen between the keratinocytes in the stratum granulosum, in particular in aged mice (F – 6 months old mouse). Bars: D,E: 150 nm, F: 250 nm. D: desmosome.

Supplemental Fig.S4: Time course of claudin 3, claudin 8 and occludin expression during postnatal development. RNA was isolated from the epidermis of control mice at P12, P18 and 8M and analyzed by Real-Time RT-PCR for the levels of claudin 3, claudin 8 and occludin mRNAs. Amplification of Rps29 cDNA was used for normalization. N=3-5 per time point. Expression levels at P12 were arbitrarily set as 1. Error bars represent mean +/- SD.

To determine if the down-regulation of claudin/occludin expression is a cell autonomous effect, we analyzed the expression of these tight junction components in immortalized keratinocytes. Indeed, a strong down-regulation was observed in three independent cell lines from K5-R1/R2 mice compared to controls (Fig.9A-C). When confluent keratinocytes from wild-type mice were stimulated with FGF7, a slight increase in the levels of occludin and a stronger increase in claudin 1 and claudin 3 were observed, indicating that these genes are targets of FGFs in keratinocytes (Fig.9B). Their regulation occurs at the RNA
level, since the mRNA levels of claudin 1 and claudin 3 were 5- or 30-fold elevated in FGF7 treated keratinocytes compared to non-treated cells (data not shown). EGF also induced their expression under these conditions (data not shown), but it can obviously not compensate for the loss of FGF receptors, since expression of claudin 1, claudin 3 and occludin was reduced in keratinocytes of K5-R1/R2 mice, which were cultured in the presence of EGF (Fig.9B).

Finally, we determined if the reduction in claudin/occludin expression is functionally important. Due to the lack of a functional assay to determine tight junction permeability in adult mice, we addressed this question in vitro. For this purpose we measured the transepithelial electrical resistance (TER) of confluent immortalized keratinocytes from control and K5-R1/R2 mice, since tight junctions restrict paracellular diffusion of ions. Indeed, the TER was more than 50% reduced in three independent cell lines from FGFR-deficient mice compared to control mice (Fig.9D and data not shown). The same difference was observed on several consecutive days (data not shown).
Fig.9: Reduced expression of tight junction proteins in cultured keratinocytes from K5-R1/R2 mice reduces the transepithelial electrical resistance. A: RNAs from cultured, immortalized keratinocytes of control and K5-R1/R2 mice were analyzed by Real-Time RT-PCR for expression of different claudins and occludin. RPS29 mRNA was used for normalization. Bars represent mean from duplicate determinations. The down-regulation of these tight junction components in K5-R1/R2 cells was reproduced with two independent cell lines from control and mutant mice. B: Cultured keratinocytes of control and K5-R1/R2 mice were starved for 24h and incubated for 96h in starvation medium with or without FGF7. Lysates were analyzed by western blotting for expression of claudin 1, claudin 3, occludin, or GAPDH (loading control). C: Cultured primary keratinocytes were grown to confluency and analyzed for the expression of claudin 3 by immunofluorescence (green). Bar: 33 μm. D: TER was measured using immortalized keratinocytes from control and K5-R1/R2 mice that had been grown to confluency and incubated in differentiation medium until stable values were obtained. A representative measurement is shown using 6 independent filters with cells from each genotype. Error bars represent mean +/- SD. The result was reproduced at different days and with two independent cell lines from control and K5-R1/R2 mice.

Taken together, our results suggest that reduced expression of several tight junction components disrupts the epidermal barrier, resulting in activation of keratinocytes and γδ T cells. To further test this hypothesis, we determined if disruption of the epidermal barrier causes a similar phenotype. Treatment of wild-type mice with acetone, which damages the epidermal barrier, caused the expected hyperproliferation of keratinocytes (Proksch et al., 1991) (Fig.10A). Similar as in K5-R1/R2 mice, this was associated with a minor increase in the number of mast cells and with a significant increase in epidermal γδT cells (Fig.10A). In a complementary experiment we tested if treatment of the skin of K5-R1/R2 mice with a moisturizing cream ameliorates the phenotype. Although we could not observe a reduction in keratinocyte proliferation, the number of mast cells and of γδ T cells was significantly reduced (Fig.10B). Concomitantly,
expression of IL-1F8 was 30% reduced, whereas no obvious change in the expression of claudin 1, claudin 3 and occludin was observed (data not shown). Together, these findings support the hypothesis that a defect in the epidermal barrier initiates and maintains the cutaneous inflammatory response in K5-R1/R2 mice. This induces keratinocyte hyperproliferation through a double paracrine loop involving γδ T cell-derived IL-1F8, keratinocyte-derived S100A8 and A9 and several keratinocyte mitogens produced by dermal cells (summarized in Fig.10C).

**Fig.10:** The epidermal barrier controls the number of mast cells and γδ T cells. A: Topical treatment of wild-type mice with acetone enhanced keratinocyte proliferation and increased the number of mast cells and γδ T cells.
B: Topical treatment of K5-R1/R2 with moisturizing cream did not affect keratinocyte proliferation, but reduced the number of mast cells and of γδ T cells. Error bars indicate mean ±SD; N=7 mice for acetone and 5 mice for moisturizing cream treatment. C: Model describing the pathogenic mechanisms in the skin of K5-R1/R2 mice. Loss of FGFR signalling in keratinocytes results in reduced expression of tight junction components. The resulting defective epidermal barrier, together with the loss of sebaceous glands, causes skin dryness. This activates a stress response in keratinocytes, resulting in activation of γδ T cells and upregulation of cytokine expression in activated γδ T cells and keratinocytes. The latter stimulate keratinocyte proliferation directly (IL-1F8) and/or indirectly (IL-1F8 and S100A8/A9) through induction of a double paracrine loop involving several keratinocyte mitogens that are produced by stromal cells. In addition, invasion of foreign antigens and pathogens may further activate immune cells and accelerate the inflammatory response.

Discussion

Cooperative functions of FGFR1 and FGFR2 in keratinocytes

We identified essential roles of FGFR1IIIb and FGFR2IIIb in the maintenance of skin appendages and epidermal barrier function. The responsible ligands are most likely FGF1, FGF7, FGF10 and FGF22, which are expressed in different compartments of the skin (Steiling and Werner, 2003). Our results identified FGFR2IIIb as the most important receptor for these ligands in keratinocytes, whereas FGFR1IIIb provides a back-up function. FGFR3 is also expressed in keratinocytes, in particular in the suprabasal layers (Logie et al., 2005). However, it binds a different set of FGFs than FGFR1IIIb and FGFR2IIIb (Zhang et al., 2006), suggesting distinct functions. Although mice lacking FGFR3 have no obvious skin abnormalities (D.M.O. and S.W., unpublished data), it will be interesting to determine the consequences of the loss of all FGF receptors in keratinocytes.
**Loss of FGFR1 and 2 in keratinocytes impairs hair regeneration**

The loss of skin appendages that we observed in K5-R1/R2 mice is consistent with the stimulatory effect of FGF7 on hair follicle growth, development and differentiation (Danilenko et al., 1995). However, only a rough hair coat but no hair loss was seen in FGF7-deficient animals (Guo et al., 1996), indicating functional redundancy among FGF family members. It seems likely that FGF7 together with FGF10 and FGF22 cooperatively orchestrate hair regeneration via activation of FGFR1IIIb and FGFR2IIIb on hair follicle keratinocytes. This hypothesis is further supported by the upregulation of these FGFs in the anagen phase of the hair cycle (Kawano et al., 2005). It was recently reported that expression of FGF7 and FGF10 increases in the dermal papilla during the transition from early to late telogen. Functional in vitro studies suggested that this results in stimulation of hair germ cells adjacent to the papilla and subsequent hair cycle activation (Greco et al., 2009). The failure of K5-R1/R2 mice to regenerate hair follicles is fully consistent with this predicted function. The reason for the subsequent hair loss is presently unclear. It may well be that the progressive inflammation contributes to this phenotype, but this needs to be further studied in the future.

**Keratinocyte hyperproliferation in K5-R1/R2 mice is mediated via the stroma**

A particular striking and unexpected phenotype of the K5-R1/R2 mice is the progressive acanthosis, which results from enhanced proliferation of keratinocytes. This was surprising, since FGFs are potent mitogens for keratinocytes and since epidermal hypotrophy was observed in the skin of newborn mice lacking FGFR2IIIb in all cells (Petiot et al., 2003) as well as in the skin of adult mice expressing a dominant-negative FGFR2IIIb mutant in keratinocytes (Werner et al., 1994). In fact, we also observed a slight epidermal hypotrophy of very young K5-R1/R2 mice (Fig.3A), but this reversed upon aging, and the reversal correlated with the onset of inflammation. Therefore, we propose that loss of FGFRs in keratinocytes results in activation of immune cells, which subsequently disrupt cutaneous homeostasis.
FGFs regulate tight junction components

Several mechanisms may be responsible for the activation of immune cells, including direct FGF-mediated suppression of pro-inflammatory cytokine expression, inflammation mediated by degenerating hair follicles, or a defect in barrier function that results in dry skin and possibly invasion of irritants/allergens and bacteria. The first possibility seems unlikely, since expression levels of S100A8/A9 and IL-1F8 were similar in cultured keratinocytes from mice of both genotypes. Inflammation as a result of hair follicle degeneration has been postulated for mice lacking β1 integrins in keratinocytes (Brakebusch et al., 2000). However, the macrophage infiltrate around the hair follicles that was seen in β1 integrin deficient mice was not observed in K5-R1/R2 animals. Furthermore, the phenotype progressed upon loss of all follicles, indicating that degenerating follicles are not or at least not exclusively responsible for the development of skin inflammation. Therefore, the most likely explanation for the skin inflammation is the defect in the epidermal barrier. Surprisingly, expression of several genes involved in the formation of the cornified envelope was even increased (data not shown). This was also the case for filaggrin, a gene, which is mutated in a large percentage of patients with atopic dermatitis (Sandilands et al., 2009). In addition, the filaggrin protein was normally processed in K5-R1/R2 mice (data not shown). By contrast, expression of several claudins and of occludin was much lower in the epidermis and in cultured keratinocytes of K5-R1/R2 mice. The reduced TER in vitro as well as the increased TEWL in vivo strongly suggest that the down-regulation of these tight junction components is functionally important. Consistent with this hypothesis, changes in the tight junction composition affected their permeability (Inai et al., 1999) (Furuse et al., 2002) (Tunggal et al., 2005). Unfortunately, functional in vivo assays, such as biotin penetration, can only be performed in newborn mice, where the phenotype was not sufficiently developed. Therefore, a final proof for an in vivo deficit in tight junction permeability will await improved in vivo assays. Nevertheless, the rudimentary development of the tight junctions and the adjacent large gaps between the keratinocytes of the granular layer that we observed by electron microscopy strongly support our hypothesis of an increased tight junctional permeability that results in severe water loss. Down-
regulation of tight junction proteins was already observed at P12, whereas enhanced water loss was only seen after P18. It may well be that a certain threshold of tight junction protein down-regulation must be reached to allow significant water loss. In addition, we found that expression levels of claudins and occludin strongly declined in control mice after P18 (Suppl. Fig.S4). Therefore, a further loss in K5-R1/R2 mice might have more severe consequences in adult compared to young mice.

The down-regulation of tight junction gene expression in cultured keratinocytes from K5-R1/R2 mice together with the increased expression of these genes in wild-type keratinocytes in response to FGF7 treatment indicates that they are targets of FGFs. These results are consistent with the reduced expression of tight junction proteins in blood vessels and the defective blood-brain barrier function in FGF2/FGF5 double knockout mice (Reuss et al., 2003), with the FGF2-mediated preservation of the composition of tight junctions in organotypic cortical cultures of mice (Bendfeldt et al., 2007), and with the disassembly of adherens and tight junctions in endothelial cells upon inhibition of FGFR signalling (Murakami et al., 2008). These findings indicate that maintenance of junctional integrity is a general, but as yet poorly characterized function of FGFs.

**Keratinocyte hyperproliferation is mediated via the stroma**

The enhanced water loss seen in K5-R1/R2 mice resulted in severe skin dryness and fragility, which is likely to be aggravated by the loss of sebaceous glands that release the moisturizing sebum. It was previously shown that low humidity stimulates keratinocyte proliferation and amplifies the hyperproliferative response to barrier disruption, causing dermal mast cell hypertrophy, their degranulation and subsequent inflammation (Denda et al., 1998). In addition, it is likely to stress and damage keratinocytes, resulting in activation and proliferation of γδ T cells (Jameson and Havran, 2007; Strid et al., 2009). The role of an impaired barrier and subsequent dryness in the skin phenotype is supported by the results obtained with acetone-treated wild-type mice and by the beneficial effect of moisturizing cream on the inflammatory phenotype of K5-R1/R2 mice. In addition, it may well be that irritants/allergens and bacteria can invade into the dry and fragile skin, in particular at sites of minor injury,
resulting in immune cell activation and enhancement of the inflammatory response.

One of the responsible inflammatory mediators is most likely IL-1F8. We identified this cytokine as a keratinocyte mitogen, suggesting that γδ T cell-derived IL-1F8 contributes to the hyperproliferative phenotype through an intra-epidermal paracrine mechanism. Most importantly, it induced the expression of keratinocyte mitogens in fibroblasts. This is likely to be relevant for the in vivo situation, since these mitogens were overexpressed in the dermis of K5-R1/R2 mice. Preliminary results also showed that intradermal injection of IL-1F8 induces expression of the same growth factors in vivo. Their upregulation may then further promote keratinocyte hyperproliferation in a paracrine manner.

The role of IL-1F8 in the induction of the hyperproliferative phenotype is consistent with the cutaneous inflammation, hyperkeratosis and acanthosis seen in transgenic mice overexpressing IL-1F6 in keratinocytes (Blumberg et al., 2007). Since IL-1F6 and IL-1F8 signal through the same receptors (Towne et al., 2004), the results from Blumberg and from us identify the novel IL-1 family members as major players in inflammatory/hyperproliferative skin disease.

In summary, our study revealed novel roles of FGFR1 and FGFR2 in epidermal barrier function and cutaneous homeostasis through their regulation of tight junction components. The importance of the latter in human skin disease associated with hyperscaling and acanthosis is emerging as reflected by the ichthyosis phenotype seen in patients with a claudin 1 mutation (Hadj-Rabia et al., 2004) and by the abnormal expression/distribution of tight junction proteins in psoriasis (Peltonen et al., 2007) (Watson et al., 2007) (Kirschner et al., 2009). Future studies will reveal if abnormal expression of FGFs and/or FGF receptors and their tight junction targets is associated with inflammatory skin diseases such as atopic dermatitis, which is also characterized by impaired barrier function (Elias and Steinhoff, 2008; Proksch et al., 2006) and which shows several similarities with the phenotype of K5-R1/R2 mice, in particular with regard to the inflammatory infiltrate and the epidermal thickening. In addition,
the data presented in this manuscript suggest new therapeutic applications of FGFs in patients with impaired epidermal barrier function.
References


Towne, J.E., Garka, K.E., Renshaw, B.R., Virca, G.D., and Sims, J.E. (2004). Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF-kappaB and MAPKs. J Biol Chem 279, 13677-13688.


Own contribution

My contribution to this publication was:

• Macroscopic analysis of the mice (Fig. 2)
• Histological analysis of mouse back skin (Fig. 3A-D)
• Quantification of proliferating cells in P18 and 3M epidermis of control and K5-R1/R2 mice (Fig. 4A-B)
• Extraction of RNA from control and K5-R1/R2 mouse epidermis and dermis at the age of P5, P18 and 8M and subsequent analysis of relative mRNA expression levels by qRT-PCR (Fig. 1B, 6B, 6C, 7C, 7D, 8B, 9A, Suppl. Fig. S4)
• Analysis of RNA from FACS sorted keratinocytes and γδ T cells from epidermis of control and K5-R1/R2 mice by RT-PCR (Fig. 6D)
• Treatment of control mice with acetone and treatment of K5-R1/R2 mice with hydrophilic cream, followed by immunofluorescence analysis of the back skin from untreated and treated mice with an antibody against the TCRδ or toluidine blue staining (Fig. 5A, 10A, 10B, Suppl. Fig. S2A,B)
• Preparation of protein lysates from 12M old control and K5-R1/R2 mouse epidermis and dermis (Fig. 5C)
• Treatment of fibroblasts with IL-1F8 and subsequent RNA extraction and analysis by qRT-PCR (Fig. 7C)
• Extraction of RNA from immortalized keratinocytes of control and K5-R1/R2 mice, followed by analysis of relative mRNA expression levels by qRT-PCR (Fig. 9A)
• Immunofluorescence analysis of normal back skin of control and K5-R1/R2 mice at P36 with an antibody against CD45 (Suppl. Fig. S2C)
3.2 The role of chronic inflammation in cutaneous fibrosis: Fibroblast growth factor receptor deficiency in keratinocytes as an example

-Review-

Michael Meyer¹*, Anna-Katharina Müller¹*, Jingxuan Yang¹,², Jitka Šulcová¹, and Sabine Werner¹*

¹Department of Biology, Institute of Cell Biology, ETH Zurich, Switzerland

²Present address: The Vivian L. Smith Department of Neurosurgery, The University of Texas Health Science Center at Houston, Medical School, 6431 Fannin Street, MSB 3.000, Houston, TX 77030, USA

*Equal contribution

Short title: FGFs in skin homeostasis and barrier function

Abbreviations used:
FGF: Fibroblast growth factor
FGFR: Fibroblast growth factor receptor
IL: Interleukin
IL-1F8: Interleukin 1 family member 8
TEWL: Transepidermal water loss
TGF: Transforming growth factor
TSLP: Thymic stromal lymphopoietin
Abstract

Fibrosis is associated with a variety of skin diseases and causes severe aesthetic and functional impairments. Functional studies in rodents together with clinical observations strongly suggest a crucial role of chronic injury and inflammation in the pathogenesis of fibrotic diseases. The phenotype of mice lacking fibroblast growth factor (FGF) receptors 1 and 2 in keratinocytes supports this concept. In these mice a defect in keratinocytes alone initiated an inflammatory response, which in turn caused keratinocyte hyperproliferation and dermal fibrosis. As the mechanism underlying this phenotype we identified a loss of FGF-induced expression of claudins and occludin, which caused abnormalities in tight junctions with concomitant deficits in epidermal barrier function. This resulted in severe transepidermal water loss and skin dryness. In turn, activation of keratinocytes and epidermal γδ T cells occurred, which produced interleukin-1 family member 8 and S100A8 and S100A9. These cytokines attracted immune cells and activated fibroblasts, resulting in a double paracrine loop through production of keratinocyte mitogens by dermal cells. In addition, a pro-fibrotic response was induced in fibroblasts. Our results highlight the importance of an intact epidermal barrier for the prevention of inflammation and fibrosis and the role of chronic inflammation in the pathogenesis of fibrotic diseases.
The connection between inflammation and fibrosis in the skin

Fibrosis, the replacement of parenchymal tissue by (non-functional) connective tissue, can affect multiple tissues and organs and constitutes a severe and frequently life-threatening health problem. Important examples are fibrosis of the lung, the kidney and the liver. Fibrosis can also occur in the skin, which is most obvious in scleroderma, hypertrophic scars, and keloids (Shaw et al., 2010). Furthermore, various other skin diseases are frequently associated with fibrotic processes, including acne and acne rosacea. In the latter it manifests predominantly in the skin of the nose, resulting in the development of rhinophyma (Payne et al., 2002).

The pathomechanisms underlying the development of fibrosis have only partially been elucidated. Fibrosis is considered as the result of abnormal repair in response to chronic tissue damage, which may be caused by different insults such as viruses, bacteria, radiation, mechanical injury, allergic responses and autoimmune processes (Wynn, 2008). As a consequence, a chronic inflammatory response develops, which results in upregulation of various pro-inflammatory cytokines and chemokines. Many of them induce the expression of growth factors, which directly stimulate proliferation of fibroblasts, their differentiation into myofibroblasts as well as production of extracellular matrix by these cells. These growth factors include platelet-derived growth factor, transforming growth factors β1 and β2, as well as activin. They are overexpressed in a variety of fibrotic diseases, including hypertrophic scars and keloids (Krieg et al., 2007; Trojanowska, 2008; Werner and Alzheimer, 2006).

Several recent studies highlight the important roles of pro-inflammatory cytokines and chemokines in the pathogenesis of cutaneous fibrosis (Wynn, 2008). Thus, interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) caused inflammation and cutaneous fibrosis when intradermally injected into mouse skin (Rankin et al., 2010; Jessup et al., 2008). This was also seen in transgenic mice inducibly expressing IL-13 in keratinocytes (Zheng et al., 2009).

The role of inflammation in the development of fibrosis is further documented in wound healing. Thus, scarless healing in the mammalian foetus is associated with a strikingly reduced inflammatory response compared to the situation in the adult organism (Cowin et al., 1998; Stramer et al., 2007; Gurtner et al., 2008).
Furthermore, mice lacking macrophages and functional neutrophils due to deficiency in the PU-1 transcription factor showed strongly reduced scarring postnatally (Martin et al., 2003). Therefore, inhibition of the inflammatory response in injured tissues is considered as a promising strategy to limit the development of fibrosis (Shaw et al., 2010).

**The role of epidermal defects in cutaneous fibrosis**

Recent studies with genetically modified mice revealed a crucial role of an intact epidermis and hair follicles in the prevention of dermal scarring. For example, mice lacking the β1-integrin subunit in keratinocytes developed a progressive inflammatory response, followed by severe dermal scarring (Brakebusch et al., 2000). Loss of Notch1 in keratinocytes caused inflammatory skin disease resembling mild atopic dermatitis (Demehri et al., 2009). Interestingly, these mice also developed a hyperplastic and fibrotic dermis. This was accompanied by the development of spontaneous skin tumors (Demehri et al., 2009), a finding that is consistent with the important role of inflammation and fibrosis in the pathogenesis of cancer (Schafer and Werner, 2008). The wound-like microenvironment that was established in the Notch1 deficient mice resulted from a defect in epidermal barrier function, which caused upregulation of cytokines, including TSLP (Demehri et al., 2009). The latter, when overexpressed in keratinocytes of transgenic mice, was sufficient to provoke an atopic dermatitis-like skin disease and dermal fibrosis (Yoo et al., 2005).

In the following we report on a recent study from our laboratory that highlights the tight connection between barrier function impairment, inflammation and dermal fibrosis. These abnormalities occurred in mice lacking fibroblast growth factor receptors (FGFR) 1 and 2 in keratinocytes. Our results demonstrate that a defect in keratinocytes can initiate a strong dermal response, consistent with the important role of stromal-epithelial interactions in the skin (Werner et al., 2007).
Fibroblast growth factors (FGFs) and their functions in the skin

The FGF family includes 22 polypeptides that control proliferation, migration and survival of different cell types through activation of four transmembrane tyrosine kinase receptors (FGFR1-4) (Beenken and Mohammadi, 2009; Ornitz and Itoh, 2001). These functions of FGFs are also important in development, repair and disease of the skin (Steiling and Werner, 2003). In particular, FGF7, FGF10 and FGF22, which activate specific splice variants of FGFR1 and FGFR2 on keratinocytes (the FGFR1-IIIb and FGFR2-IIIb splice variants), are strongly expressed in normal and particularly in wounded skin (Komi-Kuramochi et al., 2005; Werner et al., 1992; Werner et al., 1993). This is functionally important, since transgenic mice expressing a dominant-negative FGFR2-IIIb mutant in keratinocytes showed epidermal atrophy, hair follicle abnormalities, impaired wound reepithelialization and progressive dermal fibrosis (Werner et al., 1994).

Since the dominant-negative FGFR mutant blocks the action of all FGF receptors in response to common FGFs, the type of FGFR that is responsible for these abnormalities remained to be identified. To address this question, we generated mice lacking FGFR1 and/or FGFR2 in keratinocytes by breeding of mice with floxed \textit{fgfr1} and \textit{fgfr2} alleles with transgenic mice expressing Cre recombinase under the control of a keratin 5 promoter.

The consequences of the loss of FGFR1 and FGFR2 in keratinocytes

Whereas loss of FGFR1 had no obvious consequences, loss of FGFR2 caused mild skin abnormalities, including loss of sebaceous glands and hair follicle abnormalities (Grose \textit{et al.}, 2007; Yang \textit{et al.}, 2010). A much more severe phenotype was seen in the double knockout mice (designated K5-R1/R2 mice). These animals were much smaller than control littermates and they progressively lost their hairs. This resulted in complete baldness by the age of 2-4 months (Yang \textit{et al.}, 2010) (Fig.1A).
Fig.1: **Macroscopic and histological abnormalities in FGFR mutant mice.** a: Pictures were taken from control (left panel) and K5-R1/R2 mice (right panel) at the age of 5 months. b: Paraffin sections from the back skin of control and K5-R1/R2 (5 months old) were stained using the Masson trichrome procedure. D: Dermis, E: Epidermis, HF: hair follicles. Bar: 200 µm.

Histological analysis revealed that hair morphogenesis is not impaired, which is consistent with the finding that the loss of FGFR1 and FGFR2 was only completed after birth. However, the double knockout mice failed to regenerate their hair follicles and remained in the telogen stage instead of initiating a new hair cycle. This finding is consistent with the upregulation of FGF7 and FGF10 in the dermal papilla during the transition from early to late telogen. This was suggested to be important for the stimulation of hair germ cell proliferation and subsequent hair cycle activation (Greco et al., 2009). The failure of K5-R1/R2 mice to regenerate hair follicles supports this predicted FGF function. Following the first hair cycle, the remaining hairs were completely lost and no follicle remnants could be detected in the dermis at the age of 3-4 months (Fig.1B) (Yang et al., 2010). Long-term labelling with 5-bromo-2′-deoxyuridine revealed that this hair follicle loss correlated with a complete loss of hair follicle stem cells (unpublished data).
A second abnormality that we observed in the FGFR1/R2-deficient mice was the progressive epidermal hyperthickening (acanthosis) (Fig.1B). This was not due to impaired keratinocyte differentiation or reduced apoptosis, but resulted from keratinocyte hyperproliferation in the basal layer of the epidermis. The observed increase in keratinocyte proliferation upon aging suggests that the hyperproliferation is not a cell-autonomous phenotype but mediated via the stroma. Consistent with this hypothesis, cultured primary keratinocytes from FGFR1/R2-deficient mice showed a similar proliferation rate as cells from control mice (Yang et al., 2010). This finding suggested that loss of FGFR1 and FGFR2 in keratinocytes results in activation of stromal cells, which in turn express keratinocyte mitogens. Expression studies using dermal RNA from these mice indeed revealed a strong upregulation of hepatocyte growth factor, transforming growth factor α, granulocyte macrophage colony stimulating factor and others, which are likely to be responsible for the increased keratinocyte proliferation.

To determine if inflammatory cells are directly or indirectly responsible for the upregulation of the above-mentioned growth factors, we stained skin sections from control and K5-R1/R2 mice with antibodies against different immune cells. A particularly striking finding was the strong increase in the number in epidermal γδ T cells (also called dendritic epidermal T cells; DETCs), which was already seen in very young animals. DETCs are able to initiate a “stress-surveillance” response and they are believed to quickly limit the dissemination of infected or malignant cells to sustain tissue integrity (Hayday, 2009). Therefore, the increase in these cells suggests the presence of stressed/abnormal keratinocytes in K5-R1/R2 mice.

In addition, there was an increase in the number of mast cells and of dermal αβ and γδ T cells in adult K5-R1/R2 mice. Finally, B cell activation occurred in these mice as reflected by the deposition of immunoglobulins (Ig)G1, G2a and E in the dermis and the presence of high levels of IgE in the serum. The progressive inflammation was further documented by the upregulation of various pro-inflammatory cytokines in the skin of adult K5-R1/R2 mice (Yang et al., 2010).
Concomitant with the appearance of an inflammatory infiltrate, a progressive dermal fibrosis developed. This is reflected by the severe dermal thickening as determined by Masson trichrome staining (Fig.1B), and by the increase in connective tissue density over time (data not shown). These findings highlight the important role of inflammation in the development of dermal fibrosis.

To next determined if various cytokines are differentially expressed in the epidermis of control and K5-R1/R2 mice. Mice at P18 were used for this purpose, since the loss of FGFR expression was almost complete at this stage, but the epidermis was not yet hyperthickened. We found a strongly increased expression of the genes encoding S100A8, S100A9, and interleukin 1 family member 8 (IL-1F8) in the epidermis of K5-R1/R2 mice. Real-Time RT-PCR analysis of RNA from cells that had been isolated by fluorescence activated cell sorting of epidermal cell suspensions revealed that IL-1F8 is predominantly expressed by γδ T cells, whereas S100A8 and S100A9 are mainly expressed by keratinocytes. Therefore, both cell types contribute to the pro-inflammatory phenotype (Fig.2). It has previously been shown that S100A8 and S100A9 are important chemoattractants for inflammatory cells (Gebhardt et al., 2006), and their upregulation in K5-R1/R2 mice is likely to contribute to the inflammatory process in these mice. An important role of IL1-F8 in the phenotype of K5-R1/R2 mice is suggested by our finding that this cytokine stimulates the expression of IL-6 and of various growth factors in fibroblasts, including the growth factors that are upregulated in the dermis of K5-R1/R2 mice (see above) (Yang et al., 2010). Taken together, our results revealed that loss of FGFR1 and FGFR2 in keratinocytes activates these cells as well as γδ T cells, and they in turn produce S100A8, S100A9 and IL-1F8. These cytokines attract immune cells and activate fibroblasts, which then produce additional pro-inflammatory cytokines as well as growth factors that stimulate keratinocyte proliferation. In addition, inflammatory cell-derived factors induce a pro-fibrotic phenotype in dermal fibroblasts, resulting in progressive dermal fibrosis (Fig.2).
Fig. 2: Model describing the pathomechanisms in the skin of FGFR1/R2-deficient mice. Loss of FGFR1 and FGFR2 in keratinocytes resulted in reduced expression of claudins and occludin. The resulting defect in the epidermal barrier, together with the loss of sebaceous glands, caused skin dryness. This induced a stress response in keratinocytes, resulting in activation of γδ T cells and enhanced expression of cytokines in the epidermis. The latter stimulate keratinocyte proliferation directly (IL-1F8) and/or indirectly (IL-1F8 and S100A8/A9) via a double paracrine loop that involves several keratinocyte mitogens that are produced by various cell types in the dermis. In addition, invasion of irritants, allergens and pathogens may further activate immune cells and accelerate the inflammatory process. Concomitantly, this enhances expression of pro-inflammatory cytokines and growth factors and stimulates production of extracellular matrix proteins in fibroblasts, resulting in dermal fibrosis.

Cutaneous inflammation as a result of impaired epidermal barrier function

What are the mechanisms underlying the onset of an inflammatory response in K5-R1/R2 mice? A direct FGF-mediated suppression of pro-inflammatory
cytokine expression seems unlikely, since S100A8/A9 and IL-1F8 were expressed at similar levels in cultured keratinocytes from control and K5-R1/R2 mice. A second possibility is inflammation as a result of hair follicle degeneration. Although this cannot be fully excluded, it seems also unlikely, since the phenotype progressed upon loss of all follicles. By contrast, our findings strongly suggest that the cutaneous inflammation is the consequence of impairments in the epidermal barrier. While expression of various components of the cornified envelope was not reduced in K5-R1/R2 mice, mRNA and protein levels of several tight junction components, including claudins 1, 3, 8 and occludin, were much lower in the epidermis and in cultured keratinocytes of K5-R1/R2 mice compared to control littermates. This resulted in the formation of defective tight junctions as indicated by ultrastructural analysis of the epidermis and by the reduced transepithelial electrical resistance of a confluent keratinocyte monolayer formed by cells from K5-R1/R2 mice compared to control mice (Yang et al., 2010). It has previously been shown that alterations in the composition of tight junctions affect the junctional permeability and cause a defect in barrier function (Inai et al., 1999; Furuse et al., 2002; Tunggal et al., 2005). The latter was confirmed in our study, since K5-R1/R2 mice showed a strong transepidermal water loss, which progressed with age. This is consistent with the high consumption of drinking water by K5-R1/R2 mice as well as with the dry and fragile appearance of their skin. Together with the loss of sebaceous glands, which normally produce the moisturizing sebum, this causes severe skin dryness. The latter stimulates proliferation of keratinocytes and causes dermal mast cell hypertrophy, their degranulation and subsequent inflammation (Denda et al., 1998). In addition, dryness was shown to stress and damage keratinocytes, which in turn results in activation and proliferation of γδ T cells (Jameson and Havran, 2007). The role of an impaired barrier and subsequent dryness in the skin phenotype of K5-R1/R2 mice is supported by our results that topical treatment of the skin of these animals with moisturizing cream reduced the number of mast cells and γδ T cells (Yang et al., 2010). In addition to the water loss, it may well be that irritants/allergens and microorganisms can invade into the fragile skin, in particular at sites of minor injury, resulting in immune cell activation and enhancement of the inflammatory response. As a consequence,
inflammatory cell-derived cytokines activate fibroblasts, which in turn acquire a pro-fibrotic phenotype and deposit large amounts of extracellular matrix, resulting in dermal fibrosis. 

Taken together, our findings reflect the importance of an intact epidermal barrier for the prevention of fibrotic processes and highlight the important role of inflammation for the development of dermal fibrosis.
References

expression of interleukin.


3.3 FGF receptors 1 and 2 are key regulators of keratinocyte migration in vitro and in wounded skin

Michael Meyer¹*, Anna-Katharina Müller¹*, Jingxuan Yang¹**,
Daniel Moik², Gilles Ponzio³, David M. Ornitz⁴, Richard Grose⁵,
and Sabine Werner¹

¹Department of Biology, Institute of Molecular Health Sciences, ETH Zurich, Switzerland
²Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany
³INSERM U 634, Faculté de Médecine Avenue de Valombrose, 06107 Nice Cedex 02, and Université de Nice Sophia-Antipolis, 28 Avenue Valrose, F-06103 Nice, France
⁴Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA
⁵Queen Mary University of London, Barts Cancer Institute, Barts & The London School of Medicine and Dentistry, Institute of Cancer, London EC1M 6BQ, UK.

*Equal contribution

**Present address: The Vivian L. Smith Department of Neurosurgery, The University of Texas Health Science Center at Houston, Medical School, Houston, TX 77030

Running title: FGFs in keratinocyte migration and wound repair

Key words: FGF, focal adhesion, migration, reepithelialization, wound
Abstract

Efficient wound repair is essential for the maintenance of the skin’s integrity. The repair process is controlled by a variety of growth factors and cytokines, and their abnormal expression or activity may cause healing disorders. Here we show that wound repair is severely delayed in mice lacking fibroblast growth factor receptors (FGFR) 1 and 2 in keratinocytes. As the underlying mechanism we identified impaired wound contraction and a delay in reepithelialization that resulted from impaired keratinocyte migration at the wound edge. Scratch wounding and transwell assays demonstrated that FGFR1/2 deficient keratinocytes had a reduced migration velocity and impaired directional persistence due to inefficient formation and turnover of focal adhesions. Underlying this defect, we identified a significant reduction in the expression of major focal adhesion components in the absence of FGFR signalling, resulting in a general migratory deficiency. These results identify FGFs as key regulators of keratinocyte migration in wounded skin.
Introduction

The skin comprises the largest organ in humans and covers the entire body surface. Due to its essential role in the formation of a barrier against the environment, every defect in the skin needs to be rapidly and efficiently repaired. Upon injury, a complex healing process is initiated, which involves blood clotting, inflammation, reepithelialization and granulation tissue formation, and finally tissue remodelling. Under normal conditions, this leads to complete repair of the injured body site, although a scar remains that lacks all epidermal appendages and exhibits reduced tensile strength and elasticity (Gurtner et al., 2008; Martin, 1997; Menke et al., 2007; Sen et al., 2009).

Unfortunately, the healing process is frequently impaired, resulting in the formation of chronic, non-healing ulcers. This is a particularly common problem in the elderly population, in diabetic patients, and in patients treated with anti-inflammatory steroids or chemotherapy (Menke et al., 2007). Because impaired healing causes significant morbidity and generates an enormous financial burden for the health care system, it is of major importance to identify the factors that control the normal wound healing process and to identify the mechanisms underlying impaired healing.

The wound repair process is orchestrated by a large number of growth factors and cytokines (Werner and Grose, 2003). Of particular importance are fibroblast growth factors (FGFs), which comprise a family of 22 polypeptides. Most of them exert their functions through activation of four receptor tyrosine kinases, designated FGFR1-4 (Beenken and Mohammadi, 2009; Ornitz and Itoh, 2001). Further complexity is achieved by alternative splicing of FGFR transcripts. Most importantly, alternative splicing in the third immunoglobulin-like domain of FGFR1-3 generates two alternative FGFR variants, designated IIIb and IIIc. Epithelial cells, including keratinocytes, express predominantly or even exclusively the IIIb variants, whereas stromal cells produce mainly the IIIc variants. Significantly, the IIIb and IIIc variants of each receptor are characterized by different ligand binding specificities (Beenken and Mohammadi, 2009; Ornitz and Itoh, 2001).
We and others previously demonstrated that different members of the FGF family contribute to the wound repair process. Whereas FGF2 is particularly important for wound angiogenesis and granulation tissue formation (Broadley et al., 1989; Ortega et al., 1998), ligands activating FGF receptors on keratinocytes control reepithelialization. This was reflected by the severe delay in this process in mice expressing a dominant-negative FGFR2-IIIb mutant in keratinocytes (Werner et al., 1994). FGFR2-IIIb expressed by keratinocytes is activated by FGF1, FGF7, FGF10 and FGF22, which are expressed in normal and particularly in wounded skin (Steiling and Werner, 2003). In addition, FGF1, FGF10 and FGF22 activate FGFR1-IIIb, another receptor expressed on keratinocytes (Beer et al., 2000; Zhang et al., 2006). Through the ability of FGFR1-IIIb to form heterodimers with FGFR2-IIIb in response to common ligands, FGFR1-IIIb may also be inhibited by a dominant-negative FGFR2-IIIb mutant. Therefore, the contributions of specific FGF receptor(s) and their ligands to wound reepithelialization remain to be determined, and the underlying mechanisms have not been characterized.

To unravel the function of FGFR1-IIIb, FGFR2-IIIb and their ligands in the skin, we recently generated and characterized mice lacking one or both receptors in keratinocytes. The double mutant mice showed a complete loss of epidermal appendages and they developed mild, but progressive cutaneous inflammation. This was caused by a defect in the epidermal barrier as a consequence of reduced expression of different claudins and occludin and concomitant formation of abnormal tight junctions. The chronic inflammation caused keratinocyte hyperproliferation through induction of a double paracrine loop that involved production of pro-inflammatory cytokines and chemokines by epidermal cells and secretion of keratinocyte mitogens by cells of the underlying dermis (Yang et al., 2010). These findings revealed important roles of FGFR1 and FGFR2 in appendage regeneration, epidermal barrier function and cutaneous homeostasis. In this study we determined the consequences of the loss of these receptors in keratinocytes for cutaneous wound repair and for keratinocyte migration in vitro.
Results

To determine the role of FGFR1 and FGFR2 in keratinocytes for the wound healing process, we generated full-thickness excisional wounds on the back of mice lacking FGFR1 (K5-R1 mice), FGFR2 (K5-R2 mice) or both receptors (K5-R1/R2 mice) in keratinocytes. Since the double mutant mice develop mild but progressive inflammation (Yang et al., 2010), we performed the experiments with mice at the age of 1.5, 3 or 5 months to determine whether the inflammation affected the healing process. K5-R1 mice showed normal wound healing at all ages, confirming previous results with mice lacking FGFR1-IIIb in all cells (Zhang et al., 2004). K5-R2 mice showed a slight, but non-significant delay in healing (Suppl. Fig.S1A). However, wound healing was severely impaired in K5-R1/R2 mice (Fig.1A-C). The phenotype was already observed in young mice (6 weeks of age) and did not increase in severity upon aging (data not shown), indicating that the mild, but progressive inflammation seen in these mice (Yang et al., 2010) is not responsible for the healing defect.
Fig. 1. Delayed wound closure in K5-R1/R2 mice. (A) Paraffin sections from the middle of 3-day (3dW), 5-day (5dW) and 14-day (14dW) wounds were stained with H/E. Cl: Clot; D: Dermis; E: Epidermis; Es: Eschar; G: Granulation tissue; HE: Hyperproliferative wound epidermis; HF: Hair follicle. Bars = 500 μm. Arrows point to the tip of the migrating tongues of the wound epidermis. (B,C) H/E stained wound sections were analyzed morphometrically, and wound closure (B) and wound diameter (distance between the borders to the non-injured dermis) (C) were determined. An average of 2 wounds each from at least 6 mice per genotype were analyzed per time point. Bars represent mean ± s.e.m.

Histological analysis with subsequent histomorphometry confirmed the impaired healing in K5-R1/R2 mice (Fig.1B,C). The different components of the wound are schematically shown in Suppl. Fig.S1B. At day 3 after injury, the appearance of the wounds was similar in control and K5-R1/R2 mice. In mice of both genotypes, wounds were filled with a clot, and the granulation tissue comprised only a small area at the wound edge (Fig.1A). However, a delay in wound closure was detected in K5-R1/R2 mice as determined by morphometric measurement of the distance between the epithelial tongues at both wound edges in relation to the wound diameter (distance between the borders of the non-injured dermis) (Fig.1B). This was obviously due to impaired
reepithelialization, since the wound diameter, which reflects wound contraction, was similar in mice of both genotypes (Fig.1C).
At day 5 after wounding, a dense granulation tissue had formed in control mice, whereas wounds in K5-R1/R2 mice were still predominantly filled with a clot (Fig.1A). Most importantly, wound closure was strongly delayed at this time point (Fig.1B, middle panel). This was at least in part a result of impaired wound contraction. Thus, reduction of the wound diameter occurred between day 3 and day 5 in control mice, demonstrating the onset of contraction. However, this was not the case in K5-R1/R2 mice where the wound diameter even increased further due to the movements of the mice and obvious lack of contraction. As wound contraction is mainly achieved by myofibroblasts, we stained sections of 5-day wounds with an antibody against α-smooth muscle actin (α-SMA). However, we could not detect obvious differences in the area of granulation tissue populated by myofibroblasts and in myofibroblast location in the wounds of K5-R1/R2 mice (Suppl. Fig.S1C), indicating that the abnormal contraction is not the consequence of myofibroblast abnormalities.
Suppl. Fig. S1. Wound closure, smooth muscle cell differentiation, inflammation and scar formation in control and K5-R1/R2 mice. (A) Sections from 5-day wounds were stained with H/E. Wound closure was determined using histomorphometry. An average of 2 wounds each from at least 6 mice per genotype were analyzed. Bars represent mean ± s.e.m. (B) Different components of a 5-day wound are shown schematically. The region, where
myofibroblasts were analyzed by staining with an antibody against α smooth muscle actin (SMA), is indicated by a green rectangle, the region were neutrophils were analyzed by staining with an antibody against Ly-6G is indicated by a red rectangle. (C) Sections from 5-day wounds from control and K5-R1/R2 mice were analyzed by immunofluorescence for the presence of cells expressing α smooth muscle actin (green). Representative sections are shown. α-SMA positive myofibroblasts at the wound edge are indicated with an arrow. The border between hyperproliferative wound epidermis and dermis/granulation tissue is indicated with a dotted line. Staining in the wound epidermis results from unspecific autofluorescence. (D) Sections from 5-day wounds from control and K5-R1/R2 mice were analyzed by immunofluorescence for the presence of Ly-6G positive neutrophils (red dots). Representative sections are shown. (E) Sections from 13-day wounds were stained using the Masson Goldner protocol. Deposited collagen appears green. An overview of one wound halve is shown on the left hand side and a higher magnification of the area of granulation tissue indicated by the rectangle is shown on the right hand side. All scale bars represent 200 µm.

We also did not observe an excessive inflammatory response at any stage of healing as revealed by immunostaining with an antibody against the neutrophil marker Ly6G (Suppl. Fig.S1D). The number and distribution of these inflammatory cells was not obviously altered in the FGFR mutant mice. Most importantly, there was no granulomatous reaction that could impair contraction. At day 14 after wounding, mice of all genotypes were fully healed (Fig.1A and B, right panel), and the density of the late granulation tissue/early scar tissue was comparable between mice of both genotypes as revealed by Masson Goldner staining, where a similar intensity of collagen staining was observed (Suppl. Fig.S1E; green). However, the wounds were still much larger in the double mutant animals, and they had an extended area of granulation tissue (Fig.1A and C).

Taken together, wound healing was significantly delayed in K5-R1/R2 mice. During the early phase of repair the delay was predominantly caused by
impaired reepithelialization, whereas reduction of wound contraction was responsible for the delay at later stages.

We next focused on the impaired reepithelialization, since this defect was likely to be a direct consequence of the loss of FGFR1 and FGFR2 in keratinocytes. Interestingly, the area of the hyperproliferative wound epithelium was similar in control and K5-R1/R2 mice at day 3 after wounding and showed only a mild, but non-significant reduction at day 5 after injury (Fig.2A). The rate of keratinocyte proliferation was even increased within the hyperproliferative wound epithelium of 5-day wounds as determined by 5-bromo-2’-deoxyuridine (BrdU) incorporation studies (Fig.2B,C). This increase was already observed in non-wounded skin and resulted from the enhanced expression of keratinocyte mitogens in the dermis of K5-R1/R2 mice (Yang et al., 2010). In spite of the similar area of the wound epithelium in K5-R1/R2 mice, its length was significantly reduced in double knockout mice at day 3 after injury (Fig.2D, left panel). At this time point, the length of the wound epidermis reflects the ability of the keratinocytes to migrate into the wound tissue, since only few keratinocytes in the wound epidermis proliferate at this early stage of repair. At day 5 after wounding, the length of the wound epidermis was only slightly reduced in K5-R1/R2 mice compared to control, most likely reflecting compensation by enhanced proliferation (Fig.2D, right panel). These results suggest that keratinocyte migration is impaired in the healing wounds of K5-R1/R2 mice. This hypothesis is further supported by the morphological appearance of the keratinocytes at the tip of the migrating epidermal tongue: Whereas keratinocytes from wild-type mice formed a flat epithelial tongue with elongated keratinocytes at the front, the epithelial tongue in K5-R1/R2 mice was generally thicker, and the cells at the tip did not have the flattened appearance (Fig.2E, indicated by arrows).
Fig. 2. Keratinocyte migration is impaired in wounded skin of K5-R1/R2 mice

(A) The area of the hyperproliferative wound epidermis was determined morphometrically using H/E-stained sections from 3-day and 5-day wounds. An average of 2 wounds from at least 6 mice were analyzed per time point and genotype. (B) Cell proliferation was analyzed by BrdU incorporation at day 5 after wounding. The wound is on the right hand side of the sections. Representative sections from control and K5-R1/R2 mice are shown for 5-day wounds. (C) The number of BrdU positive cells/area wound epidermis was determined at both wound edges. An average of 2 wounds from at least 3 mice was analyzed per time point and genotype. (D) Sections from 3-day and 5-day wounds of control and K5-R1/R2 mice were analyzed morphometrically. The length of the wound epidermis (distance from the wound edge to the tip of the migrating tongue) was determined at both wound edges. An average of 2
wounds from at least 7 mice was analyzed per time point and genotype. (E) Representative H/E-stained sections from 3-day wounds showing the tip of the wound epidermis. Note the flattened wound tongue in control mice, but the broad, non-flattened tongue in K5-R1/R2 mice (indicated by arrows). Bars in A-D represent mean ± s.e.m. Scale bars represent 200 μm (B) or 50 μm (E). G: Granulation tissue; HE: Hyperproliferative wound epidermis.

**FGFR1 and FGFR2 are required for efficient keratinocyte migration**

To further study the migratory capacity of keratinocytes lacking FGFR1 and FGFR2, we performed migration experiments with primary and immortalized keratinocytes from control and K5-R1/R2 mice (Fig.3A-C and Suppl. Fig.S2–S4). In a modified transwell assay, primary (Fig.3A) and immortalized keratinocytes (Suppl. Fig.S2A) from K5-R1/R2 mice showed a strongly reduced migration rate in defined keratinocyte serum-free medium. Addition of FGF7 and FGF10 further stimulated migration of keratinocytes from wild-type, but not from K5-R1/R2 mice. Surprisingly, cells from K5-R1/R2 mice even showed a reduced migratory capacity in the presence of high concentrations of epidermal growth factor (EGF), demonstrating that EGF can only partially substitute for FGFs (Fig.3A). This was not due to a reduced responsiveness of the FGFR1/2-deficient cells to EGF (Yang et al., 2010), but rather reflects the intrinsic migratory deficiency of the cells that was also seen in the absence of an exogenous growth factor.
Fig. 3. Loss of FGFR1 and FGFR2 affects migration of cultured keratinocytes. (A) Primary keratinocytes from control and K5-R1/R2 mice were analyzed for their migratory capacity using a modified transwell assay. FGF7, FGF10, EGF or vehicle (Ctrl.) was added to the culture medium. Cells at the bottom side of the membrane were counted 24h after seeding of cells onto the upper side. Bars represent mean ± s.e.m. (B). Immortalized keratinocytes from control and K5-R1/R2 mice were grown to confluency. A scratch wound was inserted into the monolayer, and the cells were then incubated in the presence of mitomycin C. Pictures were taken at different time points after scratch wounding. (C) The area devoid of cells was determined 4h and 24h after wounding. Bars represent mean ± s.e.m. N≥12.

The severe migratory defect of primary and immortalized K5-R1/R2 keratinocytes was confirmed in scratch assays (Fig.3A-C and Suppl. Fig.S2B and S3). The difference between FGFR-deficient and control keratinocytes was still observed in the presence of mitomycin C, which inhibits cell proliferation (shown for immortalized keratinocytes in Fig.3B,C) as well as on collagen I-coated dishes (Suppl. Fig.S2B).
Suppl. Fig. S2. The migratory capacity is reduced in immortalized keratinocytes from FGFR1 and FGFR2 deficient mice. (A) Immortalized keratinocytes from control and K5-R1/R2 mice were analyzed in a transwell assay for their migratory capacity. The migrated cells on the bottom side of the membrane were counted 24h after seeding. The graph shows cells counted per field. Bars represent mean ± s.e.m. (B) Immortalized keratinocytes from control and K5-R1/R2 mice were grown to confluency on collagen I coated dishes. A scratch wound was inserted into the monolayer, and pictures were taken immediately after scratch wounding (0h) and 21h later.

Since the scratch closure depends on the migratory speed (velocity), the direction of migration, and the directional persistence (Hartwig et al., 2007), we
analyzed these parameters in our in vitro system (Fig. 4A-E). Live-cell recordings of migrating, immortalized keratinocytes were taken after scratching the cell layer using two independent lines of immortalized keratinocytes from control and K5-R1/R2 keratinocytes (Suppl. movies 1 and 2). K5-R1/R2 cells consistently migrated with a reduced velocity compared to control cells (Fig. 4B). Furthermore, their directional persistence was severely impaired, and they did not migrate into the scratch as efficiently as control cells (Fig. 4C-E).
**Fig. 4. Loss of FGFR1 and FGFR2 reduces velocity and impairs directional migration of cultured keratinocytes.** Immortalized keratinocytes from control and K5-R1/R2 mice were subjected to scratch wounding and analyzed by live cell imaging for 13 h. Eight cells from a minimum of 5 movies were analyzed for each genotype, including 4 cells from the front row and four cells from the fifth row behind the front. All results were reproduced with an independent cell line from control and K5-R1/R2 mice. (A) Schematic illustration of parameters analyzed for the quantification of migration. The migrating cells were analysed for (B) velocity, (C) displacement (linear distance between starting and endpoint; “b” in (A)), (D) persistence coefficient (“b”/”a” in (A); Hartwig et al., 2007) and (E) perpendicular movement (“c” in (A)). Bars represent mean +/- S.E.M. (F,G) Migrating keratinocytes were stained with an antibody against giantin to label the Golgi apparatus (green) together with rhodamine-conjugated phalloidin (red) and counterstained with Hoechst (blue). Note the polarization of the Golgi apparatus in cells from wild-type mice, but the equal distribution of giantin around the nucleus in cells from K5-R1/R2 mice. The scratch is at the top of the pictures. Scale bar: 50 µm. The percentage of cells with polarized Golgi among all cells is shown in (G). Bars represent mean +/- S.E.M. A minimum of six different pictures from two different cell lines per genotype was analyzed. (H) A representative picture of a migrating cell with a prominent ruffle is shown on the right hand side. Ruffles are indicated by a white dot. Pictures of a scratch assay experiment were taken to count cells with ruffles. 4 time points of the live imaging experiment were chosen. The percentage of cells with ruffles among all cells was determined at the indicated time points. Bars represent mean +/- S.E.M. N≥5.

These differences were observed for cells at the migrating front as well as for cells in the fifth row behind the front, with the exception of velocity, which was only significantly different for cells in the front row (Suppl. Fig. S3). The defect in directional migration was further reflected by the impaired Golgi polarization upon scratch wounding as determined by immunostaining with an antibody against the Golgi marker protein giantin. This protein was concentrated in front
of the nucleus of migrating cells from wild-type mice (Fig.4F, left panel), but it was evenly distributed around the nucleus of cells from K5-R1/R2 mice (Fig.4F right panel and G). Again, this difference was seen for cells in the front row as well as for the cells in the rows located further behind (Fig.4G and data not shown).

It has previously been shown for cultured human keratinocytes that enhanced formation of membrane ruffles reflects inefficient migration (Borm et al., 2004). Consistent with this observation, there were significantly more cells from K5-R1/R2 mice with ruffles compared to cells from wild-type mice, and this was particularly obvious at later time points after scratching of the monolayer (Fig.4H).
Suppl. Fig. S3. Loss of FGFR1 and FGFR2 reduces velocity and impairs directional migration of cultured keratinocytes at the leading edge of the scratch and further behind. Immortalized keratinocytes from control and K5-R1/R2 mice were subjected to scratch wounding and analyzed by live cell imaging for 13 h. Eight cells from a minimum of 5 movies were analyzed for each genotype – 4 cells from the leading edge (front row) and 4 cells located further behind (fifth line). All results were reproduced with an independent cell line from control and K5-R1/R2 mice. (A) Schematic illustration of parameters analyzed for the quantification of migration. The migrating cells were analysed for (B) velocity, (C) displacement (linear distance between starting and end point; “b” in (A)), (D) persistence coefficient (“b”/“a” in (A)), and (E) perpendicular movement (“c” in (A)). Bars represent mean +/- s.e.m. Note the similar migratory behaviour of cells in the front row and in the fifth row, with exception of velocity, where a difference between genotypes was only observed for cells in the front row.

Impaired attachment and focal adhesion formation of K5-R1/R2 keratinocytes

Upon passaging of primary and immortalized keratinocytes we noticed that their adhesion to the substrate and subsequent spreading was delayed compared to control cells. To quantify this finding, we performed adhesion assays with immortalized keratinocytes. To limit the deposition of laminin-322, which mediates efficient keratinocyte adhesion (Nguyen et al., 2000), we analyzed the attachment within a short (45 min) period. The reduction of adhesion of FGFR1/R2-deficient keratinocytes on PBS-coated dishes or on dishes coated with different concentrations of fibronectin was significantly reduced compared to cells from control mice (Fig.5A). Only a minor reduction was observed on collagen I-coated dishes. Subsequently, we analyzed the actin cytoskeleton of these cells at different time points after seeding into collagen I/fibronectin coated plates by staining with rhodamine-coupled phalloidin (Fig.5B; red). Concomitantly, formation of focal adhesions (FA) was analyzed by immunofluorescence staining with antibodies against total or phosphorylated paxillin (Fig.5B; green). Twenty minutes after seeding, control cells reproducibly
showed efficient attachment and spreading, and FAs were uniformly distributed at the cell periphery. By contrast, the vast majority of cells from K5-R1/R2 mice were still rounded and exhibited only a few FAs. Representative examples are shown in Fig.5B. Ninety minutes after seeding, cells of both genotypes had attached, but there were far fewer FAs in K5-R1/R2 cells compared to controls. The FAs that had formed were unevenly distributed, and approximately 25% of the cells had between 1 and 10 very large FAs (Fig.5B and C). In contrast, less than 10% of control cells formed such large FAs. This finding suggests that FA turnover is also impaired. Since spreading and migration require the rapid formation and turnover of FAs, these findings provide a likely explanation for the migratory deficiency.
Fig. 5. Impaired adhesion and focal adhesion formation of K5-R1/R2 keratinocytes. (A) Immortalized keratinocytes from control and K5-R1/R2 mice were analyzed for adhesion on BSA, collagen I, or fibronectin as described in Materials and Methods. Three different concentrations of fibronectin were used (1, 2.5 and 5 µg/ml as indicated). Bars represent mean +/- s.e.m. N=4 wells per genotype. (B) Immortalized keratinocytes from control and K5-R1/R2 mice were allowed to attach for 20 or 90 min on collagen I / fibronectin coated dishes. Cells were stained with antibodies against total or phosphorylated paxillin (green). The actin cytoskeleton was visualized using rhodamine-conjugated phalloidin (red). Cells were analyzed by confocal microscopy. Bars indicate 50 µm. Cells representative for the different genotypes are shown. FAs in control cells are indicated with arrows. (C) The percentage of cells with large FAs (between 1 and 10 per cell; indicated by asterisks on cells from K5-R1/R2 mice) among all cells was determined. N=7 photomicrographs per cell line, including at least 680 cells. The result was reproduced with independent cell lines. Bars represent mean +/- s.e.m. Scale bars: 100 µm.

Reduced expression of focal adhesion kinase (FAK) and paxillin in K5-R1/R2 keratinocytes

We next determined the mechanisms underlying the impaired adhesion and FA formation of keratinocytes from K5-R1/R2 mice. For this purpose we first determined if loss of FGFR1 and FGFR2 on keratinocytes affects the expression of major keratinocyte integrins. Flow cytometry analysis of immortalized keratinocytes revealed no significant difference in surface expression levels of the fibronectin-binding integrin subunits αv and β1 in control versus K5-R1/R2 keratinocytes, and there was also no difference in integrin β1 activation as
assessed by binding of the activation-specific monoclonal antibody 9EG7 (Fig.6A).

Fig. 6. FGFs control expression of FAK and paxillin, but not of integrins in keratinocytes

(A) Immortalized keratinocytes from control and K5-R1/R2 keratinocytes were stained with antibodies against the integrin subunits αv, α6, β1, or active integrin β1 (β1*) or unspecific isotype controls and analyzed by flow cytometry. Numbers indicate the ratio of the mean fluorescence intensity (± relative s.e.m.) between cells from K5-R1/R2 mice and those from control mice. Results are representative for triplicate measurements repeated at least three times. (B) Protein lysates were prepared from exponentially growing immortalized keratinocytes from K5-R1/R2 and control mice grown in migration medium (see Materials and Methods). 30 μg of the lysates were analyzed by western blotting for the levels of total and phosphorylated paxillin and FAK or β-Actin (loading control). Band intensities from triplicate experiments (using samples from 3 independent wells) were scanned. Bars represent mean ± s.e.m. (C) HaCaT keratinocytes were grown to confluency, starved for 20h in serum-free medium and subsequently treated for 26h with 10 ng/ml FGF7 or vehicle. Protein lysates were prepared from vehicle-treated and FGF7-treated keratinocytes, and levels of total FAK and paxillin were determined by western blotting. Probing of the membrane with an antibody against GAPDH was used as a loading control. Quantification of the band intensities is shown in the bar graph. Expression levels in vehicle-treated cells were arbitrarily set to 1 (indicated by dashed line).
Integrin subunits α1, α2, α5, and β3 were undetectable or barely detectable, indicating low expression levels (data not shown). We next determined if the impaired formation and possibly turnover of FAs results from reduced expression and/or phosphorylation of major FA components. We focused on paxillin and FAK, since these proteins were shown to be required for hepatocyte growth factor induced cell spreading and migration (Ishibe et al., 2004). Western blot analysis of exponentially growing primary and immortalized keratinocytes revealed that levels of total FAK and paxillin were indeed strongly reduced in immortalized and primary K5-R1/R2 keratinocytes compared to control cells (Fig. 6B and Suppl. Fig. S4A). As a consequence, levels of phosphorylated paxillin (Y-118) and phosphorylated FAK (Y-397) were reduced to a similar extent. When starved human HaCaT keratinocytes or immortalized keratinocytes from control mice were treated with recombinant FGF7 and analyzed for the levels of total FAK and paxillin, we reproducibly found an increase in the levels of these proteins within 12-24h after addition of FGF7 (Fig. 6C and Suppl. Fig. S4B). These findings demonstrate that FGF7 controls their expression in keratinocytes.

Suppl. Fig. S4. FGFR signaling controls expression of paxillin and FAK, but not of MMPs in keratinocytes
(A) Protein lysates from exponentially growing primary keratinocytes from K5-R1/R2 and control mice were analyzed for levels of total and phosphorylated paxillin and FAK or β-actin (loading control). (B) Immortalized keratinocytes from control mice were starved and subsequently treated with FGF7 for 12 or
24h. Protein lysates were prepared from untreated and FGF7-treated keratinocytes at the same time points and levels of total FAK and paxillin were determined by western blotting. Probing of the membrane with an antibody against GAPDH was used as a loading control in. Quantification of the band intensities is shown in the bar graphs. (C) Expression and activation of MMP9 and MMP10 was analyzed by casein zymography of cell culture supernatant of immortalized keratinocytes from control and K5-R1/R2 mice. A representative gel is shown. The experiment was performed in triplicate with 3 independent cell lines per genotype.

Discussion

Chronic, non-healing wounds are a major health problem in an aging society (Sen et al., 2009). Although rarely life threatening, they severely affect the quality of life of the affected individuals and cause enormous costs to the health care system. Therefore, there is a strong need to develop novel and efficient strategies for the treatment of these disorders, and this requires a thorough understanding of the underlying cellular and molecular mechanisms. Here we show that loss of FGFR1 and FGFR2 in keratinocytes results in a wound healing defect, which resembles the abnormalities seen in chronic human skin ulcers. This phenotype is characterized by severely impaired reepithelialization combined with reduced wound contraction (Usui et al., 2008). The latter effect is obviously indirect in the K5-R1/R2 mice, since the loss of FGFR1 and FGFR2 occurred specifically in keratinocytes and not in cells of the dermis/granulation tissue. It seems likely that the loss of FGFR1 and FGFR2 in keratinocytes results in the production of factors that affect the underlying dermis/granulation tissue. Indeed, we previously showed a strong upregulation of S100A8 and S100A9 as well as of interleukin-1 family member 8 in the non-wounded epidermis of K5-R1/R2 mice, which strongly affected immune cells and fibroblasts in the dermis. Therefore, these and possibly additional factors that are abnormally expressed in the wound epidermis of K5-R1/R2 mice and control animals are likely to affect wound contraction in a paracrine manner. However, these factors did not
obviously affect differentiation of myofibroblasts, the cell type that is mainly responsible for wound contraction (Rungger-Brandle and Gabbiani, 1983). Furthermore, we also did not observe a granulomatous reaction that could impair contraction. Rather, it seems likely that the alterations in the connective tissue that develop in these mice (Meyer et al., 2011) are responsible for the impaired wound contraction.

Whereas the impaired contraction is obviously a non-cell autonomous phenotype, the delayed reepithelialization seen in K5-R1/R2 mice is a direct consequence of the loss of FGFR1 and FGFR2 in keratinocytes. Impaired reepithelialization was also observed in transgenic mice expressing a dominant-negative FGFR mutant in keratinocytes (Werner et al., 1994), although this approach did not allow the identification of the responsible FGF receptor(s). The results of our new study revealed that loss of a single FGFR does not significantly affect the wound healing process, whereas the combined loss of FGFR1 and FGFR2 is deleterious. We previously showed that keratinocytes express mainly or exclusively the IIIb splice variants of these receptors (Yang et al., 2010), which are activated by FGF1, FGF7, FGF10 and FGF22 (Zhang et al., 2006). All of these growth factors are expressed in healing skin wounds, and at least FGF7 and FGF22 are strongly upregulated at the wound site (Werner et al., 1992; Beer et al., 1997; Beyer et al., 2003). This expression pattern, together with the results presented in this study, strongly suggest that activation of FGFR1-IIIb and in particular of the more abundant FGFR2-IIIb by the above-mentioned FGFs is crucial for efficient wound reepithelialization.

Reepithelialization is achieved by a combination of keratinocyte proliferation and migration. Interestingly, the rate of keratinocyte proliferation was not impaired in wounded skin of K5-R1/R2 mice, and even a slight hyperproliferation was observed. The latter was already seen in non-wounded skin and shown to result from the progressive inflammation that develops in these animals (Yang et al., 2010). Consistent with this finding, mice expressing a dominant-negative FGFR2-IIIb mutant in keratinocytes, which did not show cutaneous inflammation, exhibited reduced keratinocyte proliferation at the wound site (Werner et al., 1994). Furthermore, cultured keratinocytes from K5-R1/R2 mice did not show hyperproliferation (Yang et al., 2010), demonstrating
that this phenotype is not cell autonomous. In spite of the normal or even enhanced keratinocyte proliferation in K5-R1/R2 mice, wound reepithelialization was delayed as a result of impaired keratinocyte migration. At later stages of the repair process, however, the strong proliferation of keratinocytes obviously compensated for the impaired migration.

The data presented in this manuscript reflect the importance of FGF receptor signalling for keratinocyte migration and are consistent with the potent effect of exogenous FGF7 on migration of human keratinocytes (Ceccarelli et al., 2007). Surprisingly, keratinocytes from K5-R1/R2 mice did not only show impaired migration in response to FGFs but also to other mitogens, including EGF, suggesting non-redundant functions of FGFs in keratinocyte migration. The reduced pro-migratory effect of EGF was not due to a generally reduced responsiveness to this mitogen, since EGF strongly activated the Erk1/2 and PI3K signalling pathways in keratinocytes from control and K5-R1/R2 mice (Yang et al., 2010). Finally, migration of cells from K5-R1/R2 mice in a transwell assay was still reduced in the absence of exogenous growth factors. This finding may well be explained by ligand-independent activation of FGFR2 as previously shown for mechanically wounded keratinocytes (Li et al., 2009) and/or by the difference in the expression levels of paxillin and FAK.

Our functional studies revealed that the migratory defect of keratinocytes from K5-R1/R2 mice did not result from impaired expression or alterations in the activation of keratinocyte integrins. Furthermore, expression and activation of major keratinocyte matrix metalloproteinases was not obviously affected by the loss of FGFR1 and FGFR2 (Suppl. Fig.S4C). Rather, we identified a defect in cell attachment and formation of FAs as a consequence of reduced levels of total and phosphorylated FAK and paxillin. These proteins were selectively affected, since levels of vinculin, another FA component, were not altered in the absence of FGFR1 and FGFR2 (data not shown). Interestingly, treatment with FGF7 increased the total mRNA and protein levels of FAK and paxillin (Fig.6 and data not shown), demonstrating that FGFs control the expression of these major FA components in keratinocytes.

The reduced levels of major FA proteins provide a likely explanation for the abnormalities in FA formation and turnover that we observed in the absence of
FGFR1 and FGFR2. Thus, FA formation was delayed upon seeding of the cells, and the FAs that formed were strongly enlarged. Large FAs were shown to inhibit cell migration through formation of less dynamic anchoring structures and limited generation of propulsive forces (Beningo et al., 2001), a finding that is consistent with the migratory defect of K5-R1/R2 keratinocytes. Such enlarged adhesions were shown to result from reduced tyrosine phosphorylation of paxillin (Zaidel-Bar et al., 2007), and we indeed observed reduced paxillin-Y118 levels in FGFR1/R2-deficient keratinocytes.

It has previously been shown that the presence of activated FAK at FAs is required for FA disassembly at the base of extending lamellipodia, a process required for efficient directed cell migration (Webb et al., 2004). This process requires concomitant activation of Rac (Ishibe et al., 2004), and our preliminary data suggest that this is also impaired in K5-R1/R2 keratinocytes (data not shown). It remains to be determined if additional alterations contribute to the migratory deficiency of K5-R1/R2 keratinocytes, and major proteinases that are controlled by FGF7 in epithelial cells (Putnins et al., 1995) are obvious candidates that should be analyzed in future studies.

A defect in keratinocyte migration was also observed in mice lacking other growth factors or their receptors, and a particularly severe phenotype was seen in the absence of the hepatocyte growth factor receptor c-Met. In mice lacking c-Met in keratinocytes, only keratinocytes that had escaped recombination were able to contribute to wound reepithelialization, demonstrating that c-Met signalling is essential for this process (Chmielowiec et al., 2007). Such a severe phenotype was not observed in K5-R1/R2 mice, indicating that FGFR1/FGFR2-deficient keratinocytes are still able to reepithelialize the wound. Indeed, migrating cells from K5-R1/R2 mice were tested for recombination in cell culture and shown to be true knockouts for the receptors as determined by Real-Time RT-PCR (data not shown). Therefore, FGFR1 and FGFR2 are obviously not essential for keratinocyte migration, but their loss strongly impairs this process.

Impaired keratinocyte migration combined with normal or even enhanced proliferation of these cells is a hallmark of chronic human skin ulcers (Harsha et al., 2008; Usui et al., 2008). Therefore, it will be interesting to determine if reduced expression of FGF receptors or their ligands occurs in these ulcers or if
FGFR signalling is impaired in the keratinocytes of these patients, in particular at the wound edge. This would be a prerequisite for the development of novel and more efficient strategies for the improvement of these severe wound healing disorders by activating FGFR signalling pathways.
References


My contribution to this publication was:

- Transwell assay with immortalized keratinocytes from control and K5-R1/R2 mice (Suppl. Fig. S2A)
- Cell adhesion assay with immortalized keratinocytes from control and K5-R1/R2 mice (Fig. 5A)
- Live cell imaging experiments (Fig. 4 A-E, Fig. 4H, Suppl. Fig. S3)
- Cell attachment assay with immortalized keratinocytes from control and K5-R1/R2 mice and subsequent immunofluorescence analysis with antibodies against paxillin, p-paxillin, FAK and p-FAK (Fig. 5B, 5C), combined with fluorescence analysis of Cy3-conjugated phalloidin
- Scratch assay with immortalized and primary keratinocytes from control and K5-R1/R2 mice on uncoated and coated dishes, followed by microscopic analysis of the scratch healing (Fig. 3B, 3C, Suppl. Fig. S2)
- Scratch assay with immortalized and primary keratinocytes from control and K5-R1/R2 mice and subsequent immunofluorescence analysis using an antibody against giantin (Fig. 4F)
- Protein extraction from exponentially growing primary and immortalized keratinocytes from control and K5-R1/R2 mice, followed by analysis of proteins by western blotting (Fig. 6B, Suppl. Fig. S4A)
- Treatment of immortalized keratinocytes from control mice and of human HaCaT cells with FGF7 with subsequent preparation of protein lysates and analysis by western blotting (Fig. 6C)
- Analysis of active MMPs in supernatant of immortalized control and K5-R1/R2 keratinocytes using gelatin and casein zymography (Suppl. Fig. S4B)
- Immunofluorescence analysis of wounds from control and K5-R1/R2 mice with antibodies against α smooth muscle actin and Ly6G (Suppl. Fig. S1C-D)
3.4 The roles of receptor tyrosine kinases and their ligands in the wound repair process

Anna-Katharina Müller*, Michael Meyer*, and Sabine Werner

*Equal contribution

Institute of Molecular Health Sciences,
Department of Biology, ETH Zürich, 8093 Zürich, Switzerland

Running head: Receptor tyrosine kinases in wound healing
Abstract

Cutaneous wound repair is a tightly regulated and dynamic process involving blood clotting, inflammation, formation of new tissue, and tissue remodeling. Gene expression profiling of mouse and human wounds as well as first proteomics studies have identified a large number of genes and proteins that are up- or downregulated by skin injury, and some of them have been functionally characterized in animal models of wound repair. Among the key regulators of wound repair are growth factors, which control migration, proliferation, differentiation and survival of cells at different stages of the healing process. This review summarizes the results of functional studies performed in mammals that have identified important roles of receptor tyrosine kinases and their ligands in wound repair.
Key words:
Wound healing, growth factor, receptor tyrosine kinase, reepithelialization, granulation tissue, scar

Abbreviations
Ang: Angiopoietin
DETC: Dendritic epidermal T cell
EGF: Epidermal growth factor
FGF: Fibroblast growth factor
FGF-BP1: Fibroblast growth factor binding protein 1
HB-EGF: Heparin-binding epidermal growth factor
HGF: Hepatocyte growth factor
IGF: Insulin-like growth factor
MSP: Macrophage-stimulating peptide
PDGF: Platelet-derived growth factor
PLGF: Placenta growth factor
TGF: Transforming growth factor
VEGF: Vascular endothelial growth factor
1. Introduction

Injury to mammalian skin initiates a well-coordinated series of events that culminate in the reconstitution of the injured body site. Within seconds after injury, soluble mediators released from degranulating platelets, from damaged cells at the wound edge or from the serum, stimulate migration and proliferation of resident cells in the wound tissue and also attract and activate hematopoietic cells. In parallel, blood clotting occurs, and the resulting fibrin clot provides a barrier against invading pathogens. The clot also forms a provisional matrix for immune cells, fibroblasts and endothelial cells that are attracted to the wound site from the circulation or migrate into the clot from the wound edges. The first invading immune cells are neutrophils, followed by monocytes, mast cells and lymphocytes. These immune cells are an important source of various cytokines and growth factors, which are released upon injury or produced in response to signals present in the wounded tissue. Immune cells, in particular neutrophils and macrophages, also secrete proteolytic enzymes and reactive oxygen species as a defense against invading microorganisms, and they efficiently phagocytose cell debris and apoptotic cells [1, 2].

After the initial inflammatory phase, the formation of new tissue is initiated. Keratinocytes of the injured epidermis and hair follicles migrate into the wound, followed by massive proliferation of these cells that allows the coverage of the wound with a new epidermis. The latter is initially hyperplastic and poorly differentiated, but re-differentiation eventually occurs, which is accompanied by re-formation of a functional epidermal barrier. In parallel, dermal repair occurs, which involves migration and proliferation of fibroblasts at the wound edge and to a lesser extent invasion and subsequent proliferation of bone marrow-derived fibrocytes. A large percentage of the wound fibroblasts differentiate into myofibroblasts, which strongly contribute to wound contraction. Endothelial cell sprouting occurs at the wound edge, resulting in the formation of a new vascular network. These multiple new capillaries are responsible for the granular appearance of the newly formed stromal tissue, which is therefore called granulation tissue. In parallel to the formation of new blood vessels,
lymphangiogenesis occurs at the wound site to re-establish the lymphatic vasculature, and nerve sprouting results in re-innervation of the new tissue. The final phase of the wound healing process is characterized by apoptosis of excessive numbers of fibroblasts and endothelial cells and continuous remodeling of the extracellular matrix, which can last for several months. Unfortunately, wound healing in mammals does not result in regeneration of the injured dermis. Rather, the result of the repair process is a scar with reduced mechanical and elastic properties compared to normal skin and lack of epidermal appendages [1, 2].

Gene expression profiling using microarrays as well as proteomics experiments have identified a large number of genes and their products, which are expressed at the wound site and orchestrate the repair process [3-6]. Some of them have been functionally characterized using knockout, knock-down or overexpression approaches or by application of neutralizing antibodies. These studies identified important functions of different growth factors in the control of migration, proliferation, differentiation and survival of cells in the wound tissue. In this review, we will summarize the results of functional in vivo studies that revealed the roles and activities of different peptide growth factors in wound healing of mammals (Table 1). Due to space limitations, we focus on the “classical” peptide growth factors that act via receptor tyrosine kinases and their roles in mammalian wound repair. The receptor tyrosine kinases, which are covered in this review article, are shown schematically in Fig.1. We also briefly mention the sites of expression of the growth factors and their receptors, which are summarized in detail in a previous review of our laboratory to which we frequently refer [7].
Fig. 1: Schematic representation of receptor tyrosine kinases and their ligands for which a functional role in wound healing has been demonstrated.

2. Roles of receptor tyrosine kinases in wound repair

2.1 Platelet-derived growth factor (PDGF) receptors and ligands

PDGFs comprise the homodimers PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD as well as the heterodimer PDGF-AB. They bind and activate three transmembrane tyrosine kinase receptors, which are homo- or heterodimers of an α- and a β-chain [8]. PDGF (PDGF-AB in humans) is stored in platelets and released upon their injury-induced degranulation. Therefore, it is present at the wound site during the early phase of healing. During the course of wound repair, PDGF is predominantly expressed by keratinocytes, whereas the receptors are expressed on immune cells, fibroblasts and perivascular cells. Therefore, PDGF acts predominantly in a paracrine manner [9]. The reduced expression of PDGF and its receptors in impaired wound healing of mice and humans [7] suggested that
normal expression of these genes is required for an efficient wound healing process.
To determine the function of PDGFs and their receptors in the wound healing process, the PDGF receptor (PDGFR) β gene was deleted in fibroblasts. Proliferation and survival of these cells was severely impaired, and their migratory capacity in culture was completely abrogated [10]. Consistent with this finding, treatment of human wound fluid with a neutralizing PDGF antibody reduced its mitogenic effect for cultured fibroblasts [11]. In vivo, systemic treatment of mice with the PDGFR β inhibitor imatinib mesylate caused a strong delay in wound closure and granulation tissue formation. Blood vessel maturation was also impaired as reflected by the strong reduction in the number of pericytes around the vessels in the granulation tissue [12]. The important role of PDGF in granulation tissue formation was supported by a recent gain-of-function study, where expression of a hyperactive PDGFR β variant in fibroblasts enhanced proliferation and migration of these cells in vitro and granulation tissue formation in vivo after full-thickness skin wounding [13] (Fig.2).
To determine the importance of PDGF from hematopoietic origin versus PDGF produced by resident cells for the wound healing process, bone marrow chimeras were prepared by replacing the hematopoietic system of normal adult mice by that of PDGFR β-deficient mice. Surprisingly, granulation tissue formation was not affected in the chimeric mice and vascularization was even increased. Therefore, production of PDGF by resident cells is obviously sufficient for normal repair [14].
Finally, a potential role of PDGF-DD in wound healing was investigated in a gain-of-function approach where this growth factor was overexpressed in keratinocytes of transgenic mice. These animals showed increased cell density in the granulation tissue and enhanced recruitment of macrophages after full-thickness excisional wounding [15]. A potential role of endogenous PDGF-DD in wound healing remains to be determined.
2.2 Epidermal growth factor (EGF) receptors and ligands

The EGF family of mammals includes the founding member EGF, as well as heparin-binding EGF (HB-EGF), transforming growth factor-a (TGF-a), amphiregulin, epiregulin, epigen, betacellulin and the more distantly related neuregulins (heregulins, neu differentiation factors, NDF 1-4). The biological activities of these growth factors are mediated by homo- or heterodimers of four transmembrane receptor tyrosine kinases, designated EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) [16].

EGF, TGF-a and HB-EGF have been detected in wound fluid [7], and various inflammatory cells and resident cells, in particular, keratinocytes, express TGF-a, HB-EGF, and/or epiregulin [17]. They mainly act via EGFR, which is highly expressed in different cell types of the healing wound, in particular in keratinocytes. The latter also express ErbB2 and ErbB3 [17], indicating that different members of the EGF/EGFR families are involved in reepithelialization (Fig.2). An important role of the EGFR in this process was confirmed by wound healing studies in EGFR knockout mice. These mice exhibited a strong delay in wound repair as a result of impaired reepithelialization and wound contraction.

A detailed analysis of the healing wounds revealed that the EGFR controls various processes during wound healing, including keratinocyte migration and proliferation as well as inflammation and angiogenesis [18]. The responsible ligands of the EGFR have as yet not been identified. However, it has been shown that their shedding is important for efficient wound healing. EGFR ligands are produced as membrane-anchored forms, which can activate receptors on neighboring cells in a juxtacrine manner. These precursors can be proteolytically processed to soluble forms, which then act in an autocrine or paracrine manner [16]. Inhibition of this cleavage by a chemical inhibitor suppressed keratinocyte migration in vitro and in wounded skin in vivo, resulting in a severe delay in reepithelialization [19].

In a search for the responsible ligands, wound healing studies in mice lacking individual EGFR family members were performed. When TGF-a knockout mice were subjected to full-thickness excisional wounding or tail amputation, no obvious wound healing abnormalities were observed [20, 21]. However, a role of
TGF-a in reepithelialization was identified in a partial thickness ear wound model, where healing is mainly achieved by reepithelialization [22]. Under these conditions, the early phase of reepithelialization was delayed. These results demonstrate the importance of the chosen wound model for the analysis of growth factor function in wound repair. Mice with a keratinocyte-specific knockout of HB-EGF exhibited a marked delay in wound closure, in particular due to a severe delay in keratinocyte migration [23] (Fig.2). Wound healing studies in mice lacking other members of the EGF family have as yet not been published. However, a gain-of-function study suggests a role of betacellulin in wound angiogenesis. Mice overexpressing this growth factor under control of a ubiquitously active promoter had normal wound closure, but there was a strong increase in the formation of new blood vessels [24] (Fig.2).

2.3 Fibroblast growth factor (FGF) receptors and ligands

The FGF family includes 22 members in mammals, which control proliferation, differentiation, migration and survival of various cell types. Most of these FGFs bind and activate four transmembrane protein tyrosine kinases, designated FGF receptors 1-4 (FGFR1-4). Additional complexity in the FGFR family is achieved by alternative splicing in the extracellular domains of FGFR1-3, which dramatically affects their ligand binding specificities [25]. Several FGFs are present at the wound site and some of them, in particular FGF7, are strongly upregulated upon skin injury [26, 27]. Studies with FGF2 neutralizing antibodies or FGF2 knockout mice revealed a role of this type of FGF in the early phase of angiogenesis and in granulation tissue formation [28, 29] (Fig.2). The phenotype was not aggravated by concomitant loss of FGF1, demonstrating that the latter does obviously not compensate for the lack of FGF2 at the wound site [30].

In contrast to FGF2, which activates the IIIc splice variants of FGFR1 and FGFR2 that are predominantly expressed by stromal cells, FGF7, FGF10 and FGF22 target epithelial cells, including keratinocytes. These cells express the IIIb variants of FGFR1 and FGFR2. FGF7 and FGF10 are predominantly produced by
stromal cells, whereas FGF22 is produced by keratinocytes [27]. To test the importance of these ligands and their receptors for the wound healing process, mice lacking FGF7, FGF22 or FGFR1-IIIb were subjected to full-thickness wounding. Whereas the individual loss of these genes did not affect the wound healing process [31-33], mice expressing a dominant-negative FGFR2-IIIb mutant in keratinocytes had a strong delay in reepithelialization [34]. This receptor mutant inhibits signalling through all FGF receptors in response to common ligands. Therefore, it seems likely that FGFR1 and FGFR2 collaborate to stimulate reepithelialization, and recent results from our laboratory using mice lacking these receptors in keratinocytes support this hypothesis (our unpublished data). Similarly, compensation and/or redundancy among the ligands seem likely as suggested by the impaired reepithelialization in mice lacking dendritic epidermal T cells (DETC). They are an important source of FGF7 and FGF10 in the wound, and the reduction in the levels of these growth factors in wounds of DETC-deficient mice provides a likely explanation for their defect in reepithelialization [35].

Whereas loss of FGF action in healing wounds is detrimental, enhancement of FGF activity strongly promoted the wound repair process. This was demonstrated in a transgenic mouse model where FGF binding protein 1 (FGF-BP1) was inducibly overexpressed under control of a ubiquitously active promoter. FGF-BP1 binds several members of the FGF family and enhances their activities by facilitating their release from the extracellular matrix, allowing their access to the signaling receptors. Overexpression of FGF-BP1 enhanced macrophage invasion, cell proliferation and angiogenesis during wound healing, resulting in a strong acceleration of the wound healing process [36].

2.4 Met, Ron and their ligands

The small family of scatter factors encompasses hepatocyte growth factor (HGF)/scatter factor and macrophage-stimulating protein (MSP; scatter factor 2). They are also designated plasminogen-related growth factors 1 and 2 due to their homology with plasminogen. Both factors are secreted as inactive
precursors, which are proteolytically cleaved to produce the active heterodimers [37].

HGF is predominantly produced by mesenchymal cells, and it exerts its biological activities through binding and activation of a high-affinity transmembrane tyrosine kinase receptor (Met) [37]. Expression of HGF and Met increases upon skin injury, in particular in endothelial cells and keratinocytes at the wound edge. By contrast, HGF is mainly produced by fibroblasts, but it is also transiently expressed in keratinocytes upon wounding [7, 38]. An important function of HGF in wound repair was first suggested from results obtained in a gain-of-function approach. Thus, transgenic mice expressing HGF under the control of the metallothionein promoter showed strongly enhanced granulation tissue formation after full-thickness excisional wounding, in particular due to increased angiogenesis. The latter effect may be direct, but could also result from increased expression of vascular endothelial growth factor (VEGF-A) [39].

A recent study revealed that Met is phosphorylated and therefore activated in keratinocytes and dermal cells of normally healing human wounds, but not of non-healing chronic wounds. This was due to degradation of HGF by neutrophil elastase and plasma kallikrein in the chronic wound environment, which strongly inhibited its biological activity. These findings suggest that high levels of intact HGF are required for normal healing [40]. This hypothesis is supported by results obtained in a mouse model, where application of neutralizing antibodies to HGF strongly impaired wound angiogenesis, granulation tissue formation and wound reepithelialization, resulting in a severe delay in wound closure [41] (Fig.2).

To study the role of HGF and its receptor in wound reepithelialization, mice lacking Met in keratinocytes were generated. Loss of this receptor did not affect normal skin development and homeostasis. However, the healing process of full-thickness excisional wounds was strongly delayed, although the wounds finally healed. Characterization of wound keratinocytes that had been isolated by laser capture microdissection revealed that all keratinocytes that finally contributed to reepithelialization were cells that had escaped recombination and therefore still expressed Met. Thus, Met-deficient keratinocytes are obviously not able to contribute to reepithelialization. This was mainly due to a migratory deficiency
of these cells as demonstrated by *in vitro* migration studies [38]. Taken together, this study shows that HGF and Met have a unique function in wound reepithelialization and their loss cannot be compensated by other growth factors.

MSP is a liver-derived protein that is released into the serum as inactive precursor. Proteolytic activation usually occurs at the surface of its target cells. This allows the interaction with RON, its only known high-affinity receptor [37]. MSP was originally detected in wound exudates and found to stimulate activation of macrophages. These cells as well as various other cells in the wound tissue also express the Ron receptor [7]. This expression pattern suggested a role of MSP in the wound healing process. Indeed, macrophage activation was delayed in MSP-deficient mice (Fig.2), but healing of incisional wounds was not obviously affected [42].

![Schematic representation of a full-thickness excisional skin wound during the phase of new tissue formation](image)

**Fig. 2:** Schematic representation of a full-thickness excisional skin wound during the phase of new tissue formation. Growth factors, which had been shown to affect certain cell types in functional *in vivo* wound healing studies, are indicated, together with their target cells. D: Dermis; E: Epidermis, Es: Eschar; HE: Hyperproliferative wound epidermis; HF: Hair follicle.

### 2.5 Insulin-like growth factor (IGF) receptor and its ligands

IGFs I and II stimulate proliferation and survival of various cell types. They signal through a transmembrane receptor tyrosine kinase, the IGF receptor. IGF-II also
binds to the IGF type II/mannose-6-phosphate receptor, resulting in internalization and subsequent proteolytic degradation of this ligand [43]. IGF-I and IGF-II are expressed at low levels in normal skin, but their expression strongly increases upon skin injury. Interestingly, abnormal expression of IGFs and IGF receptor is a hallmark of poorly healing wounds as seen in diabetic mice or glucocorticoid-treated mice. Similarly, reduced expression of IGF-I was also seen in the skin of diabetic patients compared to healthy individuals [7].

An important effect of IGF-I on wound repair was shown in gain-of-function studies. For example, exogenous liposomal IGF-I cDNA gene transfer enhanced angiogenesis, granulation tissue formation and reepithelialization in rats [44] (Fig.2). In another study, expression of a locally acting IGF-I variant (mIGF-1) in keratinocytes of transgenic mice accelerated the wound healing process due to increased migration and proliferation of keratinocytes. By contrast, inflammation, granulation tissue formation and scarring were not obviously affected [45].

Up to now, loss-of-function studies have not been performed to address the role of IGFs and their receptor in wound healing. However, the wound healing defect seen in mice lacking DETCs was associated with reduced activation of the IGF-1 receptor at the wound site, since these T cells are an important source of IGF-1 [46]. Interestingly, epidermal T cells from acute human wounds but not from chronic wounds also produce high levels of IGF-I [47], suggesting that IGF-1 produced by epidermal T cells is important for normal healing.

2.6 VEGF receptors and their ligands

The VEGF family comprises 5 members in mammals, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PLGF). They bind and activate the transmembrane tyrosine kinase receptors VEGFR-1, VEGFR-2 and VEGFR-3 [48]. Expression of VEGF-A strongly increases upon skin injury, in particular in keratinocytes and macrophages. This upregulation is much less pronounced in animal models for poorly healing wounds, suggesting that the levels of VEGF-A are rate limiting under these conditions [49]. In support of this hypothesis, proteolytic cleavage of VEGF-A was shown in chronic human wounds [50] and in
wounds of genetically diabetic db/db mice. In the latter, plasmin was identified as the responsible proteinase, and the delayed wound repair was reversed by topical application of a plasmin-resistant VEGF-A mutant [51]. Adenoviral overexpression of VEGF-A in the same diabetic mouse model also accelerated the healing process (Saaristo et al., 2006).

The importance of VEGF-A for normal wound healing was demonstrated when neutralizing VEGF-A antibodies were applied to porcine wounds. This treatment strongly impaired wound angiogenesis and formation of granulation tissue [52]. When wound fluid from acute human wounds was incubated with neutralizing antibodies against VEGF-A, the angiogenic activity of the wound fluid that was obtained at later stages of healing was significantly reduced [53]. Finally, a delay in wound healing was observed in mice with a keratinocyte-specific knockout of VEGF-A [54]. Consistent with these findings, inhibition of VEGF function by retroviral delivery of a dominant-negative VEGFR-2 mutant to mouse skin wounds strongly reduced wound angiogenesis and granulation tissue formation [55]. A similar effect was seen upon adenoviral delivery of a soluble VEGFR-2 [56]. In both cases, however, wound closure was not affected, demonstrating that a reduction in angiogenesis can be tolerated in normally healing animals. In contrast, targeting VEGF is more detrimental in models of impaired healing, in particular in diabetic animals. Indeed, adenoviral expression of a soluble VEGFR-2-Ig in wounds of genetically diabetic mice caused a significant delay in wound closure [57].

The above-mentioned studies revealed that targeting VEGF-A in skin wounds mainly affects wound angiogenesis (Fig.2). However, an additional effect on keratinocytes cannot be excluded. These cells were shown to express VEGFR-1, and treatment of excisional wounds with a neutralizing antibody to this receptor reduced keratinocyte proliferation in vitro and delayed the wound reepithelialization process in vivo [58].

While granulatont tissue formation may be affected by targeting VEGF, inhibition of VEGF-A in normally healing wounds can even have a beneficial effect on the outcome of the healing process as demonstrated by the reduced scarring that was observed when wounds from normal adult mice were treated with VEGF-A neutralizing antibodies [59]. Therefore, the excessive angiogenesis that occurs in
normally healing wounds enhances the production of extracellular matrix and concomitantly the scarring response.

In contrast to VEGF-A, VEGF-B is obviously dispensable for wound healing, since incisional wounds generated in VEGF-B-deficient mice healed normally and angiogenesis was not affected [60]. However, its therapeutic potential for enhancement of wound angiogenesis was demonstrated in transgenic mice expressing VEGF-B in endothelial cells, which displayed enhanced vascular growth [61]. In contrast to these findings, adenoviral overexpression of VEGF-B in wounds of db/db mice had no effect on the wound repair process [57]. The different results obtained in these studies may be due to different expression levels of VEGF-B or to the use of diabetic versus normal mice.

PLGF is highly expressed in migrating keratinocytes and endothelial cells of wounded skin [7]. This is functionally important, since PLGF knockout mice showed impaired angiogenesis and wound closure [62] (Fig.2). A synergy between PLGF and VEGF-A was detected in these experiments, suggesting that the presence of both VEGF family members is required for normal wound angiogenesis.

In contrast to the thoroughly studied process of blood vessel angiogenesis, less is known about the regulation of lymphangiogenesis in healing wounds. Lymphatic vessels express VEGFR-3, which is activated by VEGF-C or VEGF-D [48]. When VEGF-C was overexpressed in wounds of db/db mice via an adenoviral vector, angiogenesis and lymphangiogenesis were significantly accelerated in comparison to control wounds [57]. In addition, monocytes/macrophages and other inflammatory cells were recruited (Fig.2). The importance of endogenous VEGF-C and VEGF-D was also demonstrated, since adenoviral expression of a soluble VEGFR-3-Ig significantly delayed the rate of wound closure in the db/db mouse model [57]. VEGF-D is expressed in the skin and upregulated upon injury, but the loss of this protein in mice did not obviously affect the wound healing process [63], possibly due to compensation by VEGF-C.
2.7 Tie-2 and angiopoietins

Angiopoietins comprise a family of four ligands that activate or inhibit signaling through the transmembrane tyrosine kinase receptor, Tie2, which is predominantly expressed by endothelial cells. Unlike VEGFs, angiopoietins do not directly affect endothelial cell migration and proliferation. Rather, angiopoietin-1 (Ang-1) induces the association of new vessels with pericytes and vascular smooth muscle cells, resulting in their stabilization. By contrast, Ang-2 acts as an antagonistic ligand of the Tie-2 receptor under most circumstances and therefore induces destabilization and remodelling of vessels [48]. Ang-1 and -2 as well as the Tie-2 receptor are expressed in skin wounds, and a transient upregulation of Ang-2 and Tie-2 was observed upon injury [7]. The potential role of Ang-1 in wound repair was first addressed using a gene therapy approach with an adeno-associated virus that expresses the Ang-1 gene. Overexpression of Ang-1 in incisional wounds of db/db mice stimulated reepithelialization, collagen maturation, wound breaking strength and angiogenesis [64]. The consequences of inducible overexpression of Ang-1 in keratinocytes in combination with VEGF-A for the wound healing process have recently been determined. Combined overexpression of both proteins strongly accelerated wound repair in normal mice, but this effect was abrogated under diabetic conditions [65]. Although these studies suggest that Ang-1 has a positive effect on wound healing, the roles of endogenous angiopoietins in this process remain to be determined.

2.8 Trk and nerve growth factor (NGF)

NGF, the prototype for the neurotrophin family of growth and differentiation factors, controls survival of certain sympathetic and sensory neurons and it is also a modulator of inflammatory processes. A role of NGF in wound healing has first been suggested by the finding that removal of the submandibular glands impaired the contraction of mouse wounds. In a follow-up study it was shown that release of NGF from salivary glands resulted in increased serum NGF levels after full-thickness excisional wounding in mice [66]. Therefore, NGF is available at the wound site upon vessel injury. It is also produced by myofibroblasts and keratinocytes of the healing wound. Further elevation of NGF levels in wounds is
obviously beneficial, since licking of wounds and thus contact with NGF-containing saliva resulted in faster healing [66]. Furthermore, exogenous NGF promoted the wound repair process in normal and healing impaired mice. NGF is likely to affect different processes in healing wounds, such as re-innervation, proliferation of keratinocytes and microvascular endothelial cells, migration of fibroblasts and their differentiation into myofibroblasts [7]. However, this hypothesis is based on in vitro activities, and elucidation of the role of NGF and other neurotrophins in wound repair will await loss-of-function studies with cell-type specific knockout animals.

<table>
<thead>
<tr>
<th>Receptor tyrosine kinase / ligand</th>
<th>Strategy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-B</td>
<td>Hematopoietic knockout chimera</td>
<td>[14]</td>
</tr>
<tr>
<td>PDGF receptor β</td>
<td>Expression of a gain-of-function mutant in fibroblasts</td>
<td>[13]</td>
</tr>
<tr>
<td>PDGF-D</td>
<td>Overexpression in keratinocytes – K5 promoter</td>
<td>[15]</td>
</tr>
<tr>
<td>FGF2</td>
<td>Knockout</td>
<td>[70]</td>
</tr>
<tr>
<td>FGF7</td>
<td>Knockout</td>
<td>[31]</td>
</tr>
<tr>
<td>FGF22</td>
<td>Knockout</td>
<td></td>
</tr>
<tr>
<td>FGF1/FGF2</td>
<td>Double knockout</td>
<td>[30]</td>
</tr>
<tr>
<td>FGFR2-IIIb</td>
<td>Dominant negative mutant - K14 promoter</td>
<td>[34]</td>
</tr>
<tr>
<td>FGFR1-IIIb</td>
<td>Knockout</td>
<td>[32]</td>
</tr>
<tr>
<td>FGF-BP1</td>
<td>Tetracycline-inducible overexpression in all cells – CMV promoter</td>
<td>[36]</td>
</tr>
<tr>
<td>TCRg (FGF7 and FGF10)</td>
<td>Knockout</td>
<td>[35]</td>
</tr>
<tr>
<td>TGF-a</td>
<td>Knockout</td>
<td>[20-22]</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Keratinocyte-specific knockout</td>
<td>[23]</td>
</tr>
<tr>
<td>Betacellulin</td>
<td>Overexpression – CMV enhancer/β-actin promoter</td>
<td>[17]</td>
</tr>
<tr>
<td>Receptor</td>
<td>Modification</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EGF receptor</td>
<td>Knockout</td>
<td>[18]</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>Overexpression in all cells - metallothionein promoter</td>
<td>[39]</td>
</tr>
<tr>
<td>Met</td>
<td>Keratinocyte-specific knockout</td>
<td>[38]</td>
</tr>
<tr>
<td>MSP</td>
<td>Knockout</td>
<td>[42]</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Overexpression in keratinocytes (K14 promoter)</td>
<td>[45]</td>
</tr>
<tr>
<td>TCRg (IGF-1)</td>
<td>Knockout</td>
<td>[46]</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Keratinocyte-specific knockout</td>
<td>[54]</td>
</tr>
<tr>
<td>VEGF-A and Ang-1</td>
<td>Inducible overexpression in keratinocytes</td>
<td>[65]</td>
</tr>
<tr>
<td>VEGF-B</td>
<td>Overexpression in endothelial cells – Tie2 promoter</td>
<td>[61]</td>
</tr>
<tr>
<td>VEGF-B</td>
<td>Knockout</td>
<td>[60]</td>
</tr>
<tr>
<td>PLGF</td>
<td>Knockout</td>
<td>[62]</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>Knockout</td>
<td>[63]</td>
</tr>
<tr>
<td>Discoidin domain receptor 2</td>
<td>Knockout</td>
<td>[69]</td>
</tr>
</tbody>
</table>

**Table 1: Use of genetically modified mice to study the function of receptor tyrosine kinases and their ligands in wound healing**

### 2.9 Discoidin domain receptors (DDRs)

Although most transmembrane tyrosine kinases transduce the signal of soluble growth factors, a few others are activated by components of the extracellular matrix. Of particular interest for the wound healing process are DDRs, which act as non-integrin-type receptors for native collagens in their triple helical form [67]. DDR2 expression was shown to increase in dermal burn wounds, suggesting a role of this receptor in the wound repair process. To test this hypothesis, mice lacking DDR2 were subjected to full-thickness incisional wounding. In a first study, reduced keratinocyte proliferation was seen at the wound site [68]. A second study revealed that wound healing in DDR2-deficient mice was strongly delayed compared to wild-type controls. The wounds showed
a reduction in tensile strength, in the number of myofibroblasts, and in collagen expression and cross-linking. These data revealed a role of DDR2 in collagen processing during wound healing [69].

3. Conclusions

The possibility to modulate the expression/activity of growth factors and their receptors at the wound site using biochemical or genetic approaches has unraveled important and sometimes even essential functions of receptor tyrosine kinases and their ligands in the wound healing process. Particularly useful is the possibility to overexpress or inhibit genes in a cell-type specific manner in transgenic and knockout mice. Ideally, expression of the transgene or loss of the endogenous genes is inducible, allowing a temporal control of growth factor/receptor overexpression or inhibition. This strategy allows to circumvent systemic defects of the laboratory animals as well as abnormalities in non-wounded skin. Therefore, it can be excluded that a wound healing defect is simply the consequence of a generally poor health status of the animals or secondary to a defect that is already present in non-injured skin.

In most cases, the consequences of the loss of a single growth factor were relatively mild, suggesting redundancy or compensation. However, some growth factors seem to exert unique functions. The most remarkable example is HGF, since loss of its receptor on keratinocytes completely inhibited migration and proliferation of the receptor-deficient cells in wounded skin. The scatter factor family only includes two ligands, and HGF is the only ligand of Met. Therefore, there is obviously no redundancy in this system. By contrast, various members of the EGF or FGF families are expressed at the wound site and this may well explain why loss of individual members of these families did not result in impaired wound repair. Therefore, it will be interesting to determine the consequences of the loss of two or more members of one growth factor family on the wound healing process. Alternatively, studies with dominant-negative receptor mutants or with soluble receptors that block the activity of several members of a growth factor family will help to unravel possible redundancies.
among related growth factors. As an example, expression of a dominant-negative FGFR2-IIIb mutant in keratinocytes of transgenic mice demonstrated an important role of FGFR signaling in reepithelialization, whereas mice lacking individual ligands, including FGF1, FGF7 and FGF22, did not have obvious wound healing abnormalities. Likewise, our preliminary data suggest that mice lacking either FGFR1 or FGFR2 in keratinocytes heal normally, whereas the combined loss has a severe effect (unpublished data).

In spite of the extensive knowledge on the role of certain growth factors and their receptors in wound healing that has been gained in recent years, there is still little information on the function of some other growth factors in wound healing. Examples are insulin-like growth factors, neurotrophins, and angiopoietins, for which no or only few loss-of-function studies have been performed. In addition, the functions of most transmembrane tyrosine kinase receptors, which do not bind the classical growth factors, remain to be determined. Such studies will undoubtedly unravel exciting functions of some of these molecules in different phases of the wound healing process.
References


Regulation of vascular endothelial growth factor expression in cultured...


4. General discussion

Cutaneous wound healing involves a large number of tightly regulated processes, which are orchestrated via growth factor and cytokine signals. One of the factors that are highly upregulated at the wound edge in epidermal γδ T cells and in fibroblasts of the dermis, dermal papilla and granulation tissue, is FGF7 (Werner et al., 1992). FGF7 as well as FGF10 and FGF22 exert their functions through FGFR2-IIIb and FGFR1-IIIb, which are expressed on keratinocytes. Surprisingly, wound healing studies with FGF7 and FGF22 knockout mice revealed no obvious wound healing abnormalities, most likely due to compensation by other FGFs of this subfamily (Guo et al., 1996; Jarosz et al., 2012). Transgenic mice expressing a dominant-negative FGFR2-IIIb mutant in basal keratinocytes, in contrast, showed severe wound healing defects, reflecting the importance of the FGFRs in this process. In addition, these mice have hair follicle deformations, a thinner epidermis and a fibrotic dermis (Werner et al., 1994). As FGFRs are able to homo- but also heterodimerize, the dominant-negative approach did not elucidate the type of FGF receptor or ligand that was responsible for the observed phenotype. To specifically shut down signaling via one particular receptor, our laboratory generated mice lacking FGFR1, FGFR2 or both receptors in keratinocytes. The phenotype of these mice should provide insight into the function of each of these receptors in normal and wounded skin and also unravel a potential role of FGF signaling in skin disease.

The most striking macroscopically detectable phenotype in K5-R1/R2 mice is the progressive hair loss. With the onset of the second hair cycle these mice lose their hair and it does not grow back. Together with the fur, all the other skin appendages disappear as well (Yang et al., 2010). Previously generated FGF7, FGF10 and FGFR2-IIIb knockout mice had a hair phenotype as well (Grose et al., 2007; Guo et al., 1996; Suzuki et al., 2000) and thus this aspect of the phenotype of double knockout mice was not surprising. In addition, recent studies showed that FGF7 and FGF10 are growth and survival factors for hair follicle stem cells (Greco et al., 2009). Lack of the receptors for these signals in hair follicle
keratinocytes in K5-R1/R2 mice can explain the loss of the fur as well as the loss of sebaceous glands, as the two structures are derived from common progenitor cells and form a closely connected pilosebaceous unit (Sotiropoulou and Blanpain, 2012). The arrector pili muscle might degenerate due to the loss of the hair follicle. To elucidate the exact mechanism of the loss of the pilosebaceous unit, a detailed analysis of the time period from first to second hair cycle needs to be performed, including histological analysis, determination of cell proliferation and apoptosis as well as expression analysis of differentiation-specific markers. Furthermore, a fate mapping of the individual cell types would be required (Ro, 2004).

In contrast to the almost expected hair phenotype of the K5-R1/R2 mice, the progressive cutaneous inflammation, which was already seen at the age of P18 as well as the acanthosis were rather surprising. A 1.5 fold increase in the number of dendritic epidermal T cells (DETC; epidermal γδ T cells) was found in the epidermis, and mast cells and αβ T cells accumulated in the dermis (an almost 2 fold increase for both cell types). In addition, there was a strong deposition of IgG 1, IgG 2a and IgE in the dermis of double knockout mice, and IgE levels in the serum were highly up-regulated. The inflammatory response in K5-R1/R2 animals is also reflected by strong activation of nuclear factor κB (NF-κB) in the epidermis, since this transcription factor is activated by various pro-inflammatory cytokines (reviewed in Wullaert et al., 2011). Real-time PCR analysis indeed revealed a much higher expression of inflammatory cytokines like TNF-alpha, interleukin-1β (IL-1β) and IL-1 family member 8 (IL-1F8) in the skin of double knockout mice. S100A8 and S100A9, chemoattractants for immune cells, were also up-regulated in K5-R1/R2 epidermis and dermis. By analyzing mice at P18, when the skin is not yet hyper-thickened but when the FGFR1/FGFR2 knockout in keratinocytes is already complete, we found a strong upregulation of S100A8, S100A9 and IL-1F8 at this early stage in K5-R1/R2 mice. These findings indicate a causative role of these cytokines in the phenotype of the double mutant mice. Semi-quantitative RT-PCR of FACS-sorted cells of the epidermis identified keratinocytes as the major producers of S100A8 and S100A9 mRNA, whereas γδ T cells express IL-1F8. However, keratinocytes of K5-
R1/R2 mice in culture did not show upregulation of these mRNAs, suggesting that their increase is not a direct consequence of the loss of FGFRs, but a secondary effect seen in vivo and involving the stroma. Activated keratinocytes in the hyperproliferative epidermis of psoriasis patients express S100A8 and A9 (Nukui et al., 2008), and a similar increase was seen in mouse models of psoriasis, e.g. in mice lacking c-Jun and JunB in keratinocytes (Zenz et al., 2005). Epidermal γδ T cells are the surveillants of keratinocytes, and these cells react on changes in the condition of keratinocytes through recognition of an as yet unknown antigen exposed on stressed keratinocytes (Chodaczek et al., 2012). Therefore, it seems likely that the keratinocytes in K5-R1/R2 mice are stressed and release S100A8 and A9. In addition, activation of γδ T cells seems to occur, which then release IL-1F8. mRNA levels of IL-1F8 are also up-regulated in the skin of psoriatic patients and in a mouse model of psoriasis (Barksby et al., 2007; Johnston et al., 2011). Treatment of psoriatic patients skin with Etanercept, a drug that binds and neutralizes TNF-α and thereby down-regulates the inflammation, suppressed the expression of IL-1F8. Psoriasis patients suffer from a defect in the barrier function of the skin as the stratum granulosum is lost and thereby tight junctions are altered (Kirschner et al., 2009). K5-R1/R2 mice have a defect in the barrier function of the skin as well, which was shown by increased trans-epidermal water loss (TEWL), reduced expression of major tight junction (TJ) proteins, lower transepithelial electrical resistance of cultured keratinocytes, and abnormal ultrastructural appearance of tight junctions in these mice. This defect could explain the mild inflammation seen in double knockout mice, since it was shown that exposure to a dry environment leads to increased keratinocyte proliferation in vivo and induction of inflammation markers (Denda et al., 1998). Consistent with a role of skin dryness in the inflammatory phenotype, topical treatment of the back skin of K5-R1/R2 mice with a moisturizing cream reduced the number of mast cells and γδ T cells in the skin. Whether expression of the pro-inflammatory cytokines and chemokines is also down-regulated after cream treatment still needs to be determined.

The hyperproliferation of keratinocytes in the epidermis of K5-R1/R2 mice may result at least in part from the enhanced expression of IL-1F8. Thus, intradermal IL-1F8 injections into wild-type mice resulted in a higher proliferation rate of
epidermal keratinocytes, and this cytokine also enhanced keratinocyte proliferation in vitro. Additionally, treatment of cultured fibroblasts with IL-1F8 enhanced the expression of different keratinocyte mitogens (FGF7, HGF, TGF-α, IL-6) in these cells. Most importantly, dermal cells of aged knockout mice expressed the same growth factors in vivo (HGF, FGF7, TGF-α, G-CSF). These results led to the hypothesis that a double paracrine loop is activated in the skin of K5-R1/R2 mice: Keratinocytes are stressed and activated because of the disturbed barrier and they secrete S100A8 and A9. γδ T cells detect the barrier defect and release IL-1F8, resulting in keratinocyte hyperproliferation. Fibroblasts in the dermis secrete cytokines and growth factors upon the stimulation by IL-1F8, S100A8 and S100A9 produced by γδ T cells and keratinocytes in the epidermis (Yang et al., 2010). The released signals from fibroblasts and immune cells in the dermis trigger the keratinocytes to proliferate even more and might also have an influence on the tight junctions due to further down-regulation of claudins and occludin (Kirschner et al., 2009). The exact molecular trigger, which results from the defective barrier function and acts on the γδ T cells, remains to be determined. It might be the change in osmotic pressure in keratinocytes because of water loss. Alternatively, it may involve pathogens, irritants or allergens that can penetrate the skin upon damage of the barrier. To test a possible role of bacteria, the double mutant mice should be transferred to a germ-line facility. A role of allergens could be tested by topical treatment of the skin with the model antigen ovalbumin (OVA). If the barrier is impaired, the mice should develop an immune response against this protein. These experiments have been initiated and will be continued in the future.

In addition to tight junctions, the cornified envelope of the stratum corneum in combination with the associated lipid bilayer are important components of the epidermal barrier. Electron microscopy pictures of skin sections of K5-R1/R2 mice did not reveal obvious abnormalities in the cornified envelope, and major components of this structure, including filaggrin and loricrin, were normally expressed and processed in K5-R1/R2 mice. Furthermore, there was a normal appearance of lamellar bodies – the organelles in the stratum granulosum, which secrete lipids, hydrolytic enzymes and proteins. In addition, lipid biosynthesis
enzymes were normally expressed in the epidermis of young double knockout mice (unpublished data). These results suggest that the barrier defect does not result from lipid alterations, although a detailed characterization of the *stratum corneum* lipids will be required to clarify this issue.

The combination of inflammation, barrier defect and hyperproliferation of keratinocytes in K5-R1/R2 mice highly resembles the symptoms seen in patients with atopic dermatitis (AD) (Kubo et al., 2012). Interestingly, reduced expression of claudins has recently been found in a subset of patients with AD (De Benedetto et al., 2011), indicating that tight junction abnormalities as seen in K5-R1/R2 mice may indeed be involved in the pathogenesis of this common human skin disease. To determine if this is due to impaired expression and/or activation of FGFRs, biopsies of such patients should be tested for the expression levels of FGFR1 and FGFR2 and their ligands using real-time PCR. In any case, our K5-R1/R2 mice could possibly serve as a useful model to study several aspects of AD.

The identification of tight junction components as targets of FGFs in keratinocytes is a particularly interesting finding of our studies. This regulation is obviously direct, since treatment of keratinocytes from wild-type mice with FGF7 for 96 hours enhanced the expression of claudin 1, claudin 3 and occludin at the RNA and protein level. This result is consistent with previous studies showing reduced expression of tight junction proteins in blood vessels of the brain in FGF2/FGF5 double knockout mice (Reuss et al., 2003). Furthermore, inhibition of FGFR signaling led to disassembly of adherens and tight junctions in endothelial cells (Murakami et al., 2008). All these findings point to a general role of FGFs in the maintenance of the junctional integrity. More studies need to be performed in the future to further understand this function and to identify the FGF-regulated signaling pathways that control the expression of junctional components.

Additional knockout of the *Fgfr3* gene in our double knockout mice would reveal if tight junction proteins are even further down-regulated upon loss of this
remaining FGF receptor in keratinocytes. It would also reveal if FGFR3-IIIb, which is activated by a different set of ligands than FGFR1-IIIb and FGFR2-IIIb, can at least partially compensate for the loss of FGFR1 and FGFR2. Therefore, we started breeding Fgfr3 total knockout mice with K5-R1/R2 mice.

K5-R1/R2 mice do not only show an impairment in skin homeostasis, but also in wound healing. Thus, healing of full-thickness excisional wounds was strongly delayed in these mice. Nevertheless, the wounds finally healed, demonstrating that FGFR1 and FGFR2 in keratinocytes are important, but not essential for wound healing. Wound closure is a combination of reepithelialization and wound contraction and both processes were affected in K5-R1/R2 mice. Immunofluorescence stainings with an antibody against α smooth muscle actin showed that there is no difference between the number and distribution of myofibroblasts, the cell type with a particularly important role in contraction, between knockout and control animals. Furthermore, there was no massive inflammation, which could hinder contraction. Between the two genotypes no difference was detected in the density of the late granulation tissue/early scar tissue in 14 day wounds. Therefore, the defect in contraction is most likely due to the alterations in the connective tissue that develop in these animals (Yang et al., 2010). The latter is a secondary effect of the knockout of FGFR1 and 2 in keratinocytes and most likely results from the inflammation with subsequent alterations in connective tissue composition and/or remodelling.

The process of reepithelialization involves proliferation and migration of keratinocytes. Surprisingly, proliferation of keratinocytes was even enhanced in normal and wounded skin of K5-R1/R2 mice, indicating that the impaired reepithelialization results from a defect in migration. Consistent with this hypothesis, the average length of the wound epidermis was significantly reduced in the mutant mice at day 3 after injury. Furthermore, the tip of the epidermal tongue in the wound lacked a migratory front, and a clump of cells seemed to be pushed forward. This defect in migration of keratinocytes from double knockout mice is cell autonomous as demonstrated by means of Boyden chamber and
scratch healing assays in vitro. In both assays a strong migratory deficiency of the FGFR1/R2-deficient cells was observed.

In order to migrate a cell needs to assemble and disassemble contacts to the surface and has to be polarized to be able to keep a direction. These capabilities were impaired in FGFR1/R2-deficient cells. Attachment and spreading on different matrices were severely reduced and delayed. The cells migrated, but with lower speed than the wild-type keratinocytes, and directional persistence and polarization of these cells were impaired. Impaired migration of keratinocytes in wounded skin had previously been shown for other receptors, including the HGF receptor c-Met and the EGFR (Chmielowiec et al., 2007; Repertinger et al., 2004). Nevertheless, FGFRs obviously have non-redundant functions in this process as revealed by the impaired reepithelialization in K5-R1/R2 mice. However, in contrast to c-Met, FGFR1 and FGFR2 are not essential for keratinocyte migration in wounded skin, since closure of the wounds was achieved and the FGFR1/R2-deficient keratinocytes were able to finally reepithelialize the wound.

The molecular protein complex that accomplishes the attachment of the cell to the extracellular matrix (ECM) is called focal adhesion (FA). This complex involves various proteins, including paxillin and FAK. Paxillin is a scaffold protein with many docking sites for other proteins. It binds to integrins and thereby links the ECM to the cytoskeleton (Deakin and Turner, 2008). Paxillin can be phosphorylated on multiple serine, threonine and tyrosine residues. FAK binds to paxillin and phosphorylates it on tyrosine residue 118. FAK, activated by integrin and receptor tyrosine kinase signaling, also phosphorylates other downstream targets, thereby transducing the signal from the ECM to the cell interior (Mitra et al., 2005). Cultured K5-R1/R2 keratinocytes showed a strong reduction in the levels of paxillin and FAK mRNA and protein, indicating that these focal adhesion proteins are novel FGF targets in keratinocytes. Indeed, treatment of cultured control mouse cells as well as of human, immortalized (HaCaT) keratinocytes with FGF7 induced the expression of paxillin and FAK in these cells. The signaling pathways responsible for this activation remain to be determined.
Taken together, the migration phenotype observed in K5-R1/R2 keratinocytes obviously results from the down-regulation of the focal adhesion proteins paxillin and FAK. By contrast, major keratinocyte integrins, which could also have an impact on migration, are expressed at normal levels in FGFR1/R2-deficient keratinocytes, and the amount of active matrix metalloproteinases (MMP) 9 and 10, which degrade the ECM and play a crucial role in migration of cells in tissue, is also not altered. Our findings are in line with reports about other receptor tyrosine kinases (EGFR, PDGFR) influencing migration behavior of cells through FAK that acts as an integrator of different stimuli from the outside of the cell (Schlaepfer and Mitra, 2004). However, in these cases phosphorylation and activation of FAK were affected, whereas FGFs seem to control FAK and paxillin at the transcriptional level.

The wound healing phenotype of the K5-R1/R2 mice is similar to the abnormalities seen in chronic skin ulcers in humans. Wounds of these patients are characterized by reduced keratinocyte migration and hyperproliferation of these cells (Harsha et al., 2008). Therefore, the expression levels of FGFR1 and 2 as well as of their ligands should be determined in these wounds. Such a study could generate new knowledge about the mechanisms underlying the pathogenesis of chronic skin ulcers and help to develop novel therapeutic strategies. Although previous studies did not show a significant improvement of wound healing by applying basic FGF (FGF2) (Buchberger et al., 2010), FGF7 (Zheng et al., 2009) or FGF10 (Robson et al., 2001) to chronic ulcers, these factors may still be beneficial for wound healing upon modification of the mode of application. This could include application in combination with proteinase inhibitors, via a slow release system or via a gene therapy approach, such as application of FGF-encoding viruses. A gene therapy strategy could even be used in the case of FGFR deficiency. Therefore, a thorough analysis of FGF and FGFR expression and function in chronic skin ulcers is an important task for the future.
References


Acknowledgments

I thank all the people who contributed to this work and helped me during the course of my PhD thesis and therefore made my time at the Institute of Cell Biology such a delightful one. Especially I would like to thank

Sabine Werner, for providing this most interesting topic to work on, for her enthusiasm and unremitting support as well as for her constant availability;

Cornelia Halin Winter, for her support as a co-examiner and her suggestions and interest in the scientific topic;

Hans Smola, for being my external co-examiner and for his scientific advises as well as personal ones;

Jingxuan Yang, for teaching me how to deal with cell culture issues and a lot of other things in the lab and for being such a pleasant-natured person to work with;

Michael Meyer, for all his great scientific ideas, for his positivity, for his support in all kind of things and for being the ideal person to work with for me;

Friederike Böhm, for her help and patience and for the good humor she brought to HPM D27;

Uli Köhler, for the cheerful and enjoyable breaks we had; for enriching my time at the Institute and making it a very pleasant one;

Katharina Birkner, Tamara Ramadan and Phillipe Bugnon, for their help with mouse experiments;

Christiane Born-Berclaz, Nicole Hallschmid, Andreia Fernandes and Mario Gysi for their technical help;

and all the lab members for creating this good atmosphere we had in the lab and for organizing a lot of amusing activities together.
Publications


*Equal contribution