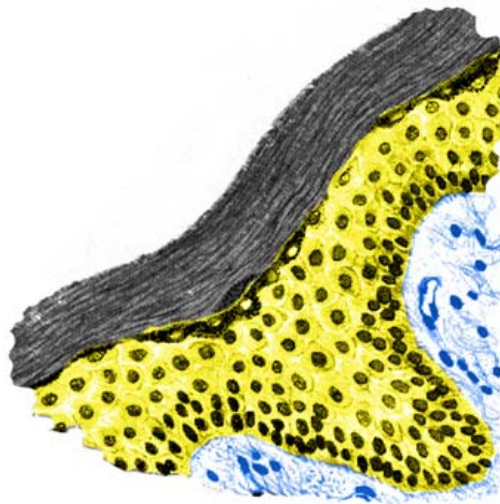


## Symposium

“Keratinocytes – Proliferation and Differentiation in the Epidermis”

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## **Comparison of CD8+ CTL responses using three different cutaneous DNA vaccination strategies with a luciferase fusion protein as model antigen**

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The skin is an attractive target for antigen-specific vaccination. Here we present three different cutaneous vaccination strategies and their ability to induce antigen-specific CD8+ cytotoxic T cells (CTL), which are of particular importance for the defense of viral infection and tumors. A fusion protein between EGFP, the H2-Kb-binding peptide OVA<sub>Aaa257-264</sub> and luciferase (gLuc) was applied to compare gene gun immunization with intracutaneous injection of adenovirus (Ad) or adenovirus-transduced DC (Ad-DC). Interestingly, we found that gene gun immunization with plasmid DNA was considerably less effective in stimulating CD8+ T cells than intracutaneous injection of recombinant adenovirus or adenovirus-transduced DC. Employing the novel technique of in vivo bioluminescence imaging, we could rule out that the comparatively weak T cell stimulatory capacity of the gene gun was simply due to inefficient in vivo gene transfer. As an alternative explanation, we determined that gene gun immunization only induced a weak non-specific activation of the immune system when compared with direct injection of recombinant adenovirus. Adjuvant administration of synthetic immunostimulatory CpG oligonucleotides led to activation of CD8+ T cells and NK cells but did not significantly enhance antigen-specific CD8+ T cell expansion or cytotoxicity following gene gun immunization. We believe that these results reflect immunological functions of the epidermis where adaptive immunity must be tightly controlled because the skin is continuously exposed to a large number of potentially immunogenic proteins. Furthermore these findings will be of great importance for the rational development of cutaneous DNA vaccination strategies.

## Metabolic fate of sphingolipids in submerge cultured keratinocytes

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Epidermal differentiation results in the formation of an extracellular lipid barrier in the stratum corneum, which consists mainly of ceramides, free fatty acids, and cholesterol. Differentiating keratinocytes of the stratum granulosum synthesize a series of complex long-chain ceramides and glucosylceramides with different chain lengths and hydroxylation pattern. Formation of complex ceramides parallels the transition of keratinocytes from the stratum granulosum to the stratum corneum, where their precursors, e.g. complex glucosylceramides, are exposed to lysosomal lipid hydrolases. In order to investigate the metabolism of the major epidermis-specific lipids during keratinocyte differentiation, we developed a simple *in vitro* culture system for the differentiation of keratinocytes under submerged conditions. Starting from the well defined system using MCDB media, we investigated the effects of additional factors such as calcium, linoleic acid, serum and vitamins on the metabolism and morphology of keratinocytes.

At low calcium ion concentrations (0.1 mM), keratinocytes proliferate to confluency and synthesize Cer(NS), small amounts of Cer(NP) and Cer(AS). After increase of the calcium concentration in the medium to 1.1 mM, keratinocytes differentiate and start with the formation of all epidermal ceramides found in the epidermis. Further addition of 10  $\mu$ M linoleic acid caused a 2-fold increase of the ceramide content after one week. After 8 days, also the mRNA levels of the differentiation markers keratin 10, profilaggrin, ceramide glucosyltransferase, and of acid sphingomyelinase increased.

A further factor of importance in the metabolism of keratinocytes is the extracellular pH. In experiments using sequential tape stripping of human epidermis, the pH of the extracellular space of the SC was found to be acidic at a pH of approximately 5.5. A pH gradient from the SC surface (pH  $\approx$  5) to the viable epidermis (pH  $\approx$  7.4) has been reported by Öhman and Vahlquist, 1994 and Turner *et al*, 1998. The acidic environment of the cells in the SC has been described to be favorable for the processing of pro-barrier lipids to barrier lipids by hydrolytic lysosomal enzymes (Mauro *et al*, 1998), and therefore for the formation of a functional permeability barrier.

In an additional experimental sequence, we studied the effect of an acidic extracellular pH on the cellular sphingolipid metabolism. For all conditions, we investigated the sphingolipid pattern of the cells, their morphology and the expression levels of genes encoding enzymes and other proteins involved in sphingolipid metabolism. We find that a regimen using 1.1 mM calcium and 10  $\mu$ M linoleic acid in combination with a pH shift to pH 6 leads to a good yield of barrier lipids while at the same time maintaining cell viability. A further reduction of the extracellular pH to pH 5.5 increases the yield of barrier lipids dramatically, but leads to changes in cell morphology and some cell death and leakage.

## **Cathepsin B is essential for regeneration of scratch-wounded HaCaT and normal human epidermal keratinocytes**

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Migration, proliferation and differentiation of keratinocytes are of importance during processes of regeneration from wounding of the skin. Here, we focussed on proteases that contribute to extracellular matrix (ECM) remodelling as a prerequisite of keratinocyte migration. In particular, we assessed the significance of the mammalian cysteine peptidase cathepsin B for human keratinocytes during regeneration from scratch-wounding. The rationale for this approach derived from our previous work where we have shown that HaCaT keratinocytes secrete cathepsin B into the extracellular space during spontaneous and induced migration. We now verified the results gained with HaCaT cells by using normal human epidermal keratinocytes (NHEK) and in addition show that cathepsin B-specific inhibitors delayed full regeneration of the monolayers from scratch-wounding in both cellular systems, HaCaT and NHEK. Thus, we conclude that cathepsin B is indeed involved in ECM-remodeling. Cathepsin B might directly cleave ECM-constituents or it may initiate proteolytic cascades that involve other proteases with the ability to degrade ECM-components. Furthermore, administering one single treatment of inhibitors of cathepsin B's proteolytic activity directly after scratch-wounding of keratinocytes demonstrating that cathepsin B is essential during initial stages of wound healing while its contribution to the subsequent processes of proliferation and differentiation of the keratinocytes might be of less significance. Because cathepsin B is essential for enabling migration of both, HaCaT cells and NHEK, our results support the notion that HaCaT keratinocytes are an excellent cell culture model for human epidermal skin keratinocytes.

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**Lipoprotein receptor-related protein 1 serves as binding partner to localize secreted cathepsin B to the surfaces of migrating HaCaT keratinocytes.**

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We showed that cathepsin B is secreted after scratch-wounding of HaCaT keratinocytes. Subsequently, it re-associates with the plasma membrane where it is vital for remodelling of extracellular matrix to enable keratinocyte migration. Hence, we proposed a cathepsin B-binding protein at the surface of migrating keratinocytes. Because annexin II heterotetramer (Allt) serves this task within caveolae of tumor cells, we tested whether Allt can also serve as receptor for secreted cathepsin B at the surface of migrating keratinocytes. We now show that cathepsin B and caveolin-1 colocalized only rarely, indicating that the protease is not present in caveolae of keratinocytes. Furthermore, neither pro- nor mature cathepsin B colocalized with Allt. As an alternative candidate, we, therefore, tested lipoprotein receptor-related protein 1 (LRP1) that is expressed at the basolateral plasma membrane domain of most epithelial cells, where it serves as scavenger receptor for many ligands including proteases. Colocalization of LRP1 and cathepsin B was indeed observed intracellularly and at the surface of migrating keratinocytes. RAP, receptor-associated protein, recognizes all ligand-binding domains of LRP-receptors. When RAP was added to the culture media of migrating keratinocytes, lower amounts of cathepsin B were present at the plasma membrane and soluble cathepsin B became detectable, suggesting that RAP competed with cathepsin B for binding to LRP1. From this we conclude that cathepsin B binding to cell surfaces differs in tumor cells and keratinocytes. Whereas Allt serves as procathepsin B-receptor of breast carcinoma cells, LRP1 is a promising candidate for a cathepsin B-binding protein at the surfaces of keratinocytes migrating during wound healing.

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## **Normal phase LC/APCI-MS for separation and profiling of stratum corneum ceramides in skin and keratinocytes.**

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Numerous reports in dermatological literature emphasize the significant role of ceramides in maintaining the barrier function of skin, which is located in the stratum corneum. In addition, important skin diseases such as psoriasis and atopic dermatitis are associated with impairments in the lipid pattern, particularly regarding the ceramides. Accordingly, investigation of the molecular composition using mass spectrometry can shed light on the detailed function of each of the up to date known 9 ceramide classes as well as the mechanisms underlying the mentioned skin diseases.

GC/MS has long been the method of first choice in obtaining structural information about stratum corneum ceramides. However, ceramide analysis using GC/MS include some disadvantages such as the laborious sample preparation steps. Alternatively, off-line combination between TLC and reversed phase LC/ESI-MS has been attempted. Still, pronounced lipophilic ceramide classes such as Cer [EOS] could not be detected.

A recently published method using normal phase LC (Liquid Chromatography) hyphenated to APCI-MS (Atmospheric Pressure Chemical Ionization-Mass Spectrometry) enabled not only the separation of the known stratum corneum ceramide classes but also the online detection of the corresponding species within each class. Furthermore, it overcame many limitations of the mentioned methods. Here, we demonstrate the benefits of this new approach for analyzing ceramides in skin as well as in keratinocytes.

## Evidence for the involvement of the endogenous cannabinoid system in the regulation of contact hypersensitivity

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Cannabis preparations have been used in traditional medicine for the treatment of inflammatory diseases. The major active constituent of the plant *Cannabis sativa* is  $\Delta^9$ -THC (tetrahydrocannabinol). Two specific receptors mediate the effects of cannabinoids (CB<sub>1</sub>- and CB<sub>2</sub>-receptor). The discovery of endogenous CB-receptor ligands proved the existence of an endogenous CB-system with many physiological functions. A participation of CB-receptors in the downregulation of inflammatory processes was demonstrated in experimental models for atherosclerosis or colitis. Therefore, we investigated the role of the CB-system in experimental contact hypersensitivity (CHS). CHS was induced in C57Bl/6, C57Bl/6-CB1<sup>-/-</sup>, -CB2<sup>-/-</sup> and -CB1/2<sup>-/-</sup> mice by application of 0,2 % DNFB on the shaved abdomen and elicited by painting the ears with 0,3 % DNFB. Ear swelling was measured after 24h, 48h and 72h. Additionally, wildtype mice were treated s.c. with the CB-antagonists SR141716 and SR144528 or the CB-agonist  $\Delta^9$ -THC. Histopathological analyses were performed on inflamed skin. Contact allergy in mice lacking the CB-receptors was increased more than 100% in comparison to wildtype mice. Pharmacological blockade of CB-receptors with SR141716 and SR144528 also induced an increased ear swelling in wildtype mice. Histological analysis demonstrated elevated numbers of infiltrating Gr-1<sup>+</sup>- and MHC-II<sup>+</sup>-cells in CB-receptor deficient and SR141716- or SR144528-treated mice.  $\Delta^9$ -THC reduced ear swelling and tissue infiltration of inflammatory cells. Our results demonstrated that the endogenous CB-system is involved in downregulating cutaneous hypersensitivity responses. Future experiments will have to address how cannabinoids participate in this regulation. Furthermore, novel CB-receptor ligands may be developed for the treatment of inflammatory skin disease.

## **Filopodia dynamics of migrating human keratinocytes depend on the substrate composition and are regulated by mitogenic growth factors**

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The actin assembly-driven formation of membrane protrusions, i.e. lamellipodia and filopodia which originate from the front lamella, is considered the initial step during the cyclic process of cell migration. In contrast to the sheet-like lamellipodia, the molecular details of the initiation and maintenance of the finger-like filopodia are only just beginning to be understood and it is an educated guess that they fulfill sensory or exploratory functions. Here we used migrating epidermal keratinocytes to study the dynamics of filopodia on different extracellular matrix components such as fibronectin and collagen and depending on the mitogenic growth factors EGF and TGF. By combining live cell imaging microscopy with the high resolution SACED motility assay we determined the correlation of filopodia length, number and density with the dynamics of lamellipodia and the migration velocity. Our studies revealed that filopodia length and number are correlated in a negative fashion with the persistence of lamellipodia, i.e. the period a protrusion lasts before it is retracted. As lamellipodia persistence is a direct measure for the efficiency of lamellipodia adhesion which in turn is a prerequisite for efficient migration, our results point to long filopodia as indicators of inefficient adhesion and migration.

## **Laminin 5 deficiency results in an adhesion defect in human keratinocytes that inducing a saltatory mode of migration**

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Laminin 5 is a major matrix component of the epidermal basement membrane involved in adhesion and migration of keratinocytes. To investigate the role of laminin 5 in keratinocyte migration we analyzed cells of patients with a lethal variant of junctional epidermolysis bullosa that are deficient for laminin 5. Normal migrating keratinocytes adopted monopolar morphologies and employed a continuous mode of translocation. In contrast, laminin 5 deficient cells adopted an extended fibroblast-like shape with two or three lamella regions and migrated in a saltatory manner characterized by significantly decreased directionality and velocity as a consequence of inefficient lamella persistence. This specific phenotype apparently resulted from a defect in the formation of substrate adhesions which were completely missing in the cell body and less stable in the lamella regions. Accordingly in normal keratinocytes, the fibroblast-like shape and the saltatory migration mode could be induced by blocking laminin 5. Growing deficient keratinocytes on fibronectin resulted in the adoption of a wild-type-like morphology but did not allow overcoming the inefficient lamellipodia persistence leading to inefficient migration. Our findings clearly point to an essential role of laminin 5 in cell-substrate adhesion and cell migration in epidermal keratinocytes.

## Subpopulations of human dendritic cells display a distinct phenotype and bind differentially to proteins of the extracellular matrix.

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Langerhans cells (LC) precursors were generated *in vitro* from monocytes (monocyte-derived DC, MoDC) or from CD34<sup>pos</sup> stem cells (CD34<sup>pos</sup> cell-derived DC, CD34DC). Four DC precursor subpopulations were characterized by their differential expression of the monocytic marker CD14 and the DC marker CD1a. The expression of chemokine receptors CCR1, CCR2, CCR6 and CX3CR1 was monitored at distinct time points during differentiation and allowed discrimination of monocytes from peripheral blood and CD14<sup>pos</sup>/CD1a<sup>neg</sup> CD34DC. Both precursor subtypes expressed the alpha integrins LFA-1, Mac-1, CR4, VLA-4, VLA-5 and the beta 2 integrin CD18. CD34DC and MoDC were negative for VLA-3, whereas MoDC, but not CD34DC expressed VLA-6. Classic protocols for *in vitro* differentiation of DC from peripheral blood monocytes involve culture periods for 7 days and longer. However, phenotypic and functional characterization of DC at earlier time points revealed that DC at day 3 of culture may reflect the *in vivo* situation more closely. Adhesion of LC precursors to endothelial cells and to components of the extracellular matrix is a prerequisite for their migration towards the epidermis. To this end, we investigated adhesion of CD34DC and MoDC to proteins of the extracellular matrix. Distinct DC subsets also showed a distinct binding pattern to proteins of the extracellular matrix. MoDC and CD34DC bound preferentially to laminin 5 and to fibronectin, but only weakly to laminin 1 or to collagens. Staining of adherent cells with CD14- or CD1a-specific antibodies revealed that CD14 positive cells bound preferentially to laminin 5; in contrast, CD1a positive cells adhered to fibronectin.

CD34DC subpopulations at day 7 of culture were sorted in four subpopulations according to their expression of CD14 and CD1a. Reculturing the cells in the presence or absence of GM-CSF or TGF- $\beta$ 1 induced the CD14<sup>neg</sup>/CD1a<sup>neg</sup> DC and the CD14<sup>pos</sup> population to become CD14<sup>pos</sup>/CD1a<sup>pos</sup> cells. Furthermore, under the influence of TGF- $\beta$ 1, the CD1a<sup>pos</sup> and the CD14<sup>pos</sup>/CD1a<sup>pos</sup> cells started to express the Langerhans cell specific marker Langerin/CD207 or expressed Langerin more strongly.

In summary, the comparison of the different DC precursors revealed that the subpopulations of CD34DC and MoDC are related to each other, but not identical. Whether these cells raised *in vitro* are equivalent to different subtypes of Langerhans cells which have been encountered *in vivo* remains to be determined.

## **Chemotactic response of human dendritic cell subpopulations towards signals from skin-derived endothelial cells, fibroblasts and keratinocytes.**

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Langerhans cells (LC) are a paradigmatic dendritic cell (DC) population from the epithelial layer in the skin. LC precursors can be generated *in vitro* from monocytes (monocyte-derived DC, MoDC) or from CD34<sup>pos</sup> stem cells (CD34<sup>pos</sup> cell-derived DC, CD34DC). On their route from the blood stream into the epidermis, LC precursors are guided by chemotactic signals provided by cutaneous structural cells. We examined the chemotactic response of LC precursors towards supernatants of skin-derived endothelial cells, fibroblasts and keratinocytes. Especially, we turned our attention to the differential migration patterns of CD34DC and MoDC subpopulations. We compared chemotaxis of monocytes, MoDC cultured for 3 days (d3 MoDC) and MoDC cultured for 7 days (d7 MoDC). Fibroblast supernatants and endothelial cell supernatants induced rapid monocyte migration, in particular of the CD14<sup>high</sup> /CD16<sup>low</sup> monocyte subset. For slow monocyte migrants, only supernatant from keratinocytes was a potent chemoattractant. Monocytes express the chemokine receptor CCR2, which is rapidly lost during *in vitro* culture. We found that trans-endothelial migration rescued CCR2 expression by monocytes, which would leave them further receptive for inflammatory signals.

CD34DC migrated specifically to all supernatants. The CD14<sup>pos</sup> subpopulation migrated strongest to all supernatants and most strongly towards supernatants from fibroblasts. Specific migration of CD1a<sup>pos</sup> cells was only slightly enhanced compared to spontaneous migration. Due to the expression of CCR2 on CD14<sup>pos</sup> cells and of CCR6 on all subpopulations, we applied specific antibodies against human CCL20 (MIP-3 $\alpha$ ) and CCL2 (MCP-1) in the supernatants. We could not detect an inhibitory effect of the CCL20 antibody in KC supernatant. In EC supernatant, the CCL20 antibody suppressed only the specific migration of the CD14<sup>pos</sup> cells. An antibody against CCL2 in KC supernatant inhibited the specific migration of the CD14<sup>pos</sup>.

To mimic the role of CCL20 and CCL2 for migration of LC precursors in the skin, we tested human recombinant CCL20 and CCL2 in a trans-endothelial migration assay. All LC precursor subpopulations migrated towards CCL20, and specific migration could be inhibited by an anti CCL20 antibody.

Migration towards CCL2 revealed a strong response of the CD14<sup>pos</sup> cells. A CCL2-specific antibody suppressed their migration, but also the spontaneous migration towards the culture medium.

These results suggest that endothelial cells, fibroblasts and keratinocytes secrete chemoattractants for LC precursors, which direct them stepwise to the epidermal compartment.

## **Functional analysis of the keratin gene family using a large-scale genomic deletion and replacement approach**

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Keratins form a multigene family of 27 type I and 27 type II keratins. In mice, type I keratins are located in a gene cluster of 1Mb on chromosome 11, except for K18, which resides in the 0.8Mb cluster of type II keratins on chromosome 15. Keratins are expressed in all epithelia, where they assemble into the intermediate filament cytoskeleton, which is responsible for the epithelial integrity. So far, very little is known about the precise function of any keratin pair. This is exemplified by knockouts of individual keratins, which have revealed overlapping functions during embryonic development.

Due to the exceptional genome organization of the keratin gene family in mammals, a unique strategy to analyze the function of individual keratins in a systematic manner is possible. To that end, we used a modified cre-loxP procedure to delete the two keratin gene families from the ES cell genome, in order to create chimeras heterozygous for the type I or type II cluster, respectively. These will be mated further to produce complete keratin knockout mice.

Here, we describe an approach to replace the endogenous keratins by just a single keratin pair, i.e. K8/K18, which are the earliest keratins expressed in embryonic development. This will reveal whether functional epithelia form in the presence of just two keratins. The procedure is based on the knock-in of K8 and K18 in combination with established and novel regulatory elements into the mouse E-cadherin locus to provide epithelial-specific expression.

## **Endothelial tubulogenesis within fibrin gels is induced by high invasive melanoma cells and requires VEGF**

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Angiogenesis is encountered in physiological and pathological situations, for example development, wound healing and tumour induced angiogenesis. Vessel maturation during angiogenesis is characterised by an endothelial cell sprouting from parental vessels, followed by migration and proliferation, alignment and finally the formation of tubular structures. All these processes involve a tightly controlled degradation of extracellular matrix components.

Cell surface receptor-mediated interactions of tumour cells with surrounding structural and cellular components of the stroma as well as soluble factors, released by the tumour cells, are likely to contribute to the activation of stromal cells leading to increased proteolysis of the matrix.

The *in vivo* formation of capillary structures can be mimicked by an *in vitro* angiogenesis assay, in which endothelial cells were cultured on top of a three-dimensional fibrin matrix. By using this assay, we could demonstrate, that addition of supernatants collected from high but not low invasive melanoma cells induced formation of tubular structures in HDMECs. These findings suggest that soluble factors, released from high but not from low invasive melanoma cells are involved in endothelial cell activation. Neutralisation of VEGF, which is highly expressed in high invasive but not in low invasive melanoma cells, resulted in reduced elongation and migration of HDMECs into the fibrin matrix. These findings pointed to VEGF as a major factor which mediates angiogenesis induced by high invasive melanoma cells.

## Loss-of-Function Mutations in the Keratin 5 Gene Lead to Dowling-Degos Disease

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Dowling-Degos disease (DDD) is an autosomal dominant genodermatosis characterized by progressive and disfiguring reticulate hyperpigmentation of the flexures. We performed a genomewide linkage analysis in two German families and mapped DDD to chromosome 12q with a total lod score of 4.42 ( $\theta=0.0$ ) for marker D12S368. This region includes the keratin gene cluster, which we screened for mutations. We identified loss-of-function mutations in the keratin 5 gene (*KRT5*) in all affected family members and in six unrelated DDD patients. These represent the first identified mutations in a keratin gene leading to haploinsufficiency. The identification of loss-of-function mutations along with the results from additional functional studies suggest a crucial role for keratins in the organization of cell adhesion, melanosome uptake, organelle transport and nuclear anchorage.

## **Generation of a novel Connexin31 F137L mutant mouse as a model for the human disease *Erythrokeratoderma variabilis* (EKV)**

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Gap junctions are intercellular conduits through which small metabolites and ions can diffuse. Gap junction channels are formed by docking of two hemichannels (connexons), each consisting of six proteins, the connexins. So far, at least 20 connexin genes were described each in the human and mouse genome.

Erythrokeratoderma variabilis (EKV) is a rare human autosomal dominant genodermatosis. Its symptoms vary from transient, fast moving erythema to persistent brown hyperkeratoses. Until now, several mutations in Cx31 and Cx30.3 genes are known to cause EKV in humans.

We have generated a conditional mouse mutant which carries the human F137L mutation in the connexin31 gene. The Cx31F137L mutation was described in patients suffering from EKV as a transdominant negative mutation. This mutation was also found in the Cx30.3 gene. Since Cx31 is expressed in the placenta of rodents and since Cx31 deficient mice show placental defects, we generated conditionally targeted mice to circumvent possible embryonic lethality.

All genotypes were proven by Southern blot hybridization. The presence of the Cx31F137L mutation was demonstrated by PCR following DNA digestion and direct sequencing. Microinjections of neurobiotin in heterozygous HM1-embryonic stem cells expressing the Cx31F137L mutation, revealed a strong decrease of intracellular coupling. After Cre mediated deletion, heterozygous Cx31+/F137L mice were found to be viable, fertile and born at the expected Mendelian frequency, whereas homozygous mice die between embryonic day 10.5 and 11.5. In rare cases acantosis was observed in mutated (Cx31+/F137L) skin, but not in wild type skin. Tail wounds in +/F137L mice healed one day earlier than in wild type mice, similar as found in Cx34 deficient adult mice (Kretz et al., 2003). This suggests direct interaction of Cx31 and C43 proteins in the epidermis. Similar expression levels and distributions of Cx26, Cx30, Cx31 and Cx43 proteins in mouse skin were shown by immunofluorescence and immunoblot analysis.

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## **Analysis of novel keratin-associated proteins using the SOS recruitment system**

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Cell architecture is largely based on the interaction cytoskeletal proteins, which include intermediate filaments (IF), microfilaments, microtubules, as well type-specific membrane-attachment structures and associated proteins.

Keratins K5 and K14 form the major IF of basal epidermis. Their primary function is to impart mechanical strength to cells, as highlighted by dominant mutations causing inherited skin disorders. Moreover, keratins are involved in additional functions, eg. mediating response to apoptosis. During the analysis of patients suffering from pigmentation disorders, we have recently identified loss-of- function mutations in K5 which shed new light on the role of keratins in melanosome uptake and transport (Betz et al., 2006). Melanosomes are melanocyte-derived organelles related to lysosomes, each melanocyte is responsible for delivering melanosomes via organelle motor myosin-V dependent transport system to numerous keratinocytes. The uptake of melanosomes into keratinocytes, which involves whole organelle donation to another cell, is a unique biological process and is poorly understood. In order to understand keratin-dependent melanosome uptake and transport, we have started to identify novel keratin-associated proteins using the SOS recruitment system in Yeast. In the poster we describe the identification of keratin-associated proteins and their classification.

## **Keratinocytes from APP/APLP2 deficient mice are impaired proliferation, adhesion and migration in vitro**

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Growing evidence shows that the soluble N-terminal form (sAPP<sub>N</sub>) of the amyloid precursor protein (APP) represents an epidermal growth factor fostering keratinocyte proliferation, migration and adhesion. APP is a member of a protein family including the two mammalian amyloid precursor-like proteins APLP1 and APLP2. In the mammalian epidermis, only APP and APLP2 are expressed. APP and APLP2 deficient mice die shortly after birth but do not display a specific epidermal phenotype. In this report we investigated the epidermis of APP and/or APLP2 knockout mice. Basal keratinocytes showed reduced proliferation in vivo by about 40%. Likewise, isolated keratinocytes exhibited reduced proliferation rates in vitro, which could be completely rescued by either exogenously added recombinant sAPP<sub>N</sub>, or by co-culture with dermal fibroblasts derived from APP knockout mice. Moreover APP-knockout keratinocytes revealed reduced migration velocity resulting from severely compromised cell substrate adhesion. Keratinocytes from double knockout mice died within the first week of culture, indicating essential functions of APP-family members for survival in vitro. Our data indicate that sAPP<sub>N</sub> has to be considered as an essential epidermal growth factor which, however, in vivo can be functionally compensated to a certain extent by other growth factors, e.g. factors released from dermal fibroblasts. (Supported by Deutsche Forschungsgemeinschaft)

## **HUMEC-matrix reduces the motility of metastatic breast cancer cells by a ROCK mediated mechanism**

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Migration normal breast epithelial cells (HUMEC) secrete an extracellular matrix (HUMEC-matrix) consisting mainly of the adhesion proteins fibronectin and laminin-5. When HUMEC cells were grown on surfaces pre-coated with HUMEC-matrix their migration velocity increased significantly as compared to surfaces coated with collagen I. Intriguingly, this matrix significantly reduced the migration velocity of highly metastatic breast cancer cells (MDA-MB231). The reduced migration velocity of MDA-MB231 was correlated with a marked re-distribution of focal adhesions and a re-organisation of the actin cytoskeleton. The resulting cortical actin belt with stellate-like stress fibers and the non-polarized distribution of focal adhesions are characteristic features of highly adhesive, less motile cells. As the small GTP Rho A is known to be one of the key regulators of stress fibre and focal adhesion formation, we speculated that inhibition of the Rho effector kinase ROCK might allow compensating the effect of HUMEC-matrix on MDA-MB231 migration. We therefore applied the specific ROCK inhibitor Y27632 which resulted in the full recovery of the migratory capacity of MDA-MB231 cells on HUMEC-matrix. Our results point to a central role of RhoA/ROCK signalling in the regulation of cell-substrate adhesion and migration.

## **Rapid growth of invasive metastatic melanoma in carcinogen-treated HGF/SF-transgenic mice carrying an oncogenic CDK4 mutation**

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Currently, novel mouse models of melanoma are being generated which recapitulate the histopathology and molecular pathogenesis observed in the human disease. Here we investigated whether impaired cell cycle control due to mutant cyclin-dependent kinase 4 (CDK4<sup>R24C</sup>) would synergize with deregulated receptor tyrosine kinase signalling due to overexpression of hepatocyte growth factor/scatter factor (HGF/SF) in melanomagenesis. C57BL/6 mice overexpressing HGF/SF in combination with wildtype CDK4, heterozygous mutant CDK4 or homozygous mutant CDK4 were treated with a carcinogen protocol (DMBA+TPA) and tumorigenesis recorded. Carcinogen treatment significantly promoted the development of melanomas in HGF/SF mice which survived more than 30 weeks with an average of 10 slowly growing melanocytic neoplasms. The simultaneous expression of mutant CDK4 had a dramatic effect on the number and the growth kinetics of melanocytic neoplasms. HGF/SF mice homozygous for mutant CDK4 had to be sacrificed with around 50 rapidly growing melanomas at the early age of about 12 weeks. HGF/SF mice heterozygous for mutant CDK4 lived until week 16 and developed around 30 melanomas. Importantly, mutant CDK4 also promoted growth of metastatic melanoma in the lymph nodes and the lungs in HGF/SF mice. Histopathological examinations revealed similarities with human melanomas both in the skin and the lymph nodes. We did not observe other tumors in CDK4<sup>R24C</sup> x HGF/SF mice at the time of sacrifice. Our results show that overexpression of HGF/SF and mutant CDK4 synergistically promote the rapid development of wide-spread carcinogen-induced melanomas in the skin of C57BL/6 mice which spontaneously metastasize in lymph nodes and lung. This new experimental mouse model can now be exploited to further study the biology of melanoma and evaluate new treatment modalities.

## **Analysis of keratins by cre/lox –mediated chromosomal engineering and gene replacement**

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Here we present an analysis of the function of individual keratins by a deletion-replacement strategy in vivo. The fact that all keratin genes, unlike members of many other gene families, are clustered in two contigs on chromosomes 11 and 15 in the mouse, offers a unique approach to address the above problem. We have employed the cre/lox system to delete the type I and type II keratin gene clusters spanning about 1 Mb each in mouse ES cells. From these cells, we have initiated the generation of mice which are deleted for the type I or the type II keratin cluster. With these mice, we will first investigate embryonic development in the absence of any keratins and compare this with a setting in which either the type I or the type II cluster only are deleted. This will identify the developmental stage and processes that rely on keratins and clarify whether protein aggregates or soluble keratins display important functions. Protein arrays or yeast-two-hybrid-screens will be employed in order to analyse the downstream and upstream targets of various keratins in order to understand their function better. Second, we will investigate embryonic development in the presence of one keratin pair, K8 and K18, typical of all embryonic and “simple” epithelia. This will reveal how far embryonic development proceeds and identify those stages which depend on other keratins. Moreover, conditional knockout of the keratin clusters has been initiated, wherein mice will be generated from the cis-targeted ES clones. This allows the conditional deletion of both gene clusters in a tissue-specific and temporally controlled pattern. This will help to identify the implications of the absence of keratin in certain tissue and organ type including tumor settings.

## **Lysosomal cathepsin L and nuclear cathepsin V are differentially regulated during regeneration of human keratinocytes from wounding.**

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In keratinocytes of human skin, different cysteine peptidases are expressed and contribute to maintenance of epidermal homeostasis. Here, cathepsin B was detected within lysosomes of keratinocytes of all epidermal layers and between suprabasal cells, suggesting a contribution of cathepsin B to discohesion of epidermal keratinocytes during intra-epidermal migration. In contrast, cathepsin L was restricted to lysosomes but never occurred intercellularly. Cathepsin V was found in a vesicular, and, surprisingly, in a nucleus-associated pattern in keratinocytes *in situ*. Others showed that transgenic expression of human cathepsin V rescues the hyperproliferative skin phenotype of cathepsin L-deficient mice, suggesting that both peptidases contribute to regulation of keratinocyte proliferation. Whereas the mouse genome encodes for cathepsin L only, human keratinocytes express both, cathepsins L and V. Here, through quantitative real-time PCR we observed that cathepsin V expression was up-regulated in cultured human keratinocytes within the first six hours after scratch-wounding, i.e. two hours before proliferation started as indicated by Ki-67 expression. In clear contrast, the expression of cathepsin L remained unaltered due to wounding. Immunolabelling at different time intervals after wounding revealed differential localization patterns for both enzymes characterized by a lysosomal localization of cathepsin L and a nuclear localization of cathepsin V, i.e. similar to the patterns observed in keratinocytes of human skin. These results led us to suggest differential functions of cathepsins L and V. We propose that nuclear cathepsin V directly contributes to regulation of keratinocyte proliferation while cathepsin L is likely not involved in this process during wound healing of human skin.

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## Novel functions of Connexin31.1 and Connexin30.3 in the mouse

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Connexin31.1 (Cx31.1) and connexin30.3 (Cx30.3) have been identified as two gap junction genes which are preferentially expressed in skin. Both genes are closely located on mouse chromosome 4 and oriented in the same transcriptional direction. In order to further characterize these two connexins, we generated Cx31.1- and Cx30.3-deficient mice, using NLS-LacZ as reporter gene in both cases. Both Cx31.1<sup>-/-</sup> and Cx30.3<sup>-/-</sup> mice are viable. Staining for LacZ reporter gene activity indicates that Cx31.1 and Cx30.3 are both expressed in the suprabasal layer of the epidermis. Histological comparison of the epidermis did not reveal any obvious phenotypic abnormality in Cx31.1<sup>-/-</sup> and Cx30.3<sup>-/-</sup> mice. Loss of Cx31.1 or Cx30.3 had no effect on the repair of wounded tail skin. In adult Cx31.1<sup>-/-</sup> and Cx30.3<sup>-/-</sup> mice, auditory thresholds were not increased compared to wild type control animals. However, both connexins are expressed in the olfactory epithelium and the vomeronasal organ. We performed behavioral analyses of Cx31.1- and Cx30.3-deficient mice in response to odor. Our analyses indicate that the locomotion of Cx30.3<sup>-/-</sup> mice was decreased, whereas the locomotion of wild type control mice was increased in the presence of vanilla odor (avoidance reaction).

Cx31.1 is expressed throughout the early trophoblast lineage and later in glycogen cells of placenta. The number of Cx31.1<sup>-/-</sup> offspring after interbreeding of Cx31.1<sup>+/-</sup> mice is reduced compared to the expected Mendelian frequency. In contrast, Cx30.3 is not expressed in the placenta, but in the papillary region of kidney. We conclude that Cx31.1 fulfills an optimizing function during placental development and Cx30.3 as well as Cx31.1 appear to be involved in olfaction.

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