# The role of the Met tyrosine kinase receptor in skin maintenance and regeneration

Dissertation zur Erlangung des akademischen Grades
doctor rerum naturalis (Dr. rer. nat)
im Promotionsfach Biologie
eingereicht an der Mathematisch-Naturwissenschaftlichen Fakultät I
der Humboldt-Universität zu Berlin

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Tag der mündlichen Prüfung: 26. September 2007

Die vorliegende Arbeit wurde unter Anleitung von Herrn Prof. Dr. Walter Birchmeier am Max-Delbrück-Centrum für Molekulare Medizin in Berlin-Buch angefertigt.

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## Zussamenfassung

Die in dieser Arbeit dargestellten Ergebnisse erlauben neue Einblicke in die Funktion der Rezeptortyrosinkinase Met für die Erhaltung und Regeneration der Haut. Es zeigte sich, dass Met und der korrespondierende Ligand HGF/SF im hyperproliferativen Epithelium von Hautwunden exprimiert sind. Aus diesem Grund ist es wahrscheinlich, dass der Rezeptor und sein Ligand in autokriner Weise wechselwirken und wichtige Funktionen für den Heilungsprozess der Haut besitzen. Um die Bedeutung des Met-Rezeptors für die Entwicklung, Erhaltung und Wundheilung der Haut zu bestimmen, wurde das für den Met-Rezeptor kodierende Gen spezifisch in der Epidermis unter Verwendung einer Keratin 14 Cre-Rekombinase mutiert. In der Tat zeigten die Ergebnisse, dass Met für die Re-epithelisierung in Wundschlussprozessen essentiell ist, da in den an der Wundheilung beteiligten Keratinozyten keine Rekombination des Met-Gens stattgefunden hat. In Met-Mausmutanten war der Wundschlussprozess verlangsamt, denn er erfolgte ausschließlich durch wenige (~5%) Keratinozyten, in denen die Cre-Rekombinase keine Rekombination bewirkte. Das Wundepithelium kann also nur von Zellen gebildet werden, die einen funktionalen Met-Rezeptor besitzen. Obwohl Met und HGF/SF auch im intakten Gewebe der Haut exprimiert werden, hatte der Funktionsverlust des Rezeptors weder Einfluss auf die Entwicklung und Erhaltung der Epidermis, noch auf die Regulation des Haarzyklus.

In Zellkulturexperimenten konnten erste Hinweise gefunden werden, weshalb Met-defiziente Keratinozyten nicht zur Wundheilung beitragen. In-vitro-Wundheilungsversuche (sog. Scratch-Assays) zeigten, dass kultivierte Met-defiziente Keratinozyten selbst in Gegenwart von HGF/SF die Fähigkeit zur Proliferation, zur Reorientierung und zur Migration verloren.

Zusammengefasst konnte in dieser Studie zum ersten Mal die Bedeutung des Met-Signalweges für regenerative Prozesse der Epidermis *in vivo* gezeigt werden. Met konnte als erstes Gen identifiziert werden, das absolut erforderlich für Re-epithelisierungsprozesse von Wunden ist. Diese Arbeit trägt daher wesentlich zum Verständnis der Regulation von Wundheilungsprozessen bei.

# Introduction

## Wound healing in the skin

Mammals, and especially humans, have paid a high price for climbing up the evolutionary ladder. They have lost much of the regenerative power found in lower animals. Lower animals show amazing regenerative abilities and develop three principal strategies to regenerate organs. First, cells that normally do not divide can multiply and grow to replenish lost tissue, as occurs in injured salamander hearts. Second, specialized cells can undergo a process known as dedifferentiation, replicate and later respecialize to reconstruct a missing part. Thirdly, pools of stem cells can step in to perform required renovations. On decapitation, planaria regenerates a new head within five days, using this approach (Davenport, 2004).

Throughout the course of time we have witnessed many animals, such as tritons and salamanders, with the ability to regenerate their shed or torn tails and broken jaws. Moreover, some animals also exhibit the ability to regenerate their damaged hearts, eye tissues, spinal cords and even skin. The skin of vertebrates serves as a protective barrier against the external world which highlights the need for a fast and efficient repair system. Of note, a temporary repair can be achieved by the formation of a blood clot to serve as a 'plug' at the site of the wound. In addition to providing this temporary shield and protection against invading microorganisms, the blood clot also serves as a provisional matrix for invading cells and importantly, as a reservoir of growth factors that are required during the later stages of wound healing. It is well established that within a few hours after injury, inflammatory cells are recruited to invade the wounded area. Neutrophils appear first at the site of inflammation, followed by monocytes then lymphocytes. It is the infiltrating neutrophils that mop-up the area of foreign particles and contaminating bacteria to clean the wound which is proceeded by a process known as phagocytosis, performed by the macrophages. Wounding of skin can cause damage to both epidermal and dermal structures. In order to restore the damaged dermis, fibroblasts invade the wound area to form a contractile granulation tissue. The new stroma has granular appearance owing to massive angiogenic invasion

by a network of capillary blood vessels, which supply the metabolically active wound tissue with nutrients and oxygen. Some of the fibroblasts within this granulation tissue transform into specialist contractile myofibroblasts, which has been speculated to contribute to the wound contractive force. During re-establishment of the epithelial barrier, keratinocytes, originating from outside the wound, migrate over the injured dermis and the granulation tissue (Fig.1).

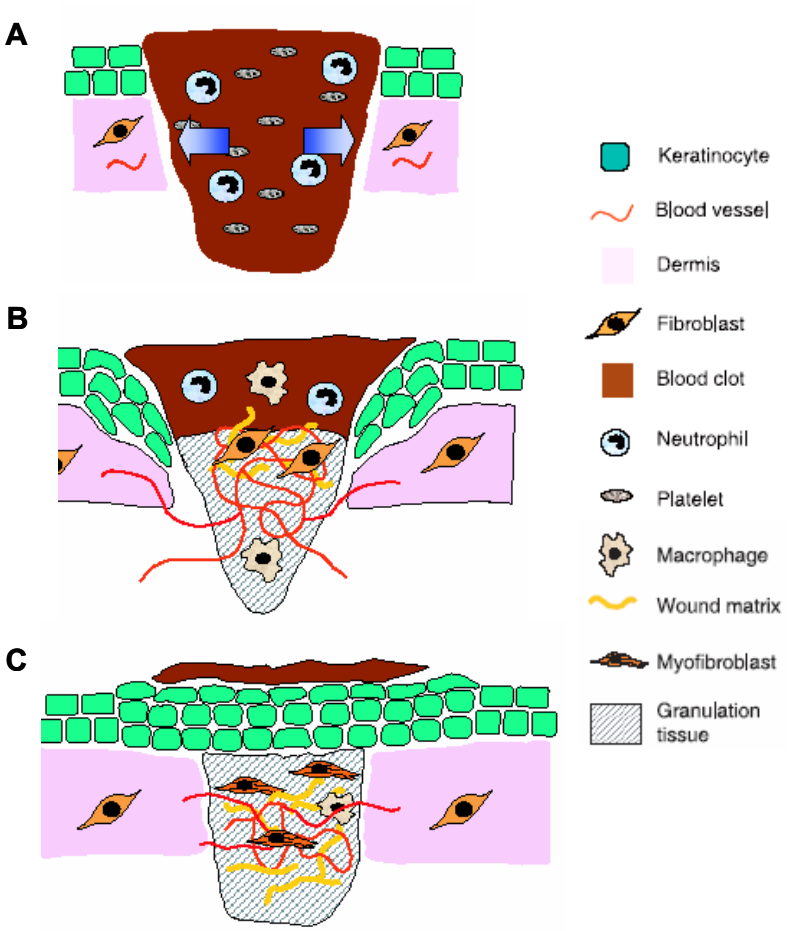



**Figure 1. Scheme of different stages of wound repair in mammals**. **A**: 12–24 h after injury the wounded area is filled with a blood clot. **B**: at days 3–7 after injury, endothelial cells migrate into the clot; they proliferate and form new blood vessels. Fibroblasts migrate into the wound tissue, where they proliferate and form extracellular matrix. The new tissue is called granulation tissue. Keratinocytes proliferate at the wound edge and migrate down the injured dermis and above the provisional matrix. **C:** 1–2 wk after injury the wound is completely filled with granulation tissue. Fibroblasts have transformed into myofibroblasts, leading to wound contraction and collagen deposition. The wound is completely covered with a neoepidermis. Modified from Werner and Grose, 2003.

At the wound edges, these keratinocytes form the so-called hyperproliferative epithelium, which strongly proliferates and migrates to replenish the wounded area with new tissue. Cells from the hyperproliferative epithelium over-time displace the fibrin clot. The hyperproliferative epithelium is characterized by the expression of keratins 6 and 16, which are normally expressed in the unwounded epidermis (Martin, 1997; Werner and Grose, 2003).

Under certain circumstances, a wound may fail to heal and develop into a chronic wound. Incidences of chronic wounds are higher among the elderly and diabetic, as well as among people with vasculature problems (Harding et al., 2002; Falanga, 2005). The epidermis of a chronic wound has a typical appearance (Fig.2). It is thick and hyperproliferative, with mitotically active cells located in the upper, differentiated layers. Furthermore, the cornified layer is hyperkeratotic (thick cornified layer) and parakeratotic (presence of nuclei in the cornified layer). Keratinocytes on a chronic wound edge are capable of proliferating, but are unable to migrate properly (Morasso and Tomic-Canic, 2005). Particularly, these types of wounds or life-threatening skin burns may require special treatments of wound mediators to accelerate healing. However, at this point in time there is not yet enough clinical data to support the routine use of such factors.

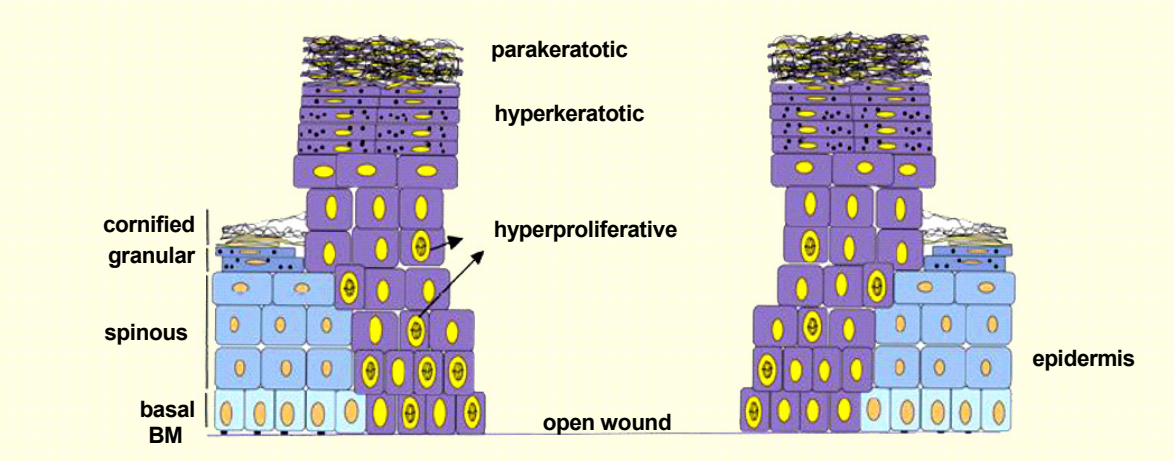



**Figure 2. Chronic wound.** Keratinocytes at the edge of the wound (purple) are hyperproliferative (indicated by mitotically active cells present throughout the suprabasal layers), hyperkeratotic (indicated by thick cornified layer) and parakeratotic (indicated by presence of nuclei in the cornified layer). BM=basement membrane.

The process of wound healing involves a complex interaction between epidermal and dermal cells. It is coordinated by many signals that trigger relatively sedentary cell lineages at the wound margin to proliferate, to become invasive, and then to lay down a new matrix. After injury keratinocytes become activated and secrete various cytokines and growth factors and, at the same time, respond to them. Keratinocytes release interleukin-1 (IL-1), which is the first signal upon wounding and has a dual function: to activate keratinocytes and to signal-alert the surrounding tissues. In the last decade *in vivo* and *in vitro* studies have provided the identification of a list of growth factors and cytokines that are important during wound repair. For example, platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Scheid et al., 2000; Werner and Grose, 2003).

The development of genetically modified mouse technologies gives new insights into the role of genes during skin repair processes. For instance, genetic evidence obtained in mice indicates that signaling via the epidermal growth factor (EGF) receptor and the keratinocyte growth factor (KGF/FGF7) receptor are important for re-epithelialization (Werner et al., 1994; Repertinger et al., 2004; Shirakata et al., 2005). Furthermore, downregulation of the transforming growth factor β (TGFβ) receptor in keratinocytes reduce the rate of re-epithelialization (Amendt et al., 2002). Smad3 is a downstream component of TGFβ signaling and, in contrast, Smad3 mutant mice show an increased rate of re-epithelialization and reduced monocyte infiltration during wound healing (Ashcroft et al., 1999). It has also been demonstrated that c-Jun and STAT3 may signaling downstream of growth factors, such as interleukins and integrins. Specifically a conditional mutation of c-Jun and STAT3 in the epidermis delayed wound closure (Sano et al., 1999; Li et al., 2003).

## Mammalian skin

The skin is the largest organ in the body, which protects against environmental insults as well as from dehydration. The mammalian skin is composed of several layers, including an underlying dermis, separated by a basement membrane from the epidermis and its appendages, including the hair follicles, sebaceous glands and sweat glands

(Fig. 3). The basement membrane is composed of extracellular matrix proteins, such as collagen IV, fibronectin and laminin 5. Both the epidermis and the dermis contribute to the synthesis of basement membrane components (DiPersio et al., 1997; Raghavan et al., 2000). The epidermis is a thin multilayer of stratified squamous epithelium that is mainly comprised of keratinocytes. The undifferentiated basal layer stratifies to give rise to differentiated cell layers of the spinous layer, granular layer, and the outer most stratum corneum. As cells withdraw from the basal layer, they stop dividing and induce a programme of terminal differentiation that will ultimately allow them to function as barrier of the skin. The epidermis originates from the outer layer of the embryo, the surface ectoderm. BMPs activate the epidermal differentiation program and induce the expression of keratin proteins via several known transcription factors (Meulemans and Bronner-Fraser, 2004; Byrne et al., 1994). The surface ectoderm proliferates and migrates from the dorsal midline to cover the embryo, and persists as a simple epithelium until approximately embryonic day 9.5 of mouse embryogenesis. At this stage basal cells begin to express keratin 5 and 14, presaging epidermal stratification, which requires the activity of a key epidermal transcription factor that also regulates epidermal fate, proliferation, and adhesion (Yang et al., 1999; Bakkers et al., 2002; Koster et al., 2004; Lechler and Fuchs, 2005). By the birth, the epidermis consists of a proliferative basal layer that differentiates to form outer layers.

To date there is considerable amount of data on the profiles of structural gene expression in the epidermis and its appendages, however much less is known about how these are established during development and what programmes are orchestrated to terminate differentiation at the transcriptional level (Fuchs and Raghavan, 2002). In normal conditions, growth and proliferation are precisely balanced and regulated processes in the epidermis. Tyrosine kinase receptors and their ligands have important role in regulating this balance. For instance, the dermal fibroblasts secrete GM-CSF and FGF7 to promote the proliferation and differentiation of overlying epidermal keratinocytes (Szabowski et al., 2000). Keratinocytes themselves are a source of autocrine growth factors that stimulate tyrosine kinase receptors, such as transforming growth factor α (TGF α), a ligand for EGFR (Luetteke et al., 1994; Sibilia and Wagner, 1995). EGFR and its downstream Ras–MAPK pathway have been implicated in epithelial proliferation (Hansen et al., 2000). Tyrosine kinase receptors can activate

phosphoinositol 3 kinase (PI3K) and the Akt cell-survival pathway, which both control epidermal homeostasis. Aberrations in these signaling pathways may result in hyperproliferative disorders of the skin, such a psorasis and basal- or squamous-cell carcinomas.

The dermis of the skin consists mostly of loosely packed fibroblasts. Mature dermis is also composed of collagen, elastin fibers and interfibrillar glycosaminoglycans (GAG)/proteoglycan gel. The dermis has a remarkable variety of embryonic origins, i.e. the ventral dermis arises from the lateral plate mesoderm, while a part of the head dermis arises from neural crest (Candille et al., 2004; Fernandes et al., 2004). However, fate mapping of dorsal dermis in mammalian embryos has not yet been described (Millar, 2005).

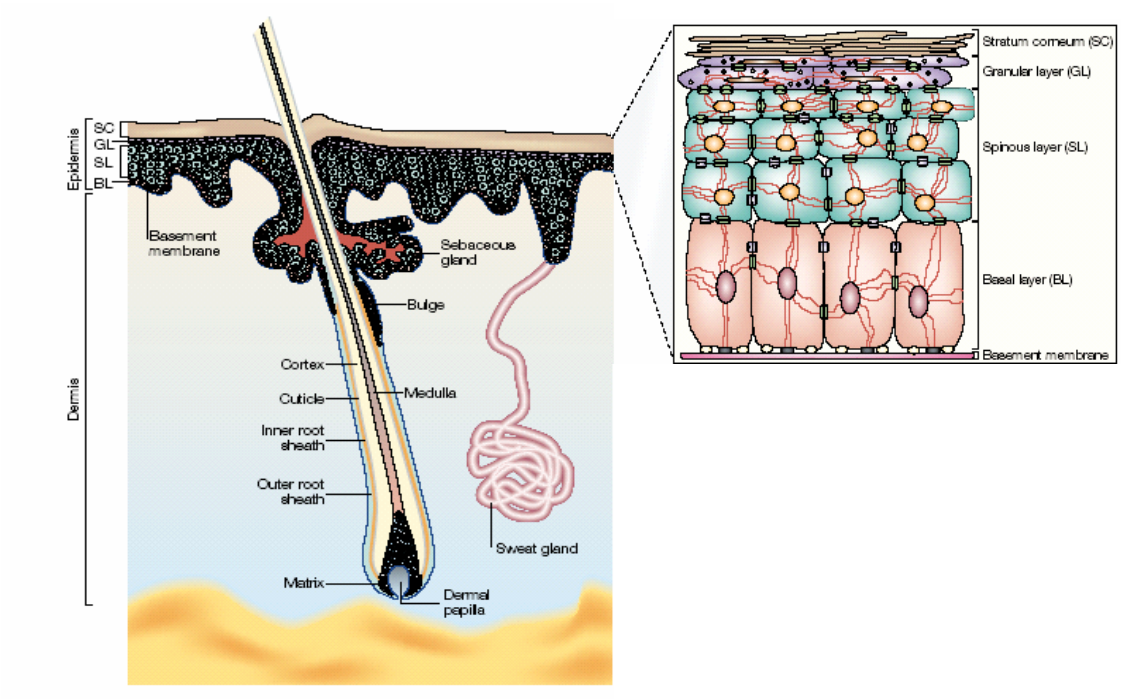



**Figure 3. Mammalian skin and its appendages.** Skin consists of the epidermis and dermis, separated by a basement membrane (BL). The epidermis is composed of is the basal layer (BL), differentiated spinous layer (SL), granular layer (GL) and the stratum corneum (SC). Also shown is a cross-section of a hair follicle, which consists of an outer root sheath that is contiguous with the basal epidermal layer, the hair bulb, made from proliferating matrix cells, and the bulge, which is part of the outer root sheath and is where epidermal stem cells reside. Modified from Fuchs and Raghavan, 2002.

**The tyrosine kinase receptor Met**

The Met receptor tyrosine kinase was first identified as an activated oncogene (Park et al., 1986). Subsequently, the cDNA of this proto-oncogene was isolated and found to encode a transmembrane receptor tyrosine kinase. The Met receptor is synthesized as a single polypeptide chain of 1436 amino acids that undergoes intracellular proteolytic cleavage into a two-chain heterodimer. This encompasses an N-terminal α chain located outside the membrane, and a C-terminal β chain that contains an extramembrane sequence, a single transmembrane domain and a cytoplasmic protein kinase domain.

Met belongs to a family of receptors that also includes mammalian Ron and the avian Sea receptors. They share the heterodimeric structural motif of an extracellular α chain and transmembrane β chain harboring the tyrosine kinase activity. The similarities are observed not only among receptors but also between ligands since Macrophage-stimulating protein (MSP), the ligand for Ron, resembles the Met receptor ligand, HGF/SF, in many aspects (Leonard and Danilkovitch, 2000). In evolutionary terms, Met is a young molecule, which appear during evolution of vertebrates for the first time (Birchmeier et al., 2003).

The ligand for Met was first identified as a factor that induces proliferation of hepatocytes and was subsequently named hepatocyte growth factor (HGF) (Miyazawa et al., 1989; Nakamura et al., 1989; Zarnegar and Michalopoulos, 1989). The activity of HGF was observed in pairs of rats with a surgically connected circulation system, which one rat had an injured liver. In the blood stream of these animals, the presence of circulating factors was having a dramatic affect on the growth of both damaged and normal liver. As a result, one of these circulating growth factors was purified form the media of primary cultured rat hepatocytes, and identified as a novel, very potent mitogen, named as HGF (Matsumoto and Nakamura, 1993). HGF was later shown to be identical to scatter factor, SF, discovered independently due to its ability to induce motility of epithelial cells (Stoker et al., 1987). The identity between HGF and SF was demonstrated by amino acid sequencing, by immunological methods, by comparison of the biological activities (Gherardi and Stoker, 1990; Weidner et al., 1990; Furlong et al.,

1991), by cDNA cloning, and by receptor binding studies (Weidner et al., 1991; Naldini et al., 1991). HGF/SF is a unique growth factor that elicits multiple cellular responses including mitogenesis, cell motility and morphogenesis. The structure of HGF/SF contains a domain that resembles that of plasminogen as well as other complex serine proteinases involved in blood coagulation and fibrinolysis in vertebrate organisms (Donate et al., 1994). They represent a family with related biological activities, termed plasminogen-related growth factors.

**Met signal transduction**

Binding of active HGF/SF to Met results in phosphorylation of the receptor. These phosphorylation events lead to the activation of the receptor and create recruitment sites for many signaling mediators. Two tyrosine residues (Tyr1349 and Tyr1356, Fig.1), together with a short amino-acid sequence motif located near the C-terminus of the intracellular domain, constitute a multi-docking site (Ponzetto et al., 1994). Studies using chimeric receptors containing the extracellular and membrane domains of other receptor fused to the intracellular portion of Met demonstrate that this docking site is both necessary and sufficient to mediate Met signal transduction and biological function (Weidner et al., 1993). Recruitment of adaptor proteins and signaling molecules to the docking site of Met enables amplification of the signal, the activation of multiple downstream signaling pathways and thus induces various cellular responses (Fig.5). Specificity at the receptor level is achieved by binding of various cytoplasmic signaling proteins to phosphotyrosines and surrounding amino acid residues of the activated receptor (Fig.4). These proteins that are recruited to activated Met include the adapter proteins Gab1, SHC, Grb2 and Crk/CRKL, along with other signal transducers, like phosphoinositol-3 kinase, PI3K and Shp2 (Pelicci et al., 1995; Ponzetto et al., 1994; Garcia-Guzman et al., 1999; Graziani et al., 1991; Weidner et al., 1996; Fixman et al., 1996).

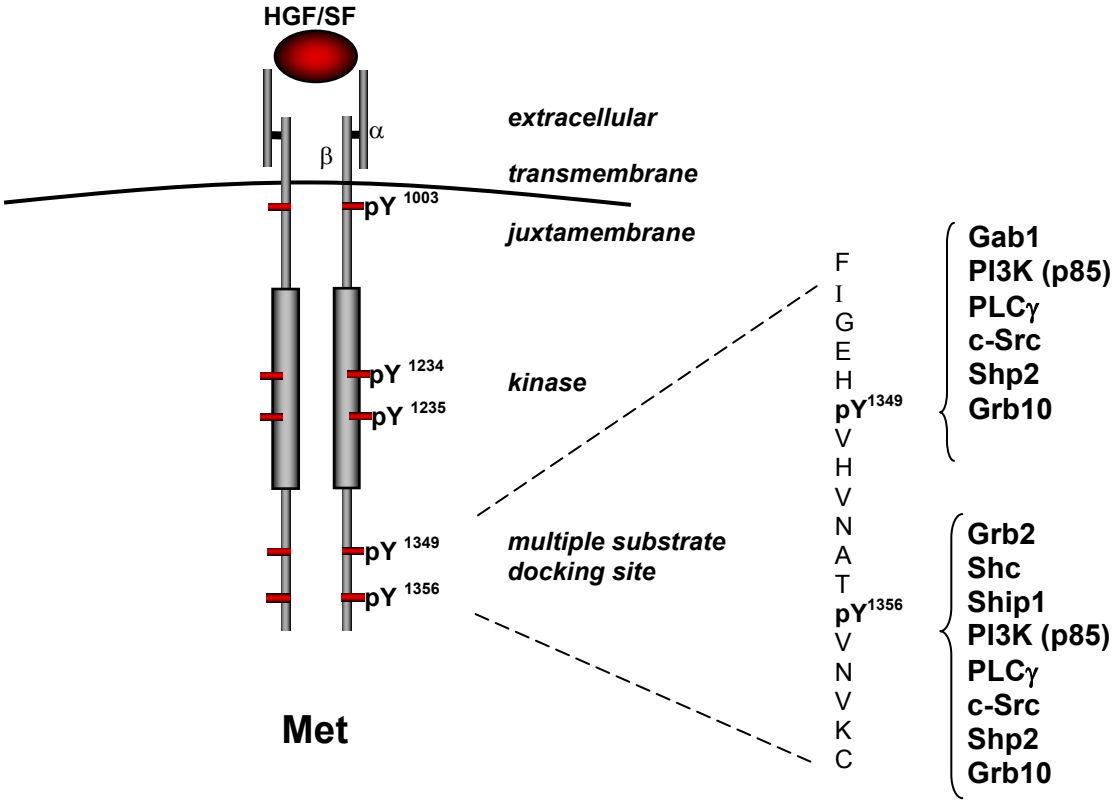



**Figure 4. Docking sites of Met**. Shown are the phosphotyrosines binding sites of Met, as well as their direct interaction partners. α and β refer to the subunits of the receptor. Gab1, growth-factor-receptor-bound protein 2 (Grb2)-associated binder 1; HGF/SF, hepatocyte growth factor/scatter factor; PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase Cγ; Shc, Src-homology-2 (SH2)-domain-containing; Shp2, SH2-domain containing protein tyrosine phosphatase 2; Grb2 and Grb10, growth-factor-receptor bound-protein 2 and 10; Ship, SH2-domain-containing inositol-5-phosphatase; p85 refers to the regulatory subunit of PI3K. Modified from Birchmeier et al., 2003.

Most of these proteins recognize specific tyrosine-phosphorylated regions of a receptor via their src-homology region 2 (SH2) domains. Thereby, this domain has a key role in relaying cascades of signal transduction (Koch et al., 1991). In contrast, the docking protein Gab1 can be recruited to the receptor directly via a unique 13-amino acids sequence, the Met-binding site (Schaeper et al., 2000). In addition to the direct association of Gab1 to Met, binding may be also enhanced indirectly by coupling Gab1 to Met via Grb2. Phosphorylated Gab1 binds several downstream signaling molecules, like PI3K, phospholipase C-γ, PLC-γ, the phosphatase Shp2, and the adaptor proteins Crk/CRKL (for recent review see Birchmeier et al., 2003). In vitro studies have shown, that binding of Shp2 by Gab1 is important for HGF/SF and Met dependent branching morphogenesis of epithelial cells (Schaeper et al., 2000; Maroun et al., 2000). Recruitment of Shp2 is critical for activation of the ERK/MAPK pathway, which plays an important role in cell proliferation, differentiation and migration (Fig.5). Met activates also other signaling branches that regulate cell motility and invasion by the phosphorylation and activation of paxillin and focal adhesion kinase (Liu et al., 2002). The driving forces for cell motility and polarity are derived from the cytoskeletal reorganization of actin, which is controled by Cdc42, Rac and Rho small GTPases (Ridley, 2001). Cdc42 promotes filopodia and microspike formation while Rac induces lamellipodia and membrane ruffling. Several effectors for Cdc42 and/or Rac have also been found to be involved in HGF/SF induced cell-cell dissociation and migration, such as Cdc42/Rac-regulated p21-activated kinase (PAK) (Royal et al., 2000). Moreover Met can also contribute to cell survival via activation of the PI3K/Akt pathway (Fan et al., 2001). In addition, other molecules such as β-catenin, integrins, and c-jun amino terminal kinase (JNK) have been reported to participate in HGF/SF/Met signaling (Monga et al., 2002; Muller et al., 2002; Chiu et al., 2002; Lamorte et al., 2000).

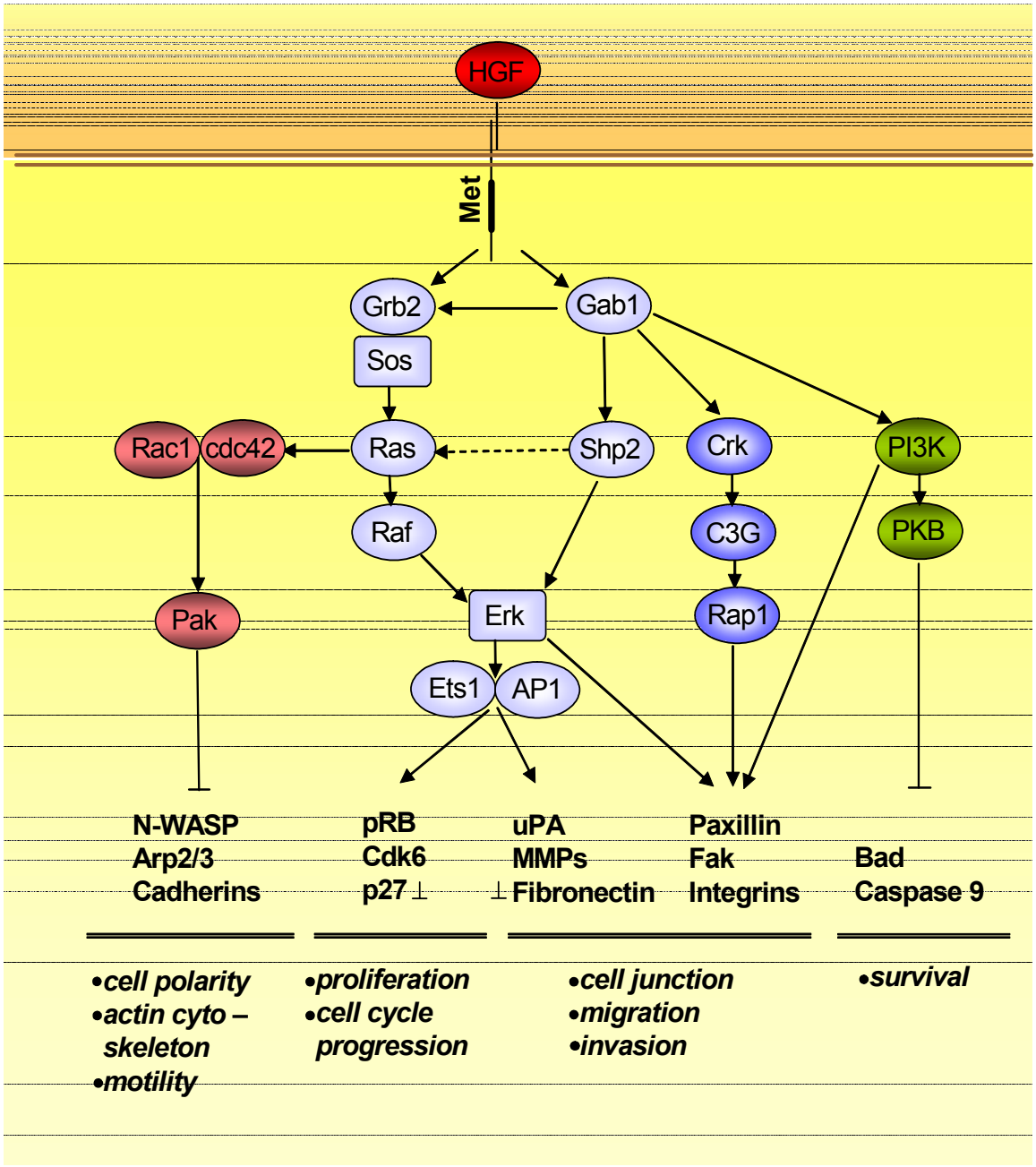



**Figure 5. Signaling by the receptor tyrosine kinase, Met**. Upon binding of HGF/SF, Met recruits various adapter proteins like Gab1 and Grb2 and activate Shp2, Ras, Erk and PI3K pathway. These pathways regulate cell adhesion, cytoskeleton, motility, cell cycle and apoptosis (Birchmeier et al., 2003).

Genetic and cell biological evidence have shown that Gab1 is the most crucial substrate for Met signaling (Maroun et al., 2000; Sachs et al., 2000). Targeted mutagenesis of Gab1 in mice revealed similar phenotypes of Gab1 and Met null mutants, which proofs the essential role of Gab1 in Met signaling. Gab1 null mutation embryos die between embryonic day 13.5 and 17.5 and are characterized by a placenta defect, a small liver and lack of muscle of limbs and diaphragm (Sachs et al., 2000). Furthermore, Gab1 null mutation mice display skin and heart defects, which could be related to defective signaling downstream of EGFR (epidermal growth factor receptor), PDGFR (platelet derived growth factor receptor) or gp130 signaling cascades (Itoh et al., 2000; Cai et al., 2002), since Gab1 is also adaptor protein for these receptors. Gab1 contains a pleckstrin homology domain (PH) at its N-terminus, a unique Met receptor tyrosine kinase binding site (MBS) and two Grb2-binding sites. The central region of Gab1 protein is rich in prolines and contains multiple tyrosine residues, which when are phosphorylated, they bind the SH2 domains of many downstream signaling proteins. Pleckstrin homology domains can recognize membrane components, and therefore contribute to the membrane targeting of Gab1 (Fig.6). Interaction of Gab1 with the adaptor protein Grb2 is important for coupling Gab1 to activated EGFR (Lock et al., 2000). Gab1 binds constitutively the SH3 domains of Grb2 via proline-rich sequences, while phosphotyrosine residues of activated EGFR bind the SH2 domain of Grb2. EGFR mutants deficient in binding Grb2, are unable to recruit and activate Gab1 (Rodrigues et al., 2000). Binding of Grb2 is also important for coupling Gab1 to other tyrosine kinases, like the FGFRs (fibroblast growth factor receptors) (Ong et al., 2001; Lamothe et al., 2004). In the FGF receptor pathway, Gab1 phosphorylation occurs via an additional scaffolding adaptor, FRS2. Upon receptor activation, FRS2 becomes tyrosine phosphrylated and binds to GRB2, which in turn recruits Gab1 (Hadari et al., 2001). Many extracellular stimuli like insulin, IL3 (interleukin 3), IL6, Epo1 (Erythropoetin1) as well as B-cell receptor activation induce Gab1 phosphorylation and association with Shc, PI3K, PLC-γ and Shp2 (Lecoq-Lafon et al., 1999; Ingham et al., 1998). Gab1 binding with PI3K has been shown to be important for prevention of apoptosis in response to NGF (nerve growth factor) stimulation (Holgado-Madruga et al., 1997).

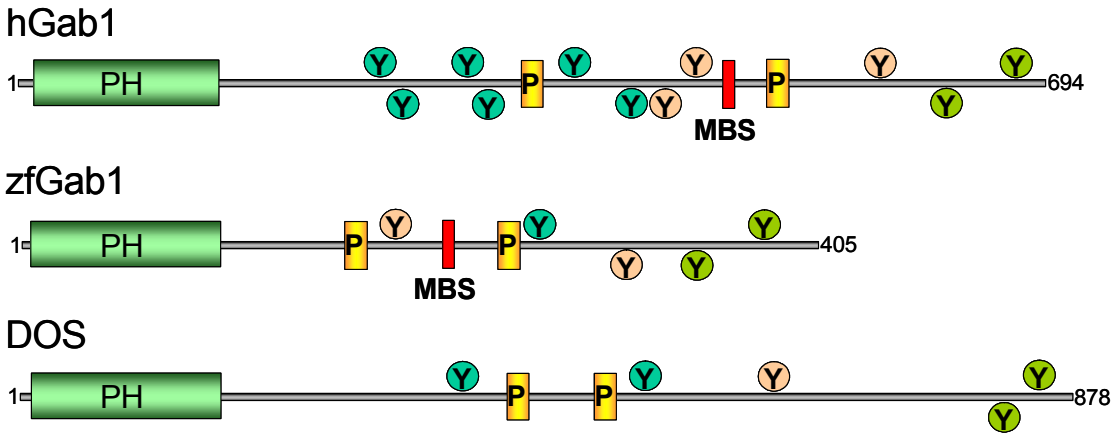



**Figure 6. Gab family proteins**. Schematic domain structures of mammalian Gab1 proteins, zebrafish Gab1 (zfGab1) and their invertebrate orthologs in Drosophila (DOS) are shown. All Gab family proteins consist of N-terminal pleckstrin-homology (PH) domain, proline-rich domains (P) and multiple tyrosines. The unique Met-binding site (MBS) that allows for direct interaction with Met is also indicated.

The Gab1 docking proteins are evolutionary conserved from worms to mammals and homologues have been identified in vertebrates, like zebrafish (zfGab1), but also invertebrates, like Drosophila (Dos, Daughter of Sevenless) and Caenorabditis elegans (Soc1, Suppressor of Clear, Fig.6) (Gu and Neel, 2003).

**Met signaling during development**

The importance of HGF/SF and Met signaling system in development has been assessed by genetic analyses in the mouse. Mice that carry a null mutation of either HGF/SF or Met die in uterus between embryonic day 12.5 and 16.5 due to placenta defect. The placental labyrinth layer formed by epithelial trophoblast is significantly reduced, which leads to an impairment of oxygen exchange and nutrients between the maternal and embryonic bloodstream (Schmidt et al., 1995; Uehara et al., 1995; Bladt et al., 1995). Ablation of Met or HGF/SF results in complete absence of the muscle groups in the mouse embryo that derive from migrating precursor cells,

whereas other muscle groups form normally. The migrating progenitors delaminate by epithelial-mesenchymal transition from the dermomyotome and migrate to the limb, tongue and diaphragm, where they differentiate into skeletal muscle. In HGF/SF and Met mutant mice, these migrating cells do not detach and do not emigrate from the dermomyotome (Fig.7).

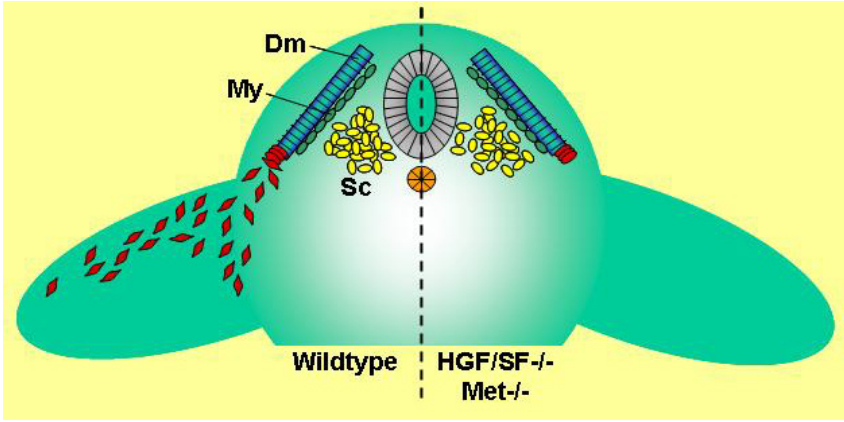



**Figure 7. Migrating muscle precursor cells in HGF/SF$^{-/-}$ and Met$^{-/-}$ embryo**. During normal development muscle precursor cells (red) delaminate from the epithelial dermomyotome (Dm, blue) and migrate to the limb bud, where they differentiate into myoblasts. In HGF/SF$^{-/-}$ or Met$^{-/-}$ embryos, the progenitor cells are not released, but remain in the dermomyotome. HGF/SF, hepatocyte growth factor/scatter factor; My, myotome; Sc, sclerotome. Modified from Birchmeier and Brohmann, 2000.

During delamination and migration of muscle precursor cells, HGF/SF is expressed in a highly dynamic pattern, first in the mesenchyme close to the epithelial dermomyotome, and then along the ways and at the targets. HGF/SF and Met null mutation mice are also characterized by small livers due to decreased proliferation and increased apoptosis of hepatocytes.

The genetic analyses of HGF/SF and Met in mice revealed that the phenotypes are identical, demonstrating that HGF/SF is the only Met ligand and Met is the only functional receptor for HGF/SF *in vivo*. HGF/SF and Met arose late in evolution. This is consistent with the function carried out by this signaling system in the embryo, related to processes such as placentation and liver development, which arose also late in evolution.

**Met function in the adult**

The Met tyrosine kinase receptor and its ligand, HGF/SF are expressed not only during embryogenesis, but also in the adult. Several experimental approaches have shown that deregulation of this pathway is implicated in many human malignancies. Transgenic mice that overexpress Met or HGF/SF have been shown to develop different types of tumors. Furthermore, the receptor or the ligand are frequently expressed in human carcinomas and other types of solid tumors, as well as in their metastases. Overexpression of Met and/or HGF/SF often correlates with poor prognosis, for example, Met-activating mutations have been found in human sporadic and inherited renal papillary carcinomas (Rong et al., 1994; Takayama et al., 1997; Abounader et al., 2002; Danilkovitch-Miagkova and Zbar, 2002). It is highly probable that HGF/SF and Met signaling might participate at different stages of tumor progression, since it is implicated during several stages of tumourigenesis such as proliferation, invasion, angiogenesis and anti-apoptosis.

Under normal physiological conditions HGF/SF acts as a paracrine factor, i.e. mesenchymal cells produce HGF, which acts on epithelial and other cells. Met is predominantly expressed in the cells derived from the epithelial or endothelial origins (Birchmeier and Gherardi, 1998). In pathological situations, such as cancer, activation of Met occurs most often through autocrine, although it is possible that Met can act through paracrine mechanisms. For instance, osteosarcomas and glioblastoma multiforme express both Met and HGF/SF (Birchmeier et al., 2003). HGF/SF and Met have also been implicated in various physiological processes in the adult. For instance, during liver regeneration, HGF/SF levels in the blood stream raise, and conditional mutagenesis in mice has shown that Met signals are essential during liver regeneration and repair (Michalopoulos and DeFrances, 1997; Taub, 2004; Borowiak et al., 2004; Huh et al., 2004). Moreover, upregulated HGF/SF and Met expression is observed after tissue injury, for instance in the lung, kidney and heart (Ohmichi et al., 1996; Kawaida et al., 1994; Nakamura et al., 2000). Interestingly, application of exogenous HGF/SF to a skin wound promoted the formation of new tissue and fast healing (Bevan et al., 2004). Thus, it suggests that up-regulated HGF/SF expression might be part of a general, defensive response to tissue injury.

## The aim of the study

It has been demonstrated that HGF/SF/Met signaling has a potential role during regeneration and tissue remodelling. These data were achieved through studies carried out by biochemical and cell culture experiments *in vitro*, and by genetic liver regeneration studies. HGF/SF/Met signaling has been implicated in skin regeneration, since HGF/SF levels increase rapidly after skin injury in the serum. However, the role of endogenous HGF/SF and its receptor, Met, in skin development and wound healing has as yet not been elucidated. The targeted mutation of HGF/SF or Met causes embryonic death due to defect in placental development, which had precluded a genetic analysis of Met or HGF/SF function in the adult. To overcome embryonic lethality, the cre-loxP technology was here employed to clarify the role of Met specifically in the adult skin. Keratin14-cre knock-in mice were used to introduce a Met null mutation into keratinocytes. The present study was aimed to understand further the role of Met in the skin and during skin wound healing. For the first time, a genetic method for the analysis of the Met gene function in the skin has been developed.

## Results

### Expression of Met and HGF/SF in the skin and during skin wound healing

The role of Met and HGF/SF has been recently implicated in the skin and in the hair follicle cycle, as shown by overexpression experiments and skin organ culture (Lindner et al., 2000). Theses experiments however, do not show the endogenous *in vivo* function of Met signaling system in the skin. In order to determine the precise expression profile of Met and HGF/SF in the skin, immunohistological analysis on tail skin paraffin sections was performed. The immunohistological analysis demonstrated that Met is expressed in the epidermis and its ligand, HGF/SF, in the dermis, which may suggest a possible role of this signaling system in the skin (Fig.8A, B). This is also in an agreement with the literature (Lindner et al., 2000). The two expression domains of HGF/SF and Met are close to each other and could function in a paracrine manner.

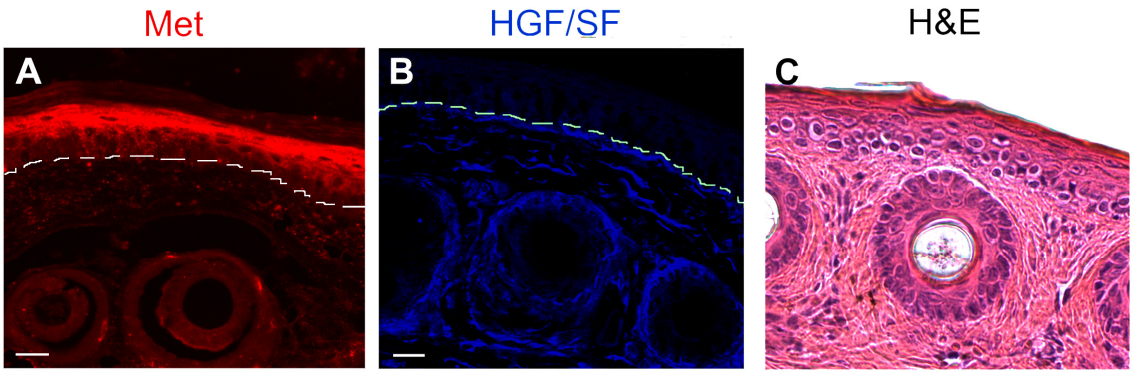



**Figure 8. Expression of the Met tyrosine kinase receptor and its ligand, HGF/SF, in the skin. A**. Immunofluorescence staining using Met antibody on a tail skin section. Met is detectable in the epidermis. **B**. Immunofluorescence staining using HGF/SF antibody on a tail skin section, showing HGF/SF protein expression in the dermis, associated with loosely packed fibroblasts. **C**. Hematoxylin/eosin staining on a tail skin section. This staining helps to recognise the morphology of tail skin. The round structure in the dermis is a hair follicle.

Furthermore, immunofluorescent staining of phosphorylated Met on skin sections was performed to investigate the functional state of the expressed Met receptors. The data from these experiments revealed that activated Met was present in both, epidermis and hair follicles, including hair bulge stem cells (Fig.9A, B). In addition, phosphorylated Met was continuously detected in whole epidermis at both early (P5, Fig.9B) and later stages (P32, Fig.9A) of skin. Using antibodies against CD34, which stain hair bulge stem cells and hematopoietic cells, colocalization with phosphorylated Met in the hair bulge stem cells could be observed (Fig.9A, B). These results clearly show that activated Met is present in the epidermis and hair follicles, further implicating a potential functional role of Met/HGF/SF signaling system in the skin.

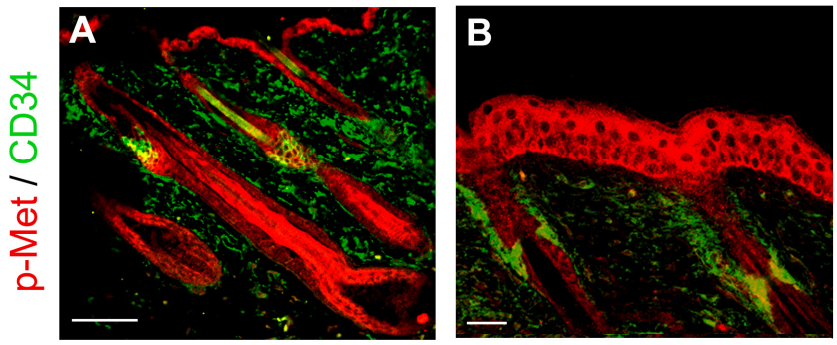



**Figure 9. Expression of activated Met in the skin**. **A, B** Immunostaining of skin section of the age P32 (A) and P5 (B) with anti phospho-Met (red) and anti CD34 (green) antibodies. CD34 is a marker of hair bulge stem and hematopoietic cells. The merged fluorescence shows that Met and CD34 are colocalized in the bulge stem cells. Scale bar 20µm

**Generation of mice deficient in Met in keratinocytes**

The previous expression studies point to a potential role of HGF/SF and the Met receptor in the skin. To further analyse the importance of the Met signaling system in the skin and in physiological processes such as skin wound repair, conditional mutagenesis of Met in the keratinocytes of mice was employed. The keratin 14-cre (K14-cre) mice were used, which express the cre recombinase in the epidermis starting on embryonic day 15, to introduce a null mutation into the Met locus in the skin. Keratin 14, and thus K14-cre, are known to be ubiquitously expressed in hair follicles and basal cells of the epidermis, as well as in tongue and esophagus(Huelsken et al., 2001). First, K14-cre mice were crossed with the conventional Met null mutation, $Met^{null}$ (Bladt et al., 1995), to generate animals with a K14-cre; $Met^{null/+}$ genotype. Then, these animals were mated with "floxed" Met mice, $Met^{flox/flox}$ (Borowiak et al., 2004), to obtain animals with the critical K14-cre; $Met^{null/flox}$ genotype, in which one allele of Met corresponds to the conventional null mutation, the other to a 'floxed' allele. Following K14-cre-mediated recombination, the exon encoding the essential ATP-binding site of Met was removed in the $Met^{flox}$ allele of the skin, and a non-functional null allele, which was denoted as $Met^{\Delta}$, was generated (see structures of non-recombined and recombined alleles in Fig.10A). This breading procedure ensured that only a single allele needs to be recombined by cre to obtain a complete ablation of Met function in the epidermis. The generated K14-cre; $Met^{flox/null}$ mice are subsequently termed "conditional Met mutant mice".

In these experiments, control animals were heterozygous $Met^{flox/+}$ mice that also carried the K14-cre allele or heterozygous $Met^{flox/+}$ and $Met^{null/+}$ mice without cre. Such controls are essential, e.g. in order to check effects caused by cre protein by itself. These control mice did not show any overt abnormalities when compared to wild-type mice. This also indicated that the inserted loxP site in the Met locus does not interfere with Met function *in vivo*. However, efficient recombination of Met was observed in the epidermis of the K14-cre; $Met^{flox/null}$ mice. Southern blot analysis demonstrated that virtually all (95%) of the cells in the epidermis had recombined the $Met^{flox}$ allele already at embryonic day 17.5 ($Met^{\Delta}$, Fig.10B). A similar high level of cells containing

the recombined allele was observed in the epidermis of young and adult animals, e.g. at P8 and at 12 weeks (Fig.10B). In other epithelial tissues like pancreas, lung and liver, recombination of Met was not observed (Fig.10C).

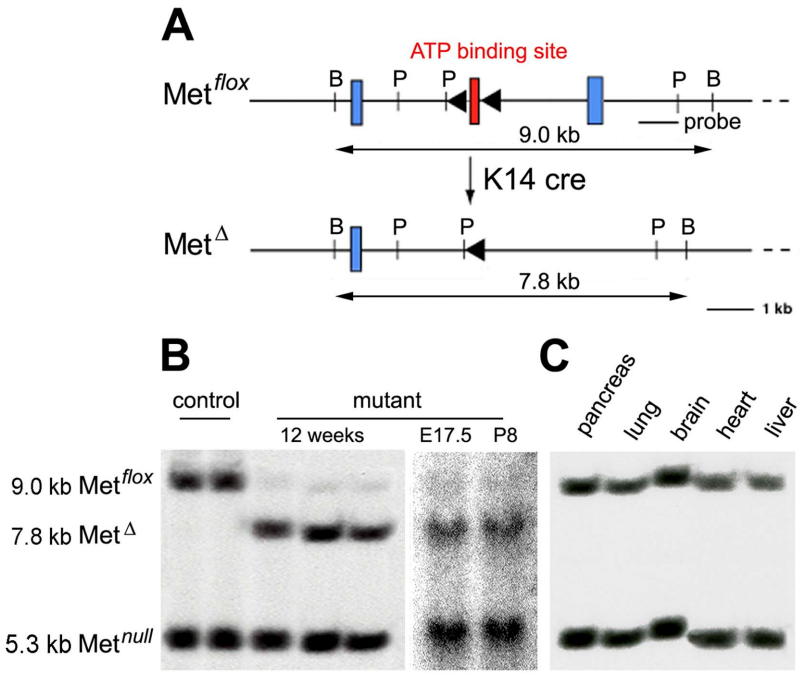



**Figure 10. Generation of skin-specific Met mutant mice. A**. Schematic representation of non-recombined and recombined alleles of Met. Exon 15 of the Met gene that encodes the ATP-binding site (red box) was flanked by loxP sites (triangles) and is excised after K14-cre-induced recombination. Blue boxes indicate exons 14 and 16. The sizes of the restriction fragments generated by BamHI digest before and after recombination are indicated. B, BamHI; P, Pst. **B**. Southern blot analysis of epidermis from control and conditional Met mutant mice of different ages (12 weeks old, E17.5, P8). **C**. Southern blot analysis of different organs of conditional Met mutant mice.

The recombination introduced by the K14-cre allele that occurred in virtually all epidermal cells, was also shown histologically using the Z/AP reporter mice. In such mice, activation of alkaline phosphatase is detectable by yellow NBT/BCIP staining in recombined cells, whereas blue LacZ staining is observed in the non-recombined cells (Lobe et al., 1999). K14-cre mediated expression of alkaline phosphatase demonstrated that the vast majority of the cells in the epidermis and hair follicles had undergone recombination, and only very small groups of non-recombined cells were detectable (Fig.11A, the enlarged picture shows a group of non-recombined cells, Fig. 11B-C). The blue LacZ staining was observed in cells that do not express K14-cre, i.e. in the dermis and the arrector pili muscles, which anchor in the dermis and insert onto the sheath of hair follicles. The non-recombined cells in the epidermis were observable with a frequency of approximately 5%, which is comparable with the K14-cre mediated recombination in the Met locus, as detected by Southern blotting.

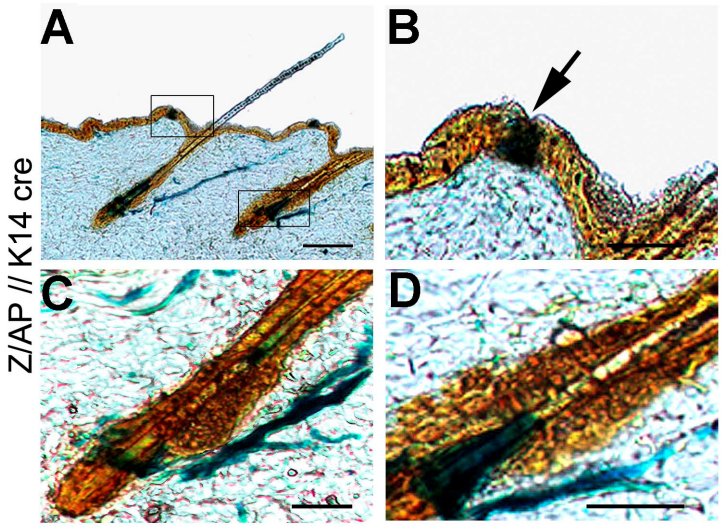



**Figure 11. Expression of K14-cre in the skin using Z/AP reporter mice**. **A**. Double staining of skin section from Z/AP; K14-cre mice for lacZ (blue, non-recombined) and alkaline phosphatase activity (yellow, recombined). **B**. A higher magnification shows an area of non-recombined epidermal keratinocytes (blue patch, marked by arrow). **C-D**. Higher magnifications show also two independent hair follicles. Arrector pilli muscle cells, which surrounded the follicle, are non-recombined (blue). Scale bar, 50μm (A, B), 20μm (C, D).

The high efficiency of recombination allowed further examination of Met deficient keratinocytes in the conditional Met mutant mice.

**Met signaling during generation and maintenance of the skin**

The conditional Met mutant mice were born in numbers predicted by the Mendelian ratio. These mice were fertile, had a normal life span and showed no overt abnormalities in the skin and other epithelial organs. The appearance of skin and hair in Met mutant mice was examined more closely by histology at birth and afterwards. No gross morphological changes in the epidermis could be detected when compared with control mice. The thickness of the epidermis was comparable and did not display any pathological alterations in the mutants. There were no apparent changes in hair cycle progression, when control and conditional mutant mice were compared. For instance, the first and second anagen phases occurred at P5 and P30, respectively. Catagen and telogen occurred at P18 and P20 (Fig.12, Paus and Cotsarelis, 1999). The conditional Met mutant mice were kept for nearly 2 years and unusual hair loss or other changes in the appearance of the aged skin could not be observed.

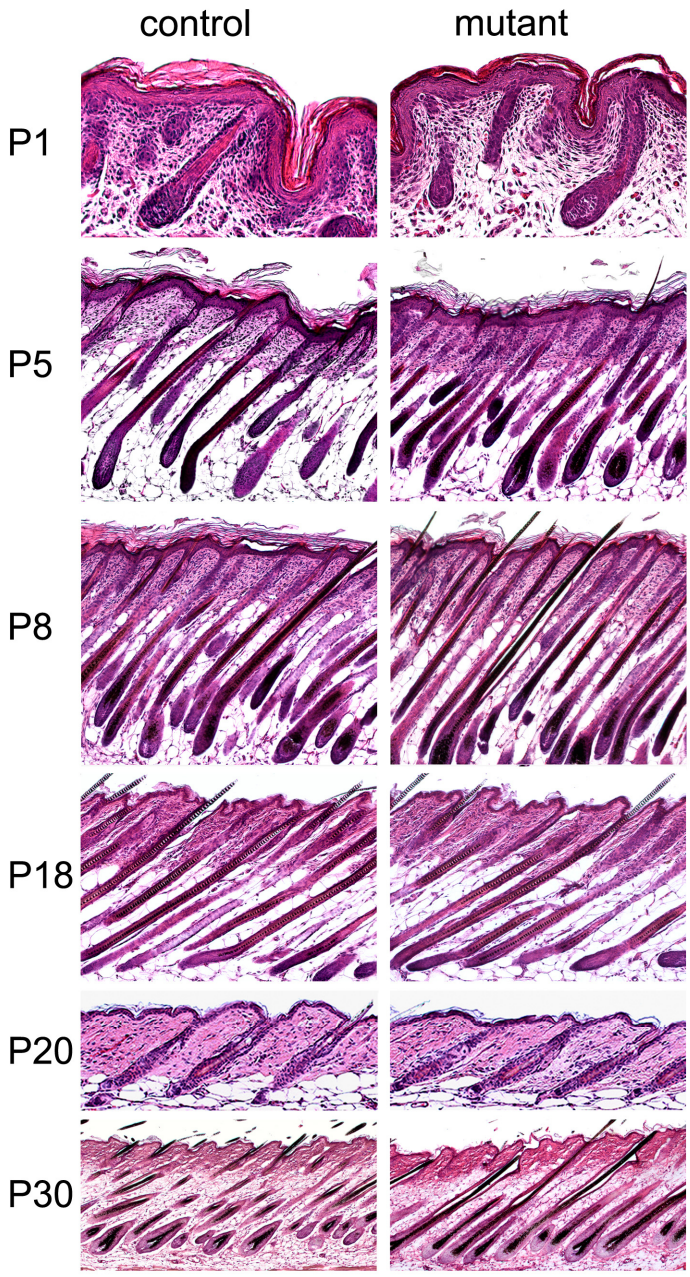



**Figure 12. Hair follicle cycle in control and conditional Met mutant mice.** Sagittal sections of control and conditional Met mutant skin stained with hematoxylin/eosin at P1 (first anagen), P5 (first anagen), P8 (first anagen), P18 (first catagen), P20 (first telogen), P30 (second anagen).

Further immunohistological analysis of the conditional Met mutant skin did not reveal essential changes in expression of markers for terminal differentiation in the epidermis, when compared to controls. Keratin 10 and loricrin continued to be expressed in the upper, differentiated layer of the mutant and control epidermis (Fuchs et al., 1992; Byrne et al., 1994). Keratin 6 was detectable only in the hair follicles in the mutants and controls (Fig.13, Fuchs, 1990;Wankell et al., 2001).

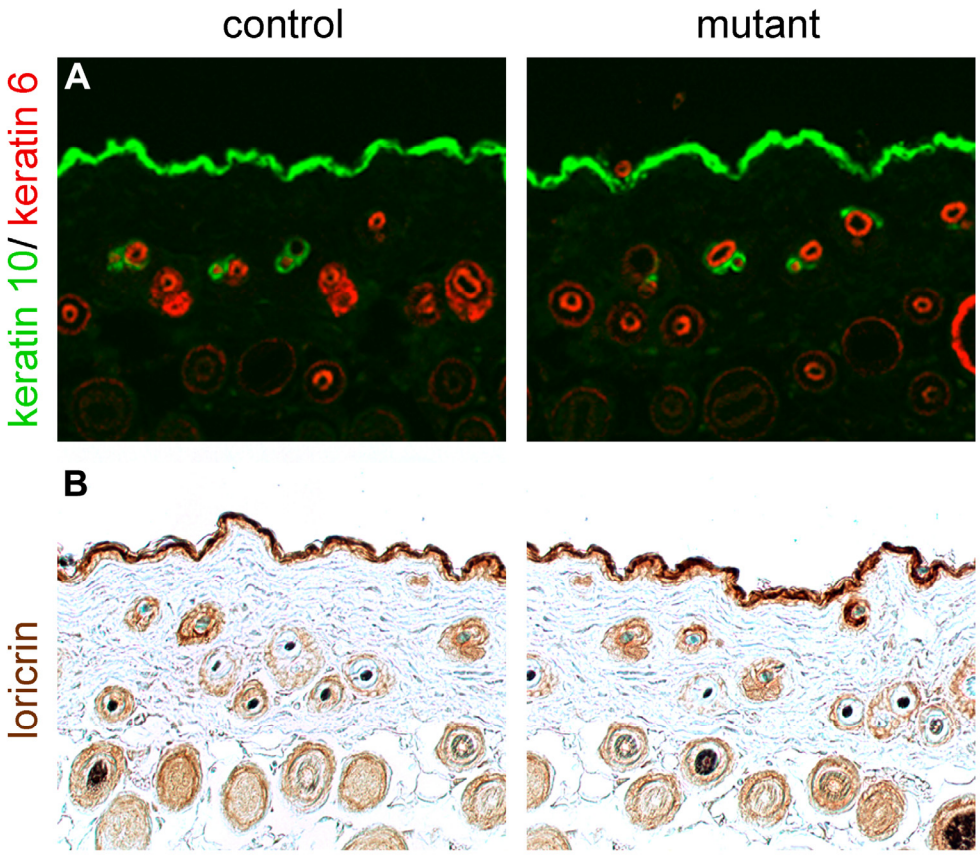



**Figure 13. Immunohistological analysis of the skin in conditional Met mice**
**A**. Immunohistological staining for keratin 10 and keratin 6 on dorsal skin paraffin sections of 2 months old mutant and control mice. Keratin 6 is constitutively expressed in the outer root sheath of hair follicles and is observed in both, control and mutant mice. Keratin 10 is present in the differentiated, upper layers of the epidermis. **B.** Immunohistological staining for loricrin on dorsal skin sections of control and mutant mice. Loricrin is expressed in upper layers of the epidermis. There are no differences in expression of these proteins in the skin between mutant and control.

The data from these experiments clearly indicate that Met is not essential for the development and the maintenance of both, the epidermis and the hair.

**Wound closure in conditional Met mutant mice**

In previous studies, Met signaling has been shown to be important during liver regeneration, and it was found that the expression levels of Met and HGF/SF increased after injury of many organs (Ohmichi et al., 1996; Kawaida et al., 1994; Nakamura et al., 2000; Borowiak et al., 2004). Wound healing of the skin is an important regenerative process in mammals (Martin, 1997; Werner and Grose, 2003), but our knowledge in this area is still rudimentary.Therefore, the function of Met in the skin under stress conditions was investigated, specifically the effect of the absence of Met during skin would healing.

Full-thickness dorsal skin wounding in control and conditional Met mutant mice was performed in a way that epidermis and underlying dermis are destroyed (Werner et al., 1994). For these experiments, only males at an age of 12 weeks were used to exclude differences caused by gender variation. First HGF/SF and Met expression during the wound healing process was analyzed, i.e. 1 to 10 days after the injury, by in situ hybridization on frozen skin sections. HGF/SF expression was initially detected in the dermis adjacent to the wound clot, an area where inflammatory cells accumulate and infiltrate the lesion (Fig.14B, only the left halves of the wounds are shown; see scheme of entire wound in Fig.14A). Three days after injury, HGF/SF was strongly up-regulated in the hyperproliferative epithelium (HE) at the edges of wounds (Fig.14C). At this time point, the development of newly formed epithelium was visible. HGF/SF was also expressed in hair follicles of skin wound sections but not in the epidermis (Fig 14B, C) (Lindner et al., 2000).The receptor tyrosine kinase Met was also shown to be strongly expressed in the hyperproliferative epithelium during the wound repair process (Fig.14E, F, (Cowin et al., 2001). Of note, Met was present in the unwounded epidermis and hair follicles shown by in situ hybridization (Fig.14E, F), further confirming the immunohistological data. Collectively, the data indicate that during wound healing, HGF/SF and Met may signal in an autocrine manner in the hyperproliferative epithelium, and that Met signaling is up-regulated during the repair process, suggesting an important role during skin repair.

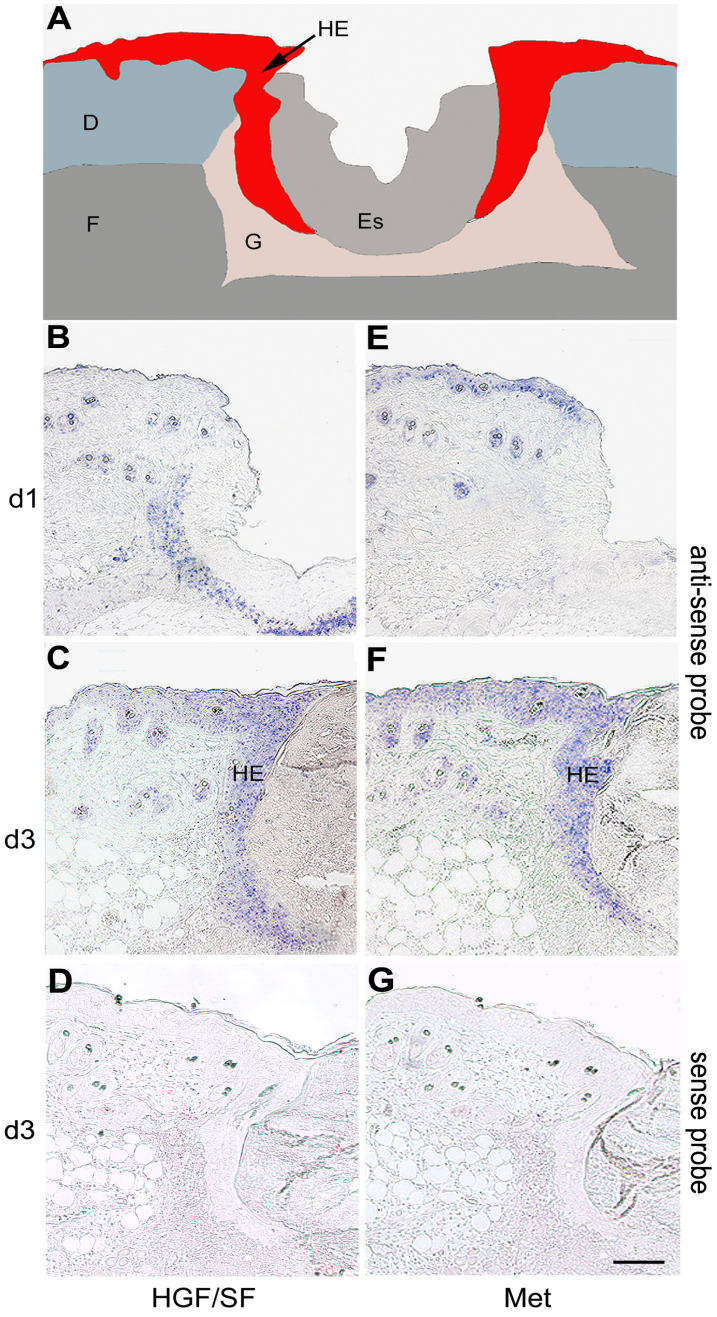



**Figure 14. Expression of HGF/SF and Met during wound healing. A**. Scheme of an entire wound 3 days after injury. Keratinocytes (red) at the wound edge proliferate and migrate down the injured dermis to form the so-called hyperproliferative epithelium (HE, marked by arrow). G, granulation tissue; D, dermis; F, fatty tissue; Es, eschar. **B and C**. In situ hybridisation of wounded skin with HGF/SF probe 1 day (B) and 3 days (C) after injury. HGF/SF is expressed in the dermis close to the clot at day 1. At day 3 after wounding, HGF/SF is highly expressed in the hyperproliferative epithelium (HE). **E and F**. In situ hybridization with the Met probe 1 day (E) and 3 days (F) after injury. Met is expressed in the epidermis and in the hyperproliferative epithelium (HE) at day 3 following wounding. **D and G** In situ hybridization with sense probes of HGF/SF (D) and Met (G). Scale bar, 50µm

The main structure responsible for wound closure is thought to be the hyperproliferative epithelium (Martin, 1997; Singer and Clark, 1999; Santoro and Gaudino, 2005). First, K14-cre was characterised in the skin wounds using Z/AP reporter mice, in order to determine whether K14-cre is expressed in the hyperproliferative epithelium and could be used for wound healing experiments. This experiment demonstrated successful recombination introduced by K14-cre, shown by activation of alkaline phosphatase and detected by yellow NBT/BCIP staining, in virtually all epidermal cells as well as in the wound epithelium (Fig.15A). Immunohistological analyses using keratin 14 antibodies confirmed that keratin 14 was strongly expressed in the hyperproliferative epithelium (Fig.15B).

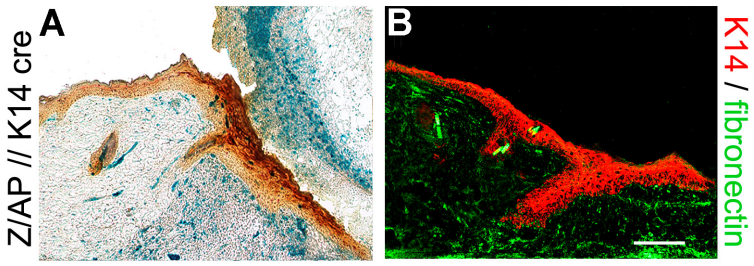
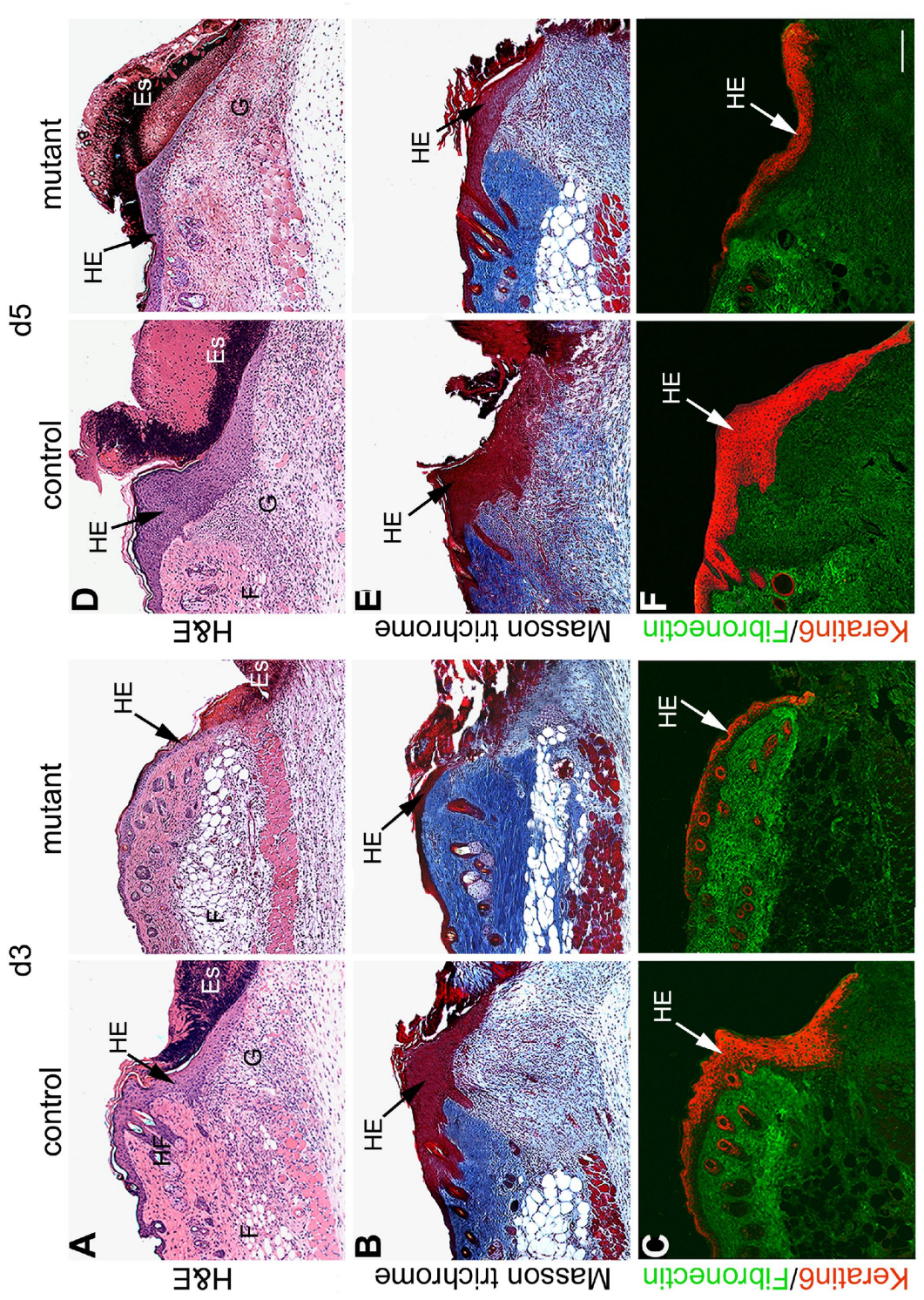



**Figure 15. Expression of K14-cre during wound healing**. **A**. Double staining of alkaline phosphatase and β-galactosidase activity of wound section from Z/AP; K14-cre mice. K14-cre-induced recombination is observed in the unwounded epidermis and in the hyperproliferative epithelium. **B**. Immunohistological analysis of a wound section from control mice using antibodies directed against keratin 14 (red) and fibronectin (green). Scale bar, 100µm

The histology of wounds 3-15 days after the injury of control and conditional Met mutant mice was analysed. The hyperproliferative epithelium was thinner and its formation was delayed in the conditional Met mutant mice, assessed by hematoxilin/eosin (Fig.16A, D) and by Masson trichrome staining (Fig.16B, E). Keratin 6 is expressed in activated keratinocytes of the hyperproliferative epithelium and in hair follicles (Fuchs, 1990; Wankell et al., 2001). Therefore, immunohistological analysis using keratin 6 antibodies was performed. These experiments demonstrated a reduction in the thickness of the hyperproliferative epithelium in the conditional Met mutant mice compared to controls (Fig.16C, F). Three days after injury, the hyperproliferative epithelium in the mutant mice consisted only of a few cell layers and was not dramatically different from the normal epidermis. However, in control mice, newly formed epithelium appeared much thicker. Five days after injury, the size of the hyperproliferative epithelium increased in both, control and mutant, but interestingly, in mutant it was more prominent than in the control (e.g. compare Fig.16B with 16E).

**Figure 16. Wound healing in conditional Met mutant mice. A and D**. Hematoxylin/eosin staining of sections of wound from control and mutant mice 3 days (A) and 5 days (C) after wounding. **B and E** Masson trichrome staining of sections 3 days (B) and 5 days (D) after injury. **C and F**. Immunofluorescence staining for Keratin 6 (red) and fibronectin (green) from control and mutant mice 3 days (E) and 5 days (F) after injury. Arrows indicate the hyperproliferative epithelium (HE). F, fatty tissue; G, granulation tissue; Es, eschar; HF, hair follicle Scale bar, 100μm ⟶


d3
d5
control
mutant
control
mutant
H&E
Masson trichrome
Keratin6/Fibronectin
A
B
C
D
E
F
HE
HF
Es
G
F

Thus, wound healing occurred in conditional Met mutant mice, but it was delayed and required about twice as much time as in control mice. The effect of wound closure was also determined as percentage of distance covered by the epidermis between the wound edges. For instance, 5 days after the injury, 50% wound closure was observed in control mice; in conditional Met mutant mice, 50% wound closure occurred in 9 days (Fig.17A). In control mice, most of the wounds were healed within 10 days, while in mutant mice it took 17 days. The formation of the hyperproliferative epithelium was indeed delayed during the repair process in the conditional Met mutant mice, as shown by quantification. Compared to control mice, the area of the hyperproliferative epithelium was reduced by 80% 3 days after injury; 5 days after injury the area was reduced by 65%, and 7 days after injury by 25% (Fig.17B). The dynamic of the growth of the wound epithelium however, was faster in the mutant than in the control, starting from day 3 after injury (Fig.17B). It was related to faster increase of cell numbers in the wound epithelium in the mutants, compared to controls (Fig.17E). In control wounds, amplification in cells number between day 3 and 5 after injury was 1.9 times, while in the mutant it was 3 times.

To test whether the delay in the formation of the hyperepithelium was correlated with keratinocyte proliferation, the numbers of 5-bromodeoxyuridine (BrdU)- and phospho-histone 3–positive keratinocytes in mutant and control wounds were counted. Indeed, the number of BrdU-positive nuclei was significantly lower in the mutant wound epithelium than in the controls (Fig.17F). However, the percentage of proliferating keratinocytes in the hyperproliferative epithelium 3 days after injury was increased in the conditional Met mutants, which could be related to the recovery of size of the hyperproliferative epithelium at later stages (Fig.17C, D). Proliferation-positive cells did not accumulate at any particular sites in the hyperproliferative epithelium or at the remnants of the hair follicles. Another possible explanation for the delayed formation of the hyperproliferative epithelium in conditional Met mutant mice was an increase in cell death. However, the number of apoptotic cells in the skin of control and mutant mice was comparable, as assessed by TUNEL staining. Thus, wound healing occurs in the skin of Met conditional mutant mice, but re-epithelialization of wounds is delayed and requires about twice as much time as in control mice.

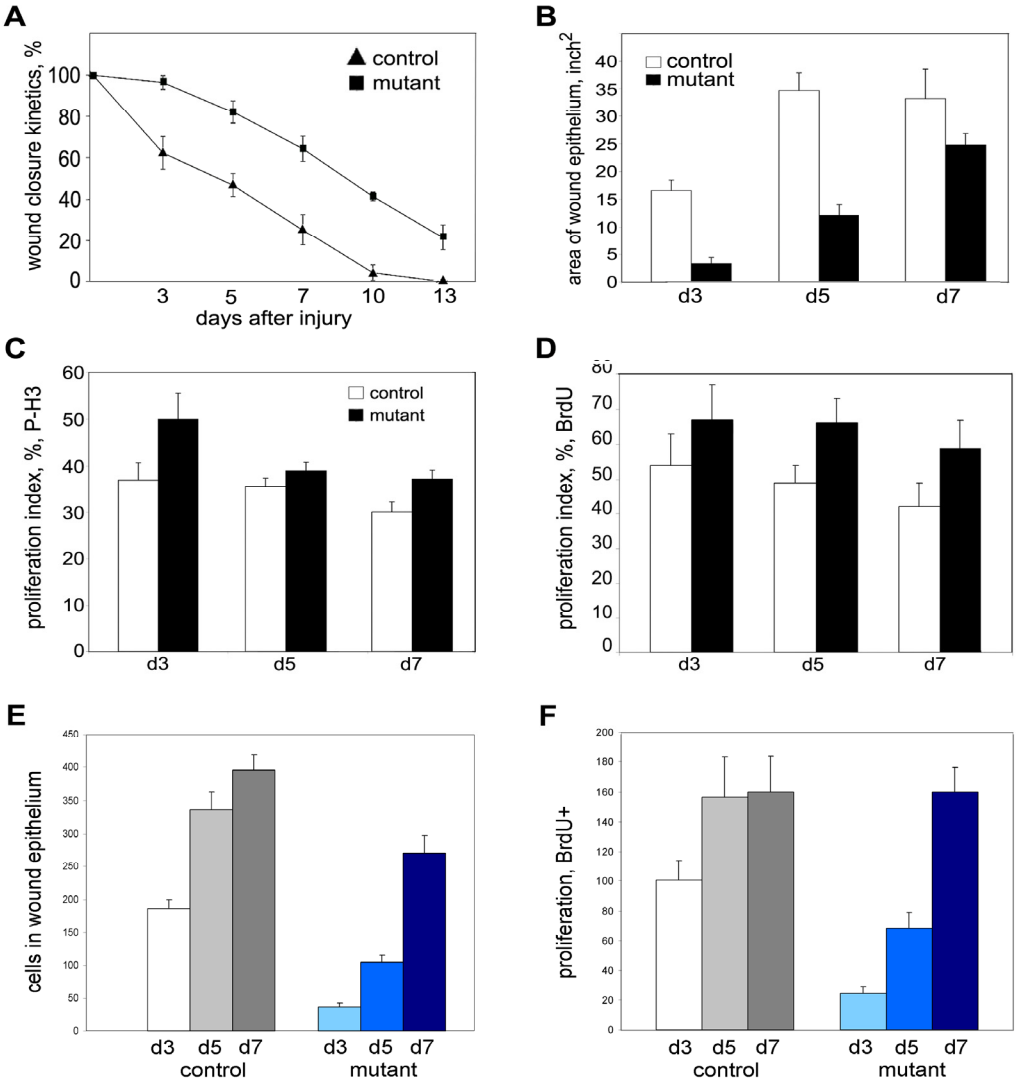



**Figure 17. Quantification of wound healing in control and conditional Met mutant mice A.** Wound closure kinetics in control and mutant mice. **B.** Quantification of the area of hyperproliferative epithelium 3, 5 and 7 days after wounding in control and mutant mice; only sections of the middle of the wounds were used for quantification. **C.** Proliferation of keratinocytes in the hyperproliferative epithelium from control and mutant mice 3, 5, and 7 days after wounding, as assessed by the proportion phospho-histone 3-positive nuclei in the epithelium. Error bars represent standard deviations. A Student's test was performed, and significant differences between control and mutant was observed 3 days after injury, P value, p=0.01. **D.** Proliferation of keratinocytes in the hyperproliferative epithelium from control and mutant mice 3, 5, and 7 days after wounding, as assessed by the proportion of BrdU-positive nuclei in the epithelium. Significant statistical differences between control and mutant was observed 5 days after injury, P value, p=0.01. **E.** Number of cells in the hyperproliferative epithelium quantified as Yopro-positive cells for control and mutant 3, 5 and 7 days after injury. Yopro is a nuclear dye. **F.** Quantification of BrdU-positive cells in the hyperproliferative epithelium of control and mutant mice at different time points after injury.

**Contribution of cells in the hyperproliferative epithelium**

To assess if the conditional Met mutant cells (approximately 95% of the keratinocytes in the epidermis) were able to contribute to the newly formed epithelium of the wounds, hyperproliferative epithelia of many control and mutant wounds were collected by laser capture microdissection and analysed by Southern blotting. The laser capture microdissection allows for precise dissection of wound epithelium. An example of a section of a wound before and after microdissection is shown in Fig.18A, B. Importantly, Southern blot analysis revealed an absence of Met-mutant cells, i.e. absence of the $Met^{\Delta}$ allele, in the microdissected hyperproliferative epithelia of mutant mice at day 5 (Fig.18C). Instead, all cells from the hyperproliferative epithelium of mutants contained the non-recombined $Met^{flox}$ allele, despite the fact that this cell population constituted only 5% in the skin prior to injury. At day 3, a 1:1 mixture of $Met^{flox}$ and $Met^{\Delta}$ cells was seen, indicating that the wounded epithelium at early time points after injury consisted of recombined and the non-recombined cells. In the unwounded mutant epidermis, only the recombined $Met^{flox}$ allele, i.e. $Met^{\Delta}$ was detected.

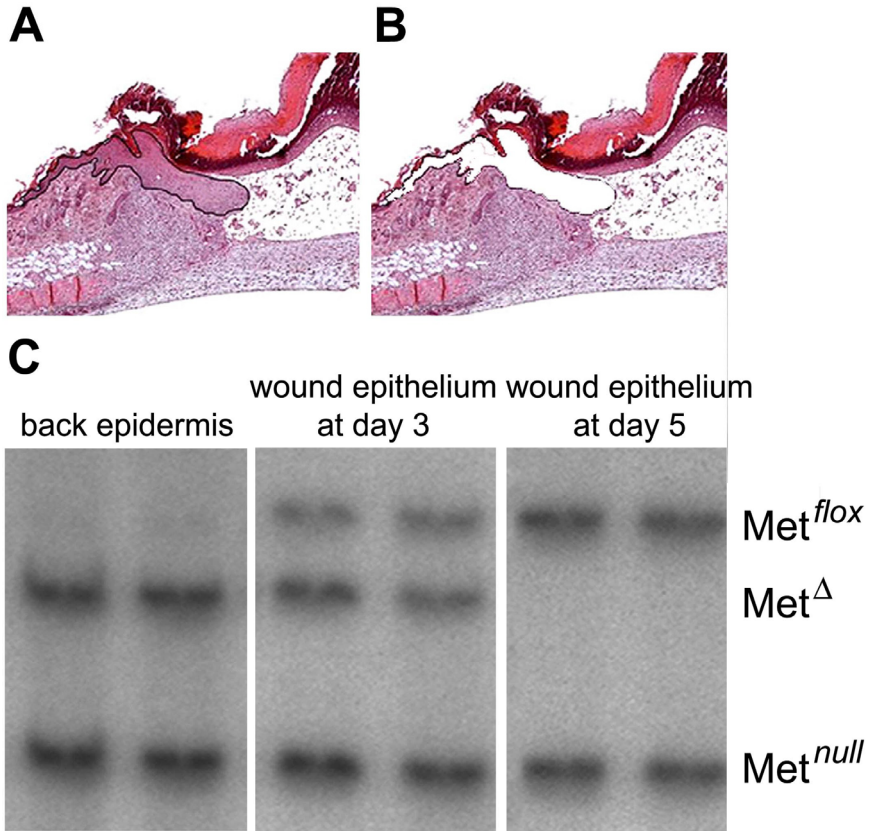



**Figure 18. Only residual Met positive keratinocytes contribute to the hyperproliferative epithelium of wounds in conditional Met mutant mice A, B.** Isolation of hyperproliferative epithelium by laser capture microdissection. A wound section before (A) and after laser capture microdissection (B) is shown. **C.** Southern blot analyses of back epidermis and hyperproliferative epithelia from conditional Met mutant mice. Microdissected hyperproliferative epithelia of wounds 3 days (middle) and 5 days (right) after injury were collected. Southern blotting of two preparations from different pools of microdissected tissues is shown. The hyperproliferative epithelium 5 days after injury in conditional Met mutant mice is formed exclusively by cells, which contain the non-recombined $Met^{flox}$ allele. At day 3, a 1:1 mixture of recombined and non-recombined cells are seen (middle).

The hyperproliferative epithelium at day 5 was examined by immunofluorescence and immunohistochemistry using anti-phospho-Met antibodies. The data revealed that indeed, the majority of cells contained the activated Met receptor in both the control and mutant skin (compare Fig.19A, C with B, D). Positive Met staining was more pronounced in the upper, already differentiated layers of the hyperproliferative epithelium, but was also visible in lower layers of the epithelia (see arrows in Fig.19A-D). It should be pointed out that activated Met was almost undetectable in the normal epidermis away from wound in the mutant (left side of Fig.19B).

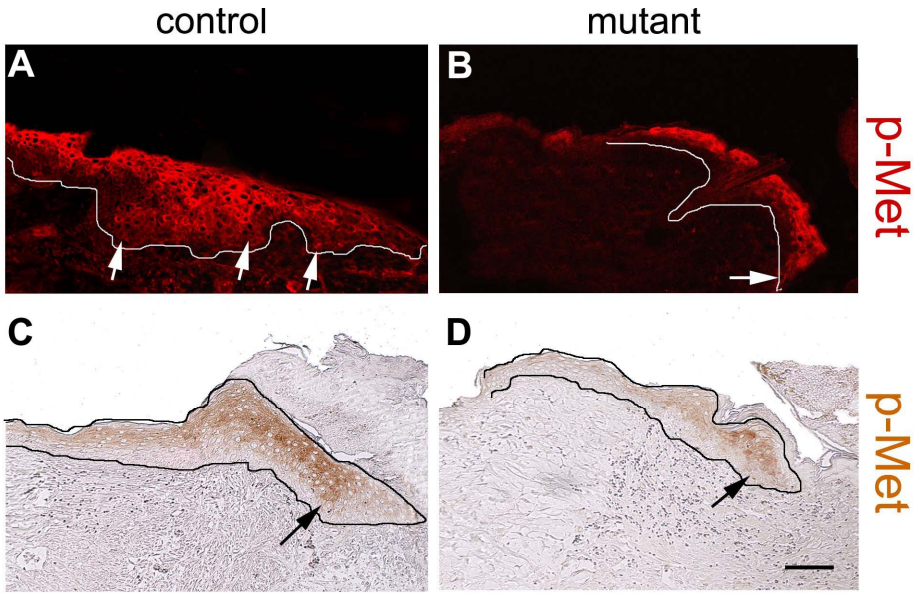



**Figure 19. Only phospho-Met positive cells contribute to the hyperproliferative epithelium in control and mutant.** Immunohistological analysis of wound sections from control and conditional mutant mice using anti-phospho-Met antibodies (red immunofluorescence in A and B, and brown immunohistochemistry in C and D). Cells in the hyperproliferative epithelium of conditional Met mutant mice (outlined) are phospho-Met positive. Arrows mark phospho-Met positive cells in the lower hyperproliferative epithelia layers. Scale bar, 100μm

It can be concluded from these data that only non-recombined keratinocytes, i.e. those that express a functional Met, can participate in the formation of the hyperproliferative epithelium. Thus, in the skin of conditional Met mutant mice the few remaining cells that escaped recombination appear to compensate and generate the entire hyperproliferative epithelia. Collectively, the data confirm that Met plays crucial functions during wound closure in the skin.

**Scratch-wound healing of Met mutant keratinocytes in cell culture**

The closure of scratch-wounds in cultured primary skin keratinocytes in response to HGF/SF was analyzed to confirm the essential role of Met in wound closure also in cell culture. Primary keratinocytes were isolated from the skin of newborn control and conditional Met mutant mice (Caldelari et al., 2000). Immunohistological staining with keratin 14 antibodies indicated that the isolated cells from control and mutant mice corresponded to keratinocytes and were not fibroblasts or other cells (Fig.20A). To confirm the expression of Met in isolated keratinocytes, Nothern blot analysis was performed on control keratinocytes stimulated with HGF/SF using the Met probe. Indeed, isolated cells expressed the Met tyrosine receptor. This again demonstrated that keratinocytes, not fibroblast or other cells were cultured, since Met is expressed in epithelial cells.

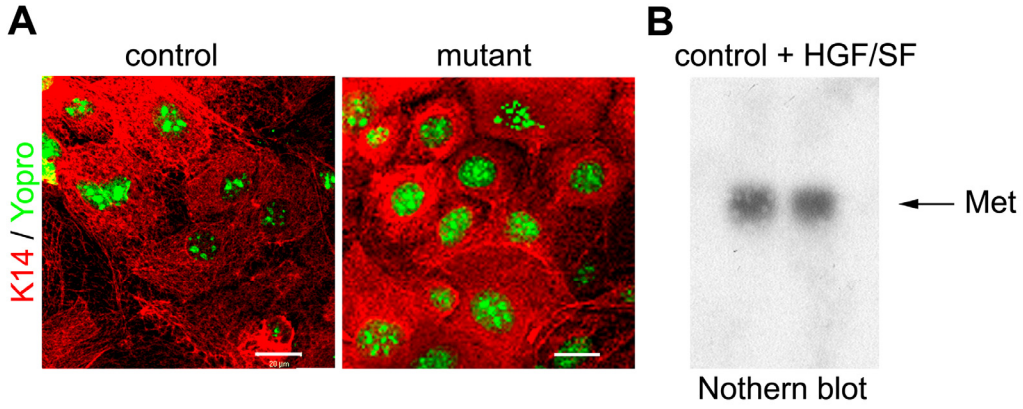



**Figure 20. Isolated keratinocytes from control and conditional Met mutant mice**. **A**. Immunostaining with anti keratin 14 antibodies on primary keratinocytes isolated from the skin of control and conditional Met mutant mice. Scale bar, 20µm **B**. Nothern blot analysis of control keratinocytes stimulated with HGF/SF probed with Met.

Isolated primary keratinocytes from the skin of newborn control and conditional Met mutant animals were cultured, and monolayers were scratch-wounded (Fig.21A-C, (Sano et al., 1999). In the presence of HGF/SF, control cells closed the wound within 48 hours. Keratinocytes isolated from Met conditional mutant mice did also close the scratch-wounds in the presence of HGF/SF, but only within 96 hours. However, keratinocytes isolated from Met conditional mutant mice that were stimulated with TGFα, closed wounds already after 48 hours. A strong proliferative response towards HGF/SF was observed in control cells close to the wound edges at 24 hours, but such a response was not observed in the mutant keratinocytes (Fig.21D, quantification in 21E). 24 hours after scratching, keratinocytes isolated from mutant skin did not proliferate, only single cells were phospho-histone 3-positive. The deficiency of proliferation in the culture of mutant keratinocytes stimulated with HGF/SF led to less density of cells close to the wounds, compared to control cultures (Fig.21D, compare control to mutant).

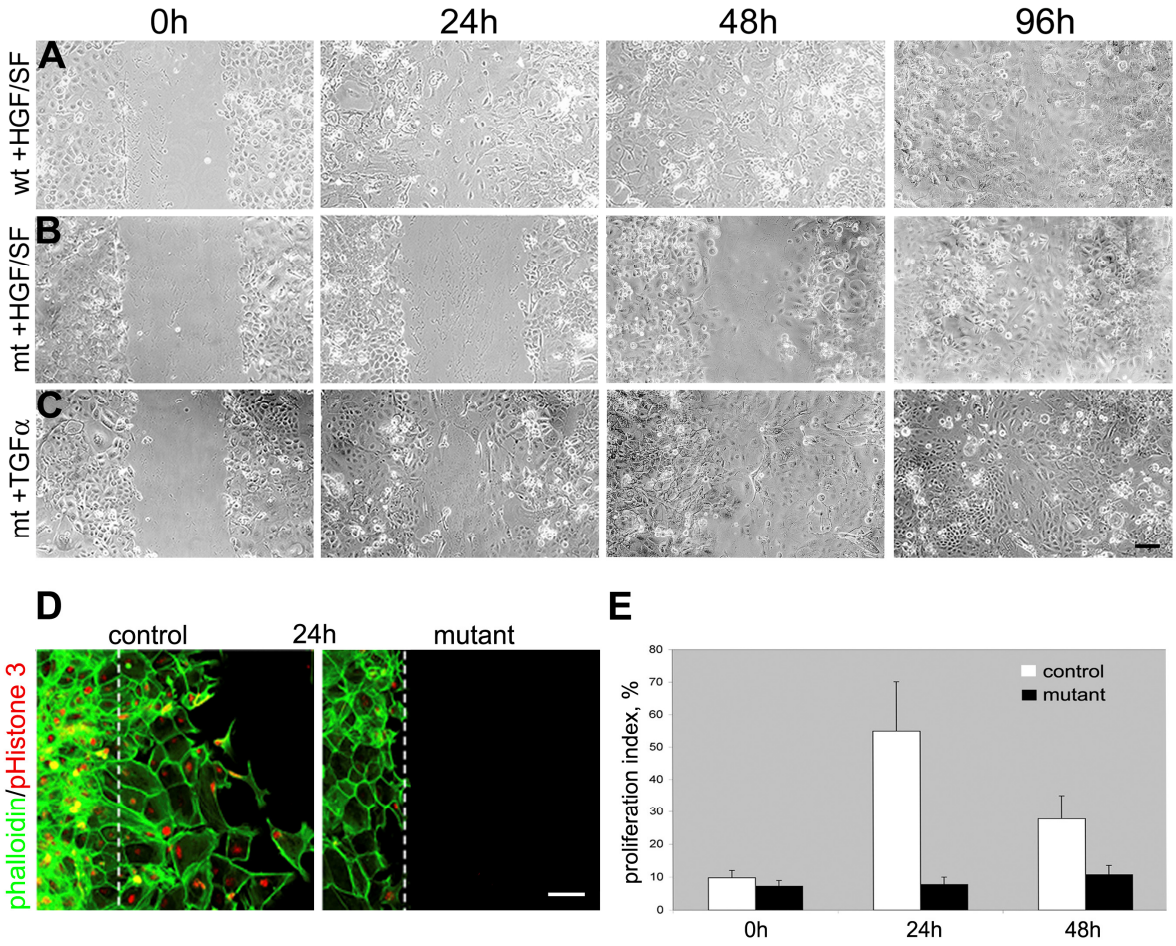



**Figure 21 Scratch-wound healing in cell culture of primary keratinocytes:** Primary keratinocytes were isolated from newborn skin of control **(A)** and conditional Met mutant mice **(B-C)**. After scratch-wounding, cells were further cultured in the presence of HGF/SF or TGFα. Photos were taken 0, 24, 48 and 96 hours after scratch-wounding. Wounds in the cultures derived from conditional mutant mice did only close after 96 hours in the presence of HGF/SF. Scale bar, 100µm. **D.** Proliferation of primary keratinocytes from control and conditional Met mutant mice 24 hours after stimulation with HGF/SF, as assessed by phospho-histone 3 antibody staining (red). A dashed line marks the scratch edge. Counterstaining was performed with phalloidin (green). Scale bar, 100µm **E.** Quantification of proliferation of primary keratinocytes at wound edges stimulated with HGF/SF in the experiments described in D. Error bars represent standard deviations.

Next, the remaining non-recombined keratinocytes from the skin of conditional Met mutant mice were tested for their ability to contribute to wound closure in culture. Primary keratinocytes were stained with anti-phospho-Met antibody at different stages of scratch-wound closure. Phosphorylated Met could be detected at the membranes of control cells, and very rarely in keratinocytes isolated from conditional mutant mice 24 hours after cultured with HGF/SF (Fig.22A, B). When keratinocytes isolated from Met mutant skin were cultured for 48 hours and longer in the presence of HGF/SF, phospho-Met positive cells accumulated exclusively at the wound edges, and after 96 hours, the majority of the cells that had closed the scratch-wounds contained phospho-Met (Fig.22B). Thus, as in skin wounds *in vivo*, the scratch-wound area in the mutant culture were finally closed with Met-positive cells. Therefore, these data indicate that only Met-positive, non-recombined cells, participate in wound closure *in vitro* and *in vivo*.

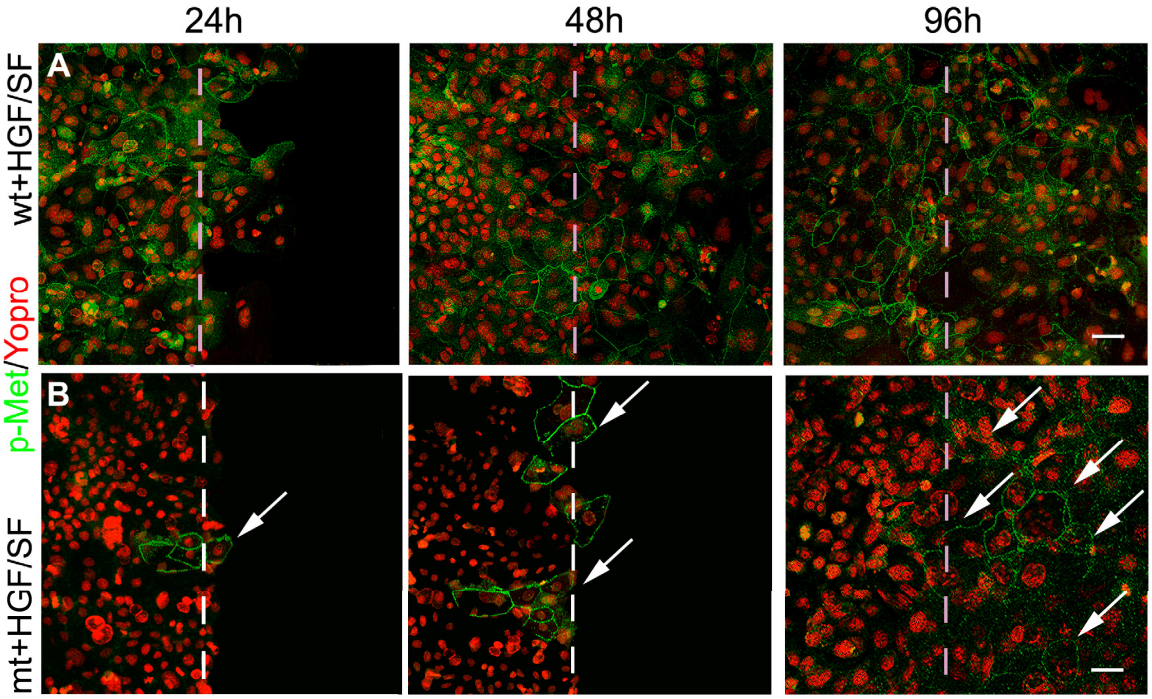
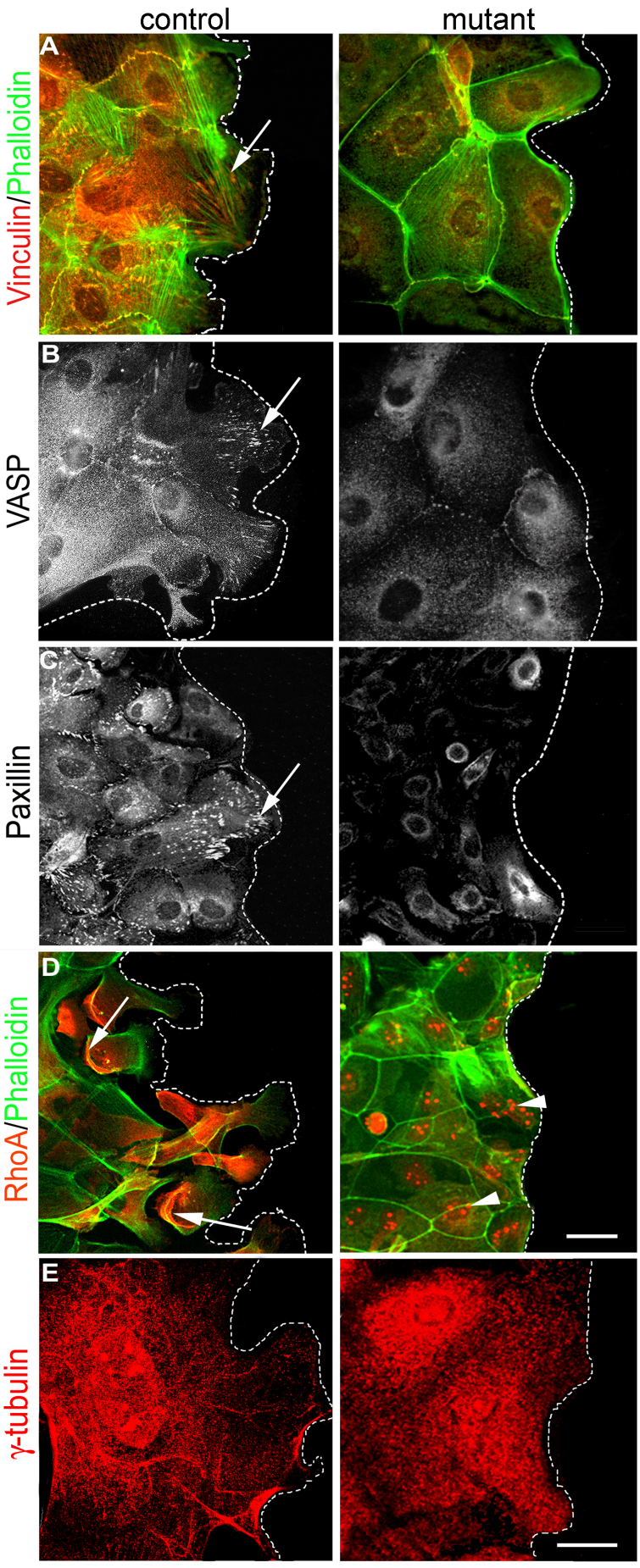



**Figure 22 Only Met positive primary keratinocytes migrate into the scratch-wounds in cell culture. A, B.** Primary keratinocytes isolated from control (A) and conditional Met mutant (B) skin were scratch-wounded and further cultured with HGF/SF. After 24, 48 and 96 hours cells were stained with phospho-Met antibodies (green). Nuclei were visualised by Yopro staining (red). In mutant cell population, phospho-Met containing cells were initially rare, but finally, after 96 hours, occupied the entire scratched area. The original edges of the scratch-wounds are marked with a dashed line. Scale bar, 50µm

**Cytoskeleton rearrangement in cultured scratch-wounded keratinocytes**

The properties of the cells at the wound edges were further examined by immunofluorescence staining for proteins that are important in directed cell migration like vinculin, paxillin, and VASP (Mitchison and Cramer, 1996; Rottner et al., 1999; Rottner et al., 2001; Raghavan et al., 2003). In the presence of HGF/SF, control keratinocytes showed increased numbers of focal adhesions as well as lamellipodia at the wound edges, and these structures pointed directly towards the wounds (Fig.23A, B, C, left pictures). Actin stress fibres, which were stained by phalloidin, were also oriented toward the wounds (Fig.23A, D). Control cells at the wound edges displayed a preferential location of RhoA staining at the rear of cells, and such localization is a characteristic feature of migrating cells (Fig.23D, left; see also Nobes and Hall, 1999; Raftopoulou and Hall, 2004). These control cells at the edges of the wound also reoriented their microtubules, which were demonstrated by γ-tubulin staining. The major arrangements of microtubules were not centrosomal in keratinocytes (Fig.23E). In contrast, Met mutant keratinocytes did not rearrange the proteins, which are known to be important during cell motility (Fig.23A-E, right pictures). In the Met mutant cells, RhoA staining appeared punctuated cytoplasmatically, but was also perinuclear. Keratinocytes from conditional Met mutant mice displayed only few new focal contacts and stress fibers, and these were not oriented towards the wounds.

**Figure 23. Met mutant keratinocytes are unable to rearrange their focal contacts and their cytoskeleton at the scratch-wound edges following HGF/SF treatment**
Keratinocytes derived from control and conditional Met mutant mice were stained 24 hours after scratch-wounding with antibodies directed against vinculin **A**., with phalloidin **A, D**, antibodies directed against VASP **B**, paxillin **C**, RhoA **D** and γ-tubulin **E**. Arrows mark the newly formed focal contacts (A-C) and RhoA at the rear of the cells (D). Arrowheads mark cytoplasmatical and perinuclear localization of RhoA in mutant. The dotted line indicates the edges of the wounds. Scale bar, 50μm (A-D), 20μm (E).


control
mutant
A
Vinculin/Phalloidin
B
VASP
C
Paxillin
D
RhoA/Phalloidin
E
γ-tubulin

**Signal transduction in primary keratinocytes**

The isolated primary keratinocytes from the skin of control and conditional Met mutant mice were used to study signal transduction by molecules that are crucial for cell proliferation and cell migration (Rubin et al., 1991; Morimoto et al., 1991; Hartmann et al., 1994; Ridley, 2001; Khwaja et al., 1998). In the presence of HGF/SF and TGFα, such molecules like Erk1/2, Akt, Gab1 and PAK1/2 were activated in control primary keratinocytes (Fig.24A). In contrast, Erk1/2, Akt, Gab1 and PAK1/2 in mutant keratinocytes following stimulation by HGF/SF, were not activated. However, TGFα in mutant cells did activate these signaling molecules. The phosphorylation of PAK1/2 was quantified in control and mutant cells that were activated with HGF/SF or TGFα, and showed that stimulation with HGF/SF did not change the activation level of mutant keratinocytes, but did upon TGFα. The peak of PAK1/2 activation was observed after 10min stimulation of TGFα in control and mutant, and the same peak was detected after HGF/SF stimulation, but only for the control (Fig.24B).

Taken together, these *in vitro* data demonstrate that HGF/SF and Met signaling is important for the induction of proliferation and migration of primary keratinocytes in cell culture. Activation of this signaling pathway results in major re-organization of adhesion and cytoskeleton complexes like focal adhesions, lamellipodia, and stress fibers, which allows cells to move into the scratch-wounds.

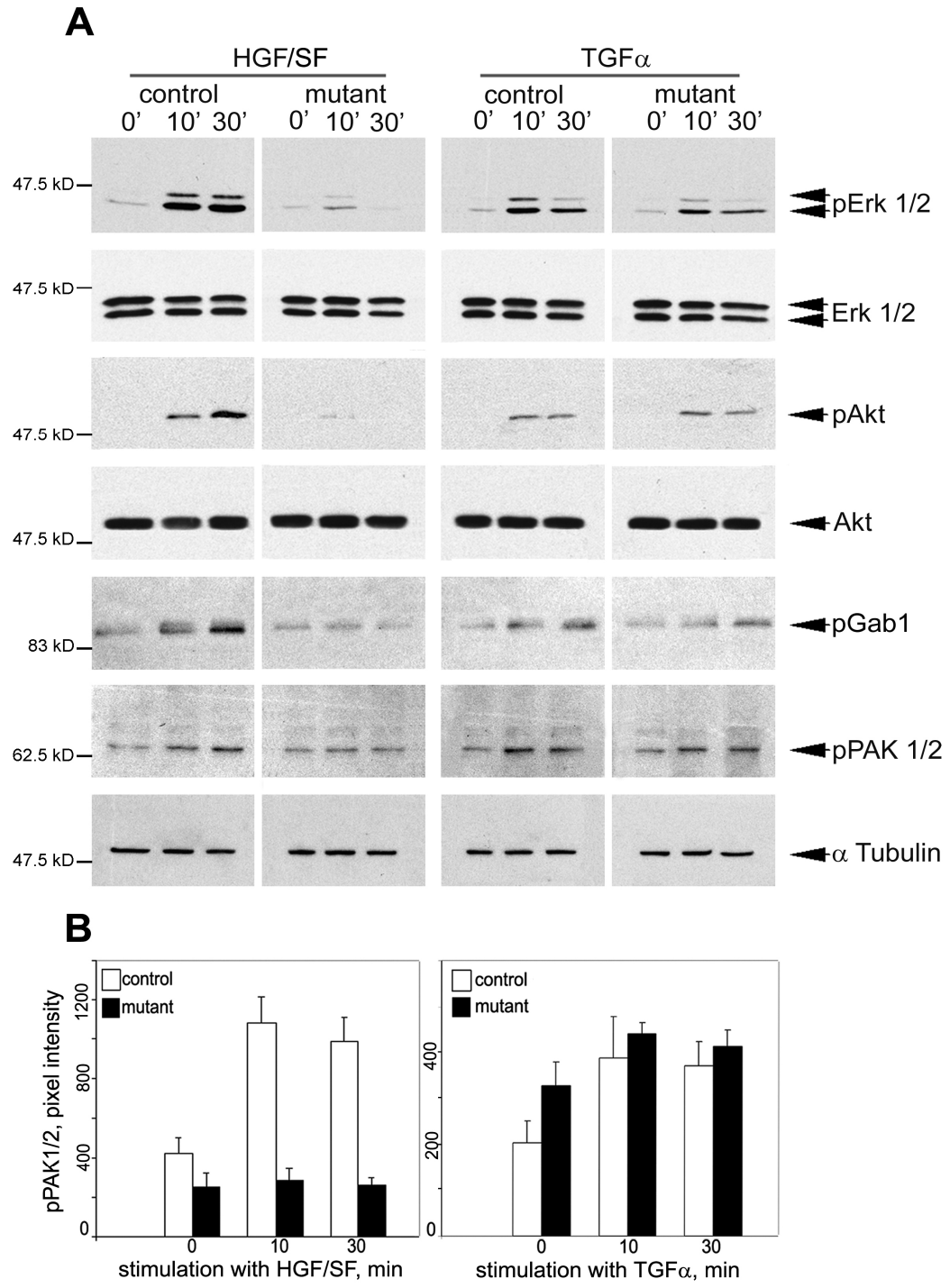



**Figure 24 Signaling is blocked in keratinocytes derived from conditional Met mutant mice that are treated with HGF/SF, but not with TGFα. A.** Western blot analysis of phospho Erk1/2, total Erk1/2, phospho Akt, total Akt, phospho Gab1 and phospho PAK1/2 in keratinocytes derived from control and conditional Met mutant mice. Cells were stimulated with HGF/SF or TGFα for 0, 10 or 30 minutes. Erk1/2, Akt, Gab1 and PAK1/2 are not activated (phosphorylated) in cultured keratinocytes from the conditional mutant mice after HGF/SF stimulation. **B.** Quantification of the phospho-PAK1/2 signal on Western blots (A) as assessed by pixel intensity.

# Discussion

The results generated from this research project provide novel insight into Met function during skin regeneration. Here it is shown that Met and HGF/SF expression is induced in the hyperproliferative epithelium, which is the major structure responsible for wound closure of the skin. The expression of both, Met and HGF/SF in the hyperproliferative epithelium suggests an autocrine system, which could play an important function during the process of skin regeneration. Through the use of genetic analysis, it was indeed shown that Met is important for re-epithelialization of wounds, since Met mutant keratinocytes could not contribute to the generation of the hyperproliferative epithelium. Furthermore, wound epithelium was formed only of cells that expressed a functional Met receptor. Although Met and HGF/SF are expressed in unwounded skin neither alteration in development and maintenance of epidermis, nor in the hair cycling is observed following conditional mutagenesis of the Met receptor. Analysis of cultured keratinocytes during the closure of scratch-wounds, in the presence of HGF/SF, indicates that the primary deficit in the mutant cells is caused by the inability of the cells to proliferate, to re-orient themselves, and to migrate into the wounded area. Thus, it is shown here the importance of Met signaling in the skin regenerative process *in vivo*. Met is thus the first gene, which is absolutely required for re-epithelialization of wounds. This work therefore, contributes significantly to our understanding of wound healing regulation.

### Conditional mutagenesis to investigate Met function in the skin

The function of the Met signaling system in skin wound healing in mice was previously investigated by blocking endogenous HGF/SF with a neutralizing antibody (Yoshida et al., 2003). However, such results have to be carefully interpreted, since cross-reactivity of the antibodies with related proteins is possible. Furthermore, it is unclear, whether neutralizing antibodies gain sufficient access to the tissue. Since this

model has clear limitations, genetic models are preferred to accurately study skin development and repair. Therefore, the aim of this project was to analyze the Met function in the skin using conditional knockout mice.

A conditional knockout approach was employed utilizing mice with a null mutation of the tyrosine kinase receptor Met in the skin. Keratin 14-cre was used allowing for the examination of Met function specifically in the epidermis. It should be pointed out that conditional mutagenesis is essential since the inactivation of Met by conventional knockout leads to an early lethal phenotype. In addition, previous knockout studies have shown that complete deletions of the Met receptor and/or its ligand, HGF/SF, in mice resulted in an absence of the muscle groups in the limbs, reduced liver size, a defective development of the placenta, and hence early embryonic lethality. Conditional mutagenesis permits the examination of genetic analysis in adult stages, circumventing early lethal phenotypes and moreover, it allows for the detailed study of gene function in particular cell lineages.

The ability to targeting specific genes in mice is based on the combination of pluripotent embryonic stem (ES) cells and the introduction of mutations by homologous recombination (Koller et al., 1990). Moreover, site-specific recombination can be induced by the Cre-loxP technology. The Cre recombinase of the P1 bacteriphage recognizes specific short consensus DNA sites and catalyses recombination between them (Gu et al., 1993; Dymecki, 1996). The efficiency of recombination depends on at least two parameters: the first is the distance between the two loxP sites along a chromosome; the further the two loxP sites are apart, the less often they are likely to collide, leading to lower rates of recombination. The second parameter is position variability and the local chromatin structure; as a result, recombination can be locus dependent. A comparison of recombination frequencies of different alleles *in vivo* showed marked differences. In this study, recombination of the $Met^{flox}$ allele in the epidermis, using Keratin14 cre, was 95%. Only 5% of keratinocytes did not undergo recombination and retained the functional Met receptor. Usually, such a recombination is acceptable and sufficient to analyse loss of function of a particular gene. The distance between loxP sites in the $Met^{flox}$ construct is 1.2 kb, which is within the optimal range for Cre recombinase. Another technical problem using conditional mutagenesis is Cre expression, which can provoke mutagenesis through strand breakage or recombination

at cryptic lox sites in the genome (Schmidt et al., 2000). It is essential to be aware of the potential for undesired Cre-mediated mutagenesis to influence the experimental outcome. For experiments using conditional mutagenesis, a comparison of the Cre transgene in the heterozygous allele background with the Cre transgene in the homozygous allele background is an essential control.

**The role of the tyrosine kinase receptor Met in the skin**

Many signaling pathways that involve tyrosine kinase receptors and ligands like FGF, EGF and TGFα are important for development and maintenance of the skin. In previous studies, mice deficient in the FGF receptor 2-IIIb, which is expressed in the epithelia of ectodermal and endodermal organs, show an extremely thin suprabasal layer, however with epidermal differentiation and establishment of unaffected barrier function. Furthermore, mice deficient for FGF10 (the main ligand of the FGF receptor 2-IIIb) display a similar, but less severe epidermal phenotype (Petiot et al., 2003). These data suggest that stem cell division in the basal layer is FGF/FGFR2-IIIb independent; however, receptor-ligand interaction is required for epidermal stratification. The EGF receptor on the other hand, is important for the regulation of the development of the epidermis and its appendages (Luetteke et al., 1994). In mammals and birds, overexpression or systemic administration of the ligand, EGF, can arrest epidermal appendage development and promote epidermal thickening (Moore et al., 1985; Kashiwagi et al., 1997). Furthermore, the EGF receptor can contribute to epithelial carcinogenesis, with elevated EGF receptor or its ligands, of which expression is reported in many types of epithelial cancers. TGFα involvement in the skin, shown in transgenic mice, reveal that overexpression leads to the development of skin tumors after treatment with the carcinogens 7,12-dimethylbenz(a)anthracene (DMBA) and/or 12-O-tetradecanoylphorbol-13 acetate (TPA) (Kiguchi et al., 1998). To date, the existence of an *in vivo* model to study the role of the Met receptor in the skin has not been established. However, in the present study Met and HGF/SF have been shown to be expressed in the hair follicles, and the function of the receptor in hair cycling has been analysed *in vivo*.

Hair follicles and other epithelial appendages develop as a result of interactions involving the embryonic ectoderm and specialized fibroblasts in the dermal papilla, which is located in the proximal mesenchyme underneath. It was shown that Met is expressed in the epithelium of hair follicles, whereas HGF/SF is produced in the dermal papilla (Lindner et al., 2000). Epithelial-mesenchymal interactions have been speculated to play an important role in hair growth. To date, the key signaling pathways involved in these epithelial-mesenchymal interactions are the Wnt/β-catenin, Sonic hedgehog, Notch, TGF-β superfamily and the FGF and EGF pathways. The secreted signaling molecules Sonic hedgehog (Shh) and bone morphogenetic proteins (Bmps) are of central importance in the regulation between proliferation and differentiation in postnatal hair follicles. Shh promotes proliferation, and Bmps promote differentiation. In the adult epidermis, Shh expression is restricted to cells at the distal portion of the growing follicle. Inhibition of Bmp signaling by the Bmp antagonist Noggin, is required for new hair growth in postnatal skin, and the growth-inducing effect of Noggin is mediated, at least in part, by Shh. Other factors that are believed to regulate the balance between epidermal proliferation and differentiation include the transcription factors Forkhead-box n1 (Foxn1) and nuclear factor κB (NF-κB) (Niemann and Watt, 2002). EGFR null mice do not survive longer than P20, and the skin and hair phenotypes could not be properly analysed in these mutant mice. Therefore, mice in which the endogenous EGFR is replaced by a human EGFR cDNA ($hEGFR^{KI/KI}$) were useful to analyse the role of the EGFR in hair follicle differentiation and cycling, because the early lethality has been overcome. After the first hair cycle, hair follicles of $hEGFR^{KI/KI}$ mice fail to enter into catagen and remain in aberrant anagen, indicating that EGFR signaling is necessary to regulate hair cycle progression (Sibilia et al., 2003). Overexpression of a dominant negative mutant of the EGFR in the skin induces striking alterations in hair cycling. These changes progressively lead to hair degeneration (Murillas et al., 1995).

The overexpression of HGF/SF in the skin is a topical debate since previous groups were able to demonstrate the importance of this growth factor in the hair cycle (Lindner et al., 2000). However, data generated from the present study cannot support such a function. Ablation of Met in the skin does not affect skin development and maintenance under normal conditions. Hair follicle cycling is unchanged in conditional

Met mutants, which has been believed to be dependent on HGF/SF and Met (Lindner et al., 2000). Histological analyses of the skin sections of P1, P5, P8, P18, P20, P30, which represent all phases of first and second hair cycle, as well as 3-months old animals did not reveal any alteration. In addition, in conditional Met mutant mice, which were kept for nearly 2 years, no unusual hair loss or no other changes in the appearance of the skin were observed.

In contrast to normal hair cycling and skin development, wound healing was severely perturbed in conditional Met mutant mice. The events involved during the closure of a wound represent a classic example of a physiological process that has characteristics of both development and organ regeneration. For instance, coordinated proliferation coupled with migration and induction of cell polarity can be executed by epithelial cells during wound healing, when cells at the wound edge start dividing and moving over the provisional matrix to reconstitute tissue integrity. This invasive growth is observed during development, but in fact, is also a requirement for organ regeneration and in carcinoma progression. Therefore, an interesting aspect to investigate would be the function of Met during skin cancer, especially in metastasis stages, where migration plays important functions.

**Only non-recombined cells contribute to wound haeling**

In general, HGF/SF and Met are expressed in different cell types, although they may be closely apposed to allow an exchange of signals. For instance, HGF/SF is expressed primarily in mesenchymal cells, while Met is generally expressed in nearby epithelia (Sonnenberg et al., 1993; Yang and Park, 1995; Birchmeier and Gherardi, 1998). Moreover, Met is expressed in the epithelial dermomyotome and in migrating muscle progenitor cells that derive thereof, whereas HGF/SF is expressed in mesenchymal cells close to the somites and along the route of the migrating cells (Bladt et al., 1995; Birchmeier and Gherardi, 1998). In the cerebellum, granule cells express HGF/SF, while surrounding Bergmann glia cells express the Met receptor (Jung et al., 1994). In tumours, however, autocrine HGF/SF and Met signaling is frequently observed, e.g. in epithelial cells in human prostate cancer (Kurimoto et al., 1998;

Birchmeier et al., 2003). Furthermore, the data presented in this study point to an autocrine signaling function of HGF/SF/Met in normal physiological process such as the healing of skin.

Several mouse models have recently been developed in which the function of particular molecules implicated in wound healing have been altered to study their genetic involvement (Ashcroft et al., 1999; Munz et al., 1999; Krampert et al., 2004; Reynolds et al., 2005; Munz et al., 1999; Ashcroft et al., 1999). Results from these studies demonstrate that the majority of factors involve in wound healing act in a paracrine manner and mediate cross-talk between mesenchymal and epithelial cells. All mutant skin cells described in these models were able to execute wound closure, although the wound healing process was either accelerated or delayed. For example, wound closure is delayed in mice that carry a targeted mutation in the gene encoding the fibroblast growth factor 2 (FGF2), which is produced by macrophages and endothelial cells and has major effects on fibroblast proliferation and angiogenesis in the skin (Ortega et al., 1998). In contrast, wound healing is accelerated in mice that are mutant for the gene encoding transforming growth factor β1 (TGFβ1), which is released from platelets and serves as a chemoattractant for macrophages and fibroblasts (Sellheyer et al., 1993; Koch et al., 2000; Amendt et al., 2002; Reynolds et al., 2005). TGFβ has been shown to induce antiproliferative actions in processes such as liver regeneration. Smad3-null mice (Smad3, a downstream component of TGFβ signaling) displayed an increased rate of re-epithelialization and reduced monocyte infiltration during wound healing (Ashcroft et al., 1999). On the other hand, conditional mutation of c-Jun and STAT3 in the epidermis, which participate in the signaling of several growth factors and interleukins, of integrins or leptin, exhibited delayed wound closure (Li et al., 2003; Sano et al., 1999). Lastly, ablation of the placental growth factor (PlGF) gene retarded wound angiogenesis, and delayed wound healing (Carmeliet et al., 2001). In contrast, mice that lack the Met receptor in the epidermis are capable of re-epithelialization under wounded conditions; this however was demonstrated to be related to the overproliferation and migration of a small proportion of residual Met positive cells. Interestingly, no other paracrine or autocrine systems can compensate for a loss of Met function in skin regeneration. Met is thus the first gene identified, which is

essential for the formation of the hyperproliferative epithelium and the closure of skin wounds.

**The role of HGF/SF and Met in development and regeneration**

In development, HGF/SF and Met control placentation, liver growth and muscle precursor cell migration, which are processes that appear late in evolution (Birchmeier et al., 2003). Genetic analyses demonstrate that Met is also important in regeneration of adult tissues, which is shown here for skin repair after wounding, and which was previously demonstrated for liver regeneration after damage (Borowiak et al., 2004; Huh et al., 2004). The conditional mutation of Met in the liver was established using the inducible Mx-cre transgene, and from these mice a portion of the liver was removed by partial hepatectomy. Liver regeneration in these mice was severely impaired, with the liver-to-body-weight ratio particularly affected. The observed defect included downregulation of hepatocyte proliferation and altered cell cycle progression (Borowiak et al., 2004). Impaired regeneration is also characteristic of Met mutant mice in the skin. These data suggest that Met signaling could be part of a general physiological response to tissue injury.

In the present study, only keratiniocytes that express functional, non-recombined Met are capable of participating in the hypreproliferative epithelium. It is still puzzling as to how the non-recombined keratinocytes in conditional Met mutant skin are able to contribute to wound closure. *In vivo*, this process was delayed, but occurred and required only twice as much time, despite the fact that the vast majority (95% mutant cells) did not participate in the process. Moreover, the overall kinetics of wound closure was similar in both, control and mutant wounds, but the rate of closure was decrease in the mutants, which may indicate that the identity of cells contributing to the hyperproliferative epithelium was the same. Re-epithelialization was delayed in the skin of the conditional mutant mice (3 days after injury), but recovered fast, since the proliferation of the keratinocytes in the hyperproliferative epithelium was increased during early wound healing. The proliferation index, for instance at day 3, was quite

impressive. It shows 20% increase when compared to the controls. Although it should be noted that at day 3 the relative number of proliferating Met-positive cells only reached 50%. Thus, nearly 100% of the keratinocytes that escaped recombination in the mutant population proliferate at day 3, while only 35% proliferate in the case of control. Moreover, in the control skin, proliferation of keratinocytes comes to a halt 5 days after the injury, but was still increasing at that time in the conditional Met mutant mice. By this, a selection of Met positive over Met negative cells occurs within a few days *in vivo*, which results in an overproliferative compensation mechanism to complete wound healing. If only 5% cells escaped recombination, 4.125 cell cycles of these would be sufficient to generate the same amount of cells that can contribute to the wound epithelium (100%) in control mice. Compensation by overproliferation in mosaic animals has been previously observed and can provide an astoundingly efficient compensatory mechanism (Riethmacher et al., 1997). Aside from these findings, another interesting aspect is the issue of the critical mass, which relates to the amount of cells necessary and sufficient to heal the wound. It is known that for some organs that this number can be relatively small, for example 500 of MTS24+ epithelial cells are enough to restore a whole thymus in nude mice (Gill et al., 2002). In other regenerative organs, e.g. the liver, the opposing situation occurs, in that the regeneration process can be triggered by only the certain number of cells, which are beyond the critical number (approximately 30% of total number, see (Michalopoulos and DeFrances, 1997). As a consequence, the recombination over time in the skin of conditional Met mutant mice was examined and revealed that the percentage of recombined cells remained constant in normal, unwounded skin.

Regeneration of the epidermis after wounding involves activation, migration and proliferation of keratinocytes from the surrounding epidermis, but also keratinocytes derived from hair follicles and sweat glands may participate in the healing process. Epidermal stem cells may also contribute to the wound epithelium since they constitute an unlimited source of cells that contribute to tissue morphogenesis, homeostasis, and also injury repair (Cowan et al., 2004). It is generally thought that each of the epithelial compartments (the interfollicular epidermis, the hair follicle, and the sebaceous gland) has its own specialized stem cells capable of sustaining tissue growth independently. Furthermore, it has been demonstrated that at times such as rapid growth or injury; hair

follicle stem cells can leave their niche and contribute to the hyperproliferative epithelium. Although it has been demonstrated that hair follicle stem cells are capable of such contributions, it is less apparent that stem cells from the sebaceous gland and the interfollicular epidermis exhibit similar abilities. Additionally, studies suggest that hair follicle stem cells contribute only transiently to the interfollicular epidermis compartment, and thus are not capable of replenishing this stem cell compartment (Miller et al., 1998; Ito et al., 2005; Levy et al., 2005). The bulge cells of the skin may therefore provide another potential source of cells that reconstitute the injured epidermis. The K14-cre-mediated recombination also occurs in the hair bulge leading to cre-mediated deletion in the bulge as in the epidermis. Of note is that Met expression is not excluded from the hair follicle stem cells. Moreover, dividing cells did not form any clusters that could correspond to cells that had escaped recombination close to hair follicle remnants. This indicates that repopulating cells that escaped recombination do not derive from a particular site. It was observed that keratin 6-positive cells form continuous layers in the wound epithelium and are not only preferentially located close to the hair follicle. Repopulating cells that escaped recombination could thus originate from both, cells of the epidermis and of the hair bulge. The rapid, but transient contributing nature of the bulge cells to repopulate wounded skin is also reminiscent of the behaviour of embryonic stem cells injected into myocardium. Early after injection, embryonic cells are plentiful, but they quickly disappear (Fraidenraich et al., 2004). However, the signals leading to the recruitment of bulge cells to the epidermis after wounding are not known. Identification of these signals could eventually lead to treatment for wound and other skin disorders, such as epidermal atrophy seen in aging, by identifying therapeutic targets for enhancing the movements of bulge cells into the epidermis. It is possible that the underlying dermis also contributes to this compensatory process, with increases of other growth factors or cytokines.

A further mechanism that might interfere with regeneration in Met mutant mice is an increase in apoptosis. Met, like other receptor tyrosine kinases, provides anti-apoptotic signals by activating the Akt kinase. Furthermore, previous studies reported that Met can directly interacts with the Fas receptor and can therefore prevent Fas-induced apoptosis (Wang et al., 2002). However, apoptosis rates in the regenerating skin of control and conditional Met mutant mice were comparable, indicating that

the lack of the anti-apoptotic function of Met is not a dominant mechanism that accounts for the impaired regeneration.

**Only Met-positive keratinocytes contribute to healing of scratch-wounds in vitro**

The effect of Met signaling on migration of primary keratinocytes was analysed utilizing an *in vitro* based system. Primary keratinocytes were isolated from the skin of control and conditional Met mutant mice and were examined for their ability to close scratch-wounds. Indeed, only cultured Met positive cells initially migrated towards the scratch-wounds in the presence of HGF/SF. In addition, at later stages Met positive cells exclusively could be detected in the scratched area. It was also observed that control keratinocytes at the edges of the scratch-wounds re-oriented themselves, i.e. focal adhesions and stress fibres pointed rapidly towards the wound edges, and RhoA accumulated at the retracting ends of the cells, which has previously been reported for other cell types (Ridley et al., 1995; Nobes and Hall, 1999). In contrast, re-orientation of the cells did not occur in scratch-wounds of Met mutant keratinocytes that were exposed to HGF/SF. The major arrangement of microtubules was not centrosomal in keratinocytes, which is in agreement with a previous study (Yvon et al., 2002). Primary keratinocytes after HGF/SF stimulation extend numerous filopodia, which were packed with actin cytoskeleton. Keratinocytes can actively move forward and slide along each other into the scratch-wound. It is already established that exogenous HGF/SF is capable of accelerating wound closure in Madin-Darby canine kidney (MDCK) epithelial cell monolayers (Sponsel et al., 1994). Other ligands for receptor tyrosine kinases such as insulin and IGF however, have been reported to promote single cell keratinocyte migration while these ligands were unable to promote colony scattering (Ando and Jensen, 1993).

The mutation of Met did not disturb the re-orientation of cells in response to other growth factors, and re-orientation occurred in the presence of TGFα, which signals via the EGF receptor. The genetic data indicate that the signals provided by HGF/SF are the only ones capable of re-orienting kerationocytes at the wound edges

and contribute to the hyperproliferative epithelium *in vivo*. This might be reflected by a limited availability of other growth factors *in vivo* that can elicit similar responses as HGF/SF. Met signaling in keratinocytes activates Erk1/2, Akt, Gab1, and PAK1/2 for the motility response. The phosphorylation of PAK1/2 might be of particular importance, since PAK1/2 is a target of Rho signaling that causes remodeling of actin cytoskeleton and focal adhesion sites (Frost et al., 1998; Royal et al., 2000).

**The Met receptor as a therapeutically target**

In the last decade, several growth factors have been implicated in wound healing, for instance FGFs, factors that signal via the EGF receptor, and members of the TGFβ superfamily (Werner and Grose, 2003). Ablation of these factors or their receptors in mice affected the kinetics of wound healing, but mutant cells contributed to the newly formed epithelium. As yet, Met is the only example of a receptor, in which the loss of its expression in skin cells make them unable to contribute to wound epithelium and is an essential factor required for efficient wound healing. The application of HGF/SF and/or HGF/SF variants in the therapy of wounds therefore is an attractive possibility (Bevan et al., 2004). Treatments of wounds with exogenous growth factors accelerate healing in normal animals. Topical administration of HGF/SF to wounds of diabetic mice enhanced neovascularization and formation of new tissue. It is also worth notice that HGF/SF was proven to prevent fibrotic disorders or facilitate resolution in liver cirrhosis, rental fibrosis or lung fibrosis (Matsuda et al., 1997; Mizuno et al., 1998; Ueki et al., 1999). The improvement of tissue repair processes after acute injury or chronic inflammatory disease, the reconstruction of damaged organs as well as the treatment of devastating diseases associated with tissue remodeling are major challenges in medical science. Until recently, progress in this area has been hampered by the fact that these repair and disease processes are based on complex interactions between different cell types and between cells and the extracellular matrix, which are still poorly understood. In the future, growth factors may be administrated sequentially, in combination, or at timed intervals to more closely mimic the normal healing process. The knowledge from this study will offer more information for clinical

intervention and the design of new therapeutic targets for wound treatments, from molecular diagnostic of Met to therapeutics tailored to the genetic background of each patient.

## Materials and Methods

Chemicals, enzymes and kits for molecular biology, oligonucleotides, or antibodies were purchased from the following companies, unless indicated otherwise: Amersham-Pharmacia (Freiburg); Biotez (Berlin); Biozym (Hess. Oldendorf); Cell Signaling, Dianova (Hamburg); Gibco/BRL (Karlsruhe); Heraeus-Kulzer (Wehrheim); Invitek (Berlin); MBI Fermentas (St. Leon-Rot); Merck (Darmstadt); MWG-Biotech (Ebersberg); New England Biolabs (Frankfurt); Oncogene Pan-Biotech (Aidenbach); Promega (Mannheim); Qiagen (Hilden); Roche (Mannheim); Roth (Karlsruhe); Santa Cruz, Serva (Heidelberg); Shandon (Frankfurt); Sigma (Deisenhofen).

Apart from the techniques detailed in the following part of this section, standard procedures for molecular cloning, sequencing and targeting vector construction were carried out according to "Molecular Cloning" by Sambrook and Russel or manufacturers instructions.

### Extraction and Purification of DNA

*Extraction of Plasmid DNA*

*E.coli* cells containing plasmid DNA were grown in autoclave sterilized LB-medium (10g bacto-tryptone, 5g yeast extract, 10g NaCl in 1l $H_20$) with an appropriate antibiotic, ampicillin (100µg/ml) or kanamycin (30µg/ml), overnight at 37°C. Small-scale preparations (mini-preps) were performed by the alkaline lysis method (Birnboim and Doly, 1979). Medium (25ml) culture and large (100ml) culture scale preparations of plasmid DNA were carried out by means of the respective Plasmid Midi- and Maxi-Kit from Qiagen (Hilden), according to the manufacturers' protocol.

*Isolation of genomic DNA from mouse tissue*

To genotype mice by PCR, DNA was isolated from ear holes. The tissue was lysed at $55^0$C in 50µl of lysis buffer containing proteinase K (1mg/ml). To inactivate the proteinase K, the digests were incubated at $95^0$C for 5min. Lysates were diluted with water and used for PCR.

For Southern blot analysis, lysates from epidermis, tail, or other organs were extracted with phenol/chloroform. The DNA was precipitated with 2 volumes of ice-cold 100% ethanol and dissolved in $H_2O$ at a final concentration of 1mg/ml. The concentration and purity of the DNA were determined by UV-spectrophotometer.

**Polymerase chain reaction (PCR)**

The polymerase chain reaction (Saiki et al., 1985) was used to genotype littermates and each specific PCR was established according to general rules. A list of primers used for genotyping is presented below.

| | |
|---|---|
| $Met^{flox}$ | mfs1: 5´-AGCCTAGTGGAATTCTCTGTAAG -3´ |
| | mfas2: 5´-CCAAGTGTCTGACGGCTGTG -3´ |
| $Met^{null}$ | Wmet5: 5'-CACTGAGCCCAGAAGAGCTAGTGG-3' |
| | neo1L: 5'-CCTGCGTGCAATCCATCTTGTTCAATG-3' |
| Cre | crenew1: 5'-GAACGCACTGATTTCGACCA-3' |
| | crenew2: 5'-AACCAGCGTTTTCGTTCTGC-3' |
| Deleter | Deleter1: 5'-CGCCATCCACGCTGTTTTGACC-3' |
| | Deleter2: 5'-CAGCCCGGACCGACGATGAAG-3' |
| K14-cre | K14cres: 5'-CTTGCGAACCTCACTACTCG-3' |
| | K14creas: 5'-AGGGATCTGATCGGGAGTTG-3' |

**Southern blotting**

Between 5 and 10µg genomic DNA were digested overnight with 20 U of restriction enzyme. The digested DNA was resolved on a 0.8% agarose gel containing ethidium bromide. To confirm complete digestion of the genomic DNA, gel was exposed to UV-light and photographed. The gel was depurinated in 0.25 M HCl solution for 10 to 15min. Then the gel was rinsed in distilled water and denatured by two 30min incubations with gentle shaking in a solution of 1.5 M NaCl and 0.5 M NaOH. Finally the gel was then rinsed in 10X SSC and blotted overnight using 20X SSC, so as to transfer the DNA onto a nylon membrane (Hybond N+, Amersham-Pharmacia). After transfer, the membrane was cross-linked using UV-light at 120mJ/cm2. Subsequently, the membrane was hybridized with specific radioactive probes. DNA probes (20-50ng) were radioactively labeled with 50µCi $\gamma^{32}$P-dCTP (Amersham-Pharmacia) using the 'Prime-It RmT Random-Primed Labeling Kit' (Stratagene). The labeled probes were purified over Sephadex-G50 spin columns (Probe Quant G50, Amersham-Pharmacia). Before hybridization, probes were denatured by boiling for 5 min.

The membranes were saturated in 20-25ml hybridization solution (6x SSC, 5x Denhardt's solution, 0.5% SDS, 100µg/ml denatured salmon sperm DNA) at $65^0$C for at least 2 hours in the hybridization oven (Biometra). The denatured probes were then added to the tubes incubating the membranes in prehybridization buffer. Hybridization was carried out at $65^0$C for 16-24 hours. In order to remove the non-specifically bound probe, the following washing steps were carried out in a shaking water bath at 65°C: 2x 15min in 2x SSC, 0.1% SDS, 1x 30min in 0.1x SSC, 0.1% SDS. The membranes were then sealed in plastic bags and exposed to a Biomax MS autoradiographic films (Kodak) at –80°C for overnight or exposed to a Phosphoimager (Fujix, BAS 2000) for several hours. If a membrane should be reused for hybridization with a different probe, the old probe was stripped by boiling the membrane in 1% SDS for 30min.

## Cell culture

*Culture of murine primary keratinocytes*

Newborn mice (0-3 days old), under aseptic conditions, were decapitated, limbs and tail were amputated and then the body washed with 1% iodine solution and 70% ethanol. The skin was peeled off, washed in PBS antibiotic solution (gentamycin, GIBCO), carefully laid flat in a sterile cell culture plate, dermis side down, in dispase medium (defined keratinocyte SFM, GIBCO, penicillin/streptomycin, Sigma, dispase II, Roche) and incubated overnight at 4°C with shaking to separate epidermis from dermis. Afterwards the epidermis was separated from the dermis using forceps, incubated with trypsin for 10min at 37°C with vigorously shaking until the solution became opaque. After centrifugation, pellet was washed two times with medium containing FCS and cells were plated on collagen IV coated dishes. Medium containing growth factors was changed every day.

## Wounding of skin

Eight weeks old mice of the same sex were anaesthetized by intraperitoneal injections of ketamine/xylazine (90mg/kg of ketamine and 10mg/kg of xylazine). They were shaved on the back. Two full-thickness excisional wounds, 5 mm in diameter, were made on either side of the dorsal midline by excising skin and panniculus carnosus as described previously (Werner et al., 1994). The wounds were left undressed after injury. For histological analysis, the complete wounds, including 2 mm of the epithelial margins, were excised and either directly embedded in “Tissue-Tec” without prior fixation or fixed overnight in 4% formaldehyde and embedded in paraffin.

## Immunohistochemical techniques

*Preparation of paraffin sections*

Animals were killed by cervical dislocation; back skin was shaved, samples dissected and fixed with 4% formaldehyde at 4°C overnight. After washing with cold PBS, the skin was dehydrated in an ethanol series: 50%, 70%, 80%, 96%, and 100%. After dehydration, it is necessary to replace the ethanol with an agent miscible with

paraffin; therefore, skin was incubated in toluol two times for half an hour. Then the skin was incubated in paraffin (Roti-Plast, Roth, Karlsruhe) overnight at $56^0$C, then embedded into cassettes at room temperature, and the resulting solid molds were used for sectioning. The 10µM sections were spread out onto glass slides (Menzel, Braunschweig). The paraffin sections were stained with hematoxylin and eosin (H&E), as well as used for antibody and TUNEL staining.

*Preparation of methacrylate sections*

For histological analysis skin was also embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim), which is a cold-polymerizing resin. Therefore, skin was fixed and dehydrated in series of graded alcohols. Then skin was incubated in Technovit 7100/100% Ethanol (1:1) for 4-6h at RT followed by overnight pre-infiltration in Technovit 7100. Afterwards, the tissue was incubated in infiltration solution (1g of hardener I/100ml Technovit 7100) for 2h up to 2 days at 4°C. Skin was embedded in infiltration solution/hardener II (15:1), which was degassed shortly in vacuum chamber. After overnight polymerization, the blocks were mounted with Technovit 3040 and stored at RT until sectioning. 4-5µm semi-thin sections were cut using Microm HM360 (Walldorf), dropped into a warm water-bath for spreading and collected onto slides (Roth, Karlsruhe).

*Preparation of frozen sections*

Laser capture microdissections were performed from frozen sections. The wound fields were excited, snap-frozen in liquid nitrogen and embedded in "TissueTek" („OCT-Compound"; Sakura, Zoeterwoude, Nederland). The 8µm sections were cut on a cryostat (Microm HM560, Walldorf) and collected onto membrane slides for laser capture microdissections (Molecular Machines & Industries). The sections were fixed in 70% EtOH for 10s and stained with hematoxylin and eosin by immersion using the following protocol: 10s deionized $H_2O$, 30s hematoxylin, 10s deionized $H_2O$, 10s 70% EtOH, 1min Eosin Y (alcoholic), 10s 95% EtOH, 10s and 100% EtOH. LCM was performed using an Arcturus PixCell II apparatus, with a 15mm laser beam, power settings of 50–90mW, and laser pulse duration of 6–7mS. This system is based on laser microdissection pressure catapulting technology. A high-pressure laser beam ejects the

selected sample and catapults it into an Eppendorf cap used with an inverted microscope. A slide with tissue was placed under the microscope, and the wound epithelium was selected using the computer program. The laser cut out the cells and the captured cells were collected into an Eppendorf tube.

*Hematoxylin/eosin (H&E) staining on paraffin sections*

Hematoxylin solution was prepared by dissolving 4g of hematoxylin in 25ml 95% ethyl alcohol and 40g/400ml $NH_4Al(SO_4)_2 \cdot 12\ H_2O$. After one-week exposition to air and light, the solution was filtered and mixed with 100ml of glycerin and 100ml of methyl alcohol. Then the solution was exposed to light until it becomes dark (6-8 weeks). Directly before use, the hematoxylin solution was diluted with an equal volume of distilled water.

For H&E staining slides were dewaxed by three incubations in xylene for 10min. Afterwards, slides were hydrated by 5min washes in a series of ethanol (100%, 95%, 80%, 70%) and washed for 2min in distilled water. Such prepared paraffin section as well as methacrylate sections were stained with hematoxylin for 2min and washed in tap water for 10min to allow differentiation. Afterwards, the slides were stained with eosin (0.25% eosin Y, 0.1 M acetic acid) for 5min. After washing, sections were rapidly dehydrated in ethanol series. Finally the sections were washed three times for 5min in xylene, mounted in “Entellan” (Merck, Darmstadt) and coversliped.

*Immunostaining*

The skin sections were dewaxed and rehydrateted, antigen retrieval was performed by boiling samples in sodium-citrate buffer ($C_6H_7O_7Na$, 10μM, pH 6.0). Keratinocytes plated on collagen IV coated coverslips were fixed in 4% formaldehyde in PBS for 10min and then subjected to immunostaining. Unspecific binding of antibodies was blocked by incubation with 10% inactivated horse serum/PBT (HS/PBT) for 1-2h at RT. Afterwards, slides were incubated with the primary antibody diluted in 10% HS/PBT O/N at 4°C with rocking or alternatively, the incubation was performed at room temperature for 1h. The following antibodies were used: anti-vinculin (Sigma), anti-RhoA (Santa Cruz Biotechnology), anti-paxillin (BD Transduction Laboratories), anti-phospho-Met and anti-VASP (Cell Signaling Technology), anti-keratin 6 (Covance,

Berkeley, CA, USA), anti-keratin10 (Sigma), anti-phospho-histone H3 (Upstate Biotechnology), and anti-PCNA (Oncogene Science). The sections were washed 4 times with PBT for 10min to remove unbound antibodies, and then the sections were incubated with Cy2 or Cy3-conjugated secondary antibodies (diluted in 10% HS/PBT) for 1h at room temperature. Sections were washed extensively. Nuclei were visualized by the DNA specific dye-DAPI or Yopro (Molecular Probes) added to the secondary antibodies solution. Finally, slides were covered with "Immunomount" (Shandon, Frankfurt).

*Detection of cell proliferation and apoptosis*

To detect keratinocytes proliferation, animals were injected intraperitoneally with 75µg of BrdU (5-Bromo-2'-deoxy-uridine) per gram of body weight. BrdU is a thymidine analog and is incorporated into DNA only in mitotically active cells and can be detected using anti-BrdU antibodies. After 1 hour of chasing time, skin samples were embedded in "TissueTek", as described for preparation of frozen sections. Sections were postfixed in 4% PFA for 15min at RT and then washed with PBS three times for 10min. DNA was denaturated by incubation in 2.4 M HCl for 30min at $37^0$C. Afterwards sections were washed as above and incubated with 20µg/ml proteinase K (Roche, Mannheim) in PBS at RT for 10min to ensure good penetration of the antibody. Afterwards, sections were blocked and immunohistochemistry was performed as described above.

Extensive DNA degradation occurs very often during early stages of apoptosis. Therefore, apoptosis was detected by terminal deoxynucleotidyle transferase nick-end labeling (TUNEL ;(Gavrieli et al., 1992). During the TUNEL assay, blunt ends of double stranded DNA breaks are enzymatically labeled with flourescin. The 3-end labeling of DNA breaks was performed using an '*In situ* Cell Death Detection Kit, Flourescein' (Roche, Mannheim) with minor modifications. Before the procedure, the specimens were heated at 60°C for 1 hour. After deparaffinization in xylene and rehydration through graded ethanol series, the sections were incubated with 20µg/ml proteinase K (Roche, Mannheim) in PBS at RT for 20min. Then the slides were processed according to manufacturer's instructions.

*In situ hybridization*

In situ hybridization of paraffin sections was performed using digoxygenin-labeled (DIG) probes (Roche) according to manufacturer's instructions. The anti-sense transcripts of mouse cDNAs were as follows: a 1.4 kb HGF/SF fragment that encompasses the 3'coding sequence, a 0.7 kb HGF/SF fragment that encompasses the 5'coding sequence, a 3.7 kb Met fragment, mouse Limd1: RZPD clone IMAGp952H0930Q, mouse Has3 : RZPD clone IMAGp998G102025Q, mouse Igf2: RZPD clone IMAGp998M161029Q

For in situ hybridisation on slides (SuperFrost Plus, Menzel-Glaeser), samples were dewaxed and rehydrated through 75%, 50%, 25% ethanol, PBS for 5 min each. All procedures were performed at RT. Samples were postfixed in 4% PFA for 20min, bleached with 6% $H_2O_2$ for 15min, and washed 3 times with PBS for 5min each. Samples were treated with 20mg/ml Proteinase K/PBS for 10min and washed in 2mg/ml glycine /PBS for 2min. Samples were postfixed with 4% PFA/PBS for 10min followed by two PBS washes, 5min each. Samples were incubated in 100mM Tris-Cl (pH. 7.5) for 2min, in 100mM Tris-Ac (100 mM Tris-Cl (pH 7.5) supplemented with 0.25% $(C_2H_3O)_2O$) for 10min, in 2xSSC twice 5min each and dehydrated through 25%, 50%, 75% and 100% ethanol. Air dried samples were hybridised [(33% formamide, 3.3% Boehringer Blocking Reagent (Roche), 3.3 xSSC (pH 4.5), 6.6% dextrane sulfate (Sigma), 3.3mM EDTA (Merck), 0.07% Tween, 100µg/ml heparin, 100µg/ml tRNA, 1µg/ml DIG or Fluorescein–labeled RNA probe)] at $63^0$C overnight in humidified chamber.

The next day samples were washed twice with solution I (50% formamide, 5xSSC (pH 4.5), 0.1% Tween) at $70^0$C for 30min each, three times with solution II (50% formamide, 2xSSC (pH 4.5), 0.1% Tween) at $65^0$C for 30min each, three times with TBST (150 mM NaCl, 100 mM Tris-Cl (pH 7.5), 2 mM KCl, 0.1% Triton-X100) at RT, 5min each. Samples were blocked in 10% sheep serum (GIBCO BRL) in TBST at RT for 90min. After blocking, samples were incubated with the anti-DIG Fab (Roche) coupled to alkaline phosphatase or anti-Fluorescein Fab (Roche) coupled to alkaline phosphatase (1:1000) at 4°C overnight. On the following day samples were washed with TBST at RT for 8h, NTMT (100mM Tris-Cl, pH 9.5, 100mM NaCl, 50mM $MgCl_2$ (Merck), 0.1% Tween) twice for 20min each. Alkaline phosphatase was detected in NTMT solution supplemented with 4.5µl/ml NBT/INT (Sigma) and 3.5µl/ml BCIP

(Sigma) at RT. Sections were dehydrated and coverslipped with Entellan mounting media (Merck).

*Galactosidase staining*

Frozen sections were fixed in 0.2% glutaraldehyde/PBS at RT for 10min. Following fixation, samples were washed three times for 15 to 30min in lacZ wash buffer (2mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet-P40, NP-40, in 100mM sodium phosphate, pH 7.3, or PBS). Staining was carried out in 0.5mg/ml X-gal, 5mM potassium ferrocyanide, and 5mM potassium ferricyanide in lacZ wash buffer at 37°C or RT for 30min to overnight, with shaking and protection from light. When the staining was complete, slides were rinsed in PBS before dehydration through a graded ethanol series and coversliped.

**Protein biochemistry**

*Extraction of total protein*

Proteins were extracted from cultured keratinocytes. Cells were homogenized in ice-cold 2x RIPA buffer (100mM Tris-HCl, pH 7.4, 300mM NaCl, 2mM EDTA, 1% Na-deoxycholate, 2% NP-40, 2mM sodium orthovanadate and 2mM NaF) in the presences of protease inhibitors cocktail (Roche Diagnostic, Mannheim). Lysates were clarified by centrifugation for 45min at 60,000 rpm and supernatants containing proteins were aliquot, snap-frozen in liquid nitrogen and stored at –80°C. All steps were carried out at 4°C temperature.

*SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

The protein electrophoresis was carried out in polyacrylamide gels under conditions that ensure dissociation of proteins. The concentration of the separating gel depends on size of the protein of interest. The electrophoresis was carried out in a discontinuous buffer containing the nonionic detergent, SDS.

Separating Gel:

| | Final acrylamide concentration | | |
|---|---|---|---|
| | 8% | 10% | 12.5% |
| 30% acrylamide/0.8% bis-acrylamide | 1.6ml | 2ml | 2.5ml |
| glycerol | 0.5g | 0.5g | 0.5g |
| 2x resolving buffer | 3ml | 3ml | 3ml |
| $H_2O$ | 0.9ml | 0.5ml | null |
| 10% ammonium persulfate | 60µl | 60µl | 60µl |
| TEMED | 4µl | 4µl | 4µl |

Resolving buffer was composed from 0.2% SDS, 4mM $Na_4EDTA$, and 0.75 M Tris-HCl pH 8.9

Stacking gel:

In a flask 0.4ml of 30% acrylamide/0.8% bisacrylamide, 1.5ml of 2x Stacking buffer (0.25 M Tris-HCl, pH 6.7, 4mM EDTA, and 0.2% SDS) and 1.05ml of $H_2O$ was mixed. Prior to pouring 30µl of 10% ammonium persulphate and 2µl of TEMED were added.

Prior to loading the protein samples (40-60µg) were diluted 1:2 with 2x Laemmli SDS sample buffer (2% 2-mercaptoethanol, 0.2 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.004% Bromophenol Blue) and heated 5min at 100°C.

The electrophoresis was performed in 1x running buffer (made up from a 4x stock of 0.2 M Tris-HCl, 1.52 M glycine, 0.4% SDS, 8mM EDTA) for 4-5 hours at 30mA constant current.

*Western blotting*

Proteins were transferred from the gel onto membranes (nitrocellulose or nylon) by a wet-transfer method. After separation of the proteins, the gel and the nitrocellulose or nylon PVDF membrane was pre-wetted in transfer buffer (25mM Tris, 192mM glycine, 20% methanol and 0.1%SDS) for 5min. In case of PVDF membranes, they were first activated for 15sec in 100% methanol. The transfer was performed at 200mA constant current for 2 hours at 4°C (Biorad, Model 200/2.0). Afterwards, the membranes were washed three times with water and the transfer efficiency was

determined by Ponceau staining (2% Ponceau, 1% acetic acid in distilled water) for 2min at room temperature with constant shaking. For further processing, the membranes were destained with water for 20min with agitation.

*Immunodetection*

Non-specific binding sites on membranes were blocked for 1 h at RT in blocking solution (5%, w/v), skimmed milk powder in PBS and 0.05% Tween-20. The primary antibodies were diluted in blocking solution and incubated with the membrane for 2-3 hours at RT. Antibodies specific to Erk1/2, phospho Erk1/2, Akt, phospho Akt, phospho Gab1, phospho PAK1/2 (Cell Signaling Technology) were used. After washing in PBT (1x PBS, 0.05% Tween 20) four times for 10min, the horseradish peroxidase-conjugated secondary antibodies diluted in blocking solution were applied for 45min. Then the membranes were washed 4 times for 10min in PBT.

For visualization of immuno-reactive bands, the chemiluminescent detecting ECL reagent was used according to manufacturer's instructions (Amersham Biosciences, Freiburg). Briefly, detection solution was applied to the membrane to cover it evenly. After one-minute incubation, excess solution was drained from the membrane, and the membrane was placed on a flat sheet of Saran Wrap. The edges of the wrap were folded over the backside of the membrane to seal it. The membrane was then exposed to a Kodak X-ray film for varying lengths of time.

**Abbreviations**

| | |
|---|---|
| ATP | adenosine triphosphate |
| bp | base pair(s) |
| BrdU | 5-bromo-2'deoxyuridine |
| BSA | bovine serum albumin |
| d | day |
| DAPI | 4',6-Diamidin-2-phenylindoldihydrochloride |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleoside triphosphate |
| DNase | deoxyribonuclease |
| DTT | dithiothreitol |
| EGTA | Ethylene-glycol-bis(2-aminoethylether)-N,N,N',N'-tetra-acetic acid |
| *et al.* | *et altera* |
| EDTA | ethylene-diaminetetraacetic acid |
| FCS | fetal calf serum |
| g | gram |
| G418 | geneticin |
| HEPES | 4-(2-Hydroxyethyl)-piperazin-1-ethansulfonic acid |
| h | hour |
| HRP | horseradish peroxidase |
| HS | heat inactivated horse serum |
| kDa | kilodalton |
| kb | kilobase pairs |
| l | liter |
| M | molar |
| mA | milliampere |
| min | minute |
| ml | milliliter |
| mM | millimolar |

| | |
|---|---|
| µM | micromolar |
| MEM | Modified Eagle Medium |
| NaAc | sodium acetate |
| ON | over night |
| PBS | phosphate-buffered saline |
| PBT | PBS containing 0.05% Tween-20 |
| PCR | polymerase chain reaction |
| PFA | paraformaldehyde |
| pH | *potentium hydrogenii* |
| rpm | rotations per minute |
| RT | room temperature |
| SDS | sodium dodecyl sulphate |
| SSC | standard saline citrate |
| TEMED | N, N, N', N'-Tetra-methylethylenediamine |
| Tris | Tris-(hydroxymethyl)aminoethane |
| U | unit (enzymatic activity) |
| V | Volt |
| Vol. | Volume |
| W | Watt |

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**Publications:**

Jolanta Chmielowiec, Malgorzata Borowiak, Markus Morkel, Theresia Stradal, Barbara Munz, Sabine Werner, Jürgen Wehland, Carmen Birchmeier, and Walter Birchmeier, *c-Met is essential for wound healing in the skin, J. Cell Biol., 2007 Apr 9;177(1):151-62*

Ute Schaeper, Regina Vogel, Jolanta Chmielowiec, Joerg Huelsken and Walter Birchmeier, *Distinct requirements for Gab2 in Met and EGF receptor signaling in vivo, Proc Natl Acad Sci USA (submitted/in revision)*

## Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfsmittel erarbeitet und verfasst habe. Diese Arbeit wurde keiner anderen Prüfungsbehörde vorgelegt.

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Berlin den 15.05.2007