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HMGB1 stimulates bone marrow mesenchymal stem/stromal cells to accelerate skin regeneration

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High mobility group box 1 (HMGB1), which is released by necrotic tissue, mobilizes platelet-derived growth factor receptor alpha-positive mesenchymal stromal cells (PDGFRa$^+$ MSCs) into circulation as a rescue signal. However, precise roles of HMGB1-induced endogenous MSCs for skin regeneration are still unclear. In this study, we investigated functions of HMGB1/BM-MSC axis for regeneration processes of mouse skin graft. We found that intravenous HMGB1 administration induced accumulation of BM-MSCs followed by significant therapeutic changes in the skin graft, such as less inflammatory infiltration, enhanced revascularization and accelerated epithelial regeneration. To further understand precise mechanisms of HMGB1/BM-MSC axis in the skin regeneration processes, we transplanted FACS-sorted c-kit$^+$ BM hematopoietic stem/progenitor cells into the lethally irradiated mice to generate mice with reduced PDGFRa$^+$ cells in BM (ckit$^+$ BMT mice). MSC accumulation in the skin graft was significantly reduced in the c-kit$^+$ BMT mice, resulting in enhanced inflammation and retarded regeneration as compared with total BMT mice. Furthermore, c-kit$^+$ BMT mice showed impairment in HMGB1-mediated regeneration of the skin graft. We further uncovered that intravenously administered HMGB1 induced expression of CXCR4, a receptor of SDF-1, on BM-MSCs *in vivo*. Systemic administration of CXCR4 antagonist AMD3100 inhibited HMGB1-induced MSC accumulation in the skin graft, indicating that HMGB1-mediated CXCR4 up-regulation on BM-MSCs plays a crucial role for MSC accumulation in the skin graft. We finally investigated therapeutic activity of the HMGB1/MSC axis in inflammatory skin diseases by intravenously administered HMGB1 to the mice with allergic contact dermatitis. Systemic HMGB1 administration significantly suppressed the inflammatory changes of the mouse ear skin. Taken together, these data provided here illustrate a pivotal role of HMGB1/BM-MSC axis for promoting healing of the injured/inflamed skin. Our studies may provide future perspectives of HMGB1 medicine for intractable skin diseases.

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Delayed healing in a model of sickle cell disease relates to impairment of endothelial progenitor cell mobilization

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Sickle cell disease (SCD), the most diagnosed genetic disease in Europe, is secondary to mutations in the b globin gene. Leg ulcer is a major manifestation of SCD responsible for pain, infection, ankylosis and depression. Pathogenesis of these prolonged ulcers remains unknown. We were able to show that mice carrying a human b globin transgene displaying 3 mutated amino acids (SAD) presented delayed healing in old mice and represented therefore a murine model for this disease. The current study aimed to understand the mechanisms responsible for delayed healing in SCD. The delayed healing in old SAD mice was associated with lower rates of re-epithelialization and keratinocyte proliferation in wound specimens. Granulation tissue formation was impaired with a reduction of Ki67+ cells in wound beds of SAD mice. Inflammation was not affected with no changes in the neutrophils and macrophages that infiltrated the wound bed quantified by immuno-labeling and FACS analysis. In contrast, micro-vessel density was significantly decreased in SAD wounds. CD31$^+$CD45$^-$ endothelial cell studied by FACS were accordingly decreased in SAD wound beds. This reduction in wound bed angiogenesis appeared to be secondary to a decrease in the number of endothelial progenitor cells (EPCs): the CD34$^+$CD11b$^-$CD31$^+$ EPCs were significantly decreased in the blood and the bone marrow of SAD mice. VEGFa and CXCL12 mRNA levels were significantly lower in SAD than in WT wounds. The injection of young WT EPC in the SAD wound at day 2 allowed a rescue of the healing delay. Furthermore, our result of RNA-sequencing analysis of WT and SAD EPCs from bone marrow showed several changes in mRNA including the CXCL12 chemokine and its receptor CXCR4. Analysis of these data are ongoing. Altogether, our results demonstrate that the delayed healing in SAD mice results from impaired mobilization of marrow EPCs possibly linked to a deficient CXCL12 secretion in skin.

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Psoriasin contributes to stress-induced angiogenesis in psoriasis

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The S100 protein psoriasin, S100A7, is highly expressed in psoriasis. Vascular modifications occur early in the development of psoriasis and angiogenesis is one of the key features in the pathogenesis of the disease. This study aims to define the angiogenic properties of psoriasin in keratinocytes and to investigate the direct effects on dermal endothelial cells, thereby promoting angiogenesis in psoriasis. We showed that psoriasin expression, demonstrated by qPCR, is induced by hydrogen peroxide (H_2O_2) in keratinocytes and by cellular stress, such as hypoxia and cobalt chloride ($CoCl_2$). Down-regulation of psoriasin, by siRNA, decreased the H_2O_2-induced expression of VEGF, heparin-binding EGF-like growth factor (HB-EGF) and matrix metalloproteinase (MMP)-1, and counteracted the reduction of the anti-angiogenic factor thrombospondin (THBS)-1. Extracellularly psoriasin was found to induce cell proliferation, migration and tube formation to a similar degree as VEGF and to induce the pro-angiogenic factors VEGF and IL-8 in dermal endothelial cells. Furthermore, we demonstrated that psoriasin-induced migration was mediated by the phosphoinositide-3-kinase (PI3K) and nuclear factor (NF)κB signalling pathways. In conclusion, psoriasin is induced by cellular stress conditions and amplifies H_2O_2-induced expression of angiogenic factors relevant for psoriasis in keratinocytes. Moreover, psoriasin contributes to key features of the angiogenic process by and inducing proliferation, migration and tube formation and increasing pro-angiogenic factors in dermal endothelial cell. Altogether, our data suggest that psoriasin is promoted by oxidative stress and mediate angiogenesis in psoriasis.

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Dominant effect of gap junction communication in mediating wound induced calcium wave, NFAT activation, cell migration and wound closure in human keratinocytes

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Intracellular calcium $[Ca^{2+}]_i$ plays a key role in regulating normal human epidermal keratinocyte (NHEK) growth and differentiation. Wounding induces a calcium wave and disrupts the calcium gradient across the epidermis but mechanisms involved in mediating the calcium flux, downstream signalling and longer-term wound healing responses have not been fully characterised. As expected, live cell confocal imaging of Fluo-4 loaded NHEK showed an immediate increase in $[Ca^{2+}]_i$ at the wound edge that spread as a calcium wave (8.3 μm/s) away from the wound edge across at least 8 cells; the $[Ca^{2+}]_i$ gradually returned to baseline within 2 min. Cells at the wound edge showed the greatest rate of rise in $[Ca^{2+}]_i$ which decreased in cells progressively further back. Wounding in the presence of 20 μM 18α-glycyrrhetinic acid (18αGA), a gap-junction inhibitor or 50 units/ml hexokinase, an ATP scavenger, blocked the wound-induced calcium wave. To investigate the potential role of two Ca^{2+}-dependent transcription factors NFAT and NFkB expressed in NHEK, dual-luciferase receptor assays were performed following mechanical wounding of NHEK. These demonstrated that wounding in a high Ca^{2+} external environment increased NFAT but not NFkB activation, compared to unwounded cells. Treatment with 18αGA or the store-operated channel blocker (GSK-7975A) inhibited wound-induced NFAT activation, whereas treatment with hexokinase did not significantly reduce NFAT activation post-wounding. Real-time cell migration analysis, measuring wound closure rates over 24 h, revealed that 18αGA prevented wound closure whereas hexokinase had no effect on wound closure rates compared to untreated control. Together these data indicate that whilst both gap-junction communication and ATP release from damaged cells are important in regulating the wound-induced calcium wave, long-term transcriptional and functional responses are dominantly regulated by gap-junction communication.

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iRhom2: a novel regulator of wound healing

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iRhom2 is a highly conserved catalytically inactive member of the Rhomboid family of pseudoproteases. We recently described dominant mutations in the gene encoding iRhom2, *RHBDF2*, associated with the inherited cutaneous and oesophageal cancer susceptibility syndrome, Tylosis with oesophageal cancer (TOC). Patient-derived tissue harboring mutant iRhom2 shows increased processing and activity of ADAM17, which upregulates shedding of its substrates such as EGF-family growth factors and pro-inflammatory cytokines. Desmosome processing is increased and immature epidermal desmosomes are present, suggesting a constitutive wound healing phenotype. To date, little is known about the mechanisms regulating iRhom2. We investigated putative regulators using Bioinformatic software to analyse iRhom2 sequence from UCSC Genome browser and found that iRhom2 contains putative p63-like responsive consensus binding site in its promotor. To support this, we have shown increased expression of p63 in tylosis cells compared with matched normal keratinocytes by both immunohistochemistry and immunoblot. p63 is thought to be involved in epidermal wound healing and repair. To identify novel interacting binding partners of iRhom2, a yeast two-hybrid experiment was performed, using a bait construct containing iRhom2 against a human skin library. This identified keratin 10, 14 and 16 as plausible preys. To confirm this interaction we performed co-immunoprecipitation and co-localisation immunohistochemistry in tissues derived from TOC patients. Keratin 14 and 16 expression is increased within the tylotic epidermis in comparison with normal controls. When immortalized cells derived from TOC patients undergo mechanical stretch, enhanced Keratin 16 expression is seen. Keratin filament cycling is a feature of wound healing, and appears upregulated by mutant iRhom2. Elucidating the interaction of iRhom2 with keratins and p63 may shed light on its role in wound healing and response to mechanical injury and identify novel therapeutic targets in abnormal wound healing states.

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A new fetal skin bioconstruct for allogeneic cell-based wound therapy

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We recently highlighted the therapeutic potential of a co-application of fetal fibroblasts and keratinocytes with immunosuppressive properties for allogeneic cell-based wound therapy and manufactured two clinical batches of fibroblasts and keratinocytes starting from a fetal skin sample. Here, we described the manufacturing process of a new cell-based skin dressing combining fetal fibroblasts and keratinocytes in a type I collagen matrix. This new device was then characterized in term of cell viability, phenotype and stability. Functional analysis was also performed by studying the wound healing growth factors and cytokines secretion profile by multiplex cytokines array system. Preparation of the fetal bioconstruct consists in a simple two steps procedure. Fetal fibroblasts and keratinocytes are thawed and mixed at the optimal ratio of 1:1. Then, cell suspension is dropped on the pre-saturated collagen type I matrix and incubated during three days. By LDH releasing assay, we demonstrated that cell viability was not altered during the time course of incubation. Structural analysis of the fetal bioconstruct revealed that cells are homogeneously distributed in the collagen matrix and strongly adhere to collagen fibers. Consistently, we showed by confocal microscopy that keratinocytes and fibroblasts strongly expressed b_1, a_2 and a_3 integrins. Keratinocytes was also found positive for keratin 14 and no keratin 10 positive cells was found, suggesting that in the matrix, keratinocytes are maintained in a metabolically active undifferentiated state. Finally, the new fetal skin bioconstruct was found to secrete high amount of wound healing growth factors and cytokines. To conclude, we developed and characterized a new fetal skin bioconstruct, acting as a reservoir of growth factor and cytokines that could be of therapeutic value for cell-based therapy of skin defects.

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ADRB2 bearing mast cells as putative effectors of propranolol vascular remodeling

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Infantile hemangioma (IH) is a vascular tumor which grows rapidly during the first trimester, and involutes in a few years. At all stages, IH is richly infiltrated by mast cells. The non-selective beta-blocker propranolol is currently the first therapeutic option for severe IH. The dramatic effect of propranolol in IH seems beta adrenergic receptor specific, because obtained at pharmacological doses and with other betablockers, but both its cellular target and mechanism of action in IH remain unclear. Our immunohistochemical study of the 3 beta-adrenergic receptors (ADRB1, 2 and 3) found the same patterns of expression in all evolution stage of IHs or treatment status. Based on their high ADRB2 expression, mast cells were the most relevant targets for propranolol, which is more selective for ADRB2. Our hypothesis is that propranolol induces an angiogenic switch on mast cells via ADRB2, as IH mast cells secrete pro-angiogenic factors such as VEGF, histamine and chymase during the proliferative stage, and the antiangiogenic factor TGF-beta during involution. In order to elicit that mechanism in vivo, we decided to study propranolol vascular effects in a xenograft tumor model, using U-87 MG, a human glioblastoma cell line, injected subcutaneously in NOG mice. We show that propranolol improves tumor vascular architecture. In vitro, VEGF-A secretion by tumor cells was reduced after exposure to propranolol, suggesting a role for VEGF-A in the propranolol-induced normalization of tumor vascular architecture. Our data suggest potential therapeutic benefits for adjuvant treatment of cancer by vascular bed remodeling betablockers. We are now investigating the potential role of mast cells in the propranolol tumor response in our xenograft model. First by immunohistochemistry and then by using mast cell depleted mice.

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Assessment of the performance and fate of adipose-derived mesenchymal stem cells forming part of bioengineered skin equivalents *in vitro* and *in vivo*

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Autologous and allogeneic cultured skin substitutes are currently being used in the treatment of cutaneous wounds due to severe skin loss (e.g. extensive burns) or in impaired healing situations (e.g. chronic ulcers). We have recently showed that skin fibroblasts forming part of fibrin-based dermal equivalents improve granulation tissue formation, which in turn enhance wound closure as demonstrated in a skin humanized model of delayed wound healing. Given their immune modulatory and anti-inflammatory properties, adipose-derived mesenchymal stem cells (ADMSC) have turned into a prime candidate for replacing fibroblasts in bioengineered skins to further enhance their performance as healing aids. To assess the potential benefits of ADMSCs for transient and permanent skin regeneration, we have assessed the function and regenerative performance of ADMSCs in comparison to that of human dermal fibroblasts. In addition, we assessed the persistence of "stemness" of ADMSCs under challenges imposed by interaction with biomaterials and other cell types. Studies were performed on dermal equivalents and bioengineered skins before (*in vitro*) and after engraftment in immunodeficient mice (*in vivo*). Dermal matrix remodelling, keratinocyte maturation and angiogenesis promotion were studied. In addition, the ability of ADMSCs to maintain their differentiation abilities towards adipose and osteocyte linages was evaluated *in vitro* and upon recovery from engrafted tissue at different time points. Overall, our results indicate that ADMSCs maintain their stem-cell nature and show enhanced performance than dermal fibroblasts in all tests. Further studies are required to determine whether ADMSCs, forming part either of autologous or of allogenic bioengineered skins, are beneficial under challenging clinical situations involving sustained cutaneous inflammation with concomitant impaired wound healing.

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Characterization of skin redness in healthy volunteers by analyzing the blood vessel and skin conditions

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Skin redness is one of the skin concerns, particularly in Caucasians. Therefore, we examined the blood vessel and skin conditions in volunteers presenting with skin redness to elucidate the mechanisms of its development. At first, we calculated hemoglobin (Hb) content by skin absorption spectra in Caucasian volunteers and found that the Hb level was significantly higher in the volunteers with red skin as compared to that in those without red skin. The Hb content was positively correlated with the transepidermal water loss value. The skin redness could be roughly divided into three types based on the appearance: the erythema-like type (70%), the telangiectasia-like type (18%), in which capillary vessels could be seen, and the acne-like type (12%). In the erythema-type skin, examination under a video microscope revealed that the redness was present mainly in the skin around pores, extending to the skin between the pores in individuals with a greater severity of skin redness. Presence of vascular alterations like angiogenesis and vasodilatation and increased expression of vascular endothelial growth factor (VEGF)-A in the epidermis were confirmed immunohistochemically in biopsy specimens of the affected skin obtained from Japanese volunteers. The expression level of VEGF-A was even larger in the stratum corneum specimens obtained from the affected skin of Caucasian volunteers by tape stripping. These results suggest that skin redness progresses from the skin around the pores, and that skin roughness, increased expression of VEGF-A in the epidermis, and angiogenesis and vasodilatation in the dermis may be involved in the development of skin redness.

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Differential gene expression in normal versus keloid dermal fibroblasts

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Scars are an inevitable consequence of trauma and surgery, and thus affect millions of people worldwide. Mild scars may be a minor aesthetic problem however the fibrotic response to skin wounding can continue unabated leading to disfiguring and painful keloid scars. To date the aetiology of keloids has not yet been clearly defined. Although there are many therapeutic approaches developed against keloids, most have not yielded satisfactory clinical results and we believe that the fundamental therapeutic aim should be to suppress the formation of keloids rather than focussing on surgical removal of the scar itself. We propose that investigating phenotypic differences between normal and keloid fibroblasts *in-vitro* may help identify potential therapeutic targets. To this end we have characterised the transcriptional differences between fibroblasts isolated from normal skin with those derived from keloid scars. The mRNA transcript profile of 84 genes important for cell-cell and cell-matrix interactions was compared between four normal dermal fibroblast cell populations and four keloid fibroblast cell populations using the Human Extracellular Matrix & Adhesion Molecules RT^2 Profiler PCR Array (SA Biosciences). Expression of multiple genes changed in keloid cells relative to normal cells, including increased expression of ADAMTS1, CTNND1, FN1 and TGFBI and decreased expression of COL14A1. The protein expression from these 5 genes is under evaluation by Western blot and these results will be discussed. In summary we describe differential gene-expression between normal and keloid fibroblasts which shed light on potential pathogenic processes.

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Disturbances in angiogenesis in granulomatosus skin diseases

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Necrobiosis lipoidica (NL) and granuloma annulare (GA) belong to the granulomatous skin diseases with unclear pathogenesis. Despite the quite common occurrence of these entities in dermatological practice, research into the subject is limited. Thus, we decided to perform an immunohistochemical analysis of skin biopsies in order to assess the expression of selected proteins involved in angiogenesis. The study group consisted of 21 patients with NL and 23 with GA, selected from the database at the Department of Dermatology, Medical University of Łód. Six healthy subjects served as the controls. Skin sections were stained with monoclonal antibodies directed against VEGF and CD34. The intensity of expression of epidermal VEGF, and the number of CD34$^+$ dermal blood vessels were assessed. The mean intensity of VEGF immunoexpression in GA patients was 0.91, and was significantly higher than in either the NL patients (0.45; $p < 0.01$) or the control group (0.14; $p < 0.009$). The mean CD34+ microvessel density per mm^2 in the GA group was 79.04, which was significantly higher than in the NL group (64.84; $p < 0.009$) and in the controls (52.03; $p < 0.001$). The obtained results confirm the similarity of the histological features of NL and GA. However, in GA, the biopsy changes in angiogenesis were more marked in the GL than in the NL group. In conclusion, based on our results we can assume that imbalance in the process of angiogenesis is one the factors involved in development of both NL and GA.

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Effects of Experimental Psoriasis-like Skin Inflammation in Atherosclerosis-prone Mice

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Epidemiological studies have shown that patients with psoriasis have increased risk of atherothrombotic disease. Psoriasis and atherosclerosis are both characterized by low-grade inflammation, but it is unclear whether psoriasis per se increases atherosclerosis. The purpose of this study was to develop an experimental mouse model combining the two diseases and investigate whether psoriasis-like skin inflammation affects the development of atherosclerosis. Thus, the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA, 0.01%) or acetone (control) was applied twice weekly for 6-8 weeks on both ears of mice from two different atherosclerosis-susceptible strains, i.e. low density lipoprotein receptor knockout (LDLr-/-) and apolipoprotein E knockout (apoE-/-) mice. In both atherosclerosis models, TPA application induced skin inflammation as measured by a 2-fold increase in ear thickness and by increased interleukin-17F levels in ear protein lysates. FACS analyses of spleens (n=13-15 mice/group) showed approximate 50% more CD11b+ cells ($p<0.001$) in mice treated with TPA, thus indicating that topical TPA application had systemic inflammatory effects. In a pilot study (n=5-7 mice/group) we found that TPA-treated LDLr-/- mice had more inflamed atherosclerotic plaques than controls as measured by the relative amount of monocytes and macrophages in histological sections of the aortic root ($p=0.01$). In apoE-/- mice we found no effect of TPA on the atherosclerotic lesions. In conclusion, dermal application of TPA on the ears of LDLr-/- (but not apoE-/-) mice may be a feasible experimental model to investigate mechanistic links between psoriasis and atherosclerosis.

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Epithelial mesenchymal transition acceleration of keratinocytes at leading edge during wound healing process supplied with basic fibroblast growth factor

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During reepithelialization of the wound healing process, keratinocytes at leading edge (KLE) of the wound edge are migrating in association with a transition into spindle cells and a reduction of cell-cell contact. These phenomena of KLE are thought to belong to epithelial mesenchymal transition (EMT). Basic fibroblast growth factor (bFGF) is available in clinical use for the wound healing acceleration, which stimulates granulation formation as well as angiogenesis. We examined bFGF effects on EMT of KLE using mouse dorsal skin. Histopathological approaches revealed that KLE formed a single layer of the epithelia and migrated toward the wound center. On the other hand, in a group with daily bFGF application, KLE formed multilayered epithelia with morphological transition to spindle shape. In addition, we found some KLE reduced E-cadherin and migrated individually toward the wound center. PCR array using RNA extracted from the skin wound tissues demonstrated that EMT related components such as TGF-ß1, CTNNB1, Notch1, Sox10, FZD7, SNAI3 and PDGFRß were significantly upregulated in the bFGF-treated wound compared to the control wound, suggesting that bFGF contribute reepithelialization through enhancement of EMT.

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Histological and Molecular Analysis of Long-Pulse 1,064-nm Nd:YAG Laser Irradiation on the Ultraviolet-damaged Skin of Hairless Mice: Association with Pulse Duration Changes

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Nonablative lasers are widely used to improve photodamaged skin, although the mechanism underlying dermal collagen remodeling remains unclear. To investigate the effects and the molecular mechanisms of long-pulse neodymium-doped yttrium aluminum garnet (Nd:YAG) laser irradiation on dermal collagen remodeling in association with pulse duration changes. Five hairless mice were pretreated with ultraviolet B irradiation to produce photodamage. A long-pulse 1,064-nm Nd:YAG laser was then used to irradiate the dorsal quadrant of each mouse at pulse durations of 1 ms, 12 ms and 50 ms with a constant fluence of 20 J/cm^2. The levels of dermal collagen, mRNAs of procollagen, matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMPs), platelet derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), and basic fibroblast growth factor (bFGF) in the laser treated skin were analyzed at 4 weeks following two sessions of irradiation. Long-pulse Nd:YAG laser treatment increased the dermal collagen level. A substantial induction in the level of procollagens, MMPs, TIMPs and various growth factors was also observed. A statistically significant increase of the expression of procollagen-3, TIMP-1,-2 and PDGF was noted at 12 ms, and of procollagen-1,-3, MMP-3, TIMP-1 and TGF-β1 was noted at 50 ms. A trend toward a maximal increase at 12 ms was observed. Long-pulse 1,064-nm Nd:YAG laser irradiation promotes collagen remodeling irrespective of the pulse duration, while trend of a maximal increase was noted at 12 ms. This wound healing process is characterized by the induction of growth factors and subsequent increase of MMPs and TIMPs, followed by matrix remodeling as confirmed by new procollagen production.

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Human fibroblast-derived matrix production in 96-well microplates

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Human dermal fibroblasts can be cultured *in vitro* to produce 3D extracellular matrices that are quite similar in structure and composition to dermis *in vivo*. The present study describes for the first time the production of human fibroblast-derived matrices (FDMs) in 96-well microtiter plates. Primary human dermal fibroblasts were seeded into tissue culture plates, which were either uncoated or coated with gelatin or fibronectin. They were then incubated for 4 to 21 days with medium in the presence and absence of different matrix promoting supplements (vitamin C, insulin, EGF). FDM production was measured in fixed cultures via a sensitive and robust protein assay employing sulforhodamine B staining. Gelatin coating led to stable matrix formation without contraction-induced distortion or peeling off during the fixation step. The combination of all matrix promoting factors proved more effective to induce FDM formation than vitamin C alone or the combination EGF plus insulin. Addition of exudates from chronic ulcers had FDM enhancing or inhibiting effects depending on the sample and the dilution. The addition of rapamycin inhibited FDM formation, while dexamethasone and the metalloproteinase inhibitor TNF484 had no or minor inhibitory effects, depending on the growth supplement used, and the ALK5 inhibitor SB413542 was not inhibitory. This 96-well microplate assay represents a convenient format to characterize the effects of compounds and biological samples on human FDM production.

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Human primary macrophage and neutrophil activation *in vitro* by wound exudate collected from chronic ulcers

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The major obstacle to healing of chronic wounds is an uncontrolled self-sustaining inflammation. Exudates of chronic pressure and venous ulcers contain activated neutrophils and macrophages as well as a mixture of inflammatory mediators and proteases. Since removal of wound exudates (WE) can improve wound healing we reasoned that factors contained within WE critically impede wound healing through activation of innate immune cells. Therefore, we tested the effect of WE on activation of primary human macrophages. By dividing WE into groups that mediated strong and weak activation of human macrophages, we found significantly elevated levels of inflammatory cytokines such as IL-1α, IL-1β and TNFα in macrophage-activating WE, with IL-1α showing a particularly strong statistical association ($p< 0.00005$) followed by IL-1β ($p<0.005$) and TNFα ($p<0.05$). No such association was found for a number of other immune mediators such as IL-6, IL-8, IL-10, CLL2, CCL3 or CCL4. Furthermore, levels of IL-1α, IL-1β and TNFα were low in WE with low stimulatory potential. Pharmacological inhibition of MEK1/2 and p38α kinases was equivalent or even superior to dexamethasone in inhibiting WE-mediated macrophage activation of immune mediator secretion, while inhibition of IL-1α by a specific monoclonal antibody had no effect. The latter finding and the observation that levels of IL-1 receptor antagonist in WE did not correlate with their low or high macrophage stimulatory potential argues against the notion of a direct involvement of IL-1α or IL-1β in macrophage stimulation. These data constitute the basis for future investigations aimed at the identification of WE-components that should be particularly targeted for therapy of non-healing chronic ulcers.

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Hydrated polyurethane polymers increase hepatocyte growth factor bioavailability – implications for wound healing

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Cutaneous tissue repair is a highly coordinated cellular process. During inflammation, granulation tissue formation and epithelial wound closure the different cell types interact via diffusible growth factors. Exogenous application of growth factors has been explored. An alternative strategy aims to increase the bioavailability of endogenous growth factors contained in the wound exudate. We here analyzed the potential of hydrated polyurethanes (PU) which in contrast to conventional polyurethane foam wound dressing contain large amounts of water. Hydrated polyurethanes were generated by different combinations of polyetherpolyol (Jeffamine), propylenglycol and isocyanate. This produced soft, clear, water-containing gels which can be molded into slab dressings. These polymers can swell and absorb fluids. We tested the absorption capacity with tissue culture medium containing 5% serum as surrogate for wound fluid. For functional studies Hepatocyte Growth Factor (HGF) was spiked to the artificial wound fluid. After 18 hours, the PU-gels absorbed from 0.995 ml/g (35,6% polymer content, PC) to 1.176ml/g (31,7% PC) decreasing to 1.119 ml/g (31,3% PC). Total protein in the supernatant above the PU gels increased to 171% (35,6% PC) to 180% (34,2% PC) decreasing to only 127% (31,3% PC) when compared to the start. Spiked HGF increased to from 20 ng/ml to 63 ng/ml + 1.9 ng/ml after 24 hours absorption and with a 1.8 ratio (v/w) of medium to gel. In scratch assays with HaCaT keratinocytes HGF reduced the scratch wound to 69% + 10% of controls (without PU-gel absorption) to 26.2% + 8.6% (after PU-gel absorption; p=<0,001). These results indicate that hydrated polyurethane can enhance epithelial wound closure. In acute as well as chronic wounds when normal wound healing mechanisms are reactivated by correcting the underlying pathology hydrated polyurethanes can be beneficial by increasing the relative concentration of growth factors.

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Life on the edge: Wound edge hair follicles proliferate more rapidly in respond to injury during anagen phase

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Over the last decade a great deal of interest in the wound repair field has focussed on the hair follicle (HF). Several groups have exploited lineage tracing to show that different HF stem cell populations contribute to re-epithelialisation, although there remains a paucity of data detailing the earliest phases of repair, when re-epithelialisation is initiated. It is also known that hair cycle growth phase influences speed of re-epithelialisation, though this has not been well characterised. Thus, the purpose of this study was to examine the early injury response within wound edge HFs, and to assess for differences across the hair growth cycle. We have collected wound tissue at multiple times from 2-24 hours post injury from male C57BL/6 mice, wounded in telogen or anagen VI. We have perfomed a histomorphometric assessment of un-injured longitudinal HFs adjacent to wounds for several wound processes, though our cell proliferation data is particularly intriguing. Here, we find that in telogen skin a wound induced proliferative burst within the HF outer root sheath (ORS) commences at 12-16 hours post injury, a pattern which is mirrored within the basal layer of the interfolicular epidermis (IFE). However, during anagen VI phase, the ORS of the upper anagen HF (i.e. permenent HF) displays a more rapid proliferative burst, with twice as many proliferative cells at 16hrs. Interestingly, this phenomenon is restricted to the HF, with the basal IFE showing an identical proliferative response across hair cycle. We also examined ORS proliferation within the lower anagen VI HF (excluding hair matrix), where we also detect a wound induced proliferation. These analyses characterise the kinetics of the early HF wound response and indicate that the epithelium of anagen HFs is poised to respond quickly in the event of injury, thus increasing its capacity to provide keratinocytes towards the neo-epidermis during repair. This may further explain why wound healing is accelerated in anagen skin.

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Lysophospholipid signalling as a therapeutic target for human skin wound healing: role of GSK-3β/β-catenin signalling

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Novel therapies are required for cutaneous wound healing defects which constitute a growing health burden. Lysophospholipid signalling is a potential therapeutic target because: 1) lysophosphatidic acid (LPA) is released by platelets into acute wound sites and is detectable in blister fluid; 2) LPA induces reepithelialisation *in situ* in animal models and 3) LPA promotes migration of human epidermal keratinocytes (HEKs). LPA is known to activate NFAT and β-catenin signalling but the mechanisms regulating wound-healing responses are incompletely understood. This study utilised an *ex vivo* human skin culture (HESC) model to investigate the effects of topical LPA treatment on wound healing. Reepithelialisation of wounded HESCs was significantly reduced from a mean (±SEM) of 9±0.7 days to 4±0.5 days following 10 μM LPA treatment ($p<0.001$, N=6). To investigate the signalling mechanisms involved, scratch wounding assays were performed on HEK monolayers. Both wounding and treatment with 1 μM LPA resulted in a 4-fold induction of activated β-catenin, detected by western blotting, accompanied by increased phosphorylation of glycogen synthase kinase-3β (GSK-3β), the upstream regulator of β-catenin (N=9). Consistent with this finding, dual luciferase reporter TOPflash assays revealed a 5-10 fold increase in β-catenin mediated transcriptional activity at 3-6 hours post wounding (N=9, $p<0.001$); LPA treatment also elicited a similar increase in TOPflash activity (N=9, $p<0.001$) with no additional response when added to wounded HEKs. To study the functional interaction between LPA and GSK-3β/β-catenin, real-time migration rates of HEK monolayers were determined over 24h. As expected, LPA rapidly induced HEK migration (N=6, $p<0.001$); however, this response was abolished by co-treatment with the selective GSK-3 inhibitor CT99021 (N=6). These data further support a key role for LPA in the regulation of human epidermal wound healing and reveal a complex time-dependent interaction between wounding and GSK-3/β-catenin signalling during wound induced HEK migration.

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Maternal wound healing triggers peculiar fetal subpopulations mobilisation that participate in skin angiogenesis

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Previous studies have demonstrated that progenitor cells are transferred from the fetus to the mother, nest in the bone marrow and are well tolerated by the maternal immune system. These cells can be selectively recruited by the injured maternal tissue. Our team has recently shown the constant implication of fetal stem cells that participate to the angiogenesis in maternal wound healing.This project aims to analyse the fetal subpopulations triggered by maternal wound healing as well as their signaling molecular pathways Wound healing kinetics was accelerated in pregnant females as compared with non gestant controls indicating a favourable effect of pregnancy. C57Bl6 WT females were mated with congenic homozygous eGFP males. Skin wounds were done at day E15.5.. Increase in GFP+ cells peaked at day 1 (2.3% of total PBMC). These were mainly CD11b+CD34+CD31+ (43%) and CD117-CD105+ (12%, fetal mesenchymal stem cells). Classical adult EPC phenotype CD11b-CD34+CD31+ was not found within fetal cells. GFP+ fetal cells decreased at day 2 (0.8%) but interestingly increased again (1.3%) at day 3 with emergence of CD11b-CD34+CD31+ (17%). A hight output qPCR for cytokines and chemokines pathways was done on mRNA obtained from fetal cells sorted from pregnant mice with or without wounds. Only 5 genes were differentially expressed. CCR2 was the most upregulated transcripted mRNA in fetal cells coming from wounded mice. Experiences targetting the role of this pathway as well as cultures of various sorted fetal subpopulations are ongoing In conclusion, fetal progenitors are triggered from marrow and participate to the maternal wound healing. There are various subpopulations that corresponds to EPC and MSC. Fetal EPC appear to express a different phenotype than their homologous adult progenitors. Ongoing experiments focus to determine which signaling pathway may specifically target migration of fetal cells to wounds in order to improve delayed maternal healing.

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Modulation of inflammatory functions of human M1 macrophages by starPEG–heparin hydrogels

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Biomaterials capable to modulate inflammatory macrophage (M1) activation and to promote inflammatory resolution represent a promising approach for treatment of non healing wounds. Since cellular functions are known to be influenced by physicochemical and biomolecular cues of the extracellular matrix (ECM) biomaterials that are capable to modulate such extracellular signals are of great interest within this context. Biohybrid hydrogels formed by crosslinking of amine functionalized star-shaped poly(ethylene-glycol) (starPEG) and carbodiimide/N-hydroxysulfosuccinimide (EDC/s-NHS)-activated heparin have been shown to encourage angiogenesis. Since both vascularization and inflammatory resolution are fundamental for successful wound healing we tested starPEG–heparin hydrogels with respect to their capability to modulate inflammatory functions of M1 macrophages. For the preparation of the hydrogels different heparin derivates were utilized as multifunctional crosslinker including native and a variety of selectively desulfated heparins. Human CD14+ monocyte-derived M1 macrophages that were seeded on the gels showed good survival and adhesion on all starPEG–heparin hydrogels. Activation of M1 macrophages by challenging with LPS revealed reduced expression and release of the inflammatory cytokines TNF, IL-12 and IL-23 for all starPEG-heparin hydrogels compared to hydrogels made of starPEG only. Moreover, reduction of the cytokine response was more pronounced in M1 macrophages cultured on hydrogels formed out of desulfated heparin compared to hydrogels made of native heparin. These findings suggest that i) starPEG-heparin hydrogels provide an immunomodulating capacity and that ii) immunomodulation by starPEG-heparin hydrogels may be fine-tuned by usage of heparin derivatives with adjusted levels of sulfation.

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Nuclear IL-33/ST2 is positively involved in wound healing in mice

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The skin is important as the barrier to external environment. Prompt skin wound healing is essential for host defense. The wound healing process is comprised of complex events, and its mechanism has not been fully elucidated. Our recent study with mouse wound model revealed high nuclear expression of IL-33 in keratinocytes around the wound site. IL-33 is a member of the IL-1 family of cytokines and is a ligand for IL-1 receptor-like-1 or ST2. IL-33 is an important cytokine expressed by keratinocytes and endothelial cells in inflammatory skin disease. In this study, we aimed to reveal the role of IL-33 signal in skin wound healing. Wild type (WT), soluble ST2 transgenic (sST2 Tg) mice, and IL-33 knockout (IL33KO) mice were wounded by 4mm biopsy punch after removal of hair on the back. At the 1, 2, 4, 7, 10 days after injury, wound area were measured for comparing the rate of healing. Wound sites were harvested by 6mm biopsy punch. These skin samples were examined histologically and total RNA was extracted from them to analyze the cytokine expression with RT-PCR. IL-33 was time-dependently induced in wound site revealed by RT-PCR, and keratinocytes in wound re-epitheliazation site expressed IL-33 in the nucleus shown by immunostaining. We found no difference in the rate of healing between WT and sST2 Tg, but skin wound healing of IL33KO was delayed compared to WT. The mRNA expression of IL1β, IL6, and CXCL1 were markedly enhanced in IL33KO compared to WT, while there was no difference in expression level of these cytokines between WT and sST2 Tg. Our previous data and others demonstrated that nuclear IL-33 suppressed cytokine production through suppressing activation of NFκB. We speculate that the nuclear IL-33 is positively involved in the wound healing, probably by suppressing excessive inflammation through inhibition of NFκB. The enhanced expression of inflammatory cytokines in IL33KO mice may aggravate the inflammation, and cause the delay in wound healing.

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Persistent inflammation impairs wound healing in a new preclinical porcine ulcer model

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A shared feature among ulcers of different etiologies is the inflammation of wound bed and wound edges. We thought to determine if wound inflammation is pathomechanistically linked to the persistence of ulcers. To test this hypothesis, persistent wound inflammation for > 5 days was induced in skin wounds in pigs by topical application of the TLR7,8 agonist R848. Inflamed wounds displayed enhanced exudate formation and a persistent infiltrate predominantly composed of macrophages and neutrophils. When comparing wound closure between the inflamed and placebo treated control group, inflamed wounds displayed greatly reduced wound closure (67.3% wound closure in non-inflamed wounds 10 days postwounding vs 6.7% wound closure in inflamed wounds). Interestingly exposure of wounds to wound fluids gained from patients suffering from non-healing wounds similarly resulted in wound inflammation and delayed wound closure (54.7% wound closure in non-inflamed wounds 9 days postwounding vs. 33.5% wound closure in inflamed wounds). Thus wound inflammation impairs healing. We next asked if prolonged inflammation might render wound edges non responsive to wound closure. To this end wounds that were inflamed over >5days were either left untreated or the wound edges were excised by sharp debridement. Interestingly the debrided wound displayed enhanced healing kinetics as compared to the non-debrided wound. Thus it appears that prolonged inflammation may render exposed tissue non-responsive to healing. In conclusion, wound inflammation delays healing and this state is preserved even when inflammation is abolished. This status of unresponsiveness parallels chronic wounds in humans and provides attractive testing modalities for future therapies.

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Pointers from frog skin organ culture to the identification of novel wound healing promoters: Esculentin to the front

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Adult frog epidermis is histologically similar to that of humans, and frog skin shows perfect scar-less wound repair. It also harbours numerous antimicrobial peptides (AMPs) and neuropeptides, some of which are known to promote wound healing. To obtain pointers to novel, evolutionarily conserved wound healing promoters, we have established a serum-free organ culture model of wounded, adult *Xenopus tropicalis* skin. We show that simple histochemistry (H&E, toluidine blue), lectin immunofluorescence microscopy, EdU incorporation, and TUNEL provide instructive, quantifiable wound healing read-out parameters, and that 17β-estradiol is an excellent positive control as promoter of reepithelialisation in this model. We have tested the wound healing-promoting effects of the amphibian skin-derived AMP, esculentin (Esc) (1-21). This AMP is characterized by an unusually broad spectrum of strong antimicrobial activity, and is related to human cathelicidin (LL-37), but in contrast to LL-37 it preserves its bactericidal activity at physiological salt concentration, and is significantly less toxic than LL-37 at high concentrations. Esc(1-21) promotes reepithelialisation of wounded frog skin in our model, and may increase keratinocyte proliferation in situ. It also promotes the migration of cultured human HaCaT keratinocytes in an epidermal growth factor receptor-signaling dependent manner. These limited data already suggest that frog skin-derived AMPs and frog skin organ culture provide valuable pointers to the identification of novel candidate wound healing promoters, and identify Esc(1-21) as an interesting candidate cross-species wound healing promoter with favourable antimicrobial properties. We are currently testing the effects of Esc(1-21) in wounded human skin organ culture.

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Sensory neurons and their neuropeptides as well NGF-β promote human skin wound healing

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Close interactions exist between primary sensory neurons of the peripheral nervous system (PNS) and skin cells, and the PNS is implicated in the modulation of different skin functions such as wound healing as patients with PNS defects show retarded wound healing. Therefore, we have studied the influence of sensory neurons on human cutaneous wound healing in injured human skin explants that were stimulated with major neuropeptides found in human skin (vasoactive intestinal peptide [VIP], calcitonin gene-related peptide [CGRP], substance P [SP]) or nerve growth factor-beta (NGF-ß) at various concentrations. Then, we evaluated the effects of these manipulations on the proliferative and extracellular matrix (ECM) remodeling phases of wound healing, dermal fibroblast adhesion and differentiation into myofibroblasts. These experiments showed that all investigated neuropeptides increased fibroblast and keratinocytes proliferation *in situ*. They also changed the expression ratio between collagens type I and III in favor of collagen I, particularly between the 3rd and 7th day of culture. Furthermore, the enzyme activities of matrix metalloprotesases (MMP-2 and MMP-9) were increased during the first days of the wound healing process. Finally, the adhesion of human dermal fibroblasts and their differentiation into myofibroblasts were promoted after incubation with CGRP, SP, VIP, or NGF-ß. Interestingly, the lowest concentrations of the tested neuropeptides, which corresponded to physiological concentrations, showed the strongest wound healing-promoting properties. In summary, these data suggest that the neuropeptides that are mainly released by their intracutaneous nerve fibers as well as NGF-ß promote human skin wound healing *in situ* on multiple different levels.

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The effect of ryanodine receptors on dermal wound healing

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Ryanodine receptors have an important role as calcium-induced calcium channels in the regulation of intracellular calcium levels in the nervous system and muscle. It has been recently proven that ryanodine receptors influence keratinocyte differentiation and barrier homeostasis. Our goal was to examine the in vivo role of ryanodine receptors in the healing of full-thickness dermal wounds. The experiments were performed on male SKH-1 hairless mice using skin fold chambers in the dorsal region. A standardized (4 mm diameter) circular wound was made on one side of the skin fold. Photographs were taken for the determination of the wound closure rate during the 20-days observation period. In the control group 1 (n=36), the wound was treated with sterile saline, the animals in group 2 (n=36) received ryanodine receptor agonist 4-chloro-m-cresol topically (0.5 mM), while in group 3 (n=36) dantrolene, an antagonist of ryanodine receptors was applied topically (100 μM). Different parameters of microcirculation were monitored by means of intravital videomicroscopy. Tissue biopsies were taken for routine histology after days 4, 8, 12, 16 and 20, respectively. Treatment with 4-chloro-m-cresol did not influence significantly the studied parameters, while dantrolene accelerated the wound closure rate and improved both epidermal and dermal regeneration. The inhibition of ryanodine receptors increased the vessel diameters in the wound edges during the process of healing and increased the blood flow in the capillaries at all times of measurement. Moreover, application of dantrolene decreased leukocyte-endothelial cell interactions during the inflammatory phase. Inhibition of ryanodine receptor-mediated effects positively influence wound healing. Thus, dantrolene may be of therapeutic potential in the treatment of wounds.

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Unexpected role for the (Pro)renin receptor in the maintenance of blood-lymphatic separation during skin development

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Apart from being a component of the tissue renin-angiotensin system, recent findings indicate that the (pro)renin receptor (PRR) plays essential functions in the maintenance of cellular homeostasis. PRR is a transmembrane protein, which also generates by intracellular cleavage a soluble form of completely unknown function. PRR is broadly expressed during early development and becomes restricted to the central nervous system and to epithelial tissues including the epidermis at E11.5 onwards, although its functions in these tissues remain elusive. To study specific role played by PRR in the skin, we have generated animals carrying epidermis-specific-deletion of PRR, by using the Cre/loxP system. We report that conditional deletion of PRR from basal keratinocytes lead to unexpected embryonic lethality around E15.5. This is to the best of our knowledge, the only known case of invalidation targeting basal epithelial cells that induces embryonic death. Overall, mutant basal keratinocytes are flattened and show a decreased proliferation rate as well as a reduced ratio of asymmetric division. However, surprisingly, the embryonic lethality shown by the mutant mice is associated with changes in the cellularity of the dermis including lymphatic hyperplasia and blood-lymphatic shunts. These observations indicate that during skin development, PRR produced by the keratinocytes plays an active role in the maintenance of blood-lymphatic separation, in the dermis. In addition, our data present evidence that PRR produced by the epidermis acts in a non-cell autonomous fashion. The mechanisms of this PRR-dependent communication between epidermis and dermis are not understood but may involve the soluble form of PRR.

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Whole mount scanning for the quantitative analysis of wound closure in an *ex-vivo* pig skin model

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Ex vivo wound models based on porcine skin have been described and are valuable tools to study wound healing biology and to test pharmaceutical compounds to accelerate wound closure. However one major limitation is the time and resource consuming histologic analysis to assess wound closure and re-epithelialization. We developed an alternative analytical method based on the whole mount labeling and analysis of skin explants that significantly enhances throughput. Fluorescent cytokeratin-5 staining of whole mounts and subsequent whole mount tissue scanning on a LI-COR device permitted measurement of epithelial ingrowth. Quantitative analysis of whole mount scans revealed a good correlation with conventional histologic analysis. In preliminary experiments, we have successfully tested the new system using growth factors or chronic wound fluid, which improved or impaired re-epithelialization, respectively. Therefore, *ex-vivo* pig skin wounds together with refined analysis may be a suitable replacement for in-vivo models for the analysis of wound healing.