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Nesprin-2 declines during skin aging and its loss negatively impacts fibroblast and keratinocyte behavior *in vitro*

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Nesprins are large multi-domain proteins that form a physical link between the cytoskeleton and nuclear membrane resulting in a stable physical connection which is essential for a wide range of cellular functions including nuclear positioning, cell polarization, motility, and mechanosensing. Mutations in the nesprins impair intracellular force transmission and are linked to muscular dystrophies and cardiomyopathies. In this study we investigated the expression and functional role of nesprin-2, the predominant isoform in human skin. The goal is to determine if nesprin-2 expression is affected by chronologic and photo aging and the functional consequences of nesprin-2 downregulation on human skin fibroblasts and keratinocytes. We found that nesprin-2 staining decreased in skin biopsies from sun-exposed regions versus sun-protected controls and that nesprin-2 expression was significantly lower in older-age skin cells versus young controls (80% decrease, $p<0.01$). To investigate functional implications of nesprin-2 downregulation we used small interfering RNA to knockdown nesprin-2 expression in fibroblasts and keratinocytes. Knockdown was confirmed by nesprin-2 protein staining and dramatically altered the actin and microtubulin cytoskeletal structure. Furthermore, the size of nuclei tended to increased (12.9%, $p=0.07$, $n=30$-36) and the circularity of nuclei decreased significantly (2.2%, $p=0.005$, $n=30$-36) in nesprin-2 knockdown cells. Knockdown resulted in misaligned collagen deposition in 2D fibroblast culture, reduced contractility in a fibroblast populated 3D collagen gel (20% less contraction, $p<0.05$, $n=3$), and slower migration/proliferation in a wound healing assay for both fibroblasts (46% less closure, $p=0.003$, $n=10$) and keratinocytes (55% less closure, $p=0.002$, $n=10$). Our results demonstrate that nesprin-2 downregulation, which occurs during both chronological and photo aging, has an important role in skin cell functionality *in vitro* and may be a relevant cellular target for treatment of aging skin conditions *in vivo*.

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Interferon-γ (IFN-γ) impedes wound healing by slowing keratinocyte migration through the upregulation of SHIP-2 and phospho-cofilin

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Wound healing is a complex process that is influenced by multiple factors, including the wound's immune environment. Our previous studies showed IFN-γ's inhibitory effect on keratinocyte migration [JID 134: S133, 2013]. Herein, we investigated the mechanism by which IFN-γ impaired keratinocyte migration, by examining its effects on SH2-containing inositol 5′-phosphatase (SHIP-2) and phospho-cofilin (p-cofilin). SHIP-2 is a docking protein for the cytoskeleton and p-cofilin is an inactive form of cofilin, the enzyme which severs actin polymers into monomers; upregulation of these proteins has been associated with impaired keratinocyte migration. We also examined the effects of IFN-γ on keratinocyte proliferation. SHIP-2, cofilin and p-cofilin expression was assessed by western blot in HaCaTs, an adult, immortalized keratinocyte cell line, treated without or with IFN-γ at 20 ng/mL. Flow cytometry was performed to examine changes in cell cycle; the percentage of cells in each phase was quantified (n= at least 3 for all experiments). We found that SHIP-2 and p-cofilin expression was significantly upregulated in the IFN-γ treated group relative to control (SHIP-2 increased 2.8 ±0.6 fold ($p<0.05$); p-cofilin increased 2.0 ±0.3 fold ($p<0.05$)). Cofilin expression did not significantly change. Flow cytometric analysis showed that following IFN-γ treatment, there was a significantly increased percentage of cells in G1, and a decreased percentage of cells in S and G2, relative to control. We show here that IFN-γ upregulates both SHIP-2 and p-cofilin. These findings extend our previous data regarding IFN-γ regulation of keratinocyte migration. In addition, we showed that IFN-γ inhibits keratinocyte proliferation. Both of these effects may inhibit wound healing, and suggest that IFN-γ itself, or its downstream proteins, may be therapeutic targets for influencing wound healing dynamics.

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Vitamin D and calcium regulation of epidermal wound healing

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Wound healing is essential for survival. This is a multistep process involving a number of different cell types. In the skin wounding triggers an acute inflammatory response, with the innate immune system contributing both to protection against invasive organisms and to triggering the invasion of inflammatory cells into the wounded area. These cells release a variety of cytokines and growth factors that stimulate the proliferation and migration of dermal and epidermal cells to close the wound. In particular, wounding activates stem cells in the interfollicular epidermis (IFE) and hair follicles (HF) to proliferate and send their progeny to re-epithelialize the wound. b-catenin and calcium signaling are important for this activation process. . Mice lacking the CaSR and/or the VDR when placed on a low calcium diet have delayed wound healing. This is associated with a blunted innate immune and inflammatory response to wounding in the epidermis and reduced proliferation and b-catenin transcriptional activity in the cells at the leading edge of wound closure. These effects are opposite to the roles of vitamin D and calcium signaling during keratinocyte differentiation. Surprisingly, deletion of the VDR coactivator Med1 accelerates wound healing, associated with increased proliferation and b-catenin signaling. These data suggest that vitamin D and calcium signaling are necessary components of the epidermal response to wounding, likely by regulating stem cell activation through increased b-catenin transcriptional activity.

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Reconstitution of three-dimensional skin composed of keratinocytes, fibroblasts and melanocytes induced from Muse human pluripotent stem cell

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Mulitilineage-differentiating stress enduring (Muse) cells exist among human somatic tissues and are non-tumorigenic pluripotent stem cells that have self-renewal ability and can differentiate toward tridermic cells like as ES and iPS cells. We have reported in SID2014 that functional melanocytes were inducible from Muse cells derived from human adipose tissue (Muse-MC). Muse-MC could reside in the basal layer of epidermis in reconstituted 3D cultured skin (3D skin) and expressed melanocyte related proteins. To generate 3D skin sheets composed of cells derived from Muse cells, we aimed to induce fibroblasts (FB) and keratinocytes (KC) from Muse cell. We cultured Muse cells obtained from adipose tissue (hASC-Muse) in medium containing 0.3 mM ascorbic acid and 10 ng/mL TGFβ2 and observed that FB-like spindle-shaped cells after 4 weeks (Muse-FB). The Muse-FB expressed collagen 1 and 3, CD10 and CD73, representative genes of normal FB. We also generated KC from hASC-Muse by culturing with 100 μM all-trans retinoic acid and 30 ng/mL BMP4 for 5 weeks (Muse-KC). Muse-KC expressed genes related to KC such as keratin 1 and 14 and Desmoglein 3. For the next step toward future clinical usage of Muse derived skin components, we generated 3D skin using Muse-MC, -FB and -KC. A type 1 collagen layer containing Muse-FB was deposited onto insert chamber membranes and incubated for 4 days. Muse-KC and Muse-MC at the ratio of 5:1 were seeded on the collagen layer. They were incubated in keratinocyte medium for 6 days and then the Ca^{2+} concentration of the media were gradually increased. The 3D skins were cultivated for another 7 days at the air-liquid interface and then fixed for evaluation. We observed the well-organized distribution of the Muse-MC, -FB and -KC in the 3D skin. These data showed that Muse cell have potentials to differentiate into major cells in human skin and suggested that Muse cells can be used to treat skin defects and to enhance wound healing.

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High glucose environment increased thrombospondin-1 expression in keratinocytes via epigenetic regulation: Metabolic memory of impaired angiogenesis during diabetic wound healing

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Diabetes is an important health issue due to its increasing prevalence and association with occurrence of chronic complications. Metabolic memory described the condition in which diabetic complications still occur despite good glycemic control. Impaired healing is an important complication of diabetes associated with metabolic memory that still poses clinical challenges in many situations. In this study, we demonstrated that the expression of thrombospondin-1 (TSP1), a keratinocyte-derived anti-angiogenic molecule known to delay wound healing, is significantly increased in high glucose environment. Mechanistically, we showed that DNA hypomethylation at TSP1 promoter was responsible for increased TSP1, and this epigenetic change was induced by increased oxidative stress but not advanced glycation endproduct. Similar findings were validated in a diabetic rat model. Moreover, early antioxidant administration normalized TSP1 expression and its DNA promoter methylation status in diabetic rat skin as well as improved diabetic wound healing *in vivo*. Since oxidative stress contributed to TSP1 DNA hypomethylation, early recognition of diabetic condition and timely administration of antioxidant are logical approaches to reduce complications associated with diabetes since alterations in epigenome may not be reversible by normalization of glucose levels during later stages of the disease course, contributing to occurrence of metabolic memory. More importantly, epigenetic profiling and modulation of epigenome may provide important prognostic information and offer preventive therapy, respectively, regarding complications associated with diabetes.

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Targeted genetic alteration in hyaluronan catabolism delays wound healing in mice

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The breakdown and molecular size of Hyaluronan (HA) has been hypothesized to influence wound healing, but direct evidence of this has not been obtained. To test if the breakdown of HA acts as an endogenous signal of injury we generated transgenic mice that conditionally overexpressed Hyaluronidase 1 (HYAL1). When HYAL1 was constitutively expressed in early embryogenesis (EIIa/HYAL1), these mice showed extensive degradation of HA in the dermis yet were morphologically normal. When these mice were wounded by full-thickness 6 mm punch biopsy, wound closure was delayed by approximately 2 days in EIIa/HYAL1 compared to control mice ($p<0.001$). Delayed repair may result from abnormal macrophage polarization in wound tissues 48 hours after wounding as we found a significantly higher M1/M2 macrophage ratio in EIIa/HYAL1 mice ($p<0.05$). Consistant with a role for HA fragments in this response, we showed treatment with specific sized HA fragments induced the M1 marker Nos2 but not the M2 marker Arg1 in bone marrow-derived macrophages (BMDM). Histological and flow cytometirc evaluation of the skin wounds at 96 hours showed significantly more inflammatory cell infiltration in EIIa/HYAL1 mice ($p<0.05$). MIP-2 mRNA and protein levels were also significantly higher in EIIa/HYAL1 ($p<0.001$). Furthermore, mRNA expression of CD44, a receptor of HA, was significantly higher in EIIa/HYAL1 before wounding ($p<0.05$) and immunohistochemistry confirmed the higher expression of CD44 in endothelial cells. Responses to HA catabolism were dependent on CD44 as the delay of wound closure was rescued by deletion of CD44. These data show that abnormal degradation of hyaluronan modulates wound healing in a CD44-dependent manner and suggests targeting this pathway may be therapeutically useful in treatment of abnormal wound repair.

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Collagen XVII regulates actin dynamics and traction forces in motile keratinocytes

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During wound healing keratinocytes disassemble hemidesmosomes and reorganize their actin cytoskeletons in order to move directionally over and exert traction forces on the dermis. The hemidesmosome component collagen XVII (C17) is found in an actin-rich lamella, subjacent to the leading lamellipodia of the cells. Since C17 binds directly to actinins (actin-associated proteins), we tested the hypothesis that C17 regulates motility via effects on the actin cytoskeleton. We evaluated actin dynamics, adhesion protein localization and traction force generation *in vitro* in live keratinocytes and keratinocytes deficient in C17 moving on a gel substrate of 5kPa stiffness, equivalent to soft dermis/a wound matrix. Traction forces were mapped by bead displacement in the gel. 60% of control keratinocytes exhibit a fan shape with a single lamellipodium. A set of actin arcs, along which C17 codistributes with actinins, is present at each lamella. Kymography reveals that the arcs are stable as cells move forward with traction forces being generated close to the edges of the actin arcs, sites enriched in paxillin, a focal contact protein. Approximately 40% of control cells are elongated, lack actin arcs and form multiple lamellipodia. Traction forces localize close to each lamella and are directed centripetally such that they cancel each other out. These cells do not move directionally but, over time, change direction and convert to a fan-shape. Only 25% of C17-deficient cells are fan-shaped but fail to move directionally. Traction forces exerted close to the lateral edges of their actin arcs are diminished with their actin arcs being significantly more dynamic than controls. The majority of mutant keratinocytes are elongated with multiple lamellipodia, lack actin arcs although their traction forces are comparable to those of control elongate cells. Unlike controls, however, these cells fail to convert to a fan-shape. In conclusion, our data indicate a novel function for C17. C17/actinin complexes restrict actin dynamics which is key to the force generation necessary for directed migration.

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Cadherin endocytosis, adhesion, and cytoskeletal linkage cooperatively regulate collective cell migration

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Endothelial cell adhesion is dynamically regulated during angiogenesis, a process important for development and for wound healing in adult organisms. Angiogenesis occurs by migration and proliferation of endothelial cells, activities that require tight regulation of adhesion dynamics. Yet, the mechanisms that control junctional plasticity are not fully understood. The adherens junction protein VE-cadherin mediates adhesion through homophilic *trans* interactions via a conserved tryptophan (Trp2) in its extracellular domain. *Trans* binding is followed by *cis* interactions that laterally cluster the cadherin in junctions. Through the VE-cadherin cytoplasmic tail, the cadherin associates with catenins, which stabilize the cadherin at the cells surface, link the cadherin to the cytoskeleton and increase adhesive strength. Previously, our lab determined that mutation of specific amino acids in the VE-cadherin cytoplasmic tail prevents endocytosis of the cadherin and inhibits collective endothelial cell migration. Interestingly, we now report that the inhibition of migration by this mutant is dependent upon both adhesion and cytoskeletal linkage. Mutation of the critical Trp2 residue disrupted adhesion and increased VE-cadherin endocytosis relative to wild type cadherin. Additionally, we observed that forced lateral dimerization of VE-cadherin prevented endocytosis even in the absence of homophilic adhesion. Importantly, dimerized VE-cadherin also inhibited migration in an adhesion dependent manner. Lastly, inhibition of VE-cadherin endocytosis disrupted both Golgi reorientation in scratch wound assays and reorientation of endothelial cells in response to shear flow, processes that are important for directed cell migration and for normal vessel homeostasis. These findings suggest that endocytosis, adhesion, and cytoskeletal linkages of VE-cadherin cooperate to control endothelial cell migration by regulating endothelial cell polarity.

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Keloid pathogenesis: Potential role of EDA domain of cellular fibronectin

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Fibronectins (FNs) are high molecular weight glycoproteins present in extracellular connective tissue matrices (ECM) and extracellular fluids, including blood plasma. The human FN gene consists of 45 exons, and the primary mRNA transcripts are alternatively spliced to form up to 20 different mRNA variants. One of the alternatively spliced exons encodes the extra domain A (EDA), that is regulated developmentally and is found exclusively in cellular fibronectin (cFN) but not in plasma fibronectin (pFN). Recent evidence has implicated a crucial role for FN-EDA not only in normal wound healing but also in fibroproliferative disorders characterized by increased production and deposition of ECM, such as in scleroderma. Our study seeks to elucidate whether FN-EDA may play a role in the development of keloid lesions. Tissue specimens were obtained from patients with keloids or from control skin in the context of cosmetic surgery procedures. Relative quantitative RT-PCR indicated dramatic, up to 70-fold ($p = 0.005851$) increase in FN-EDA mRNA in keloid tissues in comparison to controls ($n = 5$ both groups). Western blotting of the protein from keloids revealed a significantly enhanced level of FN-EDA of the molecular weight of ~220 kDa, corresponding to a FN monomer, while very little, if any, protein was found in extracts of control specimens. Localization of FN-EDA in keloid tissues was determined by immunofluorescence staining with the same antibody as used for Western blotting, and indicated heavy decoration of the surfaces of the type III collagen fibers with FN-EDA antibody in keloids in contrast to complete absence of FN-EDA in control skin. Although the basic cause of the high level of FN-EDA expression in keloid tissue and its virtual absence in normal tissue remains to be determined, its presence may explain in part the continuous excess matrix production in the development of keloids.

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Paracrine regulation of wound angiogenesis through cooperation of epidermal integrins

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While roles for individual integrins in wound healing are well known, it remains unclear how different integrins cooperate to regulate wound healing, hindering the development of clinical therapies to target integrins. To address this issue, we used a genetic approach to test combinatorial roles of two epidermal integrins, $\alpha3\beta1$ and $\alpha9\beta1$, since their extracellular matrix ligands (includ laminin-332 and cellular fibronectin, respectively) are important for wound healing. As an in vivo model, we used Cre-Lox to generate mice with epidermis-specific deletion of $\alpha3\beta1$ ($\alpha3$eKO), $\alpha9\beta1$ ($\alpha9$eKO), or both integrins ($\alpha3/\alpha9$eKO). We also derived cultured mouse keratinocytes (MK cells) that express $\alpha3\beta1$ or $\alpha9\beta1$, alone or together. We showed previously that $\alpha3$-null MK cells display reduced secretion of factors that induce endothelial cell migration, and that $\alpha3$eKO mice display reduced wound angiogenesis, indicating that epidermal $\alpha3\beta1$ promotes angiogenesis through paracrine stimulation of endothelial cells. Our current work shows that $\alpha9\beta1$ in MK cells inhibits $\alpha3\beta1$-mediated paracrine stimulation of endothelial cell migration or survival, indicating a cross-suppressive role for $\alpha9\beta1$. Moreover, wounds of $\alpha9$eKO mice show persistent angiogenesis and delayed vascular normalization, due in part to reduced endothelial apoptosis. Together, our findings suggest an $\alpha9\beta1$-dependent "brake" on ability of epidermal $\alpha3\beta1$ to promote wound angiogenesis. Consistently, microarray analysis of our MK cell panel revealed many instances in which $\alpha9\beta1$ cross-suppresses $\alpha3\beta1$-mediated gene regulation. Our results reveal a novel cooperation between epidermal integrins and suggest that targeting $\alpha9\beta1$ to control functions of $\alpha3\beta1$ may be an effective therapeutic strategy to treat wound healing defects.

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Scar from minor burns: Focus on cutaneous nerve destruction and regeneration

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The extent of nerve damage after cutaneous trauma and its subsequent regeneration plays a vital role not only in wound repair but also in the sensory function of the resulting scar. This study investigated the extent of damage to cutaneous nerves after a non-severe burn injury and the anatomical and functional changes in cutaneous innervation. Using a rat model, changes in TRPV1(c-fibre marker increased in thermal hyperalgesia), TrkA(c- fibre marker increased in mechanical hyperalgesia) and NF200 expression (myelinated fibre marker mediating innocuous mechanical stimuli) were quantified. In post-burn scar, TRPV1 and TrkA expression was significantly increased ($p=0.004$ and 0.004 respectively) while NF 200 expression decreased ($p=0.002$) indicating an increase in peptidergic c- fibre nociceptor density and decrease in touch pressure sensibility in post-burn skin. Analysis of neurons in the dorsal root ganglia (DRG) corresponding to the site of burn injury revealed a significant decrease in total DRG cell count ($p=0.038$) and large cell count ($p=0.009$) ipsilateral to the injury. There was a trend for reduced cell counts contralateral to the burn injury. Electrophysiological activity of neurons isolated from the DRG was assessed using multi electrode array (MEA). This revealed impaired electrophysiological activity in neurons isolated from burn injured animals compared to non-injured controls. These findings show anatomical and functional impacts of burn injury on peripheral nerves and at the level of the cell bodies in the DRG. These results will help in the future development of targeted interventions for improved sensory function and scar outcome.

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Comparison of the transcriptomes of mouse skin derived precursors and SKP-derived fibroblasts by RNA-Seq

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Skin-derived precursors (SKPs) from dermis possess the capacities of self-renewal and multipotency. *In vitro* and *in vivo* studies demonstrated that they can differentiate into fibroblasts. However, little is known about the molecular mechanism of the differentiation of SKPs into fibroblasts. Here we compare the transcriptomes of mouse SKPs and SKP-derived fibroblasts (SFBs) by RNA-Seq analysis, trying to find differences in gene expression between the two kinds of cells and then elucidate the candidate genes that may play important roles in the differentiation of SKPs into fibroblasts. A total of 1971 differentially expressed genes (DEGs) were identified by RNA-Seq, which provided abundant data for further analysis. Gene Ontology enrichment analysis revealed that genes related to cell differentiation, cell proliferation, protein binding, transporter activity and membrane were significantly enriched. The most significantly up-regulated genes *Wnt4*, *Wisp2* and *Tsp-1* and down-regulated genes *Slitrk1, Klk6, Agtr2, Ivl, Msx1, IL15, Atp6v0d2, Kcne1l* and *Thbs4* may play important roles in the differentiation of SKPs into fibroblasts. KEGG analysis showed that DEGs were significantly enriched in the TGF-β signaling pathway, Wnt signaling pathway and Notch signaling pathway, which have been previously proven to regulate the differentiation and self-renewal of various stem cells. These identified DEGs and pathways could facilitate further investigations of the detailed molecular mechanisms, making it possible to take advantage of the potential therapeutic applications of SKPs in skin regeneration in the future.

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Epigenetic regulation of the wound healing: the role of Polycomb Cbx4 gene in the epithelial regeneration

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The ability of the skin to repair wounds requires immediate changes in expression of the genes controlling epidermal proliferation, migration, cell adhesion and differentiation. The epigenetic regulatory mechanisms play a key role in the control of gene activation and silencing during epidermal development and keratinocyte differentiation. However, their role in gene expression changes accompanying the process of wound reepithelization is largely unknown. To determine dynamic changes in gene expression in the regenerating wound epithelia at different time-points after injury, laser capture microdissection (LCM) and global microarray analyses were employed. Regenerating wound edge showed dramatic and dynamic changes in expression of the genes involved in reorganization of chromatin structure compared to the unwounded epidermis. Among them were genes encoding Polycomb group (PcG) proteins (Pcgf5, Bmi, MEL18, Ring1A/B, RNF2, Cbx, Ezh2, and Eed), genes involved in regulation of covalent histone modifications (Hdac2, Hdac5, Kdm5b, Kat2b), ATP-dependent chromatin remodeling (Chd2, Chd7, Chd8) and genome organizer Satb1. Key component of the Polycomb repressive complex 1 Cbx4 gene showed dynamic expression in the wounded epithelia. To study the role of Cbx4 in the control of epidermal regeneration, conditional Cbx4 flox/flox/K14-CreERT were generated. Cbx4 knockout mice showed significant retardation of the wound healing process accompanied by alteration of proliferation in the wound epithelium compared to controls. Cbx4 ChIP-Seq data revealed significant enrichment of the genes that encode cyclin-dependent kinase inhibitors (Cdkn2a/p16 and Cdkn1c/p57) among the list of direct Cbx4 targets. These data suggest Cbx4 as a key determinant regulating keratinocyte proliferation in the injured epidermis and raise a possibility to develop novel approaches for modulation of the wound-induced skin regeneration via targeting Cbx4.

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Fibroblasts from the elderly fail to deposit sufficient extracellular matrix to generate connective tissue *in vitro*

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Elderly patients are more prone to skin trauma and are slower to heal resultant wounds. The poor state of dermal collagenous matrix is largely responsible for both the propensity to injury and slowness to heal. Fibronectin (FN), a protein found in the extracellular matrix (ECM), which is synthesized by fibroblasts and plays key roles in wound repair as well as morphogenesis and embryogenesis, is well known to provide a critical link between stromal cells and ECM. Less appreciated is the requirement of FN matrix deposition for collagen matrix deposition. Although fibroblasts from elderly individuals (EHDF) synthesize as much, or more, FN than fibroblasts from adult individuals (AHDF), few studies have addressed the ability of EHDF to deposit FN in the pericellular matrix and ultimately in the ECM. We have found that cultured EHDF deposit 30 to 40% less FN matrix than AHDF as judged by quantitative immunofluorescence (IF). The IF finding was validated by sequential detergent extractions of pericellular FN matrix and ECM FN matrix by 1% deoxycholate and 4% sodium dodecyl sulfate (SDS), respectively, followed by ELISA and Western blot quantification. Both extracted fractions from EHDF demonstrated up to 45% decrease compared to AHDF extracted fractions. Finally, using an in vitro AHDF system that generates connective tissue in one week as judged by 3-D layers of FN and collagen matrix (FN/Col) intercalated with fibroblasts, we found that EHDF failed to generate enough FN/Col to make connective tissue. Our findings strongly suggest that the dermal collagenous matrix deficit observed in the elderly is a result in part by deficient FN/Col matrix deposition in addition to the well known increased FN/Col degradation by matrix metalloproteinases.

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IRF1 protein levels depend on microRNA miR-31 and reduced levels of IRF1 inhibit keratinocyte migration

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MicroRNAs fulfill important functions in the skin by regulating gene expression posttranscriptionally. miR-31 is one of the few microRNAs associated with the activated state of keratinocytes. It negatively regulates a core set of target genes such as STK40, LATS2 or EMP1. A key function of miR-31 appears to be to regulate the balance of proliferation, migration and innate immunity in activated keratinocytes. Interestingly, miR-31 is located adjacent an interferon cluster on chromosome 9 and is within the same intro of the miR-31 home gene LOC554202 as is interferon epsilon (IFNE). Therefore, we were interested whether miR-31 can influence the expression of certain interferon-regulated genes. IRF1 is a predicted miR-31 target gene and is constitutively expressed in keratinocytes *in vitro*. Although miR-31 can modestly reduce the expression levels of IRF1 mRNA, inhibition of miR-31 in keratinocytes leads to a significant reduction of IRF1 protein levels. This surprising result indicates that miR-31 is required for IRF1 but not IRF2 protein expression. To test what function IRF1 may have in the miR-31 regulated network, we focused on the ability of miR-31 to regulate keratinocyte migration: miR-31 can increase migration in scratch assays of HaCaT cells and loss of IRF1 leads to a loss of migratory behavior. Our data indicate a link a surprising link between miR-31 and the regulation of IRF1. Although generally regarded as exceptions, we show here that inhibition of the activated keratinocyte microRNA miR-31 leads to the loss of IRF1 protein expression and that miR-31 can function to maintain protein expression levels of putative target genes.

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The pivotal role of periostin in RDEB scarring

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Recessive dystrophic epidermolysis bullosa (RDEB) wounds heal with excessive scarring. RDEB is caused by mutations in the gene that encodes type VII collagen (C7). Extracellular matrix components not only serve as structural matrices but also as active regulators of tissue fibrosis. We showed previously that RDEB patients display increased pro-fibrotic TGF-beta signaling and a distinct pro-fibrotic gene expression program. In this study, we show that periostin, a "matricellular protein", is markedly increased in the skin and sera of 20 RDEB patients. RT-PCR and immunoblot analysis of RDEB fibroblasts revealed 3-30 fold elevated mRNA levels and 3-80 fold higher protein levels of periostin compared with normal human fibroblasts (NHFs). Two RDEB siblings with the same C7 mutations had marked differences in scarring severity that correlated with their expression of periostin. Elevated periostin in RDEB patients is due to their paucity of functional C7 since reduction of C7 in NHFs via siRNAs led to increased expression of periostin, while re-introducing C7 into RDEB fibroblasts via molecular engineering caused a marked decrease in periostin. The siRNA–silencing of periostin in RDEB fibroblasts dramatically reduced the expression of fibrosis markers [collagen I, fibronectin, connective tissue growth factor (CTGF), and alpha smooth muscle actin (α-SMA)] and reversed the characteristic RDEB cellular phenotype of collagen lattice hypercontractability and increased cell migration. Concordantly, the addition of periostin to NHFs resulted in increased fibronectin, collagen 1, CTGF, and α-SMA. Lastly, inhibition of TGF-beta activity with angiotensin II type 1 receptor antagonists significantly decreased the expression of periostin, collagen I, fibronectin and α-SMA in RDEB fibroblasts and reduced their hypercontraction of collagen lattices. In conclusion, loss of C7 in RDEB patients up-regulates periostin, which causes exuberant RDEB fibrosis by enhancing myofibroblast differentiation and collagen synthesis. Taken together, periostin is a previously unrecognized pivotal component in the genesis of RDEB scarring.

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Prolonged local vasodilation following topical application of nitric oxide releasing nanoparticles

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Nitric oxide (NO) is a potent vasodilator which, due to its ephemeral nature, is difficult to manipulate both for therapeutic use and in studies of blood vessel physiology. Current NO donors suffer from limitations including paradoxical vasoconstriction. To overcome these setbacks, we evaluated the feasibility of a nanoparticle system capable of releasing NO over time (NO-np) to induce prolonged cutaneous vasodilation *in vivo*. The vasodilatory effects were evaluated in: a hamster dorsal window chamber and a rat transected cavernous nerve induced erectile dysfunction (ED) model. On SEM, NO-np had a diameter of 55.6+/-14.8 nm, and a hydrated diameter of 226.5 nm by DLS. Using ozone chemiluminscence, NO-np were shown to release ~ 190 ppb for up to 5 hours, with a tapered release lasting up to 8 hours. Using the chamber model, NO-np treated vessels demonstrated vasodilatation and a 1.4-fold increase ($p<0.05$) in blood flow compared with blood vessels treated with control nps. Significant ($p<0.05$) increases in blood flow were sustained for 1.5 hours. In the ED model, genital application of NO-np increased intracorproral pressures ($p<0.05$) and resulted in return of spontaneous erections without perturbation of systemic blood pressure. These results open up a new methodology for studying the effect of a prolonged increase in NO on cutaneous blood vessel physiology where the concentration of tissue NO can be manipulated independently of its upstream regulators (e.g. cyclic GMP) and delivered locally to specific sites. The results of our preclinical model of erectile dysfunction show that NO-np can increase blood flow to isolated areas of soft tissue, offering clinical implications well beyond erectile dysfunction, but overall diseases of vascular dysfunction or dysregulation.

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The guanine nucleotide exchange factor β-PIX regulates the speed of motile keratinocytes

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Successful epithelialization of skin wounds requires coordinated changes in the keratinocyte cytoskeleton and its matrix adhesions. Specifically, in moving keratinocytes, focal complexes (nascent focal contacts) assemble close to the plasma membrane and leading edges of cells and regulate signals that promote migration, directionality, and cytoskeletal rearrangement. They do so, in part, via the activity of proteins known as guanine nucleotide exchange factors (GEFs) which exchange GDP for GTP on Rho family small GTPases. β-PIX (ARHGEF7), a Cdc42/Rac GEF, promotes focal contact formation and membrane ruffling and has been implicated as a regulator of adhesion dynamics and signaling processes. Intriguingly, in single, fan-shaped keratinocytes moving in a directed fashion, β-PIX localizes to the leading edge of the cells, co-distributing with focal complexes and contacts. In small cell clusters, antibodies against β-PIX stain focal contacts at the perimeter of the cell group (i.e., at their 'free' edges) while staining diffusely along cell-cell and cell-matrix contact sites of cells internal to the cluster. Moreover, although there is no change in β-PIX levels during wound healing of keratinocyte monolayers *in vitro*, there is a dramatic change in its localization. In confluent keratinocyte monolayers antibodies against β-PIX generate diffuse staining. In sharp contrast, 8 hours after scratch wounding, β-PIX localizes to the leading front of those keratinocytes moving into the wound site in association with focal complexes and focal contacts. To investigate β-PIX functions in keratinocytes, lentiviral-mediated delivery of shRNA was used to knock-down its expression. Clones exhibiting greater than 65% loss in β-PIX were generated and then assayed for motile behavior. The knock-down cells move significantly slower than their control counterparts with the loss in cell speed correlating with the degree of β-PIX protein knock-down. In summary, our results indicate that β-PIX is recruited to focal complexes and contacts in actively migrating cells where it regulates their speed.

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Human skin equivalents with perfusable three-dimensional microvessels

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Advances in biomimetic in vitro human skin models have the potential to increase the efficiency of drug screening studies for dermatologic diseases. The current human skin equivalents (HSEs) are typically maintained under static conditions, which do not allow for studying drug transport between the skin and circulation to mimic systemic delivery. We employed two separate strategies to address this limitation: (i) developing an HSE-on-a-chip platform that can create physiologically relevant flow rates; and (ii) incorporating three-dimensional (3D) perfusable microvasculature into HSEs to recapitulate the endothelial barrier function. In the first strategy, we designed and developed a HSE-on-a-chip platform that has the capability to recirculate the medium at physiological flow rates without the need for pump or external tube connections. We demonstrated that the platform can be used to maintain HSEs for three weeks with proliferating keratinocytes and intact skin barrier function. We also validated the capability of the HSE-on-a-chip platform to be used for drug testing purposes by examining the toxic effects of doxorubucin on skin cells and structure. In the second strategy, we demonstrated the first HSEs with embedded 3D perfusable microvessels, which allow for the transport of molecules between the skin and blood compartments. The inner walls of these microvessels were lined by endothelial cells, establishing cell-cell adherent junctions and forming the endothelial barrier. In addition, we developed a mathematical model to estimate the diffusion rates of molecules through both skin and endothelial barriers and confirmed the capability of both platforms to be used in drug testing studies. Overall, both systems developed in this study will enable the study of topical and systemic delivery of drugs using a physiologically relevant in vitro human skin model.

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Keratinocyte progenitor cells in human subcutaneous adipose tissue

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Adipose-derived stem cells (ASCs) are among the most promising multipotent mesenchymal stem cell populations for therapeutic applications, since human adipose tissue is easily obtained in large quantities with little donor site trauma. Although ASCs can potentially generate an infinite number of somatic cells of any type, their differentiation towards epithelial lineages has yet to be demonstrated by a standardized method. The identification of keratinocyte progenitor cells is necessary and their potential for differentiation from ASCs must be determined before they can be considered for use in skin regeneration. Thus we investigated whether keratinocyte progenitor cells are present in the ASC population. ASCs isolated from subcutaneous adipose tissue were cultured and examined for the expression of the keratinocyte progenitor markers p63 and desmoglein 3 (DSG3) by immunofluorescence microscopy and flow cytometry. In addition, p63 and DSG3 expression levels were assessed before and after differentiation of ASCs into adipocytes by real-time PCR and western blot analysis, as well as in subcutaneous adipose tissue by real-time reverse transcriptase polymerase chain reaction. Both markers were expressed in ASCs, but were downregulated after the differentiation of ASCs into adipocytes. ASCs co-cultured with human fibroblasts and incubated with all-trans retinoic acid and bone morphologic protein 4 showed an upregulation in DSG3 level, which was also increased in the presence of type IV collagen. They also showed an upregulation in cytokeratin-5 level only in the presence of type IV collagen. These results demonstrated that keratinocyte progenitor cells reside in human subcutaneous adipose tissue, suggesting that this tissue has the capacity to produce keratinocytes.

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Repair versus regeneration: *Msx2* is required for epidermal competency during wound induced follicular neogenesis

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Following injury, adult mammalian wounds heal by repair, whereas amphibian limbs and newborn mouse digits heal by regeneration. Large full thickness wounds in adult mouse skin induce hair follicular neogenesis (WIHN). We show this requires the transient expression of epidermal *Msx2*. *Msx2* expression occurs in two phases: early in the wound margin and later in the center of the wound bed. Genetic ablation of *Msx2* inhibits WIHN in both totally null (*Msx2*$^{-/-}$) and tissue-specific (*Krt14creMsx2*$^{fl/fl}$) null mice. An *Msx2* promoter with a mutant Mad/Smad factor binding site failed to drive lacZ reporter gene expression during WIHN. However, mice over expressing noggin from the chicken *Krt14* promoter exhibited a minimal increase during WIHN. Together, these data suggest a role for the Bmp pathway in Msx2 expression and wound healing, although it is insufficient by itself. We propose that *Msx2* modulates epidermal competence by delaying wound maturation, enabling more time for regeneration. The results suggest that repair and regeneration undergo a competition in wounds. Topologically, the repair response dominates regions near the wound margin while the regenerative response dominates the center of the wound. This work provides new insight into how epidermal stem cells can be reprogrammed by endogenous mechanisms to regenerate hair ectodermal organs, and it offers a potential approach to modulating repair versus regenerative wound healing.

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Improving stretch mark pathophysiology knowledge by specific *in vitro* models

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Common during pregnancy, stretch marks or Striae Distensae (SD) are the result of many factors and look like atrophied scars. Despite their frequency of occurrence, the physiopathogenic pathways involved in their formation are not fully understood. The dermal fibroblast is the target cell under biomechanical and hormonal influences. In order to improve our understanding of the mechanisms involved in SD development, we have developed several *in vitro* study models. We used normal dermal fibroblasts under different treatment conditions mimicking mechanical distension or hormonal environment, as well as fibroblasts isolated from SD, and study their behaviour and synthesis potential. Fibroblasts exposed to TGF-beta1 transformed to myofibroblasts. Corticosteroids inhibited their secretion of collagen 1. Scratch-test assay coupled with TGFβ1 or corticosteroid demonstrated opposite results on proliferation. SD fibroblasts exerted a stronger contractile force under tension than classic ones. Their protein synthesis was decreased (collagen 1 and 3 -20%, fibronectin -14% and elastin -16%). Whole-transcriptome analysis of SD fibroblasts compared to normal fibroblasts from the same donors tended to demonstrate increased inflammation and elastin degradation factors as well as altered repair process. To go further, we used a specific model that mimics extra-cellular matrix changes as they occur in the stretch marks formation: a dermal injury was conducted on a full-thickness skin model (Phenion®). The injury had an impact on dermal and dermal-epidermal junction markers as it induced a decrease in collagen I, VII, elastin and beta1-integrin gene expression. These in vitro models allowed us to understand the different phases involved in SD formation, they provide useful tools to evaluate the biological properties of active ingredients and anti-stretch marks topical products.

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Influence of adipose-derived extra-cellular fraction on skin repair

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Fat grafting has assumed an increasingly important role as both an adjunctive and a primary procedure in aesthetic and reconstructive surgery. Whilst it is clear that mesenchymal stem cells have significant therapeutic potential via their ability to differentiate toward different lineages the therapeutic potential of secreted factors is becoming increasingly evident. We investigated the potential use adipose-derived paracrine factors as an innovative therapy to promote tissue self-repair and correct skin irregularities. We demonstrated that treatment of melanocytes, keratinocytes and fibroblasts with adipose-derived secretome leads to a significant dose-dependent increase of cell proliferation at 3 and 5 days. Adipose-derived secretome exerted its mitogenic activity both as adjuvant factor of regular cell culture medium and as single treatment in starved medium. Similar results were also observed on adipose-derived stem cells suggesting that extra-cellular fraction, currently discarded as a by-product of fat transfer procedures, could improve stem cells engraftment. To characterize bioactive molecules of adipose complex we performed quantitative immunoenzymatic analysis for EGF, bFGF, SCF, VEGF, EPO and GMCSF. Results defined a specific secretion profile that does not share similarity with peripheral blood-associated secretome. Since all these components could also play a relevant role in epidermal cells implantation we recently successfully used lipofilling in combination with autologous non-cultured epidermal cell suspension transplantation to correct dystrophic skin scarring occurring following skin cancer resection. Records at six and twelve months follow-up after surgery demonstrate a fully integrated skin graft and a good restoration of the treated area, presenting the same texture and pigmentation of the adjacent untreated skin. Based on *in vitro* evidences we propose that whole living fat complex could be used not only to correct tissue depression but also to offer a natural source of nutrients for cells improving the clinical outcome of epidermal cells graft.

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A novel animal model for lichen planus

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Mammalian skin is composed of functionally and anatomically distinct compartments such as the hair follicle (HF), sweat glands, and interfollicular epidermis (IFE). The human disease lichen planus (LP) is characterized by epidermal acanthosis and occasional scarring hair loss (lichen planopilaris, LPP), likely as a result of an autoimmune attack on the basal epidermis. The pathophysiology of this condition is unknown, which has hampered the development of directed therapies to treat LP/LPP. The aims of this project are to understand the responses of keratinocytes in the IFE and HF during chronic basal cell depletion. We hypothesize that there are both immune- and keratinocyte-specific responses to cell loss, resulting in proliferation of IFE cells, and recruitment of HF stem cells. We postulate that stem cell compartments are maintained by cell-cell interactions, and this is disrupted during cell depletion. We have generated a mouse model of basal cell depletion using a doxycycline-inducible diphtheria toxin in the basal epidermis (under the Krt14 promoter). Experimental animals exhibit IFE thickening with increased basal proliferation, and a subset also have hair loss. Histopathological examination revealed an increased CD45^{+} immune infiltrate in close association with the the basal epidermis, making this a novel model for human LP/LPP. Cellular and molecular mechanisms of IFE hyperplasia will be examined. We will perform lineage tracing and gene expression profiling of bulge stem cells to examine the contribution of the HF to the regenerative process. Using a multi-color Confetti reporter driven by Cre recombinase expressed under the Krt14 (IFE) or Krt15 (HF) promoters, we will study the compartment boundaries within the hairy skin of mice, and observe their responses to basal cell ablation. Understanding these aspects of stem cell biology will inform us on novel and specific therapies for LP and LPP, and also on the physiology of wound healing.

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Inhibition of Apoptosis signal-regulating kinase 1 alters differentiation of the wound epithelium to enhance tissue regeneration

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In this study, we show the knockout or inhibition of the mitogen-activated protein kinase, kinase, kinase (MAPKKK) Apoptosis Signal-regulated Kinase-1 (ASK1), confers the ability to regenerate ear tissues. Measurement of the hole created by a 2 mm punch through the ear was performed over a 32 day (D) period comparing the non-regenerating C57BL6 wild-type (WT), heterozygous ASK1 (HET) and ASK1 knockout (KO) mice. From D11 to D32, the KO and HET ear hole openings significantly closed (83%) as compared to WT (46%, $p < 0.001$). Compared to the HET, the KO ears showed significantly greater closure from D21 to D32. Similar results were observed in the WT ears after topical treatment with the ASK1 inhibitor (NQDI-1; 76% closure, $p < 0.001$). Regeneration of the elastic cartilage, muscle, skin and sebaceous glands was confirmed by histological analysis. Interestingly, at D7 the ASK1 KO ear the wound epithelium appeared thickened with enlarged columnar basal cells, the presence of widen spaces between the cells of spinous layer and lacked a clear granular layer or keratin hyalin granules, suggesting improper terminal differentiation. To further confirm abnormal differentiation in the ASK1 KO mice, tissues were immunostained for the terminal differentiation marker filaggrin, a late differentiation marker. In the control filaggrin was abundantly produced in the granular layer of the epidermis, but in the ASK1 KO the filaggrin staining pattern was significantly reduced. By D32 both epithelia appeared the same. In support of the effect of ASK1 inhibition on terminal differentiation, we have recently reported slowed terminal chondrocyte differentiation in the ASK1 KO mouse results in an increase in trabecular bone deposition. In conclusion, we propose the absence or inhibition of ASK1, induced a modification of the wound epithelium which promoted enhanced tissue regeneration in the murine ear punch model.

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Dielectric barrier discharge plasma treatment increases tissue oxygenation during murine ear regeneration

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Studies have shown that Dielectric Barrier Discharge (DBD) plasma can aid wound healing, promote differentiation and encourage tissue development. This study investigated the DBD-plasma effect on *in vivo* vascular changes associated with the ear tissue regeneration with and without DBD-plasma treatment. A 2-mm through-and-through hole was made in the center of the cartilaginous part the ear of 8-week-old C57BL/6 non-regenerating mice using biopsy punch. After punching, DBD-plasma treatment was performed daily for 5 days on the left ear, while the right ear was untreated. To visualize vascular changes photoacoustic (PA) imaging was performed at Day (D) 0 pre and post-punch, D4, D16 and D25 using the Vevo 2100 ultrasound/PA scanner. At D0 post-punch, both control and DBD-plasma treated ears showed a significantly elevated blood flow (BF), oxygenation (Ox) and hemoglobin /vessel area (H/VA) above the initial pre-punch values. Ox in the control remained at these levels through day 25, while a sharp increase in Ox was observed at D4 in the DBD-plasma ear; which decreased to control levels by D25. At D4, less BF was observed in both control and treated ears as compared to D0. However, the DBD-plasma ear showed increased H/VA, while BF showed the opposite trend, compared to the control. Of interest, decreased swelling was observed between D4 and D7 in the DBD-plasma treated ear. Both ears showed a return to control values of BF and H/VA by D25. At D32, the control ear hole had closed 49% while the DBD-plasma treated ear had closed 69% ($n = 10$, $p=0.01$). Histology confirmed regeneration of the cartilage within the plasma treated ear. This study demonstrates the potential of DBD-plasma treatment to affect vascular oxygenation early after injury and enhance ear regeneration. Our ongoing investigation explores the possibility that DBD-plasma can be used to regenerate tissues after injury.

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Ephrin-A ligand loss enhances keratinocyte migration via ligand-independent EphA2 action

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EphA2 is a receptor tyrosine kinase capable of restricting keratinocyte migration while promoting differentiation when engaged by ephrin-A ligand. To gain insight into which of the five ephrin-A ligands acts on EphA2 to regulate these cellular processes, we first examined their relative abundance in human keratinocytes using RNA-Seq by comparing healthy skin with primary keratinocytes maintained as: 1) undifferentiated cells in low density/calcium, 2) differentiated cells in high density/calcium, or 3) 3D epidermal raft cultures. Ephrin-A1 and ephrin-A3 represented the most prominent ligands in skin with ephrin-A1 abundant in all keratinocyte culture models and ephrin-A3 most concentrated in differentiated keratinocytes; these results were validated using qRT-PCR and Western blot analysis. To determine the relative contribution of ephrin-A1 and ephrin-A3 to keratinocyte migration and differentiation, we silenced these ephrins individually or in combination and performed linear scratch wound or calcium-induced differentiation studies. Knockdown of either ephrin marginally enhanced keratinocyte migration and reduced differentiation whereas dual silencing of these two ligands increased wound closure by > 2-fold compared to controls and profoundly impaired differentiation. Interestingly, keratinocyte migration but not differentiation was normalized by pharmacological or genetic ligand restoration, suggesting the enhanced migration of ephrin-deficient keratinocytes was not due to alterations in their differentiation state. Instead, ligand-independent actions of EphA2, as assessed biochemically by Akt phosphorylation on Ser897 of its cytoplasmic domain, was required for accelerated migration since this phenotype was no longer evident upon EphA2 silencing. These findings identify combinatorial actions of ephrin-A ligands on EphA2 that serve to restrain keratinocyte migration with relevance to cutaneous wound healing.

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Topical application of mesenchymal stem cells accelerates healing of wounds in a scleroderma mouse model

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Systemic sclerosis is an autoimmune disorder that affects the skin and internal organs and may cause skin ulceration. Difficult-to-heal digital ulcers occur in approximately 60% of scleroderma patients. We used tight skin (Tsk) mice, a common experimental model for human scleroderma. The mouse's skin tightness is due to hyperplasic thickening of subcutaneous loose connective tissue and abnormal organization and distribution of skin microfibrillar arrays. We employed a full-thickness tail wounding model, previously reported by our group as a model for delayed wound closure, to determine the effects of a topical application of bone marrow-derived mesenchymal stem cells (MSCs) to the wound beds. MSCs (500,000) were applied to each of the 3X10 mm full-thickness dorsal tail wounds using a fibrin spray system. Controls received fibrin only. The wounds were harvested for histological analysis 7 and 14 days after wounding. Using light microscopy and computerized planimetry, we then measured the newly formed epidermis of each sample and calculated the reepithelialization rate. It was found that the MSC application led to accelerated wound healing at both time points compared to the fibrin-only groups. The reepithelialization rate increased 35% by day 7 and 18% by day 14, compared to their respective controls. However, a Masson's Trichrome stain did not show a qualitative significant difference in collagen deposition in and around the wound bed. Our findings illustrate the use of the Tsk tail wounding model to determine the value of MSCs in wound closure.

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A novel assay for evaluating 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 (EpiDermFT). This model exhibits stratified epidermal components and a fully developed basement membrane and resembles in vivo skin in regard to both morphology and barrier function. Small epidermal-only wounds were induced in the model using a 3-mm punch biopsy and subsequently evaluated at various recovery time points by two methods. Historically, EpiDermFT has been used to evaluate re-epithelization of the wound by: a) manually bisecting the tissues through the center of the wound, b) staining with hematoxylin and eosin, and c) quantifying migration from the wound origin. Accurate bisection of the wound in difficult and often leads to variability in assay results. Here we describe a novel method of visualizing wound re-epithelization in situ simplifying analysis and reducing introduction of variables inherent in tissue processing that could potentially confound data. Following wounding, tissues were fixed and immunostained with markers of epidermal differentiation as well as a marker of fibroblasts allowing simultaneous visualization of migrating keratinocytes (keratin 14), differentiated suprabasal cells (involucrin), and dermal fibroblasts (vimentin) within the wound. Histological and immunohistochemical analysis showed keratinocyte migration at 2 days following wounding. In both methods, wounded tissues cultured without growth factors (2% human serum) had a reduced healing rate in which keratinocytes did not cover the entire wound within a 6 day timeframe. In contrast, wounded tissues cultured with growth factors demonstrated a dramatic increase in healing rate as keratinocyte migration completely covered the wounded area by day 6. In conclusion, this novel method of evaluating re-epithelization by utilizing immunohistochemical markers of differentiation is a quicker and more reproducible method of analyzing wound healing.

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Acute immune effects and tissue destruction in mice following skin exposure to sulfur mustard

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Sulfur mustard (SM) has been used historically as a weapon in chemical warfare and continues to pose a threat. SM causes rapid local skin vesication followed by late-onset systemic toxicity. Our goal was to develop an SM-induced mouse skin contact model that recapitulates hallmark features of SM toxicity, specifically exacerbated skin tissue destruction with delayed wound healing, disruption of hematopoiesis and death. We tested 8 different concentrations of SM and demonstrate that a single skin exposure to SM (in DMSO) leads to a dose dependent increase in wound area size on the dorsal surface of mice from 4-70 mm^2. The challenge dose range (4.8–116 mg/kg SM) was selected in order to both reduce the confidence limits around LD_{90} and obtain a statistically significant probit slope. Wounds progressed with visible erythema and remained enlarged resulting in full thickness necrosis at higher SM concentrations (40-116mg/kg). Daily progressive body weight loss of 4-6% was recorded and wasting was observed in SM-exposed mice. Blood smear analyses revealed a 60% decrease of peripheral red blood cells (261 cells/HPF) in SM-exposed mice compared to control mice (640 cells/HPF) ($p<0.005$). H&E of bone marrow shows that at challenge doses of 45mg/kg and higher SM caused acute loss of cellularity. Interestingly, we observe no mortality with exposure to SM at doses ≤ 40 mg/kg, although doses ≥ 45mg/kg appeared to be a critical threshold for death resulting in an LD_{90} at 50 mg/kg. Previously we demonstrated that 25-hydroxyvitamin D (25(OH)D) has efficacy against nitrogen mustard. Therefore we tested whether 25(OH)D has protective effects against SM. A single intraperitoneal administration of 5ng 25(OH)D administered 1 h post SM exposure resulted in 40% protection from mortality ($p=0.01$, $n=34$, assessed by log rank test). Mitigation of SM-induced damage by 25(OH)D suggests a novel potential countermeasure against SM skin exposure that may have further application in vulnerable tissues following vesicant exposure.

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Long-term type 2 diabetes induces dysfunctions in epidermal keratinocytes with impact on the interaction with nerve fibers in organotypic skin models

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Cutaneous manifestations are common diabetes-induced complications. While most of the chronic complications of diabetes have been extensively studied, mechanisms leading to skin complications are poorly investigated. The objective of this study was to analyze the influence of long-term type 2 diabetes on human skin at the cellular level. Human skin cells were isolated from punch biopsies obtained from healthy and type 2 diabetic donors. These skin cells were cultured under normoglycemic conditions and used to generate diabetic skin models. Our findings clearly show, that diabetic keratinocytes retain a metabolic memory *in vitro*, which is characterized by decreased epidermal expression of insulin receptor, insulin receptor substrate 2 and a reduced insulin-stimulated glucose uptake capacity of monocultured cells. As insulin resistance is linked to increased inflammation as well as oxidative stress, epidermal expression of pro-inflammatory cytokines was increased (e.g. TNFα, IL1β, IL6) while expression of cytoprotective factors (e.g. Nrf2, HO1, NQO1) was decreased in diabetic models. Moreover, diabetic skin models displayed reduced epidermal NGF mRNA expression, which was also confirmed *in vivo* by analyzing epidermal gene expression and NGF protein levels in suction blister fluids in diabetic subjects. To analyze the effect of reduced skin cell-derived NGF, we established a unique air-exposed skin model consisting of diabetic skin cells, and innervated by porcine dorsal root ganglion neurons. In innervated skin models, diabetic skin cells showed a reduced capacity to induce neurite outgrowth. Furthermore, we provide evidence that the innervation of skin models controls cytokine expression pointing to an aggravation of cutaneous inflammation by small-fiber neuropathy. In conclusion, we show in a translational approach that keratinocytes from diabetic patients retain a metabolic memory in culture with impact on cutaneous innervation, inflammatory state and cytoprotective capacity.

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Dermal fibroblasts derived from human venous ulcers show high migratory and proliferative activity *in vitro*

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It is generally thought that dermal fibroblasts activity (both proliferation and migration) is important in the healing of cutaneous wounds. In this report, we focused on human chronic non-healing venous ulcers of the lower extremity. We cultured (DMEM plus 0.5 % or 10 % FBS, 95 % air and 5 % CO_2 at 37°C) chronic wound fibroblasts (obtained by explant technique) and compared their *in vitro* activity to freshly wounded (Day 3) normal skin and to unwounded normal skin (Day 1), both of them derived from the ipsilateral thigh of the same patient. We established confluent cultures and made scratch wounds in the cell monolayer. The Incucyte system (Ann Arbor, MI, USA) was used for time-lapse photography and for assessment of cell migration and confluence up to 48 hours within the *in vitro* gap. For proliferation analysis, we used MTT testing. Surprisingly, we found that chronic wound fibroblasts cultured in 0.5 % FBS showed approx 50 % greater proliferative and migratory activity when compared to unwounded normal skin (Day 1) fibroblasts. We observed a statistically significant increase in the proliferation and migration activity ($p<0.05$) within individual patients. Also, we determined that fibroblasts migration capacity is higher when we cultured them in 10 % FBS compared to 0.5 % FBS in unwounded normal skin (Day 1) and in acute wound fibroblasts (Day 3). However, DMEM plus 0.5 % or 10 % FBS had the same effect on chronic wound fibroblasts migration capacity. These results suggest that fibroblasts derived from human venous ulcers may not be "dormant" or less active than normal fibroblasts. Indeed, this raises the possibility that greater energy requirements from cellular activity may play a pathogenic role in impaired healing.

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A novel stress-response mechanism by Hsp90α and Hsp90β to cope with hypoxia and nutrient paucity during wound healing

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Tissue damage and repair are a constant challenge throughout the life in humans. Failure in the repair or healing processes could lead to catastrophic outcomes such as chronic tissue inflammation, open ulcers, fibrosis and cancer. When tissue is wounded and blood vessels are clotted, the local environment becomes ischemic – lack of an adequate supply of oxygen and glucose. The heat shock protein-90 (Hsp90) family of proteins play an important role in protecting tissues from various environmental insults and participate in the subsequent repair of damaged tissues. Nevertheless, the mechanism of the repair action of Hsp90 remains elusive. Herein, we report a novel hypoxia-responsive mechanism by which the two cytoplasmic Hsp90 isoforms, Hsp90α and Hsp90β, work together to promote cell motility *in vitro* and wound healing *in vivo*. Unlike the current understanding of the so-called intracellular chaperones, our study demonstrates that Hsp90α and Hsp90β have distinct and non-overlapping functions both inside and outside cells. We show that it is only intracellular Hsp90β that acts as a chaperone to stabilize the LDL Receptor-Related Protein-1 (LRP-1) at the cell surface. In contrast, the sole function of Hsp90α is carried out by its secreted extracellular form, where Hsp90α promotes cell motility and wound healing via the LRP-1 cell surface receptor. The intracellular role for Hsp90β and the extracellular function for Hsp90α are isoform-specific and non-exchangeable. Furthermore, we propose that this repair mechanism by Hsp90α and Hsp90β applies broadly to repair damage to other non-cutaneous tissues.

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Effects of diet modification on the formation of heterotopic ossification in the mutant *Abcc6*$^{-/-}$ mouse following thermal injury

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The mutant *Abcc6*$^{-/-}$ mouse has recently been established as a reliable model for heterotopic ossification (HO), the abnormal formation of bone within soft tissues after thermal injury to ≥30% of total body surface area (TBSA). The *Abcc6*$^{-/-}$ mouse, when placed on an "acceleration diet" of twice the normal murine daily amount of phosphorus, increases ectopic mineralization (EM) and enhances HO formation. Increased magnesium, 5 times that of a normal murine diet, has been shown to decrease EM in the *Abcc6*$^{-/-}$ mouse. Here we tested the ability of the magnesium oxide (MgO), or "protective diet", in decreasing the formation of HO after thermal injury. *Abcc6*$^{-/-}$ mice were separated into 4 groups: of the burned mice, 7 were placed on "acceleration diet", 5 on "protective diet", and 5 on normal diet. Five sham mice were placed on the "acceleration diet" to act as control. A 30% TBSA burn wound was created on the dorsum of animals. Mice were imaged via micro-computed tomography (μCT) at two months after injury followed by necropsy and histopathology, and assay of serum calcium, magnesium, and phosphorus. Twenty-two mice survived the procedure; mortality was as expected at 14%, n=3. All burned mice on the acceleration and normal diet groups developed HO, but mice in the acceleration group were diffusely more affected. The MgO diet was found to be protective with minimal formation of EM. Sham mice developed EM as expected, and specifically, 2 mice were noted to have EM in the back. These μCT findings were validated by histopathology. There were no significant differences in serum magnesium or calcium. We have previously established the *Abcc6*$^{-/-}$ mutant mouse to be a valid model for the characterization of HO after thermal injury, and in this study the MgO diet is shown to be protective against development of HO. This finding forms the basis for possible clinical applications in the electrolyte management of burn patients.

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Topically delivered allogeneic mesenchymal stems cells accelerate healing

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Bone marrow-derived mesenchymal stem cells (MSCs) offer a promising topical treatment for accelerating healing. Our prior work showed that autologous MSCs, delivered in a fibrin spray, are effective for the treatment of chronic human wounds. However, autologous MSCs are specific to each patient and do not have the desirable "off-the-shelf" property of treating many patients. With the goal of expanding the use of MSC, we studied allogeneic mouse MSCs in a murine, full-thickness tail wounding model. Our data suggest that, like autologous MSCs, allogeneic MSCs accelerate wound closure. At 2 weeks post wounding, allogeneic MSC were more effective than autologous MSC in promoting wound healing (86.5% re-epithelialized in allogeneic MSC group vs. 62.3% in autologous group, p= 0.025). Both groups promoted 100% re-epithelialization by 3 weeks. We next examined how allogeneic MSCs might accelerate wound healing. Using GFP^+ labeled MSCs applied to murine tail wounds and TUNEL staining, we found that nearly all allogeneic MSCs noted within a wound bed are apoptotic, while most autologous MSC do not apoptose**.** This was noted 1, 2, and 3 weeks following treatment. As has been previously been reported, MSC, and in particular allogenic MSC, may apoptose but still have a paracrine effect on wound healing. Interestingly, GFP+ autologous MSCs, and to a lesser extent, allogeneic MSC were also found within the blood vessels at the wound base, and were $CD31^+$. This suggests that autologous MSC may differentiate into endothelium. In summary, we found that allogeneic MSCs, like autologous MSCs, accelerate wound healing in a murine model. However, the mechanisms of action of this finding may be based on paracrine effects as the allogeneic stem cells apoptose after application. In contrast, autologous MSC may act, in part, by differentiating into endothelium. While allogeneic MSC appear to be an effective treatment for chronic wounds, additional work is needed to better characterize how these cells promote wound healing.

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Increased TSLP expression in keloids: Dose increased expression of TSLP promote keloid pathogenesis?

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Keloids or hypertrophic scars are caused by pathologic wound healing and characterized by excessive synthesis of extracellular matrix and frequent relapse after surgical excision. Recently, thymic stromal lymphopoietin (TSLP), which is well known in allergic diseases, has been reported in fibrotic diseases, such as idiopathic pulmonary fibrosis and atopic dermatitis fibrosis. In this study, we attempted to investigate the role of TSLP in pathogenesis of keloid. The expression level of TSLP was measured using immunohistochemistry in keloid tissues. Normal and keloid fibroblasts were treated with TSLP, and the expressions of collagen I, III, TGF-β and SDF-1α were measured with immunoblot analysis and qRT-PCR. The cellular localization of SDF-1α and infiltration of fibrocytes were confirmed using immunofluorescence. We observed that TSLP expression was increased more in keloid tissue than normal tissue. Furthermore, TSLP treatment induced increased collagen I, III, and TGF-β expressions in both normal fibroblasts and keloid fibroblasts, but greater increase of expressions was seen in keloid fibroblasts. Also SDF-1α, which is recently highlighted in wound healing process, was increased after TSLP treatment and most of SDF-1α was expressed in α SMA^+ fibroblasts. CXCR4, which is a SDF-1α receptor, expressing fibrocytes were more significantly increased in keloid tissue than normal tissue. Therefore, we suggest that TSLP might be the potent inducer of collagen and TGF-ß production in keloid fibroblasts and also the activator of CXCR4/SDF-1 axis to increase the infiltration of fibrocytes into keloid tissue.

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Keloid pathogenesis: Gene expression profiling in a mouse model for keloids

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Keloids occur after injury in susceptible individuals, but the underlying pathogenesis leading to keloid formation is largely unknown. In this study we sought to establish and animal model for keloids. Keloid and normal skin samples, obtained from patients undergoing surgical excision for cosmesis and panniculectomy, were divided into two groups. In the first group, total RNA was extracted from tissue using commercially available kits and reverse transcribed to cDNA. Oligo-primers were designed to Lysyl Oxidase (LOX), LOX-L1, LOX-L2, LOX-L3, and LOX-L4, Connective Tissue Growth Factor (CTGF), TGF-β, Collagen 1 (Col1), Col3, Hypoxia-Inducible Factor-1α (Hif-1α), and RT-qPCR was performed to produce a baseline gene expression profile. TGF-β (2.5 fold), Col1 (5.8 fold), Col3 (7.3 fold), LOX (3.3 fold), LOX-L1 (16 fold), LOX-L2 (11.2 fold), Hif-1α (2.8 fold) were upregulated compared to controls. In the second group, fibroblasts isolated from keloids and control tissue were plated on 75-cm^2 tissue culture flasks, and then grown in DMEM supplemented with 20% FBS, 1% penicillin-streptomycin, and incubated with 5% CO_2 atmosphere. Upon confluency, fibroblasts were passaged 4-6 times and then dynamically seeded onto polylactic acid (PLA) bioscaffolds for 4-5 days in a rotary cell culture chamber. Scaffolds were implanted subcutaneously onto the backs of Rag1 immunodeficient mice and allowed to grow for 2 weeks after which scaffolds were harvested and analyzed for gene expression in a similar fashion as tissue samples in group 1. We found upregulation of TGF-β (1.4 fold), CTGF (1.7 fold), LOX (10.4 fold), LOX-L1 (5.6 fold) and LOX-L2 (2.1 fold). Thus, in our *in vivo* keloid model, several candidate genes maintain or increase their expression (TGF-β, CTGF, LOX, LOX-L1, LOX-L2) while others reduce their expression (Hif-1α, Col1, Col3) compared to the original keloid tissue. Our results indicate that the fibrotic cascade is possible to maintain in an *in vivo* model of keloids, providing a platform for pharmacological studies to counteract the growth of these fibrotic lesions.

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CAGE sequencing reveals MAFB as an early VEGF-C induced transcription factor that mediates cutaneous lymphatic vessel differentiation and development

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Cutaneous lymphatic vessels play major physiological roles in maintaining skin fluid pressure and in mediating immune responses. Surprising recent evidence indicates that activation of lymphatic vessels by the lymphangiogenesis factor VEGF-C, via activation of VEGFR-3, potently inhibits chronic inflammation of the skin, as well as rheumatoid arthritis and inflammatory bowel disease. We aimed to identify transcriptional factors that mediate the VEGF-C effects on lymphatic endothelium, utilizing a mutant form of the VEGF-C protein, VEGF-C156S, which specifically activates VEGFR-3. Human lymphatic endothelial cells (LECs) were treated with VEGF-C156S and mRNA was obtained at 16 time points (15 min to 8 h), followed by cap analysis of gene expression (CAGE) sequencing. We identified 16 immediate early response transcription factors (TF) whose transcripts were up-regulated in LECs within the first 30 to 80 min after VEGFR-3 activation. The transcription factor MAFB was found to be specifically and rapidly active in VEGF-C156S-stimulated LECs, based on data set specificity analyses and transcription factor activity analyses. Adenoviral gain- and loss-of-function studies revealed that MAFB regulates the expression of the lymphatic differentiation markers Prox1, podoplanin, Sox18 and LYVE-1, and also activates KLF4. ChIP analyses revealed MAF binding sites in the promoter of these genes, with an increase in the ChIP signal after MAFB overexpression. Importantly, MAFB was specifically expressed by lymphatic but not blood vascular endothelium in vitro and in vivo, and knock-down of the lymphatic-specific transcription factor Prox1 strongly reduced MAFB expression. Studies in MAFB knockout mice revealed a role of MAFB in the embryonic patterning of lymphatic vessels in the skin. These results reveal for the first time that MAFB plays an important role in mediating lymphatic vessel activation.

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Phosphorylation of SMAD2 linker site Thr220 is a marker for quiescent stem cells

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TGFbeta is a powerful inhibitor of keratinocyte proliferation. Signaling initiated by TGFbeta involves the phosphorylation of R-SMADs. These are R-SMADs are SMAD2 and 3. Activation of R-SMADs is generally attributed to their phosphorylation at the c-terminal tail by TGFbeta receptors. However, phosphorylation at the so-called linker region, a region connecting the two globular domains of SMADs is also critical for activation and regulation of SMADs. This linker phosphorylation is induced by TGFbeta but not performed by TGFbeta receptors. A group of related proliferation-independent CDKs are responsible for this phosphorylation, mainly CDK8 and CDK19, and enhance transcriptional activity of SMADs. Since TGFbeta signaling is generally associated with inhibition of proliferation in keratinocytes, we asked the question whether linker phosphorylation may be an indicator of quiescent stem cells in epithelial tissues. Using antibodies against phospho-SMAD2 (Thr220), we evaluated the relationship between this mark, proliferation and stemness in squamous epithelia and hair follicles. We find that phospho-SMAD2 (Thr220) is inversely correlated with proliferation in squamous epithelia, demarcates hair follicle stem cells and quiescent basal cells of squamous epithelia. Furthermore, components of the CDK8/CDK19/Cyclin C mediator complex are co-expressed with phospho-SMAD2 (Thr220). These data indicate that TGFbeta-induced linker phosphorylation of SMAD2 is a novel marker for quiescent stem cells.

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Estrogen receptor alpha-mediated control of growth factor production from nipple fibroblasts

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The development and maintenance of specialized epidermis requires inductive signals from the underlying dermal connective tissue. However, the specific signaling pathways that mediate these processes in many sites of specialized epidermis including the nipple remain to be defined. Using grafting that permits recombination of the nipple-like tissues from the ventral skin of K14-PTHrP mice with those from wild type mice, we found that dermal cells are sufficient to induce a thickened hairless epidermis with appropriate markers. To identify potential inductive signaling molecules produced by nipple fibroblasts, gene expression was profiled using a Mouse Gene 2.0 ST microarray (affymetrix) on sorted PDGFR+ ventral K14-PTHrP and wild type fibroblasts. Gene ontology analysis of 285 transcripts with >2-fold ($P<0.05$) differential expression in nipple fibroblasts identified pathways associated with matrix production and turnover, WNT, TGFβ, TNFα, and estrogen receptor alpha (ERa) signaling. Since many of the key pathway nodes have been reported to be regulated by estrogen, we hypothesized that the hormone may be central in modulating nipple fibroblast matrix production, as well as growth factor induced epidermal proliferation/differentiation. Nipple fibroblasts which express a four fold increase in ERa protein have reduced levels of TGFβ-1 mRNA and protein both *in vivo* and *in vitro*. Phospho-smad 2,3 levels were also reduced in the dermis of nipple relative to surrounding ventral skin using virgin mice as well as human sample, several smad regulated genes expression are also altered. Estrogen represses TGFβ-1 at mRNA and protein levels in nipple fibroblasts, but not in those from ventral dermis. Studies that overexpress TGFβ-1 in nipple connective tissue are currently underway. In summary, the fibroblasts that underlie the nipple have elevated levels of ERa, suggesting hormonal control of local growth factors that regulate both matrix production and epidermal differentiation/proliferation.