# 001

**Modulation of Vascular Endothelial Growth Factor Activity During Wound Healing Affects Granulation Tissue Vascularization But Not the Rate of Wound Closure**

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Tissue repair has been generally thought to be dependent on sufficient wound vascularization, and we and others have previously shown that the epidermal expression of vascular endothelial growth factor (VEGF) is strongly upregulated during the early phases of cutaneous wound healing. Thus, it has been suggested that enhanced VEGF levels might improve wound angiogenesis and closure. To test this hypothesis, 6-mm full-thickness wounds (n = 30) were created on the back of wildtype mice and of transgenic mice with skin-specific overexpression of VEGF. In addition, wildtype mice were wounded and were treated systemically either with control rat IgG or with rat antimouse flk-1 antibody DC101, a function-blocking antibody against the VEGF receptor flk-1 (VEGFR-1). Wound closure, granulation tissue formation and wound angiogenesis were monitored for up to 4 weeks. Increased granulation tissue formation and enhanced redness of granulation tissue were observed in VEGF overexpressing transgenic mice; however, the rate of wound closure was identical to that observed in wildtype mice. Quantitative image analyses revealed that the vascularity of wound granulation tissue was significantly enhanced in VEGF transgenic mice. Conversely, treatment with the VEGF function-blocking antibody DC101 resulted in reduced redness of the granulation tissue and in decreased wound vascularity but no delay of wound closure was observed under DC101 treatment. These results demonstrate that VEGF indeed potently enhances the vascularity of wound granulation tissue. However, wound angiogenesis does not appear to act as a rate-limiting factor for wound healing in mice.

# 002

**Akt Overexpression Confers a Tissue Invasive Phenotype in Endothelial Cells, but is Not Required for *In Vivo* Growth of Angiosarcoma**

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We have previously shown that phosphoinositol-3-kinase (PI-3-K) is the primary mediator of angiogenesis in angiosarcoma cells (*PNAS* 94:861–6, 1997). Akt is a kinase oncogene which is one of the primary mediators of PI-3-K activity, and is involved in the prevention of apoptosis and transformation. In order to determine the effect of akt modulation in endothelial cells, we overexpressed akt in immortalized murine endothelial cells. Akt overexpression led to a distinct tissue invasive phenotype, but did not lead to overt malignant transformation. We then studied the effect of dominant negative akt expression on fully malignant SVR angiosarcoma cells. High level expression of green fluorescent (GFP)-tagged dominant negative akt *in vitro* and *in vivo* was achieved by infection of SVR cells with high titer adenovirus followed by *in vivo* injection. Confirmation of transgene expression was observed by decreased phosphorylation of glycogen synthase kinase (GSK3) by an *in vitro* kinase assay. Down-regulation of akt failed to decrease *in vivo* tumor growth. Thus, PI-3-K regulates tumor growth and angiogenesis in angiosarcoma by akt independent pathways, which may include bcl2 and protein kinase C zeta.

# 003

**Regulation of Endothelial Barrier Function and Growth by the Armadillo Family Proteins Plakoglobin and β-Catenin**

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Plakoglobin and β-catenin are members of the armadillo (arm) gene family and play dual roles in intercellular junction assembly and in the regulation of intracellular signaling pathways in epithelial cells. However, very little is known about the function of these proteins in dermal microvascular endothelial cells. To directly test the role of these proteins in endothelial cells, we expressed myc-tagged plakoglobin and beta-catenin polypeptides in the immortalized endothelial cell line HMEC-1 using a replication deficient retroviral system. In stable HMEC-1 cell lines, myc-tagged plakoglobin (Pg.myc) colocalized at intercellular junctions with VE-cadherin. Interestingly, HMEC-1 cell lines expressing Pg.myc exhibited increased barrier function relative to control cell lines expressing empty retroviral vector. Furthermore, HMEC-1 cells expressing Pg.myc exhibited increased growth rates in low serum. The increase in HMEC–1 barrier function and growth in cell lines expressing Pg.myc was associated with the displacement of endogenous β-catenin from intercellular junctions. Therefore, we generated HMEC-1 cell lines expressing two different β-catenin mutants lacking the amino-terminal GSK-3 phosphorylation sites. Similar to HMEC-1 cell lines expressing Pg.myc, HMEC-1 cell lines expressing either of the two β-catenin mutants exhibited increased growth compared to controls when cultured in low serum. These results raise the possibility that plakoglobin and β-catenin play important roles in the regulation of endothelial barrier function and growth during cutaneous disorders that involve inflammation or angiogenesis.

# 004

**Nuclear Translocation of Phosphorylated STAT3 is Essential for Endothelial Cell Migration Induced by Basic FGF: STAT3 Phosphorylation is Involved in Tube Formation**

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Basic FGF (bFGF) and VEGF are essential in angiogenesis since they stimulate endothelial cell (EC) migration and proliferation. The mechanism of these growth factors in EC migration has not been well characterized in contrast to that of EC proliferation. We studied the signal transduction pathway of bFGF in human dermal microvascular endothelial cell migration and report the involvement of STAT3 in keratinocyte migration. We hypothesize that STAT3 phosphorylation plays a critical role in EC migration. First, the effect of bFGF on EC migration was examined by the Boyden chamber assay. bFGF enhanced EC migration in a dose-dependent manner, with a 2.5-fold optimum at 10 ng per ml in 7 h. Then, nuclear translocation of phosphorylated STAT3 was assessed by con-focal microscopy using antiphosphorylated STAT3 antibody. No phosphorylated STAT3 was observed in nuclei before the addition of 10 ng per ml of bFGF. However, translocation of phosphorylated STAT3 into nuclei was found 30 min after the addition of bFGF and reached an optimum at 45 min. To prove the functional involvement of STAT3 phosphorylation in EC migration, transfection of dominant negative STAT3 adenovirus vector was performed. Transfection of dominant negative STAT3 adenovirus at 5 MOI completely abolished bFGF-induced EC migration as well as nuclear translocation of phosphorylated STAT3. Then, the effect of dominant negative STAT3 adenovirus vector on tube formation was studied. ECs were plated on collagen-coated plates. After reaching confluence, 10 ng per ml bFGF was added and tube formation was confirmed morphologically after 48 h. Transfection of dominant negative STAT3 adenovirus at 5 MOI suppressed tube formation significantly compared to controls transfected with adenovirus vector containing the b-galactosidase gene. These data demonstrate that nuclear translocation of phosphorylated STAT3 is essential for bFGF-induced EC migration and tube formation.

# 005

**The Endogeneous Inhibitor Thrombospondin-1 Prevents Ultraviolet-B-Induced Skin Damage and Wrinkle Formation in the Skin of Transgenic Mice**

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Chronic UVB irradiation of the skin results in epidermal hyperplasia, degradation of extracellular matrix molecules, and wrinkle formation. To characterize the role of the cutaneous vascular system in the pathogenesis of UV-mediated skin damage, we treated Skh-1 hairless mice with light over a period of 10 weeks with UVB irradiation (cumulative UVB dose: 5.62 J per $cm^2$) and performed quantitative image analysis of cutaneous blood vessels. Besides wrinkle formation, cutaneous vascularization was dramatically increased after chronic UVB exposure with a significant increase of both vessel number and vessel size, most prominently in the papillary dermis. These changes were associated with marked upregulation of vascular endothelial growth factor in the hyperplastic epidermis. To directly study the biological function of angiogenic vessels in UV-induced skin damage, transgenic mice with skin-specific overexpression of the endogenous angiogenesis inhibitor thrombospondin-1 (TSP-1) and their wildtype littermates were subjected to 10 weeks of UVB irradiation regimen. TSP-1 transgenic mice showed a significantly reduced skin vascularization, decreased endothelial cell proliferation rates and increased endothelial cell apoptosisrates, as compared to wildtype littermates. Moreover, wrinkle formation was absent and dermal photo-damaged was greatly reduced in TSP-1 transgenic mice. Enhanced MMP-9 activity was found in wildtype mice but not in TSP-1 transgenic mice after a single exposure to UVB. These results reveal an important role of the cutaneous vascular system in mediating UVB-induced skin damage and suggest inhibition of skin angiogenesis as a promising new approach for the prevention of chronic photo-damage.

# 006

**Thrombospondin-1 Overexpression in the Skin of Transgenic Mice Suppresses Tumor Development, Growth and Metastasis**

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To investigate whether the endogenous angiogenesis inhibitor thrombospondin-1 (TSP-1) might play a protective role in skin carcinogenesis, we generated a transgenic mouse model for the targeted overexpression of TSP-1 in the basal epidermis of the skin, using a keratin 14 expression cassette. We then subjected TSP-1 transgenic and wild-type mice to a standard two-step skin carcinogenesis protocol using DMBA for tumor initiation and PMA for tumor promotion. K14-TSP-1-transgenic mice exhibited a delayed formation of skin papillomas with a significantly longer latency period of 14 weeks as compared to 10 weeks in wildtype mice. Moreover, the number of papillomas in TSP-1 transgenic mice averaged five papillomas per mouse at the end of the promotion stage, 2-fold less than that of control mice. Malignant conversion of papillomas to squamous cell carcinomas (SCC) was first seen at 20 weeks after first PMA promotion in TSP-1 transgenic mice, 4 weeks later than in wild-type mice. At 32 weeks, the incidence of SCC in TSP-1 transgenic mice was 60% of that observed in control mice and the average number of SCC per mouse was decreased 2-fold. Only 4% of the TSP-1 transgenic mice developed lung metastases as compared to 21% of the wild-type mice. In summary, these results demonstrate that targeted overexpression of TSP-1 in the skin results in a significantly reduced susceptibility to chemically induced skin carcinogenesis and they suggest a protective role for TSP-1 in multistep carcinogenesis and metastasis.

# 007

**Mice Overexpressing Placenta Growth Factor in the Skin Exhibit Enhanced Vascular Density and Permeability**

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Placenta growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family, but its biological role in angiogenesis remains controversial. In a previous study, we have shown that PlGF is induced in migrating keratinocytes during wound healing, concomitantly with the active angiogenic phase of wound repair. To clarify the biological role of PlGF in the skin, we have produced a transgenic mouse model constitutively overexpressing this factor in basal keratinocytes, by using a keratin 14 expression cassette (K14-PlGF). PlGF transgenic mice showed visibly redder skin since birth. Whole mount preparations of ear skin from *L. esculentum* lectin-perfused mice showed that dermal vessels were strikingly more numerous, branched and enlarged in K14-PlGF mice compared with wild-type littermates. PECAM/CD31 and smooth muscle actin immunohistochemistry on back skin biopsies confirmed the enhanced vascularization in transgenic mice. Vascular leakiness, measured after injection of Evans blue dye into the tail vein, was also markedly increased in K14-PlGF mice. Whole mount analysis of ear skin from *R. communis* lectin-perfused mice and ultrastructural analysis of dermal capillaries revealed that the augmented permeability was due to increased number of leakage sites in the endothelium. These data demonstrate that PlGF is able to induce angiogenesis and vascular permeability in the skin. Whether the phenotype of transgenic mice is due to a direct or indirect effect of PlGF, possibly mediated by VEGF, is currently under investigation.

# 008

**Lack of Vascular Endothelial Cell Growth Factor (VEGF) in Murine Epidermal Keratinocytes Results in Retardation of Angiogenesis-Dependent Processes in the Skin**

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Vascular endothelial cell growth factor (VEGF) is a major inducer of angiogenesis, and its expression is essential for normal fetal growth and development. In the adult, VEGF has been implicated in angiogenesis-dependent physiological and pathological processes, such as wound healing and neoplasia. Since in the skin the main source of VEGF is the epidermal keratinocyte (KC), we investigated the contribution of KC-derived VEGF to angiogenesis-dependent processes in this organ. We have therefore generated mice in which VEGF is inactivated specifically in KC by means of the Cre/Lox-p system, in which the floxed exon 3 of the VEGF A gene is deleted by a keratin 5 promoter driven Cre recombinase. Recombination of genomic DNA derived from epidermal KC of these mice was demonstrated by PCR and Southern blotting, and when cultured, these cells produced no detectable VEGF. Mice expressing both the K5-Cre transgene and homozygous for VEGF flox (designated VEGF f/f K5-Cre +) were generally 10–20% smaller than nontransgenic littermates (VEGF f/f). Wound-healing of 5 mm full-thickness punch biopsies and depilation-induced anagen were retarded by 3–4 days in mutant mice compared to controls. Preliminary immunohistochemical analysis of the wounds suggests a reduced microvessel density immediately below the regenerating keratinocyte layer. DMBA initiation and 15 weeks of treatment with PMA has so far not yielded squamous papillomas in either mutant or control mice. However, black macular lesions began to develop at week 6 after start of PMA treatment, and these developed more rapidly, and until week 9 were significantly more numerous ($p < 0.05$) in control compared to mutant mice. We thus show that, although probably not the only factor involved, keratinocyte-derived VEGF plays a significant role in angiogenesis-dependent processes in the skin.

# 009

**Antipodal Roles of Vascular Endothelial Growth Factor and Angiopoietin-1 in the Control of Delayed-Type Hypersensitivity Reactions Elicited in the Skin of Transgenic Mice**

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In addition to their prominent roles in malignant tumor growth, angiogenesis and increased vascular permeability are also characteristic features of cutaneous inflammation. We have previously shown that overexpression of vascular endothelial growth factor (VEGF) or of angiopoietin-1 (Ang1) in the skin of transgenic mice results in increased cutaneous vascularity, with a predominant effect of VEGF on vessel numbers and of Ang1 on vessel size. To directly investigate the biological roles of VEGF and Ang1 in the mediation of experimental inflammation, cutaneous delayed-type hypersensitivity (DTH) reactions were elicited in the ears of wildtype, VEGF or Ang1 transgenic mice by topical sensitization and challenge with oxazolone. Ear thickness was measured daily and skin angiogenesis was evaluated by quantitative, computer-assisted image analysis of CD31- or collagen IV-stained vessels. In wildtype mice, pronounced increases of dermal blood vessel size coincided with marked ear swelling. DTH reactions in VEGF transgenic mice were characterized by prolonged and significantly increased ear swelling, vascularity and inflammatory infiltrates, as compared with wildtype controls. Ang1 transgenic mice showed significantly reduced ear swelling which lasted for only 3 days, as compared with 7 days in wildtype mice. Moreover, inflammatory infiltrates were less pronounced. Skin vascularity was only slightly increased over the already elevated levels observed in the unchallenged skin of Ang1 transgenic mice. These results demonstrate antipodal effects of the angiogenesis factors VEGF and Ang1 on experimental cutaneous inflammation which are most likely related to their recently established opposing effects on microvessel permeability and maturation.

# 010

**Inverse Regulation of the Angiogenesis Factor VEGF and the Angiogenesis Inhibitors Thrombospondin-1 and TSP-2 in Human Epidermal Keratinocytes**

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Thrombospondin (TSP)-1 and TSP-2 act as endogenous cutaneous angiogenesis inhibitors, whereas vascular endothelial growth factor (VEGF) potently stimulates skin angiogenesis. We investigated the expression and regulation of TSP-1, TSP-2 and VEGF in normal human keratinocytes *in vivo* and *in vitro*. In normal skin, TSP-1 and TSP-2 were expressed in the basal epidermal layer whereas VEGF was weakly expressed in the spinous layer, as assessed by immunohistochemistry and *in situ* hybridization. *In vitro*, TSP-1 and TSP-2 mRNA were strongly expressed in proliferating keratinocytes cultured under serum-free conditions but were dramatically down-regulated in confluent cultures. Conversely, VEGF expression was low in proliferating cultures and increased along with cell density. Whereas TSP-1 and TSP-2 mRNA expression was inhibited by vitamin D3, TPA and high-Ca++ conditions, VEGF mRNA was potently induced. Western blot and/or ELISA analyses of conditioned media confirmed these results on the protein level. In contrast, all three factors were induced by addition of fetal calf serum. Interestingly, TGF-β induced TSP-1 expression but inhibited expression of TSP-2. The predominant expression of both TSP-1 and TSP-2 in proliferating keratinocytes and their down-regulation under conditions that induce keratinocyte differentiation provide an explanation for their selective expression in the basal epidermal layer where both factors contribute to the natural anti–angiogenic barrier separating the avascular epidermis from the vascularized dermis. Our results also demonstrate the capacity of epidermal keratinocytes to rapidly modulate their angiogenic potential by inverse regulation of pro-angiogenic and antiangiogenic factors.

# 011

**Systemic Inhibition of Cutaneous Tumor Growth and Angiogenesis by Thrombospondin-2 Using Cell-Based Antiangiogenic Gene Therapy**

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Endogenous antiangiogenic factors are generally thought to maintain the quiescence of the mature cutaneous vasculature by counterbalancing the activity of angiogenesis inducers. Malignant skin tumors have to overcome the activity of angiostatic factors in order to induce and to sustain angiogenesis which is essential for tumor growth, invasion and metastasis. Several endogenous antiangiogenic factors, including angiostatin and endostatin have been shown to inhibit tumor angiogenesis in experimental tumor models. Recent studies indicate that continuous delivery of endogenous angiogenesis inhibitors for cancer treatment is more potent than intermittent dosing, suggesting a potential role of gene therapy in antiangiogenic tumor therapy. We established a tissue-engineered implant system for the continuous *in vivo* production of thrombospondin-2 (TSP-2) a 420-kDa matricellular glycoprotein and a recently identified, potent endogenous inhibitor of tumor growth and angiogenesis. Fibroblasts were retrovirally transduced to overexpress TSP-2 and were seeded onto biodegradable polymer scaffolds. After transplantation into the peritoneal cavity of nude mice, bioimplants maintained high levels of TSP-2 secretion over extended time periods, resulting in increased levels of circulating TSP-2. Bioimplant-generated TSP-2 potently inhibited tumor growth and angiogenesis of human A431 squamous cell carcinomas and B16F10 malignant melanomas that were implanted orthotopically at a distant site. These results provide the first proof-of-principle for the feasibility and therapeutic efficiency of cell-based antiangiogenic gene therapy using tissue engineered bioimplants. While these results have been obtained in an experimental mouse tumor model, cell-based antiangiogenic gene therapy may also represent a promising new strategy for the treatment of human cancers, using autologous, patient-derived skin fibroblasts.

# 012

**Multiple Functions for β4 Integrin in Human Microvascular Endothelial Cells**

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α6β4 integrin expression has been observed in the microvasculature of the skin and other tissues *in vivo*. However the expression of α6β4 integrin is rapidly lost *in vitro* during the culture of primary microvascular endothelial cells (MEC). To determine the role of this molecule in MEC physiology, we used a retrovirus to stably introduce full length of β4 integrin cDNA in MEC and tested the function of this integrin on MEC attachment, proliferation, and wound healing/scratch assay and chemotaxis/Boyden chamber assays. MEC were isolated from human foreskin dermis following incubation with dispase and propagated in Iscove's growth medium supplemented with cyclic AMP, isobutyl methyl xantine and growth factors VEGF, FGF, EGF and hydrocortisone. MEC were purified by binding to Ulex-coated magnetic beads, yielding cells 99% positive for PECAM-1 and Factor VIII by IDIF analysis. The retroviral vector LZRS containing IRES blasticidin resistance gene and the full length β4 cDNA construct or LacZ cDNA as a control were used to infect purified MEC in the first passage followed by transient selection with 5 μg per ml blasticidin. The stable and uniform expression of β4 integrin in treated MEC was confirmed by FACS analysis, IDIF microscopy and Western blotting. β4 integrin in these cells was shown by IDIF to colocalize with both α6 integrin and plectin, indicating a functional gene product. β4MEC showed a proliferation rate 20% higher than control MEC assessed by MTT assay and direct cell counting. β4 MEC showed significantly increased binding to laminin 10 compared to control MEC in short-term cell attachment assays. β4 MEC showed significantly increased migration/spreading during *in vitro* wound healing/scratch assays compared to control MEC. β4 MEC showed approximately 100% greater migration in Boyden chamber chemotaxis assays compared to control MEC. In addition, β4 MEC showed both delayed tubule formation and delayed apoptosis in an *in vitro* tubule formation assay, compared to control MEC. These are the first studies to provide information on a role for β4 integrin in the microvasculature and suggest that β4 integrin may promote certain important functions during angiogenesis, including proliferation, attachment and migration.

# 013

**Role of Tyrosine Kinase in Endothelial Cell Migration and Angiogenesis**

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Increased angiogenesis is closely associated with the pathology of both invasive and noninvasive skin diseases (e.g. Kaposi's sarcoma, melanoma, and psoriasis). Strongly implicated in the morbidity of these diseases are the growth factors VEGF, FGF, PDGF and their cognate tyrosine kinase receptors. To better understand how tyrosine kinases control the growth and function of the skin microvascular system, we have used two indolinone-based inhibitors that block PDGF-,VEGF-, and FGF receptor kinase activity (from SUGEN Inc.) in models designed for maximal endothelial cell proliferation. Microvascular endothelial cells were isolated from the dermis of foreskin tissue by dispase digestion and propagated in Iscove's growth medium supplemented with cyclic AMP, isobutyl methyl xantine and growth factors VEGF, FGF, EGF and hydrocortisone. Endothelial cells were purified by binding to Ulex-coated magnetic beads. Under these growth conditions, microvascular endothelial cells maintain their epithelioid morphology in all subsequent passages and display the typical conversion to a spindle-shaped morphology when activated by PMA. When confluent monolayer cultures of endothelial cells are injured by a 0.9-mm scratch produced by a plastic micropipet tip, complete repair of the scratch takes place within a 24-h time interval. In the presence of 20 nM of either tyrosine kinase inhibitor, the repair of the scratch is completely inhibited. Removal of the inhibitor results in a normal repair process. When the extent of cell migration is measured in Boyden chambers, inhibition of tyrosine kinase also inhibits cell migration. In contrast, when new vessel formation is induced by collagen activation at the luminal surface of a confluent culture at the same concentration of inhibitors that completely blocks cell migration, a minor delay of new vessel formation is observed. These results demonstrate that nontoxic concentrations of two inhibitors of tyrosine kinase each strikingly interfere with the migration of microvascular endothelial cells, one of the earliest events of angiogensis *in vivo*.

# 014

**Caracterization of the Capillary-Like Network of a Human Skin Reconstructed *In Vitro***

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The faith of a grafted organ such as a dermal-epidermal skin substitute depends on its rapid vascularization by the wound's capillary bed. As demonstrated by earlier studies, the presence of pre-existing blood vessels in the dermal moiety of the grafted skin accelerates the neovascularization process. Our goal was to produce a reconstructed skin without exogenous or synthetic extracellular matrix components that contained a capillary-like network to eventually graft this skin substitute on full thickness wounds. In order to achieve this goal, we used the self-assembly technique developed in our laboratory in which the isolated cells secreted their own extracellular matrix that they reorganized into a tissue structure.The dermal portion of our reconstructed skin is made up of superposed sheets of dermal fibroblasts and endothelial cells (HUVEC) cultured for 5 weeks in the presence of ascorbic acid. Keratinocytes are seeded on top of the dermal moiety and grown to confluence. Maturation of the epithelium was achieved by raising the reconstructed skin at the air–liquid interface. Histological examination of the tissue engineered skin showed the presence of hollow tubules in the dermis, and the progressive differentiation and cornification of epithelial cells. Immunofluorescence staining demonstrated the endothelial nature of the tubules (von Willebrand factor and PECAM), the presence of basal membrane proteins (type IV collagen and laminins) surrounding the capillary-like structures and at the dermal-epidermal jonction, and the presence of other components of the extracellular matrix (type I collagen and fibrillin). Ultrastructural observations revealed the presence of a lumen in the reconstructed capillaries and of angiogenic sprouts. Confocal microscopy analysis confirmed the presence of angiogenic sprouts, the hollow nature of the reconstructed tubules, and the branching between capillary structures, thus confirming the three dimensionnal and complex nature of the reconstructed network.

# 015

**Expression of Angiogenic Cytokines in Intestine and Skin Lesions of Inflammatory Bowel Diseases**

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Pyoderma gangrenosum (PG) occurs as a cutaneous manifestation associated with inflammatory bowel diseases (IBD). Recent evidence increasingly suggests that IBD are the result of dysfunctional immunoregulation manifested by inappropriate production of mucosal cytokines. To elucidate the medanism of ischemic change in IBD, we asessed serum levels of vascular endothelial growth factor (VEGF) and basic firbroblast growth factor (b-FGF), and plasma level of endo thelin-1 (ET-1). We also investigated the expression of VEGF, b-FGF and transforming growth factor β1,2,3 (TGF-β1,2,3) in intestine and skin lesions by immunostaining. Blood samples wre obtained from 11 patients with asthma or diverticulitis. The cytokine levels (pg per ml) were as follows; for ET-1, UC: 127 ± CD: 167 ± 35, and controls [asthma: 38.5 ± 23.8 ($p < 0.01$), diverticuluitis: 40.5 ± 25.6 ($p < 0.01$)], for b = FGF, UC:9.2 ± 1.9, CD: 9.1 ± 1.5, and controls [asthma: 5.0 ± 0 ($p < 0.01$), diverticulits:5.0 ± 0 ($p < 0.01$)], for VGEF, UC 660 ± 181, CD: 740 ± 182, and controls [asthma: 194 ± 59 ($p < 0.01$), diverticulitis: 200 ± 60 ($p < 0.01$). The levels of VGEF and b-FGF were significantly higher in active IBD than ntose in the controls. A significant positive correlation among the serum levels of VEGF and b-FGF and plasma level of ET-1; i.e. elevated VEGF and b-FGF and ET-1 levels correlated well with each other. Immunohistochemical studies showed increased venula in the submucosa and lamina propria. Overexpression of VEGF and b-FGF in endothelial cells was revealed and TGF-β2,3 were found in inflammatory cells of active IBD, but no change was observed around the vessels in the controls. VEGF was strongly stained on the increased vessels and b-FGF and TGF-β2,3 were stained on the inflammatory cells of the involved skin of PG.

# 016

**The Angiogenesis Inhibitor Thrombospondin-2 Inhibits Tumor Growth and Angiogenesis of Human Malignant Melanomas**

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We have recently shown that thrombospondin-2 (TSP-2), an endogenous antiangiogenic factor, inhibits the orthotopic tumor growth of transplanted human cutaneous squamous cell carcinomas. Moreover, loss of TSP-2 resulted in a significantly enhanced susceptibility to chemically induced epidermal carcinogenesis in mice. To investigate the role of TSP-2 for the growth of malignant melanomas, human MeWo melanoma cells were stably transfected to overexpress TSP-2 and were transplanted, along with control-transfected clones, intradermally into nude mice. TSP-2 expression potently inhibited the *in vivo* growth of malignant melanomas by more than 50%, as compared with control-transfected clones. Moreover, tumor angiogenesis was strongly inhibited by TSP-2, with a significant reduction of the size of angiogenic tumor blood vessels. Northern blot analysis, *in situ* hybridization and immunofluorescence analyses confirmed that efficient TSP-2 mRNA and protein expression was maintained in tumor transplants over at least 5 weeks. Importantly, the expression of the major tumor angiogenesis factor, vascular endothelial growth factor, was unchanged by TSP-2. These results demonstrate that inhibition of tumor angiogenesis potently inhibits malignant melanoma growth, and they identify TSP-2 as a promising new candidate for melanoma treatment.

# 017

**Lymphangiogenesis in Melanoma, Squamous Cell Carcinoma and Breast Carcinoma**

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The lymphatic system serves as the primary route for the metastasis of melanoma, squamous cell carcinoma and breast carcinoma and the extent of lymph node involvement is a key prognostic factor for the outcome of the disease. Whereas the significance of angiogenesis for tumor progression has been well documented, the ability of tumor cells to induce the growth of lymphatic vessels (lymphangiogenesis) and the presence of intratumoral lymphatic vessels have been questioned. We investigated the role of lymphangiogenesis and the lymphangiogenic factor VEGF-C in experimental skin and breast cancer. By using the specific markers for lymphatic endothelium, LYVE-1 and VEGFR-3, we demonstrated the occurrence of intratumoral lymphangiogenesis within these cancers. Onset of tumor angiogenesis in squamous cell cancer was accompanied by induction of lymphangiogenesis, indicating coordinated regulation of angiogenesis and lymphangiogenesis during carcinogenesis. Stable overexpression of VEGF-C in melanoma cells resulted in infiltration of tumors with lymphatic vessels as well as in enhanced tumor angiogenesis. Moreover, VEGF-C induced chemotaxis of macrophages *in vitro* and *in vivo*, indicating role of VEGF-C in regulating immune function in melanoma. VEGF-C overexpressed in genetically fluorescent breast cancer cells, also potently increased intratumoral lymphangiogenesis, what resulted in significantly enhanced metastasis to regional lymph nodes and to lungs. Importantly, the degree of tumor lymphangiogenesis was highly correlated with the extent of lymph node and lung metastases. These results demonstrate the occurrence of intratumoral lymphangiogenesis in multiple tumor types and reveal the biological functions of VEGF-C in skin and breast cancer.

# 018

***In Vivo* Revascularizttion of Acellular Dermis with Human Endothelial Cells**

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The clinical utility of engineered skin equivalents is limited by a lack of a vascularization. Acellular dermis is a potentially useful material for construction of synthetic skin. To improve the survival of such constructs, a methodology for vascularizing acellular dermis with genetically modified human endothelial cells was developed. Retroviral transduction was utilized to express either a caspase resistant form of the survival gene Bcl-2, or a control transgene, EGFP in human umbilical vein endothelial cells (HUVEC). These cells were then seeded on acellular human dermis. Within 3 days, it appeared that existing vascular channels became repopulated with the HUVEC. Dermis seeded with either Bcl-2 or EGFP transduced HUVEC (n = 9) were implanted subcutaneously into SCID/beige mice. To assess the effect of the presence of fibroblasts on revascularization, 5 of the constructs in each group were also seeded with human dermal fibroblasts. One month after implantation the grafts were harvested, revealing that the majority contained perfused vascular profiles. UEA-1 staining was utilized to confirm that observed vascular structures were lined by human endothelium. Immunostaining demonstrated continued transgene expression *in vivo*. Blinded scoring of vascular density on a 0–5 scale revealed that the implants seeded with Bcl-2 transduced HUVEC had a higher mean score (3.0 ± 0.7) compared to the EGFP controls (1.6 ± 0.6). No beneficial effect of additionally seeding with fibroblasts was observed. In summary, we have developed a methodology for vascularizing skin equivalents, which demonstrates that endothelial overexpression of Bcl-2 enhances vascularization.

# 019

**Effects of Synthetic Peptides on Angiogenesis and Wound Healing**

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Angiogenesis, the growth of new blood vessels, represents a critical step in the wound-healing cascade. Numerous polypeptides exist that are known to be important mediators that facilitate angiogenesis. Among them, ACT 2 is an ubiquitous polypeptide found in a variety of tissues and cell types. ACT 1 is a novel peptide also believed to mediate cell proliferation and migration *in vitro*. The purpose of our study is to examine the effects of these peptides on angiogenesis, and more importantly, to help elucidate the roles of ACT 1 and ACT 2 in wound healing. *In vitro* assays were conducted to determine the effects of ACT 1 and ACT 2 on human microvascular endothelial cell (HMEC) migration. These include Boyden chamber and scratch wound closure assays. In addition, MTT(tetrazolium) assays were performed to elicit the effects of ACT 1 and ACT 2 on endothelial cell proliferation and cell viability. *In vivo* wound gap closure assays were conducted on full thickness punch biopsy wounds on rat skin. Our results demonstrated that ACT 1 and ACT 2 act as chemottractants for endothelial cells, stimulating the migration of HMECs in Boyden chambers. These peptides revealed heightened responses at 10–100 ng per ml when compared to positive controls (VEGF, PDGF, FGF) assessed by *in vitro* scratch wound closure assay. Furthermore, cell proliferation was stimulated 3 fold by ACT 2, and 2 fold by ACT 1 over that with medium alone when 10 ng per ml of peptide was used. Addition of ACT 1 topically or intraperitoneally increased reepithelizlization ($15 \pm 3\%$, $42 \pm 9\%$, respectively) over negative control. Our finding clearly demonstrate that synthetic peptides significantly promote angiogenesis both *in vivo* and *in vitro* by stimulating cell migration and proliferation. Peptides with a comparable effect on angiogenesis such as ACT 1 and ACT 2 are likely to play critical roles in the wound healing process when applied topically. In turn, the implications for these two polypeptides are immeasurable.

# 020

**The Angiogenic Factors VEGF, bFGF and IL-8 as New Prognostic Markers in Serum from Malignant Melanoma Patients**

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The homeostasis of pro- and antiangiogenic factors plays a major role for tumor growth and metastasis formation. In the present study we investigated the concentration of the pro-angiogenic factors angiogenin, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and interleukin-8 (IL-8) in serum from 125 melanoma patients of different stages of disease with or without current therapy including interferon (IFN)-$\alpha$ and/or different cytostatics in comparison to 30 healthy control subjects using enzyme-linked immunosorbent assay (ELISA) technique. Serum levels of angiogenin ($p < 0.0005$), VEGF ($p = 0.037$), bFGF ($p = 0.004$), and IL-8 ($p = 0.004$) were significantly increased in melanoma patients compared to healthy controls. Elevated serum concentrations of VEGF, bFGF, and IL-8 were associated with advanced disease stages and tumor burden. Cytostatic therapy of patients was accompanied by increased serum levels of angiogenin, bFGF and IL-8. As shown by univariate analysis, elevated serum levels of VEGF ($p = 0.0001/p = 0.0036$), bFGF ($p < 0.00005/p < 0.00005$), and IL-8 ($p < 0.00005/p < 0.00005$) were strongly correlated with a poor overall/progression-free survival. Multivariate analysis revealed stage of disease ($p = 0.0238$), tumor burden ($p = 0.0347$), VEGF ($p = 0.0036$), bFGF ($p = 0.0252$), and IL-8 ($p = 0.0447$) as independent predictive factors of overall survival. Tumor burden ($p = 0.0081$), VEGF ($p = 0.0245$) and IL-8 ($p = 0.0089$) were found as independent predictive factors of progression-free survival. Our data suggest that the angiogenic serum factors VEGF, bFGF, and IL-8 are useful predictive serum markers for overall and progression-free survival in melanoma patients.

# 021

**Close Relation of Reduced Expression of Actin-Binding Proteins, Calponin-h1 and h-Caldesmon, in the Vascular Smooth Muscle Inside Human Malignant Melanoma with the Poor Prognosis**

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We previously observed the decrease of a smooth muscle actin ($\alpha$SMA), and calponin-h1 (CNh1) in the blood vessels of malignant melanomas. For the metastasis, penetration of tumor cells through a blood vessel is supposed to depend on the structural integrity of the blood vessel. We stained various types of human melanocytic tumors with antibodies specific for endothelial cells and vascular smooth muscle cells. Significant suppression of $\alpha$SMA, CNh1 and h-CD in the blood vessels of malignant melanomas was observed compared with both benign melanocytic tumors and normal tissues. Furthermore, the degree of suppression was much higher in CNh1 and h-CD than in $\alpha$SMA. In particular, the expression of h-CD was inversely correlated with the frequency of metastasis and positively correlated with the survival rate in patients with malignant melanoma. These findings suggest that the nature of tumor vessels is one of the important factors for prognosis of malignant melanoma, and that the suppression of $\alpha$SMA, CNh1 and h-CD in the blood vessels in malignant melanoma reflects the structural fragility of the blood vessels, leading tumor cells to easily penetrate the vessels. Actually, CNh1-deleted mice exhibited morphological fragility of blood vessels; significantly more cancer metastasis occurred in the CNh1-deleted mice than in the normal mice. These molecules can be good markers for malignant transformation of melanocytic tissues, and in particular, h-CD is a good marker of malignant melanoma for metastatic potential and prognosis as well as tumor thickness.

# 022

**Characterization of Mouse Pemphigus Vulgaris Model Using Immunoelectron Microscopy**

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Recently, we have generated a novel active disease mouse model for pemphigus vulgaris (PV) which was produced by transfer of splenocytes from desmoglein3 (Dsg3)–/– mice, immunized with recombinant mouse Dsg3, to Rag2–/– mice that express Dsg3. These mice stably produced anti-Dsg3 IgG and developed disease phenotype including oral erosion with suprabasilar acantholysis. The purpose of this study is to elucidate whether this mouse model has the same histogenesis as that of human PV using transmission electron microscopy (EM) and immuno-EM. Samples were obtained from skin and mucosa of the model mice and Rag2–/– mice without adoptive transfer as a control, and observed under transmission EM. For postembedding immuno-EM, samples from skin and mucosa were cryofixed with liquid propane cool at –190Âé followed by freeze substitution with methanol. The samples were embedded in Lowicryl K11M and ultrathin sections were immunolabeled. As results, the samples from the mouse model showed cellular detachment at the suprabasal layer resulted in ''row of tombstones'' like appearance by transmission EM. Separated attachment plaques of desmosomes, which was also referred to ''half desmosome'' were abundantly observed. These findings in transmission EM were identical to those of human PV. By postembedding immunogold EM, *in vivo* deposited IgG to the mouse model epidermis was shown to localize mainly at the extracellular portion of desmosomal attachment plaques. Similarly, *in vitro* binding sites of serum IgG obtained from the mouse model to the control mouse epidermis were localized mainly at the extracellular portion of desmosomal attachment plaques. These results were consistent with those of human PV, which were reported previously. These data provide evidence that our mouse PV model properly reflects the histogenesis of human PV.

# 023

**Desmoglein 2 is a New Antigenic Target of Pemphigus Autoantibodies**

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Pemphigus is an autoimmune blistering disease whereby circulating antibodies cause intraepidermal split. The mechanism regulating cell acantholysis in pemphigus is controversial and unresolved, but can be attributed to pathogenic autoantibodies targeting several antigens, including the desmosomal cadherins, desmogleins 1 (Dsg1) and 3 (Dsg3), the plakin family of proteins, such as desmoplakin and envoplakin, and pemphaxin. Nevertheless, the general dogma implicates that in pemphigus foliaceus (PF), the target antigen is Dsg1 and the antibodies cause blisters in the superficial epidermis where Dsg1 is expressed without concomitant Dsg3. In pemphigus vulgaris (PV), the antibodies are directed against both Dsg1 and Dsg3 resulting in deep suprabasilar blisters in both skin and mucous membranes. However, little is known of the role of desmoglein 2 (Dsg2) in pemphigus. In the normal epidermis Dsg2 is weakly expressed in the basal cell layer but is up-regulated in afflicted pemphigus lesional and nonlesional skin. To demonstrate that there are pathogenic antibodies against Dsg2, our strategy was to produce GST-fusion proteins of the extracellular domains (EC; E1, E2, E3, E4, and EA) and characterized PV and PF sera. By immunoblotting analysis, both PF and PV sera contained antibodies that recognized Dsg2. Immunoepitopes were found, at varying degrees of intensity, in four of five EC domains, with E1 and E3 being the most immunogenic domains for PV and PF, respectively. Interestingly, most PV sera did not recognize the E2 domain of Dsg2 unlike published results showing that the E3 domain of Dsg3 is the least immunogenic. Characterization of Dsg2 as an autoantigen in pemphigus contributes to our understanding of the pathophysiology of this complex group of diseases.

# 024

**Interspecies Conservation and Differential Expression of Mouse Desmoglein Gene Family**

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Epithelial cell adhesion is mediated by intercellular junctions, called desmosomes, and desmogleins (Dsg; Dsg1, Dsg2 and Dsg3) are calcium-dependent transmembrane adhesion components of the desmosomes. While Dsg1 and Dsg3 are mainly restricted to stratified squamous epithelia, Dsg2 is expressed in essentially all desmosome-containing epithelia. In this study we have cloned and characterized mouse Dsg1 and Dsg2 genes. The Dsg1 full-length cDNA (5.5 kb) contains an open reading frame of 3171 bp encoding a precursor protein of 1057 amino acids. The Dsg2 cDNA (6.3 kb) has an open reading frame of 3366 bp coding for a precursor protein of 1122 amino acids. Mouse Dsg2 protein shares 76% identity with human Dsg2 but only 26% and 33% to mouse Dsg1 and Dsg3, respectively. Computer analysis predicted Dsg1 and Dsg2 polypeptides to contain a transmembrane domain as well as several putative calcium-binding and N-glycosylation sites. Similar to Dsg3, Dsg1 contains the RAL tripeptides, a potential site for cadherin interactions. Dsg2 contains the tripeptide YAL, also present in human DSG2. Analysis of intron/exon organization of the desmoglein genes revealed that some exons are highly conserved while others show significant divergence. The mRNA expression patterns of these desmogleins during mouse embryonic development and in various adult tissues are variable. While Dsg2 and Dsg3 are expressed in all developmental stages, Dsg1 expression is delayed until day 15 of mouse embryos. In adult mouse tissues, Dsg2 is widely expressed while the expression of Dsg1 and Dsg3 are restricted to select tissues. In summary, the divergence at the level of gene organization and the spatially and temporally regulated expression of these desmogleins may contribute to their significant role in cell-cell adhesion during development.

## 025

**Keratinocyte Cell-Cell Adhesion is Altered in Psoriasis**

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The purpose of this study was to ascertain keratinocyte cell-cell adhesion in psoriatic uninvolved (PS-U) and involved (PS-I) skin. Biopsies from 4 patients with widespread plaque psoriasis were studied by electron microscopy (EM) and by immunocytochemistry (ICC) for adherens junctions (E-cadherin, P-cadherin, β catenin and F-actin) and desmosomes (desmogleins). EM of PS-I revealed widening of intercellular spaces between keratinocytes at both the basal and suprabasal layers and reduction of intercellular bridges, which appeared thin and elongated. ICC, some by confocal laser microscopy, revealed a reduction of E-cadherin, P-cadherin, β catenin and F-actin. Neutral, tris-HCl buffer extracts without detergents (titron- ×100) were subjected to Western blots for E-cadherin and beta catenin. This study showed solubilization of β catenin but not of E-cadherin. Alterations in cell-cell ahhesion in psoriatic epidermis raise the question of their role in the regulation of keratinocyte differentiation and proliferation.

## 026

**Detection of IgG and IgA Autoantibodies Reactive with Human Desmocollins 1, 2 and 3 by Enzyme-Linked Immunosorbent Assays Using Baculovirus-Expressed Recombinant Desmocollins**

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We have previously shown that human desmocollin (Dsc) 1 is reacted by IgA antibodies of subcorneal pustular dermatosis type of IgA pemphigus. However, the presence of IgG anti-Dsc autoantibodies is still controversial, and antibodies to Dsc2 and Dsc3 have not been clearly identified. To solve these problems, we have successfully produced recombinant proteins containing entire extracellular domains of human Dsc1, 2 and 3 by baculovirus-expression system, and subsequently established an enzyme-linked immunosorbent assays (ELISA) for both IgG and IgA antibodies using these recombinant desmocollins. By this ELISA, none of the 45 sera of classic types of pemphigus showed IgG antibodies to any Dsc, while 3 of 21 Brazilian pemphigus sera and 2 of 22 paraneoplastic pemphigus sera showed IgG antibodies reactive with Dsc1–3. In addition, one atypical pemphigus serum showed both IgG and IgA antibodies to Dsc1, which were completely absorbed by incubation with Dsc1 baculoprotein. Furthermore, this ELISA detected IgA anti-Dsc3 antibodies and IgA antibodies to both Dsc2 and Dsc3 in one each of atypical pemphigus case. This reactivity was confirmed by the positive IgA immunofluorescence staining with human Dsc2 and Dsc3 expressed on COS-7 cells transfected with cDNA of each Dsc. These results suggest that, both IgG and IgA autoantibodies against all of Dsc1, 2 and 3 are present in the sera of particular nonclassical types of pemphigus, although they are rarely detected in classic pemphigus.

## 027

**Acantholysis in Pemphigus Vulgaris: A Plakoglobin-Mediated Event**

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Binding of pemphigus vulgaris (PV) antibodies to Dsg 3 provokes loss of desmosome-mediated adhesion between keratinocytes. To investigate the underlying molecular mechanism we recently established and described long-term cultures of wild-type and plakoglobin knock-out ($PG^{-/-}$) mouse keratinocytes. This dual *in vitro* system had allowed us to attribute a central role to plakoglobin in lesion formation. Here we further refined this role by assessing the involvement of plakoglobin in the desmosomal break-down at the plasma membrane. Using immunofluorescence studies we found that PV IgG induced clustering of all investigated desmosomal components in wild-type but also in $PG^{-/-}$ cells. The only exception was desmoplakin which showed only rare clusters in $PG^{-/-}$ keratinocytes. PV-Fab reproduced this clustering event concurrently with keratin retraction in wild-type cells. In contrast, despite binding of PV-Fab to $PG^{-/-}$ cells, clustering was abrogated. In summary, using our dual *in vitro* system we defined two as yet undescribed activities of pemphigus antibodies: (i) a cross-linking activity exerted by bivalent PV IgG, which is not required for lesion development and is independent of plakoglobin, and (ii) the pathogenic activity of PV-Fab which depends on plakoglobin and appears central to PV blister formation as it causes disruption of the entire desmosomal organization in wild-type keratinocytes. Collectively our results provide the basis for the establishment of a novel model explaining the mechanism leading to blister formation in PV, which includes a bipartite pathogenetic activity of plakoglobin at the plasma membrane and in the nucleus. Assessment of plakoglobin's role in keratinocyte differentiation from this disease-based perspective opens a new window on the function of this plaque protein in surveying intact epithelial architecture, hopefully leading to a better understanding of this mechanism in profit of the PV patient.

## 028

**Development of Anti-Desmoglein 3 (Dsg3) Pathogenic Monoclonal Antibody Using Active Disease Mouse Model for Pemphigus Vulgaris (PV)**

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PV is an autoimmune blistering disease caused by anti-Dsg3 IgG autoantibodies. Recently we developed a PV murine model by adoptive transfer of splenocytes from immunized Dsg3−/− mice to Rag2−/− immunodeficient mice that express Dsg3. Purpose of this study is to develop anti-Dsg3 pathogenic monoclonal IgG antibody using PV model mice. Splenocytes from PV model mice, which showed gross phenotype of the active disease including weight loss due to extensive oral erosions and hair loss, were fused with mouse myeloma cell line to isolate hybridoma clone producing antimouse Dsg3 monoclonal antibody. We obtained 10 monoclonal IgG antibodies. All of them, except one clone, reacted exclusively with mouse Dsg3, but not mouse Dsg1, as determined by enzyme-linked immunosorbent assay. Moreover, these antibodies bound to the native Dsg3 on living cultured mouse keratinocyte, PAM212, when they were added to culture media. When one of the monoclonal antibody (AK19) was injected into neonatal mice, AK19 alone could cause microscopic blisters in the skin with suprabasilar acantholysis, typical histologic finding of PV. Furthermore, when AK19 was combined with a small amount of IgG from PF sera (containing anti-Dsg1 IgG which was insufficient to cause blisters by itself), neonatal mice developed gross blisters in the skin with suprabasilar acantholysis in histology. These findings indicate that AK19 is anti-Dsg3 specific monoclonal IgG antibody which can play a pathogenic role to inhibit adhesive function of Dsg3 with resultant blister formation *in vivo*. These monoclonal antibodies will be a valuable tool to dissect the molecular mechanism of blister formation in PV as well as to develop epitope-specific therapeutic strategies.

## 029

**Expression of Desmoglein 1 Can Compensate for Genetic Loss of Desmoglein 3 in Anchorage of Telogen Hair**

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The desmoglein (Dsg) compensation hypothesis, namely that one Dsg can compensate for antibody-induced loss of function of another, has been proposed to explain the pathology of pemphigus. To validate this hypothesis genetically, we used Dsg3 knockout mice (Dsg3−/−) that lose their telogen hair prematurely due to loss of adhesion (acantholysis) between keratinocytes of the telogen hair club and the outer root sheath. In normal mice only Dsg3 is expressed in this region therefore in Dsg3−/− mice there are no Dsgs there. We asked whether Dsg1 could substitute for the function of Dsg3 in telogen hair by engineering transgenic mice that express Dsg1, with a FLAG epitope tag, driven off the keratin 14-promoter. The transgene (tg) was bred into Dsg3−/− mice. Immunofluorescence (IF) with anti-FLAG antibodies indicated expression of Dsg1 in the telogen club of Dsg3−/−tg+ but not Dsg3−/−tg− mice. Dsg3−/−tg− mice ($N = 4$) lost telogen hair with each wave of telogen. In contrast Dsg3−/−tg+ mice ($N = 7$ from 2 tg founders) had markedly delayed, and decreased, hair loss in the first telogen cycle, and did not lose hair in subsequent cycles. Dsg3−/− mice also show low weights due to blisters in the oral mucosa from loss of Dsg3 in the basal layer. Surprisingly, Dsg3−/−tg+ mice showed similar weight curves, suggesting that Dsg1 was not compensating for loss of Dsg3 in the mucous membrane. This result was explained when immunoblotting with anti-FLAG antibodies indicated that the tg, although expressed in skin, was not expressed in oral mucous membranes. These studies show that Dsg1 can compensate for loss of Dsg3 adhesion, and provide genetic evidence confirming the desmoglein compensation hypothesis.

## 030

**Tissue Distribution and Cell Type-Specific Expression of p120ctn Isoforms**

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Cadherin-mediated adhesion complexes play a major role in cell-cell adhesion. The intracellular domain of cadherins interacts with β-catenin and another armadillo protein, p120ctn, a component of the adhesion junction, whose function has not been defined at the molecular level. As a catenin, p120ctn has been localized to the cell adhesion borders in complexes with cadherins and with α- and β-catenin. Several p120ctn isoforms, resulting from differential splicing and utilization of the alternative translation initiation codons, have been identified. In this work we demonstrate that several mouse tissues express at least two major p120ctn isoforms, although in slightly different ratio. However, IIF of mouse tissues and organs revealed that p120 isoforms are expressed in cell-type specific manner. The long isoform, detected by a monoclonal antibody 6H11, is expressed in low turnover cells and epithelia, such as brain, cornea, lung, and serosal epithelium of stomach, but is present in low or nondetectable levels in rapid turnover epithelia, such as skin, tongue, mucosal membranes of esophagus, forestomach, and small intestine. The tissue- and cell-type specific expression of p120ctn isoforms suggests a specific role for the amino-terminus of the long p120 isoform in epithelia with different turnover rates.

# 031

**Central Role of the Plakoglobin-Binding Domain for Desmoglein 3 Incorporation into Desmosomes**

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To determine if the cytoplasmic domain of desmoglein 3 (Dsg3) is sufficient for its incorporation into desmosomes, we produced stable transfectants of A431 cells with cDNA encoding the extracellular domain of E-cadherin (Ecad) and the cytoplasmic domain of Dsg3 with a myc epitope on the carboxy-terminus (Ecad-Dsg3myc). Confocal immunofluorescence (IF) with antimyc and antidesmoplakin (DP) antibodies showed colocalization, indicating that the Dsg3 cytoplasmic domain is sufficient for targeting to desmosomes. Controls of transfected A431 cells expressing full length Ecad with a myc tag showed that myc staining was clearly distinct from DP staining. We then constructed cDNA encoding chimeric Ecad-Dsg3 with sequential deletions of the cytoplasmic tail, all with a myc tag at the carboxy-terminus. Expression in A431 cells followed by IF staining for myc and DP indicated that the desmoglein specific sequences at the carboxy-terminus were not necessary for desmosome targeting. With the loss of the next 87 amino acids, which are thought to contain the plakoglobin-binding domain, the chimeric molecule localized to the plasma membrane, but now colocalized with β-catenin rather than DP. Immunoprecipitation of these chimeric molecules, followed by immunoblotting for plakoglobin, confirmed the loss of these 87 amino acids resulted in loss of plakoglobin-binding. These data underscore the importance of plakoglobin-binding for desmosome localization, and suggest that absent this binding, the extracellular domain of Ecad may localize to adherens junctions, probably through lateral homophilic interactions.

# 032

**Contrasting Effects of PKCδ and PKCα on Regulation of α6β4 Integrin in Skin Keratinocytes**

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α6β4 integrin is a transmembrane glycoprotein heterodimer important in skin biology. α6β4 protein, localized to the hemidesmosomes, directly connects the keratin cytoskeleton of the proliferative basal cells to the laminin 5 enriched basement membrane, mediating outside-in and inside-out signaling events. Calcium induced differentiation of primary keratinocytes is associated with loss of α6β4 expression, loss of attachment to basement membrane components, and induction of differentiation. One of the important signal transduction pathways activated during keratinocyte differentiation, which participates in regulation of integrin function is the Protein Kinase C (PKC) family, represented by PKCs α, δ, η, ε and ζ in skin. Thus, in the present study we investigated the role of PKCs in regulation of α6β4. By utilizing recombinant PKC adenoviruses, we have identified opposing effects of PKCδ and PKCα on regulation of α6β4. PKCδ but not PKCα was able to serine phosphorylate α6β4 integrin. Specifically, overexpression of PKCδ induced downregulation of α6β4 protein, decreased localization of α6β4 to the hemidesmosomes, reduced attachment to laminin 5 and induced detachment of keratinocytes from the culture dish. In contrast, overexpression of PKCα led to increased α6β4 expression and localization to the hemidesmosomes and increased cell attachment to laminin 5. In order to further explore the interactions of these two isoforms with the α6β4 integrin, we have constructed an α6 recombinant adenovirus. Overexpression of α6 subunit in keratinocytes increased expression and coimmunoprecipitation of the endogenous β4 subunit, whereas other integrin subunits remained unchanged. In α6 overexpressing keratinocytes, attachment to laminin 5 was significantly increased, cell proliferation was not affected, but calcium induced differentiation was abrogated. This was associated with PKCα activation but not of other PKCs expressed in skin. Moreover, α6 immunoprecipitates revealed PKCα as the exclusive isoform activated in the complex. Furthermore, overexpression of α6 resulted in elevated tyrosine and serine phosphorylation of PKCα. In conclusion, activation of PKCδ and PKCα results in contrasting effects on α6β4 phosphorylation, protein expression and cell attachment to the underling matrix. Furthermore, PKCα specifically interacts with α6β4 integrin, in both inside-out and out-side in signaling, in skin.

# 033

**p120ctn Isoforms Constitute a Protein Family With Diverse Functions**

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p120 catenin belongs to the armadillo family of proteins, which have previously been implicated in regulation of cell-cell adhesion and gene expression. Alternative splicing and translation start sites enable expression of multiple p120ctn isoforms from a single gene. Previously, overexpression of the long isoform of p120ctn (isoform 1A) has been shown to result in a branching phenotype of both mesenchymal and epithelial cells in culture. In this study we describe divergent expression patterns of p120ctn isoforms in different cell lineages, different subcellular localizations of various p120ctn isoforms, and discrete effects of these p120ctn isoforms on cellular morphology upon overexpression. Specifically, human melanocytes prominently expressed a p120ctn isoform with a long N-terminus (isoform 1) whereas keratinocytes preferentially expressed three p120ctn isoforms with progressively shorter N-termini (isoforms 2, 3, and 4). Both the N-terminal and C-terminal sequences were essential to the branching phenotype. The presence of an alternatively spliced 3′ exon (exon B) abrogated the branching phenotype induced by overexpressed isoform 1. Nuclear localization of p120ctn was necessary but not sufficient for the establishment of the branching phenotype. This interpretation is supported by the findings that (a) branching was observed only when transiently expressed p120ctn was present in the nucleus (isoforms 1A-3A), (b) no branching was induced by the p120ctn isoforms which are excluded from the nucleus (isoform 4 and constructs containing exon B) and (c) C-terminally truncated isoform 1 constructs accumulated in the nucleus but did not induce branching. In summary, this study establishes that p120ctn consists of a family of proteins with distinct subcellular distribution patterns and functions.

# 034

**A Specific Sequence of G4 Domain of Laminin α3 Chain Supports Cell Adhesion Through Syndecans and Cooperative Involvement of Integrin β1**

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We analyze the heparin binding and cell adhesion activities in α3 chain carboxyl terminal globular domain (LG1~LG5 domain) utilizing recombinant proteins made in mammalian cells as well as synthetic peptides. Heparin binding activity was found in both LG3 and LG4 domains by affinity chromatography wit heparin-sepharose. In LG4, a specific heparin binding site, #75′:NSFMALYLSKGR was identified by competition assays. Serine-substituted mutants of LG4 demonstrated that basic residues Lys and Arg of #75′ were essential for the heparin-binding activity. Immortalized human keratinocyte line HaCat cells and human dermal fibroblasts bound to LG4 domain specifically and in a dose-dependent manner. By inhibition studies, the heparin-binding site #75′ inhibited cell adhesion to LG4 and the sequence #83 also weakly inhibited. Cell adhesion to LG4 and #75′ was inhibited by heparin or by heparitinase I treatment of cells, suggesting that cell binding to the #75′ within LG4 was mediated by cell surface heparan sulfate proteoglycan. Unexpectedly, anti-β1 antibody also inhibited cell adhesion. Affinity chromatography revealed that syndecan-2 from fibroblasts bound to LG4 fusion protein. Solid-phase assays demonstrated that syndecan-2 interacted with the #75′ and weakly with #83Ō. Cloned 293T cells expressing syndecan-2, –4 specifically attached to #75′ as well as to LG4 in heparin and integrin β1 dependent manner. These data indicate that syndecan-2 as well as syndecan-4 mediate cell adhesion and cooperative involvement of integrin β1 is necessary for cell adhesion to the laminin α3 LG4 domain.

# 035

**Non-Radioisotope Immunoprecipitation Assay for Detection of Anti-Laminin 5 Autoantibody**

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Cicatricial pemphigoid (CP) is autoimmune bullous disease that primarily affects mucosal tissues. Recent studies have identified most of CP patients have IgG autoantibodies that recognize laminin 5 (Lam5). Since radioisotope immunoprecipitation (RI-IP) has high sensitivity to detect anti Lam5 autoantibody, we have examined CP patient sera with this assay. However autoradiography is time-consuming and the use of radioisotope is restricted. Therefore we tried to develop the assay in which radioisotope are not used to detect autoantibodies. Sera from 10 CP patients that were diagnosed by RI-IP were incubated with solubilized SCC25 cell fraction for 16 h. The precipitated proteins were subjected to immunobloting. The blots were incubated with anti α3, β3 and γ2 subunit of Lam5 rabbit serum as first antibody and developed by ECL kit. Anti-β3 antibody could detect precipitated subunit of Lam5 by two patients' sera on nitrocellulose membrane. The sensitivity of non radioisotope immunoprecipitation assay is lower than RI-IP and treatment to enhance sensitivity (such as biotinylation of cellular protein) is now in progress.

# 036

**BP180 Protein Segments Essential for Triple Helix Formation are Located in the Membrane-Proximal One-Third of the Extracellular Domain**

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BP180 is a member of the collagen protein family [collagen XVII] and a transmembrane constituent of the dermal-epidermal anchoring complex. Our group has previously reported on the expression and structural characterization of recombinant proteins consisting of wild type and mutant forms of the BP180 ectodomain. Findings from these studies, as well as data from other groups, have established that the BP180 ectodomain forms an elongate, homotrimeric complex which adopts a helical structure similar to that of the classical collagens. While triple helix assembly in the classical collagens is initiated via interaction between the C-terminal propeptides, the C-terminal noncollagenous domain of BP180 lacks cysteines and is therefore not likely to be the nucleation site for helix formation. To begin to investigate the role of specific BP180 protein domains in the assembly of the triple helix, a truncated form of the BP180 ectodomain – composed of Col-15, the largest collagen domain, and its flanking noncollagen domains – was expressed in stably transfected 293-EBNA cells. The resulting protein, designated sec180-tC14, was structurally analyzed using a combination of immunological and biochemical techniques, including chemical cross-linking and gel filtration chromatography. When increasing amounts of DSP, a thiol-cleavable cross-linker, was added to sec180-tC14, increasing amounts of a cross-linked complex of a size consistent with a collagen-like triple helix were detected. These data are also consistent with a recent report by Tasanen *et al* [*JBC* 275:3093, 2000], in which a recombinant form of the BP180 Col-15 domain exhibited a CD spectrum suggestive of a collagenous structure. In conclusion, the findings from this study indicate that the protein segment(s) essential for triple helix assembly in BP180 are present in the membrane-proximal one-third of the ectodomain.

# 037

**Co-Localization of β1-Integrins and EGF-Receptors in Stretch Induced Integrin Clusters of Human Keratinocytes**

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Stretching induces activation of MAP-kinases via β1-integrins in human keratinocytes [Kippenberger *et al*, *J Invest Dermatol* 114:408–412, 2000]. Recent studies suggest a functional and spatial association of integrins and EGF-receptors in cell adhesion. In the present study we tested the hypothesis of a colocalization between β1-integrins and EGF-receptors under cell stretch using immunohistochemistry and confocal laser scanning microscopy. We found that in most of the stretched cells β1-integrins were assembled in clusters at the basal cell membrane. Such clusters were seen rarely in controls. In stretched cultures of human keratinocytes the frequency of β1-integrin-clusters increased about 3 fold compared to controls. Immunohistochemically we found a colocalization of β1-integrins and EGF receptors after stretching, which occurred mostly in cluster areas. Concomitantly, the application of cell stretch increased cell adhesion as tested in an usual adhesion assay. This finding suggest the regulation of integrin adhesiveness via an "inside-out signaling", namely the shift from a low affinity to a high affinity state of the fibronectin-receptor. Our results show an enhancement of the adhesion properties of stretched keratinocytes resulting in an increase of focal contacts. The immunhistochemical colocalization of integrins and EGF receptors lead us to assume that β1 integrins interact with EGF receptors and might induce a ligand-free activation of the EGF receptors.

# 038

**The Physiologic Control of Keratinocyte Adhesion Involves Regulation of Cadherin and Desmoglein Expression via α9 Acetylcholine Receptor**

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One of the acetylcholine receptors (AChRs) in human keratinocytes (KC) is formed by α9 subunits targeted by pemphigus autoantibodies. α9 AChR is the first representative of a novel class of ionotropic-and-metabotropic receptors/$Ca^{2+}$ channels with dual muscarinic-and-nicotinic pharmacology regulating intracellular $Ca^{2+}$ metabolism. Signaling through α9 AChR sustains polygonal shape and maintains intercellular adhesion of KC, and its blockade by pharmacologic antagonists or antibodies causes acantholysis *in vitro*. To elucidate the link between keratinocyte adhesion and α9-mediated signaling, we in this study investigated expression of the adhesion molecules in epidermis of α9 knockout mice. The staining of tissue samples of α9–/– mice was compared to that of α9+/+ mice from the progeny of a heterozygous α9–/+ mouse. The α9–/– genotype was associated with a significant ($p < 0.05$) decrease of the relative amounts of both classical and desmosomal cadherins. In the epidermis of α9–/– mice, the total amount of cadherins was 52 ± 7 vs. 94 ± 12 in α9+/+ mice, and that of desmogleins was 24 ± 3 vs. 44 ± 3. Using semiquantitative immunofluorescence assays, we also found that an increased expression of adhesion molecules during differentiation of normal human KC in epidermis and cultures incubated for 24 h at high, 1.8 mM, $Ca^{2+}$, is associated with a reciprocal increase of α9. The receptors were seen accumulated at the areas of the cell membrane that mediate cell-to-cell contacts. The cholinergic drugs that act upon α9 AChR changed both the transmembrane flux and intracellular concentration of $Ca^{2+}$ in human KC. These results indicate that signaling through α9 AChR may regulate assembly and disassembly of the adherence and desmosomal junctions by KC, and that $Ca^{2+}$ plays a role in α9-mediated regulation of the keratinocyte adhesive function.

# 039

**Dynamic Movement of the β3 Integrin in Focal Contacts**

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Endothelial cells assemble a novel focal contact (FC)-related structure enriched in the α v β 3 integrin heterodimer, an α4 laminin subunit-containing ligand in the matrix, and plectin that we hypothesize mediates cell surface anchorage of both vimentin and microfilaments. It plays a role in endothelial cell migration and branching morphogenesis, essential elements of angiogenesis. To gain insight into the function and assembly of FCs in endothelial cells, we compared the localization of green fluorescent protein-tagged β3 integrin (GFP-β3) with that of the α v β 3 complex and vinculin in transfected endothelial cells. GFP-β3 colocalizes precisely with the endogenous α v β 3 integrin complexes in FCs both towards the cell center and at the cell periphery. In contrast, although at the cell periphery GFP-β3 colocalizes with vinculin, towards the cell center, at least three distinct types of FCs exist: GFP-β3-positive/vinculin-positive, GFP-β3-negative/vinculin-positive, and GFP-β3-positive/vinculin-negative. We monitored dynamics of β3 integrin-containing FCs in live cells. Live cell assays were performed at 370C on a confocal laser scanning microscope. Images were taken at 5 min intervals for periods of up to 2 h. The GFP-β3 integrin containing FCs show considerable movement in migrating endothelial cells. We have also analyzed β3 dynamics using fluorescence recovery after photobleaching (FRAP). Images of cells were taken immediately before photobleaching, immediately after photobleaching, and subsequently at 2 min intervals over periods of up to 1 h. Recovery of a detectable signal in FCs took at least 1 h in instances when the entire FC was bleached. However, when a narrow stripe of fluorescence is bleached within an individual FC, recovery is remarkably rapid and is complete within 10 min. These results suggest that endothelial cells assemble multiple types of FCs that are dynamic in the plane of the membrane. Furthermore, although incorporation of new integrins into established FCs is a relatively slow process, within a FC integrin heterodimers are free to move. We propose that the latter is crucial to the process that permits a cycling of receptor/ligand interactions as endothelial cells migrate over or through the extracellular matrix.

# 040

**Characterization of Molecular Mechanisms Underlying Mutations in Dystrophic Epidermolysis Bullosa Using Site-Directed Mutagenesis**

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Type VII collagen (COLVII) is the major component of anchoring fibrils, structures which mediate epidermal-dermal adherence. COL VII gene (COL7A1) mutations cause dystrophic epidermolysis bullosa (DEB), a genetic mechano-bullous disease. The biological consequences of specific COL7A1 mutations and the molecular mechanisms leading to DEB clinical phenotypes are unknown. We previously reported the expression and characterization of a recombinant full-length COL VII α chain in human cells. In this study, we introduced 4 individual substitution mutations, R2008G, G2671V, G2749R, and M2749K into the COL VII expression vector using site-directed mutagenesis. All four of these mutations have been reported to result in a total absence of anchoring fibrils in recessive DEB patients. These four mutant constructs were transfected into human 293 cells and stably transfected cell clones isolated. All four clones synthesized and secreted a 290-kDa mutant COL VII α chain at levels similar to or higher than wild type COL VII α chain. Biochemical characterization of purified mutant COL VII proteins and structural and functional comparisons with wild type COL VII demonstrated: (1) The R2008G mutation in a RGD-cell binding site within the triple helical domain produced a mutant protein with reduced ability to support keratinocyte adhesion. (2) The G2749R mutation in the triple helical domain resulted in mutant COL VII with an increased sensitivity to protease and a decreased ability to form trimers compared with wild type COL VII. (3) The G2671V mutation within the triple helical domain completely prevented COL VII to assemble into disulfide-bonded trimers. (4) The M2749K mutation which resides 4 amino acids upstream of 2 highly conserved cysteine residues within the NC2 domain impaired the intermolecular association and antiparallel dimer formation of COL VII, a necessary step for the assembly of anchoring fibrils. We conclude that mutations within COL7A1 are located within motifs of different importance for the function of the COL VII polypeptide. Protein processing, folding, stability, and cell-matrix binding may be altered by

# 041

**Integrin Expression is Hair Cycle-Dependent, and Integrin Blockers Stimulate Human Hair Growth *In Vitro***

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Integrins have been shown to play major roles during hair follicle morphogenesis. However, the precise expression patterns and functional role of integrins during hair follicle cycling remain to be fully characterised. Here, we have investigated the spatiotemporal distribution of the integrin chains α1, α2, α3, α4, α5, α6, and β1 during the depilation-induced hair cycle in C57BL/6 J mice, and have analysed the effect of functional integrin blockers such as anti-β1 integrin antibodies, RGD peptides and the disintegrin echistatin on human hair growth *in vitro*. In comparison with hair follicles from the resting phase (telogen), during murine hair growth (anagen) a marked increase in β1 IR was detected in the outer root sheath (ORS), dermal papilla (DP), perifollicular connective tissue sheath (CTS), arrector pili muscle, and perifollicular blood vessels. α1 and α3 IR was found on sebocytes as well as on ORS and basal layer epidermal keratinocytes. α2 IR was mainly found on the CTS during hair follicle regression (catagen). α4 IR was found on nerve fibers and endothelial cells as well as on the most proximal hair matrix during early anagen. α5 IR was detected, throughout the hair cycle, on DP and CTS fibroblasts, while α6 IR was found on all epidermal and distal ORS keratinocytes as well as on the arrector pili muscle, interfollicular nerve fibres and blood vessels. Interestingly, the functional inhibition of integrin binding promoted human hair growth *in vitro*: Microdissected human anagen VI hair follicles displayed significantly more hair shaft elongation compared to vehicle controls *in vitro* after administration of antib1 integrin antibodies ($p < 0.005$), RGD peptides ($p < 0.02$) or the disintegrin echistatin ($p < 0.05$). These results suggest that integrin signalling is an intriguing novel target for pharmaceutical hair growth modulation.

# 042

**Hemidesmosome Dynamics in Live Epithelial Cells**

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Hemidesmosomes (HDs) form connections with the underlying extracellular matrix and are considered to be stable anchorage structure, perturbation in which may result in dysadhesion of keratinocytes leading to skin blister formation. We have analyzed HD dynamics and monitored the fate of HDs in migratory and stationary live 804G cells using green fluorescent protein (GFP)-tagged HD proteins including BP180 and β4 integrin. Images of cells were taken at 5 min intervals over periods of up to 2 h. In approximately 60% of stationary cells at 50–70% confluency, HD proteins show no dynamic properties. Rather, HD proteins remain in stable cat-paw-like arrays along the substratum-attached surface of the cells where they coalign with laminin-5 in the extracellular matrix. In sharp contrast in 40% of stationary cells HD proteins show quite dramatic movements. Cat-paw arrays of HD proteins not only assemble and disassemble in an apparently random fashion, but also appear to move along the substratum-attached surface of the cells. To study HD protein dynamics during wound healing, we created scrape wounds in confluent monolayers of cells, maintained in 35 mm dishes, using pipetteman tips. Both at 8–10 and 15–18 h after wounding, in those cells that have begun to repopulate the wound site, HD proteins are highly motile in the plane of the membrane. In addition HD proteins assemble and disassemble into linear arrays towards the periphery of the migrating cells. At about 16 h after wounding, a cat paw-like array of HD proteins is also occasionally observed in the latter cells. Furthermore, in migrating cells HD proteins fail to colocalize with laminin-5 in the matrix of the cells. In contrast HD proteins in those cells within about 5–10 cell lengths away from the wound edge are stationary and codistribute in cat paw-like patterns with laminin-5 in the extracellular matrix. However, EM analysis reveals that cells throughout the wounded culture lack mature HDs. These results provide evidence that HDs are highly dynamic structures and that HDs undergo extensive remodeling in wounding even in cells a considerable distance from the site of the wound. Furthermore, HD proteins disconnect from the underlying matrix after wounding and that under such conditions they are free to move in the plane of the membrane. We propose that the dynamic properties of HD proteins during wound healing reflect the balance that motile cells maintain between migration over and adherence to the wound bed.

# 043

**TACE Contributes to the Release of Collagen XVII Ectodomain from Keratinocyte Surface**

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Collagen XVII plays an important role in keratinocyte adhesion and migration. It exists in two forms, as a full-length 180 kDa type II transmembrane protein and as a soluble 120 kDa ectodomain. Here we investigated the shedding process and identified the enzyme which releases the ectodomain from the cell surface. Time chase experiments with cultured keratinocytes showed that the soluble ectodomain was stable in the medium for more than 48 h. Shedding was stimulated by PMA and IL-1, stimulators of MMPs and ADAMs. The inhibitor profile included phenanthroline and several MMP- and sheddase-targeting hydroxamates such as IC3, but not a selective gelatinase inhibitor or serine protease inhibitors. Several candidate enzymes, such as MMP-2, MMP-9 and MT1-MMP, which are involved in proteolytic cascades on keratinocyte surfaces, were excluded, since in MMP-2-deficient human gastric carcinoma cells and in MT1-MMP-deficient murine keratinocytes collagen XVII shedding occurred in a normal manner. RT-PCR analysis and immunoblotting demonstrate that the prototype sheddase TACE (ADAM-17), is expressed in human keratinocytes. Recombinant TACE did not cleave purified collagen XVII *in vitro*, probably because the substrate was not membrane-bound. To verify the role of TACE in collagen XVII processing, HaCaT cells were transfected with TACE cDNA and COS-7 cells were cotransfected with cDNAs for collagen XVII and TACE. A dose dependent increase of ectodomain shedding, with a concomitant decrease of full-length collagen XVII, was observed in both cases. The results support the conclusion that TACE participates in shedding of collagen XVII from the cell surface and thereby contributes to regulation of keratinocyte adhesion and migration in physiological and pathological processes.

# 044

**Topical Application of Retinaldehyde Increases the Expression of CD44 in Mouse Epidermis**

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CD44 is a widely distributed polymorphic transmembrane glycoprotein and is the principal cell surface receptor of hyaluronate (HA). The expression of CD44 in mouse epidermis is confined to the basal, spinous and granular layers. In a recent study we have shown that two major functions of CD44 in the mouse skin are the regulation of keratinocyte proliferation in response to extracellular stimuli and the maintenance of local HA homeostasis. In this study we examined the effect of topical retinaldehyde (RAL), on the expression of CD44 in mouse epidermis by immunohistochemistry. Immunostaining of vehicle-treated tail skin of C57BL/6 mice revealed the standard expression of CD44 in basal and suprabasal keratinocytes. Topical application of 0.05% RAL for 14 days resulted in an epidermal hyperplasia and a marked increase of CD44 expression in the follicular and interfollicular epidermis. Vehicle-treated back skin of SKH1 hairless mice showed a very faint staining of CD44 in the epidermis whereas the application of RAL for 7 days significantly increased the epidermal thickness and the CD44 expression in follicular and interfollicular keratinocytes. These findings provide evidence that RAL-elicited epidermal hyperplasia is associated with an increased expression of CD44 in mouse epidermis.

# 045

**Development of Multimeric Peptide Inhibitors of Hyaluronan-Mediated Leukocyte Trafficking**

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Hyaluronan (HA), a high molecular weight glycosaminoglycan, is expressed abundantly in the skin not only in the extracellular matrix, but also on the surfaces of keratinocytes and endothelial cells. HA is known to bind to many adhesion molecules (e.g. CD44), suggesting its potential role in leukocyte homing. Using phage display technology, we have recently developed a unique 12-mer peptide (GAHWQFNALTVR), termed Pep-1, that binds HA selectively and inhibits leukocyte adhesion to HA substrate (*J Exp Med* 192:769, 2000). *In vivo* administration of Pep-1, but not a 12-mer random peptide (RP) control, inhibited allergic contact hypersensitivity responses to DNFB at the sensitization phase by blocking hapten-induced Langerhans cell (LC) migration from epidermis, as well as at the elicitation phase by inhibiting skin-directed homing of pro-inflammatory leukocytes. These results provide the first experimental evidence of the concept that HA promotes two-way leukocyte trafficking to and from inflamed skin. The clinical utility of Pep-1 is limited in the current formulation by: (a) requirement of organic solvent (e.g. DMSO) for dissolution (b) relatively low stability of Pep-1 solutions, and (c) its moderate HA-binding affinity (Kd = 1.7 μM). In this study, we sought to overcome these limitations through chemical modification, as the first step toward clinical applications of the Pep-1 technology. We prepared a dimeric form by succinic acid-bridged conjugation of Pep-1 molecules at the a-amine termini, and a tetrameric form by conjugating Pep-1 to the branched PEG derivative, bis(Polyethylene bis[Imidazoyl Carbonyl]). Both dimeric and tetrameric forms showed markedly improved solubility and stability; both were soluble in PBS at pharmacological concentrations (0.5–1 mg per ml) and they remained chemically intact during 4 weeks storage periods. Moreover, ~2-fold (dimer) and 10-fold (tetramer) improvement over the original (monomeric) form was achieved in their capacities to block leukocyte adhesion to HA-coated plates, documenting enhanced functional affinities. Upon local administration, all three Pep-1 derivatives significantly inhibited DNFB-induced LC emigration from the epidermis, albeit with different efficiencies (tetramer dimer monomer). By contrast, no biological activities were observed for RP control even in multimeric forms. These results form the basis for clinical applications of dimeric and tetrameric Pep-1 formulations to the treatment of inflammatory skin disorders.

# 046

**The Armadillo Protein p0071 Localizes to Dermal Microvascular Endothelial Intercellular Junctions and Associates with VE-Cadherin and Desmoplakin**

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p0071 is a novel member of the armadillo gene family and exhibits structural homology to the desmosomal protein plakophilin-1 and the adherens junction protein 120. We found that p0071 localizes to dermal microvascular endothelial intercellular junctions. In addition, p0071 localizes to both desmosomes and adherens junctions in A431 cells. To test for interactions between p0071 and other intercellular junction proteins, a FLAG-tagged p0071 polypeptide was transiently expressed in COS cells with either VE-cadherin or desmoplakin. p0071 colocalized with VE-cadherin at intercellular junctions. p0071 also colocalized with full-length desmoplakin and with a desmoplakin amino-terminal polypeptide (DPNTP), but failed to colocalize with a desmoplakin polypeptide lacking the amino-terminal domain (DPdeltaN). Full-length desmoplakin, but not DPdeltaN, coimmunoprecipitated with p0071. Further analysis suggests that the central armadillo repeat domain of p0071 associates with VE-cadherin whereas the nonarmadillo head domain of p0071 associates with desmoplakin. Interestingly, expression of p0071 in COS cells induces a branching morphology similar to that of p120, suggesting that p0071, like p120, regulates actin cytoskeletal dynamics via Rho family GTPases. To further investigate p0071 dynamics, stable A431 cell lines expressing a GFP-tagged p0071 polypeptide were generated. The p0071.GFP fusion protein localized to cell-cell junctions in quiescent cells but reorganized to a cytoplasmic distribution when A431 monolayers were wounded. These data suggest that p0071 is a cadherin and desmoplakin-binding protein that is dynamically regulated during remodeling of intercellular junctions. Given the importance of other armadillo family proteins in junction assembly and tissue morphogenesis, these results suggest that p0071 may play an important role in keratinocyte and endothelial intercellular junction assembly.

# 047

**Direct Observation of E-Selectin Mediated Rolling on Cutaneous Lymphocyte-Associated Antigen Using a Novel Blot Rolling Assay**

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Adhesive interactions between leukocytes, platelets and endothelia are crucial to many normal physiologic processes as well as to pathologic inflammatory and thrombotic responses. Wall shear stress plays an important role in the regulation and promotion of these interactions. Human memory T cells found in inflamed skin differentially express the carbohydrate epitope Cutaneous Lymphocyte-associated Antigen (CLA) exclusively on P-Selectin Glycoprotein Ligand-1 (PSGL-1), a constitutively expressed surface glycoprotein. Although circumstantial and indirect evidence suggests that CLA serves as a skin homing receptor via interaction with E-selectin, there has been no direct evidence that CLA/PSGL-1 is, itself, a physiologic E-selectin ligand or whether other selectin ligands are coexpressed on CLA + human T cells. To assess these issues, we have developed a novel method for real-time, direct observation of adhesive interactions between cells in shear flow and ligands immobilized on standard Western blots rendered transparent for use in a parallel plate flow apparatus. This method allows for the rapid and reproducible assessment of both known and unknown adhesion molecules within a complex mixture without the need for prior isolation or enrichment of the constituent parts beyond standard SDS-PAGE. CHO cells transfected with human E-selectin or P-selectin tethered and rolled across the CLA/PSGL-1 band on blots of CLA-positive T cell lysate under physiologic wall shear stress conditions. In contrast, blots of CLA-negative T cell lysate supported significant binding of CHO-P cells only. Treatment of blots with function-blocking anti-PSGL-1 antibody (mAb $PL^{-1}$) abrogated CHO-P cell binding with no effect on CHO-E binding. Purified native CLA/PSGL-1, obtained by immunoprecipitation from CLA+ T cells, supported both E and P-selectin mediated rolling. These findings represent the first direct evidence that T cell CLA/PSGL-1 is a functional ligand for both E- and P-selectin under physiologic shear flow conditions, and they demonstrate the utility of a novel method for the rapid and reproducible characterization of individual cell adhesion molecules within a complex mixture. We feel this method will have broad application in the assessment of both known and unknown components mediating cell-cell and cell-matrix adhesion events.

# 048

**Down-Regulation of E-Selectin on Human Umbilical Vein Endothelial Cells (HUVEC) Requires a Di-Leucine Motif and an Upstream Serine and Occurs More Slowly on Human Dermal Microvascular EC (HDMEC)**

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We previously showed that HDMEC sustain surface E-selectin expression (surface protein half-life $t_{1/2} = 4.3$ h) while cultured TNF-activated HUVEC rapidly clear E-selectin protein from their surface ($t_{1/2} = 1.6$–$2.0$ h). After retroviral gene transfer, Flag-tagged wild-type (WT) or cytoplasmic domain mutations of E-selectin were constitutively expressed on the HUVEC surface without cytokine activation and clearance of E-selectin in the presence of the translational inhibitor cycloheximide was measured by FACS analysis. Across a range of initial surface expression levels clearance of WT E-selectin on HUVEC was rapid ($t_{1/2} = 2.1 \pm 00.13$ hours), similar to that on TNF-activated HUVEC. Replacement of the E-selectin cytoplasmic tail with that of ICAM-1, or deletion of 25 of the 32 most carboxy-terminal E-selectin cytoplasmic a.a. residues, reduced the clearance rate by greater than 10-fold relative to WT, suggesting that specific a.a. residues present within the E-selectin cytoplasmic tail mediate E-selectin clearance. Mutation of a putative di-leucine internalization motif (I588A, L589A) sufficed to delay E-selectin internalization ($t_{1/2} = 3.0 \pm 00.41$ hours) as did mutation of a single upstream serine (S581A; $t_{1/2} = 3.7 \pm 00.30$ hours). In contrast, mutation of two cytoplasmic a.a. residues constituting a putative tyrosine internalization motif (Y582A, P585A) did not measurably alter E-selectin clearance compared to WT. The functions of the $IL^{588, 589}$ di-leucine motif and of $S^{581}$ are not redundant because a construct with mutations at both locations cleared much more slowly ($t_{1/2} = 5.7 \pm 00.52$ hours) than did either of these two different single site mutant constructs. Compared with HUVEC, we observed slower clearance of WT E-selectin on transduced HDMEC ($t_{1/2} = 4.0$ h in two experiments) and more rapid WT E-selectin clearance on transduced HeLa cells ($t_{1/2} = 1.1$ and $1.5$ h in two experiments). In summary, rapid E-selectin clearance on HUVEC requires both an upstream serine and a di-leucine motif, suggesting that internalization of E-selectin on HUVEC may follow a pathway other than the established di-leucine route. The relatively slower rate of E-selectin clearance by HDMEC previously observed following cytokine activation persists even in the absence of cytokine activation, suggesting that this difference is constitutive and not due to a differential response to cytokine.

## 049

**Bone Morphogenic Protein-1 Inhibitors Block Human Squamous Cell Carcinoma Invasion *In Vitro***

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Squamous cell carcinoma (SCC) is the most common metastatic cancer in the skin and body. Laminin 5 is known to concentrate at the invading margins of SCC tumors, and expression of laminin 5 correlates with invasiveness and prognosis of SCC tumors. We have previously shown that laminin 5 processing by isoforms of the bone morphogenic protein 1 (BMP-1i) family of proteins is critical for the migration of keratinocytes. The purpose of this study was to determine whether inhibition of processing of laminin 5 by BMP-1i interferes with the invasion of human squamous cell carcinoma. To this end, we used a nontoxic and highly specific hydroxamic acid based inhibitor of BMP-1i (FG-1731) to inhibit processing of laminin 5 in two SCC cell lines, SCC 25 and SCC 15. Immunoblots confirm FG1731 inhibited processing of laminin 5 $\alpha$3 and $\gamma$2 chains in each SCC line. Addition of 10 $\mu$M FG-1731 to SCC cells inhibited migration of cultured SCC cells in scratch assays. The presence of 30 $\mu$M FG-1731 also resulted in a four-fold reduction in the invasion rate of SCC 25 and 15 cells through matrigel. These results suggest that BMP-1i processing of laminin 5 may be a crucial step in regulating SCC migration and invasion. In addition, these results suggest a role for BMP-1i inhibitors as nontoxic and specific anti-SCC chemotherapeutic agents. This will be further elucidated by *in vivo* studies.

## 050

**Melanoma Movement Factors Stimulate Dissociation of Focal Adhesions**

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Formation and dissolution of focal adhesions are important components of cell migration, and are controlled by receptor mediated signaling. Multiple growth factor receptors and integrin receptors have been shown to control migration in melanoma. We have demonstrated that the mitogens ET-1, bFGF and IGF-1 are powerful inducers of melanoma migration, acting through a signaling pathway including PLA2, lipoxygenase and Hydroxyeicosatetraenoic acids (HETE). We have developed new methods to study the effects of mediators in this pathway on the dynamics of focal adhesions in malignant melanoma cell lines. In our study, we have grown the radial growth phase melanoma cell line, WM35 on laminin-coated glass cover slips and viewed the effects that multiple growth factors have on cell adhesion using interference reflection microscopy (IRM). This technique allows direct visualization of focal adhesions and measurement of the density and subcellular localization of these structures. Addition of IGF-1, bFGF, ET-1, or 12-(S)-HETE to cultured WM35 cells produced significant detachment of the cells from their extracellular matrix within a 45 minute period when compared to the appropriate media controls. Along with detachment of focal adhesions, lamellapodia retraction was also observed. These results provide interesting evidence of the important role of growth factors in determining cellular adhesion, an integral process affecting the ability of cancer cells to migrate. The data also suggest a connection between melanoma movement factors and the generation of biochemical effectors such as HETE.

## 051

**Isolation of an Antigenic Mimic of an Epitope of MUC18 by a pVIII-28aa Phage Displayed Peptide Library**

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The cell surface adhesion molecule MUC18 is strongly expressed by advanced primary and metastatic melanoma. It was first described as an integral membrane glycoprotein of malignant melanoma and is also expressed by endothelial cells of various origins. To characterize epitopes that antigenically mimic MUC18 we screened the pVII-28aa phage peptide library against the murine monoclonal antibody muc18AD5D7 which recognizes a domain of MUC18 with high specificity. After three rounds of panning the phage pool was specifically enriched. The deduced amino acid sequence of the peptides showed no homology to the amino acid sequence of MUC18 indicating that this epitope was conformational. Phage expressed peptides were selected for further studies in ELISA and immunoblot experiments. One peptide ligand was recognized specifically by the respective antibody in ELISA and dot blot experiments. Interestingly this peptide ligand blocks in a dose dependent manner the interaction of muc18AD5D7 to natural MUC18 of Mel-JuSo melanoma cell line lysate and recombinant MUC18 in ELISA and immunoblot experiments. In addition no inhibition was observed using a panel of other antimuc18 antibodies. Our results clearly indicate that this phage displayed peptide is an artificial equivalent of the muc18AD5D7 epitope of MUC18 and might be used as a substitute for further *in vitro* and *in vivo* studies of MUC18 mediated cell–cell interaction.

## 052

**Shear Stress Conditioning of *In Vitro* Cultured Cells: Implications for Tissue Engineering**

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Tissue engineering of skin and other organ systems is rapidly evolving. Many of the key components of specific tissues such as epidermal and dermal constructs are now available for clinical application. The integration of different cell types into more complete tissue engineering constructs is currently being addressed in hopes of developing viable, readily implantable, vascularized organ substitutes. Our laboratory investigated the effects of short-term shearing forces on cells cultured in a static *in vitro* environment. Confluent layers of bovine aortic endothelial cells and bovine aortic smooth muscle cells were exposed to 25 dynes per $cm^2$ of shear stress using a centrifuge adhesion assay for periods of time ranging from 5 s to 1 h. The results of this analysis indicate that both smooth muscle and endothelial cell layers undergo an initial increase in cell loss that approaches 30% of the cell monolayer after 30 min of shear stress exposure. However, after 40 min of shear stress exposure, the cell layers appear to become more resistant to shearing forces with a significant reduction in cell loss to less than 5% of the cell monolayer. Cell layers exposed to the centrifuge adhesion assay were also examined by epi-fluorescent microscopy. Phalloidin labeled cell monolayers demonstrated actin cytoskeletal rearrangement related to the length of exposure to shear stress. The transition of actin filaments from the peripheral border of statically cultured cells to filamentous extensions across the cell body directly coincided with changes in adhesive capabilities of the cells. These studies indicate that shear stress conditioning may be a critical component of tissue engineering constructs both in vascular systems as well as skin constructs where shear stress and strain play a key role in the functional capability of the tissue.

## 053

**Specific Furans From Avocado: A New Tool in Dermatology**

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Based on our know-how in the fields of lipidic extracts, we have discovered original and specific compounds from avocado, namely avocadofurans. The aim of this work is to present these molecules, and especially their biologicals activities on skin metabolism. Avocado furans are obtained from virgin avocado oil. The drying of the entire fruit is an important step in the process which allows the selective formation of these molecules which are then concentrated in a two steps molecular distillation process. Studies were first performed on normal human skin fibroblasts. Avocadofurans (20 $\mu$g per ml) were added directly in cell culture medium. After 24 h incubation, TGF$\beta$-1 production was measured in cell culture medium using a specific ELISA kit assay. In our experimental conditions, avocadofurans were able to significantly induce TGF$\beta$-1 synthesis compared to non treated cells. This increase in TGF$\beta$-1 has been related to an increase in collagen synthesis as demonstrated by the collagenase assay. This results was confirmed using antibodies against TGF$\beta$. In a second set of experiments, we have checked the effect of avocadofuranes on epidermal lipids synthesis using human skin explants. We have demonstrated that these molecules were able to stimulate specificaly the synthesis of cholesterol and ceramides 1 and 2, without any modification of the total lipid content of the epidermis. Avocadofuranes are long chain furanics lipids whitout any equivalent in the field of natural compounds which are able to stimulate skin metabolism at the dermal level through TGF-$\beta$ dependent pathway and at the epidermal level. In conclusion, our study suggest interesting application of avocadofuranes especially for the development of a new generation of topical products intended for the prevention and/or the treatment of skin aging.

## 054

**The Cell Sorted Skin Equivalent as a Model for Evaluating Effects of Telomerase on Skin Aging and Wound Repair**

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Human skin can be accurately modeled by use of a Cell Sorted Skin Equivalent (CeSSE) that relies on the inherent cell adhesive properties of keratinocytes and dermal fibroblasts to sort-out into distinct layers from a mixed cell slurry when transplanted onto SCID mice. We used this method to evaluate the effects on skin morphology of expression of the cloned telomerase catalytic subunit (hTert), which extends the lifespan of human fibroblasts and other primary cells. Since replicative senescence is accompanied by substantial alterations in gene expression, we previously evaluated characteristics of *in vitro*-aged dermal fibroblast populations before and after immortalization with telomerase, and determined biological behavior in the CeSSE. We observed increased fragility and subepidermal blistering with increased passage number of dermal fibroblasts, but the expression in late passage populations restored the normal nonblistering phenotype, correlating with restored collagen I and III levels. Overall, expression of telomerase results in mRNA expression patterns that were substantially similar to early passage cells. Thus, telomerase activity may also prevent or reverse the loss of biological function seen in senescent cell populations. We are extending our results to evaluate the introduction of telomerase. So far we find that the CeSSE does form using HPV E6-E7 immortalized keratinocytes. We will present our results of CeSSE created using keratinocytes immortalized by hTERT. The use of CeSSE grafts for drug efficacy and toxicity testing, disease modeling, and tissue transplantation may be further enhanced by the successful use of telomerase immortalized keratinocytes in the CeSSE.

# 055

**Multiple Fibroblast Subpopulations in the Papillary Dermis**

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Studies have shown that fibroblasts derived from the papillary dermis differ in several respects from fibroblasts in the corresponding reticular dermis, an indication that skin contains physiologically distinct subpopulations of these cells. In the present study, primary and first passage fibroblasts obtained by enzymatic and mechanical dissociation of the papillary dermis were cloned by limiting dilution. Fibroblast subsets were selected from cloned cells on the basis of differences in morphology and growth kinetics. All cell strains selected for further study were positive for vimentin and cell surface markers known to be present on dermal fibroblasts. These clones were stable, retaining their essential characteristics over 10 or more passages. These characteristics, in addition to growth kinetics and morphology, include differential abilities to contract type I collagen gels, differential abilites to produce and organize a fibrillar extracellular matrix, differential expression of α-smooth muscle actin, and a differential response to and production of specific growth factors and cytokines. These studies indicate that stable, heterogeneous subpopulations of dermal fibrobasts can be obtained from the papillary dermis. Some cellular subsets may have specific roles in communicating with and regulating keratinocte physiology, while other subsets may interact with other populations of cells in the dermis or serve to provide the connective tissue framework of the dermis. Identification and subsequent use of specific fibroblast subsets could permit the fabrication of new generations of skin equivalents. These studies also suggest that different fibroblasts subsets can contribute differentially to the phenotypic characteristics of normal and pathologic skin.

# 056

**The Essential Role of Pantophysin in Early Development of the Skin**

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The physin family consists of integral membrane proteins of cytoplasmic microvesicles. We showed that pantophysin is widely distributed in various cells in microvesicles and expressed ubiquitously in all cells of human and murine skin including fibroblasts. Because of the localization in constitutive transport vesicles and the expression in fibroblasts there is evidence that pantophysin is involved into transport of extracellular matrix proteins. To learn more about the importance of pantophysin and its functon *in vivo*, we generated a targeting construct to inactivate the pantophysin gene in a knock-out mouse. Investigations were performed by RT-PCR, Northern blot analysis, subcloning of DNA fragments, establishment of gene-deficient embryonal stem-cells, generation of transgenic mice, immunofluorescence and immunoelectron microscopy using polyclonal antibodies against human and murine pantophysin. Pantophysin deficiency exhibits considerable defects both *in vivo* and *in vitro*: comparing the effect of transplantation of wild type and pantophysin deficient embryonal stem cells into syngeneic nude mice, the latter show a reduced teratoma formation. There is no germ line transmission. Pantophysin deficient embryos die at periimplantation. Pantophysin deficient embryoid bodies are characterized by the lack of the outer primitive endoderm and the absence of the early basement membrane (Reichert's membrane) and its molecular components. It makes sense that pantophysin deficiency affects primitive endoderm function, because endoderm cells fulfill important secretory tasks by mass production of extracellular matrix components that are laid down as Reichert's membrane. Furthermore, other functions of endoderm cells such as migration and transport of metabolites might also rely on pantophysin dependent vesicular traffic. In conclusion, these data suggest a possible link between vesicle-mediated transport processes and survival of specific cell populations during early development. Ongoing investigations will elucidate pathobiological functions of pantophysin in human skin diseases.

# 057

**Dermal Remodeling and Nerve Regeneration in a Reconstructed Skin Transplanted on Nude Mice**

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Wound healing of deep and extensive burns can induce hypertrophic scar formation, which is a detrimental outcome for skin functionality, and a major hindrance to nerve regeneration. Innervation deficiency of skin constitutes a significant handicap to rehabilitation of burn patients. Our aim was to produce a reconstructed skin model, which could favor cutaneous nerve regeneration. We transplanted on the back of nude mice a reconstructed skin made of a collagen sponge seeded with human fibroblasts and keratinocytes and grown *in vitro* for 20 days. After 40–90 days, the reconstructed skin extracellular matrix was mostly made of collagen fibril bundles arranged in a basket weave pattern, like in normal dermis. Nerve regeneration was assessed by immunohistochemical staining of calcitonin gene-related peptide, nerve-cell adhesion molecule, neuropeptide-Y and protein S-100 (specific for Schwann cells). All markers, except neuropeptide-Y, demonstrated an axonal migration occurred in the reconstructed skin 90 days after graft. This result was confirmed by observation of nerve fibres in the graft by transmission electron microscopy. These results suggest that by promoting the remodeling of a well-organized dermal extracellular matrix, our reconstructed skin model should reduce scar formation when transplanted on deep wounds, while favoring cutaneous nerve regeneration.

# 058

**UV Irradiation Inhibits Procollagen Synthesis by Down-Regulating the TGF-β Type-II Receptor in Human Skin Fibroblasts**

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Reduced synthesis of procollagen I (COL-I) and III (COL-III) contributes substantially to impaired dermal function in photoaged human skin. Transforming growth factor-β (TGF-β)/Smad pathway, directly and indirectly through induction of connective tissue growth factor (CTGF), regulates COL-I and COL-III synthesis. Smad2, 3, and 4 are transcription factors, which are activated by TGF-β receptors, that mediate TGF-β-induced gene expression. We have investigated the effects of UV irradiation on TGF-β/Smad-mediated production of COL-I in cultured human skin fibroblasts and in human skin *in vivo*. UV irradiation (30 mJ per $cm^2$, UVB source) prior to treatment with TGF-β inhibited TGF-β-induced COL-I production in human skin fibroblasts. UV irradiation of fibroblasts expressing a Smad-regulated/luciferase reporter gene inhibited TGF-β-induced luciferase activity by 80% ($n = 3$, $p = 0.05$), indicating that UV impairs TGF-β-regulated Smad-dependent gene expression. UV irradiation of fibroblasts prior to addition of TGF-β also blocked TGF-β-induced nuclear translocation of Smad2 and 3, and formation of Smad2, 3, 4/DNA complexes ($n = 3$). UV also reduced TGF-β binding to TGF-β receptors by 60% at 8 h post UV ($n = 4$). This reduced receptor expression resulted from transient inhibition by UV of TGF-β type II receptor mRNA and protein expression ($n = 3$). Consistent with these findings, UV inhibited transcription of the TGF-β type II receptor promoter by 50% ($n = 3$). In contrast, TGF-β type I receptor mRNA and protein expression were unaffected by UV. In human skin *in vivo*, UV irradiation inhibited (60%) TGF-β type II, but not type I, receptor mRNA expression as determined by Northern analysis and *in situ* hybridization ($n = 3$, $p = 0.05$). Taken together, these results indicate that UV irradiation impairs TGF-β responsiveness by down-regulating TGF-β type II receptors, thereby reducing expression of TGF-β-regulated CTGF, COL-I, and COL-III genes in human skin fibroblasts.

# 059

**Expression of TGF β1, Collagens and Collagen-Binding Heat Shock Protein 47 in Cicatricial Pemphigoid: a Possible Role in Dermal Fibrosis**

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Cicatricial pemphigoid (CP) is a mucocutaneous disease characterized by scarring. Transforming growth factor β1 (TGFβ1) and heat shock protein 47 (HSP47) plays an important role in fibrogenesis in various tissues and organs. In the present study, we examined the role of TGFβ1 and HSP47 in dermal fibrosis in cutaneous lesions of CP. Dermal fibroblasts isolated from normal individual and patients with CP were studied for the expression of TGFβ1, HSP47, type I and III collagens both at mRNA and protein level by RT-PCR and immuno-staining. Skin biopsies from patients with CP, and normal subjects were studied for the expression of collagens by immunohistochemistry. Compared to the control skin sections, an increased deposition of collagen was seen in the skin section of CP patients. Fibroblasts isolated from skin of CP patients grown *in vitro* exhibited increased expression of HSP47, type I and type III collagens at the mRNA level, when compared with fibroblasts grown from normal skin. Weak expression of TGF-β1 was observed in the control dermal fibroblasts using RT-PCR. Markedly increased expression of TGF-β1 was noted in the dermal fibroblasts isolated from skin of CP patients. This study indicates that the up-regulation of TGFβ1 and HSP47 is associated with increased expression and deposition of collagens in skin of CP patients. There appears to be a sequential role for TGFβ1, HSP47 and collagens in the scarring process in the skin of CP patients.

# 060

**Dermal Fibroblasts Alone are Capable of Producing Type VII (Anchoring Fibril) Collagen at the Dermal-Epidermal Junction**

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We and others have shown that human keratinocytes and fibroblasts are capable of synthesizing and secreting type VII collagen, the main component of anchoring fibrils within the dermal-epidermal junction (DEJ) of human skin. Cells secrete type VII collagen α chains into the extracellular space where they are transported to the DEJ and processed into anchoring fibrils. Although both cell types can secrete type VII collagen, it is thought that keratinocytes account for type VII collagen at DEJ. We asked the question of whether or not type VII collagen secreted solely by dermal fibroblasts could be transported to the DEJ to form a linear basement membrane zone (BMZ) structure. To answer this question, we established organotypic, skin equivalent cultures composed of keratinocytes from patients with recessive dystrophic epidermolysis bullosa (RDEB), cells which can not produce type VII collagen because of a defect in the gene encoding for type VII collagen α chains, and normal dermal fibroblasts. In these cultures, all type VII collagen is derived from fibroblasts. After the cultures were composed and maintained for 6 weeks, they were subjected to routine histology and immunohistochemistry using an affinity purified polyclonal antibody to the NC1 domain of type VII collagen or normal control IgG. Histology demonstrated organotypic cultures with keratinocytes juxtaposed to a delicate "neodermis" matrix embedded with fibroblasts. Surprisingly, immuno-labelling the cultures with the antitype VII collagen antibody revealed tight linear staining at the DEJ between the RDEB keratinocytes and "neodermis" created by the dermal fibroblasts. These experiments show that human dermal fibroblasts alone are capable of synthesizing and forming a linear BMZ-like structure rich in type VII collagen. Since fibroblasts can be passaged 30–60 times *in vitro* and are easy to grow in larger numbers, this finding opens the possibility that fibroblasts from RDEB patients could be genetically modified to produce a type VII collagen DEJ structure.

## 061

**Targeted Disruption of Dermatopontin Causes Abnormal Collagen Fibrillogenesis in Skin**

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Dermatopontin (DPT) is a 22-kDa extracellular matrix protein (ECM), which widely distributes, including skin, skeletal muscle, bone, cartilage, and other tissues. DPT has been shown to interact with decorin and have cell adhesion activity, which is mediated by cell surface integrin receptor. Moreover, DPT binds to type 1 collagen and accelerates collagen fibril formation *in vitro*. To understand functions of DPT *in vivo*, we generated the DPT-null mice by gene targeting. DPT-null mice were born alive, grew to normal size and were fertile. DPT-null mice showed Ehlers–Danlos syndrome-like skin laxity and fragility, although these mice did not reveal obvious histological abnormalities in the skin. In electronmicroscopic study, the diameter of collagen fibrils in skin from DPT-null mice is remarkably greater than that of littermate conrol and individual fibrils in DPT-null mice show irregular contours. In addition, the absence of dermatopontin leads to skin fragility confirmed by tensile strength test. The values of tensile strength measurements of the skin are significantly reduced in DPT-null mice as compared with normal littermates. These results indicate that dermatopontin plays an important role in collagen fibrillogenesis *in vivo*.

## 062

**Molecular Mechanisms of Connective Tissue Alterations in Premature Skin Aging Induced by Environmental Insults: Ultraviolet Exposure and Tobacco Smoking**

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Skin aging following repeated exposures to ultraviolet (UV) irradiation and tobacco smoking results largely from the damage to cutaneous connective tissue, which is composed of collagen, elastin, and proteoglycans. Here we have further studied that the alterations of cellular component and the extracellular matrix of dermal connective tissue after exposure to UVA and tobacco smoke extracts. Expression of type I and type III of procollagen were substantially decreased and newly collagen biosynthesis was significantly reduced by 59% of the skin fibroblasts treated with 25 ml per ml tobacco smoke extracts. Fibroblasts from both UVA and tobacco smoke extracts exhibited higher levels of matrix metalloproteinase-1 (MMP-1) than those from the respective control both at the mRNA and protein level using PT-PCR and ELISA methods. Also MMP-3 and MMP-13 mRNA expression were significantly elevated dose dependently upon the both stimulations, whereas tissue inhibitors of metalloproteinase (TIMP)-1 and –3 mRNA expression remained unaltered in the skin fibroblasts. In addition, mRNA expression of tropoelastin was increased in these fibroblasts. Versican, the large chondroitin sulfate (CS) proteoglycan, has been identified in the dermis in association with elastic fibers, and decorin, small CS proteoglycan, has been shown to codistribute with collagen fibers. Versican protein levels were found to be decreased approximately two fold in cultures medium, and versican V0 mRNA levels were significantly decreased in the same cells. Dermatan sulfate prteoglycan of 45 kDa core protein levels were elevated approximately five-fold (Western blotting), whereas mRNA expression of decorin D1 was decreased as compared with sham treated cells. The deficient synthesis of collagen and elevated tropoelastin expression as well as abnormal proteoglycan assembly might thus contribute to an altered formation of the extracellular matrix resulting in the connective degradation observed in aged appearance. Moreover, treatments of antioxidants abrogated the induction of MMP-1. Taken together, our findings indicated that the skin premature aging induced by UV and tobacco smoke extracts share molecular features including abnormal regulation of extracellular matrix deposition through elevated MMPs, reduced collagen production and abnormal proteoglycan accumulation via ROS generation.

## 063

**Mice Deficient in Lysyl Oxidase-1 have a Lethal Developmental Phenotype, whereas Mice Deficient in Lysyl Oxidase-2 Develop Normally, but Manifest an Abnormal Phenotype with Increasing Age**

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Lysyl oxidases are extracellular matrix enzymes, which are crucial to the development of collagen, and elastin cross-links. Lysyl oxidases catalyze the conversion of certain lysine or hydroxylysine residues in collagen, or lysine residues in elastin, to aldehydes. These aldehyde residues then participate in covalent cross-linking. Lysyl oxidases are a gene family with at least five members. Two lysyl oxidases genes, lysyl oxidase-1 (LO-1) and lysyl oxidase-2 (LO-2), are known to be differentially expressed. To study to roles of lysyl oxidase-1 vs. –2 during matrix development and maintenance, mice with loss of function mutations of either LO-1 or LO-2 were generated by homologous recombination. Mice deficient in LO-1 die at parturition secondary to diaphragmatic rupture or aortic aneurisms. Aortic and lung desmosine is reduced 50% in mLO-1 –/– animals when compared to heterozygous or wild type animals. Conversely, mice deficient in LO-2 develop normally and are phenotypically normal at birth, but manifest abnormalities with increasing age. LO-2–/– mice, 6 months old or more, demonstrate enlarged pulmonary airways and airspaces, and a lymphocytic pulmonary infiltrate. These disparate phenotypes reveal significant novel information into the biology of connective tissue and lysyl oxidases.

## 064

**Hypoxia Increases Type I Procollagen Transcription Through TGF-β1 Dependent and Independent Mechanisms**

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Low oxygen tension has been shown to increase growth and clonal expansion of fibroblasts. We have found that exposure (hours to days) of fibroblast cultures to hypoxia (2% $O_2$) compared to standard (20% $O_2$) conditions enhances TGF-β1 synthesis and transcription (–453 to +11 bp CAT-promoter construct) and leads to TGF-β1 dependent increases in collagen synthesis and mRNA levels of α1(I) procollagen (COL1A1). The transcriptional activity of COL1A1 in hypoxic human dermal fibroblasts is up-regulated in promoter-luciferase constructs having 5′ endpoints between –804 bp and –174 bp (but not –72 bp), with greater enhancement (up to 40% more) proximal to –190 bp, once a repressive control element (cKrox; –224 to –199 bp) is removed. Antibodies to TGF-β1 block the COL1A1 transcriptional increase in hypoxia by more than 20%, pointing to the existence of additional stimulatory mechanisms which are independent of TGF-β1. Hypoxia also increases by more than three-fold the transcriptional activation of two active COL1A2 CAT-promoter constructs (–353 and –772 bp), suggesting that a program of more general collagen gene activation, on different chromosomes, is operative in hypoxia. These findings show that low oxygen tension enhances collagen synthesis and transcriptional activation of collagen genes. TGF-β1 dependent and independent mechanisms are both involved, and this needs to be considered in our understanding of fibrosis.

## 065

**Inhibition of Matrix Metalloproteinase Activities by Scavenol**

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Scavenol is a proprietary mix of antioxidant and anti-inflammatory materials designed to exert antiaging effects on the skin. To substantiate Scavenol's benefit to matrix integrity, we have investigated its effects on several matrix metalloproteinase activities. Our target enzymes were MMP-1 (interstitial collagenase), MMP-2 (72 kDa gelatinase-A), MMP-3 (stromelysin-1), MMP-8 (neutrophil collagenase), and MMP-9 (92 kDa gelatinase-B). Activities were measured via digestion of fluorogenic peptide substrates, zymography using gelatin or casein substrates, and digestion of native substrate detected with SDS-PAGE. Flurogenic peptides were Dnp-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Nma)-$NH_2$ for collagenase and gelatinase detection, and NBD-Arg-Pro-Lys-Pro-Leu-Ala-Nva-Trp-Lys(DMC)$NH_2$ for stromelysin detection. Using these substrates, 0.1% Scavenol inhibited all of the targeted MMP activities by more than 85%. This efficacy was confirmed by gelatin zymography (MMP-2 and MMP-9), casein zymography (MMP-1 and MMP-3), and by digestion of purified human collagen type I with SDS-PAGE detection (MMP-1 and MMP-8). Although Scavenol contains metal chelating compounds such as mannitol and EDTA, its anti-MMP activities are not accounted for by equivalent concentrations of these components. Hence, Scavenol is capable of inhibiting the MMPs believed to be responsible for the major structural alterations of dermal matrix that are related to an aged appearance of the skin.

## 066

**The Effect of Cyanate on Type I Collagen Expression by Dermal Fibroblasts in Culture**

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Fibrosis is a common response to various insults or injuries and can be the outcome of any perturbation in the cellular function of any tissue. Peritoneum is always exposed to the waste products during peritoneal dialysis. In aqueous solution there is partial and spontaneous decomposition of urea to ammonia, carbonate and cyanate. Cyanate reacts irreversibly with the N-terminal groups of amino acid, peptides and many proteins by a process known as carbamylation and may contribute to peritoneal injury with fibrosis. In this study, we investigated the fibrogenic effect of cyanate on dermal extracellular matrix components. Fibrosis is characterized by extracellular matrix deposition, of which collagen type I is the major constituent. The level of type I collagen gene expression in cultured dermal fibroblasts was increased when incubated with cyanate (0.1–10 μM). At pretranscriptional level, the activity of type I collagen gene promoter was also increased. Transforming growth factor-β1 (TGF-β1) is a well-known fibrogenic cytokine produced by many types of cells including dermal fibroblasts. To investigate whether this fibrogenic cytokine is involved in increment of collagen gene expression by cyanate, TGF-β1 expression was evaluated by immunihistochemical study. The TGF-β1 was highly expressed in cultured fibroblasts by cyanate. These present study suggest that cyanate can be used as an one of the therapeutic regimens induce artificial fibrosis in the some pathological condition such as pitting scar of acne or striae distense, etc.

# 067

**Maturation in Culture of an *In Vitro* Produced Human Dermal Matrix**

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We had previously described the development of a specific culture method that allowed neonatal human dermal fibroblasts to produce a well-defined and organized extracellular matrix (ECM) *in vitro* without the addition of an exogenous scaffold, under serum-free conditions. Culturing of human dermal fibroblasts under these conditions resulted in the production of a dense predominantly collagenous extracellular matrix at 21 days of culture that showed considerable similarity to the human dermis. We now extend the culture time of the human dermal fibroblasts to 63 days and report a detailed characterization of the constructs obtained. The construct thickness progressively increased over time resulting from an orderly and controlled deposition of collagen, proteoglycans, glycosaminoglycans and other matrix molecules. Maturation of the dermal ECM produced in culture was further demonstrated by scanning electron microscopy (SEM). At day 49 and beyond thick collagen fiber bundles were observed. A significant increase in collagen fibril diameter was observed at day 63 by transmission electron microscopy (TEM), similar to that observed in human skin. Combined together, these data support the production of an organized extracellular matrix that matures in culture over time.

# 068

**Collagen Stimulation by Peony Root Extract on Fibroblasts**

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During aging the skin undergoes a loss of density mainly due to synthesis collagen decrease often associated with metalloproteinase (MMP) activation. Mature skin shows an impaired hormonal balance due to oestrogen deficiency which amplifies these phenomena. Peony root extract (Paeonia lactiflora) titrated in paeoniflorin, a monoterpene glycoside with a cage-like pinane skeleton, was tested on human skin fibroblasts for collagen synthesis and metalloproteinase inhibition. Fibroblasts from mammary skin or face lifts were grown in medium 199 with Earle's salts, containing 2.5% foetal calf serum, without phenol red, in a 96 well microplate. 24 h later, cells were treated with peony extract at 12.5 and 25 μg per ml in a new medium without foetal calf serum. After a period of 72 h, secreted type I and type III collagen were determined by the ELISA method. MMP-1 protein was also determined by ELISA (Biotrak, Pharmacia). Type I and type III collagen production was, respectively, significantly increased by +31%, +40% and +34%, +58%, compared to an increase of +27% and +14% for β estradiol at $10^{-7}$ M. Peony root extract also inhibits MMP-1 protein, respectively, by –20% and –25% at the tested concentrations. These biological activities suggest that such specific Paeonia lactiflora extract can be used to reinforce the dermis density in mature skin.

# 069

**Modulation of Skin Collagen Metabolism in Aged and Photoaged Human Skin** *In Vivo*

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To the best of our knowledge no study has been conducted to date to directly compare the collagen metabolism of photoaged and naturally aged human skin. In this study, we compared the collagen synthesis, MMP-1 levels and gelatinase activity of the sun-exposed and sun-protected skin of both young and old subjects. Using Northern blot analysis, immunohistochemical stain and Western blot analysis, we demonstrate that the levels of procollagen type I mRNA and protein in photoaged and naturally aged human skin *in vivo* were significantly lower than those of young skin. Furthermore, we demonstrated, by Northern blot analysis, that the procollagen α1(I) mRNA expression of photoaged skin was much greater than that of sun-protected skin in the same individual. *In situ* hybridization and immunohistochemical stain were used to show that the expression of type I procollagen mRNA and protein in the fibroblasts of photoaged skin was greater than those of naturally aged skin. In addition, it was found, by Western blot analysis using protein extracted from the dermal tissues, that the level of procollagen type I protein in photoaged skin was lower than that of naturally aged skin. The level of MMP-1 protein and the activity of MMP-2 were higher in the dermis of photoaged skin than in naturally aged skin. Our results suggest that the natural aging process decreases collagen synthesis and increases the expression of MMPs, while photoaging results in an increase of collagen synthesis and greater MMPs expression in human skin *in vivo*. Thus, the balance between collagen synthesis and degradation leading to collagen deficiency is different in photoaged and naturally aged skin.

# 070

**Effect of Pentapeptides on the Regulation of Matrix Metalloproteinases Pathway**

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Repeated exposure to solar radiation damage human skin leading to photoaging (PA). Evidences suggest that PA results largely from UV induction of matrix metalloproteinases (MMPs) which degrade extracellular matrix components within the dermis. Thus, the development of new potent MMPs inhibitors is of interest in regard to the prevention of PA. In this work, we demonstrate the effect of White Lupin peptides on the regulation of MMPs pathway. Low molecular weight purified pentapeptides are obtained from lipid free seeds of White Lupin using a biotechnological process which eliminates the polysaccharides potentially involved in the secondary reactions of glycation. Using the Enz/ChekÓ Gelatinase/Collagenase kit from Prolabo, we demonstrate that these pure peptides have a significant inhibitory effect on MMP activity. This effect is both time and dose dependent. We also check MMP production in UVA-irradiated human skin fibroblasts. 48 h postirradiation, lupin peptides (concentrations range from 0.5 to 2%) almost completely inhibit UVA-induced-MMP production (both MMP-1, –3, and –9), as measured in cell culture medium. MMP are zinc dependent enzymes. Thus, we verify if lupin peptides are zinc chelators. As measured *in vitro*, lupin peptides do not chelate zinc, they rather act as steric inhibitor of these enzymes. In conclusion, these data suggest interesting applications of these specific pentapeptides in the development of a new generation of topical products intended for the prevention of PA.

# 071

**Ultraviolet Irradiation Inhibits Expression of Connective Tissue Growth Factor (CTGF) in Human Skin *In Vivo* and in Human Skin Fibroblasts**

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Evidence suggests that connective tissue growth factor (CTGF), which is induced by TGF-β, is the major mediator of the induction of type I procollagen synthesis by TGF-β in human skin. We have investigated CTGF gene expression and its regulation by UV in human skin *in vivo* and in cultured human skin fibroblasts. In nonirradiated human skin, CTGF mRNA was constitutively, highly expressed as determined by real-time PCR. CTGF was expressed in keratinocytes throughout the epidermis and in dermal cells, as detected by *in situ* hybridization (n = 6). UV irradiation (2MED, UVB source) inhibited CTGF mRNA levels in human skin *in vivo*. CTGF gene expression was reduced (30%) within 4 h post UV, and remained reduced (50%) 8–24 h post UV (n = 9). UV caused reduced CTGF mRNA expression throughout the epidermis and dermis. UV irradiation of cultured human skin fibroblasts also resulted in a time dependent inhibition of CTGF mRNA expression. At 24 h post UV, CTGF mRNA expression was reduced 80% (n = 3). Cultured human skin fibroblasts constitutively expressed CTGF mRNA, which was further enhanced by addition of TGF-β (10-fold by 24 h, n = 3). UV irradiation of fibroblasts prior to addition of TGF-β1 reduced TGF-β1-induced CTGF mRNA expression by 80% (n = 3). Electrophoretic mobility shift assays, using oligonucleotides containing the TGF-β response element in the CTGF gene promoter as probe, demonstrated that UV irradiation inhibited formation of DNA/protein complexes. These data demonstrate that CTGF is constitutively expressed in human skin *in vivo*, and support the hypothesis that reduced CTGF expression mediates UV reduction of type I procollagen expression.

# 072

**The Cell Cycle Profile of Epidermal Stem Cells is an Intrinsic Property and Independent of the Proliferative State of the Tissue that they Maintain**

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The epidermis is a continuously renewing tissue, maintained by small undifferentiated stem cells (SC). It has always been assumed that stem cells are held in G0, but no experimental evidence for these assumptions existed until now. Recently, we developed a new sorting method that allows us to separate the two cell populations. We determined the cell cycle profiles of murine SC and TA (transient amplifying cells) obtained from neonate back skin, adult ear and adult footpad skin. As expected, we found that both SC and TA are more proliferative in the neonate than in the adult epidermis. We also determined that TA cells from footpad proliferate twice as much as those from the ear. More interestingly, the cell cycle profile of the adult somatic SC from these two anatomic sites was similar with nearly all the cells held in G0/G1. 1% of SC compared to 10% of TA cells were incorporating BrdU after 1 h pulse. Taken together, these results strongly suggest that (1) stem cells are cycling and (2) the cell cycle profile is an intrinsic property of SC and independent of the proliferative state of the tissue that they maintain. The mechanism of how this is controlled remains to be elucidated. Supported by NIH-NIAMS-AR45259.

# 073

**Epidermal Stem Cells do not Communicate Through Gap Junctions**

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Stem cells maintain homeostasis of self-renewing tissues. In epidermis, enrichment of stem cells has been achieved using cell surface markers. Additional surface markers that could distinguish stem from transient amplifying cells is needed for obtaining pure stem cell population. The efficient isolation of pure populations of epidermal stem cells should enhance future research on the regulatory processes involved in self-renewal, and facilitate keratinocyte gene therapy. Furthermore, the availability of a pure stem cell population will enable investigation of potential keratinocyte fate change In the bulge region of human hair follicle, we identified cells with no detectable levels of gap junction protein connexin 43 (Cx43). Using flow cytometry analysis we showed that isolated human neonatal foreskin keratinocytes that lack the expression of Cx43 represent a minor subpopulation (about 10%) of basal cells. These cells were uniformly small and low in granularity. In mouse tissue most of the label retaining cells (presumptive stem cells) did not express Cx43. Microinjection studies using fluorescent low molecular weight dyes confirmed restricted gap junction communication of stem cells. These results suggest that keratinocyte stem cells can be identified *in situ* and isolated based on Cx43 expression. Furthermore, our data reveal an important feature of keratinocyte stem cells, i.e. their lack of communication through gap junctions.

# 074

**Stem Keratinocytes are Protected From Cell Death Via an Integrin Signaling Pathway, in a bcl-2-Dependent Manner**

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Keratinocytes, which rapidly adhere to the extracellular matrix (ECM), expressing highest levels of β1 integrin are considered to be stem cells. Because disruption of cell–matrix interaction induces apoptosis in other cell systems, we investigated whether a subpopulation of keratinocytes, enriched in stem cells, could be protected from cell death. To this purpose, we isolated three populations of basal keratinocytes: a population of keratinocytes that adhered to type IV collagen within 5 min (population 1), the second population that adhered overnight (population 2), and a population that did not adhere to the ECM (population 3). Population 1 expressed the highest levels of β1 integrin, the lowest levels of involucrin, and showed the greatest CFE. Adhesion to type IV collagen was blocked by preincubating keratinocytes with anti-β1 integrin. The percentage of apoptotic cells, as measured by TUNEL technique, was significantly higher in population 2 ($9.5 \pm 3.5\%$) than in population 1 ($2.1 \pm 1\%$), and was highest in population 3 ($30 \pm 7.5\%$, $p < 0.05$). The addition of anti-β1 integrin to both population 1 and 2 caused a significant increase in apoptotic cells, as compared to controls ($21 \pm 4\%$ and 17.5% vs. $1 \pm 0.5\%$ and $7.5 \pm 3.5\%$, respectively, $p < 0.01$). Bax and Bad protein expression was high in population 3, decreased in population 2, and was very low or nearly absent in population 1. On the other hand, Bcl-2, Bcl-xL and Mcl1 levels were markedly higher in population 1 than in population 2, and were absent in population 3. Caspase 8 and Bid were processed in population 2 and 3, but not in population 1. The cleaved fragment of hILP/XIAP was higher in population 3 than in population 2, and absent in population 1. The addition of anti-β1 integrin failed to induce apoptosis in HaCat cells overexpressing Bcl-2 ($30 \pm 4.2\%$ in mock, $10 \pm 3.2\%$ TUNEL positive cells in Bcl-2 transfectants). These results indicate that a subpopulation of keratinocytes enriched in stem cells are protected from apoptosis via a pathway mediated by β1 integrin, that appears to be Bcl-2 dependent.

# 075

**Murine Epidermal Label-Retaining Cells Isolated by Flow Cytometry Do Not Express CD34, Sca-1 or Flk-1**

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Keratinocyte stem cells are present in the murine epidermis, based on both *in vitro* and *in vivo* evidence, and better characterization of these cells remains an active goal. Because keratinocyte stem cells are believed to cycle slowly, a good method for identification is based on their ability to retain a nucleotide analogue, such as BrdU. Adult stem cells have been identified in other tissues, including hematopoietic, neural, and skeletal muscle, and cell surface markers of these cells have been characterized. We wanted to determine if cell surface markers that are present on both hematopoietic and skeletal muscle stem cells (CD34, Sca-1, and Flk-1) were also present on keratinocyte stem cells, and could be used to identify them. Initially, the successful BrdU labeling and identification of label-retaining cells (LRCs) in both interfollicular epidermis and hair follicle bulge areas was demonstrated by kinetic studies using immunohistochemistry and flow cytometric analysis. The cell-surface expression of stem cell markers on LRCs was compared to that of nonlabel-retaining keratinocytes. Double-labeling for FACS analysis was employed, and LRCs did not express markers that are present on other tissue stem cells. β1 integrin levels were also evaluated, and although expressed on the LRCs, high β1 integrin expression was found not to be specific for these cells. These data suggest that CD34, Sca-1 and Flk-1 are not keratinocyte stem cell markers.

# 076

**Focal Activation of a Mutant Allele Defines the Role of Stem Cells in Mosaic Skin Disorders**

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Mosaic skin disorders are characterized by the presence of at least two genetically distinct cell populations from the same differentiation lineage within the same tissue. The mechanisms that lead to clinical mosaicism are poorly understood. It remains unclear, how certain genetic defects lead to a mosaic disease, whereas others never become apparent in the same tissue. Two candidate disorders to study the molecular and cellular basis of mosaicism are the autosomal-dominant keratin disorders, epidermolytic hyperkeratosis (EHK) and epidermolysis bullosa simplex (EBS), which are caused by mutations in the suprabasal keratins K1 or K10 and the basal keratins K5 or K14, respectively. Whereas mosaic forms have been reported for EHK, where stripes of affected and unaffected skin alternate, this has never been reported for EBS. We generated a mouse model for each disorder that allows activation of a somatic mutation in epidermal stem cells in a spatially and temporally controlled manner using a ligand-inducible Cre recombinase, resulting in the respective phenotypes in treated areas. Whereas the EHK lesions persisted after 3–5 topical applications of the inducer to a circumscribed area of the skin, to date for 10 months, the induced blisters in the EBS model healed by migration of surrounding nonphenotypic stem cells into the wound bed. Our results indicate that lack of selective pressure against certain mutations in epidermal stem cells could explain why mosaic forms exist for EHK, but not for EBS. These findings have important implications for the development of new strategies for somatic gene therapy of dominant genodermatoses.

# 077

**Embryonic Mouse Keratinocytes: Low Growth Potential and High Commitment Towards Terminal Differentiation**

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Embryonic cells are generally expected to possess high growth and differentiation potential, required for organ maturation and expansion during development. However, little information is available on the intrinsic properties of embryonic cells of epithelial origin, due to the difficulties involved in their isolation and cultivation. We report here our *in vitro/in vivo* characterization of keratinocyte populations derived from mouse embryos at 15.5 days of gestation (E15.5 keratinocytes). These cells exhibit a short-term growth/differentiation behavior similar to that of newborn keratinocytes, except some differences in the expression of differentiation markers. Surprisingly, however, embryonic keratinocytes show a low long-term growth potential and become irreversibly committed to differentiation at much earlier times than newborn cells. The increased susceptibility to terminal differentiation is not a consequence of culture conditions, as it is also observed with freshly prepared embryonic keratinocytes prior to culturing and is associated with high turnover of these cells in the intact skin *in vivo*. Integrin expression has been linked not only to control of cell attachment but also cell growth and differentiation. In parallel with their propensity to differentiate, we show that E15.5 embryonic keratinocytes express significantly lower levels of integrin α6/β4 than newborn cells, both under culture conditions, prior to cultivation and in the intact skin, while levels of integrin β1 do not differ significantly. Integrin α6/β4 levels remain low up to E17.5 day, and increase substantially thereafter. Thus, contrary to expectations, E15.5 embryonic keratinocytes have a low growth potential and an intrinsically high propensity to differentiate. These properties can best be explained by low expression of integrin α6/β4 at this stage of epidermal development.

# 078

**Targeted Disruption of LIG-1 Gene Provides New Insight into Skin Stem Cells**

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Epidermis that covers the skin surface is continuously regenerated throughout the lifetime of the mammalian adult through proliferation of keratinocyte stem cells. Despite the central role of keratinocyte stem cells in tissue homeostasis, wound healing, and neoplasia, the precise *in vivo* localization of the cells is an unsettled issue because of the lack of appropriate molecular markers. Here we show that the cutaneous expression of LIG-1, a transmembrane glycoprotein of which extracellular region is uniquely organized with the leucine-rich repeats and immunoglobulin-like domains, was restricted to the bulge of hair follicles and the subpopulation of epidermal basal cells where the keratinocyt stem cells are considered to reside. The LIG-1 deficient mice developed a skin change on the tail and facial area after birth. The affected skin showed histological features of the epidermis in common with the human skin disease psoriasis. LIG-1 expression was apparently down-regulated in the psoriatic epidermis. LIG-1 appears to be a new cell-surface marker for keratinocyte stem cells and to be involved in the pathogenesis of psoriasis.

# 079

**Telomerase Extends Lifespan and Restores Wound Healing in Aging Keratinocytes**

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Telomerase, a reverse transcriptase that maintains telomeric DNA, dramatically extends the lifespan of several human cell types without inducing cancerous changes. Human skin provides a valuable model to investigate the potential role of telomerase in chronic disease. We have previously demonstrated that ectopic expression of telomerase, immortalizes dermal skin fibroblasts and microvascular endothelial cells while maintaining growth control and differentiated function. However, the third major skin cell, the keratinocyte, is reportedly refractory to telomerase immortalization unless the pRb/p16 $^{INK4a}$ pathway is inactivated. Since keratinocyte proliferation is an important component of normal skin turnover and wound healing, and loss of telomeres, proliferative capacity, and function are associated with skin aging and chronic wounds, we sought to reinvestigate the ability of telomerase to extend the lifespan and youthful function of human keratinocytes. Here we report that under appropriate culture conditions, ectopic hTERT expression alone can immortalize human keratinocytes. The lifespan extended and hTERT-expressing keratinocytes retain p16 $^{INK4a}$, pRb and p53 protein expression, respond to normal growth control, differentiate in response to high cell density, growth factor withdrawal and phorbol ester, and do not spontaneously activate c-myc. Further, both stable and short-term telomerase expression in old keratinocytes accelerated the rate of wound healing in a tissue culture model by approximately 3-fold compared to cells transduced with control vectors ($p < 0.0002$, $p < 0.001$, respectively). Thus, telomerase is sufficient for lifespan extension in human keratinocytes and may prove useful in the treatment of chronic skin ulcers where keratinocyte function is compromised.

# 080

**IκB Kinase (IKK) Complex Function Inhibits Epidermal Growth via NF-κB and Fails to Alter Epidermal Differentiation**

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In all tissues, NF-κB activation is accomplished by highly homologous kinases (IKK-α and β) that reside in the cytoplasm complexed with the regulatory IKK-γ subunit. Mice with targeted disruption of all 3 IKK genes have been generated and, in the case of γ and α subunits, display phenotypes predominantly characterized by skin abnormalities, with mutations in IKK-γ implicated in human incontinentia pigmenti. Similar to transgenic NF-κB blockade, IKK-α absence leads to epidermal hyperproliferation and increased cell death in outer epidermis but also to disrupted expression of certain differentiation genes, a process that we believe is due to abnormal cell death. Based on these findings, however, a model has been generally accepted in which IKK-β regulates inflammation via NF-κB while IKK-α regulates epidermal growth and differentiation in an NF-κB-independent manner. If true, this model predicts that IKK-α activation would lead to epidermal differentiation and growth inhibition even when NF-κB activity is blocked. To test this directly, we expressed active IKK subunits in normal human keratinocytes and in epidermis of transgenic mice. *In vitro*, both IKK-α and β phosphorylate epidermal IκB, activate NF-κB directed transcription and inhibit cell growth as expected but both, however, fail to induce differentiation. *In vivo*, IKK-β transgenic skin displays no obvious abnormalities within the first weeks of life. IKK-α transgenic skin, on the other hand, is clinically and histologically hypoplastic with decreased cell proliferation but no evidence of premature differentiation or abnormal cell death; these findings strongly resemble epidermis transgenic for active NF-κB subunits. Blockade of NF-κB function with the IκB super-repressor abrogates IKK-α induced growth inhibition, indicating IKK growth effects are NF-κB-dependent. From these findings, we feel that the current model for the roles of IKK function in epidermis may need to be revised. We propose that IKK complex function negatively regulates growth and apoptosis in an NF-κB-dependent fashion but does not affect differentiation. These findings have broad implications for understanding the functions of upstream activators of NF-κB family proteins in skin.

# 081

**Spatially Localized Ras Action Inhibits Epidermal Differentiation and Promotes Growth Capacity via the Raf Effector Pathway**

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Ras GTPases are implicated in epithelial neoplasia, however, Ras action in normal epidermis is uncharacterized. To study this, we altered Ras function by gene transfer *in vitro* and by generating multiple independent lines of mice transgenic for Ras activation [RasV12] and Ras blockade [RasN17] using promoters targeting differentiating [K1] and undifferentiated [K14] epidermis. In both cells and tissue, Ras activation induces β1 and β4 integrins while inhibiting differentiation. In contrast, Ras blockade in basal epidermis inhibits integrin expression and induces epidermal hypoplasia and differentiation. Ras function is necessary for cell proliferative capacity as Ras blockade in keratinocytes diminished the proportion of highly proliferative holoclones *in vitro*, increasing abortive colonies from 12.6 + 3 to 84.1 + 7%. Ras blockade in suprabasal epidermis exerts no effect, indicating that the main site of Ras action resides within the basal layer. Consistent with this, phosphorylated MAPK – a Ras downstream effector induced via Raf and MEK – was found only in a subset of basal layer cells within normal epidermis. To define the Ras downstream effector pathways involved, mutants selective for the 3 major Ras effector pathways Raf, RalGDS and PI3K were expressed in keratinocytes. As seen with active Ras, selective induction of the Raf effector pathway blocks calcium induction of differentiation. This was confirmed pharmacologically using specific MEK/MAPK inhibitors U0126 and PD098059. In contrast, PI3K pathway activation fails to inhibit differentiation. To further characterize downstream Ras effector mechanisms, a tamoxifen-responsive Raf-ER fusion was generated and expressed *in vitro* and in transgenic mice via the K14 promoter. Raf induction promoted epidermal growth and inhibited differentiation in a fashion similar to Ras, indicating that Raf alone is sufficient to mediate these Ras effects. Collectively these findings indicate that spatially restricted Ras signaling divides normal epidermis into an undifferentiated proliferative compartment and a differentiating postmitotic compartment and suggest a new role for Ras-Raf signaling in the homeostasis of self-renewing tissues.

# 082

**HB-EGF Activation of Keratinocyte ErbB Receptors Mediates Epidermal Hyperplasia, a Common Side-Effect of Retinoid Therapy**

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Sun-protected human skin was maintained in organ culture and treated with 3 μM all-*trans* retinoic acid (RA) in the presence or absence of reversible or irreversible pharmacological antagonists of c-erbB receptor tyrosine kinase (RTK) activity. In the absence of these inhibitors, RA induced epidermal hyperplasia comparable to that induced in intact skin by all-*trans* retinol or RA itself. Both of the c-erbB RTK antagonists prevented the hyperplastic response, and preserved normal histological features. There was a strong correlation between inhibition of epidermal hyperplasia in organ culture and inhibition of EGF-dependent keratinocyte growth in monolayer culture. In additional studies, organ-cultured skin was examined for expression of heparin-binding EGF-like growth factor (HB-EGF) mRNA in the presence or absence of 3 μM RA. The level of HB-EGF mRNA was increased approximately 4-fold in the presence of RA. An antibody to HB-EGF inhibited retinoid-stimulated epidermal hyperplasia in organ culture and reduced proliferation in cultured keratinocytes. In contrast to these results, the c-erbB-RTK antagonists and the neutralizing HB-EGF antibody were ineffective in inhibiting RA-dependent survival and proliferation of human dermal fibroblasts. Taken together, these data indicate (i) that retinoid-induced epidermal hyperplasia in human skin proceeds through c-erbB, and (ii) that HB-EGF is one of the c-erbB ligands mediating this effect.

# 083

**Ubinuclein, a Ubiquitously Expressed Nuclear Protein, Regulates Keratinocyte Proliferation and Differentiation**

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The major targets of Epstein-Barr virus (EBV) are B-lymphocytes and epithelial cells especially within the upper spinous layer of the stratified oral epithelium. EB1 is an EBV transcription factor interacting with viral and cellular promoters and involved in the initiation of the lytic cycle of EBV. Previously, we cloned a novel human cDNA through protein–protein interaction with the EB1 and designated the corresponding protein ubinuclein. Ubinuclein is ubiquitously expressed in adult and fetal tissues. In cultured keratinocytes, immunostaining with ubinuclein antibody revealed grainy or dotty nuclear staining. Overexpression of the full length ubinuclein resulted in spreading and disintegration of the transfected cells resembling terminal differentiation of the keratinocytes in culture. A functional role for ubinuclein as a transcriptional regulator is suggested by the discovery that the central region of ubinuclein is capable of interacting with the basic domains of both cellular and viral bZIP transcription factors c-Jun and EB1, respectively, and competing with binding to the AP1 consensus sequence. In order to study the effects of down-regulation of ubinuclein we established a HaCaT cell line transfected with an episomal vector coding for an antisense transcript to ubinuclein mRNA. Our results revealed that ubinuclein down-regulation accompanied the formation of giant, multinucleated cells among the population of antisense mRNA expressing cells. Up- or down-regulation of several proteins involved in the signal transduction and control of proliferation or apoptosis is shown to result in cellular growth and endoreplication due to defective cytokinesis. These results strongly suggest that ubinuclein plays a role in the processes controlling cytokinesis and is involved in the regulation of normal cell proliferation and differentiation in keratinocytes.

# 084

**Synergism in Phosphorylase Kinase Inhibitor Suppression of Psoriatic Activity**

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Elevated phosphorylase kinase (PhK) was previously reported to correlate with psoriatic activity. We tested the antiproliferative activity of curcumin, a phosphorylase kinase inhibitor, in the treatment of psoriatic plaques. Thirty patients entered the study by inclusion criteria. Ten psoriatic patients were treated topically with 1% curcumin gel for 6 weeks (C group), 10 with Dovonex 0.005% ointment for 6 weeks (D group), and 10 with curcumin for 3 weeks and Dovonex for 3 weeks (CD group). Punch biopsies taken at the start (0 week) and at 6 weeks were processed for (a) phosphorylase kinase activity and (b) Ki-67 proliferating cell nuclear antigen (PCNA) expression, a marker of psoriatic proliferative activity. Data analysis (one-way ANOVA) showed significant differences among the three groups in the following: (1) suppression of PKI activity was highest in CD group (82.2 ± 5.0%), lower in C group (54.8 ± 6.7%) and lowest in D group (19.5 ± 5.0%); $F_{2,27} = 314.7$, $p < 0.0001$; and (2) suppression of Ki-67 expression was similarly highest in CD group (96.4 ± 1.1%), lower in C group (85.6 ± 1.4%) and lowest in D group (42.8 ± 7.0%); $F_{2,27} = 464.7$; $p < 0.0001$. PhK suppression correlated with suppression of Ki-67 expression (Pearson $r = 0.93$). The results indicate that although curcumin is more effective than Dovonex alone, curcumin with Dovonex act synergistically to produce the greatest inhibition of PhK and antipsoriatic proliferative activity.

# 085

**Matrix-Independent Survival of Human Keratinocytes Through an EGF Receptor/MEK-Dependent Pathway**

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…Normal epithelial cells undergo apoptosis when they are denied contact with the extracellular matrix, a process termed ''anoikis''. Conversely, malignant epithelial cells typically acquire anchorage independence, i.e. the capacity to survive and grow in the absence of matrix interaction. Previously we and others demonstrated that engagement of the EGFR promotes survival of attached keratinocytes, at least in part, through activation of the MEK/MAPK pathway (Jost *et al*, *JBC*, in press). This observation and the fact that EGFR signaling is frequently deregulated in malignant epithelial cells led us to investigate whether EGFR engagement enhances survival of keratinocytes in forced suspension. …We demonstrate that, in suspension culture, EGFR activation markedly alleviated the requirement of matrix engagement for survival of primary and immortalized human keratinocytes. Protection of epithelial cells through EGFR activation against anoikis was associated with and required sustained MEK/MAPK signaling during the early phase of suspension culture. Interestingly, high levels of MAPK phosphorylation were not only required for EGFR mediated protection against anoikis but also occurred as a consequence of caspase activation at later stages of suspension culture. These results demonstrate that EGFR activation contributes to anchorage independent epithelial cell survival and identify MEK/MAPK activation as an important mechanism in this process.

# 086

**Galectin-7 (PIG1:p53-Induced Gene 1) Exhibits Pro-Apoptotic Function Through Mitochondrial Cytochrome C Release and JNK Activation**

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Galectin-7 is a member of a growing family of β-galactoside-binding animal lectins with diverse biological activities. This lectin is abundantly expressed in normal skin keratinocytes, but the expression is significantly down-regulated in tranformed keratinocytes and skin neoplasms, thus the lectin may function in the maintenance of normal phenotype of keratinocytes. Galectin-7 was recently found to be highly inducible by p53 and designated as PIG1 (p53-induced gene 1). In order to define the function of galectin-7, we studied transfectants of HeLa and DLD-1 cells ectopically expressing this protein and found that these transfectants were more susceptible to apoptosis than mock-transfected cells. This was observed in apoptosis induced by mechanistically distinct stimuli, such as UV irradiation, actinomycin D, etoposide, camptothecin, and TNF-α plus cycloheximide, suggesting that galectin-7 acts on a common point in the apoptosis signaling pathways. Further analyses of apoptosis induced by actinomycin D demonstrated enhanced caspase-3 activity in association with increased PARP cleavage in galectin-7-expressing transfectants. The potentiation of apoptosis by galectin-7 was completely abrogated by a caspase inhibitor, zVAD-fmk, indicating that the effect of galectin-7 is caspase-dependent. In addition, cells overexpressing galectin-7 displayed accelerated mitochondrial cytochrome c release, which was unaffected by zVAD-fmk treatment, suggesting that galectin-7 exerts its function through cytochrome c release, which itself is a caspase-independent process. Finally, the activity of JNK, a known regulator of the apoptotic pathway, was specifically up-regulated in cells expressing galectin-7, indicating that galectin-7 possibly promotes apoptosis through JNK.

# 087

**Nitric Oxide Synthase Antagonism Increases Keratinocyte Apoptosis after UVB Irradiation**

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Ultraviolet radiation induces apoptosis in keratinocytes as well as inducing nitric oxide (NO) production by both constitutive and inducible nitric oxide synthase (NOS) enzymes. Paradoxically, NO has both pro and antiapoptotic properties. In this study we determined whether keratinocyte-derived NO increased or decreased apoptosis following UVB irradiation. The E6/E7 transformed CCD1106 human keratinocyte cell line was plated at a density of $1.7 \times 10^4$ per $cm^2$ and irradiated with 0, 25, 50, 75 or 100 mJ per $cm^2$ UVB. Apoptosis was measured after 12 h by analysis of annexin V using flow cytometry. Cells were cultured with 0, 50, 200 or 1000 μM of the NOS antagonist L-NAME. Apoptosis increased with UVB doses above 50 mJ per $cm^2$ and was further increased in direct correlation with the concentration of L-NAME. (Apoptosis percentage: No NAME, 0 mJ UVB 8%; 50 mJ, 18%; 100 mJ 25%; with 50 μM NAME, 0 mJ, 11%; 50 mJ, 27%; 100 mJ 28%; with 1 mM NAME, 0 mJ, 14%; 50 mJ, 30%; 100 mJ, 38%). To assess the specificity of this finding, cells were irradiated with 75 mJ per $cm^2$ UVB, ± 1 mM NAME and the NO releasing agent SNAP (0, 10, 100, 250, 1000 μM). 10 μM and 100 μM SNAP almost totally abrogated the increased UVB apoptosis due to L-NAME. A small, dose-dependent increase in apoptosis after high dose SNAP was seen in unirradiated, non-L-NAME treated cells (0 SNAP, 6% apoptosis; 1 mM 16%). Our results demonstrate that NO released by keratinocytes subsequent to UVB irradiation autologously limits UV-induced apoptosis. This antiapoptotic effect may be essential for maintenance of the integrity of the epidermis.

# 088

**Characterization of Apoptotic Machinery Controlling Cell Death in Epidermis**

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Ironically, the functional integrity of epidermis is dependent on a ''planned cell death'' pathway by which keratinocytes (KCs) undergo terminal differentiation, and then die producing corneocytes. To explore the molecular basis for crucial steps regulating epidermal KC cell death, normal human skin was examined by immunostaining and Western blot analysis. Fractionating KCs by state of maturation using discontinuous Percoll gradients followed by immunoblotting confirmed immunohistology results in which KCs in the upper level strata – including the granular cell layer expressed high levels of TRAIL and its 2 death receptors (DRs), but not decoy receptors (DcRs), whereas low and mid level KCs expressed 2 DcRs for TRAIL. While caspases 8 and 3 were detected equally throughout all cell layers, caspase 14 was preferentially expressed in upper layer KCs. To establish a cause and effect relationship amongst these mediators of cell death, submerged living epidermal equivalents (EEs) containing stratified, multilayered but relatively undifferentiated KCs grown on a permeable membrane were raised to an air/liquid interface (A/L-I), which triggers KC terminal differentiation/corneogenesis. Western blot analysis of proteins extracted from submerged EEs vs. EEs raised to A/L-I for 2 days or 5 days revealed a 5–10 fold increase in TRAIL levels, accompanied by increased TRAIL DRs and decreased DcRs. Moreover, upon raising EEs to A/L-I triggers degradation (activation) of caspases 14, 8 and 3. Pre-incubation of submerged EEs with a cocktail of caspase inhibitors (CIs) blocked these biochemical reactions and subsequent KC terminal differentiation/cell death. To further explore a role for TRAIL as a relevant death inducing ligand in normal epidermis, cultured KCs were grown to confluence and exposed to either TNF-α ($10^3$ U per ml), soluble FasL (20 ng per ml), or LZ-TRAIL (100 ng per ml). Only TRAIL could induce rapid and prominent apoptosis of KC monolayers, which was blocked by CIs. Moreover, TRAIL induced greater apoptosis in confluent vs. nonconfluent cultures which correlated to enhanced DR and decreased DcR cell surface expression (determined by FACS analysis), which occurred in KCs upon reaching a state of confluency. These results

# 089

**The Low-Affinity Neurotrophin Receptor (p75) Mediates Apoptosis in Human Keratinocytes**

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Normal human keratinocytes express both the low (p75) and the high-affinity (trk) nerve growth factor (NGF) receptors. While trk mediates survival effects in keratinocytes, the role of p75 remains to be elucidated. p75 shares with other members of the tumor necrosis factor receptor (TNF-R) superfamily an intracellular ''death domain'', as well as an extracellular homology. Whereas the signaling pathway and the effects of TNF-R and Fas are well known, p75 transduction system is poorly understood. Activation of c-jun kinase (JNK) and NF-kB have been associated with p75-induced apoptosis. We investigated the effects of p75 in normal human keratinocytes cultivated in serum-free medium. To this purpose, cells were transfected with either wild-type or a mutant form of p75 cDNA. Apoptosis was evaluated by TUNEL technique 72 and 144 h after transfection. Both full-length p75 and mutated construct, lacking the ''death domain'', induced a marked apoptosis in human keratinocytes (19% and 22%, respectively), as compared to either controls (5%) or mock-transfected cells (7%). This seems to indicate that the ''death domain'' is not required in p75 mediated apoptosis. Because p75 signaling is enhanced in cells where trk activity is down-regulated, keratinocytes were treated with K252, an inhibitor of trk phosphorylation. The addition of K252 significantly augmented p75-induced cell death (full-length 33%, mutated construct 40%). We next investigated whether p75-induced apoptosis was mediated via the activation of JNK. Western blot analysis showed that neither full-length or mutated p75 affected JNK protein, either alone or in combination with K252. On the other hand, K252 markedly activated NF-kB. Taken together, these results demonstrate that p75 induces apoptosis in human keratinocytes, although the signaling pathway remains to be clarified.

# 090

**Caspase-14 but not Caspase-3 Expression and Activation is Regulated by Retinoids in a Differentiation-Dependent Manner**

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Caspase-14 is the first member of the caspase family of genes that shows a restricted tissue expression, being mainly confined to epidermal keratinocytes (KC) and sebocytes. In contrast to caspase-3, caspase-14 is not activated during apoptosis induced by UV light. Because caspase-14 is cleaved under conditions leading to epidermal barrier formation, we have recently suggested that caspase-14 is involved in the terminal differentiation of KC. Here we show that retinoic acid (RA), a well-known regulator of KC differentiation, strongly suppressed caspase-14 mRNA and protein expression by differentiating KC in monolayer culture. Treatment of *in vitro* reconstructed skin equivalents (SE) with RA inhibited epidermal differentiation in a time and dose-dependent manner and virtually abrogated the expression and activation of profilaggrin and caspase-14. By contrast, the constitutive expression of the apoptosis associated caspases 3 and 8 was increased and unchanged, respectively, after RA treatment of KC and SE. In untreated SE, caspase-14 but not caspase-3 and –8 activation was found by Western blot analysis. After RA treatment, caspase-3 activation was detectable when, in addition to differentiation inhibition, increased apoptosis was apparent, as confirmed by TUNEL staining and *in situ* detection of caspase-3 activation. We conclude that expression of the epithelial cell specific caspase-14 is not required for KC apoptosis. Furthermore, in contrast to caspase-3 and caspase-8, expression and activation of caspase-14 is regulated by retinoids in a differentiation dependent manner, comparable to that of epidermal proteins participating in the formation of the skin corneocyte barrier.

# 091

**Keratinocyte Apoptosis as a Parameter of Chemical Toxicity: Flow Cytometric Analysis in Different Conditions of Cultivation**

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Human cultured keratinocytes represent a good experimental system to evaluate the potential toxicity of chemicals active on the skin. They are constituted of various cell types, such as proliferating, differentiating and apoptotic cells. These last are present even in normal epidermis, but their formation and role are not yet clear. The aim of the present study was to investigate the role of culture conditions in inducing apoptosis on human keratinocytes and define their relation with some definite damaging agents. Cultures were obtained from surgical skin fragments. Keratinocytes were cultivated both as organoid cultures (OC) in DME medium supplemented with fetal calf serum and a 3T3 feeder layer, and as monolayer cultures (MC) in definite medium (MCDB 153) with low calcium level. OC were processed at confluence, while MC were processed at 75–80% or complete confluence. Some cultures were treated with damaging agents, in particular with $H_2O_2$. After treatment the cells were placed in incubator, and 2 and 4 h later were harvested for analysis. Preliminarly, cell viability was tested using MTT test and trypan blue dye exclusion assay. Monodispersed suspensions of trypsinized cells were used for cytofluorimetric analysis. Cytofluorimetric patterns of our cultures showed that human keratinocytes are constituted of different cell populations, as demonstrated by other research groups. Apoptotic cells were found more numerous in cultures at complete confluence than in subconfluent ones. However, the oxidant stress caused by $H_2O_2$ was more effective in inducing apoptosis on proliferating keratinocytes than on the nonproliferating ones. Apoptosis evaluation can be a suitable parameter to test potential toxic agents for different pharmacological uses.

# 092

**_De Novo_ Synthesized Ceramide is Critical for Ultraviolet-B Irradiation-Induced Keratinocyte Cell Death**

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Multiple pathways are involved in UV-induced keratinocyte apoptosis, such as activation of p38MAP kinase, PKC, CD95, many of which are associated with caspase activation. Ceramide (Cer) is recognized as an effector lipid in cell cycle arrest, differentiation, senescence and apoptosis in a variety of cell types, including keratinocytes. Here, we investigated the involvement of *de novo* Cer synthesis in UVB-induced apoptosis of cultured normal human keratinocytes (CHK) grown in serum-free keratinocyte growth medium. UVB (50–60 mJ per $cm^2$) inhibited CHK proliferation (85%; assessed by [$^3$H]-thymidine incorporation) at 2 h after irradiation, while both TUNEL-positive and trypan blue-positive apoptotic cells were significantly increased, coincident with the activation of caspase-3, at both 24 and 48 h following irradiation. In addition, the Cer content and synthesis were significantly increased (i.e. 1.5- and 3.2-fold vs. sham-irradiated controls, $p < 0.01$ and 0.001, respectively), coincident with an induction of the enzyme Cer synthase (1.5-fold; $p < 0.01$), 8 h after UVB; serine palmitoyltransferase activity was unchanged at this early time point. Fumonisin B1, a specific inhibitor of Cer synthase, significantly reduced both the UVB-induced Cer formation and subsequent cell death compared with vehicle-treated CHK. Finally, although fumonisin B1 did not alter the UVB-induced caspase-3 activation, coincubation of fumonisin B1 and a caspase-3 inhibitor (Ac-DEVD-FMK) further attenuated UVB-induced cell death. These results demonstrate in CHK that: (1) UVB increases *de novo* Cer synthesis; (2) the resultant increase in Cer content is critical for UVB-induced cell death; and (3) this Cer-dependent cell death pathway operates independently of caspase-3 activation. These findings also suggest a pro-apoptotic role for Cer generated *de novo* in response to specific epidermal stressors, including UVB.

# 093

**Assembly Behavior of Desmosomal Cadherins is Dependent on Isoform Specificity**

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Desmosomes contain two subclasses of cadherin, desmocollins (Dsc) and desmogleins (Dsg), each consisting of three isoforms, which are expressed in a tissue- and differentiation- dependent manner. However, rules governing the assembly of different isoforms into desmosomes are unknown. First, we examined whether the differentiation-dependent isoforms, Dsc1 and Dsg1, can substitute for the widely expressed isoforms, Dsc2 and Dsg2, in desmosome assembly. Myc-tagged forms of desmosomal cadherins were introduced into three epithelial cell lines (MDCK, Scc9, A431) that do not normally express Dsc1 and Dsg1. Dsc2myc and Dsg2myc efficiently incorporated into desmosomes in all cell types. In contrast, Dsc1a,bmyc and Dsg1myc did not colocalize with desmosomes efficiently. In addition, introduction of Dsg1myc disrupted desmosome assembly in A431 cells. Detergent solubility analysis showed that Dsc1myc remained largely in a Triton soluble pool whereas Dsg1myc fractionated into an insoluble pool. We tested whether incorporation of Dsg1 requires the presence of its normally coexpressed partner, Dsc1. Co-expression of Dsg1myc and Dsc1myc did not lead to their colocalization or assembly into desmosomes. Next we examined whether disruption of desmosomes in Dsg1myc cells is caused by sequestration of plakoglobin (PG) from E-cadherin, as PG: E-cadherin complex is thought to be critical for desmosome assembly. Co-immunoprecipitation showed that the ratio of PG: E-cadherin decreased in a Dsg1myc cell line as predicted. However, a decrease was also observed in Dsc1amyc cell lines in spite of the lack of desmosome disruption, suggesting that reduction of PG associated with E-cadherin is not sufficient for the disruption. These results demonstrate that desmosome assembly is sensitive to isoform-specific differences and highlight the importance of tight regulation of the epithelial differentiation program.

# 094

**Autoantibodies to Desmoplakin: Binding and Internalization in Human Keratinocytes**

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Autoantibodies to desmoplakin I and II have been identified recently in a subset of patients with erythema multiforme. Since desmoplakins are major constitutive proteins of the inner dense desmosomal plaque of keratinocytes and are entirely localized intracellularly the question remains how antidesmoplakin autoantibodies reach their target in living, unaltered cells. To address this problem we have used cultured human keratinocytes and characterized peptide-specific antidesmoplakin autoantibodies. For indirect immunofluorescence experiments unfixed cultured cells were incubated with antidesmoplakin autoantibodies at 4°C followed by incubation at 37°C for different time spans. Cell surface bound autoantibodies were detected in a faint, granular pattern. After warming up to 37°C most of surface bound antibodies turned to a fine but distinct dotted pattern within the periphery of the cytoplasm. With increasing time a prominent, perinuclear vesicular pattern reminiscent of endosomes and multivesicular bodies emerged. At the same time autoantibodies appeared in various intensities at desmosomal plaques of individual keratinocytes. By double immunofluorescence bound autoantibodies colocalized at different time points with antibodies to caveolin and transferrin receptor. From these findings we conclude that: (i) antidesmoplakin autoantibodies bind to the cell surface of cultured human keratinocytes, (ii) bound antibodies enter living cells by internalization via plasmalemmal vesicles to an endosomal compartment, (iii) a fraction of vesicular internalized antibodies reaches the desmosomal plaque of individual, intact keratinocytes. These observations indicate that vesicular transport may represent a relevant biological mechanism for antidesmoplakin autoantibodies to enter the cell and reach their corresponding antigen.

# 095

**Abnormalities of Keratinocyte Functions in the Epidermis of Knockout Mice Lacking the Neuronal Nicotinic Acetylcholine Receptor Subunits α3, α7 or β4**

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Keratinocytes (KC) produce acetylcholine (ACh) and express receptors that mediate autocrine and paracrine effects of ACh. During differentiation, KC change repertoire of their ACh receptors which diversifies effects of ACh. In KC, the ACh-gated ion channels, or nicotinic ACh receptors (nAChRs), are comprised of classic neuronal subunits. The α3 and β4 subunits form heteromeric channels, α3β2(β4) ± α5, and several α7 or α9 subunits assemble homomeric channels. In this study, we investigated the biological roles of different keratinocyte nAChRs using knockout mice lacking α3, β4 or α7 subunits. The epidermal morphology and expression of cell function markers were analyzed in pups delivered by a heterozygous mouse, followed by genotyping. The α3−/− genotype was associated with abnormalities of cell adhesion. In epidermis, we observed both areas of loosely attached KC and areas of microvesiculation. By semiquantitative immunofluorescence, the relative amount of keratinocyte cadherins was 50 ± 5 vs. 90 ± 14 in α3+/+ mice ($p < 0.05$), and that of desmogleins was 23 ± 5 vs. 41 ± 10 (p0.05). The KC in β4−/− mice also exhibited a decreased expression of cadherins (66 ± 5 vs. 130 ± 10, $p < 0.05$) as well as a decreased expression of the differentiation marker loricrin (59 ± 7 vs. 117 ± 6, $p < 0.05$). The α7−/− mice showed signs of prolonged epidermal turnover. The relative amounts of both filaggrin (63 ± 21) and loricrin (59 ± 6) were less than the control levels of 117 ± 10 and 95 ± 20 ($p < 0.05$), respectively, whereas the hyperproliferation marker cytokeratin 6 was increased (102 ± 5 vs. 65 ± 1, $p < 0.05$). These results suggest that while α3 nAChRs are coupled to regulation of keratinocyte adhesion, the presence of β4 couples the α3 nAChRs to rapid unfolding of the differentiation program, a function controlled by α7 nAChRs.

# 096

**Genes Encoding Hemidesmosome Proteins are Not Coordinately Regulated by TNF-α or IFN-γ**

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Hemidesmosomes (HDs) mediate attachment of basal keratinocytes to epidermal basement membrane, thereby providing a connection between the cytoskeletal elements of these cells and their extracellular matrix. HDs are composed of five proteins, namely, bullous pemphigoid antigen (BPAG) 1, BPAG2, plectin, and integrin subunits α6 and β4. The assembly of HDs is a dynamic process that is of crucial importance for development, homeostasis, wound healing, and tumor invasion. To determine if genes encoding HD proteins are modulated (and if such modulation occurs in a coordinate fashion), cultured human keratinocytes (HKs) were treated with cytokines known to induce responses in these cells (specifically, TNF-α [0.1–50 ng per ml; 24 h] or IFN-γ [(10–1000 U per ml; 24 h]). Total RNA was extracted from treated and untreated HKs, reverse transcribed, and further investigated using quantitative real-time PCR (ABI Prism® 7700) with specific primers for all five HD proteins. ICAM-1 mRNA expression was used as a positive control; all mRNA levels were normalized relative to that of β-actin. Preliminary studies identified reaction conditions in which all primer sets used in these quantitative experiments generated amplicons with equivalent efficiency. TNF-α as well as IFN-γ up-regulated ICAM-1 mRNA levels greater than or equal to 50 fold. In contrast, both TNF-α and IFN-γ decreased BPAG1 mRNA expression in HKs (3.8 and 9.4 fold, respectively). mRNAs encoding other HD proteins were not notably altered by these cytokines. Genes encoding HD proteins in HKs were not coordinately regulated by the cytokines examined in these studies.

## 097

**The Intracellular Tail Region of the Short Desmocollin Isoform Binds to Rack1, Receptor for Activated C Kinase, and Modifies the Keratinocyte Cell Adhesion**

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Desmocollins constitute the extracellular part of desmosomal complexes with another desmosomal cadherin, desmogleins. These two desmosomal cadherins consist of three distinct isoforms, Dsc 1–3 and Dsg 1–3, and each desmocollin isoform has two splicing forms, the longer Dsc1a-3a and the shorter Dsc1b-3b. Amino acid sequences of the carboxyl termini of Dsc b forms are highly conserved within three human desmocollin isoforms and among those of other mammalian species. The intracellular domain of shorter Dsc is thus expected to preserve some biological function, however, no proteins are yet known to interact with this domain. We employed yeast two-hybrid system to find out proteins binding to the carboxyl end of Dsc 2b from human skin cDNA library. Rack1, receptor for activated C kinase, was isolated as an interacting protein with the intracellular region of all Dsc b forms. The GFP-Rack1 fusion protein was observed to translocate from the cytoplasm to the plasma membrane after PMA treatment in the transfected keratinocytes. Rack1 was coimmunoprecipitated with Dscs from the keratinocyte lysates after C kinase activation. In human skin, Rack1 was expressed at the basal keratinocytes and was observed to colocalize with the intracellular desmosomal plaque proteins. Rack1 has been thought to be an anchor of activated C kinases and to translocate the kinases close to their target proteins after various stimuli. From these data, the cytoplasmic tail of desmocollins was suggested to act as an anchoring site of PKC and to have a function modifing the cell adhesion through the phosphorylation of desmosomal proteins.

## 098

**Evidence that Pemphigus Vulgaris IgG Causes no Steric Hindrance in Desmosome Formation, but Forms Desmoglein 3-Deficient Desmosomes**

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Low-$Ca^{++}$ (0.05 mM) grown keratinocytes (KC) produce no desmosomes (DS), but they form "half-DS", which can couple to form DS within 2 h when $Ca^{++}$ is switched to high (1.2 mM). We studied effects of pemphigus vulgaris IgG (PV-IgG) on this $Ca^{++}$ induced-coupling of "half-DS" to form DS. Low $Ca^{++}$ normal human (NH) keratinocytes and human carcinoma (DJM-1) cells were pretreated with PV-IgG (confirmed to be specific to Dsg3) or NH-IgG for 30 min or 30 h in low $Ca^{++}$, and $Ca^{++}$ concentration in the medium was switched to high. After 2-h incubation in high $Ca^{++}$, cells were studied by immunofluorescence (IF), immunoelectron (IEM) microscopy and Western blotting (WB) using anti-Dsg3 affinity purified polyclonal, Dsg1/2, desmocollin 3 (Dsc3) and desmoplakin 1/2 (Dpk) monoclonal antibodies. In low-$Ca^{++}$ grown cells, WB detected Dsg3, Dpk and Dsc3, but not Dsg1/2, in cytoskeleton fractions, IF showed their punctate distribution in the cytoplasm and/or on the cell membrane, but not at cell-cell contacts, and IEM revealed Dsg3 on the cell surface "half-DS". Double-staining IF revealed that high-$Ca^{++}$ switched cells after pretreated with PV-IgG for 30 min in low $Ca^{++}$ showed a punctate-linear pattern of PV-IgG, which were colocalized with Dsg3, Dsc3 and Dpk, at cell-cell contacts. When low $Ca^{++}$ cells were treated with PV-IgG for 5 min and labeled with antihuman IgG-5 nm gold for 5 min in low $Ca^{++}$ medium and followed by incubation in high $Ca^{++}$ without antibodies for 2 h, gold-labels were detected in the newly formed DS by IEM. These IF and IEM results demonstrate that PV-IgG causes no steric hindrance in DS formation. In high-$Ca^{++}$ switched cells pretreated with PV-IgG for 30 h in low $Ca^{++}$, PV-IgG and Dsg3 showed a random-punctate pattern in cells, whereas Dsc3 and DPK showed a punctate-linear pattern at cell-cell contacts. These IF results suggest the formation of Dsg3-deficient DS.

## 099

**Periplakin Tail Domain Interacts with Intermediate Filament Proteins**

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Plakin proteins are a group of cytoskeletal binding proteins that are characterized by a central coiled-coil rod domain and an N- and C-terminal globular domains. Currently, five members of plakin proteins have been identified as components of the desmosomal or hemidesmosomal plaque. Plectin localizes in nucleus, hemidesmosomes, desmosomes, and in association with intermediate filaments. BPAG1 is a component of hemidesmosomes in epidermal basal cells, but a neuronal isoform, dystonin, has also been identified. Envoplakin and periplakin have been detected in both the cornified envelope of the stratified epithelium and desmosomes. We used the periplakin tail domain as a bait in yeast two-hybrid system to search for interacting proteins. cDNA clones encoding cytokeratin 8 and vimentin were isolated through these interactions, which were also confirmed by *in vitro* interaction assays using periplakin-GST fusion protein. We also prepared green fluorescent protein (GFP) fusion proteins from the homologous linker region of each plakin protein. Transient transfection of HaCaT cells with GFP-fusion proteins showed that desmoplakin linker region had a diffuse localization throughout the cell. BPAG1 linker formed aggregates throughout the cytoplasm and on the cell membrane, while plectin and envoplakin tail-GFP were found in nucleus. Peripakin-GFP colocalized with keratin 8 along the intermediate filaments. These data suggest that periplakin, like plectin, is able to interact with intermediate filament proteins and the short C-terminal tail, with high homology to the linker domain of other plakin proteins, is responsible for the interaction.

## 100

**Regulation of Desmoplakin Dynamics During Desmosome Assembly**

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Although the modulation of epidermal adhesive junctions is essential for cell migration during wound healing and metastasis, little is known about the dynamics of individual junction components or their regulation during these processes. We have established an approach to study the dynamics of the desmosomal plaque protein, desmoplakin (DP), and a phosphorylation site variant in living cells. We previously used a truncated DP containing its C-terminal IF binding domain and showed that upon phosphorylation of a Ser residue within a PKA consensus site 23 amino acids from the DP C-terminus the construct was released from the IF; mutation of this residue to a glycine prevented this release. We hypothesized that phosphorylation of SerC23 regulates trafficking of full length DP (FLDP) during desmosome assembly. To test this hypothesis we generated full length FLAG-tagged and GFP-tagged wildtype DP and the glycine mutant and introduced these into cultured keratinocytes. Forskolin treatment facilitated the accumulation of endogenous DP at cell-cell borders in epithelial cells expressing FLDP, consistent with a role for PKA-dependent phosphorylation in promoting DP's incorporation into desmosomes. Scc12F cells transiently transfected with wild type or mutant DP were imaged live as steady state cultures or during the closing of a scrape wound before and after EGF treatment. In steady state cultures wild type DP, which was localized in desmosomes at cell-cell borders, appeared to break away from these sites as single dots, and moved in a slow retrograde manner towards the nucleus. Cell-cell contact at the leading edge of a scrape wound triggered a more rapid movement of individual cytoplasmic DP dots in an anterograde direction to cell borders where they lined up in a discontinuous, desmosome pattern. These data suggest that at least some of the DP dots represent desmosome precursors that become incorporated into desmosomes upon cell contact, in contrast with the previous suggestion that these dots are all endocytosed remnants bound for degradation. In general, movements of wild type DP were continuous, whereas glyC23 DP exhibited halting movements consistent with an inhibition of normal DP dynamics. Our results provide important insight into the mechanism of DP assembly into desmosomes and indicate a possible role for phosphorylation-dependent regulation of DP dynamics.

## 101

**Cdc42, a Member of RhoA GTPases Family, is Associated with Microtubules in Keratinocytes**

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RhoA GTPases family has been known to play an important role in actin organization. One member, rac1, was reported to be associated with microtubule structures. In this presentation, we describe the association of cdc42 protein with microtubules in keratinocytes. In keratinocytes including normal human epidermal keratinocytes, immunofluorescence staining of cdc42 after removal of cytoplasmic soluble proteins showed a pattern of strand-like organization, from the perinuclear region to the cell periphery. These fluorescence patterns almost completely disappeared when microtubule were disrupted by nocodazole. Double labelling of cdc42 and β-tubulin revealed their colocalization. In the case of rac1 staining, association with microtubule was prominent especially in platelet derived growth factor (PDGF) treated keratinocytes. Sequential fractionation of keratinocytes showed that cdc42 and rac1 proteins but not RhoA were present in tubulin-rich fraction. In nocodazole treated cells, cdc42 and rac1 together with tubulin were predominantly located in soluble fractions. Subsequent microtubule polymerization *in vitro* using soluble fraction of nocodazole treated keratinocytes lysates revealed that cdc42 and rac1 were associated with assembled microtubules. However, tubulin association of these proteins could not be demonstrated in GST pull-down assay using GST-fused GTPases. This suggests that association of cdc42 and rac1 with microtubules in keratinocytes might require the intact microtubule structure or other cofactors. In fibroblast, we could not find any evidence of microtubule association of cdc42. Thus, this association might be a keratinocyte specific phenomenon.

## 102

**Activation of RhoA and Actin Polymerization are Not Required for Cell–Cell Adhesion Induced by Microtubule Disruption in Keratinocytes**

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We have previously described that microtubule disruption can induce cell–cell adhesion through activation of endogenous E-cadherin in primary human keratinocytes in low calcium media. This cell adhesion was accompanied with stress fiber formation and increased focal adhesion, but in the keratinocyte cell-line, HaCaT, neither cell–cell adhesion nor stress fiber were induced by microtubule disruption. In this study, we analyzed the possible involvement of RhoA function in this phenomenon. Cell–cell adhesion could be induced when actin structure and microtubules were disrupted together, suggesting actin polymerization was not essential for cell–cell adhesion induced by microtubule disruption. Transient expression of RhoA mutants (RhoV12, dominant active; RhoN17, dominant negative) in NHEK showed that both mutants did not inhibit cell–cell adhesion induced by microtubule disruption, although stress fiber formation was significantly induced by RhoV12 and inhibited by RhoN17 in the nocodazole-treated case. Also it was found that cultivation of neomycin or hygromycin-resistant HaCaT cells in corresponding selection media led these cells to become inducible for cell–cell adhesion by microtubule disruption without any change of stress fiber formation or focal adhesion. Together, these results suggest that nocodazole-induced cell–cell adhesion occurs without RhoA activation, because stress fiber formation without cell–cell adhesion could be induced by RhoV12 expression and growth factor stimulation in HaCaT.

# 103

**Multi-Step Model of Cell–Cell Adhesion Induced by Microtubule Disruptions in Normal Human Epidermal Keratinocytes**

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Microtubule disruption induces cell-cell adhesion in normal human epidermal keratinocytes (NHEK) grown in low calcium conditions. However, E-cadherin immuno-staining showed heterogeneous patterns, such as double and single line of fluorescence. The double lines pattern could be observed predominantly in undifferentiated NHEK. Single line pattern was dominant in differentiating NHEK grown in high calcium up to 4 days, followed by dissociation with low calcium and nocodazole treatment. Precise time course analyses of cell–cell adhesion in moderately differentiating NHEK with E-cadherin and F-actin double staining revealed that the double line patterns were premature forms of cell–cell adhesion. Furthermore, it was found that microtubule disruption predominantly induced the intermediate form between double and single line form in supplements starved NHEK followed by 6 h culture in complete media. Using this NHEK arrested in steps of cell–cell adhesion, we analyzed various molecules which were known to be related to cell–cell adhesion. Our results showed that Rac1 played a role in initiation of cell–cell adhesion through mediation of lamellipodic protrusion over the focal adhesion plaque. In matured cell–cell adhesion (single line), rac1 was exactly colocalized with E-cadherin at cell–cell contact sites. For the maturation of cell–cell adhesion from double to single lines patterns, some tyrosyl phosphorylated proteins appeared to be involved. Together, our results suggested that cell–cell adhesion induced by microtubule disruption is a multistage process and each stage is modulated by different molecules.

# 104

**Cloning and Characterization of a Novel Short Chain Dehydrogenase/Reductase that is Abundantly Expressed in the Epidermal Keratinocytes**

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Vitamin A is known to profoundly influence the growth and differentiation of epidermal keratinocytes. The biological actions of vitamin A are throughout to be predominantly mediated by all-*trans* retinoic acid, which is biosynthesized from retinol by two oxidative reactions. The first and the rate-limiting step involves enzymatic conversion of all-*trans* retinol to all-*trans* retinaldehyde. Recently we described the first epidermal retinol dehydrogenase, a member of the short chain dehydrogenase/reductase family. Here we report the cloning of another retinol dehydrogenase which belongs to the same family of enzymes but differ substantially both in expression pattern and enzymatic characteristics. In contrast to hRDH-E, the new enzyme, hRDH-E2, is wildly expressed in both epithelial and nonepithelial tissues, including epidermis, heart, lung, placenta, brain and skeletal muscle but its transcripts are barely detectable in liver. Within the different cell types the primary transcript is processed into a variety of mature mRNA species, the predominant one in the keratinocytes being of about 1.5 kilobases. Human RDH-E2 transcripts are distributed throughout the epidermal layers but are more abundant in the proliferating basal cells. The steady state level of the epidermal transcripts is under a complex control by retinoic acid. Short treatment of keratinocyte cultures with 10 nM retinoic acid results in a transient several-fold increase in the mRNA. Prolonged incubations with retinoic acid, however, lead to a marked down-regulation of hRDH-E2 expression. When transiently expressed in keratinocytes under a CMV promoter, hRDH-E2 localizes in the microsomes. There, the enzyme is capable of converting both free- and CRBP-bound all-*trans* retinol to all-*trans* retinaldehyde, utilizing NADP as a preferred cofactor. The expression pattern and the biochemical properties of this new protein indicate that it might be one of the key enzymes that generate the active ligand for the retinoid function in the epidermal keratinocytes.

# 105

**EGF and KGF Stimulate Hyaluronan Synthesis and Inhibit Keratinocyte Differentiation in Organotypic Keratinocyte Culture**

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Hyaluronan is a major intercellular matrix molecule in the vital cell layers of skin epidermis. It has several physiochemical and biologic functions, such as matrix space filling, cell proliferation and migration, and it has been suggested to have a vital role in epidermal wound healing. An organotypic keratinocyte culture model was developed to examine the effects of the epidermal mitogenic growth factors EGF and KGF, and that of an anti proliferative factor, TGF-β, on the hyaluronan synthesis and epidermal morphology by using a rat epidermal keratinocyte cell line (REK) which exhibits histodifferentiation similar to that of the native epidermis when cultured at an air–liquid interface. EGF and KGF increased the number of vital cell layers and decreased the thickness of the stratum corneum. Furthermore, the expression of differentiation markers keratin 10 and filaggrin was reduced in these cultures. Hyaluronan synthesis was stimulated by EGF and KGF as studied with histochemical staining assay and quantitative PAGE, apparently due to up-regulation of hyaluronan synthase 2 (Has2). In contrast, TGF-β inhibited effectively the stratification of the epidermis. The expression of differentiation markers and hyaluronan synthesis were at the same level in TGF-β treated cultures as in controls. Taken together, the present data show that the agents stimulating hyaluronan synthesis support epidermal stratification but retard terminal differentiation, indicating that the expression of Has genes participates in keratinocyte differentiation.

# 106

**RelA-Associated Inhibitor (RAI) Regulates NF-κB in Differentiated Keratinocytes**

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RelA and p50, major subunits of NF-κB in keratinocytes, exist mainly in the cytoplasm of basal cells as an inactive complex with their inhibitory molecules, IκBs, and then translocate into the nucleus in suprabasal cells. We found that RelA protein was increased in the nuclei of differentiated keratinocytes under two different culture conditions: stratified keratinocytes induced by incubation with DME containing 10% FCS and suspension culture. However, NF-κB activity was not detected in these differentiated keratinocytes by gel shift assay, although the presence of RelA protein in the nuclei was confirmed by Western blot. Moreover, NF-κB activity induced by TNF-α and IL-1 was reduced in differentiated keratinocytes. This led us to hypothesize that RelA is inactivated by forming a complex with an intranuclear inhibitor. The candidate molecule is RelA-associated inhibitor (RAI). RAI was isolated in a yeast two-hybrid screen using the central region of RelA as bait. RAI is a novel protein located in the nucleus that binds to RelA and inhibits its transcription activity. We report that both cultured human epidermal keratinocytes and the epidermis expressed RAI mRNA and protein. In addition, RAI mRNA was increased about 2-fold 24 h after the induction of differentiation. The continuous and gradual increase of RAI protein in nuclei was observed from 24 (3-fold) to 96 h (5-fold), indicating that the synthesis of RAI protein is dependant on keratinocyte differentiation. Combined, these findings demonstrate that RAI is an essential regulator of NF-κB inactivation in differentiated keratinocytes.

# 107

**A Potential Role for Heparin Binding EGF-Type Growth Factors in Epidermal Morphogenesis**

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Amphiregulin, heparin binding-EGF, betacellulin, and heregulin-α and -β are heparin-binding members of the EGF family, each of which signals through one or more HER/erbB 1–4 receptors. Our previous evaluation of growth factor and receptor expression in normal and psoriatic epidermis showed that human keratinocytes labeled for each of the factors and for HER 1–3, but HER 4 labeling was absent. Colocalization by pair wise dual immunostaining of heregulins and betacellulin with the receptor HER 3 was to strata above the proliferative basal layer, suggesting a function not limited to cell proliferation but rather in epidermal morphogenesis or differentiation. We therefore characterized growth factor expression in keratinocyte cultures by regulating differentiation with medium calcium concentrations in serum free culture. Proliferation and differentiation were monitored by keratin 15 and 10 immunostaining, respectively. Imaging of immunostains was by epi-fluorescence, enhanced with deconvolution software to resolve the 3-D relationship of the factors. Confluent cultures in basal EpiLife medium with 0.06 mM calcium contained small islands of differentiated cells, whereas those switched to 0.2 mM calcium presented keratin 10-labeled, flattened cells blanketing the basal monolayer. Labeling for betacellulin was moderately, and for heregulin-α and -β exclusively, segregated to these differentiating foci. In contrast, HB-EGF labeled strongly in both compartments. The *in vitro* labeling patterns thus correlate with our prior *in vivo* data and support the general hypothesis that betacellulin and heregulins function in epidermal differentiation. The culture system should allow for experimental manipulations to further evaluate growth factor and receptor function in relation to epidermal morphogenesis.

# 108

**Ultrastructural Localization of Basigin in Normal Human Epidermal Keratinocytes**

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Using aλgt11 expression library of F9 embryonal carcinoma cells, we cloned basigin as a carrier of an oncodevelopmental carbohydrate marker which had the structure Galβ1→4(Fucα1→3) GlcNAc. It was identified to be a transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily and has been shown to play roles in intercellular recognition involved in the differentiation of various cells, tissues and organs. The purpose of the present study was to investigate whether basigin is associated with the differentiation of epidermal keratinocytes and localization of basigin. Expression was examined immunohistochemically and the ultrastructural localization was evaluated by immunoelectron microscopy using normal skin specimens ($n = 11$). By immunohistochemical staining, basigin was most strongly expressed on the membrane of basal cells, weakly expressed in the suprabasal layer, and absent in the prickle cells and horny layers, indicating a close association of basigin expression with the differentiation of keratinocytes. Immunoelectron microscopy showed the strongest labeling with gold particles on the membrane of basal cells, being consistent with those of the immunohistochemical study. Gold particles were observed on the apical and lateral sides but not on the dermal side on the membrane of basal cells. Interestingly, gold particles were mostly localized on the microvilli, especially on their tops ($p < 0.001$). There were significantly fewer gold particles on the intervillous area ($p < 0.001$) and none at the desmosomes and gap junctions. These findings suggest that basigin on microvilli participates in the differentiation of keratinocytes by coming into contact with adjacent cells.

# 109

**CD44 Interactions with $Ca^{2+}$ Signaling Molecules are Required for Keratinocyte Differentiation**

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CD44 belongs to a family of transmembrane glycoproteins known to be important in function of many cell types including keratinocytes. $Ca^{2+}$ is a major regulator of keratinocyte differentiation, and the failure of squamous cell carcinomas (SCC) to respond to $Ca^{2+}$ is a striking feature of keratinocyte transformation. In this study we have focused on CD44 interaction with several $Ca^{2+}$ signaling molecules [calcium sensing receptor (CaR) and two phospholipase C (PLC-β1 and PLC-γ1) in normal and transformed keratinocytes. Using immunoblotting, we have found that multiple species of CD44 isoforms are detected in both normal keratinocytes and SCC cell lines. In low $Ca^{2+}$ media undifferentiated keratinocytes express several CD44 isoforms (e.g. epican and several low molecular weight forms) in abundance. When keratinocytes become differentiated in high $Ca^{2+}$ media, the lower molecular weight forms cease to be expressed leaving epican as the dominant species. SCC cells either in low $Ca^{2+}$ or high $Ca^{2+}$ media continue to overexpress both epican and a number of lower molecular weight forms. Double immunofluoresence staining indicates that high $Ca^{2+}$ (but not low $Ca^{2+}$) increases the formation of CD44-associated intracellular adhesion and induces colocalization of CaR and PLC-β1/PLC-γ1 with CD44 in normal keratinocytes. In SCC cells, both PLC-β1 and PLC-γ1 are colocalized with CD44, but $Ca^{2+}$ fails to induce colocalization of CaR with CD44. These findings suggest that selective association of CD44 isoforms with certain $Ca^{2+}$ signaling molecules (e.g. CaR, PLC-β1 or PLC-γ1) in keratinocyte membranes is required for normal $Ca^{2+}$-mediated keratinocyte differentiation. Failure of CaR to interact with CD44 in SCC cells may be responsible for the inability of these cells to respond to $Ca^{2+}$ during malignant transformation.

# 110

**Activation of Notch Signaling in Keratinocytes (KCs) is Necessary and Sufficient to Create Mature Human Epidermis**

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The signaling system regulating the highly coordinated and programmed series of molecular events that generate a mature, fully differentiated and cornified epidermis is unknown. To determine if Notch ligands and receptors that regulate cell fate decisions in other organ systems may be operative in epidermis, normal human skin was initially examined by immunostaining and Western blot analysis. Fractionating KCs by state of maturation using discontinuous Percoll gradients, followed by immunoblotting confirmed immunohistology results in which KCs in suprabasal and mid epidermal levels prominently expressed a Notch ligand – Jagged 1 on the plasma membrane; which were the same KC layers coexpressing several Notch receptors; including Notch1, Notch3, and Notch4. Based on these localization studies, Jagged-1 appeared to be strategically located in epidermis to influence both early and late stages of KC differentiation. To establish a cause:effect relationship between notch signaling and KC differentiation, submerged living epidermal equivalents (EEs) consisting of multilayered but relatively undifferentiated KCs grown on a permeable membrane were raised to an air/liquid interface (A/L-I), which triggers KC terminal differentiation/corneogenesis. Western blot analysis of proteins extracted from submerged EEs vs. EEs raised to A/L-I for 2 days revealed an ~4-fold increase in levels of these notch ligands/receptors. This result prompted use of soluble notch ligands-peptides derived from the most conspicuously expressed ligand in human skin, Jagged-1. Exposing human KC monolayers to peptides synthesized to correspond to the most conserved DSL domain of hJagged1 (designated:JAG-1 or R-JAG), but not a scrambled control (SC) peptide, produced stratification and loricrin/involucrin expression. Moreover, addition of JAG-1 or R-JAG, but not SC, peptides to submerged EEs was sufficient to induce epidermal maturation resembling submerged ees raised to A/L-I. Finally, a soluble decoy notch inhibitor (rh11–12) prevented KC differentiation and corneogenesis when submerged ees were pretreated with inhibitor prior to being lifted to A/L-I. Overall, these results point to a key role for notch ligand/receptor-dependent signaling in governing the complex process of epidermal maturation.

# 111

**Global Transcriptional Changes in Keratinocyte Differentiation: DNA Array Analysis of Purified Basal and Differentiating Cells**

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Upon leaving the basal layer, keratinocytes discontinue the expression of basal and commence expression of differentiation markers. Specific markers for each cell type are known, but a systematic global analysis of the transcriptional changes has not been possible until the advent of DNA array technology. To exploit fully this powerful technique, we developed a new and efficient method for large-scale purification of basal and suprabasal keratinocytes from human epidermis. More than $10^7$ of each cell type was obtained and their purity ascertained using known markers, e.g. K10, K14 and K15 keratins, in Northern and Western blots. The two cell types differentially responded to certain extracellular signals, e.g. IL-1. We then examined the expression of 12 000 genes using the Affymetrix arrays platform. Some 5500 genes, 45% of the total, are scored as expressed in keratinocytes. Of these, over 600 are expressed differentially 2.5-fold or more comparing the basal and the differentiating population. The basal cells distinctively expressed several integrins, collagens and tenascin, expected basal markers, while the suprabasal cells expressed filaggrin, involucrin, sciellin and SPRs, known markers of differentiation. Only the basal cells expressed cell cycle proteins, reflecting their exclusive growth potential. Receptors for small diffusable factors were also predominantly expressed on the basal cells. Among markers of differentiating cells we found, inter alia, CD24, defensin, occludin, protease-activated receptors, as well as lipid metabolism enzymes. Both basal and differentiating cells express specific sets of regulatory proteins, transcription factors, protein kinases and phosphatases, presumably responsible for the observed changes in transcription. Our approach uncovered a large number of new markers for the two epidermal cell types and revealed many potential regulators of epidermal differentiation.

# 112

**Phosphatidylinositol 3-Kinase (PI3K) Regulates Early Phase Keratinocyte Differentiation**

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Previously, we showed that the apoptosis signal regulating kinase (ASK1)-p38 MAP kinase cascade regulates late phase keratinocyte differentiation. In this study, we investigate the intracellular signal transduction mechanisms of early phase keratinocyte differentiation. PI3K activates the Akt survival pathways in response to cell adhesion to the extracellular matrix. Adhesion signals are transmitted to intracellular signaling cascades via integrins through integrin-linked kinase (ILK) in a PI3K-dependent manner. Since epidermal keratinocytes undergo differentiation as they leave the basement membrane, we hypothesized that PI3K regulates early phase keratinocyte differentiation. In keratinocyte suspension culture using poly HEMA coated dishes, differentiation markers, such as keratin (K) 1, K10, involucrin, loricrin, and transglutaminase-1 mRNA were significantly induced within 24 h, as analyzed by ribonuclease protection assay. Transfection of the constitutively active form of PI3K (Myr-p110) using adenovirus vector almost completely abolished the induction of K1 and K10 mRNA in keratinocyte suspension cultures, while the induction of involucrin, loricrin, and transglutaminase-1 was not affected. This indicates that activation of PI3K prevents early phase differentiation. Furthermore, transduction of a dominant negative form of PI3K (Δp85) into keratinocytes resulted in significant morphological changes simulating differentiation. In these cells, K1 and K10 mRNA were induced 11- and 35-fold compared to the control, respectively, as analyzed by ribonuclease protection assay. On the other hand, the levels of involucrin, loricrin, and transglutaminase-1 mRNA expression were not altered. Wortmannin, a PI3K inhibitor, also enhanced the K1 and K10 mRNA expression. These data demonstrate that the activation of PI3K inhibits early phase differentiation, and that blockade of PI3K results in early phase differentiation. Therefore, PI3K is essential for the adhesion signal to suppress "default" differentiation mechanisms, which are inactivated by PI3K at the basal layer. We conclude that PI3K regulates early phase differentiation.

# 113

**Conditional Gene Targeting of APC (Adenomatous Polyposis Coli) Gene in Mouse Epidermis Reveals Critical Role of APC in Keratinocyte Differentiation/Survival and Follicular Development**

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APC protein functions as a negative regulator of β-catenin, which is a key molecule in wnt signaling pathway. Patients with APC gene mutations can develop tumors of follicular origin. Transgenic mice with β-catenin accumulation in epidermis display signs of de novo hair follicles formation. These findings suggest that APC protein plays a central role in keratinocytes growth and differentiation. To elucidate the function of APC protein in keratinocytes, conditional gene targeting was performed because germline APC gene ablation led to embryonic lethality. We disrupted the APC gene in epidermis by crossing APC conditional targeting mice with keratin14-Cre transgenic mice. Epidermis-specific APC gene knockout mice exhibited marked abnormalities with fragile skin. As a result of APC gene ablation, β-catenin was markedly accumulated in epidermis. Despite of β-catenin accumulation, mutant mice revealed defect of hair follicles. In contrast, β-catenin transgenic mice displayed de novo hair follicle morphogenesis. Moreover, aberrant terminal differentiation with apoptotic keratinocyte in epidermis was shown. In immunohistochemical analysis keratin10 and α6 integrin was markedly reduced, whereas almost normal expression of keratin14 was detected in the epidermis. We found no significant difference in keratinocyte proliferation. These observations suggest that APC protein plays an important role not only in keratinocyte differentiation/survival but also in hair follicular development.

# 114

**The Induction of Terminal Differentiation in Normal Human Keratinocytes by an Osmotic Stress**

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Terminal differentiation of normal human keratinocytes in culture has been shown to be controlled by a variety of factors. One factor that has not been investigated is osmotic stress. Since the keratinocyte is the most osmotically challenged cell in the body it must not only protect is's self from this insult but may also use this stimulus as a signal for differentiation. We have tested this hypothesis by using increasing molar sorbitol as an osmotic stress. With this we have observed that hyperosmolarity induced by sorbitol treatments will induce normal human keratinocytes to undergo terminal differentiation. Sorbitol was added to keratinocyte media to produce 50 mM, 100 mM, 200 mM and 300 mM final concentration. These concentration of sorbitol induce a omositc shock in the keratinocytes and dehydration effect. The sorbitol treatment increased the protein levels of keratin K1 and K10 and involucrin. The mRNA for these markers was also increase by the sorbitol treatment. Northern blots demonstrated an increased mRNA for SPR1 and transglutaminase in keratinocytes treated with sorbitol. RT-PCR analysis of mRNA in sorbitol and trehalose treated normal human keratinocytes appeared to have increased K1 and K10, transglutaminase, and involucrin. This data suggest that normal human keratinocyte may response to an osmotic stress by differentiating. This treatment may also prove to be a more *in vivo* like model of keratinocyte growth.

# 115

**Implication for Epithelial Sodium Channel (ENaC) in Epidermal Differentiation and Hair Follicles Development**

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The epithelial sodium channel (ENaC) constitutes the limiting step in Na+ absorption in tight epithelia such as kidney, colon and lung. This channel is also expressed in epidermis and hair follicles and its expression is enhanced as keratinocytes differentiate. Recently, we found that skin of newborn αENaC knockout mice showed epithelial hyperplasia, premature secretion of lipids, abnormal keratohyaline granules, and changed expression of differentiation markers, suggesting an important role for this sodium channel in epidermal differentiation processes. Since the perinatal lethality of αENaC knockout mice did not allow us to further study the consequences of an αENaC-deficiency in epidermal maturation or hair follicle development, we performed grafting experiments from skin of wildtype and αENaC knockout mice onto nude mice. We now report that in addition to the previous findings in newborn knockout mice, we observed a delayed onset of hair growth in the grafts from knockout mice compared to those from wild-type mice. Paradoxically, hairs from knockout grafts were noticibly longer than hairs from wild-type grafts. Scanning electron microscopy of grafts from knockout mice revealed a hair phenotype different from wildtype mice. These data suggest that ENaC expression in keratinocytes modulates ionic signaling important for epidermal differentiation like synthesis or processing of differentiation-specific proteins and contributes to hair development.

# 116

**Cross-Talk Between Notch Signaling and Two Pathways Regulating Epidermal Differentiation**

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Recently we established that peptides derived from the Notch ligand-Jagged-1 could trigger complete epidermal differentiation using submerged living epidermal equivalents (EEs). Moreover, using a selective inhibitor designed to block Notch receptor activity, epidermal differentiation was also blocked when submerged EEs were raised to an air/liquid interface. To determine the molecular basis by which Notch signaling triggered epidermal differentiation, focus was directed on established pathways that impact epidermal maturation –, i.e. NF-κB and PPAR. Addition of a peptide synthesized to correspond to the most conserved DSL domain of hJagged1 (designated JAG-1 peptide), but not scrambled control peptide, rapidly induced (7–60 mins) p50 and p65 translocation to the nucleus as detected by Western blot analysis, accompanied by increased DNA binding activity of NF-κB (EMSA), and transcriptional activity (luciferase reporter assay). Supershift analysis revealed both p65 and p50 subunits were present in the protein:DNA complex. Moreover, this DNA binding returned to baseline after 2 h, despite continued presence of intranuclear p65 subunits. This suggested a possible inhibitor of p65-mediated DNA binding activity. Since PPARγ has been previously observed by others to inhibit NF-κB transcriptional activity in monocytes, and because we previously observed that JAG-1 peptide could induce PPARγ in adipocytes, the ability of JAG-1 to induce PPARγ was explored. Addition of JAG-1 peptide to KCs also induced increased nuclear levels of PPARγ and PPARα between 30 min and 6 h. Moreover, in separate experiments in which both p65 and PPARγ were elevated, immunoprecipitation followed by Western blot analysis demonstrated physical association between p65 and PPARγ. Furthermore, using a dominant negative retroviral vector for NF-κB (i.e. IκBαDN), there was inhibition of PPARγ- induction indicating a link between NF-κB and PPARγ. Thus, we propose a series of reactions in which JAG-1 peptide initially activates Notch signaling, followed by NF-κB activation, and then PPARγ. A negative feed-back loop can be envisioned whereby the PPARγ can bind to p65 and thereby prevent excessive or uncontrolled NF-κB activation. These complex biochemical pathways suggest extensive cross-talk amongst several mediators of epidermal differentiation – Notch, NF-κB, and PPAR.

# 117

**Role of PKC Phosphorylation on Dlx3 Homeoprotein Function**

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The Dlx3 homeodomain protein is expressed in the granular layer of the stratified murine epidermis and potentially functions as a transcriptional activator. Reports have shown the essential role of the Protein Kinase C (PKC) pathway in the up-regulation of late differentiation structural genes. We have investigated whether PKC can modulate the function of the Dlx3 protein. Using *in vitro* kinase assays, we show that recombinant PKC enzymes phosphorylate Dlx3. Using keratinocytes nuclear extracts for the kinase reaction, we determined that Dlx3 protein is phosphorylated, and this event is inhibited by the PKC-specific inhibitor GF109203X, suggesting that Dlx3 is phosphorylated by PKC *in vivo*. From the PKC isoforms present in the epidermis (α, δ, ε, η, ζ), we show that Dlx3 is primarily phosphorylated by recombinant PKCα. By deletional, mutational and mass spectrometry analysis of the Dlx3 protein, we have identified the serine residue $S^{138}$ located in the homeodomain region of Dlx3 to be specifically phosphorylated by PKC. The phosphorylation of purified Dlx3 by PKCα decreased the formation of complexes between Dlx3 protein and DNA. Furthermore, the transcriptional activation of Dlx3 was reduced in transfected keratinocytes overexpressing PKCα. Altogether, these results suggest that Dlx3 protein can be directly phosphorylated by PKC and this phosphorylation affects the DNA binding and transcriptional activity of Dlx3.

# 118

**Overexpression of Smad7 in the Epidermis of Transgenic Mice Results in Developmental Defects in the Epidermis and its Appendages**

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Biochemical studies have shown that Smad7 blocks signal transduction of the transforming growth factor β (TGF β) superfamily. However, the physiological functions of Smad7 are largely unknown. We have shown that Smad7 is transcriptionally elevated by TGF β in keratinocytes, and its expression is increased during skin carcinogenesis. To determine the functions of Smad7 in the skin, we have generated transgenic mice expressing Smad7 in the epidermis, utilizing a targeting vector based on the truncated keratin K5 promoter. The Smad7 transgene was predominantly expressed in the epidermis and hair follicles. K5.Smad7 mice were born with sparse and curly whiskers. In comparison with nontransgenic controls, neonatal transgenic skin exhibited fewer and smaller hair follicles, which showed delayed expression of hair follicle specific keratin K6. By day 7, K5.Smad7 pups exhibited a thickened and wrinkled skin, and histologically the epidermis was markedly hyperplastic and hyperkeratotic. K5.Smad7 pups also showed retarded growth and most founders died within the first week of life. Only the low expressors have survived to adulthood. The adult K5.Smad7 mice also exhibited severe retarded growth. The first coat of hair, normally apparent by 1 week of age in nontransgenic littermates, was delayed until approximately 3 weeks of age in K5.Smad7 mice. K5.Smad7 mice began to lose their hair and whiskers at 5–8 weeks of age, which never grew back. Epidermal hyperplasia worsened in adult K5.Smad7 mice. In contrast to neonatal K5.Smad7 skin, the number and size of hair follicles in adult K5.Smad7 skin were increased, however, they were disoriented. K5.Smad7 mice also exhibited hyperplasia in sebaceous gland, and the lobules grouped around the enlarged gland duct were increased. BrdU labeling in K5.Smad7 epidermis, hair follicles and sebaceous glands showed a significant increase compared to controls. However, plucked hairs from K5.Smad7 mice showed a thinner hair shaft compared to nontransgenic controls. Our study provides *in vivo* evidence that deregulation of Smad7 in the skin perturbs proliferation, differentiation and development of the epidermis and its appendages, possibly by blocking signaling of the TGF β superfamily members.

# 119

**Expression of Caspase 14 During Murine Fetal Development**

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Caspase 14 is a skin specific caspase that is expressed in the granular layer of the epidermis and in the infundibulum of the hair follicle. In cultured keratinocytes, caspase 14 is expressed in conditions that stimulate differentiation, as determined by expression of suprabasal keratins and profilaggrin, but not in proliferating basal keratinocytes. Caspase 14 is not activated in submerged, cultured keratinocytes, but rather is activated in skin and organotypic cultures, indicating that it functions late in terminal differentiation. During murine development, caspase 14 mRNA is detected briefly and in low abundance at embryonic day 7, then again at day 15 through birth at high levels. To characterize the tissues where caspase 14 protein is expressed during mouse embryogenesis, we performed immunohistochemistry on whole embryos at days 14, 16, and 18 days of development. Caspase 14 protein appeared between embryonic day 16 and 18 in the suprabasal layers of cornified epithelium including dorsal skin, tail and paws. No caspase 14 protein was detected in any other organs, including other epithelia, nor was any caspase 14 detected in mucosal epithelia. Caspase 14 expression appears at the same time that murine skin begins to fully differentiate into a cornified epithelium and is not expressed in noncornified epithelia. The expression pattern of caspase 14 during mouse development strongly suggests it functions during stratum corneum formation.

# 120

**PPAR-δ Ligands Stimulate Epidermal Differentiation**

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The PPARs are members of the nuclear hormone receptor superfamily that heterodimerize with RXR. Previous studies demonstrated that ligands of PPAR-α stimulate epidermal differentiation and inhibit keratinocyte proliferation. Another member of the PPAR family, PPAR-δ, is present in keratinocytes, but its function is unknown. Here, we report the effect of a PPAR-δ ligand, GW1514, on keratinocyte growth and differentiation. In cultured keratinocytes, the PPAR-δ ligand increased mRNA (determined by Northern analysis) and protein levels (Western) of involucrin and transglutaminase-I up to 5-fold, indicating that this compound stimulates differentiation. However, DNA synthesis (thymidine incorporation) was not altered in cultured keratinocytes treated with the PPAR-δ ligand. Topical treatment of mice with GW1514 resulted in increased expression of filaggrin and loricrin (detected by immunohistochemistry) coupled with an increase in epidermal thickness. Yet, PCNA labeling, a marker of cell proliferation, was unchanged. Similar changes were seen when GW1514 was applied to PPAR-α (–/–) knockout mouse skin, indicating the specificity of this ligand for PPAR-δ. As seen in previous studies, repeated barrier disruption by topical acetone treatment induced epidermal hyperplasia and reduced the expression of keratinocyte differentiation markers. Treatment of mice with GW1514 normalized the expression of the differentiation markers loricrin and filaggrin, but GW1514 did not decrease epidermal hyperplasia. These results demonstrate that PPAR-δ ligands stimulate epidermal differentiation without altering proliferation. PPAR-δ ligands may be useful in the treatment of skin disorders characterized by impaired differentiation.

## 121

**The *Srcasm* Molecule: A Src-Family Tyrosine Kinase Substrate with Features of an Adaptor Molecule**

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The Fyn tyrosine kinase is important for proper keratinocyte differentiation *in vitro*. To identify molecules which interact with Fyn and potentially constitute a signaling pathway controlling differentiation, we performed a yeast two-hybrid screen and identified a novel substrate for Src-family tyrosine kinases (SFKs) called Srcasm: Src activating and signaling molecule. *In situ* hybridization analysis for mRNA demonstrates that Srcasm is coexpressed with Fyn in differentiating keratinocytes. Immunofluorescence studies show colocalization of Srcasm with Fyn in cytoplasmic punctate structures. The N-terminus of Srcasm encodes a VHS domain which localizes proteins to intracellular membrane compartments, including endosomes. Srcasm also contains closely spaced motifs in its C-terminus that form ligands for the SH2 and SH3 domains of SFKs. Using GST pulldown assays, we demonstrate that binding between the Srcasm ligand motifs and Fyn SH2 and SH3 domains is synergistic and dependent on Srcasm phosphorylation. Based on the structure of SFKs, it has been postulated that an activator of the kinase will contain colocalized SH2 and SH3 ligand motifs as is found in Srcasm; these motifs activate Fyn by displacing the C-terminal negative regulatory tyrosine, allowing the molecule to open into its active configuration. As predicted, addition of Srcasm to *in vitro* kinase assay stimulates Fyn kinase activity in a dose dependent manner. Additional GST pulldown experiments suggest that Srcasm interacts in a phosphorylation dependent manner with Grb2 and the regulatory subunit of PI3-kinase, p85, which are important regulators of cell proliferation and differentiation. Thus, the interaction of Srcasm with Fyn may be an important step for initiating keratinocyte differentiation.

## 122

**Expression of Estrogen Receptor β in Human Skin**

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A second estrogen receptor, ER β, was discovered in 1996. The expression of ER α and ER β vary in different organ systems. The importance of estrogen signaling in human skin is uncertain, despite the many changes that occur in skin with hormonal stimulation. Immunohistochemical studies have shown some evidence for the presence of ER β in skin. We isolated keratinocytes, fibroblasts and melanocytes in tissue from normal skin obtained during circumcisions, breast reduction and abdominoplasty surgeries. Tumor cell lines were either purchased commercially or isolated at the time of excision. Protein purified from tissue culture cell lysates was analyzed with Western blots using a rabbit polyclonal antibody (Upstate Biotechnology) directed toward the N-terminal region of human ER β, which is common to all of the five isoforms. ER β protein was detected in adult and neonatal keratinocytes and fibroblasts. The breast cancer cell lines, HCC and MCF-7, served as positive controls. mRNA was isolated from the same cell types in tissue culture and a single strand of cDNA was synthesized using reverse transcriptase. Polymerase chain reaction (PCR) was performed using an ER β primer pair located within a common region of the gene to detect expression of any of the five potential isoforms. PCR confirmed the expression of ER β in neonatal and adult epidermal keratinocytes and fibroblasts, and in two melanoma cell lines, WM115 and WM266-4. The presence of ER β in normal skin and some skin cancer cell lines provides a foundation for further research into steroid hormone regulation of cutaneous neoplasms and other skin disorders. Furthermore, modulation of signaling through estrogen receptor pathways may provide a molecular target for future therapeutic interventions.

## 123

**Differences in Phospholipase D Activation Profiles in Response to Inducers of Primary Mouse Epidermal Keratinocyte Differentiation**

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Two isoforms of phospholipase D (PLD), PLD1 and PLD2, have been identified and characterized *in vitro*. However, the methods utilized to monitor PLD activity in intact cells have to date largely prevented the measurement of a single PLD activity in cells possessing both isoforms. We have previously shown that *in vitro* PLD2 can metabolize phospholipids in the presence of glycerol to yield phosphatidylglycerol. Further, we demonstrated that a 24-h treatment of primary murine epidermal keratinocytes with elevated extracellular calcium levels, but not 1,25-dihydroxyvitamin D, elicited a PLD activity that could generate radiolabeled phosphatidylglycerol from radioactive glycerol. Our present data indicate that the inability of 1,25-dihydroxyvitamin D to elicit phosphatidylglycerol production is not the result of differences in glycerol uptake. Thus, while 1,25-dihydroxvitamin D did indeed inhibit glycerol uptake, elevated extracellular calcium inhibited uptake to the same extent. This inhibition of glycerol uptake by both agents was likely due to their observed ability to inhibit the expression of aquaporin-3, a water/glycerol channel. Moreover, short-term treatment of keratinocytes with the phorbol ester, 12-O-tetradecanoyl-phorbol 13-acetate (TPA), failed to activate the glycerol-utilizing PLD activity. Since (1) we have shown that 1,25-dihydroxyvitamin D increases the expression/activity of PLD1 and (2) TPA is thought to activate PLD1 to a greater extent than PLD2, we speculate that in keratinocytes radiolabeled phosphatidylglycerol production upon exposure to glycerol is a measure of PLD2 activation. Thus, this assay may provide a means to monitor the activity of a single PLD, PLD2, in an intact cell system possessing both PLD isoforms.

## 124

**Hyaluronan Production by Epidermal Keratinocytes: Up-regulation During Differentiation of Rat Keratinocytes in an Artifical Epidermis at the Air–Liquid Interface**

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Hyaluronan (HA) is an ubiquitous extracellular macromolecule that acts as a space-filler and matrix organizer. HA, abundant in the dermis, is now also recognized as a prominent component in the epidermis, where it plays an important role in maintaining intercellular spaces that facilitate the trafficking of nutrients, growth factors, and immune cells. To further understand the role of HA during epidermal formation, we studied HA biosynthesis in a rat keratinocyte (REK) cell line that differentiates in organotypic culture. Cells were seeded on reconstituted collagen fibrils (rat, type I) polymerized on polyester membranes (3 micron pore size) in a plastic culture insert. Cells were grown submerged until they reached confluence, at which time they were maintained at the air–liquid interface. Within 5 days, the REKs differentiated into a multilayer epidermis displaying well-defined basal, spinous, and granular layers, and a well-formed stratum corneum. To establish a time course of differentiation and of HA production, cultures were harvested daily over 5 days. Specimens fixed and treated for H&E staining and immunohistochemistry demonstrated well-accepted markers of epidermal differentiation (keratin 10, loricrin, and involucrin, all in the suprabasal layers) as well as the presence of hyaluronan between the cells of the basal, spinous, and (to a lesser extent) granular layers. Time-dependent increases in K10, loricrin, and involucrin were confirmed by protein extraction and Western blotting. HA contents in the cell layer and in the medium compartment were assessed using Fluorescence-Assisted Carbohydrate Electrophoresis (FACE) technology. The amount of HA in the cell layer increased markedly during the differentiation process, rising from 65 to 387 pmoles disaccharide/culture (or 1.7–4.0 pmoles disaccharide/microgram of DNA) between day 1 and 5, respectively. Interestingly, the largest effect on HA biosynthesis was evident in the first 24 h of lift culture (increasing from 65 to 162 pmoles disaccharide/culture), prior to the first morphological evidence of differentiation. Thus, our data suggest that HA production increases markedly as an early effect of keratinocyte differentiation, and may play a part in the subsequent events (upward migration, stratification of keratinocytes) during epidermal formation.

## 125

**Epidermal Growth Factor (EGF) Stimulates Keratinocyte Hyaluronan (HA) Synthesis by Increasing Has2 mRNA Level**

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HA is an abundant and rapidly turning over major matrix molecule between the vital cell layers of epidermis. HA is involved in several cellular functions, such as stimulation of proliferation and migration, and produces an environment favorable for migration. EGF induced a coat of HA, and a 3–5 fold increase in its synthesis rate in a rat epidermal keratinocyte cell line that has retained the ability for differentiation. EGF also increased HA in perinuclear vesicles, suggesting concurrent enhancement in HA endocytosis. Cell associated HA was most abundant in cells stimulated to migrate by EGF. RT-PCR showed no significant changes in Has1 and 3, while Has2 mRNA increased 2–3 fold in less than 2 h after addition of EGF. The average level of Has2 mRNA increased from ~6 copies/cell in cultures before change of fresh medium, up to ~54 copies/cell after 6 h in EGF-containing medium, as estimated by quantitative RT-PCR with a truncated Has2 cRNA internal standard. A control medium with 10% serum caused a maximum level of ~21 copies/cell at 6 h. The change in the Has2 mRNA levels and the stimulation of HA synthesis followed a similar temporal pattern, a finding in line with a predominantly Has2 dependent HA synthesis in epidermal keratinocytes.

## 126

**Gene Expression of the Water Channels, Aquaporins, in Human Keratinocyte and Skin-Equivalent Cultures, and Osmotic Stress Induction of Aquaporin-3 mRNA in Cultured Keratinocytes**

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Aquaporins (AQPs) are water channel proteins expressed in diverse tissues, and 10 members of the AQP family (AQP0-AQP9) have been cloned. The mRNA expression and their regulation of AQP1, AQP3, AQP4 and AQP9 in human keratinocyte and skin-equivalent cultures were examined by an RNase protection assay. AQP3 mRNA was expressed in growing and differentiating cells under monolayered conditions, while AQP9 mRNA was only detected in differentiating cells. Epidermis in a skin-equivalent culture expressed both AQP3 and AQP9 mRNA. However, neither AQP1 nor AQP4 mRNA was detectable in either the monolayered or skin-equivalent culture. Incubation of keratinocytes in sorbitol-added hypertonic medium at both growth and differentiated phases increased AQP3 mRNA expression. This was confirmed by using an ionic solute, NaCl, and other nonionic solutes such as mannitol, glucose and sucrose at the growth phase, whereas the addition of urea, a relatively permeable solute, decreased the AQP3 expression. The effect of sorbitol was reversible and maximal at 24 h after the addition and 200 mM of the concentration. These findings indicate that an osmotic pressure gradient is required for induction. However, the mRNA expression of AQP1, AQP4 and AQP9 was unchanged under any of the hypertonic conditions examined. Based on these findings, we propose that AQP3 in human keratinocytes may play a role in maintenance of intracellular osmolality and cell volume against the possible water loss in epidermis.

# 127

**Isolation of RNase P Activity from Human Epidermal Keratinocytes**

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Ribonuclease P (RNase P) is a key enzyme in tRNA biogenesis that catalyzes the endonucleolytic cleavage of nearly all tRNA precursors to produce the mature 5 end. RNase P activity has been found in prokaryotic and eukaryotic cells. RNase P enzymes are composed of protein and RNA; in bacteria and some archaea the latter component alone is sufficient for activity. So far, human RNase P has been partially characterized only from HeLa cells and there is no available information on tRNA biogenesis in human skin. We found it therefore of interest to attempt to isolate RNase P activity from normal human epidermal keratinocytes (NHEK) grown in serum-free keratinocyte growth medium. NHEK cell pellets were homogenized in a glass homogenizer. After centrifugation the nuclear pellets were resuspended- in ice-cold buffer and vigorously shaken. Nuclear extract was loaded on an ion-exchange column and RNase P was eluted with a linear gradient of 50–500 mM KCl. The substrate for RNase P assays was an *in vitro* 32P labeled transcript of the S. pombe tRNASer gene SupS1. Enzyme assays were carried out at 37°C for 30 min in 20 µl buffer (30 mM Tris-HCl pH 7.5, 100 mM $NH_4CL$, 5 mM $MgCl_2$, 0.1 mM â-mercaptoethanol). Reaction products were resolved on denaturing 10% polyacrylamide/8 M urea gels and visualized by autoradiography. The isolated RNase P activity represents an experimental system for *in vitro* and *in vivo* studies on tRNA biogenesis in human epidermis. This system may allow the determination of the role of this enzyme in the pathogenetic mechanisms of keratinization disorders and its response to therapeutic agents.

Acknowledgements: This work was supported in part by the Greek Government (General Secretariat of Research and Technology, YPER grant).

# 128

**Expression and Regulation of Polyspecific Membrane Influx and Efflux Transporters and CYP-Enzymes in Proliferating Human Epidermal Keratinocytes**

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The aim of this study was to analyze the expression pattern of polyspecific membrane transporters such as OATP A-E, P-gp and MRP1-6, and CYP-enzymes in human epidermal keratinocytes under constitutive conditions and after induction with various inducers and cytokines. RT-PCR analysis of transport proteins revealed the constitutive expression of MRP1, MRP3, MRP4, MRP5 and MRP6, as well as OATP-B, -D and -E and CYP1A1, 1B1, 2B6, 2E1 and 3A5 in human epithelial keratinocytes and was negative for MDR1, MRP2, OATP-A and OATP-C. Expression of MDR1 and CYP3A4 was seen after induction with dexamethasone. RT-PCR results were confirmed by northernblots, cDNA microarrays, immunoblots and immunohistology. The localization of all CYP-enzymes was restricted to the cytoplasm of the keratinocytes and transport proteins were expressed in the cell membrane. Constitutive activity of CYP 1A1, 2B, 2E1 and 3A enzymes was measured by catalytic assays. In contrast to hepatocytes and tumor cell lines incubation of keratinocytes with various cytokines revealed no down-regulation of transport protein- and CYP-enzyme- expression and band shift assays showed a STAT1 pathway activation for INFa, IFN gamma and TNFa. These results show that keratinocytes of the human skin express various transport associated- and detoxifying metabolic enzymes. This is extremely interesting, because previous studies have revealed, that CYP enzymes and transport-associated proteins play complementary roles in drug disposition by uptake (phase 0), biotransformation (phase I) and antitransport (phase III). Especially the discovery of the expression of organic anion transporting polypeptides seems to be a complete new factor in human skin barrier.

# 129

**The Effects of Vitamin D3 Analogues on Heat Shock Hsp70, mRNA and Protein Levels**

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Heat shock proteins (HSPs) are a family of conserved proteins that can be induced in all organisms upon exposure to stressful conditions such as elevated temperatures. A number of toxic compounds have been shown to induce HSPs. This list includes sodium arsenate, cadmium salts, sodium salicylate and alcohol (Liu *et al* 1994). 1,25-dihydroxyvitamin D3 has been shown to increase heat shock protein and response in human monocytes (Kantengwa *et al* 1990, Krane *et al* 1990, Healy *et al* 1987). In cell culture experiments, 1, 25-dihydroxyvitamin D3 is well documented to inhibit growth and increase keratinocyte differentiation. 1,25-dihydroxyvitamin D3 has been shown to increase p53 and c-fos expression in human keratinocytes (Sebag *et al* 1994). We investigated the effects of a variety of vitamin D3 analogues; 2-methylene-19-nor-(20S)-1a, 25(OH) 2-D3, 1a, 25(OH) 2–22E-dehydro-24-dihomocalciferol, 1-a, OH-homopregnacalciferol, 19-nor-20 (S)-1a, 25(OH) 2-D3, 19-nor-14-epi-1a, 25(OH) 2-D3, to induce HSP protein mRNA in normal human keratinocytes. The keratinocytes were treated for 3 h with the vitamin D3 analogue. 1a, 25(OH) 2–22E-dehydro-24-dihomo-calciferol ($10^{-6}$ M) and 1aOH-homopregnacalciferol induced Hsp70A by 60% and 45%, respectively. Little or no effect was observed for the other vitamin D3 analogues on HSP70A. However all vitamin D3 analogues tested increased mRNA for Hsc 70 and, Hsp90β, Hsp90a. The largest increases (140%) were observed in the Hsp90a. The levels of Hsp27 mRNA were also increased by the vitamin D3 analogues but more modestly. The effects of these vitamin D3 analogues on heat shock protein mRNA may reflect on the inherit toxicity of these compounds and help delineate their mechanism of action topically.

# 130

**Decrease of Environmental Humidity Down-Regulates Filaggrin Expression via Oxidative Stress and Induces Skin Surface Dryness**

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Effects of drastic decrease of environmental humidity were studied on filaggrin and amino acid processing in hairless mice epidermis and human keratinocytes. Skin surface conductance and free amino acid contents in the stratum corneum of mice, which were transferred from humid (80% relative humidity) to dry (< 10% relative humidity) environment, were significantly lower than those of the mice transferred from normal environment (relative humidity = 40–70%) to dry environment. Moreover, filaggrin, which is the main precursor of free amino acids in the stratum corneum, also decreased in the epidermis of the mice transferred from humid to dry environment. In the cultured human keratinocytes, both protein and mRNA of profilaggrin decreased significantly when the cultured cells were exposed to air. Oxidative level in the keratinocytes was increased by the air-exposure. Furthermore, antioxidants, vitamin C and glutathione inhibited the down-regulation of filaggrin induced by the air-exposure in the cultured keratinocytes. These results suggested that drastic decrease of environmental humidity down-regulated filaggrin expression in the keratinocytes via oxidative stress, and that consequently skin surface dryness was induced by the reduction of free amino acids in the stratum corneum.

# 131

**Abnormal Processing of Filaggrin in GPI-Anchor-Deficient Epidermis**

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Glycosylphosphatidylinositol (GPI)-anchored proteins are widely distributed on the exterior surface of eukaryotic plasma membranes, including both epithelial and endothelial cells of the skin. In GPI-anchor-deficient (Pig-a null) mice, we have found a disruption of the epidermal barrier function associated with abnormal lamellar membrane structures in the stratum corneum. This study focused on epidermal proteins in Pig-a null mice. Western blotting analyses showed a defect of filaggrin monomer with normal expression of profilaggrin in the epidermis of Pig-a null mice, indicating impaired conversion of profilaggrin to the monomeric form. However, two cornified envelope proteins, involucrin and loricrin, were expressed normally. In addition, the catalytic activity of protein phosphatase 2A (PP2A), which is thought to be implicated in the processing of profilaggrin to filaggrin, was significantly decreased in Pig-a null mice, while the actual amount of PP2A remained unchanged. These results suggest that the GPI anchor or GPI-anchored proteins are involved in the processing of profilaggrin to filaggrin in the epidermis. Moreover, in these findings and in previous results, it was confirmed that the epidermis of Pig-a null mice showed a number of pathological changes coincident to those in Harlequin Ichthyosis (HI), namely, hyperkeratosis, defective lamellar granules, the absence of normal SC lamellae, defective filaggrin monomer, and a decrease in PP2A catalytic activity. Pig-a null mice may be a useful animal model for understanding the role of filaggrin in normal epidermis and human skin disorders such as HI.

# 132

**Expression of Filaggrin mRNA and Protein During Skin Aging**

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Filaggrin is a component protein of keratohyalin granules (KHG) and a precursor of amino acids in the stratum corneum. Amino acids are one of natural moisturizing factors of the stratum corneum. We examined immunohistochemical change of filaggrin protein during skin aging and got the result that filaggrin protein decreased in the aged skin, especially in the extensor surface of lower leg. In order to investigate the change of the expression of filaggrin during skin aging, RT-PCR, immunoblotting analysis and immunohistochemical studies using young and aged epidermis were performed. The expressions of filaggrin mRNA in young and aged skin were similar but the immunoblotting analysis using antihuman filaggrin monoclonal antibody revealed that the amount of filaggrin protein in the aged epidermis was lower than that in the young epidermis, which was the same result with immunohistochemical observation using antifilaggrin antibody. Because these findings suggested that the decrease of filaggrin protein might be caused by the promotion of filaggrin degradation, the localization of cathepsin L which is a protease for degradation of filaggrin protein was compared between the young and aged skin using anticathepsin L antibody. The positive reaction by anticathepsin L antibody was observed in the aged skin where the positive reaction by antifilaggrin antibody was not observed, and the reaction by anticathepsin L antibody was not observed in the young skin. Therefore, the decrease of filaggrin protein in the aged skin might be not suppression of filaggrin synthesis but promotion of filaggrin degradation.

# 133

**Withdrawn**

# 134

**Ultrastructural Analysis of Keratin 19 and Keratin 14 in Mouse Vibrissae Revealed Distinct Organisation of the Intermediate Filament Network**

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Keratins (K) are intermediate filaments present in epithelial cells and they are expressed in a cell-type and a differentiation-specific manner. We have already shown that, in skin epithelium of mice and humans, the expression of K19 is restricted to basal keratinocytes with stem-cell characteristics. K14 is expressed in basal cells. The aim of this study was to determine the ultrastructural organization of the filament network in cells expressing K19 and/or K14 in mouse skin. Double immunostaining of longitudinal frozen sections of FVB/N mouse vibrissae were performed for K19 and K14 with secondary antibodies coupled to 5 or 10 nm colloidal gold particles. The ultrastructural observations revealed that K19-expressing cells are located in the bulge region of the vibrissae, and possess a loose filament network. Moreover, these K19+ cells expressed a very low level of K14. Alternatively, K14-expressing cells possessed rather a denser intermediate filament network with small bundles of keratin. These observations support the data obtained with human cutaneous cells indicating that the K19 expression correlates with a lower level of K14 and a loosely arranged keratin filament network. They are consistent with previous biochemical studies showing that K19 possesses distinct assembly properties. In conclusion, K19 probably contributes to the undifferentiated stem cells phenotype of the cells expressing it.

# 135

**Cloning and Characterization of a Novel Keratin Binding Protein, Occulibin, which Links Keratin Filaments to the Nuclear Envelope**

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Keratins are the major cytoskeletal filaments, which spread all through the cytoplasm of the epithelial cells. Keratin intermediate filaments (KIF) bind to the desmosomal and hemidesmosomal apparatus at cell periphery, however, few proteins are yet identified to interact with KIF at the nuclear surface. We thus attempted to find out such keratin binding proteins by yeast two-hybrid system. Employing the head domain of type I keratin as a bait, we screened the human skin cDNA library and obtained a candidate clone. The isolated cDNA consists of approx. 4.0 kbp including relatively large 5Õ untranslating region of 1.0 kbp. The predicted gene product is a 108-kDa protein encoded by 970 amino acids lacking for any significant homology or similarity with known proteins. This protein, designated as occulibin, contains one signal sequence motif at its N-terminus, and several nuclear localization signals as well as one putative transmembrane segment. Northern blot analysis revealed that occulibin was expressed not only in the epithelial tissues including skin but also in nonepithelial tissues such as heart and muscle. Occulibin shows the binding capability with type I keratins, K14 K16, K18, but not with type II keratin, K5, K6, K8 nor vimentin in the yeast system. When occulibin tagged with green fluorescence protein (GFP) or FLAG, was forced to express into cultured keratinocytes, the GFP and FLAG signals are observed predominantly along with the nuclear envelope. The perinuclear staining was overlapped with keratin localization at the nuclear surface in the confocal-microscopic analysis. These data suggest that this newly identified gene product, occulibin, might encode an outer nuclear envelope protein, which binds the KIF to the nuclear membrane.

# 136

**Mass-Spectroscopic Identification of a Mutant Keratin 1 Protein Assosiated with Ichtyosis Hystrix Curth-Mackin**

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Recently we identified a severe form of epidermal hyperkeratosis known as ichthyosis hystrix Curth-Macklin. The is caused by the first known pathogenic mutation in the variable tail domain (V2) of a keratin gene (KRT1). The mutation results in a frameshift and early termination, producing a truncated alanine-rich tail domain instead of the wildtype glycine-rich motif. This is expected to cause a profound abnormality of the cytoskeletal architecture in suprabasal keratinocytes. Here we demonstrate that the truncated form of keratin 1 (K1) is indeed expressed and present in the skin of an affected patient. Keratins were extracted from skin scales and resolved on an 8% SDS gel. After in-gel digestion with trypsin, peptide fragment maps from individual bands were obtained by analysis of peptide mixtures on a MALDI-TOF spectrometer. A truncated form of K1 was identified at 60 KDa, yet peptides from the C-terminal 80 amino acids were not observed – due to *in vivo* proteolysis or lack of digestion sites. Double digestion with trypsin and endoproteinase Glu-C in the presence of 5% acetonitrile, 0.01% SDS was performed resulting in enhanced recovery of peptides. Digests were spotted on a MALDI-TOF spectrometer sample plate coated with nitrocellulose. Interfering salts were washed away and spectra were obtained. Two peptides with masses 1073.53 and 1453.81 were identified, corresponding to mutant peptides VAAAAMAATAPE and AAAAAALAAGALAAGALE. The presence of the extended hydrophobic alanine-rich tail domain is expected to result in self-aggregation of keratin filaments, and/or altered interactions with other glycine-rich proteins including keratin 10 and loricrin.

# 137

**Evidence for Protofibrils in Keratin Intermediate Filaments**

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The packing of the constituent molecules in some fibrous proteins such as collagens and intermediate filaments (IF) is thought to consist of several hierarchical levels, the penultimate of which is the organization of subfilamentous units termed protofibrils. However, to date only indirect evidence, such as electron microscopic images of unraveling fibers or the existence of mass quanta, has been adduced in support of the existence of protofibrils. We have re-examined this issue in IF. Crosslinks have been induced in reduced and oxidized forms of Type Ia/IIa trichocyte keratin IF, and reduced K5/K14 cytokeratin IF using improved conditions for assembly and peptide fractionation. New crosslinks have been identified and characterized. Several crosslinks were shown to correspond to links between molecules four rows apart on two-dimensional surface lattice models. In addition, in the reduced IF, crosslinks were found between any four rows of molecules in groupings of six or more. Thus our data affirm and provide robust support for the concept of an eight-chain (four-molecule) protofibril substructure in keratin IF. Furthermore, there may be redundancy or promiscuity in this arrangement since any adjacent grouping of four rows of molecules could bundle together to form a protofibril. Such data might explain the difficulty of prior reproducible visualization of protofibrils in microscopic images. Finally, our data are consistent with IF most commonly containing four such protofibrils.

# 138

**Induction of Keratin K6 by IFNγ Occurs not only Through STAT1 but also Through IL-1α Released from Keratinocytes and Downstream NFκB**

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Keratinocytes contain various cytokines, which are produced and released upon injury by external stimuli to initiate inflammation. IL-1α is, among such cytokines, the major cause of early phase of inflammation. Not only mechanical injury but also cytokines and growth factors cause keratinocytes to release IL-1α. We have shown previously that IFNγ induces keratin K6 in epidermal keratinocytes. In order to elucidate the signaling pathways of IFNγ on keratinocytes, we measured supernatant IL-1α concentration of HaCaT keratinocytes stimulated by IFNγ. Up to 50 pg per ml of IL-1α was detected in the supernatant. Next to investigate the role of IL-1α in keratin K6 induction by IFNγ in HaCaT keratinocytes, we tested antibodies, dominant negative constructs, and antisense oligonucleotides to block the signal transduction pathways initiated by IL-1α and IFNγ. Western blotting using the insoluble keratin fractions from HaCaT cells revealed that anti-IL-1α antibody inhibited expression of keratin K6 protein. Dominant negative STAT1, antisense oligonucleotides for NFκB inhibited K6 induction by IFNγ, but antisense oligonucleotides for C/EBPβ did not. These results suggest that induction of K6 by IFNγ needs not only STAT1, but also IL-1α released from keratinocyes and its downstream NFκB.

# 139

**A Hot-Spot Mutation Alters the Mechanical Properties of Keratin Filament Networks**

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Keratins 5 and 14 polymerize to form the intermediate filament network in the progenitor basal cells of many stratified epithelia including epidermis, where it provides crucial mechanical support. Inherited mutations in K5 or K14 result in epidermolysis bullosa simplex (EBS), a skin fragility disorder. The impact that such mutations exert on the intrinsic mechanical properties of K5-K14 filaments is unknown. To address this issue we compared the mechanical properties of wild-type K5-K14 filaments with those of K5-K14 Arg125→Cys using rheology and a particle tracking-based assay. The mutation chosen affects a highly conserved amino acid residue within the keratin α-helical rod domain. Inherited mutations at the corresponding codon account for 40% of known incidences of keratinopathies. Differential interference contrast microscopy shows that this hot spot mutation in K14 greatly reduces the ability of reconstituted mutant filaments to bundle under crosslinking conditions. Yet, rheological assays measure practically identical small-deformation mechanical responses for crosslinked solutions of wild type and mutant keratins. The mutation, however, dramatically reduces the resilience of crosslinked networks against large deformations. Single-particle tracking, which probes the local organization of filament networks, shows that the mutant polymer displays highly heterogeneous structures compared to wild type filaments. These results imply that the fragility of mutant keratin expressing epithelial cells results from an impaired ability of keratin polymers to be crosslinked into a functional network.

# 140

**Mice Deficient for MK6a and MK6b Reveal the Presence of Another MK6 Isoform**

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Keratin 6 (K6) is expressed in several, remarkably diverse, epithelia, for instance in hair follicle, oral mucosa, palmoplantar epidermis and nail bed. Another interesting characteristic of K6 is the existence of several K6 isoforms. Mice are known to possess two K6 genes, MK6a and MK6b. We had previously generated MK6a knockout mice, which, unlike knockouts of other keratins, did not display any signs of epithelial fragility. We then generated mice deficient for both isoforms. The majority of MK6a/b−/− mice die of apparent starvation within the first 2 weeks of life. This is due to a localized disintegration of the lingual epithelium in the back of the tongue, which results in the build up of a plaque of cell debris which severely impairs feeding. However, approximately a quarter of MK6a/b −/− mice do not show this phenotype, and survive to adulthood. Remarkably, the surviving MK6a/b−/− mice have normal hair, hair follicles, and nails. To our surprise we discovered MK6 staining both in the hair follicle and the nail bed of MK6a/b−/− mice, indicating the presence of a third MK6-like gene. We cloned this previously unknown murine keratin gene and found it to be highly homologous to HK6hf, a human K6 isoform known to be expressed in the hair follicle. We therefore termed this gene MK6hf. The presence of MK6hf in the MK6a/b−/− follicles and nails might explain the absence of a defect in these structures in the double knockout animals.

# 141

**Regulators of Expression of K15 Keratin Gene Promoter**

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Mitotically active keratinocytes of all stratified squamous epithelia express K5 and K14, ''acidic'' or type I keratins, which assemble into basal keratin filament network. Mutations in K14 gene give rise to Epidermolysis Bullosa Simplex, a disorder that causes blistering in the basal layer of stratified epithelia due to cell fragility in the absence of a major keratin filament network. Keratin K15 can pair with keratin K5 to form a filament network substituting for the absence of K14, thus alleviating the symptoms in nonkeratinizing stratified epithelia in patients with K14 mutations. In expectation that increasing the content of K15 may alleviate the epidermal symptoms in such patients, we cloned the promoter of the K15 gene and determined the molecular regulators of its expression. We investigated the role of thyroid hormone, retinoic acid and glucocorticoids, their receptors, transcription factors C/EBPβ, AP-1, NF-κB, and of IFNγ in regulation of K15 expression. Using cotransfection, gel mobility shift assays and DNAse I footprinting, we have identified the transcriptional activators of the K15 promoter and their binding sites. We found that K15 promoter was potently and very specifically induced by thyroid hormone in the presence of its receptor and by IFNγ. We conclude that the K15 gene has unique mode of transcriptional regulation by thyroid hormone and its receptor, different from all other epidermal keratins. In contrast, transcription factors C/EBPβ, and AP1 strongly, but nonspecifically induced, while NFκB and retinoic acid and glucocorticoid receptors suppressed the K15, as well as other epidermal keratin promoters. These findings suggest that treatments based on thyroid hormone and IFNγ could become powerful agents in the therapy of EBS patients.

# 142

**Molecular Cloning, Nucleotide Sequence of the cDNA, Properties, and Localization of Human Peptidylarginine Deiminase Type III**

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Peptidylarginine deiminase (PAD) catalyzes the post-translational n of proteins through the conversion of arginine to citrulline in the presence of calcium ions. In rodents, PAD has been classified into 4 isoforms, types I, II, III, and IV. Only type III PADs from mouse and rat were detected in epidermis and hair follicles, while characterization of human PAD type III was not performed yet. In this study, we cloned the full-length cDNA (3142 bp) from cultured human keratinocytes by RT-PCR and rapid amplification of cDNA end methods. This cDNA contained a 1995-bp open reading frame encoding 664 amino acids (Mr 74770). We constructed a plasmid and produced a recombinant human PAD type III in bacteria. The recombinant enzyme showed the catalytic activities toward structural proteins of epidermis and hair follicle, filaggrin and trichohyalin, in which the deiminations maxima of 60% and 13% arginine residues were observed in filaggrin and trichohyalin, respectively. An immunohistochemical study with a monospecific anti-PAD type III antibody revealed that the type III enzyme was localized to the inner and outer root sheaths of hair follicles in human scalp skin. Expression in the inner root sheath was notable between supramatrix and keratogenous zone and was scarcely detected in cornified hair zone. The enzyme was also expressed in the cuticle layer of hair. On the other hand, expression in the epidermis was low. These results imply that human PAD type III is the predominant isoform in hair follicles and may function as a modulator of hair structural proteins, including trichohyalin during hair and hair follicle formation.

# 143

**Hairless is Translocated to the Nucleus via a Novel Bipartite Nuclear Localization Signal and is Associated with the Nuclear Matrix**

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Hair follicle cycling is an exquisitely regulated and dynamic process consisting of phases of growth, regression and quiescence. The transitions between the phases are governed by a growing number of regulatory proteins, including transcription factors. The hairless (h) gene encodes a putative transcription factor that is highly expressed in the skin, where it appears to be an essential regulator during the regression in the catagen hair follicle. In hairless mice, as well as humans with congenital atrichia, the absence of h gene function initiates a premature and abnormal catagen due to a dysregulation of apoptosis and cell adhesion, and defects in the signaling required for hair follicle remodeling. Here, we report structure-function studies of the hairless protein, in which we have identified a novel bipartite nuclear localization signal (NLS) of the form KRA (X13) PKR. Deletion analysis of the mouse h gene mapped the NLS to amino acid residues 409–427. Indirect immunofluorescence microscopy of cells transiently transfected with hairless-green fluorescent fusion proteins demonstrated that these amino acid residues are necessary and sufficient for nuclear localization. Furthermore, nuclear fractionation analysis revealed that the h protein is associated with components of the nuclear matrix.

# 144

**Corneocytes with Immature Cornified Envelopes in Inflammatory Skin Disorders are not Always Associated with Parakeratosis**

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We have previously established a noninvasive method to evaluate maturation of cornified envelopes, and have reported appearance of immature cornified envelopes in the stratum corneum with impaired barrier function, such as that of the face. The purpose of the present study is to evaluate cornified envelopes in inflammatory skin disorders, and to clarify whether the appearance of the immature cornified envelope is associated with parakeratosis, which often used as a marker for hyperkeratosis, detected in inflammatory skin disorders, or not. Cornified envelopes were prepared from the outermost stratum corneum collected by a tape stripping. They were stained with anti-involucrin followed by FITC-labelled secondary antibody to evaluate loss of antigenicity during maturation, and with Nile red to assess their hydrophobicity. Immature cornified envelopes, which were stained with involucrin, but not with Nile red, were detected in the stratum corneum of lesional skin of psoriasis and atopic dermatitis. These corneocytes were dissociated by incubation of the tape-stripped stratum corneum in 20 mM sodium dodecyl sulfate-80 mM N, N′-dimethyl dodecyl amine oxide. They were stained with anti-involucrin followed by FITC-labelled secondary antibody, and with propidium iodide to detect the presence of nuclei. Involucrin (+) nuclei (+) cells were detected in 7% of corneocytes from psoriatic lesional area. In addition, there were also involucrin (+) nuclei (–) and involucrin (–) nuclei (+) cells (9%, 25%, respectively). Thus, involucrin-positive immature cornified envelopes were not always associated with parakeratosis. These results suggested that defect of cornified envelopes maturation may, at least in part, account for impairment of barrier function in the inflammatory skin disorders, and that the maturation of cornified envelopes and disappearance of nuclei are regulated independently.

## 145

**Short-Time Incubation Optimizes the Protocol for *In Situ* Transglutaminase Assay**

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*In situ* transglutaminase (TGase) assay is a powerful tool used in the screening of TGase activity in patients with autosomal recessive congenital ichthyosis. The protocol for *in situ* TGase assay was analysed using normal human skin obtained from various sites by immunofluorescent method with several different conditions. We found that the pattern of incorporation of monodansylcadaverine (MDC) in normal epidermis was dependent on concentrations of MDC, incubation time and body site. Preferential TGase activation in the granular cell layers was confirmed with low concentration, 0.1–0.3 μM, of MDC, or with short-time incubation, down to 2 min, in normal skin from the abdomen and buttock. In normal skin from the sole, TGase activation was observed from the suprabasal layers with incubation for only 2 min. No significant difference of fluorescence patterns was observed with reaction buffer of different pH, between 8.5 and 6.8. Specificity of *in situ* TGase assay for detecting TGase 1 (keratinocyte TGase) activity was confirmed by comparing patterns in skin sections from wild-type and TGase 1-deficient mice. In lesional skin samples from three patients with autosomal recessive congenital ichthyosis examined, TGase activity was positive after 2 min of incubation and the pattern of TGase-activation was not changed with different concentration of MDC or incubation time. These results suggest that short-time incubation with MDC may be sufficient and specific for screening TGase 1 activity in lesional skin samples as observed in patients suspected of having autosomal recessive congenital ichthyosis.

## 146

**Three-Dimensional Structures of Human Transglutaminase 3, from Zymogen to Active Form**

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The rigidity and extraordinary insolubility of the cornified cell (CE) envelope affords the mechanical barrier attributes of CEs in the epidermis. These properties are a result of extensive cross-linking of the constituent proteins. Transglutaminases (EC2.3.2.13. protein-glutamine:amine γ-glutamyltransferase) are calcium-dependent enzymes that catalyze the formation of covalent ε-(γ-glutamyl)lysine cross-links in proteins. Here we describe the three-dimensional structure of transglutaminase 3 (TG3), determined by X-ray crystallography. To understand the structure-function relationships of TG3 with its preferred substrate, we have crystallized the zymogen precursor, inactive + protease intermediate, as well as active form of TG3. Recombinant TG3 was expressed in the baculovirus system. The enzyme was isolated through a three-step purification, two columns of Source 15Q (Pharmacia) and Mono Q anion exchange column (FPLC, Pharmacia), from the pellet of insect cell culture. The active form of TG3 was prepared by 30 min treatment of neutral protease Dispase I and repurified using Mono Q anion exchange column. The crystal of the inactive precursor belongs to the triclinic space group P1 at 2.2 Å, resolution, with unit cell dimensions of a = 58.11 Å, b = 68.07 Å, c = 117.3 Å, α = 97.26°, β = 90.23°, γ = 99.0°. Furthermore, The crystal of inactive precursor + protease intermediate and active form belongs to P2(1) space group at 2.7 Å, with unit cell dimensions of a = 58.73 Å, b = 115.37 Å, c = 121.34 Å, γ = 92.66° and a = 58.73 Å, b = 115.84 Å, c = 61.96 Å, γ = 94.14°, respectively. The structures have enabled us to examine the active site pocket, catalytic triad and activation mode, which detail the structural events leading to active form of the enzyme.

## 147

**Mechanism of Cornified Cell Envelope Scaffold Formation**

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Envoplakin and periplakin are components of cell envelope barrier structures (CE) in terminally differentiating epithelial cells. Basing on (i) their early patterns of expression in differentiating keratinocytes, (ii) their localization in the protein layer of the CE, and (iii) their cross-linking to ceramides on the outer part of the CE, envoplakin and periplakin have been proposed to serve as a scaffold for the attachment of other protein components to the CE. Envoplakin and periplakin belong to the plakin family that also includes desmoplakin, bullous pemphigoid antigen 1 and plectin. Using purified proteins, we investigated the possibility of envoplakin and periplakin copolymerization and the possible mechanism of their involvement in early stages of CE assembly. The oligomeric state of recombinant envoplakin and periplakin and their complexes were investigated by chemical cross-linking. Envoplakin formes large water-insoluble aggregates, while periplakin homo-oligomers and envoplakin/periplakin hetero-oligomers were soluble in physiological conditions. Hetero-oligomers assembled from equimolar mixtures of envoplakin and periplakin were found to be as stable as periplakin homo-oligomers. Both periplakin and envoplakin/periplakin complexes were found to be able to bind to synthetic lipid vesicles, mimicking the cytoplasmic surface of plasma membrane of eukaryotic cells, and in a Ca++-dependent manner. These findings suggest that envoplakin and periplakin hetero-oligomerization might serve as the driving force for their Ca++-dependent cellular redistribution during keratinocyte terminal differentiation, an initiation of CE scaffold formation.

## 148

**Immunohistochemical Localization of Sulfhydryl Oxidase Correlates with Disulfide Bond Formation in the Rat Epidermis; the Altered Localization and Regulation Revealed by TPA, RA and UVB Radiation**

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Skin sulfhydryl oxidase (SOx), the specific enzyme catalyzing disulfide (S-S) formation, was reported to play an important role in post-translational modification of cornified cell envelope proteins in the terminal differentiation. Immunohistochemical analysis using the polyclonal antiserum proved that SOx localized specifically at the juncture of the living and cornified layers in rat skin. The prominent conversion of free -SHs to S-S is reported to occur at this juncture through the mediation of a fluorescent thiol reagent, N (7-dimethylamino-4-methyl coumarinyl) maleimide (S-S staining). Higher magnification showed that the immunopositive layers included some apoptotic nuclei, thus identifying the layers as the stratum lucidum. *In situ* hybridization assay using the antisense probe containing the N-terminal sequence of skin SOx confirmed this result. Furthermore, we attempt to demonstrate that the conversion to S-S and SOx distribution always occur together in rat epidermis even when the epidermis has been exposed to certain chemical agents, such as phorbol acetate (TPA) or all-*trans* retinoic acid (RA), or has been subjected to ultra-violet B (UVB) damage. 48 h after the local application of TPA, conspicuous expression of SOx in the stratum lucidum was observed with strong fluorescence of the whole stratum corneum evinced by S-S staining. On the other hand, decreased staining of skin SOx with faint S-S staining in the stratum corneum was observed 48 h after the local application of RA. Irradiation of rat skin with UVB up-regulated enzyme expression in the stratum lucidum. Moreover, abundant SOx expression was also evident in the cytoplasm of sun burn cells (SBCs) in the epidermis. These findings suggest that SOx may serve an important biological function for keratinocytes by being responsible for the process of structural change in the stratum lucidum and SBCs via S-S bond formation.

## 149

**Localization of Caveolins at the Stratum Granulosum–Stratum Corneum Interface and their Enrichment in an Isolated Lamellar Granule Fraction Suggest a Role in Lamellar Granule Assembly or Function**

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Lamellar granules (LGs) are sphingolipid-enriched organelles, likely intimately related to the tubulovesicular elements of the *trans*-Golgi network, that deliver the precursors of stratum corneum barrier lipids to the extracellular compartment. Caveolins are cholesterol-binding proteins that facilitate the assembly of cholesterol and sphingolipid enriched membrane domains known as caveolae. Similarities in the composition of LGs and caveolae suggest that caveolins could be involved in LG assembly and/or function. In order to explore this relationship, we have examined the expression of caveolins in epidermis, keratinocyte cultures, and an isolated LG fraction using immunolabeling, immunoblotting and RTPCR. Several antibodies show strong immunolocalization of caveolin 1 and 2 in the basal layer of human epidermis, a decline in the suprabasal layers and a reemergence of strong expression at the junction of the stratum granulosum and stratum corneum. The junctional fluorescence colocalizes with glucocerebrosidase, an enzyme known to be critical for remodeling of extruded LG contents. RTPCR and immunoblotting in keratinocyte cultures show expression of caveolin mRNA and protein, respectively, throughout differentiation, suggesting that the immunofluorescence findings may result from epitope masking in suprabasal keratinocytes. Caveolins are enriched in an isolated LG fraction that is also enriched, as we have previously described, in lysosomal acid lipase and glucocerebrosidase. These preliminary studies suggest that caveolins may play a role in LG assembly and/or function.

## 150

***In Vivo* Studies of Phytosterol Sulfate Effect on Human Skin Hydration and Barrier Function**

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Our prior studies have shown that Phytosterol sulfate (PS), the new homologue to Cholesterol sulfate, demonstrates interesting effects on enhancing the skin barrier and keratin synthesis, *in vitro*. Therefore, we investigated the *in vivo* effect of Phytosterol sulfate on human skin hydration and TEWL. PS hydration kinetic was investigated in two double blind studies. PS 1% cream formula or placebo was applied on the forearm of 10 healthy volunteers (age 23–65). Measurements of stratum corneum hydration were performed using a CM 825 Corneometer, and statistical analyses using the Wilcoxon non parametric test for matched pairs on difference for each subject were applied (significance level $\alpha \leq 5\%$). The results of the first study (3 *h*-test) were highly significant and showed an improvement of skin hydration of 58% of the Air Under Curve (AUC) over the studied time, and of 61% at 30 min ($p = 0.001$ for both). The second study was a 6-h hydration test and demonstrated a longer moisturizing effect of PS, with a very significant increase of AUC values of 40% and of 23% at time 6 h ($p = 0.002$ for both). An additional double blind study of skin surface aspect was conducted on dry skin of the legs of 4 volunteers using Visioscan camera 98. This study confirmed the results above and showed that 2 h after the products' application, the dry skin aspect was already visible in the placebo-treated zone, while PS-treated skin stayed well-hydrated up to 8 h. In order to investigate PS effect on skin barrier function, a double blind study of 3 h was conducted and hydration and TEWL measurements were performed (CM825 and Evaporimeter Cortex Technology) on the forearms of 6 volunteers after the skin barrier function was experimentally disturbed by detergent contact. Statistical results showed that PS application on the skin diminishes AUC detergent-induced TEWL by 300% ($p = 0.032$) and improves skin hydration by 127% ($p = 0.032$) compared to placebo-treated skin. These results are statistically significant, and confirm Phytosterol sulfate's interesting role in skin barrier protection from detergent aggression. These studies demonstrate that Phytosterol sulfate significantly improves skin hydration and skin barrier functions.

# 151

**Acute Barrier Disruption Activates Extracellular Signal-Regulated Kinase in Epidermis**

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It is well known that epidermal barrier disruption leads to an increase in epidermal barrier lipid production and DNA synthesis together with production of a variety of proinflammatory cytokines and altered differentiation. However, the signals involved for these biological responses after barrier disruption are only partially understood. In this study, we examined the role of three mitogen-activated protein kinases (MAPKs), p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase (Erk), and c-Jun – NH2-terminal kinase (JNK) in the signal transduction after acute epidermal barrier disruption. We tape stripped the skin of hairless guinea pigs and took biopsies at various time points after the barrier disruption. We examined the activation of the three MAPKs in the barrier-disrupted skin in the extracted proteins from biopsy specimens by Western blotting and kinase assay as well as by immunohistochemistry. The obtained results demonstrated only an increase in the phosphorylation and activation of Erk 30 min, 1 h, and 3 h after tape stripping among three MAPKs. Immunohistologically, positive nuclear staining was also detected in the epidermal cells by antiphospho-Erk antibody in a similar time course. Moreover, the immunoreactivity of epidermal cells with antiphospho-Erk antibody positively correlated with an increase in TEWL after tape stripping. Furthermore, immunohistochemically we found that artificial restoration of the barrier by Tegaderm™ Transparent Dressing(R) occlusion immediately following barrier disruption inhibited the phosphorylation of Erk in the epidermis. These data suggest the involvement of Erk in the signal transduction of acute barrier disruption.

# 152

**Identification of a Barrier Deficiency in the Stratum Corneum of the Axilla**

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The environmental conditions encountered by the stratum corneum of the axilla are unique, with low desiccation stress but high microbial loads and elevated levels of glandular secretions. To investigate the specialised nature of the axillary skin we have determined the barrier potential of the stratum corneum in comparison with other body sites. We have used corneosurfametry (CSM) as an indirect method of barrier measurement. Stratum corneum from the axilla, forearm, torso and back of the hand was sampled using cyanoacrylate adhesive and the barrier potential determined. CSM revealed that the axilla had a deficient barrier, both baseline and in response to surfactant challenge, compared to the other body regions. Analysis of the stratum corneum lipids by HPTLC demonstrated statistically elevated levels of fatty acids, ceramide and particularly cholesterol in the axilla compared with forearm and this perturbation of lipid rations may explain the barrier impairment. To investigate whether a specially formulated antiperspirant (AP) product could be used to supplement barrier function, Skin Surface Water Loss (SSWL) analysis was performed on AP treated skin. AP treatment reduced by approximately 50% the water loss from the site compared to untreated control. Conclusion: (1) axillary stratum corneum has a deficient barrier compared to other body sites; (2) the levels of axillary stratum corneum fatty acids, ceramide and cholesterol are significantly elevated compared to forearm: (3) ap product can significantly improve the water holding capacity of the stratum corneum, and promote normal barrier function.

# 153

**Novel Methods for Measurement and Localization of Stratum Corneum pH**

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We previously reported the importance of an acidic stratum corneum (SC) pH for optimal barrier recovery (*Arch Derm Res* 290:215, 1998). Subsequently, we showed that the Na+/H+ type 1 antiporter (NHE1) controls both intracellular pH in cultured human keratinocytes (CHK) and SC pH *in vivo*. A functional NHE1 is essential for barrier homeostasis, because both pharmacologic inhibition and transgenic knockout of NHE1 impair lipid processing and delay barrier repair (*JID* 114:277, 2000). However, further investigations into the mechanisms responsible for SC acidification and the role of NHE1 have been limited by current methodology for measurements of SC pH. We therefore first compared the standard method for SC pH measurement; i.e. a flat pH electrode, with a method combining topical applications of the water-soluble, pH-sensitive, fluorescent dye, BCECF, with ratiometric pH measurements on a fluorospectroscope (JYHoriba SkinSkan(r)). The SkinSkan(r) method yielded readings in normal SC comparable to flat electrode measurements. We next determined the distribution of BCECF within the SC by a third method, *in vivo* confocal microscopy, which revealed dye signal accumulation within the expected acidic compartments, i.e. the SC extracellular domains. As a fourth method, we then used fluorescence-lifetime imaging (FLIM), combined with multiphoton microscopy, which allows simultaneous capture of pH and localization within the SC. This method so far has revealed an extracellular dye distribution pattern consistent with the *in vivo* confocal microscopy results. Finally, we have begun to apply these new methods to compare SC pH in NHE1 knockout vs. wild-type animals, and have found alterations consistent with antiporter involvement in SC pH regulation. Thus, a combination of established methods with these novel approaches should not only allow more precise determinations of SC pH, but also provide additional insights into the mechanism(s) responsible for SC acidification.

# 154

**Production of Lamellar Bodies in a Novel, Vitamin C-Enriched Submerged Human Keratinocyte System: Regulation of Secretion by Extracellular Calcium**

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Prior studies have shown that extracellular calcium ([Ca]ex) plays a key role not only in the regulation of keratinocyte differentiation *in vitro*, but also in cellular processes that lead to barrier recovery following acute challenges *in vivo*. Whereas elevated [Ca]ex inhibits lamellar body (LB) secretion, a reduction of [Ca]ex stimulates fusion of LB with the apical plasma membranes of granular cells, and enhances secretion of LB contents (Menon *et al*, *JID* 102:789, 1994). To further assess the mechanism(s) by which [Ca]ex regulates the processes of LB secretion and epidermal barrier homeostasis *in vivo*, we first developed a submerged cultured human keratinocyte (CHK) model, supplemented with vitamin C (50 μg per ml), FBS, and 1.8 mM calcium. At 9–14 days postconfluency, these CHK display abundant LB. Although total lipid content did not change significantly, both GlcCer and Cer content increased progressively from 9 to 14 days, which correlated with the appearance of increased numbers of LB with increasingly mature contents. However, at high [Ca]ex few mature extracellular lamellar membrane structures were present. Whereas high [Ca]ex suppressed LB secretion, reduction of [Ca]ex in the medium of LB-enriched cultures (i.e. day 12 postconfluency), either by lowering calcium from 1.8 to 0.03 mM, or by the application of excess EGTA, triggered both the secretion of preformed LB, and the subsequent appearance of mature extracellular lamellar membrane structures. These results demonstrate directly the role of changes in [Ca]ex in the regulation of LB fusion and secretion in an immersed CHK culture system. This novel, submerged model should prove useful for studies into the mechanisms that regulate LB secretion and epidermal permeability barrier homeostasis.

# 155

**A GPI Anchor is Required for the Epidermal Permeability Barrier**

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Glycosylphosphatidylinositol (GPI)-anchored proteins are widely distributed on the exterior surface of eukaryotic plasma membranes, including those of both epithelial and endothelial cells within the skin. We previously reported both neonatal lethality and abnormal keratinocyte differentiation in mice with an epidermal-specific deficiency of GPI-anchored biosynthesis (Tarutani *et al*, *PNAS USA* 94:7400, 1997). In this study, the phenotype of epidermal GPI-anchor deficient (Pig-a null) mice was analyzed. Mice deficient in epidermal GPI displayed abnormal permeability barrier function, evidenced by a significant elevation in transepidermal water loss; i.e. 5.3-fold increase over control. The basis for altered barrier function appeared to be production of lamellar bodies (LB) with abnormal internal contents in the stratum granulosum cell layer, leading to the presence of abnormal lamellar membrane structures in the stratum corneum (SC). These morphological changes correlated with a significant accumulation of epidermal glucosylceramide, and concurrent reduction in SC ceramide (Cer) and free fatty acid (FFA). Moreover, both Cer 1 and Cer 4 (acylCer), two important components of the SC lamellar membrane structures, as well as Cer 2, a major SC Cer derived from both glucosylCer and sphingomyelin, were significantly decreased in Pig-a null mouse SC. Since the cholesterol content of Pig-a null vs.wild-type mice did not change, alterations in the proportions of the three major SC lipids, Cer, cholesterol and FFA, could explain the structural and functional abnormalities in Pig-a null mice. Together, these results suggest that either the GPI anchor itself or GPI-anchored protein(s), are essential for the formation of a normal epidermal permeability barrier. Barrier impairment in GPI-defiicient skin is explained by abnormalities in SC extracellular lamellar membrane structures. These structural alterations may be due to abnormalities of both LB assembly and postsecretory events that result in altered SC lipids.

# 156

**Negative Electric Potential Induces Epidermal Lamellar Body Secretion and Accelerates Skin Barrier Recovery after Barrier Disruption**

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Previous reports suggested that localization of ions such as calcium and magnesium in the epidermis plays a crucial role in skin barrier homeostasis. We demonstrated here that application of a negative electric potential load (–0.50 V) on hairless mice skin accelerated the barrier recovery approximately 60.7% within one hour compared with the control (37.5%) after barrier disruption by acetone treatment. Even after the loading of the negative potential, the skin showed accelerated repair for 6 h. On the contrary, the skin, which was loaded a positive potential (+0.50 V) for 1 hour, showed a significant delay in the barrier recovery (25.3%) than the control. Ultrastructural studies by electron microscopy suggested that the extent of lamellar body exocytosis into the stratum corneum/stratum granulosum interface significantly increased under the negative potential. Magnesium and calcium ion concentrations in the upper epidermis were relatively higher in the negative portion than in the portion where the positive potential load was applied. Topical application of these ions on mice skin also accelerated the barrier recovery. These results suggest that the external electric potential affects the localization of ions in the epidermis and also influences the skin barrier homeostasis.

## 157

**Abrupt Decrease in Humidity Induce Abnormalities in Permeability Barrier Homeostasis**

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Prior studies have shown that a shift from a normal to a dry environment improves barrier function while changing from a normal to a humid environment causes a progressive deterioration in barrier homeostasis over 2 weeks. Here, we assessed the impact of a more extreme shift; i.e. from a high (relative humidity 80%) to a low (relative humidity < 10%) humidity environment on barrier function in hairless mice. As described previously, transfer from a normal to a dry environment improved barrier function. In contrast, two days after transfer from a humid to a dry milieu, transepidermal water loss (TEWL) rates abruptly and paradoxically increased (6-7-fold increase), with TEWL rates returning to normal by four days. Stratum corneum (SC) hydration declined precipitously in parallel, but the decline did not trigger an increase in epidermal DNA synthesis. Whereas epidermal differentiation (loricrin and profilaggrin expression) and apoptosis did not change, the basis for the transient barrier abnormality correlated with a marked depletion of lamellar bodies (LB) from the outermost granular cell (SG) layer, with reduced deposition of secreted LB contents at the SG-SC interface, and a paucity of lamellar bilayers in the SC interstices. The present study demonstrates that a drastic change from a high to low humidity milieu provokes a profound, though transient perturbation in permeability barrier function.

## 158

**Regulation of Both Serine Palmitoyltransferase and Glucosylceramide Synthase Promoter Expression by a Common PKC- and SP1-Dependent Mechanism**

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The *de novo* synthesis of ceramide (Cer) and its subsequent glucosylation to form glucosylCer (GlcCer), are required for the generation of stratum corneum Cer species that are critical for epidermal permeability barrier structure and function. Both serine palmitoyltransferase (SPT), the rate-limiting enzyme in sphingoid base formation, and GlcCer synthase are key regulatory steps in the production of these two sphingolipids, and are both regulated by PKC-dependent mechanisms in cultured human keratinocytes (CHK). To investigate the molecular regulation of SPT and GlcCer synthase expression we determined the response of CHK, transfected with a series of luciferase-promoter deletion constructs, to tetradecanoyl phorbol 13-myristate (TPA). Northern analysis revealed that TPA (10 nM) increased mRNA levels for both LCB2, which codes for SPT activity, and GlcCer synthase (i.e. 1.8- and 4.9-fold, respectively); both of these increases were inhibited by the PKC inhibitor, BisIM. TPA also increased promoter activity in CHK transiently transfected with the putative promoter sequence for either human LCB2 (1.8 kb) or murine GlcCer synthase (0.73 kb); i.e. 3-to-5-fold and 4.2-fold, respectively. Promoter-deletion construct studies revealed regions in both LCB2 (–83 to –188) and GlcCer synthase (–473 to –537) promoters that are important for PKC-dependent activation, and contain consensus binding sites for the transcription binding factor, SP1. These results demonstrate that both SPT and GlcCer synthase expression are regulated by PKC-dependent mechanism(s) that utilize putative SP1 sites, suggesting for the first time coordinate regulation of these two key enzyme systems in the epidermis.

## 159

**Sphingolipid Production is Regulated by a PKC-Dependent Mechanism in Cultured Human Keratinocytes**

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Sphingolipids, including ceramide (Cer), glucosylceramide (GlcCer) and sphingomyelin (SM), are important components for epidermal permeability barrier homeostasis. Although prior studies have demonstrated PKC-dependent regulation of keratinoctye differentiation & GlcCer synthase activity, the molecular mechanism(s) regulating serine palmitoyltransferase (SPT), the first committed and rate-limiting step in SL synthesis, remain unknown. To address whether SPT is regulated by PKC-dependent mechanism, cultured normal human keratinocytes (CHK) were incubated with tetradecanoyl phorbol 13-myristate (TPA; 10 nM) in serum free keratinocyte growth medium. Analysis of sphingolipid content revealed that TPA significantly increased both Cer (1.2-fold) and GlcCer (2.3-fold), while SM was decreased at 24 h. These changes that were completely blocked by Bis-IM, a specific inhibitor of PKC. In addition, [3H]-L-serine incorporation into both Cer and GlcCer, but not into SM, was elevated following TPA. Moreover, SPT activity also was increased significantly by TPA; i.e. 1.3- and 1.7-fold at 8 and 24 h, respectively. Northern blot analysis revealed mRNA levels for LCB2, a key subunit of SPT, increased 1.8-fold at 24 h following TPA. Again, Bis-IM abolished the increases in both enzyme activity and mRNA levels. Finally, significantly enhanced promoter activity (3-to-4-fold increase) was evident following TPA treatment of CHKs transfected with a luciferase-promoter construct containing 1.8kb of the 5′-upstream sequence for the human LCB2 gene. These results demonstrate that TPA elevates SPT mRNA, enzyme activity, and subsequent SL production, at least in part, through up-regulation of LCB2 promoter activity. Together with our concurrent report of increased GlcCer synthase promoter expression by TPA, these results show that *de novo* Cer and GlcCer production in CHK are regulated by PKC-dependent mechanism(s), distinct from that for SM synthesis.

## 160

**Interleukin-1α Signaling is a Determinant of Permeability Barrier Homeostasis in Aged Murine Skin**

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Previous studies have shown alterations in IL-1 signaling in aged epidermis both basally and following barrier perturbation. These studies were designed to assess further the role of IL-1 signaling in the aged barrier abnormality. We first analyzed barrier recovery after tape stripping in aged mice with a knockout of the functional IL-1 receptor type I vs. age- matched wild-type controls. Mice with a knockout of the IL-1 receptor type I exhibited delayed barrier recovery compared to their age-matched wild-type counterparts. We then assessed whether intracutaneous IL-1α, 50 ng in 0.1 ml PBS, 5 min prior to barrier perturbation, would accelerate barrier recovery in aged mice. IL-1α administered intracutaneously resulted in a significant improvement in barrier recovery (40% vs. 5% recovery at 6 h, $p < 0.01$). Finally, imiquimod (10%), a stimulator of endogenous keratinocyte cytokine production (including IL-1α), was applied topically to aged murine skin twice daily for one week prior to barrier perturbation. Barrier recovery was then monitored in treated vs. control sites. Imiquimod, which resulted in a doubling of epidermal IL-1α by ELISA, but no change in the IL-1ra by western blotting, also improved barrier recovery significantly (37% vs. 9% recovery at 3 hours, $p < 0.01$)). These studies demonstrate the significance of the IL-1 family of cytokines for the barrier defect in aged skin, and further, show the ability of cytokine stimulation to improve barrier function in the aged.

## 161

**Basis for Epidermal Side-Effects of Topical and Systemic Glucocorticoids**

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Prolonged treatment with either high doses of systemic steroids or potent topical steroids can produce important cutaneous side-effects, such as skin fragility. We assessed here whether systemic and topical glucocorticoids produce alterations in epidermal structure and function that can account, in part, for these side-effects. As shown previously (*Am J Physiol* 278:R367-372, 2000), systemic steroids produced a dose-dependent deterioration in permeability homeostasis in hairless mouse skin, characterized by a delay in barrier recovery after acute insults. Topical applications of 0.05% clobetasol to hairless mouse skin also produced a localized barrier abnormality. On electron microscopy, both the production and secretion of lamellar bodies (LB) were reduced, explaining the barrier abnormality. Topical steroids also caused a local abnormality in stratum corneum (SC) integrity in both humans and mice (# strippings to achieve TEWL = 4 mg per $cm^2$ per h) and in SC cohesion (μg protein removed/stripping). The integrity abnormality correlated with a diminution in the density of corneodesmosomes in the lower SC (two-fold decline by 3 days) by quantitative electron microscopy. This study demonstrates two epidermal consequences of steroid therapy: both systemic and topical steroids compromise epidermal barrier homeostasis as a result of decreased LB production, explaining the propensity of some steroid-treated dermatoses to worsen or rebound with steroid treatment. Finally, the skin fragility associated with topical steroid use may be, in part, related to a decline in SC integrity, resulting from premature dissolution of corneodesmosomes in the outer epidermis.

## 162

**Generation of Free Fatty Acids from Epidermal Phospholipids Regulates Stratum Corneum Acidification and Integrity**

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There is evidence that the ''acid mantle'' of the stratum corneum (SC) is important for both permeability formation and cutaneous antimicrobial defense. However, the origin of the acidic pH of the SC remains conjectural. Both passive (e.g. eccrine/sebaceous secretions, proteolytic) and active (e.g. proton pumps) mechanisms have been proposed. We assessed here whether free fatty acid (FFA) pool which is derived from phospholipase-mediated hydrolysis of phospholipids (PL) during cornification, contributes to SC acidification and function. Topical applications of two chemically unrelated secretory phospholipase (sPLA2) inhibitors, bromphenacylbromide (BPB) and MJ33 produced a progressive increase in the pH of murine SC that was paralleled not only by a permeability barrier abnormality, but also altered SC integrity (# strippings required to break barrier) and cohesion (protein weight removed/stripping). Not only SC pH, but also both of these functional abnormalities normalized when either palmitic, steraric, or linoleic acid were coapplied with the inhibitor. Moreover, exposure of intact murine SC to a neutral pH for as little as three hours reproduced the $sPLA_2$-induced abnormalities in SC integrity and cohesion. Furthermore, short-term applications of an acidic pH buffer to inhibitor-treated skin also normalized SC integrity and cohesion, despite a putative decrease in FFA. Finally, the $sPLA_2$-inhibitor-induced alterations in integrity/cohesion were linked to premature dissolution of desmosomes and reduced desmoglein 1 expression in the lower SC. Together, these results demonstrate: (1) the importance of PL-to-FFA processing for normal SC acidification; and (2) the potential role of this pathway in the mediation not only of barrier homeostasis, but also the dual functions of SC integrity/cohesion.

# 163

**Disruption of Barrier Function Due to Protein Oxidation in Stratum Corneum in Atopic Dermatitis Possibly by Environmental Pollution-Induced Oxygen Radicals**

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We reported that a recent dramatic change in the features of atopic dermatitis (AD) in Japan: a marked increase in the incidence of AD and in severe and treatment-resistant AD, especially in urban and/or industrial areas. We have ascribed this phenomenon in part to increased oxygen radicals (OR) generated from environmental pollution and solar radiation. Here, we have investigated oxidative protein damage by assessment of carbonyl moieties in the skin biopsies from 75 patients with AD, using both spectrophotometric and immuno-histochemical detection of the formation of dinitrophenylhydrazone (DNP) from dinitrophenylhydrazine (DNPH). Protein carbonyl moieties in lesional skin was elevated in all of the patients with AD and correlated directly with the severity of the disease. Immuno-histochemical staining showed that the highest levels of protein carbonyl groups were found in superficial layers of stratum corneum. The data support the hypothesis that environmentally generated OR induce oxidative protein damage in the skin, especially in stratum corneum, which is exposed to environment and to solar ultraviolet light, leading to the disruption of barrier function with resultant loss of moisturization and enhancement of antigen presentation.

# 164

**Pathogenesis of the Permeability Barrier Abnormality in Epidermolytic Hyperkeratosis**

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Epidermolytic hyperkeratosis (EHK) is a dominantly inherited form of primary ichthyosis, often associated with mutations in the rod domains of keratins (K) 1 or 10. In addition to a severe disorder of cornification, patients with clinically typical EHK display an abnormality in basal permeability barrier function. However, the nature and pathogenesis of the barrier abnormality in EHK are unknown. We assessed here first both basal transepidermal water loss (TEWL) and barrier recovery kinetics in three patients with EHK. Whereas basal TEWL rates were elevated by approximately three-fold, recovery rates were faster than in age-matched controls. Despite the known occurrence of keratinocyte fragility in EHK, the water-soluble tracer, colloidal lanthanum, remained restricted to the SC interstices, with increased movement of tracer through these domains. Accordingly, we found major defects in both the quantities and the supramolecular organization of extracellular lamellar bilayers in EHK. Although K1/10 become cross-linked to the cornified envelope (CE) during terminal differentiation, we found defects neither in the CE, nor in the adjacent, cornified-bound lipid envelope in EHK. Instead, the intercellular permeability abnormality could be attributed to incomplete lamellar body (LB) secretion, presumably due to a defect in the cytoskeleton, demonstrable not only by several morphological findings, but also by a marked decrease in the delivery of the LB content marker, acid lipase, to the SC interstices. Together, these results show that the barrier abnormality in EHK can be attributed to a cytoskeletal abnormality rather than to either corneocyte fragility or to an abnormal CE/corneocyte scaffold.

# 165

**Sebaceous Gland-Derived Glycerol Regulates Hydration of Mammalian Stratum Corneum**

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Although a variety of functions have been proposed for sebum, the predominant view is that its principal role in humans relates to the provocation of acne. Whether sebum influences the key stratum corneum (SC) functions of hydration or barrier function is not known. We compared these two functions in mice with profound sebaceous gland (SG) hypoplasia, the asebia ($Scd^{ab}$/$Scd^{ab}$) mouse [asebia-J or $ab^{J}$ strain]. Whereas permeability barrier homeostasis was normal in asebia-J SC, they displayed epidermal hyperplasia and decreased (~50%) SC hydration, associated with a marked reduction in sebaceous gland-derived lipids (wax diesters, wax monoesters, sterol esters, and triglycerides [TG]). Despite the known, high rates of TG synthesis by normal SG, the TG content of control SC was also low, consistent with high rates of hydrolysis within normal SG. Topical applications of a surface lipid extract from control mice largely corrected the hydration abnormality, but comparable extracts from asebia-J mice had no effect. In assessing which SG fraction was responsible for rehydration, topical applications of synthetic, sebum-like lipids (sterol/wax esters and triglycerides) to asebia-J skin did not restore normal SC hydration, but topical glycerol, the putative product of TG hydrolysis in SG, restored normal hydration and ameliorated the epidermal hyperplasia in asebia-J mice. The importance of glycerol generation from SG-derived TG for normal hydration was demonstrated further by the absence of SG-associated lipase activity in asebia-J mice. These results demonstrate both the importance of SG-derived glycerol for the maintenance of normal SC hydration, and the increased propensity for SG-depleted skin sites to develop eczematous dermatoses, presumably triggered by decreased SC hydration.

# 166

**The Scavenger Receptor (SR-B1) is Expressed in Cultured Human Keratinocytes and Murine Epidermis: Regulation in Response to Changes in Cholesterol Homeostasis and Barrier Requirements**

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Cholesterol (Chol) is a key lipid in the stratum corneum, where it is critical for permeability barrier homeostasis. Although epidermis actively synthesizes Chol, inhibition of synthesis only modestly impairs barrier function, suggesting extra-epidermal delivery of Chol. The recently described scavenger receptor class B type 1 (SR-B1) mediates the selective uptake of Chol esters from high density lipoprotein (HDL). It also mediates Chol uptake for hepatic bile secretion and for adrenal steroidogenesis. We demonstrated here, using both Northern and Western blots, that SR-B1 is present in cultured human keratinocytes (CHK) and that $Ca^{++}$-induced differentiation dramatically decreases SR-B1 expression. Moreover, immunofluorescence staining demonstrated intense SR-B1 expression on the plasma membrane of undifferentiated, but not differentiated CHK. Additionally, $^{3}$H-Chol-labeled HDL uptake decreased markedly in differentiated vs. undifferentiated CHK. Furthermore, inhibition of Chol synthesis with simvastatin resulted in a 4-fold increase in both protein and mRNA levels of SR-B1, while 25-OH Chol suppressed SR-B1 levels by ≈50%. SR-B1 mRNA is also expressed in murine epidermis, increasing by ≈50% in parallel with Chol requirements following acute barrier disruption (tape stripping or acetone treatment). Since this increase was completely blocked by occlusion with a vapor-impermeable membrane, SR-B1 expression is regulated specifically by barrier requirements. The present study demonstrates that SR-B1: (1) is expressed in keratinocytes and regulated by changes in cellular Chol content, indicating a role for this receptor in keratinocyte Chol homeostasis; (2) localizes to the basal layer in epidermis; and (3) its increase following barrier disruption suggests that SR-B1 facilitates Chol uptake from extra-epidermal sources for lamellar body formation and barrier restoration.

# 167

**Slow Internal Release of Bioactive Compounds Under the Effect of Skin Enzymes**

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Among the enzymes involved in the maintenance of the epidermal barrier function (1), two, a β-glucocerebrosidase and an esterase can serve to release bioactive compounds. Glucoconjugation indeed confers amphiphilic properties which allow penetration through the skin via passive diffusion. The two enzyme activities are first assayed in a normal skin, using appropriate substrates. A more detailed analysis of the hydrolysis requirements of β-glucocerebrosidase was obtained after cloning and overexpressing it. Hydrolysis occurs as long as the leaving group is good enough ($pK < 10$) and there is no steric hindrance at the glycosidic bond being broken. This strategy is firstly illustrated with an antioxidant, δ-tocopherol, owing to the general importance of that class of compounds. The corresponding glucoconjugate is hydrolyzed with release of δ-tocopherol, the antioxidant activity of which is higher than that of the α-derivative. Moreover, this strategy of slow release of the compound prevents a pro-oxidant effect. Release of any compound can be obtained by introducing a spacer between the glucose unit and the molecule to be released. The spacer may also provide extra bioactivity. This situation is exemplified by the release of retinoic acid, using two different spacers, hydroquinone (depigmentent) and glycerol (control agent of water loss), the two spacers controlling the rate of the release. As the difference in rate of hydrolysis for the two diastereoisomeric forms with glycerol as spacer is not too great, a mixture of isomers can be used for dermatological applications without drawbacks. It should be noticed, in the context of practical applications, that glucoconjugation increases the thermal and photochemical stability of δ-tocopherol; it was checked that for the δ-toco-conjugate, delivery of the compound actually is observed *in vivo*, in the stratum corneum. A reservoir effect was also noticed. This strategy can be extended to diverse situations such as care and therapy of the skin, delivery of compounds which are presently internalized in the body from patches and drugs which have shown hepatic toxicity when administered orally.

# 168

**Novel Amphiphilic Cyclosporin Derivatives Show Dramatically Improved Skin Penetration Properties Challenging the "500D Rule"**

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Skin penetration of molecules after topical application is generally assumed to decrease with molecular weight (MW). Compounds with a MW over 500 are thought to hardly cross intact skin (the "500D rule"). Cyclosporin A (CyA) a cyclic undecapeptide with a MW of 1202, has indeed very poor skin penetration properties. Although CyA is highly effective in inflammatory skin diseases after oral application, it proved to be ineffective when applied topically, despite considerable efforts to improve the penetration by galenical formulations. We now prepared novel amphiphilic cyclosporin derivatives featuring neutral and charged polar substituents and investigated their penetration properties *in vitro*. Using rat and human skin in Franz-type diffusion cells, penetration into skin, by measuring skin concentrations, as well as permeation through skin, by determining permeation rates, were evaluated. Despite considerably higher molecular weights (1330–2100) the novel cyclosporin derivatives exhibited much higher permeation rates than the parent compound by a factor of up to 320 in rat skin and 47 in human skin. Skin concentrations were also increased by a factor up to 47 and 16 in rat and human skin, respectively. The main factors in determining skin penetration were the length, polarity and charge of the side chains. In summary, the introduction of polar and charged substituents to CyA has provided compounds with amphiphilic properties and greatly improved penetration properties. These results put into question the current paradigm that MW is the main factor determining skin penetration.

## 169

**Sunflower Oil Oleo Distillate for Atopy Treatment: An *In Vitro* and Clinical Evaluation**

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Sunflower Oil Oleo Distillate (a 10 concentrate solution of free fatty acid, sterols and vitamine E), obtained by molecular distillation, have chemical structure which could interacts with lipids metabolism. We investigated first the effect of this oleo distillate on epidermal lipid synthesis, using human skin explants obtained from abdominal plastic residues. A 2% oleo distillate emulsion (O/W) was topically applied during 18 h. The neosynthesis of di and triglycerides, cholesterol (–sulfate), cerebrosides, ceramides 1 and 2 was evaluated by the measurement of 14C-acetate, incorporated in the neosynthetized lipids extracted and separated by thin layer chromatography. We demonstrated that this specific oleo distillate induced a significant increase in the neosynthesis of both ceramides 1 & 2, and cholesterol. These data suggested that this oleo distillat may have potential applications in the regulation of abnormal desquamation as well as in the improvement of the skin permeability. Dry skin is found in several conditions, and especially in atopic patients, resulting in changes in epidermal barrier due to modifications in the lipid content. Thus, we investigated, in 218 children with atopic dermatitis, the effect of a 30-days twice daily application of a 2% cream, on pruritus, erythema, skin dryness, and desquamation. Clinical examination by the dermatologist clearly showed the improvement of all these parameters in treated children. The same treatment was performed on 20 adults with atopy. The 2% Sunflower oleo distillate cream was able to increase skin hydratation (corneometry) and to enhance keratinocyte cohesion as demonstrated by analysis of skin surface rubbering. In conclusion, Sunflower oleo distillate modulated epidermal lipid synthesis *in vitro*. *In vivo*, this better epidermal lipid composition was correlated with an improvement of atopic dermatitis clinical features in children, and a healthier epidermal barrier in atopic patients.

## 170

**Stat3 Deficiency Leads to Increased Tyrosine Phosphorylation of p130$^{cas}$ and Focal Adhesion Abnormalities in Keratinocytes**

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We previously reported that Stat3 plays a crucial role in transducing a signal for migration of keratinocytes (*EMBO J* 18:4659–4668, 1999). To clarify the role of Stat3 in signaling for the migration, we studied the integrin receptor to intracellular signaling pathway in the Stat3-deficient keratinocytes. Stat3-deficient keratinocytes demonstrated increased adhesiveness and fast spreading on the collagen matrix. The staining with antiphosphotyrosine antibody revealed that Stat3-deficient keratinocytes had an increased number of hyperphosphorylated focal adhesions. By using immunoprecipitation, we found that p130$^{cas}$ was constitutively hyperphosphorylated on tyrosine residues, while other focal adhesion molecules such as FAK and paxillin were not. Furthermore, impaired migration and increased tyrosine phosphorylation of p130$^{cas}$ in Stat3-deficient keratinocytes were rescued by wild type Stat3-gene transfer using adenovirus vector. These results strongly suggest that intracellular signaling of Stat3 in keratinocytes modulates tyrosine phosphorylation of p130$^{cas}$ and cell adhesiveness to the substratum leading to cell migration.

## 171

**Characterization of Cyclooxygenase in Epidermal Repair After Mild Abrasion**

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We investigated the role of cyclooxygenase (COX) in repair of the epidermis using a model of acute mild abrasion in SKH-1 hairless mice. Since regeneration of the epidermis following injury occurs via a carefully balanced program of keratinocyte proliferation and terminal differentiation, we characterized these pivotal keratinocyte responses in this model as well. Skin of SKH-1 mice was treated with ultra-fine sandpaper to produce a mild abrasive wound. Histologically this resulted in the disruption of the cornified layer of the epidermis without any changes within the dermis and no elicitation of an immune response. At various times following abrasion, the epidermis or whole skin samples were dissected. COX protein expression was analyzed by Western immunoblot and immunohistochemistry. Keratinocyte proliferation and differentiation were measured by BrdU incorporation and cytokeratin 6 immunohistochemistry. COX-2 was robustly induced within 6 h following abrasion, peaking at 72 h and returning to baseline by 120 h, whereas COX-1 expression was unchanged throughout the time course. The abrasive wound resulted in transient, but nonmalignant epidermal hyperplasia as measured by gross histology, BrdU labeling in the basal keratinocytes and by the expression of cytokeratin 6 in the suprabasal layers of the epidermis. Interestingly COX-2 was located only in the proliferating basal keratinocytes. These results indicate that Cox-2 expression is up-regulated following mild abrasive wounding.

## 172

**Biphasic Effects of Nicotine on Re-Epithelialisation in Cultured Human Keratinocyte Sheets**

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A number of studies suggest that repair mechanisms are adversely affected in patients who smoke. Nicotine has been found to influence keratinocytes via nicotinic acetylcholine receptors (nAChR) and nAChR expression increases with the differentiation state of keratinocytes. The purpose of this study was to investigate the effects of nicotine in an *in vitro* re-epithelialisation assay comparing the responses in undifferentiated and differentiated human keratinocytes. Primary keratinocytes isolated from routine abdominoplasty were cultured in collagen I coated 6 well tissue culture plates until multilayered keratinocytes sheets were obtained. Sheets were wounded by the impression of a cell scraper producing a $2 \times 5$ mm wound bed. Growth media was then supplemented with nicotine, and re-epithelialisation recorded every 24 h until re-epithelialisation was complete. Sheets of prematurely differentiated keratinocytes were produced by supplementing the growth media with 1 mM L-ascorbate 2-phosphate (LAP) for 3 days prior to wounding. A biphasic response to nicotine was noted; concentrations below 100 μM increased the rate of re-epithelialisation while 1 mM significantly reduced re-epithelialisation, increasing the time taken to reach 50% re-epithelialisation (t1/2) 1.5-fold. Induction of differentiation with LAP reduced re-epithelialisation. However, nicotine treatment of these differentiated cells gave a further 1.8-fold inhibition of re-epithelialisation. Treatment with nicotine prior to wounding gave a 2.5-fold increase in t1/2, while treatment of these pretreated keratinocyte sheets with nicotine gave a 3.6-fold increase in t1/2. We conclude that nicotine can have a biphasic affect on keratinocyte re-epithelialisation. However, the chief finding of this study was that nicotine at 1 mM significantly reduce re-epithelialisation of both undifferentiated and differentiated keratinocytes suggesting that it would be detrimental to normal wound healing.

## 173

**A Model for Studying Keratinocyte Morphology and Attachment at the Interface Between Skin and Percutaneous Devices**

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Adverse complications from medical percutaneous devices result in significant morbidity and mortality. Characterization of biomaterial/tissue interfaces by light microscopy (LM) and immunohistochemistry (IHC) is challenging since most biomaterials cannot be processed and sectioned for molecular morphology. In an effort to find the best biomaterial to use as a model to study the biomaterial/tissue interface, we systematically analyzed a number of biomaterials. Several unfixed materials including silicones, polyurethane and fluorinated polymers were directly embedded in paraffin and OCT(r) and sectioned for LM. Those biomaterials that successfully sectioned were further tested by fixation in 10% neutral buffer formalin or 1/2 Karnovskys. Two samples of polyfluorotetraethylene (PTFE) tubing, which appeared to fix and section well, were then implanted in a 6-mm punch biopsy of normal human skin to determine whether both PTFE and skin could be simultaneously sectioned without the PTFE being dislodged during sectioning. The PTFE/tissue was embedded in paraffin. Of the materials tested, the fluorinated polymers sectioned most easily, with PTFE tubing undergoing the least distortion and displacement from its original position within the embedding material. LM sections of PTFE material inserted through human skin biopsy tissue consistently produced sections with the PTFE/tissue interface intact and free of distortions. PTFE is a suitable material to implant percutaneously to study the morphology of the implant/tissue interface. It can be fixed, embedded and sectioned for LM with minimal distortion of the tissue/implant interface. This model may be used in bioengineering studies designed to improve the viability of long-term implantation of medical devices such as dialysis catheters, central venous lines and indwelling glucose sensors.

## 174

**The Active and Passive Roles of Extracellular Matrices and Growth Factors in Control of Human Keratinocyte Migration**

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Re-epithelialization of a human skin wound is a complex process involving interactions among cells, extracellular matrices (ECM) and growth factors/cytokines (GF/Cs). Despite of the importance of ECM and GF/Cs in wound repair, little is known about their specific roles in the control of human keratinocyte (HK) migration. In this study, we asked (i) if either ECM alone or GF/Cs without ECM are sufficient to drive migration; (ii) which elements play the active vs. passive roles; and (iii) what their respective relationships are in the regulation of HK migration. To investigate these questions, we used two independent well-established HK motility assays on collagen and tissue culture plastic substrata under conditions of complete and serum-depleted media. We found that collagen alone was able to drive HK migration to 50% (Migration Index/MI, ~15) of the maximum (MI, ~30) with no serum or growth factors present at all. In contrast, in the absence of collagen matrix the optimization of complete serum (10%) or any single GF/*C*-tested was unable to even initiate HK motility (MI, < 2%). This fundamental finding suggests strongly that ECM such as collagen initiates HK signal transduction leading to migration and is the primary motility factor. GF/Cs are secondary cofactors that further assist and maximize motility. Consistent with these data were the findings that the presence of GF/Cs enhanced the overall cellular protein tyrosine phosphorylation 2–3 fold as measured by densitometry scanning and cell surface focal adhesions 3–4 fold (from 17 to 29/cell to 57–94/cell). Interestingly, only the immobilized, but not soluble collagen, initiated HK motility. Surprisingly, the presence of soluble collagen to HKs apposed to collagen matrix attenuated the maximal motility by 30%. These observations suggest that the state of the collagen presented to the HK could determine different signaling outcomes initiated by the same integrin receptors and the overall balance of the cell migratory signaling. This study shows for the first time that ECMs such as collagen play an ''active'' role in HK motility while GF/Cs play secondary accessory roles that assist the predominant matrix-driven human keratinocyte migration.

## 175

**The Prefabricated Scapula Flap Consists of Syngeneic Bone, Connective Tissue and a Self-Assembled Epithelial Coating**

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The reconstruction of maxillary defects is a challenge in plastic surgery. The so called "prefabricated scapula flap" consists of syngeneic bone covered with syngeneic dermis (Holle *et al, Plast Reconstruct Surg* 98:542–552, 1996) and allows to cover large maxillary defects. After placing these flaps into the oral cavity, they are reepithelialized within an amazing short time period, raising the question of the cellular origin of the "neomucosa". We therefore performed sequential biopsies of the prefabricated flap and of the flap after being placed into the oral cavity and analyzed the keratin expression profile of epithelial cells. We expected that after placing the prefabricated flap into the oral cavity, keratinocytes from adnexal structures of the dermal component of the graft would migrate onto the surface and reepithelialize the flap. Unexpectedly, reepithelialization occurred earlier. The flap had acquired a mucosa-like epithelium at the interface between the Goretex coating and the dermis while still being positioned within the scapular region. The keratin expression profile of this epithelium was very similar to that of mucosal epithelium. Thus, the prefabricated scapula flap consisted not only of bone covered with connective tissue, but was also covered with epithelial cells derived from adnexal structures of the dermal graft. This seems to be the reasons for the rapid restoration of an intact mucosa and the excellent outcome achieved with this surgical technique.

## 176

**Two Complementary Mechanisms are Involved in Wound Reepithelialization**

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Wound reepithelialization must occur efficiently to restore rapidly the epidermal barrier function. We took advantage of the tissue-engineered wound-healing model that we have developed from human-skin cells to reevaluate the mechanisms of reepithelialization. We have produced excisional wounds in reconstructed human skins. After three days, we observed by immunohistochemistry two contributing patterns of wound reepithelialization. Initially located at the wound margin, the superficial epidermal layers were displaced over the wound bed. The inert and cohesive cells, forming these superficial layers, appeared to be passively displaced as a coherent sheet. Distinguished by their filaggrin and loricrin labelings, the cornified layer was located further toward the wound center than the granular layer, indicating that a pushing force, likely coming from the mitotic pressure, appeared to be responsible for their passive displacement. At the tip of the regenerating epidermis, the keratinocyte immediately behind and above the foremost basal one was elongating over the latter toward the wound center to contact itself with the extracellular matrix. We observed that keratinocytes expressing laminin 5 and collagen IV had a characteristic spatial distribution at the tip of the regenerating epidermis. Particularly evident for laminin 5, the labeling was characteristically present in deep and superficial cells further suggesting that superficial cells roll over basal ones to migrate toward the wound center. Therefore, reepithelialization occurs by two spatially independent and complementary mechanisms: (i) the passive displacement of the superficial layers as a coherent sheet near the wound margins and (ii) the rolling of individual keratinocytes over each others at the tip of the regenerating epidermis.

## 177

**Severely Delayed Wound Healing in TNF-Receptor p55 and IL-1 Receptor I Deficient/Double Deficient Mouse Models**

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It has been put forward that inflammatory response after cutaneous wounding is a prerequisite for healing. Inflammatory cytokines might be intimately involved in the healing process. We previously showed that TNF-Receptor p55 (TNF-R55) and IL-1 Receptor I (IL-1RI) are of crucial importance for signal transduction during skin permeability barrier repair. In the present study, we examined the healing of full thickness wounds in TNF-R55 deficient and TNF-R55/IL-1RI double deficient mice. We found that deficient mice had a highly significant delay in wound repair and required up to eight more days than wild-type controls for complete wound healing. In TNF-R55/IL-1RI double deficient mice, wound healing had completely ceased. These effects were partially related to wound contraction, which was reduced in TNF-R55 deficient mice and completely abolished in TNF-R55/double deficient mice. Reepithialization was also significantly reduced in TNF-R55 deficient and absent in TNF-R55/IL-1RI double deficient mice. Epidermal proliferation important for cell replacement was reduced in the deficient mice. A reduction also occurred in the activities of acid and neutral sphingomyelinases downstream of TNF-R55 and IL-1RI. Sphingomyelinase generates ceramide which works as a second messenger and is important for epidermal differentiation during wound repair. In summary, we found severely delayed wound healing related to diminished wound contraction, epidermal proliferation, differentiation, and sphingomyelinase activity in TNF-R55 deficient and TNF-R55/IL-1RI double deficient mice.

## 178

**Epidermal Margins of Chronic Nonhealing Ulcers Lack K10 and K2e in Contrast to Normal Acute Human Wounds**

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Keratinocytes at the margins of chronic ulcers show evidence of excessive proliferation but fail to migrate across the wound bed in contrast to normal acute wounds. We evaluated keratinocytes in the margins of chronic ulcers vs. acute wounds for differences in protein expression. Diabetic and decubitus ulcers including adjacent tissue were collected from patients undergoing amputation or surgical repair, respectively. Acute incisional 6, 24 and 72 h wounds created on normal age-matched subjects were biopsied postwounding. Tissue was frozen in OCT(r) and immunoperoxidase-labeled, using antibodies to $\alpha2\beta1$ integrin (collagen receptor), $\alpha3\beta1$ integrin (laminin 5 receptor), $\alpha5\beta1$ (fibronectin receptor), K16 (wound keratin) and keratinocyte suprabasal differentiation markers, K10 and K2e. Staining patterns for $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha5\beta1$ integrins were similar in the keratinoctyes in the margins of chronic ulcers and normal acute wounds. Keratinocytes throughout the chronic ulcer and normal acute wound margins intensely immunstained for K16. Keratinocytes on the migrating tongue of 6 h to 3 day incisional acute wounds expressed K10 and K2e. In contrast, staining for K10 and K2e was absent in the margins of diabetic and decubitus chronic ulcers. It is unclear why hyperproliferative keratinocytes in the margins of chronic ulcers do not stain for K10 and K2e despite morphologically appearing to be suprabasal. The data raises questions about alterations in normal differentiation of this hyperproliferative epithelium. The "rolling model" of wound re-epithelialization hypothesizes that differentiated suprabasal keratinocytes migrate across the wound bed. If this model is correct, the absence of normal suprabasal keratinocyte phenotype in the wound margin of chronic ulcers may be associated with defective migration.

## 179

**Heterogeneous Molecular Mechanisms Regulate Activation-Specific Transcripts Following Keratinocyte Contact with Type I Collagen**

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Keratinocytes from intact, uninjured skin express specific patterns of genes that promote proliferation and differentiation. However, in injury that disrupts the basement membrane, wound edge keratinocytes contact dermal extracellular matrix (ECM) and are activated to undergo global changes in gene expression to support re-epithelialization. The factors that induce keratinocyte activation following injury, whether soluble or matrix, remain to be fully elucidated. Type I collagen, an abundant dermal ECM protein, may play an important role in keratinocyte activation following wounding. Indeed, when keratinocytes from intact skin are plated onto fibrillar type I collagen *in vitro*, they are stimulated to express collagenase-1, a matrix metalloproteinase expressed invariantly by activated keratinocytes at the wound edge. Using differential display reverse transcription PCR, we previously identified several other activation-specific genes that are modulated in keratinocytes following contact with collagen (e.g. small proline rich protein, involucrin, $\alpha6$ integrin, ApM2, calumenin, cystatin A, ADP/ATP carrier protein, MAD immediate early gene, and Lzip transcription factor). In the present study, we sought to define the molecular mechanisms by which collagen contact mediates this altered pattern of gene expression. Interestingly, we found significant variation among transcripts with respect to their time course of up-regulation following collagen contact. Further heterogeneity was exhibited in the response to collagen structure as a subset of transcripts demonstrated a strict requirement for the native, triple helical conformation of collagen, whereas others were induced equally on collagen or gelatin (heat denatured collagen). Lastly, the intracellular signaling mechanisms required for collagen-mediated expression were transcript-specific as extracellular signal-regulated kinase 1,2 (ERK 1/2), p38 MAP kinase, and Jun kinase were each required for collagen induction of specific mRNAs. This heterogeneity underscores the complex ordered orchestration of keratinocyte gene induction upon contact with type I collagen and further supports the importance of this cell:ECM interaction in keratinocyte activation following injury.

## 180

**Co-up-regulation of Syndecan 2 and Integrin $\alpha5\beta1$ in Basal Keratinocytes During Cutaneous Wound Healing**

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We have previously reported that in cutaneous wound healing (CWH), fibronectin (FN) is a provisional matrix for keratinocytes migration and FN receptor $\alpha5\beta1$ has been induced in basal keratinocytes (BK). Our recent report on the syndecan (Syn) 2 expression has shown that Syn 2 participated selectively in the induction of stress fiber formation in an epithelial cell line in cooperation with integrin $\alpha5\beta1$ through specific binding to FN. In this context, we have studied Syn1, 2, 3, and 4 expressions in migrating epidermis in mouse full thickness CWH model by using polyclonal antibodies raised against mouse Syn 1–4 in our laboratory. In the immunohistochemical studies on normal skin, Syn1 and 4 were detected in the whole epidermal layers and Syn 2 was in BK layer, but Syn 3 was not. After wounding, Syn 1 was enhanced in whole epidermis but that of Syn 4 was not changed. The expression of Syn 2 in BK was also clearly enhanced especially in the dermal side of the cells. The intensity of Syn 2 expression was increased by degrees toward the migrating epidermal edges. The expression of $\alpha5\beta1$ was also induced in the migrating epidermal BK with the gradient increase of the intensity toward the migrating edges, which was comparable to the Syn 2 expression. Semi-quantitative RT-PCR studies on the Syn mRNA expressions in murine cultured normal keratinocytes revealed that the expressions of Syn 1,2 and integrin $\alpha5\beta1$ were significantly up-regulated in the cultured keratinocytes, compared with the freshly isolated keratinocytes. The up-regulated Syn 2 and integrin $\alpha5\beta1$ expressions in the cultured BK were down-regulated after confluency to the basal levels. These observations show that the expressions of Syn 2 and integrin $\alpha5\beta1$ are cooperatively regulated in the epidermal BK in CWH, which may suggest that the Syn 2 expressions play important roles on integrin $\alpha5\beta1$ mediated BK cell adhesion during CWH.

## 181

**Glutathione Treatment Accelerates Diabetic Wound Healing**

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Cutaneous wound healing is relatively slow in diabetic patients. We hypothesized that this defect in healing of diabetic patient wounds results from skin fibroblast and epidermal keratinocyte dysfunction. Therefore, the contraction of fibroblasts in a fibroblast populated collagen lattice, and keratinocyte apoptosis in the presence and absence of esterified GSH (glutathione) were investigated. The effect of topical esterified GSH on the wounds of the mutant C57BLKS/J-m+/+Lepr db diabetic mouse with impaired healing was also determined. Adult human fibroblasts and keratinocytes were grown in Dulbecco's growth medium and serum and lipid-free MCDB 153, respectively. Primary cultures of human dermal fibroblasts were used to test the hypothesis that the lack of wound contraction in diabetic patients is a result of fibroblast dysfunction and that the defect(s) in fibroblast contraction is related to disrupted intracellular GSH homeostasis. The fibroblast populated collagen lattice (FPCL) is an *in vitro* contraction model where fibroblast gel contraction is measured. High glucose medium (hyperglycemia) reduced the contraction ability of fibroblasts ($p < 0.05$). The normalization of glucose medium concentrations for hyperglycemic fibroblasts did not restore the contraction capacity. The percentage of apoptotic keratinocytes was statistically higher in hyperglycemic cells ($p < 0.05$). Non-diabetic control mice showed a 71% reduction in wound size 15 days post injury. In the diabetic mice, the wounds actually enlarged to 150% the area of the original wound. In contrast, treatment of wounds in diabetic mice with 100 mM esterified GSH decreased the size by 48% ($p < 0.05$). These data suggest that restoration of intracellular GSH level in diabetic patients could shorten the healing time of diabetic wounds.

## 182

**Characterization of Human Epsin 3, a Novel Gene Induced by Contact with Type I Collagen that is Specific to Wounded Epithelia**

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In injuries that disrupt the basement membrane, wound edge keratinocytes contact dermal extracellular matrix (ECM) and are activated to undergo global changes in gene expression, switching to a program that supports repair of the tissue defect. Type I collagen, an abundant dermal ECM protein, plays an important role in keratinocyte activation as changes in cell:matrix interactions induce transcripts required for re-epithelialization. Using ddRT-PCR we have identified epsin 3, a novel transcript markedly induced (20-fold) following keratinocyte contact with type I collagen. The 1.9 kb epsin 3 cDNA codes for a 632 amino acid protein closely related to, but distinct from other members of the epsin protein family. Epsins 1 and 2 are constitutively and ubiquitously expressed proteins purported to function as molecular bridges required for clathrin-mediated endocytosis. Interestingly, and in contrast to other known epsins, epsin 3 was expressed specifically by collagen-activated keratinocytes, in wounds or pathologies that exhibit a disrupted basement membrane (e.g. chronic wound, ulcerative colitis), and basal cell carcinoma. Epsin 3 expression was not found, however, in any homeostatic tissues examined or cutaneous pathologies that lacked basement membrane disruption (e.g. psoriasis). Induction of epsin 3 mRNA in keratinocytes occurred 12–14 h after keratinocyte contact with collagen and the native, triple helical conformation of collagen was required to mediate this response. Epsin 3 protein, as detected by Western immunoblotting, was not expressed in keratinocytes freshly isolated from intact skin, but was markedly induced in cells 24 h following collagen contact. Localization of epsin 3 by immunofluorescence demonstrated a punctate, vesicular pattern reminiscent of endocytic vesicles throughout the cytoplasm of migrating keratinocytes. Indeed, epsin 3 labeled structures colocalized with clathrin, suggesting a role for this protein in endocytosis. Thus, we have identified a novel epsin that is induced specifically in epithelial wounds or pathologies exhibiting a disrupted basement membrane, suggesting that this transcript serves an important role during tissue repair and morphogenesis.

## 183

**Inhibiting Damage by Reactive Carbonyl Species and Advanced Glycation Endproducts in Human Skin Cells**

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Human skin is directly exposed to many chemical oxidants, air pollutants, and UV irradiation, all of which have the potential to induce reactive oxygen species (ROS), and reactive carbonyl species (RCS). Both ROS and RCS are known to play important roles in human skin deterioration leading to the visible effects of skin aging and ultimately to actinic keratosis and carcinogenesis. Although skin damage is known to originate from the complex interactions between ROS and RCS, most research has focused on identification of potent inhibitors of ROS to limit the damage, e.g. topical application of vitamin E. Very little work has been done to prevent RCS damage in skin. To this end, we have used human skin fibroblasts and keratinocytes as a model system to search for RCS inhibitors. Cells were exposed to increasing concentrations (0–600 μM) of glyoxal (G) and methylglyoxal (MG), both potent inducers of dicarbonyl stress, for 72 h, in the absence or presence of the potent dicarbonyl scavenger, D-penicillamine. L-Alanine was used as a negative control, as this compound would not be expected to scavenge dicarbonyls. Using this model, 1 mM D-penicillamine showed no toxicity during a 72-h exposure to either keratinocytes or fibroblasts. In both cell types, G and MG caused concentration dependent growth inhibition, with the highest concentration of 600 μM inhibiting growth by 90%. As expected, coincubation with 1 mM L-alanine showed no protective effects against G and MG. In contrast, coincubation with 1 mM D-penicillamine resulted in nearly complete protection of cells against the growth inhibitory effects of G and MG. Using the same model system, we assessed the ability of these two compounds to cause DNA strand breaks using the comet assay. G induced DNA strand breaks whereas MG caused extensive DNA cross-linking. Furthermore, D-penicillamine protected cells from DNA strand break formation. These data establish D-penicillamine as a potent dicarbonyl scavenger able to scavenge equimolar concentrations of dicarbonyls. We conclude that compounds with structural features similar to D-penicillamine are good candidates for topical delivery to decrease the effects of protein and subsequent DNA damage in skin.

## 184

**Biomarkers to Assess Prevention and Repair of Oxidative and Carbonyl Stress in Skin**

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Skin deterioration results from molecular damage to tissue macromolecules, such as proteins, lipids, and DNA. Reactive oxygen (ROS) and reactive carbonyl species (RCS) are key intermediates of oxidative and carbonyl stress leading to cellular damage and disruption of genomic integrity. In skin, formation of ROS and RCS result from various endogenous and exogenous sources, e.g. exposure to solar light, xenobiotics, and oxygen metabolism. Enzymatic and nonenzymatic defence mechanisms have evolved to reverse the detrimental effects of ROS and RCS. Anatomically, epidermal skin cells are at the end of the body's nutrient supply line. A variety of oral and topical antioxidant formulations have been developed to support skin cell maintenance and repair. To evaluate the effectiveness of skin treatments designed to suppress the consequences of oxidative and carbonyl stress, we have developed an analytical method to measure intranuclear protein damage. Formation of histone-bound carboxymethyllysine (CML) and carboxyethyllysine (CEL), advanced glycation end products and cellular markers of glycoxidative stress were detected in the human keratinocyte cell line, HaCat, exposed to the biogenic RCS, glyoxal, and a key intermediate of lipid peroxidation and glycation. Intranuclear accumulation of CML and CEL were quantified after hydrolytic release from histone, enrichment by ion exchange chromatography, and OPA-derivatization followed by HPLC using fluorescence detection. Our data show that these biomarkers can be used to detect with high sensitivity and selectivity damage resulting from ROS and RCS. This method should be useful in assessing the effectiveness of therapeutic and preventive agents that target repair of glycoxidative skin cell damage.

## 185

**Functional Proteasome Alterations in Aging Human Keratinocytes**

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Aging cells accumulate damaged DNA, lipids and proteins, leading to various alterations that are strongly believed to contribute to the aging process. In particular, proteins constitue a target for the attacks of reactive oxygen species that generate protein carbonyl derivatives, thus impairing their functional properties. It is now well established that the turnover of oxidized and misfolded proteins is mainly achieved by the multicatalytic protease, or "proteasome". *In vivo*, the central catalytic complex of proteasome (the "20S" particle) is found in association with different activator complexes, namely the "PA700<inch> or "19S" complex that contains ATP-ase activities and is mainly represented in the nucleus, and the "PA28" or "11S" complex which is present mainly in the cytoplasm. Results from several laboratories strongly suggest that impairment of proteasome function could contribute to the age-related accumulation of altered proteins. We analyzed the status of proteasome function in aging human keratinocytes, in cell cultures and in epidermis samples. We showed that glycated and oxidized proteins accumulated with the number of cell divisions in cultures, and with the age of donors *in vivo*. We showed that this phenomenon could be correlated with a decrease in proteasome amounts and activities in soluble cellular extracts, compared with total protein content. This suggested that proteasome may be involved in cellular aging in keratinocytes. For a better understanding of the *in vivo* pathway by which proteasome may be involved, we partially separated different forms of proteasome from human keratinocytes by 100 000 g centrifugation of soluble extracts, followed by gel filtration (Superose-6, Sephacryl-500) of the resuspended pellet. This allowed to define differential assay conditions for different kinds of proteasome complexes. In parallel, we developed an *in situ* method to measure proteasome activity within human permeabilized keratinocytes. The *in situ* assay was combined with differential assay conditions to evaluate the status of proteasome activity in aging cultures of human keratinocytes. Results showing quantitative and qualitative changes in proteasome activity upon cellular aging will be presented and discussed.

## 186

**Gelatinases in Stratum Corneum of Healthy Human Facial Skin**

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Gelatinases, which belong to the family of matrix metalloproteinases (MMPs), and degrade a wide range of substrates including gelatin, type IV collagen, elastin, laminin, and fibronectin, may be involved in photoaging. We have demonstrated that gelatinase activity exists at the basal layer of the epidermis and we have observed histologically the decomposition of basement membrane structures. We show here that gelatinases are present in healthy human facial stratum corneum (SC). MMP-2 and MMP-9 were measured by immunostaining, gelatin zymography, and enzyme-linked immunosorbent assay (ELISA) kits specific to their proenzyme forms. Extracts of SC sheets detached from sunburnt skins contained pro-MMP-2 and, more abundantly, pro-MMP-9. Positive immunostaining of pro-MMP-9 was also observed in SC stripped from facial skin without artificial UV-exposure, but not in SC from nonexposed skin of the back, chest and the inside of the arms. In order to detect gelatinases in SC, we developed a special film coated with gelatine and colloidal silver which sensitively reacts with gelatinase propeptide. Ninety percent of SC from facial skins of 61 healthy women (45–55-year-old) showed positive for gelatinases on the film. These results strongly suggest that gelatinases are constantly produced in the facial epidermis of most middle-aged woman during their daily life and therefore might be associated with the aging-related degeneration of the matrix fibers of the basement membrane and upper layer of the dermis.

# 187

**UV-B Induces Phosphorylation of the Small Heat Shock Protein (HSP27) in Human Corneal Epithelial Cells**

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Acute exposure to ultraviolet B radiation (280–315 nm, UV-B) is known to cause photokeratitis and shedding of corneal epithelial cells. The 27 kDa small heat shock protein (HSP27) is thought to protect cells from varied classes of stress including UV-B exposure. Recent studies have demonstrated that it is the phosphorylation of HSP27 that plays an important role in cell survival after stress. We investigated whether UV-B irradiation induces phosphorylation of HSP27 in human corneal epithelial cells. Corneal epithelial cells were cultured and exposed to UV-B radiation at a dose of 30 mJ per $cm^2$. Isoelectric focusing (IEF) followed by immunoblotting was carried out on cell lysates to identify HSP27 phosphorylated isoforms. UV-B irradiation increased the level of bi-phosphorylated HSP27B and tri-phosphorylated HSP27C isoforms and decreased the level of the nonphosporylated HSP27A isoforms proportionally. UVB-induced phosphorylation was reversible after two hours of recovery. UV-B irradiation also induced a heterogeneous relocalization of HSP27 to the peri-nuclear area. UVB-irradiation also activated the p38 MAP kinase in these cells, and inhibiting this kinase diminished UVB-induced phosphorylation of HSP27, indicating a p38-mediated mechanism for UVB-induced HSP27 phosphorylation. Normal cornea was also found to express HSP27 within the cytoplasm of limbal epithelial cells. These findings demonstrate that HSP27 is normally present within corneal epithelial cells, and that UVB stress initiates p38-mediated signaling which results in HSP27 phosphorylation which may provide a protective mechanism to UVB-exposed cells.

# 188

**UV-B Light Suppresses the All-*trans*-Retinoic Acid-4-Hydroxylase (CYP26) and Induces AP-1 and p53 in HaCaT Keratinocytes**

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UVB-light is know to exert a variety of effects on the expression of nuclear factors in skin. The proto-oncogen AP-1 and also the p53 are known to be induced in normal epidermal keratinocytes upon UVB-irradiation. However, only little is known concerning the effect of UVB on the expression of enzymes of the cytochrome p 450 multigene family. These enzymes catalyze the oxydation of various xenobiotics and endogenous molecules. One member of this family is the all-*trans*-retinoic acid-4-hydroxylase (Cytochrome 26, CYP26). It catalyzes the 4-hydroxylation of all-*trans* retinoic acid. The CYP26 activity is essential for the inactivation of the hormone active retinoid all-*trans*-retinoic acid. In the present study, the effect of UVB exposure on the expression of AP-1, p53 and the CYP26 in human epidermal HaCaT keratinocytes was determined by immunoblotting, semiquantitative RT-PCR, and on the biochemical level by reverse phase high performance liquid chromatography (RP-HPLC). RP-HPLC analysis of UV-B-irradiated cells (0,1, 1, 6, 12, and 20 mJ per $cm^2$, respectively) revealed a dose dependent decrease of 4-hydroxy metabolites of retinoic acid while the intracellular levels of all-*trans* retinoic acid remained stable. Semiquantitative RT-PCR revealed an abolished basal expression, and a decreased inducibility of the CYP26 mRNA expression, while the beta-actin expression (internal control) remained unchanged. Simultaneously, AP-1 and p53 protein were found to be induced by UVB-radiation. These results demonstrate that UVB radiation can alter the metabolism of retinoids in a dose dependent fashion in skin cells. The induction of AP-1 and p53 indicates that the CYP26-suppression is a cell specific event and not simply due to UVB-induced cell damage.

# 189

**The Role of the Epidermal Growth Factor Receptor in the Response of Human Keratinocytes to Ultraviolet B-Irradiation**

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The epidermal growth factor receptor (EGF-R) is a critical molecule in a wide range of signal transduction pathways. In human keratinocytes, the function of the activated EGF-R is thought to depend on the proliferation status of the cell. For example, in proliferating keratinocytes the activation of the EGF-R by ligands of the EGF-family results in the stimulation of mitotic signal transduction pathways. In contrast, stimulation of the EGF-R in keratinocytes that cannot replicate leads to the induction of differentiation. Activation of the EGF-R has also been demonstrated to occur in the absence of direct ligand binding. Exposure of human keratinocytes to UVB radiation leads to tyrosine phosphorylation of the EGF-R. Since the outcome of EGF-R activation by EGF-family members is dependent on the growth status of keratinocytes, we asked if the activation of the EGF-R by UVB has different cellular consequences depending on the growth potential of the irradiated keratinocyte. Normal primary human keratinocytes were grown *in vitro* such that they were maintained as actively growing cells or they were allowed to become confluent on the culture dish, inducing cell-contact dependent growth inhibition. These keratinocytes were then irradiated with UVB and treated with the EGF-R tyrosine kinase inhibitors DAPH or AG 1478. Inhibition of EGF-R activation in actively growing keratinocytes renders them resistant to UVB-induced apoptosis. In contrast, if the EGF-R is inhibited in nonproliferating keratinocytes, then the keratinocytes are more sensitive to UVB-induced apoptosis. Therefore, the growth potential of keratinocytes determines what role the EGF-R will play following exposure to UVB radiation.

# 190

**An *In Vitro* Epidermal Lift Culture Model Using BALB/MK Mouse Keratinocytes Cultured on MDCK Cell-Derived Basement Membranes and Other Substrates**

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We are interested in developing *in vitro* 3-D models that can help to dissect early vs. late events during differentiation of mouse keratinocytes. Although reconstruction of a complete mouse epidermis has been reported using dead devitalized dermis as a substrate, this system has its drawbacks in terms of the ability to isolate individual factors essential to the differentiation process. Simpler systems with mouse cells, in the absence of a dermal substrate, have been problematic because mouse keratinocytes tend to attach and grow poorly on conventional support matrices. The purpose of this study was to adapt a previously established rat keratinocyte 3-D system (REK), for use with mouse keratinocytes. Following the REK approach, mouse BALB/MK keratinocytes were cultured on a reconstituted collagen gel (type I collagen, rat) for one week. The results showed scattered foci of keratinocytes, indicating failure of cells to adhere. In order to create an environment conducive to cell adhesion, we pretreated the collagen matrix by first depositing a native basement membrane using MDCK (canine kidney) cells. MDCK cells were plated on the collagen at a density of $2 \times 10^5$ cells/insert, grown for two weeks and then lysed, washed, and replaced with BALB/MK cells at $2.5 \times 10^5$ cells/insert. After achieving confluence in submerged culture, BALB/MK cells were raised to the air–liquid interface. On MDCK-derived basement membranes, the keratinocytes were healthy, but unlike the REK model, showed only incomplete stratification. We also tried depositing a number of substrates directly on the polyester inserts including (1) MDCK-basement membranes, (2) mouse laminin, (3) mouse collagen IV, and (4) human fibronectin. All allowed formation of a BALB/MK monolayer. These cultures showed incomplete differentiation, exhibiting scattered islands of stratified cells. Of note, inserts coated with MATRIGEL failed to permit cell adhesion. These findings are important initial steps in establishing an *in vitro* mouse epidermal raft culture model that will allow, in the absence of feeder fibroblasts or other undefined factors, the introduction of defined growth factors and gene products into the system. We are currently exploring the suitability of this system for use with primary keratinocytes from knockout and transgenic mice.

# 191

**Keratinocytes are the Instructive Cells in Engineered Skin and Gingival Tissue Substitutes**

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Grafting skin into the oral cavity is commonly used for oral mucosal defects resulting from excision of malignancies. However, skin grafted into the oral cavity maintains its original structure and function, and often patients experience hair growth and sweating in the grafted areas. In order to determine whether fibroblasts are instructive or permissive to these grafts, we engineered tissue substitutes using fibroblasts and keratinocytes isolated from skin or gingiva. Each type of fibroblast was allowed to remodel collagen gels for two weeks, then either skin or gingival keratinocytes were added, and the four combinations were grown at the air/liquid interface for four weeks. The reformed epithelia resembled the original epithelia and expressed the proteins found in the keratinocyte's original tissue despite the type of fibroblast present in the collagen gel. K14, Loricrin and Filaggrin were expressed by both skin and gingival keratinocytes, whereas K1 was expressed by the skin keratinocytes only, and K4 and K19 by gingival keratinocytes only. Using a newly designed miniscale isoelectric focusing unit in combination with SDS-PAGE, we performed two dimensional electrophoresis analysis of proteins secreted into the collagen gel. This novel approach allowed us to perform the first step in a liquid medium, making pH determination and sample recovery easy and precise. The fractions were analyzed on second dimension SDS-PAGE for the identification of differentially expressed proteins. Profiles of secreted proteins changed in response to the presence of different keratinocytes. Thus the keratinocytes appear to be the governing cell in our *in vitro* tissue engineered model. These recombination experiments suggest that keratinocyte differentiation is intrinsic, whereas fibroblast protein secretion is influenced by the type of keratinocyte present.

# 192

**A Retinoid-Containing Protein-Free Defined Medium for the Culture of Normal Human Keratinocytes**

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Commercial serum-free media designed for the culture of normal human keratinocytes are typically supplemented with EGF, insulin and a panoply of undefined protein factors present in BPE. There is clearly a need to simplify these growth factor requirements and, if possible, eliminate them altogether. Toward this goal, we have designed a proprietary keratinocyte culture medium designated HECK 109. This medium has superior clonal and batch culture growth performance due to its physiological osmolarity (280–300 mOsms), reduced organic buffer toxicity (20–25 mM, Hepes), and selective optimization of six amino acids (his, isoleu, met, phe, tryp, tyr). Extensive growth studies with this medium have validated its use for both research and product development purposes. This medium only requires EGF and IGF-1 to achieve confluent cell densities. It was successfully employed to generate autologous epidermal skin grafts for a clinical trial testing its safety and efficacy in the treatment of chronic leg ulcers. Here, we report that sustained proliferation occurs in this medium without exogenously added growth factors when supplemented with physiological amounts of retinyl acetate (RAc). Proliferating keratinocyte cultures initiated in HECK 109 medium supplemented with EGF and IGF-1 were refed growth factor-deficient standard HECK 109 medium, i.e. supplemented with 0.15 mM $Ca^{+2}$, hydrocortisone ($5 \times 10^{-7}$ M), ethanolamine ($10^{-4}$ M), and phosphoethanolamine ($10^{-4}$ M) or standard HECK 109 medium containing $3 \times 10^{-8}$ M RAc. Two and four days later the living cultures were photographed with phase contrast optics, then fixed and stained with crystal violet. As expected, cultures refed standard medium only were growth arrested and assumed a more compact colony morphology. By contrast, cultures refed RAc in standard medium grew at ~ half the rate of control cultures refed media replete with protein growth factors, and displayed a loose colony morphology typical of proliferating cultures. We propose that Rac blocks growth factor-dependent commitment to terminal differentiation.

## 193

**Refinements of Long-term Culture of Mouse Epidermal Keratinocytes**

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We previously described a method that allows the growth, subculture, freezing, and reconstitution of total mouse epidermal keratinocytes (MEK) from single newborn pups. The technique involves growth of MEK on collagen IV coated dishes in medium conditioned by primary cultures of murine fibroblasts with 0.06 mM calcium and added growth factors (CM1). However, production of CM1 is expensive, requiring multiple newborn pups. The purpose of this study was to compare medium conditioned by cell lines with CM1. Medium was conditioned by actively growing, subconfluent 3T3 cells, human fibroblasts, and 1st and 2nd passage newborn mouse fibroblasts. MEK were grown to confluence, subcultured, frozen as viable stocks, and induced to differentiate in all media except that from human fibroblasts, which did not support growth. Growth rates in all mouse cell-conditioned media did not differ significantly. Primary MEK cultures were initiated successfully in all mouse media, and primary MEK initiated in CM1 could be passaged or thawed into other conditioned mouse media. Stratified keratinocyte cultures expressing keratin 1 and profilaggrin were induced in all mouse media through passage 10 by first purging the monolayer cultures of growth factors, then adding medium with 0.15 mM calcium. The new mouse media have been used successfully for the study of MEK from the spontaneous mutant mice, flaky tail *(ft/ft)* and harlequin ichthyosis *(ichq/ichq)*. These refinements simplify the production of conditioned media and facilitate long-term culture of MEK.

## 194

**Behavior of Different Types of Keratinocytes in the Formation of Skin Equivalent**

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Skin Equivalent (SE) is well established model to study skin regeneration, wound healing, drug testing and, etc. More reproducible SE model is a desirable tool for most of these topics. Formation of SE involves the ability of keratinocytes (Ks) to establish an epidermal monolayer followed by epidermal stratification and differentiation. We showed in our previous work (*JID*, 1996) that Fibroblasts (Fbs) plays an important role in the full thick epidermis development. The role of Ks is not clear. We used 4 Fbs lines: 3T3, Adult Normal, Keloid (Kl) and Foreskin Fbs and 5 Ks lines: Adult Nl, Kl, Psoriatic (Ps), transformed psoriatic (gift of Dr I. Leigh lab) and Foreskin Ks. We combined each line of Fbs with each line of Ks. We did 6 SEs for each combination (total 120 SEs). Fbs lines at passage 3–4 were placed in collagen gel in 1 cm insert. Ks at passage 1 were added after 24 h. FAD media were changed every 24 h. SEs were sacrificed on day 14. In our case only foreskin Ks on top of DE with any type of Fbs (24 SEs) were able to develop full stratified epidermis (except for Kl Fbs which developed SE with abnormal stratification). All other Ks fail to produce normal differentiation and keratinization. Since Retinoids play an important role in epidermal differentiation we added Retinoids in FAD media and fed SE constructed with foreskin Fbs and the same 5 types of Ks. Again, we did not see a big difference in epidermal formation. Transformed Ks formed bubbly abnormal multilayer epidermis without layers of differentiation regardless of Retinoid's presence. Ps Ks fail to form full monolayer. We showed that transformed or psoriatic Ks can not be use to create easily reproducible SE model. Unfortunately the best SEs are formed with Normal Foreskin Ks at p1, demanding permanent source of young Ks.

## 195

**New Skin Equivalent as a Model for Evaluation of Cutaneous Irritants**

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Recently, in our laboratory a new skin equivalent model using a dermal substrate that combines de-epidermized dermis with fibroblast-populated collagen matrix was developed. In this study the new skin equivalent was used to study irritating effect of sodium lauryl sulfate (SLS) and compared with the results of previous skin equivalents (RE-DED, LSE). After a 24-h exposure to SLS (0–5%) the extent of cytotoxicity was evaluated on the basis of morphology and the expression pattern of differentiation markers. In the new skin equivalent SLS induced dose-dependent changes in epidermal morphology. When SLS was applied topically in concentration of 1% on new skin equivalent and RE-DED and 0.5% on LSE, significant changes in epidermal morphology and changes in the expression and localization of differentiation markers were observed. These results indicate that the new skin equivalent has similar susceptibility to SLS compared to RE-DED. LSE seems to be more susceptible to SLS than new skin equivalent. Thus, new skin equivalent may be used as a model for evaluation of cutaneous irritants.

## 196

**Reconstruction of a Human Skin Equivalent using Epithelial Cells Derived from Umbilical Cord**

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Umbilical cord epithelia (UCE) is usually described as consisting of a single epithelial layer. In fact, stratified regions often occur, in which the expression of differentiation-specific protein markers such as keratins 1 and 10, loricrin, involucrin and transglutaminase can be detected as in the epidermis. We have therefore hypothesized that UCE cells have the potential to differentiate in the same manner as the epidermis. In this study, we attempt to establish air-exposed cultures to form a multilayered epithelial sheet using the cells derived from the umbilical cord (UC), grown on artificial substrates immersed in culture medium, and then cultivated with an air–liquid interface. By this method, we succeeded in stimulating the UC cells to differentiate in a manner similar to cutaneous keratinocytes, four cell layers, while the cornified layers displayed orthokeratosis. The granular layers with keratohyaline granules stained positively when exposed to filaggrin antibody. We attempted to graft UCE cells on the back of nude mouse, and found that the morphology and immunohistochemistry of the transplanted tissue showed the development of both a granular and a horny layer almost like normal rat epidermis but without any hair follicles, appendages, or rete ridges. These interesting features of UCE cells may provide us not only with useful information about epithelial differentiation, but also with beneficial laboratory-grown substitutes for epithelial graft for clinical practice.

## 197

**An Improved and Rapid Method to Construct Skin Equivalents from Human Hair Follicles and Fibroblasts**

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To produce sufficient amounts of high quality skin equivalents (SE), either allogenic for dermatopharmacological and dermatotoxicological studies or autologous for transplantation purposes, we established a rapid, easy and cost effective three-dimensional SE model on the basis of human dermal fibroblasts, collagen and freshly plucked hair follicles. Acidic liquid collagen was polymerized with sodium hydroxide in the presence of fibroblasts to form a dermal equivalent (DE) resembling normal human dermis. 24 h later, freshly plucked hair follicles were implanted into the surface of these DEs after cutting their bulbs off. Another 48 h later, the surface of the SEs was lifted to the air–liquid-interface. 14 days after implantation, outgrowing keratinocytes from the outer root sheath of the hair follicles completely covered the surface of the SE and built a fully developed, multilayered and cornified epidermis. Histology and immunofluorescence studies with specific antibodies directed against components of keratinocytes, fibroblasts, cell-adhesion molecules, different extracellular matrix and basement membrane proteins revealed the similarity of our three-dimensional SEs to the *in vivo* situation in normal human skin. Using autologous cell sources and cell culture media enriched with serum from the respective cell donor, it will be possible to use these SEs for autologous transplantation, thereby reducing the risk of transplant rejection.

## 198

**Carrier Surfaces for Culture of Keratinocytes and Melanocytes for Clinical Use**

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Methodologies for culture and transfer of laboratory-expanded keratinocytes and melanocytes to the patient remain a challenge. The aim of this study was to produce a chemically defined well characterised surface suitable for keratinocyte and melanocyte attachment and proliferation from which subconfluent cells could be transferred for epithelialisation and repigmentation. Plasma polymerisation was used to produce surfaces containing acid, amine and alcohol functional groups and their interactions with keratinocytes examined in terms of attachment (MTT activity 24 h postseeding) and proliferation (DNA analysis over 7 days). For keratinocytes, the most effective attachment (approaching that achieved for keratinocytes on collagen I) was achieved with a low percentage (2–5%) of acid functional groups. Cells proliferated well on these surfaces and retained the ability to transfer to an *in vitro* wound bed model based on sterilised de-epidermised acellular human dermis. Transfer of cells from acid functionalised surfaces to this dermis was 80% as efficient as for cells cultured on collagen I. As we have shown that high density keratinocytes can down-regulate both melanocyte number and pigmentation, our next challenge was to develop a surface to support subconfluent keratinocytes and melanocytes for grafting of patients with vitiligo. Our preliminary data indicate that amine functionalised surfaces are appropriate for coculture of keratinocytes and melanocytes and that cell interactions with these surfaces can be positively influenced by the media used. In conclusion, we report that it is possible to produce chemically defined surfaces suitable for the culture and subsequent transfer of keratinocytes and melanocytes for clinical use.

# 199

**Comparison of Human Skin or Epidermis Models with Human and Animal Skin in *In Vitro* Percutaneous Absorption**

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For the study of *in vitro* skin penetration of candidate drugs, excised animal skin is frequently used as replacement for human skin. Reconstructed human skin or epidermis equivalents have been proposed as alternatives. We compared the penetration properties of human, pig and rat skin with the Graftskin(tm) LSE(tm) (living skin equivalent) and the Skinethic(tm) HRE (human reconstructed epidermis) models using four topical dermatological drugs (salicylic acid, hydrocortisone, clotrimazole and terbinafine) with widely varying polarity. In agreement with published data, pig skin appeared as the most suitable model for human skin: the fluxes through the skin and concentrations in the skin were of the same order of magnitude for both tissues, with differences of at most two- or four-fold, respectively. Graftskin(tm) LSE(tm) provided an adequate barrier to salicylic acid (flux of 45 μg per $cm^2$ per h as compared to 22 μg per $cm^2$ per h for human skin), but was very permeable for the more hydrophobic compounds (e.g. about 900-fold higher flux and 50-fold higher skin concentrations of clotrimazole as compared to human skin), even more than rat skin. In the case of the Skinethic(tm) HRE we found similar concentrations of salicylic acid as in human skin (745 μg per g) and an about 7-fold higher flux (153 μg per $cm^2$ per h). In contrast, the permeation of hydrophobic compounds through the epidermal layer was vastly higher than through split-thickness human skin (up to a factor of about 800). To conclude, currently available reconstituted skin models cannot be regarded as generally useful for *in vitro* penetration studies, while pig skin is a suitable model for human skin for this purpose.

# 200

**Altered Sebaceous Gland Morphology and Function in Parathyroid Hormone-Related Protein Transgenic Mice**

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Murine transgenic models that either overexpress or lack parathyroid hormone-related protein (PTHrP) in the epidermis suggest that the peptide influences both the development and maintenance of adnexal structures in the adult. In this study we evaluated sebaceous gland morphology and function in both the K14-PTHrP mice and the collagen II-PTHrP (rescued)-PTHrP-knockout mice that overexpress or lack the peptide, respectively. In the adult mouse skin, the most intense PTHrP mRNA and protein labeling was observed in the upper portion of the external root sheath of the hair follicle close to where the sebaceous gland intersects the structure. During the first hair cycle (1–3-weeks), sebaceous gland morphology and function in the two PTHrP transgenic models did not appear to differ greatly from that of wild-type littermates. By 8–12-weeks, differences in coat texture became apparent in both the K14-PTHrP and the rescued PTHrP-knock out mice as compared to sex matched wild-type littermates. The dorsal coat of the 12–16-week-old K14-PTHrP mice appears dry, neutral lipids in the hair were decreased by ~50% and the average sebaceous gland area was increased 27 + 5% as compared to sex matched wild-type littermates. The coat of the 12–16-week-old rescued PTHrP-knockout mice appears to be matted and greasy, neutral lipids in the hair were increased ~2 fold and the average sebaceous gland area was decreased by 53 + 6% as compared to sex matched wild-type littermates. Morphologic analysis of the oldest skin samples available form both of the models (6 months for the rescued PTHrP-knockout and 1.5 years for the K14-PTHrP) suggest that the respective trends in the size of the sebaceous glands appear to become more pronounced with age. Taken together these findings suggest that PTHrP may function to regulate sebocyte precursor proliferation/differentiation in the adult mouse.

# 201

**Activators of Peroxisome Proliferator-Activated Receptors (PPARs) Alter the Profile of Lipid Synthesis in Rat Preputial Sebocytes**

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Excess of sebum production is one of the major factors contributing to the formation of primary lesions in acne. Androgens play an important role in acne due to their effects on sebaceous gland growth and differentiation. Recently, activators of another subfamily of nuclear receptors, PPARs, have been shown to induce sebocyte differentiation *in vitro* as judged by counting the number of lipid forming colonies stained with Oil Red O. In the present work, we describe the effects of Methyltrienolone (R1881) a specific agonist of the androgen receptor (AR) and selective activators of the three PPAR subtypes (PPARα, PPARδ and PPARγ) on the profile of lipid synthesis in rat preputial sebocyte cultures. Lipid synthesis was analyzed by incorporation of $^{14}C$-acetate followed by thin layer chromatography. Polar lipids represented approximately 80%, triglycerides 2–3%, cholesterol 7–8%, fatty acids 2–4%, diglycerides 2%, cholesterol- and wax esters 0–2% of total lipids synthesized by sebocytes. Treatment of sebocytes with the specific AR agonist R1881 at $10^{-8}$ M did not alter the profile of lipid synthesis. BRL49653, a specific agonist of PPARγ, induced a 2- and 4-fold increase of triglyceride and cholesterol/wax ester synthesis, respectively, while synthesis of diglycerides, cholesterol and fatty acids remained unchanged. A synergistic effect on the triglyceride (5-fold increase) and cholesterol/wax ester synthesis (12-fold increase) was observed when sebocytes were treated with BRL49653 and R1881. The effects of selective agonists of PPARα (Wy14 643) and PPARδ (L-165041) on the lipid synthesis profile of cultured rat preputial sebocytes will also be presented. In conclusion, rat preputial sebocytes respond synergistically to AR and PPAR agonists by an increase in the synthesis of lipids observed in sebaceous glands, namely triglycerides and cholesterol/wax esters.

# 202

**The Skin as a Steroidogenic Tissue: Enzymes and Cofactors Involved in the Initial Steps of Steroidogenesis are Expressed in Human Skin and Rat Preputial Sebocytes**

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Cytochrome P450 cholesterol side chain cleavage enzyme (P450scc) catalyzes the rate-limiting step in steroid hormone synthesis. In concert with its cofactors, adrenodoxin and adrenodoxin reductase, and transcription factor, steroidogenic factor-1, this enzyme converts cholesterol to pregnenolone. Translocation of cholesterol from the outer to the inner mitochondrial membrane is regulated by the steroidogenic acute regulatory protein. Although the human sebaceous gland can synthesize cholesterol from two carbon fragments and can metabolize steroids such as dehydroepiandrosterone into potent androgens, the de novo production of steroids has not been demonstrated in human skin. The goal of this study is to delineate the steroidogenic pathway upstream from dehydroepiandrosterone by documenting the presence of members of the P450scc system. Immunoreactivity of an antibody to P450scc and cytochrome P450 17-hydroxylase was noted in human facial skin, cultured human sebocytes and keratinocytes, and rat preputial sebocytes. These enzymes were expressed in the epidermis, hair follicles, sebaceous ducts, sebaceous glands, and eccrine glands of human facial skin. Immunoreactivity of antibodies to the steroidogenic acute regulatory protein, adrenodoxin, adrenodoxin reductase and the steroidogenic factor-1 colocalized to areas of cytochrome P450scc and cytochrome P450 17-hydroxylase expression. Results of immunohistochemistry were confirmed with Western blotting using protein extracts of human sebaceous glands. Biochemical activity of cytochrome P450scc and cytochrome P450 17-hydroxylase was documented in rat preputial sebocytes as detected by radioimmunoassay. These data suggest that the skin can synthesize steroid hormones de novo. These steroids in turn may mediate skin diseases such as acne, androgenetic alopecia and hirsutism.

# 203

**Human Specific Sebaceous Lipid Synthesis and Sebum Secretion in Human Skin-Scid Mouse Chimera**

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Human facial skin from postmenopausal females contains relatively small sebaceous glands mostly associated with sebaceous or vellous hair follicles. Split thickness grafts of such skin, transplanted onto male SCID mice 5–6 weeks of age, revealed presence of small glandular structures within the first two months post transplantation (0.5% of total skin area occupied by glands – TOG). A significant increase in the gland size was noticed between 8 and 12 weeks post transplantation (4.3% TOG). Following this period of time, the glands remained approximately the same size or decreased slightly during the 4–8 weeks. Compared to the skin sample prior to grafting, the glands post transplantation enlarged approximately 6.5 fold. Sebum secretion was first detected by SEBUTAPE four weeks post grafting. There was a gradual increase in sebum secretion that paralleled the observed increase in gland size. Maximal secretion was at 12 weeks. The levels were approximately the same at 20 weeks post grafting. In parallel experiments, when the same skin was transplanted onto female mice, the gland enlargement and sebum production was not observed. However, topical treatment of such grafts with androgens (DHT) resulted in increased gland size and sebum production, similar to that seen in the male mice. Thus, the observed increase in gland size and sebum production was presumably initiated by androgens produced by the male mice. Ex-vivo labeling with 14C acetate, followed by high performance thin layer chromatography (HPTLC), showed increased sebaceous lipid synthesis in 3-month-old transplants when compared to the pretransplantation levels. Interestingly, the sebaceous lipids synthesized in the transplanted skin were human specific, showing the presence of squalene and wax esters and other sebum components, i.e. triglyceride. In addition to human specific lipid production other sebocyte expressed markers, i.e. epithelial membrane antigen, β-endorphin and melanocortin-5 receptor were all detected in the human sebaceous glands in SCID mice. The presented data provide evidence for functional sebaceous glands displaying a human phenotype in the SCID mouse. This

# 204

**Metabolic Fate and Selective Utilization of the Major Fatty Acids in Sebaceous Glands**

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Wax esters are one of the major products of the lipid secreting sebaceous gland of mammalian skin. The synthetic pathways and enzymes involved have not yet been determined. Towards the understanding of their synthesis, the metabolism of exogenously supplied saturated (16:0, 18:0), monounsaturated, Δ9 (16:1, 18:1) and the linoleic Δ9, 12 (18:2) fatty acids (FAs) was followed in cultured sebaceous glands. Explanted glands from human facial skin and hamster ear were incubated with the above radiolabeled FAs or acetate for 24 h. Acetate and 16:0 were incorporated into all of the cellular and sebaceous specific lipids in both hamster and human systems. The 16:0 was elongated to 18:0 by both species but the hamster glands desaturated them to 16:1 and 18:1, respectively. In both species 16:1 was incorporated more extensively into polar lipids than triglycerides, but not into sebaceous specific lipids and was elongated to 18:1 (Δ11). As proven by HPTLC analysis 18:0 and 18:1 were both incorporated into the cellular lipids but at a lower rate than the 16:0 into wax esters. In the hamster gland 18:0 was desaturated to 18:1 but was not further metabolized to other fatty acids. Furthermore, addition of exogenous 18:1 was not further metabolized. In human explants, linoleic acid was the only fatty acid that appeared to be β-oxidized. That oxidation was specific since it did not induce a general oxidation of other FAs. The ability of the explants to synthesize sebaceous lipids correlated with the putative β-oxidation of linoleic acid. In addition the oxidation of linoleic acid was not detected in hamster explants. Thus the oxidation of linoleic acid is specific for the human sebaceous cells and correlated with their function and differentiation. Our results support the hypothesis that the sebaceous gland selectively utilizes fatty acids.