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WITHDRAWN

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The development of a point-of-care diagnostic of wound status

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Chronic wounds typically arise from a disruption in the normal healing process which results in a prolonged or continuous inflammatory phase. They are often a symptom of an underlying physiological condition such as venous insufficiency, diabetic neuropathy or through limited mobility which sees a prolonged application of pressure. Chronic wounds affect millions of people inflicting significant pain and reducing quality of life for the patient as well as placing a significant burden upon the health care system. The objective of this Wound Management Innovation CRC project is to develop a quick and easy to interpret diagnostic tool that can aid the clinician in providing an accurate prognosis of a wound's chronicity. This will assist in ensuring that a correct diagnosis of the wound is given and that appropriate care is provided in a timely manner, therefore improving healing time and patient quality of life. It is hoped that this will significantly improve patient outcomes in a system that faces significant economical burden from chronic wounds each year. Plasma polymerisation was used to engineer a surface with amine chemistry which will form the platform of a multiplexed ELISA. Two monomers were optimised for their amine functionality and the resultant plasma polymer films were analysed via X-Ray Photon Spectroscopy (XPS). These films were subjected to aging and stability studies in solutions across a pH range to assess suitability for use with native wound fluid. The plasma polymer films as prepared are relatively stable and suitable for the aqueous environment that ELISA assay requires. The ELISA is currently being optimised for potential biomarkers of wound status including VEGF, in complex solutions. It is expected that this technology will be adaptable and transferrable to a range of different formats and biomarkers present within wound fluid that are indicative of wound healing or chronicity.

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Collagen I matrices containing high-sulfated HA modulate phenotype and function of human pro-inflammatory M1 macrophages

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In chronic wounds sequence of normal wound healing progressing through inflammation, granulation tissue formation and remodelling often fails due to aberrant prolongation of the inflammatory phase. Unopposed activation of inflammatory macrophages (M1) is assumed as major cause for persistent inflammation. Thus biomaterials capable to modulate macrophage activation and to promote inflammatory resolution represent a promising approach for treatment of non healing wounds. Since native ECM is known to guide functions of immune cells we tested artificially compounded ECM (aECM) on their capability to modulate the function of inflammatory M1. Artificial ECM were composed of collagen I and the glycosaminoglycan hyaluronan (HA) which was additionally modified by introduction of sulfate groups resulting in low- and high-sulfated HA derivatives. Testing functions of M1 revealed reduced inflammatory activities of macrophages differentiated on matrices composed of collagen I and high-sulfated HA (hsHA) as seen e.g. by reduced release of TNFα and IL-12 due to impaired activation of NFkB. Moreover, these macrophages are capable to secrete immunregulatory IL-10 typically not produced by M1. Cytokine ratio of IL-12/IL-10 created by macrophages on this aECM is similar to that produced by regulatory M2 macrophages. Since these macrophages also show reduced activity of transcription factors STAT1 and IRF5 both controlling macrophage polarization to M1-subsets we conclude that collagen I matrices containing hsHA dampen inflammatory macrophage function by impeding signaling pathways crucial for polarization and activation of pro-inflammatory M1. We therefore suggest this aECM as promising biomaterial for modulating inflammatory macrophage functions during wound healing.

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Sulfation of hyaluronan in regenerative biomaterials can control TGFβ1 induced myofibroblast differentiation due to competition with TGFβ1 receptor binding

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Myofibroblasts (MFb) are key players during wound healing, since they restore tissue integrity, close the wound and produce a permanent scar. The fibroblasts to MFb differentiation requires stimulation with TGFβ1 and results in the expression of α-smooth muscle actin (αSMA), collagen I (coll) and the ED-A splice variant of fibronectin (ED-A FN). To prevent hypertrophic scars or fibrosis MFb finally undergo apoptosis. Thus, controlling MFb differentiation and apoptosis are essential in wound healing. Sulfated glycosaminoglycans (GAG) are reported to interact with and modulate the bioactivity of growth factors and putatively could modify the action of TGFβ1 on fibroblasts. Within the scope of testing chemically modified matrix components for their potential use in treatment of non-healing wounds we investigated the influence of hyaluronan (HA) and chemically high-sulfated HA (hsHA) on MFb differentiation in the absence and presence of TGFβ1. Therefore, human dermal fibroblasts (dFb) were cultured on matrices of coll, coll with HA or coll with hsHA. The TGFβ1 stimulated induction of MFb markers αSMA, coll and ED-A FN was attenuated in the presence of hsHA. Furthermore, preincubation of hsHA with TGFβ1 impaired the Smad2/3 translocation to the nucleus in dFb while HA did not. In silico docking experiments with HA- and hsHA-tetrasaccharides suggest a significant difference in their distribution on the surface of TGFβ1 molecule, whereby hsHA occupies the TGFβ1-RI binding site on the TGFβ1 molecule. Consequently, hsHA hinders dFb to MFb differentiation probably by the occupation of the TGFβ1-RI binding site on TGFβ1 and thus prevents receptor binding, signal transduction and the expression of MFb markers proteins. Thereby, introduction of hsHA could be a therapeutic approach for hypertrophic scars and fibrosis.

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Phytochemical incorporated biomaterials for wound healing

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Efficacy of natural compound encapsulated biomaterials in wound care management is gaining importance now a days. Systems capable of delivering bioactive agents into cutaneous/subcutaneous levels are of great interest as therapeutic or cosmetic approaches for effective treatment and acceleration of skin wound healing. C-phycocaynin, a chromo-protein from cyanobacteria was impregnated/deposited/cross-linked with PLL-PLL (GA) into collagen solution to form biofilms, bioscaffolds and bio-based cryogels by polymerization, interlinking and cryogelation techniques respectively. Tensile strength, swelling test, drug release rate, porosity and in vitro biodegradation rate were evaluated. In vitro and in vivo models of wound healing were carried out by standard methods. Rate of fibroblast migration and proliferation was calculated during the treatment period. In vitro studies on human dermal fibroblasts proved that C-pc encapsulated biofilms and scaffold could retain the cellular cyto-compatibility and effectively accelerate cell migration and proliferation through cyclin dependent kinase pathways and changes in cell cycle patterns. In vivo animal test further revealed that scaffold could sufficiently support and accelerate the fibroblast infiltration from surrounding tissue to wounded area and also accelerate wound contraction. Among all biomaterials tested, cryogel proved to be better in terms of tensile strength and hydro-absorption rate. The cell infiltration rate was higher than in the bio films and scaffold.These results suggest that collagen/c-phycocyanin scaffold cross-linked with PLL(GA) is a potential candidate for dermal equivalent with enhanced bio-stability and cryogels have higher bio-compatibility with faster biodegradability. The suitability of cryogel was also observed in fibroblast-keratinocytes co-culture condition which closely mimics the artificial skin.

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Human dermis-derived ABCB5-positive mesenchymal stem cells accelerate mouse skin full-thickness excisional wound healing in part by the secretion of interleukin-1 receptor antagonist

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Mesenchymal stem cells (MSCs) feature many characteristics, such as tissue regeneration capacity and immune modulation, beneficial for therapeutic applications in injury and trauma. With respect to skin wound healing, MSCs have been proposed to suppress inflammatory processes and stimulate repair mechanisms such as myofibroblast differentiation and matrix deposition, angiogenesis, as well as re-epithelialisation. Although MSCs are present in all connective tissues of the body including the dermis, these studies have mainly focused on MSCs isolated from either bone-marrow or adipose tissue. Here we describe the isolation of an ATP-binding cassette sub-family B member 5 (ABCB5) positive plastic-adherent dermal cell subpopulation and its characterization as bona-fide MSCs. ABCB5+ dermal MSCs contributed to full-thickness excisional skin wound healing in mice to a comparable level as GMP-isolated and cultured bone-marrow derived MSCs. Furthermore, we demonstrated that ABCB5+ dermal MSCs secreted interleukin-1 receptor antagonist (IL-1RA) in response to inflammatory stimulation, which in turn inhibited classical macrophage activation with TNF-alpha release. IL1-RA inhibits the activity of IL-1 cytokines by binding to the IL-1 receptors without activating signal transduction. The importance of MSC-secreted IL-1RA for the observed accelerated cutaneous wound healing in mice was substantiated by a siRNA-mediated gene-silencing approach. In conclusion, human dermal ABCB5+ sorted MSCs represent an easy accessible source for cell-based therapy of skin wounds that accelerates healing at least in part by the secretion of IL-1RA.

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Ratite oils promote keratinocyte cell growth and inhibit leukocyte activation

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Emu oil has been used as a popular traditional wound healing treatment and anti-inflammatory remedy by Australian aborigines. Several published animal studies suggest emu oil treatment can promote reepithelialization in wound areas and may have anti-inflammatory properties. We investigated effects of 6 different individual emu oils along with oils from other ratites (3 ostriches and 1 rhea), duck, tea tree and olive oil on immortalized human keratinocytes (HaCaT cells) in vitro with 0%, 0.5% and 1.0% oil concentrations in culture medium. Human peripheral blood mononuclear cells (PBMC) were subjected to phytohemagglutinin (PHA) activation and exposed to each oil at 0.5% concentration in culture medium for 48 hours incubation to evaluate their impact on PBMC's survival rate and IFNγ production with ELISpot Assays. Shorter population doubling time durations were observed for HaCaT cells cultured in emu oil (1.18X faster), ostrich oil (1.25 X faster), and rhea oil (1.14X faster) when compared to no oil control. Whereas cells cultured with tea tree oil and olive oil showed prolonged population doubling time durations; 1.13X slower and 3.08X slower respectively. In contrast, majority of the oils reduced PBMC viability, with tea tree oil having the most significant adverse impact. ELISpot results showed that although the degree of IFNγ inhibiting effect varied among different oils, individual emu, ostrich, rhea, and duck oil, bestowed a high degree of inhibition. This preliminary investigation suggests that emu oil has potential to aid wound healing in humans by impelling growth rate of keratinocytes and confirms its anti-inflammatory properties on PBMC. With this combination, ratite oils may be useful as a component in treatments for wound healing and inflammatory skin conditions.

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Inhibition of NF-κB signaling by hypochlorite rejuvenates skin

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Hypochlorite, the active ingredient in Clorox, is used worldwide for cleaning. As a clinical treatment, 0.005% hypochlorite baths (the same concentration used in swimming pools) improves atopic dermatitis, which may be mediated by antimicrobial and/or anti-inflammatory effects. We've discovered that hypochlorite inhibits signaling governed by nuclear factor (NF)-κB, thereby inhibiting inflammation and reversing aging programs in skin. In cultured cells, hypochlorite exposure leads to oxidation of regulatory cysteines and inactivation of IκB kinase (IKK), a key regulator of NF-κB activation. In aged mice, topical hypochlorite reduced skin NF-κB signaling in vivo, and attenuated age-dependent expression of p16INK4a and other genes, leading to a striking re-acquisition of juvenile skin phenotypes. These included enhanced epidermal thickness and proliferation, which reverted with hypochlorite withdrawal. Our work defines a new mechanism for a commonly used dermatologic treatment. Discovery of chemical methods that reversibly modulate IKK signaling should advance our ability to control diverse physiological and pathological skin processes regulated by the NF-κB pathway.

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Impaired β_2 integrin-dependent activation of NOX2 in macrophages delays wound healing in *CD18*$^{-/-}$ mice

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Leukocyte Adhesion Deficiency type1 (LAD1) patients with reduced levels of β_2 integrins due to mutations in their common β chain (CD18) suffer from severe wound healing defects. In the *CD18*$^{-/-}$ murine LAD1 model we previously found β_2 integrins to control phagocytosis of apoptotic neutrophils (PMN) by macrophages (MΦ) with subsequent release of reactive oxygen species (ROS) and active TGF-β_1 at wound sites and thus normal wound healing. The NADPH oxidase NOX2 is the main source of ROS release in MΦ. We here investigated whether impaired phagocytic activation of NOX2 in MΦ is causal for insufficient ROS release, reduced TGF-β_1 activation and impaired wound healing in CD18 deficiency. *In vivo* imaging and wound healing experiments revealed *CD18*$^{-/-}$ wounds to mount significantly reduced amounts of ROS compared to wildtype (WT) wounds. Further, the wound healing defect of *CD18*$^{-/-}$ mice was rescued by injection of the oxidative burst inducer Rotenone. Rotenone significantly increased active TGF-β_1 release at wound sites and from MΦ upon phagocytosis of apoptotic PMN *in vitro*. NADPH based fluorescence lifetime imaging revealed NOX2 to be activated in WT but not in *CD18*$^{-/-}$ MΦ upon adhesion to apoptotic PMN. Similar to *CD18*$^{-/-}$ mice NOX2-deficient *p40phox*$^{-/-}$ mice presented significantly delayed wound healing compared to WT mice. Importantly, injection of WT but not *p40phox*$^{-/-}$ MΦ around *CD18*$^{-/-}$ wounds fully restored the LAD1 wound healing defect. Taken together, β_2 integrin-dependent NOX2 activation is essential for sufficient ROS production with subsequent TGF-β_1 activation by MΦ and normal wound healing. Targeting β_2 integrins and the downstream target NOX2 may prove promising to therapeutically tilt MΦ function in MΦ-driven inflammatory disorders.

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Fractional laser-assisted changing of tissue shape

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There is a strong need to change the shape and to predictably move soft tissue in a particular direction, for example to relieve tension on burn scars, close a wound or treat skin conditions. Conventional fractional ablative laser typically removes 10-30% of the skin volume by ablating very small channels deeply into skin. These microscopic holes "fill in" by fibroblast migration into the channels and new tissue is formed. Instead of letting the holes "fill in", we tested the hypothesis that closing them (similar to macroscopic wound closure) would reduce the skin area, and move skin in the direction of hole closure. Ex-vivo human skin samples were treated with a prototype fractional CO2 laser device operating at 3-10 ms exposure duration to produce arrays of holes of 2 mm depth. We found that the residual thermal damage layer (RTD) surrounding each hole, typical of fractional lasers, was stiff and prevented hole closure. A contact ZnSe window apparatus was designed and used to entrap laser-induced steam inside the skin tissue, breaking up the RTD. This produced a significant increase in hole eccentricity ($p<0.00$) and circumference ($p=0.027$), with fracturing of the RTD seen by histology which allowed easier hole closure. We then stretched and applied an adhesive, elastic surgical dressing to provide hole closure by elastic recoil of the dressing. In an in-vivo swine study, epilated abdominal test sites were marked with sterile ink micro tattoos prior to fractional laser treatments with and without (control) hole closure. At 7, 14 and 28 days there was significant reduction in skin area due to hole closure, specifically in the direction of hole closure. There was no scarring. In conclusion, we show that a modified fractional ablative laser treatment followed by closure of microscopic holes, can reduce skin area and move skin in a preferred direction. This approach appears promising for clinical applications.

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Interest of Rhealba® oat plantlets on epidermal reparation

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Epidermal reparation is a crucial step in cutaneous restoration of irritated skin and in wound healing. It involves the combination of two cellular processes, proliferation and migration, temporally and locally orchestrated by specific molecular mediators (growth factors, cytokines, collagen, hyaluronic acid, etc). The restoration of barrier function is also important for epidermal reparation. ABCG1, a lipid transporter, is then induced during keratinocyte differentiation to assure lipid barrier formation. In order to evaluate Rhealba® oat plantlets on epidermal reparation, we tested this extract on keratinocyte proliferation, on hyaluronic acid (HA) expression, on collagen IV (dermal-epidermal junction protein) expression, and on ABCG1 mRNA expression. Normal human keratinocytes were submitted to 48h-treatment with increasing concentrations of Rhealba® oat plantlets. Cell proliferation was assessed by the measurement of BrdU incorporation. Then we performed a cell culture with Rhealba® oat plantlets to assay the expression of molecular mediators involved on epidermal regeneration at the protein level by ELISA and immunolabeling, or at the transcriptional level by real-time RT-PCR. Finally we analysed the influence of a cream containing Rhealba® oat plantlets on the lipids neosynthesis on human reconstructed epidermis. Our results showed that Rhealba® oat plantlets were able to stimulate keratinocyte proliferation. We also showed that, following Rhealba® oat plantlets treatment, the expression of HA and collagen IV were induced. Gene expression analysis showed that ABCG1 was induced, suggesting that the Rhealba® oat plantlets favour barrier function restoration by enhancing lipid synthesis, which was further supported by the data obtained on human reconstructed epidermis. Together, the present data, obtained in vitro, support the positive action of Rhealba® oat plantlets on skin reparation.

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Keloid fibroblasts exhibit hyper-responsiveness to mechanical stimulation: The role of cell softening and nuclear Runx2

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Keloids prone to form in the body area with increased skin tension. We hypothesize that keloid results from the hyper-responsiveness of keloid fibroblasts (KFs) to mechanical stimulation. The purpose of the study is to understand the mechanical properties of KFs and the role of nuclear Runx2, an osteogenic and chondrogenic transcription factor, in their responsiveness to mechanical stimulation. By using atomic force microscopy, we found that KFs (1,205±70 pascal) tended to be softer than normal fibroblasts (NFs) (1,521±145 pascal) (N=7, P=0.0744), while keloid tissue and normal skin tissue were measured at 16,570±1,648 and 1,503±250 pascal, respectively (N=3, P≦0.001). We plated KFs and NFs on collagen-coated polyacrylamide gel of different stiffness simulating tissue microenvironment, and found that KFs produced more fibronectin than NF when cultured on a stiff gel (20,000 pascal) while both KFs and NFs produced equally low level of fibronectin on a soft gel (2,000 pascal). Tissue protein analysis revealed that keloid tissues were abundant with fibronectin, type 1A1 collagen, type 3A1 collagen and skeleton-associated protein collagen type 11A1. From our immunohistochemical study, the upstream transcription factor, Runx2, was detected in the nuclei of fibroblasts within keloid lesions. Knocking down Runx2 in KFs using RNA interference decreased the ECM production, including fibronectin, type 3A1 collagen and type 11A1 collagen. On polyacrylamide gel culture system, the nuclear expression of Runx2 in KFs correlated with substratum stiffness. In conclusion, KFs and NFs display different profile of biomechanical properties and response to changes in substratum stiffness. Ectopic expression of Runx2 possibly plays a role in the hyper-responsiveness of keloid to mechanical stimulation.

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High concentration of glucose activates migration and proliferation of human skin keratinocytes through inducing active release of HMGB1

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High-mobility group box 1 (HMGB1) is a nuclear factor and a secreted protein. During inflammation, HMGB1 is secreted into the extracellular space where it can interact with the receptor for advanced glycation end products (RAGE) and trigger proinflammatory signals. This protein has also been shown to function as a cytokine and to promote keratinocyte scratch wound healing. In the present study, we investigated the effect of a high glucose concentration on secretion of HMGB1 in cultured human skin keratinocytes. A high concentration (10 mM) of glucose decreased the high concentration of glucose induced HMGB1 release in human skin keratinocytes and promoted phosphorylation of ERK1/2 but not that of p38 or JNK. The MEK1/2 inhibitor PD98059 also suppressed HMGB1 release induced by 10 mM glucose. The high concentration of glucose activated migration and proliferation of human skin keratinocytes, and antibodies to HMGB1 inhibited these glucose-induced phenomena in vitro. These results suggest that a high glucose concentration induces HMGB1 release from skin keratinocytes and may enhance wound healing in the skin.

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Rapid construction of a three-dimensional multilayered dermis with a vascular tube network by polymeric nanofilm coating on cell surfaces for a living skin equivalent

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The development of artificial three-dimensional (3D) tissues possessing a similar structure and function to natural tissues is a key challenge in regenerative medicine. A living skin equivalent consists of an epidermis and dermis; in particular, it takes a long time to prepare the dermal component. To address this, we developed a simple and unique approach using nanometer-sized films consisting of fibronectin and gelatin (FN-G) as a nano-extracellular matrix. The FN-G nanofilms were prepared directly on the cell surface, and they acted as a stable surface for adhesion. The FN-G nanofilms prepared on individual cell surfaces provided an interactive property via $\alpha 5\beta 1$ integrin receptor in the cell membrane; thus, adhesion between the seeded cells could be induced at the same time in three dimensions. This rapid approach readily provided >10-20 layers after only 1 day of incubation. Next, we attempted to construct a thick multilayered dermis with endothelial tube networks by embedding human umbilical vein endothelial cells (HUVECs) in 3D tissues composed of normal human dermal fibroblasts (NHDFs). We performed a sandwich culture of HUVECs between 4L-NHDFs. After 7 days of incubation, highly developed capillary networks and a tubular morphology were clearly observed for the HUVECs. Moreover, when human lymph endothelial cells (LECs) were used together with the HUVECs, individual tubular networks of HUVECs and LECs were obtained. To our knowledge, such widespread and dense blood and lymphatic capillary networks in 3D multilayered tissues have not been reported. In conclusion, a cell accumulation technique is valuable in developing 3D cellular architectures using multiple cell types to control the location and types of cells. Our technique represents one solution for the development of 3D tissue models such as skin.

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Apelin inhibits subcutaneous adipose tissue accumulation by enhancing lymphatic and blood vessel integrity

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Recent publications featured the lymphatic function on adipose tissue research including skin. Chy mice, a naturally occurring mouse model of lymphedema due to heterozygous inactivating mutations in VEGFR-3, exhibit adipose layer accumulation in skin. In humans, excess adipose tissue is frequently found in lymphedema patients, suggesting that the impairment of lymphatic function resulted in adipose tissuce accumulation. We have recently shown that apelin/APJ signaling promotes the integrity of blood and lymphatic vessels, resulting in attenuation of UVB-induced inflammation. Here we first describe a link between adipocytes and the integrity of blood/lymphatic endothelial cells in skin. Apelin-deficiency resulted in increased subcutaneous layer of adipocytes, whereas apelin overexpression inhibited its accumulation by enhancing vessel integrity. The co-cultures of lymphatic endothelial cells and adipocytes revealed that adipocyte differentiation was potently induced by the plasma from a high-fat diet through the endothelial monolayer, whereas no significant difference was found in the absence or presence of plasma from a regular diet. Moreover, oleic acid, one of fatty acids rich in a high-fat diet induced hyperperpermeability of endothelial cells with loss of VE-cadherin localization on cell-cell junctions, causing adipocyte differentiation, whereas apelin inhibited vascular hyperpermeability by promoting vascular stabilization. These results indicate that apelin inhibits high-fat-diet-induced obesity and subcutaneous adipose tissue accumulation by enhancing vessel integrity. Apelin could serve as a therapeutic target for treating adipose tissue accumulation and its related diseases.

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Polyphenol enriched botanical extract pretreatment protects keratinocytes against oxidative stress

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Oxidative stress is elevated in both acute and chronic wounds and impairs keratinocyte motility, an integral part of wound healing. Botanical extracts possess antioxidant properties. We examined if pretreatment with botanical extracts enriched in polyphenolics could protect keratinocytes from oxidative stress induced impairment of cell motility. Antioxidant activities of aqueous and non-aqueous botanical extracts were tested by the ferric reducing antioxidant power (FRAP) assay. Keratinocytes were either pretreated or post-treated with botanical extracts, before exposure to hydrogen peroxide, and single cell migration was measured. To determine if effects could be ascribed to particular phytochemical subgroups, keratinocytes were pretreated with vitamin C (control), D-limonene (terpene), ellagic acid (EA, polyphenol), or epigallocatechin gallate (EGCG, polyphenol) prior to hydrogen peroxide exposure. The FRAP assay revealed that aqueous extracts of Aloe Vera and Echinacea had 16-fold and 21-fold ($p<0.001$) elevated antioxidant activity compared to turmeric, respectively. Acetone and ethanol extracts of aloe vera respectively had 1.7 and 2.3-fold enhanced antioxidant activity ($p<0.001$) while similar extracts of Echinacea had 8 and 4-fold decreased antioxidant activity ($p<0.001$) in comparison to aqueous extracts. Hydrogen peroxide impaired keratinocyte migration in both a dose and time dependant manner. Pretreatment with aqueous extracts of aloe vera and Echinacea protected keratinocytes from hydrogen peroxide mediated impairment in motility by 49% ($p<0.001$) and 23% ($p=0.042$), respectively. Post-treatment provided no protection. While vitamin C, EA, and D-limonene did not protect the keratinocytes, EGCG partially protected them from impaired cell migration by 20% ($p<0.02$). Pretreatment with polyphenol enriched extracts or specific polyphenolic phytochemicals protects keratinocytes against oxidative stress mediated reduction in motility. The solvent of extraction plays an important role in determining the final antioxidant activity of the extract.

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Cytoplasmic sequestration of keratinocyte GLUT1 by ganglioside GM3 mediates impaired diabetic wound healing

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We have previously shown that ganglioside GM3, which accumulates in diabetic skin, blocks insulin signaling and that knockout (KO) of GM3 synthase (GM3S) reverses the impaired wound healing in diabetic mice. Excess glucose prevents normal keratinocyte (KC) proliferation and migration, but stimulates cell migration and proliferation in GM3-depleted KCs. We hypothesized that depletion of GM3 promotes glucose uptake, even in the presence of excess glucose, energizing KCs to be more mobile and proliferative, thus facilitating wound healing in diabetics. Using spherical nucleic acid-gold nanoparticle (SNA) gene suppression to modulate GM3S expression and purified GM3 as a biochemical supplement, we explored the effect of ganglioside content on glucose uptake in human KCs (NHEKs). In 24h scratch wounds, NHEKs lacking GM3S had greater cell proliferation ($p<.001$), and migration ($p<.01$) compared with untreated and scrambled SNA-treated controls, particularly in the presence of increased glucose ($p<.001$ by 12h post-scratch). In normoglycemic medium, SNA-induced GM3S depletion increased glucose transporter 1 (GLUT1) translocation to the plasma membrane. In addition, glucose uptake was increased 5-fold (with no growth factor), 11-fold (with insulin), and 15-fold (with IGF-1 stimulation) (all $p<.001$). The ratio of membrane:cytoplasmic GLUT1 in GM3S SNA-treated NHEKs was increased 10-fold by excess glucose. SNA-induced prevention of GM3 metabolism using SNAs to increase GM3 or excess glucose inhibited NHEK proliferation ($p<.001$), migration ($p<.01$), GLUT1 translocation to the membrane, and glucose uptake ($p<.01$). Adding GM3 to GM3S SNA-treated NHEKs prevented GLUT1 translocation and reversed increases in cell proliferation ($p<.001$), migration ($p<.01$) and glucose uptake ($p<.01$). These data suggest that GM3 mediates impaired diabetic wound healing by suppressing glucose transport and supports the development of strategies to deplete cutaneous GM3 as a novel therapeutic modality for diabetic ulcers.

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Vascular hyperpermability and severe impairment of lymphatic vessels contribute to the formation of pressure ulcers in mouse ischemia-reperfusion injury

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Ischemia-reperfusion (IR) injury plays a major role in developing pressure ulcers. However, it remains unclear whether IR injury affects the structure and function of blood or lymphatic vessels in the skin. To address the question, we investigated the biological impact of IR injury on cutaneous vasculature in a mouse IR injury model utilizing ceramic magnets. We initially subjected wild-type mice to the standard regimen, and found that cutaneous IR injury develops marked edema and subsequent ulceration. Therefore, we next quantitated the vascular leakage in vivo by intravenous injection of fluorescence-labeled polyion complex nanoparticles which are 100nm in diameter. Two hours after IR, marked increase of fluorescence was found in the areas of IR injury, indicating that vascular hyperpermeability was induced in the experimental cutaneous IR injury. Second, we quantitated the lymphatic drainage in vivo using indocyanine green (ICG). Intradermal injection of ICG into the adjacent area of IR injury revealed that the lymphatic transport of ICG was severely impaired in the areas of injury 24 hours after IR, whereas sham-treated skin showed the complete transport of ICG by normal lymphatic vessels. Double immunofluorescence stains for blood and lymphatic vessels demonstrated that blood vessels are markedly enlarged, whereas no lymphatic vessels are found in the areas of IR injury, indicating that the lymphatic vessels are totally abolished. These results demonstrate that the increased leakage from blood vessels and the lack of lymphatic drainage contribute to the formation of cutaneous edema and ulceration in the mouse IR injury model.

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Smad7 accelerated healing of cutaneous wounds associated with chronic inflammation

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We have previously shown that overexpression of the TGFβ1 antagonist Smad7 in mouse epidermis accelerates skin wound healing. In this study, we found increased TGFβ1 expression and inflammatory cell infiltration in chronic ulcers of diabetes patients. To assess if Smad7 has the potential to be used to treat defective wound healing associated with chronic inflammation, we cross bred K5.Smad7 transgenic mice with K5.TGFβ1 mice; the latter exhibits a chronic inflammatory skin phenotype and delayed healing of excisional wounds. We found that bigenic K5.TGFβ1/Smad7 mice had reduced inflammation and delayed wound healing. Next, we produced recombinant human Smad7 protein with a Tat-tag (Tat-Smad7) that rapidly permeates the cell membrane and enters the nucleus. In vitro tests show that Tat-Smad7 rapidly entered keratinocytes in culture and remained in cells for at least 36 hours. Tat-Smad7 treatment was capable of blocking TGFβ1-induced phosphorylation of Smad2 in keratinocytes. Subcutaneous injection of Tat-Smad7 into the skin of K5.TGFβ1 mice significantly alleviated skin inflammation. When Tat-Smad7 protein was topically applied to 6-mm wounds on K5.TGFβ1 transgenic mice, it accelerated healing by promoting re-epithelialization and reducing inflammation. Molecular analyses revealed that Smad7 directly bound to the promoter of Rac1 and transcriptionally upregulated Rac1 expression, resulting in accelerated keratinocyte migration. In addition, Smad7 treated wounds have shown reduced staining of pSmad2 and NFκB p50, suggesting that Smad7 blocking of both TGFβ and NFκB signaling pathways is sufficient to overcome chronic inflammation, thus improving the wound microenvironment for healing. Our study suggests that Tat-Smad7 could be developed into a novel therapeutic agent to treat chronic inflammation-associated wounds by accelerating re-epithelialization and inhibiting inflammation.

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IFC-CAF, extract from Cryptomphalus aspersa's eggs significantly promotes skin homeostasis and migration and survival of skin cells *in vitro*

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Regenerative properties of skin decrease with age and, thus, the search of substances that minimize the cutaneous aging is increasing in the last few years. IFC-CAF is a patented natural extract that bears regenerative properties when applied topically. Objectives: To study the in vitro effects of IFC-CAF on skin homeostasis and cell migration, as well as on cell-cell and cell-substrate adhesion proteins expression. Moreover, its effects on cell survival and MMPs regulation has also been explored. Methods: We have used HaCaT cells and primary dermal fibroblasts (HF). To test cell proliferation, the colorimetric MTT assay was performed. We also studied cell migration using wound-healing assays complemented with MTT assays to avoid subjectivity in results quantification, whereas Western Blot and immunofluorescence microscopy were carried out to test the expression of different cell adhesion proteins. ELISA and immunofluorescence were performed to determine fibronectin, MMPs and Collagen 1. Results: We found that IFC-CAF clearly promotes proliferation and migration of HaCaT cells in a time and dose-dependent manner facilitating tissue homeostasis. Moreover, treatment with IFC-CAF increases the migratory behavior and the expression of adhesion molecules in both HaCaT and HF. Finally, IFC-CAF also improves cell survival and promotes phosphorylation of FAK and nuclear localization of β-catenin. Conclusion: IFC-CAF promotes the epithelial tissue regeneration, resulting more effective for fibroblasts than for keratinocytes. The dermal effect seems supported by significantly increasing collagen synthesis and fibronectin production. Moreover, MMPs release is downregulated in both types of cutaneous cells. All these effects suggest a potential clinical impact in skin rejuvenation and regeneration of wounded tissues.

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Development of a clinical grade suspension of allogeneic human dermal fibroblasts with extended shelf life capable of intradermal delivery through small calibre needles

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Injectable autologous fibroblast preparations have a history of use and are approved in the US for the treatment of nasolabial folds. We report on the development and testing required for allogeneic dermal fibroblast cell banks and the development of an injectable cell suspension with an 11 day shelf life at 2-8C. This material can be shipped internationally and has been used in a series of clinical studies in the UK and US including a study for the treatment of Epidermolysis Bullosa that is presented at this meeting. This presentation will outline the GMP and US/UK regulatory requirements to produce such cell therapies with particular emphasis on the UK situation.

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Engineered wound-dressing carriers with peripheral blood-derived angiogenic factor protein mixtures: Therapeutic implications for peripheral ischaemic tissue

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Spatiotemporally-controlled delivery of hypoxia-induced angiogenic factor mixtures has been identified by this group as a promising strategy for overcoming the limited ability of chronically ischaemic tissues to generate adaptive angiogenesis. We previously developed an implantable, as well as an injectable system for delivering fibroblast-produced factors in vivo. Here, we identify peripheral blood cells (PBCs) as the ideal factor-providing candidates, due their autologous nature, ease of harvest and ample supply, and present a novel device for one-step harvesting and delivering PBC-derived angiogenic factor protein mixtures. It was found that hypoxia (3% O_2) significantly affected the expression of a range of angiogenic factors including VEGF, Angiogenin and Thrombospondin-1 relative to the normoxic baseline ($p<0.05$). VEGF expression was found to be dependent on cell-scaffold material composition, with fibrin scaffold stimulating production the most, followed by collagen and polystyrene. Cell-scaffold matrix stiffness was also an important factor, as shown by higher VEGF protein levels when PBCs were cultured on stiff vs. compliant collagen hydrogel scaffolds ($p<0.05$). PBC-produced factors could be harvested within cell-free gel carriers. While the levels of VEGF released over 24hrs from collagen, fibrin and collagen/fibrin carriers were comparable, releasates from collagen gels induced significantly higher endothelial cell tubule formation and membrane invasion in an in vitro Matrigel assay. The device shown here provides an optimized microenvironment for PBC factor production, together with a cell-free delivery system in the form of a wound dressing. It could thus have important utility at the bed-side, as an angiogenic therapy in wounds, burns and ulcers.

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Matrix metalloproteinases 1, 2, 13 and 14 are differentially expressed in keloid scars compared to normal skin

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Keloids are fibroproliferative scars that form in response to abnormal healing processes. There is evidence that ECM remodelling of the dermis in the maturation phase of normal wound healing is insufficient in keloids. Our study aims to investigate the role of certain collagenases from the matrix metalloproteinase (MMP) family, in keloid pathogenesis. Protein expression patterns for MMP1, MMP2, MMP13, and MMP14 (also known as MT1-MMP) were examined in formalin fixed paraffin embedded skin biopsies from both keloid affected patients and healthy volunteers (n=3). The gene expression levels of the same targets were analysed in the same tissues through Real-Time qPCR. MMP1 and 13 gene expressions are over 40-fold increased in keloid tissue, compared to normal tissue. MMP2 and 14 gene and protein expressions are at least 2-fold higher in keloid vs normal tissue. Immunohistochemistry for MMP2 and 14 show a very distinct pattern of protein expression, in which MMP2 and MMP14 appear to be expressed as a wave across the central, marginal, and adjacent non-keloid regions of the scar. In the dermis, MMP2 and 14 are most evident at the leading edge, whereas the epidermis reveals a strong but variable pattern along all regions. This is in contrast to healthy skin, where expression in both epidermis and dermis is much lower overall, and shows a more consistent pattern across the tissue. The striking heterogeneity of MMP expression patterns observed in this study, indicate a significant role for the epidermis in the maturation of keloid scars. Additionally, it appears the leading edge of the keloid is the most active region.

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Mast cells are dispensable for granulation tissue formation during skin wound healing and do not impact tissue fibrosis in mice

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Impaired wound healing, defective regeneration as well as fibrosis of diverse tissues are leading causes of morbidity and mortality. Studies examining the role of altered mast cell (MC) function in the pathology of defective tissue regeneration and fibrosis have obtained contradictory results, most likely due to the use of different model systems. Therefore, conclusive evidence for the functional impact of MC in tissue remodelling is still lacking. The aim of this study was to examine whether wound healing defects or tissue fibrosis are due to dysregulated MC activity. To investigate the consequences of MC depletion in murine skin with respect to the quality and kinetics of tissue repair we developed a novel mouse model of Cre-inducible diphtheria toxin receptor-mediated cell lineage ablation. In this mouse model Cre recombinase is expressed cell-type specific in MC under control of the Mcpt5-promoter. Efficient systemic ablation of connective tissue MC is achieved by repetitive intraperitoneal injections of diphtheria toxin. In response to mechanically-induced tissue damage genetic ablation of MC did not impact the kinetics of reepithelialisation, the formation of vascularized granulation tissue and the deposition of collagen in scar tissue. However, MC ablation resulted in impaired wound contraction during the early phase of repair. The recruitment of neutrophils but not macrophages during the inflammatory phase of repair was significantly attenuated. In a model of bleomycin-induced skin fibrosis genetic ablation of MC failed to prevent the development of skin fibrosis. Dermal thickness, the amount of deposited collagen and the formation of collagen crosslinks within fibrotic lesions were comparable in MC depleted and control mice. Collectively, we conclude that MC activity has no impact on the formation of vascularized granulation tissue during skin wound healing and that the absence of MC fails to prevent tissue fibrosis in mice.

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Podoplanin enhances migration of keratinocytes by downregulation of E-cadherin

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Podoplanin, a transmembrane protein, is a ligand for C-type lectin-like receptor 2 (CLEC-2) that is predominantly expressed by platelets. We and others have previously found that podoplanin is induced in epidermal keratinocytes during wound healing. However, it remains unclear whether podoplanin plays a crucial role in promoting epidermal regeneration. To address this question, we investigated the biological function of podoplanin in keratinocytes. We initially created full-thickness wounds on the dorsal skin of wild-type mice, and assessed the induction of podoplanin at differential time points. Immunofluorescence staining showed that podoplanin is markedly expressed at the leading edge of regenerated epidermis three days after wounding. We next investigated the expression levels of podoplanin in cultured normal human epidermal keratinocytes (NHEK). Podoplanin was prominently expressed during proliferation. NHEK were then subjected to migration assay by interfering podoplanin at mRNA level. Podoplanin was found to contribute to enhanced migration in cultured NHEK. Importantly, siRNA technique further revealed that the loss of podoplanin strongly increased the expression levels of E-cadherin, indicating that podoplanin plays a key role in promoting keratinocyte migration by decreasing cell adhesion molecules such as E-cadherin in the epidermal keratinocytes. Finally, to elucidate the potential regulation of podoplanin by platelets, cultured NHEK were incubated with platelets and subjected to the assessment of mRNA profiles. In response to platelets, transcriptional levels of podoplanin were markedly decreased, whereas E-cadherin mRNA levels were increased in cultured NHEK, leading to a reduced migration by NHEK. These results indicate that the epidermal expression of podoplanin contributes to keratinocyte migration that is negatively regulated by platelets during wound healing.

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The regulation of skin wound healing by MFG-E8

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We recently demonstrated that pericytes are major sources of the secreted glycoprotein and integrin-ligand MFG-E8 in B16 melanoma tumors, and that MFG-E8 promotes angiogenesis via enhanced PDGF:PDGF receptor β signaling mediated by integrin-growth factor receptor cross talk. However, functions of MFG-E8 in angiogenesis in cutaneous wound healing are not well characterized. The objective of this study was to ascertain the role of MFG-E8 in cutaneous wound healing. First, we examined the distribution of MFG-E8 in normal murine and human skin in vivo. In the dermis, accumulations of MFG-E8 were found around CD31+ blood vessels, and MFG-E8 co-localized with PDGFRβ+ pericytes. Next, we examined the role of MFG-E8 in a cutaneous wound healing mice model. Protein and mRNA levels of MFG-E8 expression in the dermis was elevated during full-thickness wound healing by immunofluorescence staining and real-time PCR. In addition, MFG-E8 localized diffusely in granulation tissue, as well as around blood vessels in the dermis. Wound healing was delayed in MFG-E8 KO mice compared with WT mice, and vessel numbers in wound areas were reduced in MFG-E8 KO mice. Inhibition of MFG-E8 production by siRNA attenuated the formation of capillary-like structure of mesenchymal stem cells and endothelial cells in vitro. Finally, we examined expression of MFG-E8 in human granulation tissue. Expression of MFG-E8 in normal granulation tissue that contained many blood vessels was higher than that in fibrous granulation tissue with scant vessels. We conclude that MFG-E8 may promote cutaneous wound healing by regulating angiogenesis. Abnormality in MFG-E8 production or function may result in intractable wound healing that cause considerable morbidity in patients with diabetic and decubitus ulcers.

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Eccrine sweat gland-derived keratinocytes rapidly express epidermal differentiation markers during repair of human wounds

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Eccrine sweat glands are skin appendages that are unique to humans and some primates. It was recently shown that eccrine sweat glands are major contributors to reepithelialization of human wounds. Specifically, loss of epidermis upon wounding stimulates formation of epithelial outgrowths above each sweat gland duct. Outgrowths expand laterally and merge with each other to form the new epidermis. Because eccrine sweat gland ducts do not express typical epidermal differentiation markers, we sought to examine the spatial-temporal expression of proliferation and epidermal differentiation markers in sweat gland-derived keratinocytes during reepithelialization of human wounds. Partial thickness wounds were generated on human volunteers' forearm skin, under local anesthesia, by CO_2 laser. Skin biopsies were taken daily during the first 7 days post-wounding and expression of proliferation (Ki67) and differentiation (involucrin, loricrin) markers was studied by immunohistochemistry. Our results show that very few eccrine gland cells are proliferative in unwounded skin. Partial-thickness wounding triggers an intense proliferative response in sweat gland ducts underlying the wound within 2 days, followed by formation of epithelial outgrowths above each duct on the third day. Parallel expression of proliferation and differentiation markers is readily observed in new outgrowths, even at the earliest stage of outgrowth formation. Proliferative cells form the most basal layers of epithelial outgrowths, whereas epidermal differentiation markers are expressed concomitantly in all other layers. Interestingly, corneocytes are present at the outgrowth stage, even before reepithelialization is complete at day 8-9. Altogether, our data show that sweat gland-derived keratinocytes acquire an epidermal phenotype after wounding, and that keratinocyte expansion and differentiation occur concomitantly during repair of human wounds. These findings extend recent observations of the regenerative potential of eccrine sweat glands in humans.

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Recipe for impaired wound healing: Cross-talk between adrenergic and toll-like receptors in mesenchymal stem cells and keratinocytes

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Previous studies demonstrate that skin wounds generate epinephrine (EPI) that can activate local adrenergic receptors (AR), impairing healing. Bacterially-derived activators of Toll-like receptors (TLR) within the wound initiate inflammatory responses and can also impair healing. Here we examined the hypothesis that these two pathways cross-talk to one another, using EPI and macrophage activating lipopeptide-2 (MALP2) to activate AR and TLR2 respectively, in bone marrow-derived mesenchymal stem cells (BMSC) and neonatal keratinocytes (NHK). Human BMSC and NHK were exposed to EPI [50nM] or the TLR2-specific ligand MALP2 [100ng/ml] for 4hrs and compared to untreated controls. Activation of the AR by EPI significantly ($P<0.05$) increased TLR2 message (7-fold in BMSC, 3.5-fold in NHK) and protein (2-fold), and expression of its downstream effector MyD88 (4-fold). Conversely, activation of TLR2 by MALP2 increased β2-AR message (6-fold in BMSC, 2.7-fold in NHK) and protein (2.5-fold), and its downstream effector, phospho-β-AR activated kinase (p-BARK, 2-fold), and induced release of EPI from both cell types (2-fold). When cells were treated with EPI and MALP2 together, as they would encounter in the wound, β2-AR and p-BARK protein expression both increased 6-fold. The combined activation of β2-AR and TLR2 impaired cell migration, resulting in a 21% inhibition in BMSC and 60% inhibition in NHK speeds ($P<0.002$). EPI and MALP2 together resulted a 10 (for BMSC) and 51-fold (for NHK) increase in release of the pro-inflammatory IL-6 ($P<0.001$), responses that were remarkably reduced by pre-treatment with a β2-AR antagonist. In vivo, EPI-stressed animals exhibited impaired healing, with elevated levels of TLR2, MyD88 and IL6 in the wounds ($P<0.05$) relative to non-stressed controls. Thus, our data describe a recipe for decreasing cell migration and exacerbating inflammation in a wound via novel cross-talk between the adrenergic and toll-like receptor pathways in BMSC and NHK.

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Angiopoietin-like 4 improves diabetic wound healing

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Poor healing diabetic wounds are characterized by an accumulation of devitalized tissue, increased/prolonged inflammation, poor wound-related angiogenesis and deficiencies in the extracellular matrix components. They are prone for infections and effective therapies have been lacking. Recent identified matricellular protein, Angiopoietin-like 4 (ANGPTL4), is up-regulated during normal wound healing and has been implicated in angiogenesis and vascular permeability. ANGPTL4 can interact with specific matrix proteins preventing their degradation, and affects cell-matrix communication by altering the availability of intact matrix proteins. ANGPTL4-null mice have delayed wound re-epithelialization, reduced matrix proteins expression, increased inflammation and impaired wound-related angiogenesis, which are characteristics of diabetic wounds. However, its expression and role in diabetic wound repair remains unclear. Using in vivo leptin receptor-deficient diabetic mouse model, we found that diabetic wound has altered expression of ANGPTL4 as compared to normal wound (Day 3 post wounding ~2.0 folds, $p<0.05$). Furthermore, topical application of recombinant ANGPTL4 enhanced diabetic wound closure, re-epithelization, wound angiogenesis and tissue granulation. Importantly, focused gene expression profiling revealed that treatment with ANGPTL4 can restore majority of the dysregulated temporal and expression levels of cytokines, growth factors and transcription factors observed in diabetic mice wounds. Our study suggested that replacement of ANGPTL4 provide adjunctive or new therapeutics avenues in diabetes-associated complications, such as diabetic foot ulcers.

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Topical controlled release of PPARβ/δ agonist from microparticles accelerates diabetic wound healing

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One of the most debilitating complications in diabetes is impaired wound healing. The diabetic wound can become a portal for infections that can lead to dehiscence, sepsis and death. Hence, understanding and elucidating the biology of wound repair and identifying novel pharmacological targets in wound healing are of paramount importance to diabetic patients. Wound healing is a high survival response to skin damage with four overlapping phases: homeostasis, inflammation, proliferation and remodelling. Diabetic wounds fail to follow these phases and do not achieve healing result. PPARβ/δ is a ligand-activated transcription factor that regulates gene expression for many functions such as lipid metabolism, energy homeostasis and epidermal differentiation and repair. However, the clinical impact of a PPARβ/δ agonist as a pharmacological agent for the treatment of diabetic wounds has not been investigated. We showed that different release kinetics of PPARβ/δ agonist encapsulated in single- or double-layered biopolymer microparticles have different effect on the wound re-epithlialization rate of full-thickness excisional splint wounds of diabetic mice.

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A novel, simplified, non-cultured autologous cellular grafting procedure for chronic non-healing ulcers

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Skin substitutes have been used as adjuvant therapy in chronic non-healing wounds. We investigated a novel, simplified non-cultured, autologous cellular grafting procedure using a '6-well plate' technique to treat chronic recalcitrant wounds. This was a prospective pilot study that involved harvesting an ultra-thin split skin graft from the patient's gluteal region which was washed, separated and prepared in 6 different wells to obtain an autologous mixture of keratinocytes, melanocytes and fibroblasts that was subsequently applied directly to the wound via a hyaluronic acid matrix. There were 9 patients (pts) with chronic recalcitrant ulcers of different etiology recruited between Apr 2008 to May 2011 at the National Skin Centre, Singapore. A total of 16 ulcers were analysed with an average size of 47cm^2 (range 0.5-149.5, SD 39.6) and mean duration of 34 months (range 3-216, SD 68.5, median 12). Outcome evaluation was performed via digital photography, direct tracing and quality of life assessment using the Cardiff Wound Impact Schedule (CWIS). At 3 months after the 1st grafting, the mean re-epithelialisation was 57.9% (range 6.8-100, SD 35) with complete healing in 2 pts, >50% re-epithelialisation in 3 pts, 25-50% re-epithelialisation in 2 pts and <25% re-epithelialisation in 2 pts. 4 and 2 pts had repeat 2nd and 3rd grafting procedures done respectively at 4 weekly intervals. At 3 months after the final graft, 4 pts had further re-epithelialisation(average 49.9%, range 26.6-95.2, SD 30.8), 2 had worsening after initial improvement attributable to active underlying disease and poor adherence respectively, 1 was lost to follow-up and 2 remained completely healed. All pts showed improvement in all domains of the CWIS post-grafting compared to pre-grafting. These results suggest that this simplified non –cultured autologous cellular grafting may be beneficial for treating chronic ulcers that have failed standard therapy. Distinct advantages of this technique include ease of use in clinical practice, economical, short preparation time and the ability to treat large wounds in a single procedure.

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Development of 3D scaffolds for the investigation of chronic wounds

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The development of a tissue engineered skin model is seen as an important step in the study of chronic, non-healing wounds. Here we are seeking to develop such a tool utilising a synthetic extracellular matrix (ECM) to support the 3D co-culture of skin cells. This model will support the development of novel pharmaceutical and cellular therapies. Plasma polymerisation techniques were used to modify surfaces to facilitate the binding of biomolecules identified as important in skin remodelling/healing. 2D studies on plasma polymerised cell culture plates have shown that glycosaminoglycans (GAGs) and basic fibroblast growth factor (FGF-2) bound to a surface, mimicking their positioning in the ECM environment, can enhance proliferation of primary fibroblast cells. With their activity on proliferation being enhanced compared to their addition to the media. FGF-2 levels are increased in stages of wound healing to repair the dermal structure and this could be utilised to infill 3D scaffolds for production of skin models. These modified surfaces are now being recreated on polymer electrospun scaffolds made from polycaprolactone (PCL) to mimic a more realistic ECM to support the co-culture of fibroblasts and keratinocytes. The plasma modified scaffolds were investigated using both XPS and ToF-SIMS with the results demonstrating a complete, homogeneous coating throughout the 3D scaffold form both polymer film and then bound GAG to which a number of important biomolecules such as FGF and VEGF can then be bound. The results to date demonstrate that such a scaffold can be modified via plasma polymerisation to bind important biomolecules. Studies to show these engineered scaffolds are non-toxic continue in parallel with the co-culture of fibroblasts and keratinocytes toward the generation of dermal and epidermal layers.

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Audible doppler signal versus ankle brachial pressure index in patients with leg ulcer

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Peripheral vascular disease (PAD) of lower extremity affects 12% of older general population. The vascular assessment is required for all leg ulcers to confirm a good vascular flow for compression therapy. The non-invasive vascular assessment tools include Ankle Brachial Pressure Index (ABPI), toe pressure and full arterial segmental leg Doppler. About 80% of people with diabetes have non-compressible arterial vessels and 20% of non-diabetics also have calcified vessels. These individuals have unreliable ABPI. There is a need for an alternative quick bedside assessment method at point of care to support clinical findings. Methodology: We conducted a study on 200 consecutive patients referred to wound care clinic. The results from a full segmental hand held arterial Doppler (HHDU) compared to the results from audible signals (monophasic, biphasic, triphasic) done by hand held doppler at bedside. Results: Triphasic and biphasic HHDU signals indicate ABPI of 0.9 or higher and monophasic signals indicate ABPI of lower than 0.9 in general population but in diabetic foot ulcer only monophasic signal indicates ABPI of less than 0.9. Audible doppler has high sensitivity and subotimal specificity in detction of significant PAD if persons with diabetes are included (specificity 39% & sensitivity 64%). Conclusion: Audible Doppler signal sounds have a high degree of accuracy for the detection or exclusion of PAD compared with the ABPI using the cut off point of 0.9 ratio or lower. The emphasis in PAD detection should be directed toward encouraging a through physical examination along with audible doppler signals.

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MiR-29b as a potential therapeutic target of radiation-induced skin fibrosis

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Excessive collagen deposition resulting in skin fibrosis is a leading cause of skin dysfunction at the late stages of radiation-induced skin injury. MicroRNAs (miRNAs) are epigenetic regulators of gene expression and have been implicated in regulation of cellular responses to ionizing radiation. The aim of the present study was to identify differentially expressed miRNAs in irradiated rat skin using high-throughput Illumina sequencing technology and to identify their post-transcriptional targets. Adult male rats were irradiated (n=6) with a single dose of 30 Gy to the skin, sham-irradiated rats (n=6) served as a control. Irradiation to the skin was limited to the full thickness of the skin only. At 48 hours or 30 days after irradiation, rats were euthanized and skin miRNA fractions were used to generate cDNA libraries which were amplified through polymerase chain reaction (PCR). Deep sequencing of miRNAs in skin resulted in approximately 1.5 gigabytes of data from each experimental sample. TruSeq detected on average 206 known mature miRNAs per sample (ranging 184 to 224) and 192 novel miRNAs per sample (176 to 244). miR-29b was identified among the top differentially expressed miRNAs. The significant miR-29b upregulation at 48 hours was followed by its downregulation at 30 days post-irradiation. These results were confirmed by qRT-PCR. Computational predictions identified collagen 1A as potential target of miR-29b. Moreover, further evaluation of collagen 1A mRNA and protein expression levels at later time points inversely correlated with miR29b expression in irradiated skin. These data suggest that miR29b plays a role in the regulation of radiation-mediated skin fibrosis and might represent a potential therapeutic target for this devastating condition.

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Novel synthesis and sctivity of solenopsin A and analogs, topical inhibitors of phosphoinositol-3 kinase/akt with ceramide-like properties

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Solenopsin A is a small molecule that is the principle alkaloid in fire ant venom (Solenopsis invicta). We have previously demonstrated that solenopsin A is an inhibitor of phosphoinositol-3 kinase/Akt signaling and angiogenesis (Blood 2007) and of Pseudomonas quorum signaling (J Infect Dis 2008). Based upon the structural similarity of solenopsin A to ceramide, a lipid signaling molecule that also inhibits Akt, we performed live-cell imaging experiments to monitor PDK1 activation, Akt membrane recruitment, and Akt activity in 3T3 cells, utilizing FRET-based PDK1 activation reporter (PARE), YFP-tagged PH domain of Akt, and FRET-based Akt activity reporter, AktAR. We observed that solenopsin A demonstrated an inhibitory activity in all 3 assays, while an inactive solenopsin analog S3 was inactive. We also devised a novel synthetic route to solenopsin, using industrially available compounds. 2,6-Dimethylpyridine was lithiated using n-butyllithiium, followed by addition of alkyl halides or aldehydes. Solenopsin and solenopsin analogs were successfully obtained after palladium-catalyzed hydrogenation of the various 2-alkyl-6-methylpyridines. The synthetic solenopsin and novel analogs was also shown to downregulate constitutive Akt expression in A375 melanoma cells. We have thus made solenopsin A and analogs amenable to large scale synthesis. Topical solenopsin and analogs have potential for treatment of cutaneous neoplasms, through mechanisms distinct from imiquimod and 5-fluorouracil.

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Collagen XVII ectodomain shedding alters keratinocyte proliferation and motility through modulation of cell adhesiveness

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The hemidesmosomal component collagen XVII is crucial for the anchorage of the epidermis to the basement membrane. We have previously demonstrated that the collagenous ectodomain of this transmembrane protein is constitutively shed from the cell surface by disintegrin-metalloproteinases (ADAMs). The physiological function of collagen XVII shedding is still unclear. To investigate this we generated transgenic collagen XVII non-shedding mice (Col17ΔNS) by introducing a deletion of 41 amino acids within the linker domain that contains the sheddase cleavage sites. Exclusive expression of this mutant did not interfere with early skin architecture, epidermal differentiation or formation of the basement membrane. To verify previously observed in vitro alterations in cell motility, standardized acute skin wounds were induced in the Col17ΔNS mice. Wound closure was significantly accelerated in Col17ΔNS mice, with increased epithelial tongues and enhanced keratinocyte proliferation, especially during early re-epithelialization. This cell autonomously driven increase in proliferation and adhesion in primary Col17ΔNS keratinocytes was linked with decreased directed migration and a flattened cell morphology. These changes were associated with enhanced activation of the Akt/mTOR pathway. Pharmacologic inhibition of Akt/mTOR in vitro strongly suppressed wound closure and proliferation of Col17ΔNS keratinocytes. Our studies provide in vivo evidence for a novel regulatory function of collagen XVII in keratinocyte motility and proliferation during re-epithelialization processes through shedding of its ectodomain and suggest that collagen XVII functions as a cell-to-matrix-sensor which regulates cell polarity in response to changes in the extracellular environment, e.g. during wound healing.

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A role for 11β-hydroxysteroid dehydrogenase type 1 in stress-induced wound healing impairment

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Systemic/local glucocorticoid (GC) excess inhibits wound healing (WH) causing prolonged patient discomfort and infection risk. Impaired WH also occurs under stress. Although the GC-activating enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) regulates local GC availability in tissues including liver, adipose, muscle and skin, its role during WH is unknown. Following ethical approval, two 5mm full-thickness dorsal wounds (day 0; d0) were generated in female SKH1-HR mice and collected at d2, d4 and d8. 11β-HSD1, cofactor-supplying hexose-6-phosphate dehydrogenase (H6PDH) and glucocorticoid receptor (GR) mRNA were analyzed by qPCR (n=8, normalized to 18S rRNA). 11β-HSD1 and GR protein were analyzed by Western blot (n=6, normalized to β-actin). Impaired WH was investigated under restraint stress (RS) involving daily 12h overnight immobilization for 48h before wounding and during healing (tissue harvested on d0, d4 and d8). 11β-HSD1 protein increased in d2 wounds, decreasing at d4 (vs. unwounded skin) and was negligible by d8; 11β-HSD1 mRNA increased 14-fold at d2, 4-fold at d4 (vs. unwounded, $p<0.05$) and decreased to unwounded levels by d8. H6PDH mRNA also increased in d4 and d8 wounds (2-fold, $p<0.05$). Interestingly, GR protein decreased by 50% and 70% in d2 and d4 wounds, respectively ($p<0.05$). RS impaired wound closure by ~20% at d2 and d4 (n=12, $p<0.01$), effects that were normalized by d8. 11β-HSD1 mRNA increased by ~50% in d4 RS wounds (vs. unstressed; n=4, $p<0.05$) with a similar trend in unwounded RS skin and comparable increases in corresponding protein levels. GR mRNA/protein were also elevated in unwounded RS skin (2-fold and 3-fold respectively, $p<0.05$). H6PDH expression was unaffected by RS. These results reveal not only changes in 11β-HSD1 levels during normal skin WH, but also increased 11β-HSD1 levels (as well as increased GR) during stress-induced delay of WH, consistent with increased local GC availability/activity during stress. 11β-HSD1 is a potential therapeutic target to accelerate WH and/or normalize impaired WH during stress.

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The presence of dermal papilla cells in bio-engineered skin substitutes improves the wound healing process in deep skin lesions in nude mice

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The bio-engineered composite skin could be a useful tool to treat deep and extensive skin injuries. We previously demonstrated that the presence of human Dermal Papilla Cells (DPC) in a composite skin with Hair Follicle Stem Cells (HFSC), using acellular porcine dermis (APD) as scaffold, induced a regular and multi-layered stratified epidermis with a high number of basal p63-positive cells and invaginations in vitro. When nude mice were grafted with these constructions, the presence of DPC favoured the epidermis survival after 14 days, probably due to an effective neovascularisation of the matrix. An extensive remodelling of porcine dermis was also observed. In the present work we compared the effect of DPC and human dermal fibroblasts (DF), as dermal component, on tissue architecture and graft take of composite skin in nude mice. For that purpose we grafted mice with APD alone, composite skin with HFSC and DF or HFSC and DPC. Mice grafted with composite skin constructions containing DPC showed, 28 days after grafted, a more mature remodelled dermis that indent the lower layer of the epidermis of the injured area, what it was not seen in skin constructions containing DF. Same features were maintained 60 days after grafted but showing a thinner epidermis. Moreover, the grafting of APD alone showed at the same time a strong contraction of the wound both at microscopic and macroscopic level, which was not seen in the presence of any dermal cellular component. In brief, the use of DPC as dermal component, in composite skin, contributed to an arranged stratified-epidermis with a high number of precursor cells and invaginations in vitro. Moreover, the efficient graft-take and the mature remodelled dermis, similar to normal skin structure, observed in presence of DPC, suggest that DPC and HFSC are promising cellular components for a permanent skin substitute.

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Age-related phenotypic and functional alteration of primary dermal fibroblasts

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Fibroblasts are recruited at the lesion site during cutaneous wound healing where they proliferate, differentiate into myofibroblasts under local cytokines and synthesize extracellular matrix. Defects of skin wound healing are observed under aging as characterized by chronic wounds, ulcers or cutaneous fibrosis. The aim of our study was to characterize the senescence status of fibroblasts from young (<45 years) and aged (>45 years) donors in order to identify molecular and functional alteration related to age-induced senescence. Fibroblast cultures were established using skin from mammary plastic surgery of young (n=10) and aged (n=10) donors. The senescent status was evaluated by detection of senescence associated β-galactosidase (SA-βgal) activity as well as analysis of senescence markers (p16, p21, p53) by quantitative PCR and western blot. A PCR array of 84 genes focusing on senescence was used to identify variations in gene expression. Functional capacities were assessed by contractile and migratory assays as well as fibroblast differentiation into myofibroblasts under TGFβ. Our results confirmed the senescent status of fibroblasts from aged donors with 63.4±16% of SA-βgal-positive cells (vs 28.9±18% for "young" fibroblasts, $p<0.001$). P16, p21 and p53 expression was correlated to SA-βgal activity and significantly increased with age, with a 65% fold increase in p16 expression in aged donors as compared with their younger counterparts ($p<0.01$). A loss of proliferative and contractile capacities as well as a loss of migratory potential under PDGF stimulation was observed in "aged" fibroblasts. Activation of fibroblasts into myofibroblasts under TGFβ decreased with donor age, as shown by lower expression of α-smooth muscle actin. In conclusion, the phenotypic and functional differences between fibroblasts from young and aged donors may contribute to the alteration of wound healing process observed during cutaneous aging.

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Interleukin 6 promotes adult de novo hair follicle organogenesis through STAT3 phosphorylation

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Adult de novo organogenesis holds great promise in regenerative medicine. As a model system of regeneration, we study Wound-Induced Hair follicle Neogenesis (WIHN), where embryogenesis is recapitulated after full-thickness excisional wounding and de novo hair follicles are counted within the healed wound. To identify causes of natural variation, we screened gene expression differences between mice with high and low WIHN levels. Interleukin 6 (IL-6) was identified as prominently upregulated in mice with increased WIHN. We hypothesized that IL-6 and subsequent STAT3 phosphorylation promotes WIHN. Both IL-6 and STAT3 phosphorylation are increased immediately after wounding and in the reepithelialized keratinocytes. Levels of IL-6 mRNA (n=4; $p<0.05$) and protein (n=3; $p<0.05$) positively correlated with regeneration ability among mouse strains. Exogenous addition of recombinant IL-6 during wound healing significantly increased WIHN in C57BL/6J mice (n=20; $p<0.01$). Paradoxically, WIHN is also significantly increased in IL-6 null mice, compared to strain-matched (C57BL/6J) controls (n=45; $p<0.01$), highlighting the complexity associated with tissue regeneration. However, phosphorylation of STAT3, a downstream mediator of IL-6 signaling, is also significantly increased in IL-6 null mice versus controls (n=4; $p=0.02$), suggesting that other IL-6 superfamily members that also activate STAT3 compensate for IL-6. Indeed, the IL-6 family member oncostatin M (OsM) is significantly increased (n=4, $p<0.01$) in IL-6 null mice compared to strain-matched controls. Underscoring its importance, pharmacological inhibition of STAT3 phosphorylation inhibits WIHN in both wild-type (n=6-9; $p=0.01$) and IL-6 null mice (n=7-9; $p=0.03$). In all, these findings demonstrate that IL-6 and STAT3 phosphorylation trigger hair follicle regeneration. These results suggest targets to promote de novo hair morphogenesis in human clinical trials and also argue against generalizations about inflammatory mediators negatively impacting regeneration.

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Management of hand pressure sores

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Pressure sores most commonly occur on the sacum, heel, and ischium. However, an uncommon location for such wounds is the palm of the hand. They typically occur in flexion contracures of the hand on the setting of spasticity or chronic contractures. Here, we report two cases of palm pressure wounds from hand contractures. Both patients' wounds were full-thickness, with one wound covering the palmar surfaces of the hands from the index to ring fingers. The second patient had a fingertip wound at the paronychia. The fingers in that patient were inflexible and dressings could not be applied secondary to the contractures. In the first patient, the contractures were the result of finger pressure from spasticity resulting from cerebrovascular accident. After the failure of splinting therapy, the patient was treated surgically by tendon release and splinting. All wounds healed completely with no recurrence. The second patient had finger wounds secondary to spasticity from a closed head injury. Splinting alone was unsuccessful. This patient was healed with botulinum toxin injections to the profunda and superficialis muscles. The hand spasticity improved and the finger wounds healed spontaneously with splinting. Hand ulcers secondary to spasticity are often difficult to treat. Botulinum toxin injections and surgery can be useful means of treating these wounds.

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Molecular mechanisms for epithelia to generate wound electric currents that promote wound healing

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Wounds in skin naturally generate electric currents, which send powerful signals for epithelium to heal. How the wound electric signals are generated remains largely unknown. Here we use cornea as a model system to elucidate the ionic and molecular mechanisms of the wound electric currents. Using a systematic screen, electrophysiological techniques, and molecular approaches, we identified an essential role for Cl- transport in the wound electric current and in healing. We first used broad-spectrum Cl- channel blockers. Most of these blocked up to 50% of the cornea wound current as measured soon after wounding. When we did timelapse experiments in the presence of Ca2+-activated Cl- channel (CaCC) blockers, measuring the wound current at regular time intervals after wounding, there was a dramatic reduction of wound current (>80%). Whole-cell patch clamp recording showed that human corneal epithelial cells had a Ca2+-sensitive Cl- current which was inhibited by ACA, a specific CaCC blocker. ACA also blocked wound healing, but did not inhibit corneal epithelial cell (CEC) electrotaxis. ANO1 (a CaCC) expression was upregulated after wounding, and ANO1 knockout mice had significantly reduced wound currents. In slow healing wound, ANO1 appeared to be downregulated in the cornea. Modulating CEC internal Ca2+ also altered the wound current. These data show that the cornea wound electric current is generated mainly by upregulation and activity of CaCC, which is necessary for cornea healing. Those data also identify a novel role for Ca2+ as in wound healing through wound electrical signal regulator. Key words: wound healing, electrical signal, ion channels. This work is supported by NIH 1R01EY019101.

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Oligosaccharide modification by N-acetylglucosaminyltransferase-V promotes skin sclerosis by inducing macrophages to shift toward M2

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Oligosaccharide modification by N-acetylglucosaminyltransferase-V (GnT-V), that catalyses the formation of β1,6 GlcNAc (N-acetylglucosamine) branches on N-glycans, is reported to be associated with various disease pathologies such as cancer metastasis, multiple sclerosis, and liver fibrosis. In this study, we demonstrate the association of GnT-V with localized scleroderma. We found that GnT-V was highly expressed in fibroblasts and infiltrating cells in the skin sections from localized scleroderma patients. Most of the GnT-V-expressing cells were CD68 and CD163 positive M2 macrophages. M2 macrophages are involved in resolution of inflammation and tissue repair, and it is recently reported that the recruitment of M2 macrophage plays an important role in localized scleroderma. To know the role of GnT-V in scleroderma, we used murine model of scleroderma induced by bleomycin (BLM) injection. The expression of GnT-V was elevated in BLM-injected sclerotic skin. We next investigated skin sclerosis in GnT-V-deficient (GnT-V KO) mice. GnT-V KO mice were resistant to BLM-induced skin sclerosis with reduced collagen type1A1 content. Moreover, the number of M2 macrophages in BLM-induced skin sclerosis was significantly fewer in GnT-V KO mice than in wild-type mice. Bone marrow-derived macrophages (BMDMs) from GnT-V KO mice were resistant to M2 shift induced by IL-4 demonstrated by significantly decreased arginase 1, Fizz1, and Ym1 expressions. Akt phosphorylation that is reported to play an important role in M2 shift was also decreased in GnT-V KO BMDMs. Taken together, our results suggest that oligosaccharide modification by GnT-V on various cell surface proteins played an important role in skin sclerosis through inhibiting M2 shift of macrophages.

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Effect of the peripheral nervous system on skin wound healing

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Close interactions exist between primary afferent fibers of the peripheral nervous system (PNS) and skin cells. The PNS may be involved in the modulation of different skin functions. In order to study this influence on wound healing, we have co-cultured human skin explants that were mechanically injured with cells from dorsal root ganglia (DRGs) with or without Nerve Growth Factor (NGF) and evaluated different parameters. Thus, we studied the effects of the DRGs in the skin on the cell proliferation (Ki-67 incorporation), on the evolution of the ratio between type I and III collagens (immunohistochemistry), and on the activity of matrix metalloproteases 2 and 9 (MMP-2/-9). ELISA assay was performed on culture supernatants to discriminate if calcitonin gene-related peptide (CGRP) and Substance P (SP) were secreted by DRGs and/or human skin during wound healing. The presence of DRGs with or without NGF increased fibroblasts proliferation and the expression levels of type I and III collagens according to incubation times, particularly between the 3rd and 7th day of culture (proliferation phase) and continue after the 10th day of culture. This last result was particularly interesting because collagen III is the first type of collagen synthetized after injury, before its progressive replacement by collagen I. The presence of DRGs increased the activity of both MMPs (2 and 9) from the 3rd to the 7th day of culture. In conclusion, we have shown that the presence of DRGs increased the secretion of both SP and CGRP for all times of culture. Thus, the PNS, through the presence of DRGs, is able to promote skin wound healing.

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Superiority of hair follicle neogenesis in engineered human skin substitutes using early keratinocyte cultures

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Information about the effect of long term *in vitro* expansion of dissociated cells on hair inducing ability may lead to the development of clinically applicable bioengineered dermal-epidermal composites that regenerate hair follicles (HF). Earlier studies showed that mouse cells lose trichogenic capacity with passage, but studies on the effect of keratinocyte passage on human HF neogenesis and quality has been hampered by the lack of a suitable model system. We recently attained *de novo* formation of human HF in grafted dermal-epidermal composites comprised of adult human dermal papilla cells and neonatal foreskin keratinocytes. Our goal in this study was to determine the effect of keratinocyte passage on HF neogenesis. Dermal equivalents were made with cultured dermal papilla cells and were overlaid with either primary (P0) or passaged keratinocytes to form dermal-epidermal composites; these were then grafted onto immunodeficient mice. Grafts using P0 keratinocytes (HFs formed in 6/6 mice) and passage 1 (P1) keratinocytes (HFs in 5/6 mice) had HF densities of 0.94±0.44 and 0.82±0.55 follicles/mm of epidermis, respectively. Grafts using P3 keratinocytes had a significantly lower HF density (0.24±0.18 HF/mm, $p<0.05$; HFs in 5/7 mice) than grafts using P0 keratinocytes. HF diameters using P0 keratinocytes (240±37 μm) were greater than those observed in composites using P1 (144±26 μm) or P3 keratinocytes (106±22 μm) ($p<0.02$). Superior HF neogenesis using early keratinocyte cultures may be the result of greater abundance and/or responsiveness of progenitor cells with a capacity for HF formation. This model system has the unique capability of examining the functional integrity and true regenerative capacity of bioengineered skin with hair follicles. This system can be used to further optimize autologous tissue engineered skin substitutes for clinical applications.

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Effect of human skin explants on the neurite growth and electrophysiological profile of the PC12 cell line

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The skin is a densely innervated organ. After a traumatic injury, the nerve growth and the recovery of sensitivity take a long time and are often incomplete. Growth factors play a crucial role in the process of neuronal growth and reinnervation. To study the impact of the skin on nerve cells and nerve fiber growth, we have developed an in vitro model of human skin explants co-cultured with PC12 cells (rat pheochromocytoma cell line differentiated in neuron) in presence of nerve growth factor (NGF). The neurites outgrowth of PC12 was measured after 2, 4, 6 and 8 days of culture. The neuritis length of differentiated PC12 cells co-cultured with skin explants increased after six days. At day 6 and 8, the length of the neurites in the presence of the skin explant was significantly increased ($p<0.01$) by factor of 1.79- and 2.01-fold, respectively, when compared with PC12 cultivated without skin explant. At day 10, we have observed that PC12 cells released neuropeptides such as SP and CRGP. Furthermore profile for sodium, potassium and calcium channels were observed for the PC12 cells by electrophysiological method using patch clamp. In addition, the TRPV1 channel of these cells was stimulated by capsaicin. These observations demonstrated the influence of trophic factors produced by the skin explant on growth of PC12 cells and the possibility of functional co-culture.

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The use of acellular dermal matrix and skin grafting in the treatment of heel pressure sores

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The heel is the second-most common site for pressure wound development and has a high prevalence in both acute and long-term care facilities. Moreover, they can be difficult to prevent and treat, especially in patients who are elderly, malnourished, or have underlying medical conditions such as diabetes and peripheral vascular disease. In many circumstances, these wounds are extensively debrided or the patient ultimately undergoes partial calcanectomy and other amputations, thereby severely limiting the patient's ambulation and overall level of functioning. Here, we present a series of two patients whose calcaneal pressure ulcers were treated with the regimen of acellular dermal matrix and split-thickness skin grafting. One patient was a middle-aged male with a foot drop who was non-compliant with his foot brace. In consequence, he developed a mid-plantar wound. The other was an elderly female who developed a posterior heel wound. Both patients were taken to the operating room where acellular dermal matrix was applied directly to their wound beds after debridement. After two weeks, an autologous split-thickness skin graft was then applied to the neodermal bed. The patients tolerated the procedure well and there were no complications. In the post-operative months, the wounds of all patients healed completely, and the patients had excellent functional outcomes with a return to ambulation. Although further research with larger sample sizes is needed, based upon our experience, we feel that the combination of Integra and split-thickness skin grafting is a beneficial means of surgically treating heel pressure sores and preventing calcaneal osteomyelitis.

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The correlation between ultrasound findings and clinical assessment of pressure related ulcers: Is the extent of injury greater than what is predicted?

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The current staging system by the National Pressure Ulcer Advisory Panel (NPUAP) classifies the stages of pressure ulcers based on clinical assessment and visual inspection. We postulate that patients presenting with clinically superficial stage I wounds will have a greater depth of injury than predicted. On admission, patients with sacral pressure ulcers were staged according to the NPUAP classification system. Patients who were classified as having a stage I or II pressure wounds or suspected deep tissue injury were assessed with high-frequency (12-MHZ) ultrasonography (US) to identify any evidence of injury to the deep tissue. Those patients classified as having stage III or IV were excluded from the study. The study included 17 patients undergoing US for pressure related ulcers of the sacrum; 9 patients with Stage I pressure ulcers and 8 patients classified as having suspected deep tissue injury. In all 9 patients with clinically superficial stage I wounds, the US demonstrated evidence of injury to the deeper tissue layers. For the 8 patients classified as suspected deep tissue injury, the US also revealed abnormal findings representing deep tissue injury. The abnormal sonographic signs indicating deep tissue injury included loss of epidermal dermal interface, disruption of facial planes, and hypoechogenicity of the subcutaneous layers. The current staging system by the NPUAP has expanded to include suspected deep tissue injury as an additional stage. In patients with suspected deep tissue injury, we have found US to be a reliable diagnostic tool that confirms the clinical suspicion of deep tissue injury. Interestingly, for the stage I pressure ulcers that appeared clinically superficial, the US revealed evidence of associated deep tissue injury. This suggests that pressure wounds classified as superficial may have a deeper tissue damage component.

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The microbiota colonizing diabetic foot ulcers is associated with glycemic control, ulcer depth, and duration

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Microbial colonization and/or infection are believed to underlie delayed healing in recalcitrant wounds, including diabetic foot ulcers (DFUs). However, little is known of those clinical factors that may influence the wound environment and thus various dimensions of the colonizing microbiota. We profiled the microbiota colonizing neuropathic, non-ischemic DFUs in 52 individuals by deep sequencing of the bacterial-specific 16S ribosomal RNA gene. Quantitative cultures, the standard of care in the clinic, vastly underrepresented DFU microbial load and diversity. DFUs partitioned into three clusters differentiated by microbial diversity and composition. We identified several clinical factors associated with features of the DFU microbiome including glycemic control, ulcer depth, and ulcer duration. Hemoglobin A1c (HgbA1c) value, a measure of glycemic control, significantly associated with DFU cluster (P=0.037), with highest HgbA1c levels partitioning to a Streptococcus- and Staphylococcus-rich cluster. Ulcer depth was positively correlated with relative abundance of anaerobic bacteria (ρ=0.33, P=0.018), but negatively correlated with relative abundance of Staphylococcus (ρ=-0.47, P=0.0005). Greater number of bacterial species and high relative abundance of Gram negative bacteria were positively correlated with ulcer duration (ρ=0.38, P=0.006 and ρ=0.41, P=0.002, respectively). This work provides the first evidence that microbiota colonizing DFUs is associated with clinical factors and provides a foundation for prospective studies aimed at delineating problematic microbiota from benign colonization that can then be used to guide clinical treatment.

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Bacterial challenge study in porous polyHEMA implants

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Percutaneous devices are critical for medical care, but are associated with significant risk of infection. In previous studies, we developed an animal model in which we showed cutaneous ingrowth in percutaneous implants of porous poly(2-hydroxyethyl methacrylate) (polyHEMA) cylindrical rods with uniform pore size. Implants survived several months without evidence of infection. When implanted rods were challenged with 10^7 colony forming units (CFU) *Staphylococcus aureus* immediately after implantation, the challenge site was infected and bacteria migrated through the implant. Our goal in this study was to determine whether allowing the skin to integrate with the biomaterial prior to bacterial challenge would prevent peri-implant infection. Porous polyHEMA rods were implanted percutaneously in the backs of C57BL/6 mice. The skin was allowed to integrate for seven days into the porous percutaneous device, at which time the entrance insertion sites were challenged with 10^7 CFU *S. aureus* biofilm grown for 24 hours. Implants were harvested seven days after bacterial challenge and evaluated morphologically and by bacterial counts. Neither scab nor inflammation was seen at the challenge site. CFUs were calculated for the skin surrounding the implants and segments along the rod from challenge sites to the unchallenged exit sites. CFU counts for the unchallenged skin were 10^3, challenged entry sites were 10^5, and the middle region of the implants 10^2. These results suggest allowing skin cells integrate with percutaneous porous implants in a sterile environment before challenging the insertion site with bacteria may limit bacteria to the challenge site and reduce bacterial infection of the implant. Current studies in which skin is allowed to integrate with the implants for both shorter and longer times prior to bacterial-challenge are being conducted to evaluate infection rate. These studies will use rods made of porous and solid silicone, a biomaterial more commonly used for medical devices.

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TAp63α promotes the transition of hair follicle stem cells to interfollicular keratinocytes

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p63 is an essential transcription factor expressed as isoforms that either contain (TA) or lack (ΔN) a transactivation domain. The ΔNp63 isoforms are constitutively expressed in the basal layer of the murine epidermis, and have been shown to orchestrate the stratification and differentiation of simple epithelia to stratified epithelia. This contrasts with the TAp63 isoforms, which are not required for epidermal development, but are instead induced upon wounding. Additionally, TAp63-null mice have a marked delay in epidermal wound healing. In order to understand the role of TAp63 during the wound response, we have generated an inducible mouse model that expresses the TAp63α isoform in the epidermis when crossed to a K5 activator and treated with doxycycline. Unexpectedly, continual treatment with doxycycline from birth resulted in the failure of TAp63α-expressing mice to develop a coat of hair. Histological analyses of TAp63α-expressing skin revealed a progressive transformation of hair follicles into cyst-like structures within the dermal compartment. Due to the dramatic hair phenotype in the TAp63α-expressing mice, we next examined hair follicle differentiation via immunofluorescence using the hair follicle markers AE13 and K75 and found that the remaining abnormal follicles were fully differentiated despite their hair shafts never protruding through the epidermis. To determine if the ectopic expression of TAp63α had an effect on the interfollicular epidermis, we examined the differentiation markers K1 and loricrin via immunofluorescence and found no differences between treated and untreated mice. Surprisingly however, we did find that both of these markers were ectopically expressed in the follicular cysts, suggesting a transdifferentiation of follicular keratinocyte stem cells into interfollicular epidermal keratinocytes. In conclusion, our data suggest that TAp63α alters the cell fate of hair follicle keratinocytes, and provide a mechanism by which hair follicle stem cells convert to interfollicular keratinocytes to accelerate wound healing.

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Challenges of tamoxifen and 4-OH-tamoxifen induction in the skin of transgenic K1.CreERT2 adult mice

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Conditional control of gene expression using tamoxifen induction of effector transgenes in mice has been a successful strategy to analyze biological processes, particularly in embryonic and newborn mice. We did not, however, anticipate the difficulty in applying this methodology to create a suprabasal keratinocyte (KC) lineage marker for wound healing studies in adult mice. Tamoxifen inducible transgenic mice with a keratin 1 (K1) promoter (K1.CreERT2) were crossed with either enhanced yellow fluorescent protein reporter mice (Rosa26EYFP/EYFP) or beta-galactosidase (βgal) reporter mice (Rosa26R LacZ). Crossing K1.CreERT2 mice with Rosa26EYFP/EYFP mice allows live animal imaging to visualize efficacy of induction, however, these mice were poor breeders and newborn pups were either cannibalized or abandoned by their mother. We then crossed K1.CreERT2 mice with Rosa26R LacZ reporter mice, since previous studies showed that tamoxifen successfully induced βgal expression in K1 KC in the skin of embryonic and newborn pups. Following induction doses (0.1-1.0 mg/5 days) of either topical 4-OH tamoxifen or i.p. tamoxifen, whole mount staining for βgal activity in the adult K1.CreERT2/Rosa26 LacZ positive mice was patchy and sparse. Commercially available βgal antibodies, which had not been affinity-purified, yielded artificial staining in the epidermis, most likely due to contamination with keratin antibodies. Additional strategies to maximize the likelihood of identifying the βgal positive K1 KC population in adult K1.CreERT2 mice include increasing tamoxifen concentration towards LD50 doses, identifying alternative βgal detection methods, βgal antibody purification or crossing the K1.CreERT2 mice with a different reporter transgenic mouse. Animal age may be a limiting factor in creating a suprabasal KC lineage model for wound healing studies.

1501

Different expression of tight junction proteins in chronic compared to normal wounds – implication on proliferation, differentiation and migration

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Tight junction (TJ) proteins are well known for their important role in barrier formation, but have also been linked to proliferation and differentiation. As these processes are essential for normal wound healing, we investigated the TJ proteins Occludin, ZO-1 and Claudin-1 in normal wounds using a porcine ex-vivo wound healing model as well as in tissue samples of human chronic wounds by immunostaining. Since chronic wounds feature increased levels of IL-1β and TNF-α we also investigated the influence of these cytokines on wound healing and TJ protein localization. In addition, we investigated the effect of knock-down of TJ proteins by siRNA in BrdU-, scratch wound-, adhesion-, differentiation- and cytokine release-assays. Immunohistological analysis revealed differences in the localization of all three TJ proteins between normal healing wounds and chronic wounds, with the latter ones often showing an at least partial loss of the investigated proteins. Cytokine application led to impaired wound healing and altered the localization of the TJ proteins but mimicked only partly the loss of the proteins. Downregulation of Occludin in primary human keratinocytes resulted in increased wound healing in scratch assays without changes in proliferation, thus likely affecting migration. In addition, it reduced cell-cell and cell-matrix adhesion and alters differentiation. ZO-1 had only a slight impact on cell scratch healing and no influence on proliferation, but affected cytokine release. Cldn-1 is also involved in differentiation. In conclusion, our results show different expression and localization of TJ proteins in normal and chronic wounds and that elevated levels of IL-1β and TNF-α are only partly responsible for these alterations. Further, we demonstrate that TJ proteins contribute to cellular characteristics important for wound healing.

1502

Application of light emitting diode and celecoxib (Cox-2 inhibitor) for the management of wounds

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Background: Wound management, especially management of chronic wounds, have always been a challenge for clinicians. Many factors come into play in order for wounds to heal properly and timely. These factors include control of infection, inflammation, stimulation of keratinocytes and fibroblast growth as well as adequacy of wound vascular bed. Of late, LEDs has been shown to upregulate keratinocyte proliferation in cell culture studies through upregulation of growth factors. Objective: Our study aims to evaluate the efficacy of Light-Emitting Diodes (LEDs) combined with topical COX-2 inhibitor in the management of wounds, using burn wound as a model. Methodology: We used a pig model of partial thickness burn injury and studied efficacy of various treatment modalities by a) wound contraction, b) Laser Doppler imaging, c) histology, and d) immunohistochemistry for Ki-67, proliferating cell nuclear antigen, and laminin. The various treatment modalities studied include LED monotherapy, Celecoxib monotherapy, LED + Celecoxib, Silverlon burn dressing and control group. Results: Our findings show that LED + Celecoxib combined treatment is very effective and achieved the best wound healing profile among all treatment groups. The improvement of wound healing is significantly better when compared to control as well as with the other treatment groups. Even when used alone, LED treatment of burn wounds showed good healing when compared to control. Conclusion: Our results suggest that treatment of burn wounds with LED in combination with topical celecoxib significantly improves wound healing. This combination showed potential to be used as a novel therapeutic intervention for the management of wounds, thus possibly opening up new avenues for the management of difficult wounds.

1503

Development of a pragmatic novel assay for studying pathological wound healing of human skin *in vitro*

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The current "ulcer epidemic" warrants the development of clinically relevant preclinical screening tools for testing candidate wound healing promoters. Yet, commonly employed human in vitro-assays fail to reproduce the pathological wound healing milieu that underlies chronic skin ulcers. Here we have asked whether normal human skin can be rapidly transformed into a state that mimics key aspects of chronic ulcer pathobiology. Full-thickness human skin was wounded by preparing a 4 mm punch biopsy and was organ-cultured for 3 days in insulin- and hydrocortisone-supplemented William's E. medium(control) under denervated, non-perfused conditions in the absence of established endogenous wound healing promoters (e.g. serum, estrogen, thyroxine). In test samples, pathological ("pseudodiabetic") wound healing conditions were impaired by hypoxia (5% oxygen), withdrawal of insulin, by the addition of high concentrations of glucose to the medium and repetitive hydrogenperoxide administration to create a ROS-enriched tissue milieu. Compared to controls, reepithelization of test samples was significantly retarded or even abrogated by day 3, along with greatly reduced epidermal proliferation and increased apoptosis (Ki-67/TUNEL quantitative immunohistomorphometry), down-regulation of wound healing-associated cytokeratin 6 and upregulation of dyskeratotic epidermal keratinocytes. Other morphological indicators of tissue damage and LDH release into the culture medium were also significantly increased in test compared to control skin. Thus, human skin can be rapidly transformed in vitro into a pathological, "pseudodiabetic" state that mimics key ulcer characteristics and substantially retards wound healing. We propose that test agents which promote human skin wound healing in this ulcer milieu-imitating preclinical assay are more likely to also promote human ulcer healing in vivo than candidate wound healing promoters identified with conventional in vitro assay.

1504

Fabrication and characterization of epithelial and mesenchymal scaffolds for engineering hair regeneration

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The field of hair follicle regeneration is advancing rapidly, and there have been a number of major achievements over the last decade. Nonetheless, most current technologies are still unable to maintain its in-vivo characteristic in-vitro on the field of hair biology. The creation of new hair follicles for the treatment of alopecia using tissue engineering is promising however never tried before. To recover and enhance the competent hair inducing ability of epithelial and mesenchymal cells, we tried three-dimensional scaffolds for making functionally working epithelial structure for human and mouse hair regeneration study. The bladder-sub mucosal sponge (BSM hybrid sponge) shows the intrinsic activation of melanocyte once we insert inductive dermal papilla sphere. It could be applicable to the study of epithelial mesenchymal signaling pathway. We found that the Fiber sheet with Keratin enhance twice the cell spreading and adhesive property compared with other supplements such as Hyaluronic acid. The human outer root sheath keratinocytes did make cluster onto the mouse newborn epithelial skin scaffold (NESS) However the distribution is uneven due to the physical damage during preparation. This study speculated the possibility of using scaffold for hair regeneration study as point of epithelial cells. The present findings could be relevant model for epithelial and mesenchymal cells to advance our understanding of the hair regeneration and cure for hair loss.

1505

Ologen® collagen matrix: A new dermal scaffold for skin tissue engineering

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Acute and chronic human skin wounds have a large impact on patient's quality of life as well as on the global cost of health care. This problem is expected to further increase with ageing of the human population, illustrating the urgent need for novel chronic wound treatment strategies such as tissue engineered skin. Various epidermal, dermal and bilayered products are currently available for skin wound healing purposes, either cellular or acellular. In this study we investigated for the first time the suitability of the biodegradable porous collagen-glycosaminoglycan (C-GAG) ologen® Collagen Matrix (ologen® CM) for full thickness skin tissue engineering purposes. We generated ologen® CM-based human skin equivalents (HSEs) by seeding ologen® CM with dermal human fibroblasts and epidermal human keratinocytes. Static and centrifugal seeding approaches were compared. Primary human skin cells successfully attached to the ologen® CM and resulting HSEs showed healthy and intact human skin morphology, proliferation, differentiation and basement membrane formation. This was shown by normal presence of proliferation marker Ki67, early and late differentiation markers keratin 10 and involucrin in the epidermis and basement membrane components collagen type IV and laminin 332 at the dermal-epidermal junction of ologen® CM-based HSEs. In conclusion, the C-GAG content of ologen® CM combined with centrifugal seeding of human dermal fibroblasts provides a functional dermal substitute closely representing native human dermis. This opens opportunities for successful applications in full thickness human skin tissue engineering.

1506

Wound healing mouse model without contraction to study epidermal migration and mechanisms of action

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We have developed a tail wound mouse model that is unique. Among the advantages of this model are: a) its simplicity and speed of wounding on the dorsal aspect of the tail, down to fascia; b) the absence of contraction and thus ability to focus on epithelialization; c) delayed healing requiring up to 21 days versus 7-8 days in traditional dorsal back contraction-dependent wounds; d) the ability to observe the wound at all times, without having to shave the hair, including with the use of live photography and the use of an IVIS system; and e) the ability to use the model in any strain of mice, including beige SCID and knock-outs. At designated experimental times, the tail wound healing is determined histologically, the extent of epithelialization is measured microscopically, and special stains and immunostaining are accomplished to measure healing parameters, molecular studies, and differentiation of resident cells and stem cell types. In this report, we show and confirm the validity and extreme usefulness of this model. We show that acceleration of healing takes place with both autologous and human (using beige SCID strains) bone marrow-derived mesenchymal stem cells (MSCs) and pluripotent very small embryonic-like stem cells (VSELs). The VSELs, derived from bone marrow, are extremely effective, and differentiate into endothelial and other cell types critical to wound healing. Moreover, unlike human embryonic stem cells, VSELs do not lead to the development of teratomas in standard in vivo assays. The number of stem cells required for achieving wound closure favors VSELs (400/wound; $p<0.01$) vs. MSCs (500,000/wound; $p<0.04$). In summary, this easy to perform mouse wound healing model appears to be ideal for studying epithelialization, molecular markers, differentiation of multipotent and novel pluripotent stem cells, and other mechanisms of action.

1507

C7 plays a dual role in skin wound healing

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Recessive dystrophic epidermolysis bullosa (RDEB) is a heterogeneous skin fragility disorder caused by mutations in the COL7A1 gene encoding collagen VII (C7), a protein required for secure attachment of the epidermis to the dermis. Patients suffer from a constant wound burden with repetitive wounding of exposed areas and formation of chronic wounds. Although clinical observations suggest that wound closure is impaired in RDEB, limited knowledge exists of C7 function in skin wound healing. Here we employed two different genetic RDEB mouse models to investigate the role of C7, and report that C7 is instrumental for closure of skin wounds by two interconnected mechanisms. First, C7 is vital for re-epithelialization through organization of its ligand, epidermal laminin-332. Loss of C7 perturbs the organization of laminin-332 at the dermal-epidermal junction zone during wound healing, which, in turn, abrogates strictly polarized expression of integrin $\alpha6\beta4$ in basal keratinocytes and has a profound effect on the laminin-332 - integrin $\alpha6\beta4$ signaling axis guiding keratinocyte migration. Second, C7 is directly involved in granulation tissue formation and dermal fibroblast functions during wound closure by supporting fibroblast migration and regulating their cytokine production. Importantly, our findings derived from the mouse models were validated in human wounds. Together, these observations reveal C7 as a key linker between epidermal and dermal wound healing and will help develop therapeutic strategies not only for RDEB but also for other chronic non-healing wounds.

1508

Cx31.1 is associated with apoptosis of scrape-wounded HaCaTs in diabetic conditions

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Modulating connexin43 (Cx43) mediated communication via gap junctions and/or hemichannels increases the wound-healing capacity of skin models in simulated normal and diabetic conditions. Cx31.1 expression is associated with apoptosis in eye and ovary. This study investigated whether Cx31.1 expression was altered in HaCaT scrape-wounded monolayers cultured in normal (NGI) and high glucose and insulin (HGI) and if this was associated with apoptosis. HaCaT cells were treated for 5 days with normal or high glucose (5.5 or 25 mM) and insulin (1 or 10 nM). 600 μm scrape-wounds were introduced and cell movement into the denuded area monitored over 48 h. Cx31.1 expression was determined by immunocytochemistry. Conditioned media was assessed for caspase 3/7 activity, with cells exposure to UV 18 h before caspase measurement (positive control). MTT assay measured cell viability. Cx31.1 expression was increased in HGI compared to NGI, and at higher levels on the membranes of apoptotic rather than healthy cells. Cx31.1 appeared to increase in wound edges at 24 and 48 h after scrape-wounding. Caspase activity in conditioned media from the scrape-wounds showed that apoptosis in NGI reduced at 24 h post-wounding and was similar to control levels at 48 h, although caspase in HGI did not alter. UV exposure significantly increased caspase in both NGI and HGI ($P < 0.05$) although HGI cells showed less caspase than NGI cells pre-UV ($P < 0.01$). In parallel cell viability was reduced post-UV exposure in NGI cells ($P < 0.001$), although not in HGI. Cell viability was not significantly different in unexposed cells under NGI or HGI. Cx31.1 expression altered during wound closure and was associated with 'diabetic' levels of glucose, insulin and apoptosis in HaCaT cells. Despite Cx31.1 being higher in HGI, caspase activity did not alter in HGI conditioned media. Cells in HGI retained viability after UV exposure to a greater extent than those in NGI. Cx31.1 is modulated in wound healing, and may be associated with tissue turn-over in diabetic wounds.

1509

Sox9 is elevated in a subset of keloid scars with cartilage-like histological features

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The fibrotic response to skin wounding can proceed out of control in a subset of the population, resulting in disfiguring and painful keloid scars. Based on the histological and physical features of keloids, as well as gene expression studies, we hypothesized that inappropriate differentiation of dermal fibroblasts towards a chondrocytic cellular phenotype underlies keloidogenesis. In order to investigate the cartilage-like characteristics of keloid scars, standard histological approaches and quantitative RT-PCR were used, and keloid samples (n=11) were compared to normal human skin (n=10). Haematoxylin and eosin staining was used to assess collagen density and arrangement, alcian blue to evaluate glycosaminoglycan (GAG) abundance. Transcript copy numbers for c-Myc and Klf4 (reprogramming and proliferation factors), as well as Sox9 (cartilage-associated transcription factor), were also compared. Semi-quantitative scoring of the histology results revealed greater collagen density in the keloid samples, with a subset of the specimens showing a matrix uniformity reminiscent of cartilage. Moreover, 3 of 11 keloid scars showed significant alcian blue positivity. Quantitative RT-PCR showed striking elevation of Sox9 expression in the three keloids histologically most similar to cartilage (high alcian blue staining). Conversely, there was significantly less c-Myc expression in the keloids relative to normal skin, and Klf4 was unaltered. The findings suggest that in mature keloid scars, such as those collected during revision procedures for this study, markers of differentiation including Sox9 may be elevated, whereas reprogramming factors (e.g. c-Myc and Klf4) have normal or repressed levels. The possibility remains that c-Myc/Klf4 may be elevated during early stages of keloidogenesis, functionally contributing to the erroneous commitment of dermal fibroblasts to a cartilage-like lineage. Our findings highlight that keloids are highly heterogeneous, perhaps reflecting unique aetiologies.

1510

Is prolactin a negative modulator of human skin wound healing?

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Human skin and its appendages are not only targets, but also sources of prolactin (PRL). While PRL potently modulates hair growth and has recently emerged as a regulator of keratin expression in human skin, little is known about the impact of PRL on cutaneous wound healing. However, hair growth and wound healing share multiple characteristics. Furthermore, given that PRL can modulate keratinocyte proliferation, human stem cell biology in situ, and exerts both pro- and anti-angiogenic effects, we tested the hypothesis that PRL modulates human skin wound healing. This hypothesis was probed by assessing the effect of PRL (400ng/ml) on key wound healing parameters in wounded, organ-cultured human skin (full-thickness, punch-within-a-punch design, serum-free William's E medium) from two female subjects aged 57 and 66 years. The tested PRL concentration was selected because it modulates human hair growth, keratin 15+ human hair follicle progenitor cells, and keratin expression and simulates hyperprolactinemia. Pooled data from our pilot assays suggested a tendency of PRL to inhibit reepithelialisation, as evidenced by reduced length and area of the regenerated epithelium, which also showed increased apoptosis (TUNEL assay). Interestingly, proliferation (Ki67) in the regenerated wound epithelium was up-regulated by PRL, followed by increased expression of the late differentiation marker, involucrin. Instead, the wound healing-associated cytokeratin 6 was initially down-regulated. Moreover, there was a trend towards PRL reducing both overall CD31 immunoreactivity and the number of CD31+ vessel lumina. These preliminary in vitro data suggest that PRL indeed impacts on human skin wound healing, where it may primarily operate as a negative wound healing modulator that could limit epithelial regeneration and angiogenesis. This, in turn, raises the question of whether endogenous, intracutaneously produced PRL may serve as a safeguard mechanism against excessive wound healing phenomena such as hypertrophic scars or keloid formation.

1511

VEGF promotes cutaneous wound healing via VEGFR-1 signaling in keratinocytes and macrophages

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Vascular endothelial growth factor (VEGF) plays an important role during wound healing by activating VEGF receptors (VEGFR) on endothelial cells, which stimulates angiogenesis. Interestingly, VEGFR-1 has now been described on other cell types critical for wound healing, including keratinocytes and macrophages. This suggests that VEGF may stimulate repair through angiogenic and non-angiogenic mechanisms. The purpose of this study was to determine whether VEGF directly affects keratinocytes and macrophages during wound repair. Healing was assessed in full-thickness excisional wounds from two unique conditional knockout (KO) mouse strains in which VEGFR-1 was ablated in either keratinocytes or macrophages. A significant delay in wound closure was observed in mice lacking VEGFR-1 in epidermal keratinocytes (epiKO). Wounds from epiKO mice contained fewer macrophages and healed more slowly than control wounds. Strikingly, only 20% of epiKO wounds were completely healed at 5 days compared to 86% of control wounds, suggesting that VEGF may mediate pro-inflammatory mediator production by keratinocytes and directly promote reepithelialization by keratinocytes. Healing was also examined in mice lacking VEGFR-1 in macrophages (macKO). Significantly fewer macrophages were observed in wounds from macKO mice compared to controls, indicating that VEGF acts as a macrophage chemoattractant during repair. Only 15% of the macKO wounds showed complete closure at 5 days compared to 42% of controls. It is likely that reduced macrophage recruitment in macKO mice contributes to the delay in wound closure, as macrophages produce a variety of factors that affect reepithelialization by keratinocytes. Overall, these results suggest that in addition to stimulating angiogenesis, VEGF affects wound healing by acting through VEGFR-1 on keratinocytes and macrophages. These studies provide new information about how VEGF contributes to dermal repair, which could be useful for the development of new strategies to prevent chronic wounds and improve healing.

1512

Development of an *in vitro* organotypic wound model & application of this model to the characterization of notch in healing wounds

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Wound healing affects millions of people annually. Following injury, keratinocytes from the wound edge proliferate, migrate & differentiate to recapitulate the 3-D structure needed to provide a barrier function. We are interested in discovering novel strategies to enhance the wound healing process. Human keratinocytes (NIKS) were grown in organotypic cultures composed of human dermal fibroblasts embedded in type I collagen. Cultures were kept submerged for 5 days, then raised to the air-interface, & maintained for an additional 15 days allowing formation of fully-stratified squamous epithelia. A full thickness wound was created in the organotypic culture & allowed to heal over 14 days. The organotypic cultures express differentiation markers & Notch comparable to human tissue via indirect immunoflorescence (IIF). Notch1 & 2 were expressed at the basal layers, while Notch3 expression extended to cells in the granular layer of the epidermis, indicating a possible role of Notch1 & 2 in proliferation & migration of basal keratinocytes & Notch3 in differentiating keratinocytes. Notch ligand Jag-1 was expressed throughout the epidermis, Jag-2 localized to the basal layer while DLL-1 localized to the suprabasal & granular layer again indicating a differential role.Our hypothesis is further supported in the organotypic wound model we have developed. The expression patterns of the Notch isoforms & ligands at the leading edge of the healing wound were observed through Day 14. While the area away from the wounded edge resembled the expression patterns for Notch & its ligand to that of static cultures, we noticed a robust expression of Notch & Jag-1at the hyperproliferative wound edge & along the leading edge. DLL-1 was not detected. We are using this novel wound healing model to investigate the role of notch in wound healing. Broader applications of this model to investigate new therapeutic interventions for wound healing are also being pursued.

1513

Wound healing in a full-thickness *in vitro* human skin model

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Cutaneous wound healing involves interactions between dermal fibroblasts and epidermal keratinocytes as well as cell-extracellular matrix interactions. The current study describes wound healing experiments conducted in a full thickness in vitro human skin model (EpiDerm-FTTM). This model exhibits stratified epidermal components and a fully developed basement membrane and resembles in vivo skin in regard to morphology and barrier function. Small epidermal only wounds (3mm biopsy punch) or full-thickness wounds (cauterizer burns) were induced in the model and cultures were processed for histological evaluation at various recovery time points. H&E stained sections of burn wounds showed a necrotic epithelium and denatured collagen matrix following burning. Beginning on day 1, matrix degradation and keratinocyte migration into the wounded area was observed. Over the course of 7 days, migrating keratinocytes covered the wounded area and fibroblasts were observed repairing the dermal matrix. Biopsy wounds were conducted with or without 2% human serum. Histological analysis showed keratinocyte migration at 2 days following wounding in both conditions. Wounded tissues cultured without growth factors had a reduced healing rate in which keratinocytes did not cover the entire wound within a 6 day timeframe. Wounded tissues cultured in 2% human serum demonstrated an increase in healing rate as keratinocyte migration completely covered the wounded area by day 6. Increased fibroblast proliferation in dermal areas directly adjacent to migrating keratinocytes was observed in cultures supplemented with 2% human serum. Gene expression profiling of the wounded area showed temporally regulated changes in mRNA expression of basement membrane components, collagens and genes involved in extracellular matrix remodeling. Fibroblast proliferation and epidermal healing in tissues cultured in 2% human serum was severely impaired in the presence of an EGFR tyrosine kinase inhibitor or a TGFα neutralizing antibody. Taken together, EpiDermFT appears to be a promising approach for analyzing cutaneous wound healing.

1514

Biological function & modulation of Thymosin β-4 in human skin

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Thymosin beta-4 (Tβ-4) belongs to a highly conserved, water-soluble, acidic polypeptide family of proteins. Although it was originally identified as an actin-sequestering molecule in eukaryotic cells, Tβ-4 plays a critical role in dermal and corneal wound healing via multiple activities: anti-inflammatory, stimulation of keratinocyte migration, regulating MMP expression, and matrix remodeling. Using a wound healing assay, we demonstrated that Tβ-4 plays a vital role in keratinocyte migration and wound repair. We postulated that this wound repair property of Tβ-4 could be compromised by skin aging since we found that Tβ-4 expression decreases with age in vivo. We initiated a screening program in order to identify active ingredients that can influence the expression of Tβ-4 in skin cells, and potentially produce a positive impact on the visual appearance of aged skin. In this poster, we report on the identification of several novel ingredients that increase the expression of Tβ-4 in skin cells in vitro. Topical skincare formulations containing these novel Tβ-4 stimulators can counteract age-induced reduction of Tβ-4 in vitro. This may help improve the appearance of aging skin in vivo.

1515

Role of kallikrein 6 during epidermal regeneration after glucocorticoid-induced cutaneous atrophy

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One of the major adverse effects of glucocorticoid therapy is cutaneous atrophy often followed by the development of resistance to steroids (tachyphylaxis). Previously we showed that within two weeks of topical fluocinolone acetonide (FA) treatment, interfollicular mouse keratinocytes acquired resistance to the anti-proliferative effects of this glucocorticoid. To search for potential mechanism(s) by which keratinocytes regain their ability to proliferate, we performed extensive DNA array analyses focusing on genes that were activated after a 2 wk treatment with steroids. Kallikrein 6 (Klk6), a trypsin-like serine proteinase known to enhance keratinocyte proliferation and migration both in vitro and in vivo was the most up-regulated (8-10 fold) gene. We confirmed the increased expression of Klk6 by RT-PCR and immunostaining. Interestingly, Klk6 was expressed in single keratinocytes in the suprabasal layer of the atrophic mouse epidermis, and these Klk6+ keratinocytes were adjacent to BrdU+ proliferating keratinocytes. Epidermal induction of KLK6 was also noted after application of glucocorticoid clobetasol propionate to human skin. We identified multiple putative glucocorticoid receptor binding sites (GRE) in the Klk6 promoter suggesting that Klk6 is a primary glucocorticoid receptor target gene. We are using Klk6 knockout (KO) animals to further assess the importance of Klk6 in skin regeneration after steroid-induced cutaneous atrophy. Our pilot experiment indicated that in Klk6 KO mice the development of proliferative resistance to FA was weaker than in wild type littermates. Collectively, our results suggest a novel mechanism of epidermal regeneration after glucocorticoid-induced cutaneous hypoplasia via activation of Klk6, which stimulates keratinocyte proliferation.

1516

Generation of 3D full-thickness skin equivalents exclusively from human induced pluripotent stem cell (iPSC)-derived keratinocytes and fibroblasts

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Cell-based therapies are limited by the number of undifferentiated cells available and/or by immune rejection. Generation of iPSCs can provide unlimited source of immunocompatible, patient-specific cells for medical applications, thus circumventing the obstacles encountered by adult and embryonic stem cells. However, the potential for iPSCs in regenerative medicine is still being realized. Here, we have generated full-thickness 3D skin equivalents using exclusively iPSC derived cells. iPSCs were generated from human dermal fibroblast cells isolated from foreskin, by exogenous expression of 4 transcription factors, Oct3/4, SOX2, MYC, and KLF4. Keratinocytes were differentiated from iPSCs using the method developed in our lab, by treatment with RA and BMP4. Fibroblast cells were derived from iPSCs using ascorbic acid and TGFB2 before culturing in DMEM supplemented with ascorbic acid and 20% bovine serum. These cells assumed fibroblast-like morphology and expressed typical CD surface markers such as CD10, CD44, CD73 and CD90. To generate 3D skin equivalents, iPSC-derived fibroblast cells were embedded in 3 mg/ml of rat tail type I collagen and cultured for 7 days before applying iPSC-derived keratinocytes on top of fibroblast-containing collagen gels. Epidermal differentiation was facilitated by lifting gels to the air interface. Hematoxylin and eosin staining revealed a well-differentiated epidermis with distinct basal, spinous and granular layers and a stratum corneum, and immunohistochemistry showed that collagen VII was expressed at the basal membrane zone. We successfully reconstituted two distinct iPSC-derived cell types into 3D human skin equivalents, which may be utilized for various applications such as high throughput screening and the development of cell-based therapies for inherited skin diseases.

1517

Light emitting diode generated red light modulates keloid-derived fibroblast proliferation and migration speed

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Keloids are a disorder characterized by increased fibroblast proliferation and extracellular matrix deposition. Cultured keloid-derived fibroblasts also demonstrate increased migration speed compared to fibroblasts derived from normal human skin. We previously found that light emitting diode-generated red light (LED-RL) can modulate normal human skin fibroblast proliferation. Here we hypothesized that LED–RL can modulate keloid-derived fibroblast proliferation and migration speed. To test these hypotheses, two different keloid-derived fibroblast lines were irradiated with LED-RL, each matched with a temperature regulated "bench control plate" (BCP), to ensure that the measured effect was a result of LED-RL treatment and not due to other environmental factors. LED-RL at fluences of either 320 J/cm2 or 480 J/cm2 significantly decreased cell proliferation at 48hrs post irradiation (14 - 29% decrease from control, temperature-matched BCP cells, $p<0.05$), with no significant decrease in cell viability, as measured by trypan blue exclusion. Keloid-derived fibroblasts also demonstrated decreased migratory speeds compared to BCPs as measured by time-lapse video microscopy imaging over a period of 4 hours at 30-minute intervals. A fluence of 320 J/cm2 decreased their speed by 15-20% of that of the BCP ($p<0.02$). Similar LED-RL-induced decreases in proliferation and cell migration were observed in 2 normal fibroblast cultures. We conclude that LED generated red light modulates keloid-derived human skin fibroblast functions that are associated with fibrosis. There are few effective treatment options for keloids and other cutaneous fibrotic diseases. We envision that our findings will serve as the foundation for future translational studies that contribute to the management of fibrotic skin disease.

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Genome organizer and AT-rich binding protein Satb1 inhibits epidermal regeneration during wound healing via modulation of cell migration and apoptosis

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Genome organiser and special AT-rich binding protein Satb1 plays an important role in the control of higher-order chromatin remodelling in keratinocyte-specific genes, which is required for the maintenance of their transcriptionally active status (J Cell Biol, 2011, 194, 825). Here, we show that Satb1 expression in mouse epidermal keratinocytes is markedly downregulated during wound healing. To study the role of Satb1 in the control of epidermal regeneration, transgenic (TG) mice overexpressing Satb1 under control of Dox-inducible K14 promoter (K14-rtTA/TRE-Satb1) were generated. TG mice showed significant retardation of the wound healing process accompanied by the decrease of the length and area of hyperproliferative epithelium compared to controls. However, cell proliferation in regenerating epidermis was not altered in TG mice versus the controls. In contrast, TG mice showed significant increase in the number of TUNEL+ apoptotic cells in the hyperproliferative epithelium compared to controls. Also, primary keratinocytes isolated from TG mice showed significant retardation of migration compared to non-TG keratinocytes. Furthermore, ChIP-on-chip and microarray data revealed that a large number of the motility-associated genes encoding the essential components of the cell migration machinery (myosins, troponins, etc.) serve as direct Satb1 targets in keratinocytes. Thus, Satb1 downregulation likely serves as a part of the protective response mechanism controlling epidermal regeneration during wound healing, while Satb1 targeting might be used as a novel approach to improve skin regeneration in chronic wound conditions.

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IL-33 is upregulated after injury and stimulates inflammation during repair

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Inflammation plays an important role in repair by eliminating potential pathogens from the wound. However, inflammation can also delay healing and stimulate scar formation in some cases. Despite the importance of inflammation in the healing process, the exact mechanisms by which inflammation is initiated after injury are not fully understood. Recently, a role for interleukin-33 (IL-33) has emerged in the skin. This cytokine is a member of the IL-1 cytokine family and has been described as an alarmin, which is a class of endogenous molecules that trigger inflammation. The goals of this study were to characterize IL-33 expression during repair and determine the role of IL-33 in wound inflammation. IL-33 expression was examined by immunohistochemistry in adult and fetal murine skin wounds. In uninjured adult skin, nuclear IL-33 staining was observed in basal keratinocytes. IL-33-positive cells began to increase in the dermis 12 hours post-injury and remained high through 48 hours. Increased IL-33 staining was observed in keratinocytes at the wound margin beginning at 24 hours. Epidermal IL-33 expression peaked at 48 hours and remained elevated until 7 days. IL-33 expression was also examined in fetal wounds. In this model, wounds created at embryonic day 15 (E15) lack an inflammatory response and heal scarlessly, while wounds created at E18 heal with robust inflammation and scarring. IL-33 expression was not observed in unwounded E15 or E18 skin. In fetal wounds, more IL-33-expressing cells were seen for a longer period of time in E18 wounds compared to E15 wounds, suggesting that IL-33 may help regulate inflammation and scar formation during wound healing. To test the importance of IL-33 in wound inflammation, wound healing studies were performed in IL-33 knockout (KO) mice. Significantly fewer neutrophils were detected in wounds from IL-33 KO mice compared to wild-type mice, suggesting that IL-33 promotes inflammation after injury. Ongoing studies are examining the effects of IL-33 on other aspects of healing, including scar formation.

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Bone morphogenetic protein signalling regulates keratinocyte proliferation and migration during wound healing in murine and human skin

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Bone morphogenetic proteins (BMPs) and their receptors (BMPRs) regulate tissue development and remodelling; however, their role in wound healing is unclear. To study the role of BMPs in skin repair, we used transgenic mice overexpressing the BMP antagonist Noggin or BMP downstream component Smad1 under the control of a K14 promoter as in vivo models, as well as a human ex vivo wound healing model. K14-Noggin mice displayed accelerated wound healing, associated with increased keratinocyte proliferation at the wound margin and a hyper-proliferative wound epithelium, as well as increased wound capillary density versus the corresponding controls. In contrast, K14-Smad1 mice exhibited retarded wound healing and significantly reduced wound epithelial surface area compared to controls. qRT-PCR showed down-regulation of BMPR-IA and up-regulation of BMPR-IB in murine wounded skin, as well as down-regulation of the BMP receptor ligands (BMP2/4/6/7). Human and mouse keratinocyte proliferation was impaired after treatment with BMP4/7 as assessed using flow cytometry, whereas treatment with BMP antagonist Noggin increased cell proliferation. siRNA silencing of BMPR-IA increased keratinocyte proliferation, whilst constitutive activation of both BMPR-IA and IB reduced cell proliferation. Keratinocyte migration was slowed after BMP4/7 treatment compared to controls. Furthermore, silencing of BMPR-IB accelerated migration compared to controls, whilst targeted overexpression of BMPR-IA/IB in conjunction with Smad1/5 attenuated migration. Topical administration of BMP4/7 to a human ex vivo wound healing model impaired epithelial closure versus the controls, whereas treatment with Noggin resulted in accelerated epithelial closure. Thus, this study demonstrates that BMPs negatively regulate keratinocyte proliferation and migration during wound healing, and raises a possibility for using BMP antagonists for the management of chronic wounds.

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TNS4 delayed wound healing through actin cytoskeletal regulation

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Tensin proteins are known to play a pivotal role in cell migration, wound healing and tumorigenesis. TNS4 is one of the four tensin family members, but little is known about its role in skin epidermal migration and wound healing. In this study, TNS4 was over-expressed in primary cultured normal human epidermal keratinocytes (NHEK) *in vitro* or epidermis *in vivo* using a GFP tagged adenoviral over-expression system. We examined the role of TNS4 in the skin through a wound healing assay. In the TNS4 over-expressed NHEK, TNS4 was observed in the peripheral cytoplasms, and the morphology and the size of the keratinocytes differed from that of the GFP controls. Consistent with the morphological change, F-actin polymerization was decreased where TNS4 was over-expressed on immunocytofluorescent staining. When in vitro scratch assay was performed, scratched area was remained significantly larger in the TNS4 over-expressed cell layer after 48 hours compared to the GFP control group (59% VS. 26%, respectively, n=3, p=0.05) indicating that cell migration was diminished by TNS4 over-expression. Cdc42 have well known associations with F-actin polymerization and cell migration. We found that Cdc42 protein level was reduced by 50% when TNS4 was over-expressed, in addition to the downstream signals of Cdc42 being reduced. Cdc42 level was restored when treated with a proteasome inhibitor was treated. When TNS4 was over-expressed, the proportion of S phase was reduced in TNS4 over-expressed group. Finally, we confirmed a delay in wound healing in vivo using mice back skin with punch injury. Four days after wounding, the injured area was 1.7-fold ($p<0.05$) wider in the TNS4 over-expressed group compared to the GFP control group. In conclusion, our study suggested that TNS4 over-expression triggers Cdc42 degradation through a proteasome pathway and eventually affects the cytoskeletal system, which leads to aberrant cell migration, proliferation, and delayed wound healing.

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Development of ECM-based biohybrid skin substitutes

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Objective: To utilize discarded adipose tissues as waste-to-resource materials for the development of in vitro biohybrid skin substitutes containing natural biological cues. Currently, adipose tissue is often discarded after plastic and reconstructive surgery in the form of lipoaspirates and grafts. A chemical-free decellularization method was used to obtain ECM from the human adipose tissues. The whole decellularization process used to treat the lipoaspirate material takes place in over an hour as compared to days taken by the commonly-used chemical and hybrid methods. H&E staining results showed no evidence of visible nuclear material, whilst the amount of dsDNA left in the dry ECM was 47.1 ng/mg. SEM analysis further confirmed the removal of both cells and lipids from the remaining fibrous ECM material. Besides, immunostaining results showed that key structural proteins such as collagen type I (Col1), collagen type IV (ColIV) and laminin remained intact in the ECM. Angiopoietin-like 4 (ANGPTL4), a matricellular protein that is implicated in wound healing and angiogenesis, is also found intact in the ECM obtained using our chemical-free decellularization method. The successful coupling of ECM with alginate was confirmed by the presence of amide I band (at 1642 cm-1) and amide II band (at 1523 cm-1) in FTIR spectrum. Alginate scaffolds after 60 min of second cross-linker treatment had relatively larger pores and thicker inter-pore connectivity compared to those without second cross-linker treatment. It was observed that the size of the scaffolds decreased with increasing duration of second cross-linker treatment. Overall, this biohybrid scaffold is a stepping-stone towards the development of tunable acellular scaffolds with cellular benefits without the need for additional biologics.

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Expression of either microRNA-198 or FSTL1 from a primary transcript is controlled by TGF-β and KSRP for efficient wound healing

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Wound healing requires the regulated integration of complex biological events including cell migration, proliferation and extracellular matrix remodeling, globally stimulated by TGF-β and other growth factors. We report a novel post-transcriptional switch that dictates the spatio-temporal and mutually exclusive expression of two alternative gene products from a single transcript. In unwounded normal skin expression of exonic microRNA-198 (miR-198), located in the 3′-untranslated region of follistatin-like-1 (FSTL1) mRNA is observed. Upon wounding miR-198 expression is shut down and expression switches to the linked open-reading-frame (ORF) of FSTL1. We demonstrate that binding of a KH-type splicing regulatory protein (KSRP) to the primary transcript determines the fate of the transcript and is essential for the processing of miR-198: TGF-β-signaling switches off miR-198 expression by down-regulating KSRP, which promotes FSTL1 protein expression. We also observed that FSTL1 expression promotes keratinocyte migration, whereas miR-198 expression has the opposite effect by targeting and inhibiting genes important for migration. A clear inverse correlation between the expression pattern of FSTL1 (pro-migratory) and miR-198 (anti-migratory) highlights the importance of this regulatory switch in controlling context-specific gene expression to orchestrate wound re-epithelialization. The deleterious effect of failure of this switch is apparent in non-healing chronic diabetic ulcers, where expression of miR-198 persists, FSTL1 is absent and keratinocyte migration, re-epithelialization and wound healing all fail to occur.