c-Met is essential for wound healing in the skin

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Wound healing of the skin is a crucial regenerative process in adult mammals. We examined wound healing in conditional mutant mice, in which the c-Met gene that encodes the receptor of hepatocyte growth factor/scatter factor was mutated in the epidermis by cre recombinase. c-Met–deficient keratinocytes were unable to contribute to the reepithelialization of skin wounds. In conditional c-Met mutant mice, wound closure was slightly attenuated, but occurred exclusively by a few (5%) keratinocytes that had escaped recombination. This demonstrates that the wound process selected and amplified residual cells that express a functional c-Met receptor. We also cultured primary keratinocytes from the skin of conditional c-Met mutant mice and examined them in scratch wound assays. Again, closure of scratch wounds occurred by the few remaining c-Met–positive cells. Our data show that c-Met signaling not only controls cell growth and migration during embryogenesis but is also essential for the generation of the hyperproliferative epithelium in skin wounds, and thus for a fundamental regenerative process in the adult.

Introduction

The mammalian skin functions as a barrier to many forms of environmental stress, therefore wounds of the skin need to be repaired efficiently (Martin, 1997; Werner and Grose, 2003). Wounding of skin can damage both epidermis and dermis; thus, wound healing requires reepithelialization of the epidermis and the formation of new dermal structures, called granulation tissue. During reestablishment of the epithelial barrier, keratinocytes from outside the wound migrate over the injured dermis and the granulation tissue. At the wound edges, these keratinocytes form the so-called hyperproliferative epithelium (HE), which strongly proliferates and migrates to replenish the wounded area with new cells. Cells from the HE displace the fibrin clot over time (Martin, 1997; Singer and Clark, 1999; Ito et al., 2005; Santoro and Gaudino, 2005). The HE (Fig. 1 A, HE) is characterized by the expression of keratins 6 and 16, which are also present in the hair follicle, but not in the uninjured epidermis (Fuchs, 1990; Takahashi et al., 1998; Wojcik et al., 2001). Impairment of wound healing, e.g., in diabetes, can result in the development of chronic wounds (Singer and Clark, 1999; Falanga, 2005; Morasso and Tomic-Canic, 2005).

Various signaling systems coordinate the wound healing process, as demonstrated by the analysis of growth factors, their receptors, and downstream signaling components (Scheid et al., 2000; Werner and Grose, 2003). For instance, genetic evidence obtained in mice indicates that signaling of the EGF receptor and the keratinocyte growth factor (KGF/FGF7) receptor are important for reepithelialization of wounds (Werner et al., 1994; Repertinger et al., 2004; Shirakata et al., 2005). Furthermore, down-regulation of the TGFβ receptor in keratinocytes reduces the rate of reepithelialization (Amendt et al., 2002). Smad3 is a downstream component of TGFβ signaling; in contrast, Smad3 mutant mice show an increased rate of reepithelialization and reduced monocyte infiltration during wound healing (Ashcroft et al., 1999). c-Jun and STAT3 participate in the signaling of growth factors, interleukins, and integrins; conditional mutation of c-Jun and STAT3 in the epidermis delays wound closure (Sano et al., 1999; Li et al., 2003).

Cell culture models have been used to simulate wound closure. In such experiments, monolayers of epithelial cells are scratch wounded, the migration of the cells is traced, and the molecular mechanisms that control migration are studied (Raghavan et al., 2003; Kodama et al., 2004). Movement of cells into scratch wounds requires modulation of cell adhesion and

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Abbreviations used in this paper: E, embryonic day; HE, hyperproliferative epithelium; HGF/SF, hepatocyte growth factor/scatter factor; KGF, keratinocyte growth factor; P, postnatal day.

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changes in the cytoskeleton, e.g., membrane protrusion and generation of new sites of substrate adhesion at the front, as well as actin disassembly and cell detachment at the rear. Small GTPases and protein kinases play essential roles in actin dynamics and cell migration processes, and closure of scratch wounds in cultured cells depends on Rho and Rac as well as c-Jun N-terminal kinase (Ridley et al., 1995; Fenteany et al., 2000; Lamorte et al., 2000; Raftopoulou and Hall, 2004). Wound closure in vitro is not only achieved by activities restricted to cells in the front row, but also involves cells further away from the wound edge (Farooqui and Fenteany, 2005). Thus, dispersion and migration of single cells at the wound edge is observed, which is accompanied by movement of back row cells that maintain their cell–cell contacts and migrate as coherent cell sheets. The cellular and molecular mechanisms during the closure of scratch wounds in vitro resemble those involved in the migration of epithelial cells during the healing of skin wounds.

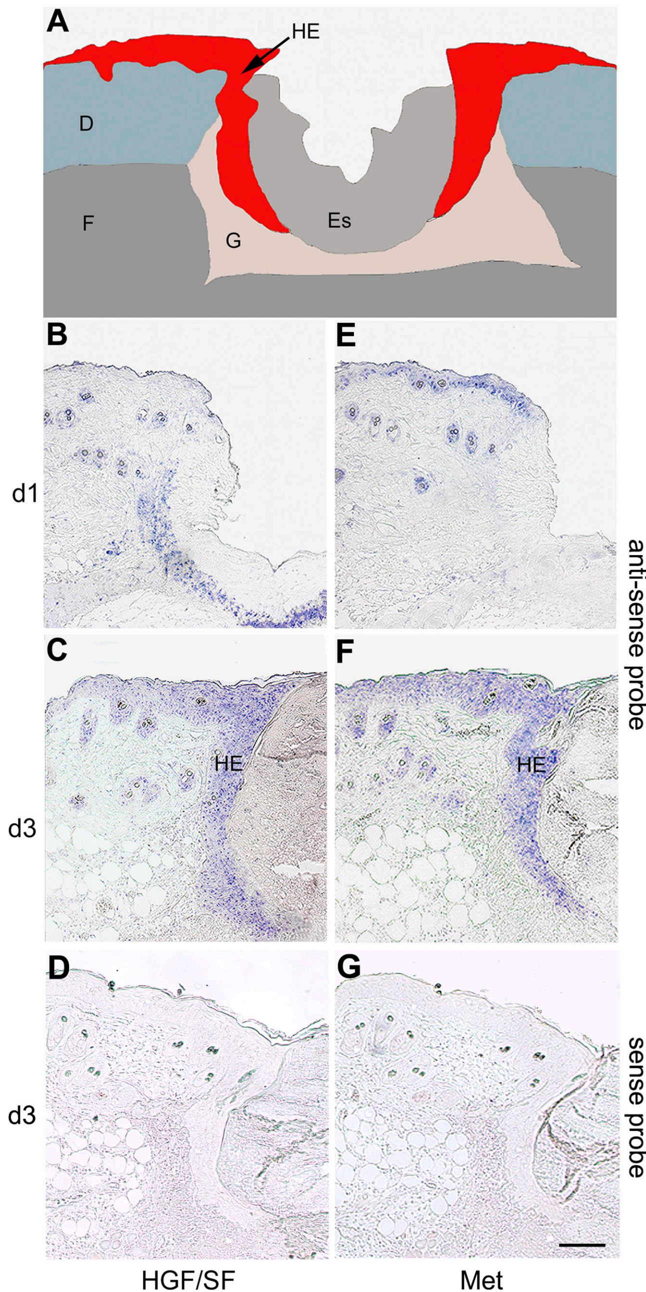

Figure 1. **Expression of HGF/SF and c-Met during wound healing.** (A) Scheme of a wound section 3–5 d after injury. Keratinocytes (red) at the wound edge proliferate and migrate down the injured dermis to form the so-called HE (arrow). D, dermis; Es, eschar; F, fatty tissue; G, granulation tissue. (B and C) In situ hybridization of wounded skin with a HGF/SF probe 1 (B) and 3 d (C) after injury. Note that HGF/SF at day 1 is expressed in the hair follicles and around the clot, which is where inflammatory cells accumulate and infiltrate the wound. 3 d after wounding, HGF/SF is highly expressed in the HE. (E and F) In situ hybridization with the c-Met probe 1 (D) and 3 d (E) after injury. Note that c-Met is expressed in the epidermis, in hair follicles, and in the HE 3 d after wounding. (D and G) In situ hybridization with sense probes of HGF/SF (F) and c-Met (G). Bar, 50 μm.

Hepatocyte growth factor/scatter factor (HGF/SF) and its receptor c-Met promote proliferation of epithelial cells in culture, and can dissociate and scatter MDCK cells, which is accompanied by an increase in their motility (Stoker et al., 1987; Miyazawa et al., 1989; Weidner et al., 1991; Nakamura et al., 1989; Bottaro et al., 1991; for review see Birchmeier et al., 2003). Mutation of the HGF/SF and c-Met genes in mice demonstrated that this signaling system is important during vertebrate embryogenesis (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). During development, HGF/SF and c-Met control cell survival and proliferation of hepatocytes, as well as the formation of the placenta. Furthermore, HGF/SF and c-Met are essential regulators of cell motility. HGF/SF signals release cells from dermomyotome, and they subsequently migrate to targets where they form skeletal muscle (Bladt et al., 1995; Birchmeier and Gherardi, 1998; Dietrich et al., 1999). HGF/SF and c-Met have also been implicated in various physiological and pathophysiological 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 the c-Met receptor is essential during liver regeneration and repair (Michalopoulos and DeFrances, 1997; Borowiak et al., 2004; Huh et al., 2004; Taub, 2004). Furthermore, up-regulated HGF/SF and c-Met expression was observed after injury of other tissues, for instance the lung, kidney, heart, and skin (Kawaida et al., 1994; Ohmichi et al., 1996; Nakamura et al., 2000; Cowin et al., 2001; Yoshida et al., 2003). Thus, up-regulated HGF/SF and c-Met expression might be part of a general response to tissue damage, and it is interesting to note that cytokines such as interleukin 1 or 6 activate HGF/SF transcription (Michalopoulos and DeFrances, 1997). Moreover, application of exogenous HGF/SF to skin wounds promotes the formation of granulation tissue, angiogenesis, and reepithelialization, whereas neutralization of HGF/SF by the application of antibodies delays these processes (Toyoda et al., 2001; Yoshida et al., 2003; Bevan et al., 2004).

We report that both HGF/SF and c-Met are up-regulated in the HE during wound repair in mice, suggesting that HGF/SF and c-Met signal may act in an autocrine manner to promote wound healing. We generated conditional mutant mice, in which c-Met was inactivated in the epidermis by the use of a keratin 14 (K14) promoter-driven cre recombinase. This resulted in the mutation of c-Met in ∼95% of the epidermal cells. The HE of wounds was collected by laser capture microdissection from mutant and control mice, and Southern blotting was performed to analyze the contribution of c-Met mutant keratinocytes to the

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newly formed epithelium. Remarkably, we found that c-Met mutant keratinocytes were completely unable to reepithelialize the wounds. Instead, residual keratinocytes that escaped recombination (5%, c-Met–positive cells) closed the wounds, but the wound healing process was delayed. These results demonstrate that the c-Met signaling system is essential for skin wound healing. Apparently, no other signaling system is able to compensate for a lack of c-Met in this process.

Results

HGF/SF and c-Met are expressed during wound healing and may act in an autocrine fashion

We performed full-thickness dorsal skin wounding in mice in such a manner that the epidermis and the underlying dermis were damaged (Werner et al., 1994). We then analyzed HGF/SF and c-Met expression during the wound healing process by in situ hybridization of these sections, i.e., 1–10 d after the injury. Before wounding, HGF/SF was expressed in hair follicles, but not in the epidermis (Lindner et al., 2000). After wounding, HGF/SF was initially expressed in the dermis adjacent to the clot (Fig. 1 B, only the left halves of the wounds are shown; Fig. 1 A shows the scheme of entire wound; Cowin et al., 2001). 3 d after the injury, HGF/SF was strongly up-regulated in the HE at the edges of wounds (Fig. 1 C; control is shown in D). At this time point, the new epithelium is already formed and visible. The receptor tyrosine kinase c-Met is expressed in the epidermis and hair follicles of normal skin (Lindner et al., 2000), but it is also strongly expressed in the HE during wound repair (Fig. 1, E and F; control is shown in G). We also performed immunofluorescence staining of phosphorylated c-Met on normal skin

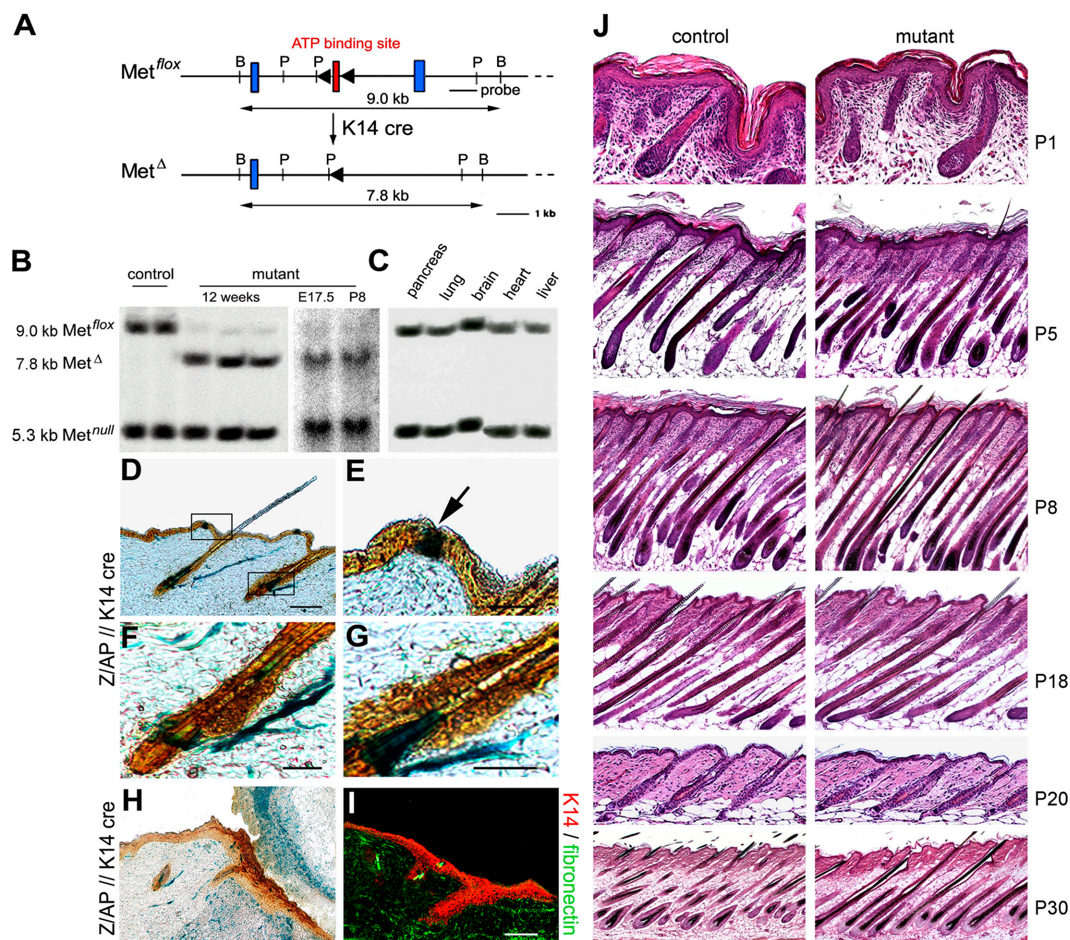

Figure 2. **Generation and analysis of skin-specific c-Met mutant mice.** (A) Schematic representation of nonrecombined allele and recombined allele of c-Met. Exon 15 of the c-Met gene that encodes the ATP-binding site (red box) was flanked by loxP sites (triangles) and is excised after K14-cre–induced 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 c-Met mutant mice of different ages (12 wk old, E17.5, and P8). (C) Southern blot analysis of different organs of conditional c-Met mutant mice. (D–G) Double staining of skin section from Z/AP; K14-cre mice (Lobe et al., 1999; Huelsken et al., 2001) for *lacZ* (blue, nonrecombined) and alkaline phosphatase activity (yellow, recombined). (E) A higher magnification shows an area of nonrecombined epidermal keratinocytes (blue patch). Higher magnifications show also two independent hair follicles (F and G); note that the bulge region contains recombined (yellow) cells. Arrector pili muscle cells, which surround the follicle, are nonrecombined (blue). (H) Double staining of alkaline phosphatase and β-galactosidase activity of wound section from Z/AP; K14-cre mice. Note that K14-cre–induced recombination is observed in the unwounded epidermis and in the HE. (I) Immunohistological analysis of a wound section from control mice using antibodies directed against K14 (red) and fibronectin (green). (J) Hair follicle cycle in control and conditional c-Met mutant. Sagittal sections of control and conditional c-Met mutant skin stained with hematoxylin/eosin at P1 (first anagen), P5 (first anagen), P8 (first anagen), P18 (first catagen), P20 (first telogen), and P30 (second anagen). Bars: (D) 50 μm; (E–F) 20 μm; (G–I) 100 μm.

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and could show that activated c-Met is present in both epidermis and hair follicles, including hair bulge stem cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200701086/DC1). Our data suggest that during wound healing, HGF/SF and c-Met may signal in an autocrine manner in the HE.

Wound closure in conditional c-Met mutant mice

We examined the function of the c-Met signaling system in the skin using conditional mutagenesis. For this, we crossed K14-cre mice, which express cre recombinase in the epidermis starting on embryonic day (E) 15 (Huelsken et al., 2001), with c-Met mutant mice, to generate animals with a K14-cre; c-Met$^{flox/null}$ genotype. In such animals, one allele of c-Met corresponded to the conventional null mutation, c-Met^{null} (Bladt et al., 1995), and the other to a "floxed" allele, c-Met^{flox} (Borowiak et al., 2004). After cre-mediated recombination, the exon encoding the essential ATP-binding site of c-Met is removed in c-Met^{flox}, and a functional null allele, which we denote as c-Met$^{\Delta}$ is generated (structures of nonrecombined and recombined alleles are shown in Fig. 2 A). K14-cre; c-Met$^{flox/null}$ mice will henceforth be called conditional c-Met mutant mice.

Efficient recombination of c-Met was observed in the epidermis of conditional c-Met mutant mice. Southern blot analyses demonstrated that 95% of the cells in the epidermis had already recombined the c-Met^{flox} allele at E17.5 (Fig. 2 B, c-Met$^{\Delta}$). A similar proportion of cells containing the recombined allele were observed in the epidermis of young and adult animals, e.g., at postnatal day (P) 8 and at 12 wk (Fig. 2 B). In other epithelial tissues, such as pancreas, lung, and liver, mutation of c-Met was not observed, i.e., the nonrecombined c-Met^{flox} allele was observed (Fig. 2 C). Recombination introduced by K14-cre in the skin was also assessed histologically, using the Z/AP reporter mice (Lobe et al., 1999); in such mice, yellow NBT/BCIP staining measures alkaline phosphatase activity, which is detectable in recombined epidermal cells, whereas blue X-Gal staining indicates β-galactosidase activity, which is observed in nonrecombined cells. The histological analysis of skin sections of these mice demonstrated that the vast majority of the cells in the epidermis had undergone recombination, and only small groups of nonrecombined cells were detectable (Fig. 2 D; the enlarged picture in Fig. 2 E shows a group of blue nonrecombined cells, which are marked by arrow). Recombination was also observed in hair follicle cells, particularly in those cells that locate in the bulge region (Fig. 2, F and G, yellow cells); recombination was not apparent in arrector pili muscle cells (Fig. 2, F and G, blue cells).

We first examined the appearance of skin and hair in conditional c-Met mutant mice by histology at birth and afterward. No gross morphological changes in the epidermis were detected compared with control mice. For instance, the thickness of the epidermis was comparable, and it did not display any pathological alterations as assessed by immunohistology using antibodies directed against keratin 10 and loricrin (Fig. 2 J and not

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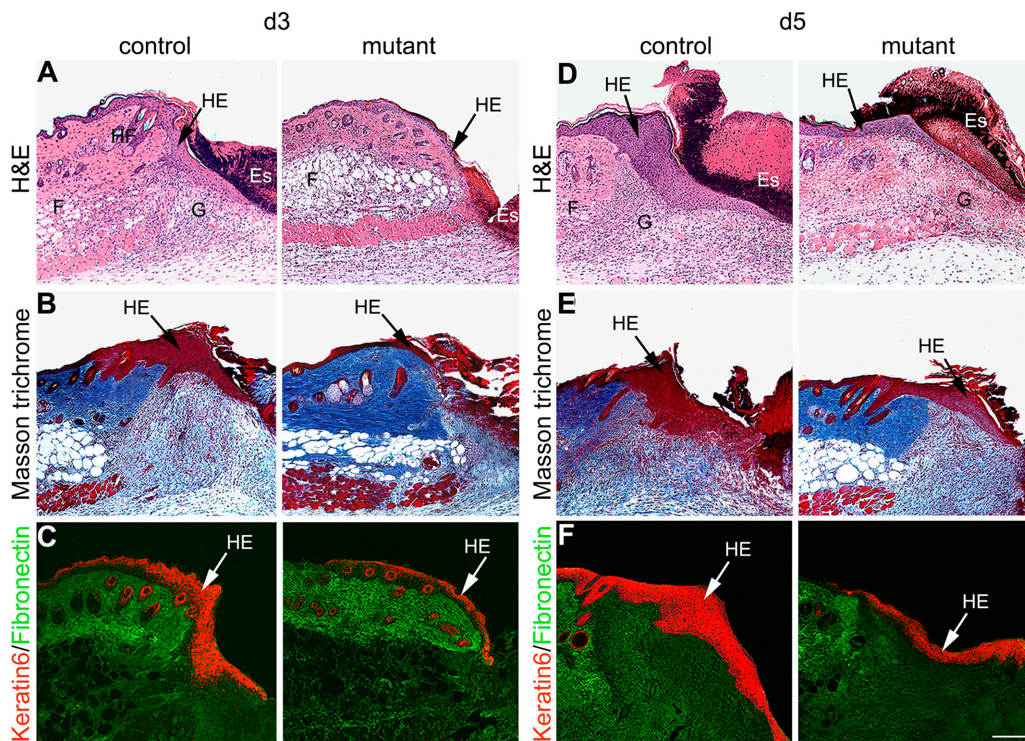

Figure 3. **Wound healing in control and conditional c-Met mutant mice.** (A and D) Hematoxylin/eosin staining of sections of wound edges from control and mutant mice 3 (A) and 5 d (D) after wounding. Only halves of the wounds are shown (for the scheme of a complete wound see Fig. 1). (B and E) Masson trichrome staining of wound sections 3 (B) and 5 d (E) after injury. (C and F) Immunohistological analysis of wound sections from control and mutant mice 3 (C) and 5 d (F) after injury using antibodies directed against keratin 6 (red) and fibronectin (green). Arrows mark the HE. Es, eschar; F, fatty tissue; G, granulation tissue; HF, hair follicle. Bar, 100 μm.

depicted; Fuchs et al., 1992; Byrne et al., 1994). Similarly, we did not observe 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 (Fig. 2 J). Catagen and telogen occurred at P18 and P20 (Paus and Cotsarelis, 1999). We have now kept conditional c-Met mutant mice for nearly 2 yr, and we have not observed unusual hair loss or other changes in the appearance of the skin.

We introduced full-thickness wounds into the dorsal skin (Werner et al., 1994) of control and conditional c-Met mutant mice at 12 wk of age, and we analyzed wound closure by histology 3–15 d after the injury. Wound healing did occur in conditional c-Met mutant mice, but was delayed and required about twice as much time as in the control mice (Fig. 3, A–F; see quantification in Fig. 4 A). For instance, 5 d after the injury, 50% wound closure was observed in control mice; in conditional c-Met mutant mice, this required 9 d (Fig. 4 A). Histological examinations showed that the HE was thinner, and its formation was delayed in the conditional c-Met mutant mice, as assessed by hematoxylin/ eosin (Fig. 3, A and D) and by Masson trichrome staining; the latter stains the epithelium in red (Fig. 3, B and E). Immunohistological analysis using keratin 6 antibodies also demonstrated a reduction in the thickness of the HE in the conditional c-Met mutant mice (Fig. 3, C and F). Keratin 6 is expressed in activated keratinocytes of the HE and in hair follicles (Fuchs, 1990; Takahashi et al., 1998; Wankell et al., 2001). Quantification showed that the formation of the HE was delayed in the conditional c-Met mutant mice; compared with control mice, an 80% reduction of the area of the HE was observed 3 d after injury, a 65% reduction 5 d after injury, and only a 25% reduction 7 d after injury (Fig. 4 B). In contrast, the proportion of proliferating keratinocytes in the HE was increased after injury in the conditional c-Met mutants, as assessed by phospho-histone 3 and BrdU staining (Fig. 4, C and D). We did not observe accumulations of proliferate-positive cells at particular sites in the HE, and, in particular, we did not observe an accumulation of proliferating cells close to the remnants of the hair follicle. We also did not observe any difference in the number of apoptotic cells in the skin of control and conditional c-Met mutant mice, as assessed by TUNEL staining (unpublished data). Thus, wound healing occurred in the skin of c-Met conditional mutant mice, but reepithelialization of wounds was delayed and required about twice as much time as in control mice.

Residual keratinocytes that escaped recombination reepithelialize the wounds in conditional c-Met mutant mice

To assess if the c-Met mutant cells (95% of the keratinocytes in the epidermis) could contribute to the newly formed epithelium of the wounds, we collected hyperproliferative epithelia of many control and mutant wounds by laser capture microdissection (see Materials and methods) and performed Southern blotting. An example of a section of a wound before and after microdissection is shown in Fig. 5, A and B. Remarkably, Southern blot analysis demonstrated the absence of c-Met mutant cells (i.e., of the c-Met$^{\Delta}$ allele) in the microdissected hyperproliferative epithelia of the mutant mice at day 5 (Fig. 5 C). Instead, all cells in the mutant HE contained the nonrecombined c-Met^{flox} allele, despite the fact that this cell population constituted only 5% in the skin before injury. At day 3, a 1:1 mixture of c-Met^{flox} and c-Met$^{\Delta}$ cells was seen. Immunofluorescence and immunohistochemistry staining with anti–phospho-c-Met antibodies revealed that the majority of cells in the HE at day 5 contained the activated and phosphorylated c-Met receptor, both in control and mutant skin (Fig. 5; compare D and F with E and G). We have observed that staining of activated c-Met is more pronounced in the upper layers of the HE, but staining is also visible in lower layers (Fig. 5, D–G, arrows). We also generated wounds in the skin of K14-cre; Z/AP reporter mice, and could show that K14-cre recombination occurred in the wound epithelium (Fig. 2 H). Immunohistological analyses confirmed that K14 was strongly expressed in the HE (Fig. 2 I). We conclude from these data that

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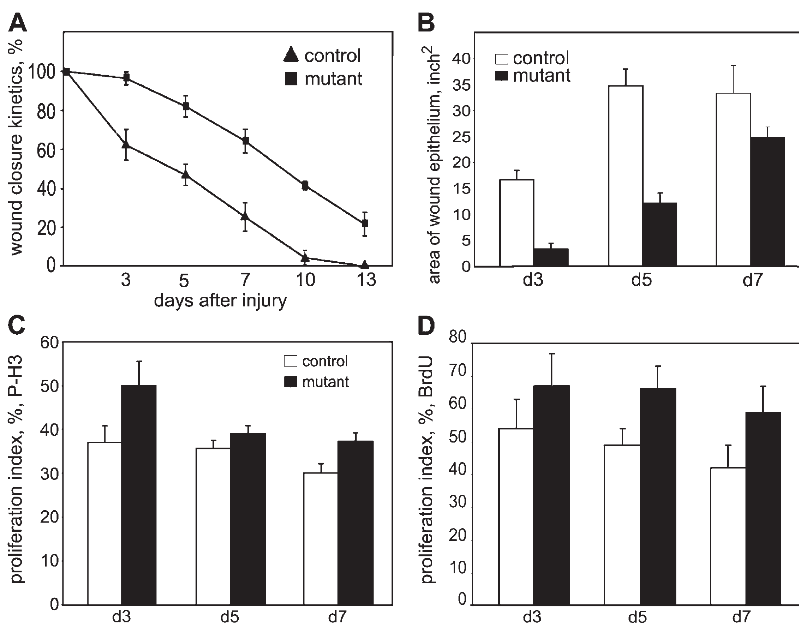

Figure 4. **Quantification of wound healing in control and conditional c-Met mutant mice.** (A) Wound closure kinetics in control and mutant mice (see Materials and methods). (B) Quantification of the area of HE 3, 5, and 7 d after wounding in control and mutant mice; only sections of the middle of the wounds were used for quantification. (C) Proliferation of keratinocytes in the HE from control and mutant mice 3, 5, and 7 d after wounding, as assessed by the proportion of phospho-Histone 3–positive nuclei in the epithelium (see Materials and methods). A *t* test was performed, and significant differences between control and mutant were observed 3 d after injury. P = 0.01. (D) Proliferation of keratinocytes in the HE from control and mutant mice 3, 5, and 7 d after wounding, as assessed by the proportion of BrdU-positive nuclei in the epithelium. Significant statistical differences between control and mutant were observed 5 d after injury. P = 0.01. Error bars represent the SD.

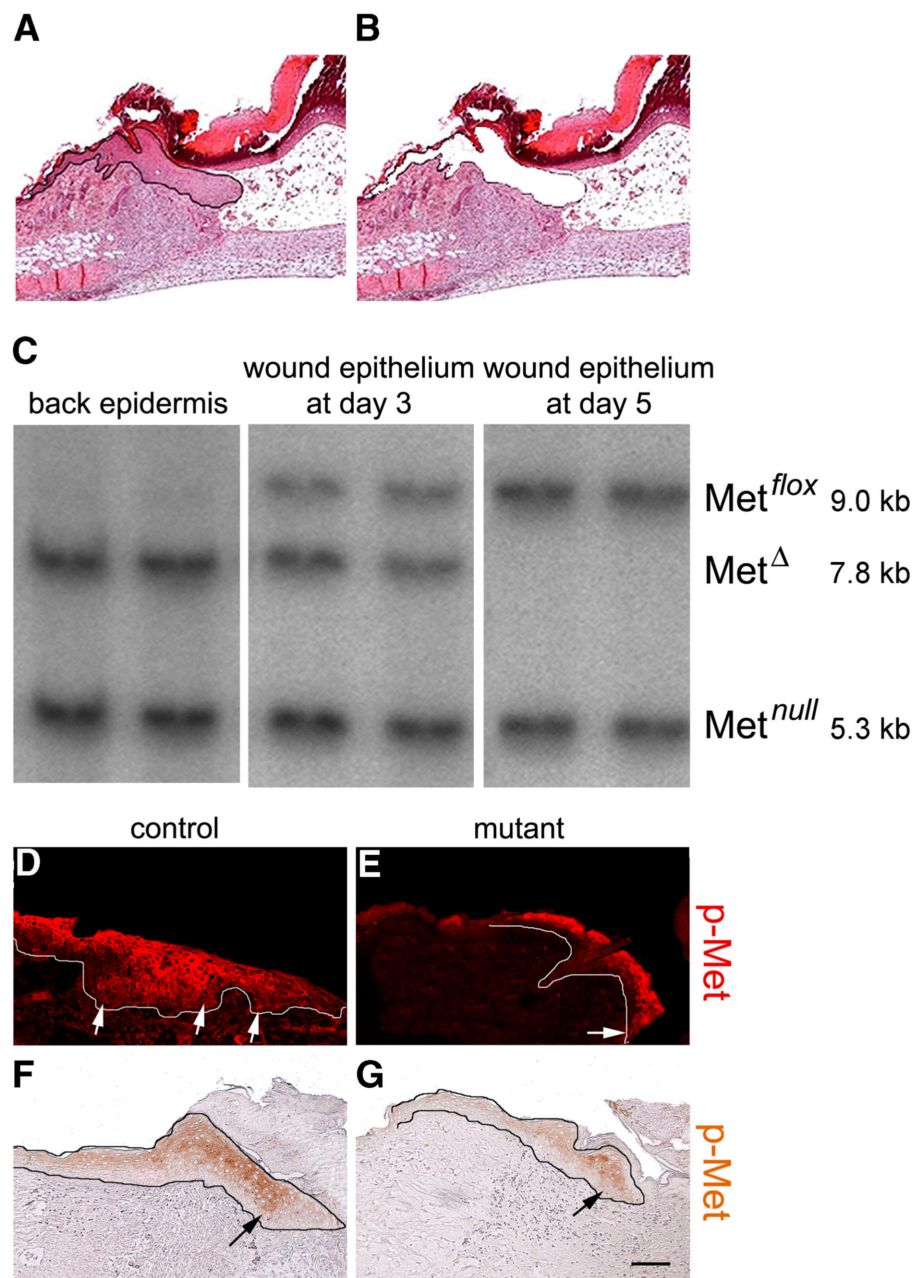

Figure 5. **Only residual c-Met–positive keratinocytes contribute to the HE of wounds in conditional c-Met mutant mice.** (A and B) Isolation of HE by laser capture microdissection. A wound section before (A) and after laser capture microdissection (B) is shown. (C) Southern blot analyses of back epidermis and hyperproliferative epithelia from conditional c-Met mutant mice. Microdissected, nonwounded epidermis from the back (left) and microdissected hyperproliferative epithelia of wounds 3 (middle) and 5 d (right) after injury were collected. Southern blotting of two independent preparations from different pools of microdissected tissues is shown. Note that the HE 5 d after injury in conditional c-Met mutant mice is formed exclusively by cells that contain the nonrecombined c-Met^{flox} allele, but not the recombined c-Met$^{\Delta}$ allele. At day 3, a 1:1 mixture of recombined and nonrecombined cells is seen (middle). The c-Met^{null} allele is also present because heterozygous c-Met$^{flox/null}$ mice were used for conditional mutagenesis. (D–G) Immunohistological analysis of wound sections from control and conditional mutant mice 5 d after injury using anti–phospho-c-Met antibodies (red immunofluorescence in D and E, and brown immunohistochemistry in F and G). Note that in the skin of conditional c-Met mutant mice, cells in the HE (outlined) are phospho-c-Met positive. Arrows mark phospho-c-Met–positive cells in the lower hyperproliferative epithelia layers. Bar, 100 µm.

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only nonrecombined c-Met keratinocytes, i.e., cells that express a functional c-Met, participate in the formation of the HE. In the skin of conditional c-Met mutant mice, the few remaining cells that escaped recombination can apparently compensate and generate the entire hyperproliferative epithelia. These data demonstrate that c-Met plays crucial functions during wound closure.

Closure of scratch wounds is impaired in cultured keratinocytes derived from conditional c-Met mutant mice

Primary keratinocytes were isolated from the skin of newborn control and conditional c-Met mutant mice (Caldelari et al., 2000), and monolayers in culture were scratch wounded (Fig. 6, A–C; Sano et al., 1999). Analysis of K14 by immunohistology indicated that the isolated cells, indeed, correspond to epithelial cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200701086/DC1). We found that in the presence of HGF/SF, primary keratinocytes from control mice closed the wounds within 48 h, as did cells from conditional c-Met mutant mice in the presence of the EGF receptor ligand TGFα (Fig. 6, A and C). However, in the presence of HGF/SF, keratinocytes from the skin of conditional c-Met mutant mice did not close the scratch wounds within 48 h, but required 96 h (Fig. 6 B). Strong proliferate response toward HGF/SF was observed in control cells close to the wound edges at 24 h, but such a response was not observed in the mutant keratinocytes (Fig. 6 D, quantification is shown in E).

We then examined if, while in culture, the few remaining nonrecombined keratinocytes from the skin of conditional c-Met mutant mice preferentially contribute to wound closure.

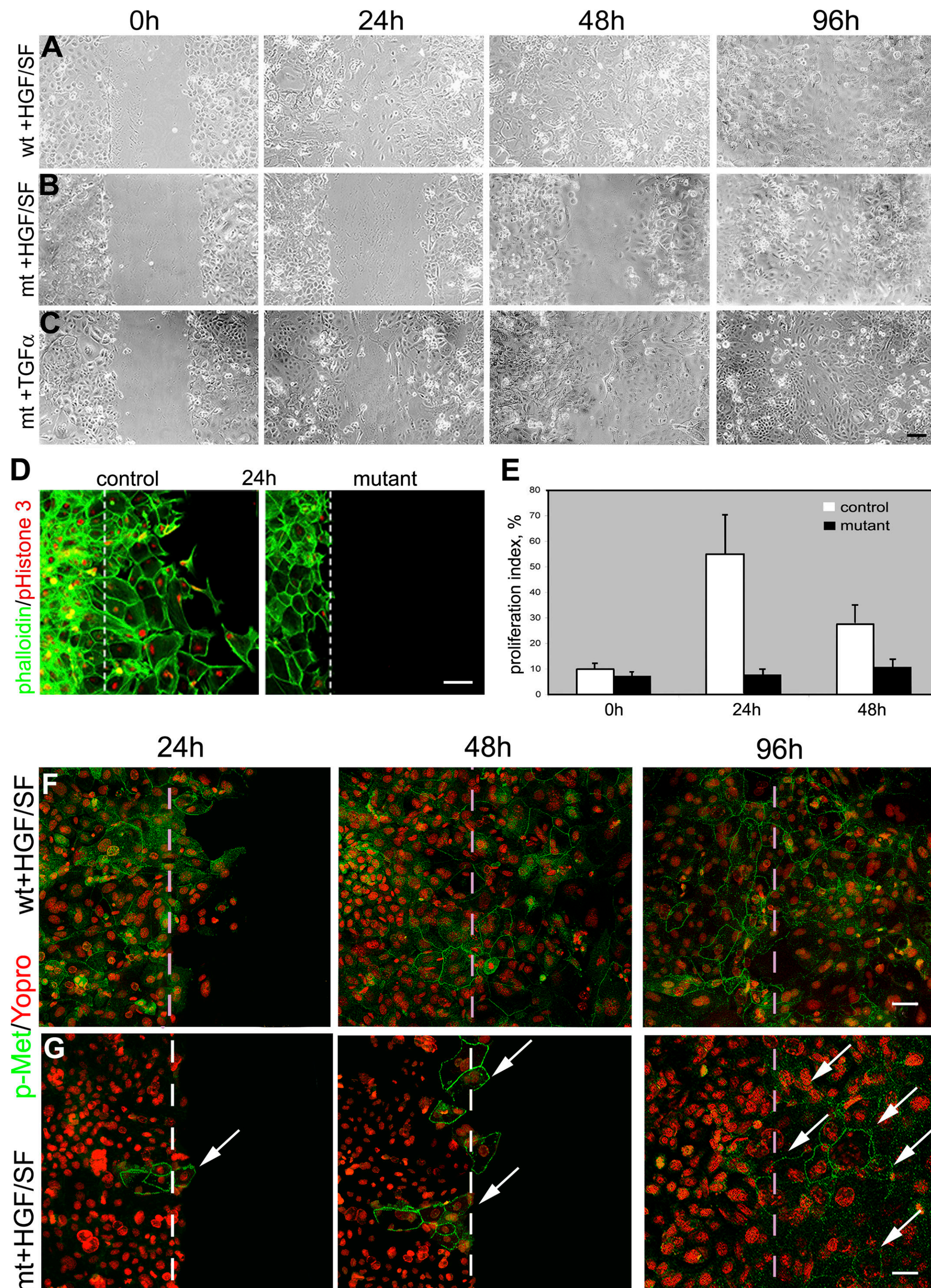

Figure 6. **Scratch wound healing in cell culture of primary keratinocytes: c-Met–positive primary keratinocytes migrate preferentially into scratch wounds.** Primary keratinocytes were isolated from newborn skin of control (A) and conditional c-Met mutant mice (B and C). After scratch wounding, cells were further cultured in the presence of HGF/SF or TGFα. Photos were taken 0, 24, 48, and 96 h after scratch wounding. Note that wounds in the cultures derived from conditional mutant mice did only close after 96 h in the presence of HGF/SF. (D) Proliferation of primary keratinocytes from control and conditional c-Met mutant mice 24 h 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). (E) Quantification of proliferation of primary keratinocytes at wound edges stimulated with HGF/SF in the experiments described in D. Error bars represent the SD. (F and G) Primary keratinocytes isolated from control and conditional c-Met mutant skin were scratch wounded and further cultured with HGF/SF. After 24, 48, and 96 h, cells were stained with anti–phospho-c-Met antibodies (green). Nuclei were visualized by YO-PRO staining (red). Note that many cells in the controls (F) showed a cobblestone pattern of phospho-c-Met staining at the plasma membrane. In populations derived from the skin of conditional mutant mice (G), cells with phospho-c-Met were initially rare (at 24 h), but after 96 h they are abundant in the scratched area (marked by arrows). The original edges of the scratch wounds are marked with a dashed line. Bars: (A–D) 100 μm; (F and G) 50 μm.

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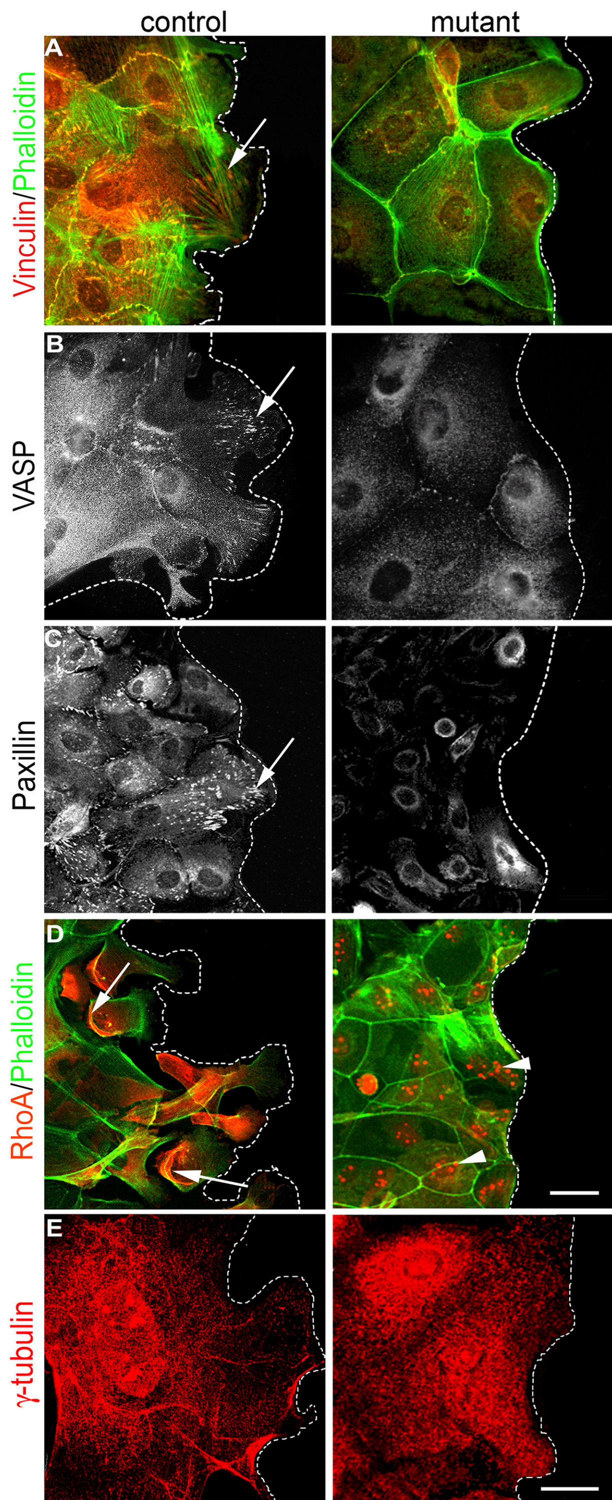

Figure 7. **Keratinocytes derived from conditional c-Met mutant mice do not rearrange their cytoskeleton at the scratch wound edges in the presence of HGF/SF.** Keratinocytes derived from control and conditional c-Met mutant mice were stained 24 h after scratch wounding, with antibodies directed against vinculin (A), with phalloidin (A and 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 cytoplasmatic and perinuclear localization of RhoA in mutant (Fig. 7 D, right). The dotted line indicates the edges of the wounds. Bars: (A–D) 50 μm; (E) 20 μm.

Primary keratinocytes were stained with phospho-c-Met antibody at different stages of scratch wound closure. In the presence of HGF/SF, control cells showed phospho-c-Met staining in a cobblestone manner (Fig. 6 F). Such a staining was associated with the membrane of virtually all cells that were close to the wounds at 24 h, and also with cells in the healed area at 48 and 96 h (Fig. 6 F). In contrast, only few keratinocytes derived from the skin of conditional c-Met mutant mice were initially phospho-c-Met positive (Fig. 6 G, left, arrow). We observed an enrichment of phospho-c-Met–positive cells at the wound edges after 48 h, and after 96 h a large proportion of cells that had closed the scratch wound contained phospho-c-Met (Fig. 6 G, arrows). Thus, residual c-Met–positive cells preferentially participate in wound closure in vitro and in vivo.

We also examined if primary keratinocytes derived from the skin of control and conditional c-Met mutant mice exhibited different properties at the wound edges. The keratinocytes were characterized by immunohistological analysis using antibodies directed against proteins that are important for directed cell migration, such as vinculin, paxillin, and VASP (Mitchison and Cramer, 1996; Rottner et al., 1999, 2001; Raghavan et al., 2003). Keratinocytes derived from control skin in the presence of HGF/SF showed increased numbers of focal adhesions at the wound edges, and these pointed directly toward the wounds (Fig. 7, A–C, left, arrows). Actin stress fibers, which were stained by phalloidin, were also oriented preferentially toward the wounds (Fig. 7 A). Control cells at the wound edges displayed a preferential location of RhoA staining that is located at the rear of the cells (Fig. 7 D, left, arrows; Nobes and Hall, 1999; Raftopoulou and Hall, 2004). These control cells at the edges of the wound also reoriented their microtubules, which was demonstrated by γ-tubulin staining (Fig. 7 E, left; Yvon et al., 2002). In contrast, keratinocytes derived from the skin of conditional c-Met mutant mice displayed punctuated, cytoplasmatic, and perinuclear staining of RhoA, which is not located at the rear of the cell (Fig. 7 D, right, arrowheads). Keratinocytes from conditional c-Met mutant mice displayed only few focal contacts and stress fibers, and these were not oriented toward the wounds (Fig. 7, A–C, right).

We also used the isolated primary keratinocytes from the skin of control and conditional c-Met mutant mice to study signal transduction by molecules that are crucial for cell proliferation and cell migration (Morimoto et al., 1991; Rubin et al., 1991; Hartmann et al., 1994; Ridley et al., 1995; Khwaja et al., 1998). Erk1/2, Akt, Gab1, and PAK1/2 were phosphorylated, and thus activated, in control cells in the presence of HGF/SF and TGFα (Fig. 8, left). In contrast, phosphorylation of these molecules in keratinocytes derived from conditional mutant mice was pronounced in the presence of TGFα, but not of HGF/SF (Fig. 8 A, left; for quantification see Fig. 8 B). These data demonstrate that HGF/SF and c-Met signaling is important for the activation of molecules that control proliferation and migration of primary keratinocytes in cell culture. Activation of this signaling system results in reorganization of adhesion and cytoskeleton complexes, such as focal adhesions and stress fibers, which allows cells to move into the scratch wounds.

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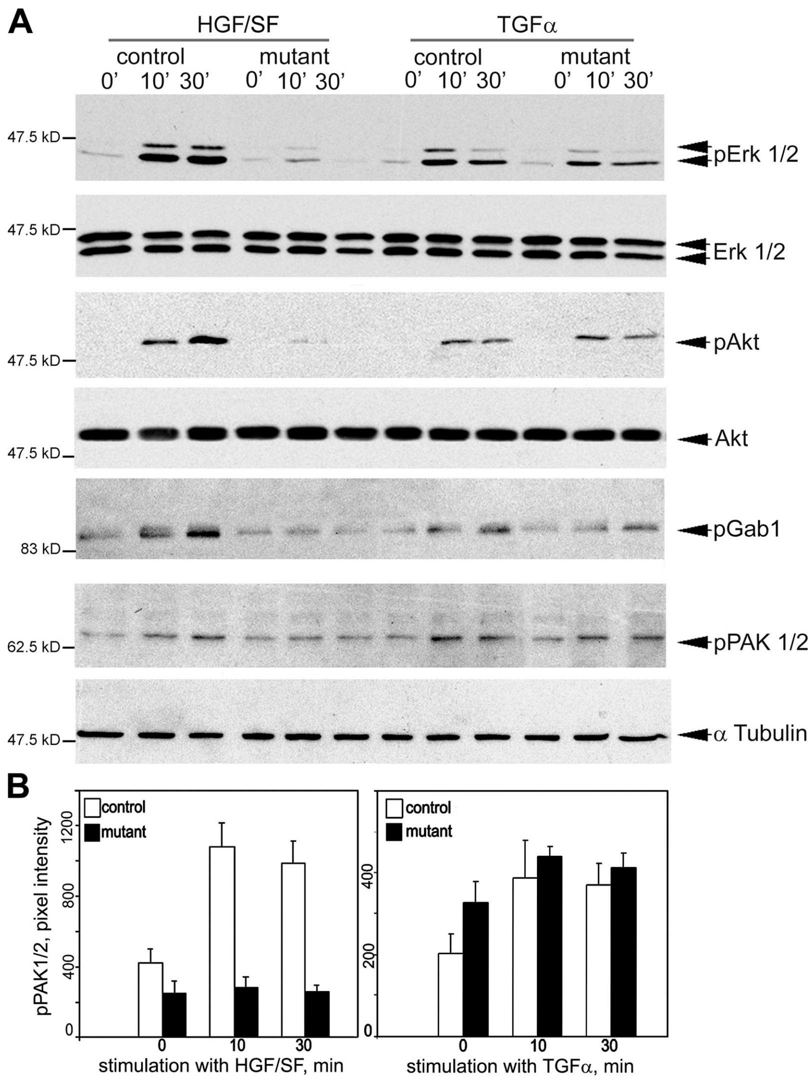

Figure 8. **Signaling is blocked in keratinocytes derived from conditional c-Met mutant mice that are treated with HGF/SF, but not with TGFα.** (A) Western blot analysis of phospho Erk1/2, total Erk1/2, phospho-Akt, total Akt, phospho-Gab1, and phospho-PAK1/2 in keratinocytes derived from control and conditional c-Met mutant mice. Cells were stimulated with HGF/SF or TGFα for 0, 10, or 30 min. Note that 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 shown in A, as assessed by pixel intensity.

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Discussion

We show that c-Met and HGF/SF expression is induced in the HE of skin wounds, indicating that receptor and ligand may act in an autocrine manner during wound healing. We also report that c-Met is crucial for reepithelialization during wound closure in the skin and in cell culture; c-Met mutant keratinocytes cannot contribute to the generation of the HE in vivo, and contribute inefficiently to the closure of scratch wounds in vitro. Analysis of cultured keratinocytes during the closure of scratch wounds indicates that the primary deficit of the mutant cells is their inability to proliferate and to migrate into the wounded area.

HGF/SF and c-Met represent an autocrine system in wound healing

In general, HGF/SF and c-Met are expressed in different cell types, although these may be closely apposed to allow an exchange of signals. For instance, HGF/SF is expressed primarily in mesenchymal cells, whereas c-Met is generally expressed in nearby epithelia (Sonnenberg et al., 1993; Yang and Park, 1995; Birchmeier and Gherardi, 1998). Moreover, c-Met is expressed in the epithelial dermomyotome and in the migrating muscle progenitor cells that derive from it, whereas HGF/SF is expressed in mesenchymal cells close to the somites and along the route of the migrating cells (Birchmeier et al., 2003). In the cerebellum, granule cells express HGF/SF, whereas surrounding Bergmann glia cells express the c-Met receptor (Jung et al., 1994). In tumors, however, autocrine HGF/SF and c-Met signaling is frequently observed (Birchmeier et al., 2003). Our expression and genetic data indicate that, also in a normal physiological process (i.e., during healing of skin wounds), autocrine HGF/SF/c-Met signaling is required.

HGF/SF and c-Met appear late during evolution and are only present in vertebrates (Birchmeier et al., 2003). In development, HGF/SF and c-Met control placentation and muscle precursor cell migration, which are processes that appear late in evolution. Genetic analyses demonstrate that c-Met is also important in regeneration of adult tissues, which we show here for skin repair after wounding, and which was previously demonstrated for liver regeneration (Borowiak et al., 2004; Huh et al., 2004). The conditional mutation of c-Met in the liver was introduced by the use of the inducible Mx-cre transgene, and a portion of the

liver was then removed by hepatectomy. In such conditional c-Met mutant mice, liver regeneration was impaired and, in particular, cell cycle progression of hepatocytes was altered (Borowiak et al., 2004). We show that c-Met signaling is also crucial for wound healing, which is a regeneration process of the skin. No other paracrine or autocrine system can compensate for a loss of c-Met function in skin regeneration because c-Met mutant cells were completely unable to contribute to the HE, which is the source of the keratinocytes that reepithelialize the wounds.

Few residual keratinocytes, which escaped recombination, close the wound in the skin of conditional c-Met mutant mice

The question arises how the few remaining nonrecombined cells in the conditional c-Met mutant skin (∼5%) could close the wound in vivo. Wound closure in vivo was delayed, but occurred and required only twice as much time, despite the fact that the vast majority, i.e., the 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 was shifted to the right in the mutants, which may indicate that the identity of cells contributing to the HE was the same. Thus, reepithelialization started after a delay in the skin of the conditional mutant mice (3 d after injury), but recovered fast because the proliferation of the keratinocytes in the HE was increased during early wound healing. Moreover, in the control skin, the size of the HE comes to a halt 5 d after the injury, but was still increasing at that time in the conditional c-Met mutant mice. A compensatory overproliferation of the control cells occurred because of this, resulting in a selection of cells that escaped recombination over mutant cells within a few days in vivo, and results in a wound that heals. Regeneration of the epidermis after wounding involves activation, migration, and proliferation of keratinocytes from the surrounding epidermis, but keratinocytes derived from hair follicles and sweat glands may also participate in the healing process. In response to injury, bulge stem cells leave their niche and contribute to the HE (Miller et al., 1998; Ito et al., 2005; Levy et al., 2005). Thus, bulge cells of the skin are another potential source of cells that reconstitute the injured epidermis. However, K14-cre–mediated recombination also occurs in the hair bulge, and we provide evidence that cre-mediated deletion is efficient in the bulge and in the epidermis. Moreover, we did not detect clusters of dividing cells that could correspond to cells that had escaped recombination close to hair follicle remnants. We also observe that keratin 6–positive cells form continuous layers in the wound epithelium in c-Met mutant skin, and do locate to areas close to hair follicles. Repopulating cells that escaped recombination could thus originate from both cells of the epidermis and of the hair bulge. It is possible that the underlying dermis also contributes in this compensatory process, e.g., by producing an increased amount of other growth factors or cytokines.

Wound healing in vitro and the importance of signal transduction and cytoskeletal changes

We also analyzed the effects of c-Met signals on wound closure of primary keratinocytes in a culture system, and analyzed if c-Met–positive keratinocytes preferentially contribute to wound closure in a culture that contains few nonrecombined (c-Met+) and the majority of mutant (c-Met−) cells. In such cultures, we observed that, in the presence of HGF/SF, nonrecombined (c-Met+) cells migrate preferentially into the scratch wounds. We also observed that control keratinocytes at the edges of scratch wounds orient themselves, i.e., focal adhesions and stress fibers pointed toward the wound edges (Ridley et al., 1995; Nobes and Hall, 1999). In contrast, such a pronounced orientation of cells was not observed in the scratch wounds of the keratinocytes derived from conditional c-Met mutant mice. However, mutation of c-Met did not disturb orientation of the cells toward the wound in the presence of TGFα. Our genetic data thus indicate that HGF/SF and c-Met are the major signal that is available to reorient cells at the wound edges in vitro, and to join the HE in vivo. In vitro, the loss of c-Met signaling can be compensated for by the administration of other growth factors like TGFα, but in vivo, the availability of other growth factors that can elicit similar responses to HGF/SF appears to be limited. We found that c-Met signaling in primary keratinocytes activates Erk1/2, Akt, Gab1, and PAK1/2. The phosphorylation of PAK1/2 might be of particular importance in the motility response; PAK1/2 is a target of Rho signaling that regulates actin polymerization and protrusion formation (Frost et al., 1998; Royal et al., 2000).

In the last decade, several growth factors have been implicated in wound healing, e.g., 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 appear to have contributed to the newly formed epithelium. As yet, c-Met is the only example of a receptor that is essential in wound healing. The application of HGF/SF and/or HGF/SF variants in the therapy of wounds (Bevan et al., 2004) therefore appears to be an attractive possibility.

Materials and methods

Wound healing experiments, histology, immunohistochemistry, and Western blotting

To mutate c-Met in the skin, the K14-cre^{neo} mouse line was used, which expresses cre after E15 of embryogenesis (Huelsken et al., 2001). 2–3-mo-old mice were anesthetized, and two full-thickness 4-mm-diam excisional wounds were made on both sides of the dorsal midline, as previously described (Werner et al., 1994). Mice were kept separately in cages to prevent fighting, and no self-induced trauma was observed in control or mutant mice. Littermates of the same sex were used for the analysis of wound closure. Wound closure was determined as the percentage of the distance covered by the epidermis between the wound edges. For histological analysis, dissected wounds were fixed overnight in 4% formaldehyde in PBS, followed by dehydratation through a graded ethanol series and embedding in paraffin. Sections from the middle of the wound were stained with hematoxylin/eosin, and immunofluorescence was performed as previously described (Huelsken et al., 2001). Histological sections were used to determine the area of HE by using the measure function of the Adobe Photoshop program. BrdU incorporation was assessed by immunohistochemistry 2 h after injection of 75 µg BrdU/g of body weight. The proliferation index represents the percentage of BrdU-positive cells within the HE. We used antibodies directed against keratin 6 (Covance), phospho-histone H3 (Millipore), PCNA (Oncogene Science), BrdU (Sigma-Aldrich), CD34 (Biozo), phospho-Met (Sigma-Aldrich), and K14 (Covance). Sections were incubated overnight with primary antibodies at 4°C, followed by fluorescent-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). TUNEL staining was performed using an in situ cell-death

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detection kit (Roche). Sections from the middle of the wound were also stained using the Masson trichrome procedure (Sigma-Aldrich). Stained tissues were analyzed on a confocal scanning laser microscope (LSM; Carl Zeiss MicroImaging, Inc.).

In situ hybridization of paraffin sections was performed using digoxygenin-labeled RNA probes (Roche; Huelsken et al., 2001). The antisense 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, and a 3.7-kb c-Met fragment. Western blot analysis was performed using antibodies specific to Erk1/2, phospho Erk1/2, Akt, phospho-Akt, phospho-Gab1, and phospho-PAK1/2 (Cell Signaling Technology).

Laser capture microdissection and Southern blotting

For laser capture microdissection, 46 wound areas were excised, embedded in Tissue-Tek OCT compound and snap-frozen in liquid nitrogen. Frozen sections (8 μm) were prepared and fixed in 70% EtOH for 60 s and stained shortly with hematoxylin/eosin. Laser capture microdissection was performed using an Arcturus PixCell II apparatus. Southern blot analysis of hyperproliferative epithelia from 400 microdissected sections was performed according to standard procedures.

Primary keratinocyte culture, in vitro scratch assay, and immunofluorescence

Primary murine keratinocytes were isolated from newborn mice, as described previously (Caldelari et al., 2000), and cultured on collagen IV–coated plates in defined keratinocyte serum-free medium (Invitrogen). The confluent monolayers of primary keratinocytes were scratch wounded by a pipette tip and further cultured in the presence of growth factors: 10 U/ml HGF/SF (Weidner et al., 1993) and 20 ng/ml TGFα (Sigma-Aldrich). For immunostaining, cells were washed with PBS and fixed in 4% formaldehyde in PBS for 15 min at room temperature. The following antibodies were used: antivinculin (Sigma-Aldrich); antipaxillin (BD Biosciences); anti–phospho-c-Met, anti-RhoA, anti–γ-tubulin (Santa Cruz Biotechnology); and anti-VASP (Cell Signaling Technology). Additional reagents used were TRITC/FITC phalloidin (Sigma-Aldrich) and YO-PRO (Invitrogen).

Online supplemental material

Fig. S1 shows the expression of c-Met in bulge stem cells. Fig. S2 shows isolated keratinocytes from control and conditional c-Met mutant mice. The online version of this article is available at http://www.jcb.org/cgi/content/full/jcb.200701086/DC1.

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