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# Epidermal Development and Wound Healing in Matrix Metalloproteinase 13-Deficient Mice

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## Abstract

Degradation of the extracellular matrix, which is an indispensable step in tissue remodelling processes such as embryonic development and wound healing of the skin, has been attributed to collagenolytic activity of members of the matrix metalloproteinase family (MMPs). Here, we employed *mmp13* knockout mice to elucidate the function of MMP13 in embryonic skin development, skin homeostasis, and cutaneous wound healing. Overall epidermal architecture and dermal composition of non-injured skin were indistinguishable from wild-type mice. Despite robust expression of MMP13 in the early phase of wound healing, wild-type and *mmp13* knockout animals did not differ in their efficiency of re-epithelialization, inflammatory response, granulation tissue formation, angiogenesis, and restoration of basement membrane. Yet, among other MMPs also expressed during wound healing, MMP8 was found to be enhanced in wounds of MMP13-deficient mice. In summary, skin homeostasis and also tissue remodelling processes like embryonic skin development and cutaneous wound healing are independent of MMP13 either owing to MMP13 dispensability or owing to functional substitution by other collagenolytic proteinases such as MMP8.

## INTRODUCTION

Matrix metalloproteinases (MMPs), a family of structurally related neutral endopeptidases, play a central role in the breakdown of extracellular matrix (ECM). ECM degradation is essential for normal connective tissue remodelling in embryonic growth and development, morphogenesis, bone growth and resorption, wound healing, and uterine involution (Matrisian, 1992; Birkedal-Hansen *et al*., 1993; Nagase and Woessner, 1999; Vu and Werb, 2000; Stamenkovic, 2003). Additionally, incorrect regulation of MMPs is proposed to contribute to a variety of pathological processes characterized by increased matrix breakdown such as rheumatoid arthritis, atherosclerosis, and tumor invasion and metastasis (Mauch *et al*., 1993; Basset *et al*., 1997; Balbin *et al*., 2001, 2003; Neuhold *et al*., 2001). All MMPs display a conserved catalytic $Zn^{2+}$ binding site and can be inhibited by specific physiologic inhibitors, the tissue inhibitor of metalloproteinase (for review, see Vu and Werb, 2000; Sternlicht and



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Werb, 2001; Stamenkovic, 2003). At present, 25 different human MMPs have been characterized at the amino-acid sequence level (for review, see Vu and Werb, 2000; McCawley and Matrisian, 2001; Lynch and Matrisian, 2002). Closely related members of the MMPs are often classified into different subfamilies of collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs (for review, see Nagase and Woessner, 1999; Vu and Werb, 2000; Sternlicht and Werb, 2001). The collagenase subfamily of human MMPs comprises three different members: the fibroblast interstitial collagenase (MMP1), the neutrophil collagenase (MMP8), and collagenase-3 (MMP13). To date, homologues in mice have been identified for MMP8 and MMP13 but not for MMP1. In mouse and rat, MMP1 appears to be functionally substituted by other collagenolytic enzymes such as MMP13, MMP14 (MT1-MMP), and McolA (Mariani *et al*., 1998; Balbin *et al*., 2001; Wu *et al*., 2003). Collagenases are the only members of the MMP family that degrade native fibrillar collagens in the triple helical domain *in vivo* (Krane *et al*., 1996; for review, see Massova *et al*., 1998; Vu and Werb, 2000; Balbin *et al*., 2001; Sternlicht and Werb, 2001; Egeblad and Werb, 2002; Stamenkovic, 2003). Therefore, collagenases are thought to execute a rate-limiting function in ECM re-organization that is essential not only for morphogenesis, but also for tissue remodelling processes in the adult organism, such as cutaneous wound healing.

This repair process, which provides an entirely restored barrier to protect the body from external environmental exposition, involves multiple distinct processes including (1) migration, proliferation, and re-stratification of keratinocytes into a multilayered structure, (2) infiltration of inflammatory cells and formation of the granulation tissue, (3) neovascularization, and (4) restoration of stable adhesive interaction with the underlying dermal stroma (for review, see Fuchs, 1990; Singer and Clark, 1999). During wound healing, the ECM undergoes a dramatic re-organization at the wound site, mainly accomplished by the action of MMPs (Clark, 1996; Ravanti and Kahari, 2000; Vu and Werb, 2000). In addition, MMP activity causes the release of cryptic information from the ECM. By cleaving large ECM components and ECM-associated molecules, MMPs liberate bioactive fragments and molecules (Schenk and Quaranta, 2003; Mott and Werb, 2004) and thereby regulate angiogenesis (Xu *et al*., 2001; Hangai *et al*., 2002) and release growth factors and cytokines (Yu and Stamenkovic, 2000; Dangelo *et al*., 2001; Mu *et al*., 2002; Belotti *et al*., 2003). Thus, ECM proteolysis executed by MMPs may be a prerequisite for proper wound repair and accurate tissue regeneration. Degradation of the ECM that enables the movement of migrating keratinocytes between the collagenous dermis and the fibrin matrix deposited within hours after injury (Singer and Clark, 1999) presumably depends on the production of collagenolytic proteases produced by the keratinocytes (Pilcher *et al*., 1997; for review, see Seiki, 2002; Beare *et al*., 2003; Stamenkovic, 2003). Moreover, secretion of different MMPs by inflammatory cells such as neutrophils and macrophages facilitates infiltration into the granulation tissue (Clark, 1996; Beare *et al*., 2003). MMPs produced by the endothelial cells of newly formed blood vessels (Hiraoka *et al*., 1998; Werb *et al*., 1999; Langlois *et al*., 2004; Warner *et al*., 2004) may participate in angiogenesis (Singer and Clark, 1999; Seandel *et al*., 2001). Although the list of MMPs found to be expressed in the wound is constantly growing (Okada *et al*., 1997; Madlener *et al*., 1998; Saarialho-Kere, 1998; Lund *et al*., 1999; Planus *et al*., 1999; Hieta *et al*., 2003), the exact spectrum of MMPs critically involved in cutaneous wound healing has not been completely unravelled so far. Clearly, collagen degradation is required for keratinocyte migration in culture (Pilcher *et al*., 1997) and *in vivo* (Beare *et al*., 2003). The strong expression of MMP13 by migrating keratinocytes at the leading wound edge in the early phase of the healing process (Madlener *et al*., 1998) and its secretion by the dermal fibroblasts in the granulation tissue (Wu *et al*., 2003) suggests that this enzyme plays an important role in wound healing.

In this study, MMP13-deficient mice were generated to analyze the physiological function of MMP13 during skin development, homeostasis, and healing of excisional skin wounds.

# RESULTS

## Generation and phenotype of MMP13-deficient mice

To determine the role of MMP13 in embryonic skin development, skin homeostasis, and cutaneous wound healing, we crossed mice with a floxed *mmp13* (*mmp13* >/>) locus (Stickens *et al*., 2004) to CMV-cre *deleter* (Schwenk *et al*., 1995) to obtain ubiquitous disruption of the *mmp13* gene. By inter-crossing offspring, which were heterozygous for the mutated *mmp13* and the *cre* transgene, we obtained *mmp13* null (*mmp13* −/− *mmp13*), heterozygous (+/−), and wild-type (*mmp13* +/+) mice, transgenic and non-transgenic for *cre*, in the expected Mendelian ratio. Floxed *mmp13* mice did not differ from wild-type mice in MMP13 expression (Stickens *et al*., 2004, and data not shown) and were used as controls in this study. Complete *cre* recombination and loss of MMP13 transcripts in all tissues of *mmp13* null mice including skin were verified by Southern blotting (data not shown) and by *in situ* hybridization (Figure 2), Northern blot (Figure 5), and reverse transcription-PCR (data not shown).

Despite MMP13 deficiency, mice developed normally, were fertile and showed no gross phenotypic abnormalities, and long-term survival rates were indistinguishable from their wild-type littermates and the floxed controls, being in agreement with published data (Stickens *et al*., 2004). Histological examination of back skin of embryonic day 16.5 embryos (Figures 1a and b) and of tail (Figure 1c and d) and back skin (data not shown) of 7- to 8-week-old mice revealed by hematoxylin and eosin (H&E) staining showed no obvious difference between *mmp13* >/> and *mmp13* −/− in overall morphology. As the epidermis of back skin comprises only 2–3 keratinocyte layers, we used tail skin sections to analyze keratinocyte proliferation and epidermal differentiation pattern in the mutant animals (Figure 1c–j, and data not shown). No significant difference was observed in terms of keratinocyte proliferation rate, as demonstrated by Ki67 staining of tail skin sections (data not shown). Likewise, expression analysis of (i) keratin-5 (Figure 1e and f), a cytokeratin protein expressed in the basal keratinocyte layer of the skin, (ii) involucrin (Figure 1g and h), detectable in all suprabasal layers, and (iii) loricrin (Figure 1i and j), a protein representative of the *stratum granulosum*, indicated normal keratinocyte differentiation in *mmp13* knockout mice. Epidermal organization and dermal composition in skin of 6- and 12-month-old knockout mice were also unchanged when compared to controls (data not shown). In addition, we did not detect obvious increases in collagen deposition, fibrosis, or any other obvious abnormalities in the dermis of tail skin (data not shown) and back skin (Figure 1k and l). These data indicate that loss of MMP13 expression compromises neither proliferation and differentiation of epidermal keratinocytes nor the function of dermal fibroblasts and other cell types in mouse skin during embryonic development and homeostasis in adult mice.

## Cutaneous wound healing in *mmp13* −/− mice

To study the physiological role of MMP13 during the healing process, we generated full-thickness excisional wounds in the back skin of 8- to 12-week-old *mmp13* −/− and *mmp13* >/> mice. In agreement with published data (Madlener *et al*., 1998), during the early phase of wound healing, we observed an upregulation of MMP13 in the keratinocytes of the leading wound edge of wild-type and floxed controls, by *in situ* hybridization (Figure 2a, and data not shown). In contrast, in wounds of *mmp13* −/− mice, MMP13 expression was extinguished (Figure 2b), confirming MMP13 deficiency in skin keratinocytes.

Macroscopic examination of wounds from *mmp13* −/− and control animals revealed a comparable healing process in all individuals tested (data not shown). To assess the re-epithelialization potential of *mmp13* −/− keratinocytes from day 1 to 10 post wounding, we applied detailed histological analysis of knockout and control wounds and studied keratinocyte differentiation pattern and wound closure kinetics (Figures 2c–l and Figure S1, and data not

shown). In all cases, 24 hours post injury wound beds of both strains were just covered by a fibrin clot, and keratinocytes had not yet formed a continuous layer (Figure 2c and d). In some instances, wounds of *mmp13* −/− as well as *mmp13* >/> mice were already re-epithelialized at day 3 after wounding. This was evidenced by a complete keratinocyte layer (Figure 2e and f) and continuous involucrin staining throughout the former wound area (Figure 2g and h). In both strains, epidermal re-organization was completed 7 days post wounding, as proven by staining with antibodies against the early differentiation marker keratin-10 (Figure 2i and j) and the late differentiation marker loricrin (Figure 2k and l). Both proteins were expressed at normal levels and within the adequate layers. Measuring the distance between the leading wound edges on sections of *mmp13* −/− and control mice at different time points after wounding revealed no significant difference in wound closure at days 2, 3, and 5 (Figure S1).

These data show that re-epithelialization after cutaneous injury is not significantly affected even though MMP13 expression is lost in the keratinocytes of the leading wound edge, suggesting either that MMP13 is not required during this process or can be functionally replaced by other MMPs.

## Granulation tissue formation and angiogenesis at the wound sites

Dermal fibroblasts of the granulation tissue express reasonable amounts of MMP13 (Madlener *et al*., 1998; Wu *et al*., 2003), suggesting a contribution of this protease in granulation tissue formation and angiogenesis. Therefore, we explored the size and composition of the granulation tissue in wounds of *mmp13* −/− and control animals. Concerning the size of the granulation tissue, which was visible in both strains 2–3 days post wounding (Figure 3c–h), no alterations were found in wounds of *mmp13* null mice compared to the floxed controls (Figure 3, and data not shown). To determine the inflammatory response, tissue sections were analyzed for the infiltration of macrophages and neutrophils. As shown in Figure 3a–d, the number of infiltrated neutrophils was comparable in wounds of *mmp13* −/− and *mmp13* >/> mice. Similar data were observed for the number of infiltrated macrophages (Figure 3g and h).

Angiogenesis, one of the important events during wound healing, was explored on wound sections of MMP13-deficient and *mmp13* floxed mice by CD31 immunostaining. No significant differences in vessel density and size were observed in uninjured skin (data not shown) or 7 days after injury (Figure 3i–l). These data imply that MMP13 deficiency interferes neither with the formation of granulation tissue nor with angiogenesis at cutaneous wound sites.

## Basement membrane and dermal re-organization at the wound sites in MMP13-deficient mice

To re-establish proper dermal–epidermal junctions after re-epithelialization, an intact basement membrane has to be reconstituted by keratinocytes and fibroblasts. Recently, components of the basement membrane, such as perlecan and laminin-5, and cell surface receptors, such as integrins and dystroglycans, which interact with the basement membrane network, have been demonstrated to be substrates of a number of MMPs including MMP13 (Mott and Werb, 2004).

To demonstrate a possible function of MMP13 in the regeneration of basal lamina, we followed the deposition of various basement membrane components during the wound healing process. Immunostaining of histological sections for laminin-5 (Figure 4a–d), α6-integrin (Figure 4e–h), perlecan, and nidogen (data not shown) revealed no differences in protein expression pattern and intensity between wounds of *mmp13* knockout and control mice. These data indicate that basement membrane re-organization after wound repair appears to be unaffected by the loss of MMP13 activity.

Large amounts of ECM proteins, most prominently collagens, are synthesized, deposited, and remodelled during wound healing until the final reconstitution of the dermal matrix is achieved. To follow the expression level and quality of the collagen network, fibrillar collagen was visualized by Picrosirius staining throughout the course of wound healing on sections of control and *mmp13* knockout mice from day 1 to 30 post wounding (Figure 4i–n, and data not shown). We could not detect any obvious abnormalities in the distribution, mass, or arrangement of the collagen fibers in the dermal compartment of the MMP13-deficient wounds. These data demonstrate that loss of MMP13 enzyme activity does not affect the formation and remodelling of fibrillar collagen during the entire wound healing process and indicate that a high degree of functional redundancy between MMP13 and other members of the MMP family occurs during initial and late phase of remodelling and tissue repair.

## Expression pattern of MMPs in wounds of MMP13-deficient mice

In wild-type and *mmp13* floxed mice, highest MMP13 expression levels were observed 1 and 2 days post wounding (Figure 5c, and data not shown; Madlener *et al*., 1998). To narrow down the potential candidates compensating for MMP13 deficiency in mutant mice during this early phase of wound healing, we determined the expression of other MMPs found in wounds shortly after injury (Figure 5).

The first candidate we analyzed was MMP9, which is frequently expressed at sites of active tissue remodelling such as the moving epidermal layer in cutaneous wounds (Salo *et al*., 1994; Mohan *et al*., 1998; Lund *et al*., 1999) and functionally cooperates with MMP13 in long bone development and homeostasis (Inada *et al*., 2004; Stickens *et al*., 2004). In agreement with published data (Lund *et al*., 1999), MMP9 expression originated predominantly from keratinocytes, which are located at the leading edge of the wound (Figure 5a and b), and completely overlapped with the pattern of MMP13-expressing cells in wild-type mice (Figure 2a). Upon quantification however, the amount of MMP9 transcripts was not significantly enhanced compared to wounds of floxed controls, as shown by Northern blot analysis of RNA prepared from day 1 and 2 wounds (Figure 5c).

Two other MMPs that are capable of cleaving native, undenatured fibrillar collagens *in vivo* are MMP8, which is predominantly expressed by neutrophils invading the wound area (Devarajan *et al*., 1991; Krane *et al*., 1996; Balbin *et al*., 1998), and McolA, the putative murine counterpart of the human interstitial collagenase MMP1 (Balbin *et al*., 2001). Although McolA expression has only been described for embryonic tissues so far (Balbin *et al*., 2001), McolA transcripts could be detected in wild-type mice from day 1 to 5 after wounding (data not shown), and in wounds of *mmp13* −/− mice its expression level was marginally reduced compared to control (Figure 5d). By contrast, expression of neutrophil collagenase MMP8 was significantly enhanced in wounds of *mmp13* −/− mice, as measured by Northern blot analysis and semiquantitative PCR (Figure 5d, and data not shown). Immunohistochemical analysis of wounds from control and mutant mice 3 days post injury revealed no sign of additional cell types expressing MMP8 at the wound sites of *mmp13* −/− mice (Figure S2).

Finally, we analyzed the expression levels of MMP2, MMP3, and MMP14 (MT1-MMP), which exhibit collagenolytic activity, at least *in vitro* (Overall and Sodek, 1992; Sodek and Overall, 1992; Ohuchi *et al*., 1997; Seandel *et al*., 2001; Tam *et al*., 2002, 2004). The amount of these transcripts was even slightly reduced in MMP13-deficient wounds except for MMP14, which remained unchanged (Figure 5d).

These data demonstrate that a series of MMPs are expressed during re-epithelialization. Some MMPs showed enhanced or reduced expression in MMP13-deficient wounds. Thus, most likely, the activity of one or multiple members of the MMP family appears to be responsible for the functional compensation of MMP13 deficiency.

## DISCUSSION

MMPs are important players in tissue remodelling and repair processes, in which they mediate proteolysis of the ECM predominantly composed of fibrillar collagens. In the mouse, *in vivo* collagenolytic activity has been widely attributed to MMP13 activity, the major interstitial collagenase in this species. In this study, we functionally determined the role of this protease during tissue remodelling and repair processes, such as skin development and cutaneous wound repair, employing the powerful knockout technology. To achieve MMP13 deficiency in mice, a targeted *mmp13* locus with floxed exons (Stickens *et al*., 2004) was inter-crossed with the CMV-cre strain (Schwenk *et al*., 1995). Despite the fact that MMP13 was suggested to be involved in postpartum involution, at least in rats (Wolf *et al*., 1996), as well as in very early embryo development (Yamamoto *et al*., 1998) and in microvessel formation (Burbridge *et al*., 2002), we found no impact of MMP13 deficiency on reproduction or embryonic survival. Yet, in line with the massive expression of MMP13 during long bone development (Gack *et al*., 1995) and homeostasis (Tuckermann *et al*., 2000), MMP13 inactivation caused abnormal skeletal growth plate development (Inada *et al*., 2004; Stickens *et al*., 2004; unpublished data). To date, MMP13 expression has only been detected during endochondral ossification but not in embryonic or newborn skin (Gack *et al*., 1995). In line with these data, the unaltered skin architecture of the *mmp13* −/− mice shows that this member of the family of interstitial collagenases obviously does not play a role during skin development and skin homeostasis.

Mice that express a collagenase-resistant version of collagen suffer from impaired tissue remodelling and spontaneously develop skin fibrosis in aged unchallenged animals (Liu *et al*., 1995), underscoring the need for collagenolytic enzymes. However, our data strongly imply that MMP13 is not the critical candidate, as no sign of fibrotic depositions was seen in the skin of the *mmp13* −/− mice, even after 12 months of age. Thus, the assigned function of MMP13, as an interstitial collagenase cleaving fibrillar collagen in skin homeostasis, may be compensated by other members of the MMP family such as MMP2 and MMP14 (MT1-MMP) in *mmp13* mutant mice. It was shown previously that both MMP2 and MP14 are expressed in unchallenged skin (Madlener *et al*., 1998) and are capable of cleaving fibrillar collagen, at least *in vitro* (Overall and Sodek, 1992; Sodek and Overall, 1992; Ohuchi *et al*., 1997; Seandel *et al*., 2001; Tam *et al*., 2002, 2004).

Similar to MMP3, MMP9, MMP8, MMP10, MMP11, and Mcol-A (Madlener *et al*., 1998; Nwomeh *et al*., 1998; our unpublished data), MMP13 expression is strongly upregulated shortly after injury and declines when re-epithelialization is completed (Madlener *et al*., 1998; Nwomeh *et al*., 1998; Lund *et al*., 1999). In the early phase of wound healing, MMP13 transcripts are found in the migrating keratinocytes located at the wound edges, and at later phases of wound repair when re-epithelialization is completed, insular fibroblasts express the enzyme in the granulation tissue (Madlener *et al*., 1998; Wu *et al*., 2003; our unpublished data). Mice expressing a collagenase-resistant version of collagen displayed a severely delayed wound healing (Beare *et al*., 2003), demonstrating the need for collagenases in healing of the injured skin, even though the identity of the specific MMP in charge is not clear. Upon performing excisional wounding experiments on the back skin of *mmp13* −/− and control mice, we observed no significant effects on the wound healing process. Angiogenesis and collagen network assembly in the area of the former wound bed are unimpaired. Although MMP13 is strongly upregulated during the early phase of wound healing, we found no changes in re-epithelialization, epidermal re-organization, granulation tissue formation, inflammatory response, re-assembly of the basement membrane, and tissue remodelling in *mmp13* −/− mice. These results are in agreement with data from wound healing studies performed in mice deficient for other MMPs. Except for mice lacking MMP14, which develop fibrosis of the dermis in unchallenged skin (Holmbeck *et al*., 1999, 2004), none of the MMP knockout mice

generated so far displayed a severe phenotype in wound healing (Bullard *et al*., 1999; Mohan *et al*., 2002; Mauch, 2003).

Based on these results, it is reasonable to postulate that skin development, homeostasis, and wound healing do not rely on a single proteinase, but rather benefit from the availability of an array of MMPs that are simultaneously expressed. Facing the fact that most MMPs exhibit broad substrate specificity and that many MMPs share the same substrates (McCawley and Matrisian, 2001; Sternlicht and Werb, 2001), redundancy among the MMPs can be postulated. Collagen type I, being the preferential substrate of MMP13, together with collagen type II and III (Knäuper *et al*., 1996; McCawley and Matrisian, 2001; Sternlicht and Werb, 2001) can also be cleaved by non-MMP proteolytic enzymes such as serine proteases (Hibbs *et al*., 1983; Dano *et al*., 1985; Kofford *et al*., 1997; Ashcroft *et al*., 2000; Behrendt, 2004; Iba *et al*., 2004; Provenzano *et al*., 2005). A role in ECM degradation and tissue remodelling during cutaneous wound healing has already been demonstrated for the serine protease plasmin, which is the effector protease of the urokinase plasminogen activator system (Romer *et al*., 1991, 1994, 1996). Hence, we suggest that collagenolytic proteases other than MMP13 may also substitute for the loss of collagenolytic activity in *mmp13* −/− mice during these processes.

One obvious candidate for the compensatory assignment by MMP family members is MMP9, which, like MMP13, is expressed by keratinocytes of the leading wound edge. Moreover, MMP9 was found in *mmp13* −/− mice to be upregulated during embryonic bone development and was shown to synergize with MMP13 during endochondral ossification (Inada *et al*., 2004; Stickens *et al*., 2004). However, monitoring MMP9 expression in wounds of MMP13-deficient mice 1 and 2 days post injury revealed no significant increases in the level of MMP9 transcripts. Furthermore, the mild epidermal wound closure phenotype observed in *mmp9* knockout mice was not further impaired by an additional loss of MMP13 in *mmp9*/*mmp13* double-knockout animals (B. Hartenstein, B. Dittrich, B. Arnold, and P. Angel, unpublished data). These data suggest that in contrast to the process of endochondral ossification, MMP9 does not compensate for the loss of collagenase activity during cutaneous wound healing in MMP13-deficient mice.

Similarly, another candidate for substitution of MMP13, MMP14, a proteinase with the capacity to cleave native collagen (Tam *et al*., 2004) and which is expressed during wound healing (Madlener *et al*., 1998) and upregulated in bones of *mmp13* −/− embryos (Inada *et al*., 2004), also remained unchanged at the mRNA transcript level in MMP13-deficient wounds.

Interestingly, we detected enhanced expression of MMP8 in the early phase of cutaneous wound healing in MMP13-deficient mice, corresponding with the induced expression level of this neutrophil-specific collagenase in bone marrow cavities of newborn *mmp13*−/− mice (Inada *et al*., 2004). Although expression of MMP8 has recently been detected in a number of other cell types (Wang *et al*., 2004), neutrophils that infiltrate the granulation tissue are postulated to be the main source of MMP8 in wounds (Balbin *et al*., 1998; Madlener *et al*., 1998; Armstrong and Jude, 2002). As quantification of the number of neutrophils in the granulation tissue of *mmp13*−/− wounds was unchanged and no additional cell types, such as keratinocytes, exhibited MMP8 protein in mutant wounds, augmented MMP8 transcript levels in skin very likely originated from enhanced expression of this gene in neutrophils by an as yet unknown mechanism. Thus, increased MMP8 activity may be one of the events during the process of cutaneous wound healing in *mmp13* null mice that counteracts the harmful consequences of reduced collagenolytic activity caused by MMP13 deficiency.

In humans, cutaneous wound healing is associated with the induction of another interstitial collagenase, MMP1, which shares a broad overlap in substrate specificity with MMP13, at least *in vitro* (McCawley and Matrisian, 2001; Sternlicht and Werb, 2001). Facing the fact that

a true homologue for MMP1 has not been identified in mice, the issue of functional redundancy in humans might be even more complicated and has to additionally take into account MMP1.

In the face of functional redundancy in skin homeostasis and remodelling, future analyses of isolated cell types, such as keratinocytes, in appropriate tissue culture systems mimicking the *in vivo* situation, as well as wound healing studies on skin of a variety of knockout mice that are deficient for multiple MMPs, will have to be performed in order to identify the nature of cooperating MMPs and to unravel the underlying molecular mechanism of this functional collagenolytic network, which is obviously absolutely required for survival of a complex higher organism.

## MATERIALS AND METHODS

### Mice

To generate MMP13-deficient mice, floxed *mmp13* mice (Stickens *et al*., 2004) were crossed to CMV-cre mice (Schwenk *et al*., 1995), ensuring ubiquitous disruption of the *mmp13* gene. The mice used were in a mixed genetic background (C57BL/6;129/SV). The recombination of the floxed *mmp13* alleles was analyzed by Southern and Northern blotting. All mice were housed in specific pathogen-free environment and under light-, temperature-, and humidity-controlled conditions. Food and water were available *ad libitum*. The procedures for performing animal experiments were in accordance with the principles and guidelines of the ATBW (officials for animal welfare) and were approved by the Regierungspräsidium Karlsruhe, Germany.

### Wounding study

Control (floxed *mmp13*) and MMP13-deficient female mice aged 7–8 weeks were anesthetized with Ketanest and four paired 4mm diameter, full-thickness, excisional wounds were made using a sterile biopsy punch through the shaved and fumigated skin on the back of each mouse. At 1, 2, 3, 5, 7, and 14 days post wounding, mice were killed by cervical dislocation and wound tissue and surrounding wound margin skin were harvested and embedded in O.C.T.™ Compound (Tissue Tek, Vogel, Giessen, Germany). For each time point, 6–8 wounds originating from different mice were analyzed. Cryosections (5$\mu$m) of the wounds were stained with H&E and documented using a Leica (DMLB) microscope.

### Immunofluorescence analyses and immunohistochemistry

Cryosections (5$\mu$m) of wounds and tail skin were stained with the following antibody solutions: rabbit anti-mouse loricrin (1:100; BAbCo, Richmond, CA), rabbit anti-mouse keratin-10 (1:250; BAbCo, Richmond, CA), rat anti-mouse CD68 (1:100; Serotec, Düsseldorf, Germany), rat anti-mouse neutrophils (1:400; Serotec, Düsseldorf, Germany), rat anti-mouse CD31 (1:100; Pharmingen, Heidelberg, Germany), rabbit anti-human MMP8 (1:500; Chemicon Europe, Hampshire, UK), rabbit anti-human laminin-5 $\gamma$2 (1:2,000; kindly provided by Dr G. Meneguzzi), rat anti-mouse α6-integrin (1:400; kindly provided by Professor A. Sonnenberg), rabbit anti-mouse involucrin (1:800; kindly provided by Professor F. Watt), and guinea-pig anti-human keratin-5 (1:500; generous gift from Dr L. Langbein).

For immunofluorescence analyses, cryosections were fixed in 100% methanol (4°C) for 1minute and acetone (−20°C) for 2 minutes (α6-integrin, laminin-5), in 80% methanol (4°C) for 5 minutes and acetone (−20°C) for 2 minutes (neutrophils, CD31, CD68), in acetone (−20° C) for 2 minutes (keratin-5, keratin-10, loricrin), in 80% methanol (room temperature) for 5 minutes (MMP8), or left unfixed (involucrin). For MMP8, cryosections were incubated for 20 minutes in citrate buffer (2m$_M$ citric acid, 10m$_M$ tri-sodium citrate, pH 6.0) at 85°C with subsequent rinsing in phosphate-buffered saline (PBS). Cryosections were then blocked with

1% BSA (Sigma, Deisenhofen, Germany) in PBS and incubated with the primary antibodies diluted in the blocking solution for 2 hours at room temperature (α6-integrin, keratin-10, loricrin, laminin-5) or overnight at 4°C (CD31, CD68, neutrophils, MMP8, involucrin, keratin-5). Subsequently, sections were washed with PBS and incubated with Cy3-conjugated goat anti-rat secondary antibody (for α6-integrin, CD31, neutrophils, CD68; 1:400; Dianova, Hamburg, Germany), Cy3-conjugated goat anti-rabbit secondary antibody (for involucrin, loricrin; 1:400; Dianova, Hamburg, Germany), Cy2-conjugated goat anti-rabbit secondary antibody (for keratin-10; 1:400; Dianova, Hamburg, Germany), Cy2-conjugated donkey anti-guinea-pig secondary antibody (for keratin-5; 1:400; Dianova, Hamburg, Germany), or Alexa-488-conjugated goat anti-rabbit secondary antibody (for laminin-5, MMP8; 1:400; Molecular Probes, Göttingen, Germany) for 1hour at room temperature. The nuclei were counterstained with Hoechst dye H33342 (1:1,000; Calbiochem-Novabiochem, Schwalbach, Germany). Finally, the cryosections were rinsed and mounted.

For immunohistochemistry, cryosections were fixed in 100% methanol (4°C) for 5 minutes and acetone (4°C) for 10 minutes. After rinsing with PBS, endogenous peroxidase was quenched with 3% $H_2O_2$ in methanol for 10 minutes at room temperature. The cryosections were then blocked with 1% BSA (Sigma, Deisenhofen, Germany) in PBS and incubated with the primary antibodies (keratin-10, loricrin) diluted in blocking solution for 1 hour at room temperature and followed by incubation with a peroxidase-coupled secondary antibody plus streptavidin–peroxidase complex and DAB staining (Vectastain ABC kit, Vector Laboratories, Burlingame, UK). Sections were counterstained with hematoxylin.

## Collagen staining

Cryosections (5$\mu$m) of wounds and back skin were stained with Picrosirius red as described elsewhere (Junqueira *et al*., 1979) and imaged on a Zeiss Axioplan microscope. Images were captured using a Hamamatsu 3 CCD cooled camera (Hamamatsu, Hamamatsu City, Japan) and Simple PCI imaging software (Compix Imaging Systems, Pittsburgh, PA).

## RNA extraction, Northern blotting, and reverse transcription-PCR

Total RNA was extracted from wounds 1 day after injury using the peqGold RNAPure™ kit (PeqLab, Erlangen, Germany) following the manufacturer's protocol. For Northern blot analysis, 10$\mu$g of total RNA was denatured in glyoxal/DMSO buffer (1M Glyoxal, 10mM $Na_2HPO_4$ (pH 6.9), and 50% DMSO) and electrophoresed on a 1.4% agarose gel in 10mM $Na_2HPO_4$ (pH 6.9), blotted onto Hybond N+ membrane (Amersham Pharmacia Biotechnology, Freiburg, Germany), and hybridized at 65°C overnight with Church-Gilbert hybridization buffer (1mM EDTA, 0.25M NaPi, 7% SDS). As probes, the complete cDNA for MMP13 (Gack *et al*., 1995) and a *Bam*HI/*Xho*I fragment of the MMP9 cDNA spanning exon 3 to exon 8 (Reponen *et al*., 1995) were used. A cDNA probe for rat GAPDH was taken as an internal control for RNA quality and quantity. Probe labelling with [$^{32}$P]dCTP was performed using the RediPrime™ Labelling kit (Amersham Pharmacia Biotechnology, Freiburg, Germany).

Single-strand cDNAs were synthesized from 1$\mu$g of total RNA using AMV reverse transcriptase and oligo(dT) as primer (Promega, Mannheim Germany); reverse transcriptase products were amplified by semiquantitative PCR for different MMPs to compare transcript amounts. All PCR primer pairs used are intron-spanning on separate exons: MMP2 (495bp) sense 5′-ccgggagcgcaagatgga-3′ and antisense 5′-gagaaaagcgcagcggagtgacg-3′, MMP3 (673bp) sense 5′-attgcatgacagtgcaaggg-3′ and antisense 5′-tggaggacttgtagactggg-3′, MMP8 (484bp) sense 5′-ggcagaggaaggcaggagagg-3′ and antisense 5′-aaggtcaggggcgatgctacact-3′, MMP14 (516bp) sense 5′-ggcctgcctgcatccatcaata-3′ and antisense 5′-actgccctcctcatccacctcaat-3′, Mcol-A (374bp) sense 5′-gaccttccccaaatcccatcc-3′ and antisense

5′-ctcttcctcacaaacagcagcatc-3′. *β*-Tubulin (401bp) was used as an internal standard using the primers sense 5′-gcgacctgcagctggaccgaatct-3′ and antisense 5′-gggcgagggcaccacactgaagg-3′. For Mcol-A and MMP8, after electrophoresis the PCR products were blotted onto Hybond N + membrane and hybridized at 65°C overnight with Church-Gilbert hybridization buffer. Hybridizations were performed with the corresponding PCR fragments as probes.

### *In situ* hybridization

For *in situ* hybridization, 5$\mu$m cryosections from wounds were treated and hybridized with $^{35}$S-UTP-labelled sense and antisense probes, as described previously (Tuckermann *et al*., 2000). MMP13 cRNA antisense probe spanning exon 5 to exon 3 (246bp including nucleotides 595 to 429 of the published sequence) was derived by sp6 *in vitro* transcription from a plasmid containing an MMP13 cDNA fragment spanning exon 1 to exon 5 linearized at an internal StuI site. MMP9 cRNA antisense probe spanning exon 8 to exon 2 (904bp including nucleotides 1,275 to 371 of the published sequence) was derived by T7 *in vitro* transcription from plasmid linearized at a NotI site.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

ECM, extracellular matrix; >, floxed allele; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline.

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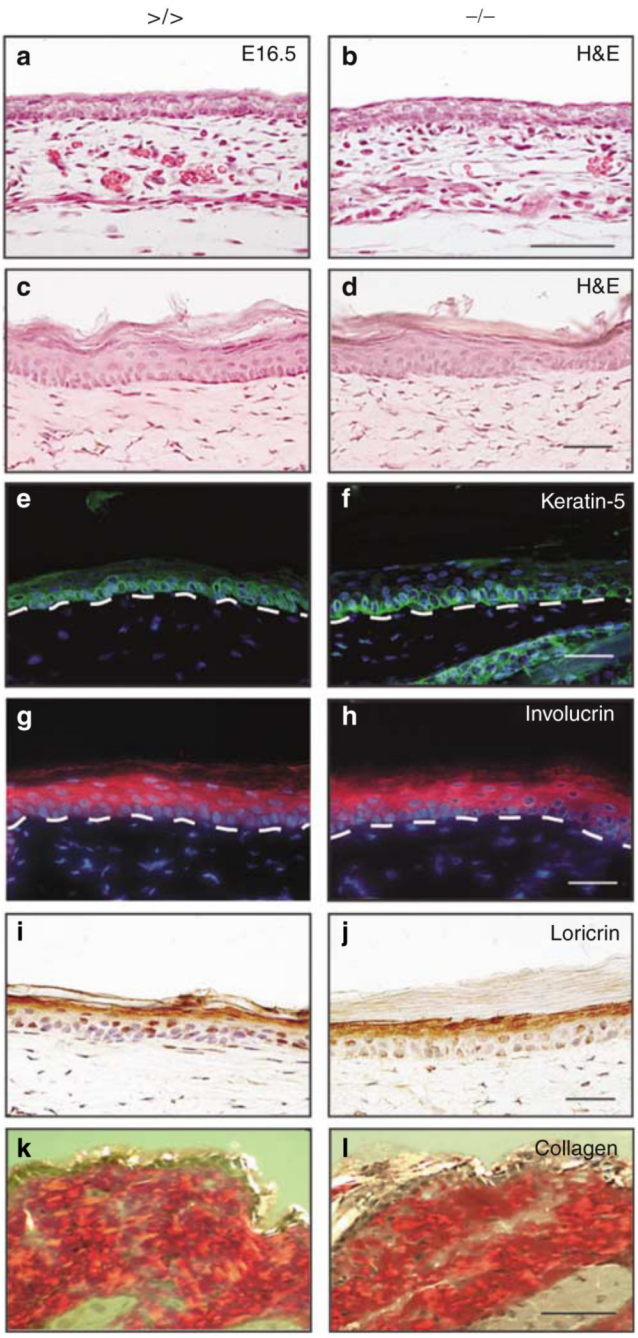


**Figure 1. Histopathological analyses of epidermal tissue morphology**

(**a, b**) H&E of sections from back skin of embryonic day (E)16.5 embryos of (**a**) controls and (**b**) *mmp13* −/− mice. Bar: 100$\mu$m. (**c–l**) Tail skin sections (**c–j**) and back skin sections (**k, l**) of adult mice (left panel: *mmp13* >/> and right panel: *mmp13* −/−) were either stained with H&E (**c, d**) or analyzed by immunofluorescence for (**e, f**, green signal) keratin-5 and (**g, h**, red signal) involucrin expression. Nuclei were counterstained with Hoechst dye (blue signal). The basement membrane is indicated by a dashed line. (**i, j**, brown signal) Loricrin was visualized immunohistochemically and, for this analysis, nuclei were counterstained with hematoxylin (blue signal). (**k, l**) Collagen fibers in the dermis of back skin were stained with Picrosirius red and visualized by crossed polarization. Bars: 50$\mu$m.

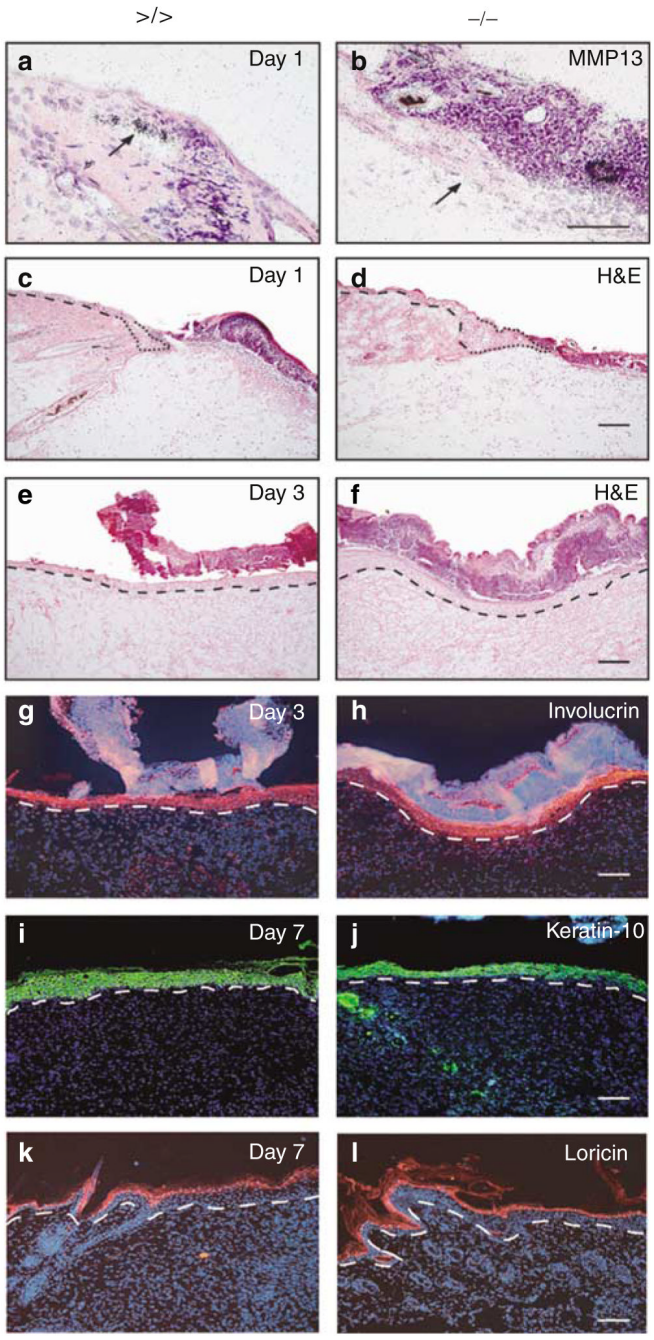


**Figure 2. MMP13 expression analysis and microscopic assessment of wounds in MMP13-deficient mice**

(**a, b**) *In situ* hybridization for MMP13 on wounds of (**a**) *mmp13* >/> controls and (**b**) *mmp13* −/− mice followed by H&E staining to detect transcripts on sections of the left-hand site of wounds 1 day post injury. The arrow points to keratinocytes of the leading wound edge. Bar: 50$\mu$m. (**c–f**) H&E-stained sections of the left-hand site of wounds (**c, d**) 1 day and (**e, f**) 3 days after injury. The dashed line indicates basement membrane. The migrating tip is marked by the dotted line. Bar: 100$\mu$m. (**g, h**) Full re-epithelialization of *mmp13* −/− and control wounds, as demonstrated by wound sections taken 3 days post injury and immunostained for involucrin (red signal). Nuclei were counterstained with Hoechst dye (blue signal), which also

stained the scab. The basement membrane is indicated by a dashed line. Bar: 100$\mu$m. (**i–l**) Sections of wounds taken 7 days post excision were immunostained for the differentiation marker (**i, j**, green signal) keratin-10 and (**k, l**, red signal) loricrin to demonstrate complete keratinocyte differentiation. Nuclei were counterstained with Hoechst dye (blue signal). The dashed line indicates basement membrane. Bars: 100$\mu$m.

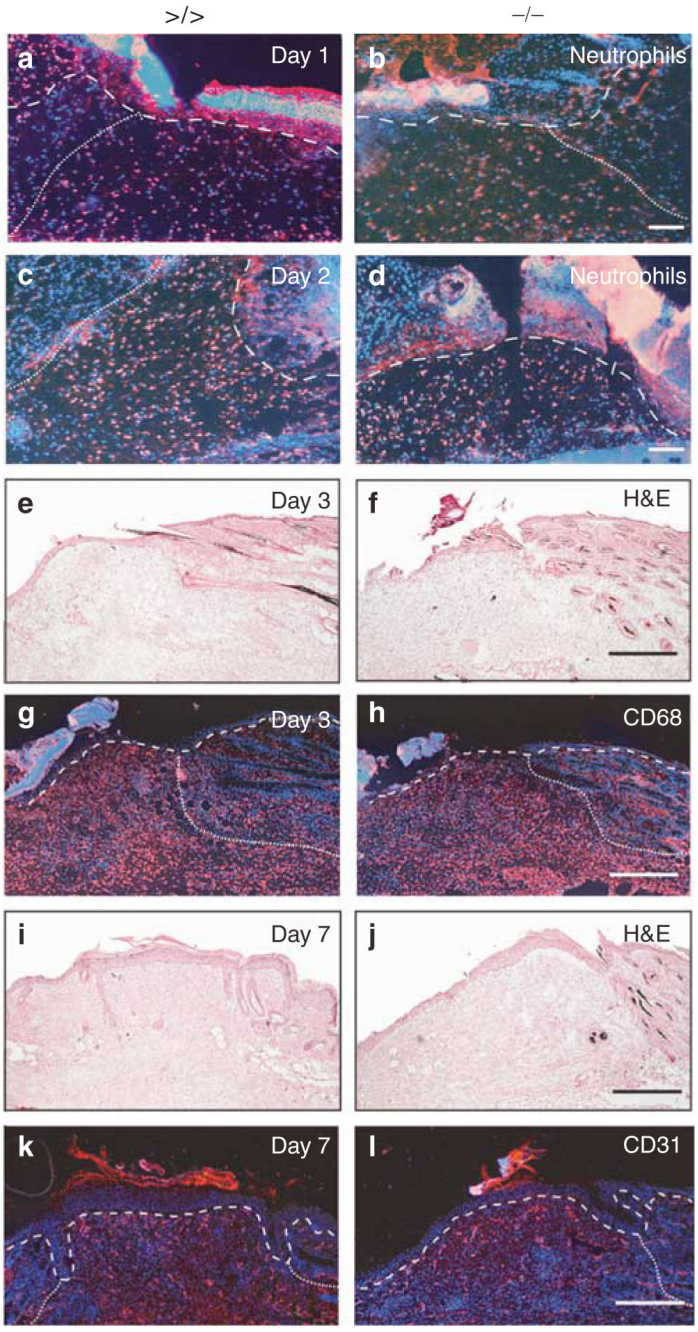


**Figure 3. Histological analyses of the granulation tissue at wound sites of *mmp13* −/− mice**

(**a–d**) Neutrophils located in the granulation tissue of (**a, b**) day 1 and (**c, d**) day 2 wounds of *mmp13* >/> (left panel) and *mmp13* −/− (right panel) mice were stained by immunofluorescence (red signal) using the antibody *neutrophils*. Nuclei were counterstained with Hoechst dye (blue signal). The basement membrane is depicted by a dashed line and the granulation tissue is highlighted by a dotted line. (**e–h**) Bar: 100$\mu$m. (**e, f**) H&E staining and staining of macrophages by (**g, h**, red signal) CD68 immunofluorescence on wound sections 3 days post wounding. Bar: 300$\mu$m. (**i–l**) H&E staining (**i, j**) and staining of endothelial cells by (**k, l**, red signal) CD31 immunofluorescence on wound sections 7 days after wounding. Bars: 300$\mu$m.

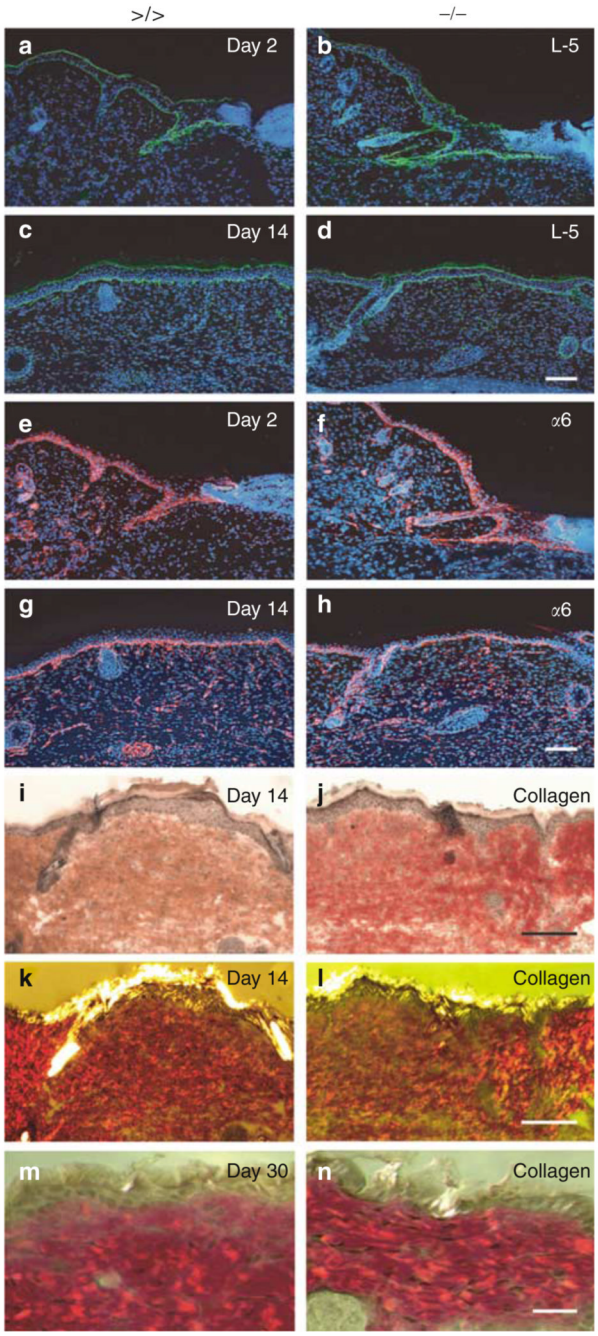


**Figure 4. Immunofluorescence analyses of basement membrane re-organization and visualization of dermal collagen fibers in wounds of *mmp13* −/− mice**

(**a–h**) Laminin-5 (**a–d**, green signal) and (**e–h**, red signal) α6-integrin were visualized by immunofluorescence on sections of *mmp13* >/> (left panel) and *mmp13* −/− (right panel) wounds analyzed 2 and 14 days after injury. Nuclei were counterstained with Hoechst dye (blue signal). Bars: 100$\mu$m. (**i–n**) To visualize collagen fibers of wounds taken at (**i–l**, bar: 50$\mu$m) day 14 and (**m, n**, bar: 30$\mu$m) day 30, sections were stained by Picrosirius red and documented by both (**i, j**) bright field and (**k–n**) crossed polarization.

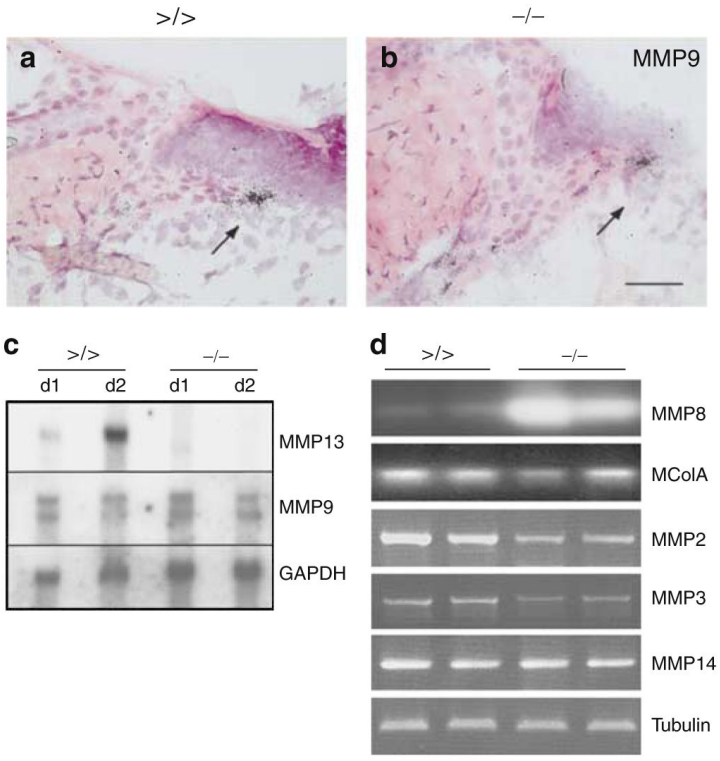


**Figure 5. MMP expression analyses in the early phase of wound healing**

(**a, b**) *In situ* hybridization followed by H&E staining to detect MMP9 transcripts on sections of the left-hand site of wounds from (**a**) *mmp13* >/> and (**b**) *mmp13* −/− mice. The arrow points to keratinocytes of the leading wound edge. Bar: 50$\mu$m. (**c**) Northern blot analyses were performed with total RNA prepared from day 1 and 2 wounds of *mmp13* >/> and *mmp13* −/− mice and hybridized to MMP13 and MMP9 cDNA. Quality and quantity of the RNA was confirmed by visualization of GAPDH. (**d**) Semiquantitative PCR of different MMPs performed on cDNA prepared on total RNA isolated from day 1 wounds of *mmp13* >/> and *mmp13* −/− mice. After gel electrophoresis, PCR products of MMP8 and McolA were blotted and hybridized to the corresponding PCR fragments. The autoradiograph was scanned and reverted. $\beta$-Tubulin was used as an internal standard.