

# Dissertation

## Regulation of Keratinocyte Differentiation and Hyaluronan Metabolism in an Organotypic Keratinocyte Culture

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Organotypic keratinocyte cultures provide an alternative to animal tests and offer a useful tool to study keratinocyte differentiation. In organotypic cultures, keratinocytes are grown on a matrix-resembling dermis, and kept at the air-liquid interface. In organotypic cultures, keratinocytes can stratify and form an apparently normal epidermis with distinct epidermal cell layers (e.g. basal, spinous, granular and cornified layer). However, a cornified layer with barrier properties comparable to that in normal skin has not been achieved in organotypic keratinocyte cultures. The primary focus of this work was to develop and characterize an organotypic keratinocyte culture model, which can be used in studies of drug penetration, keratinocyte differentiation and hyaluronan metabolism.

A continuous cell line rat epidermal keratinocyte (REK) was utilized in developing the organotypic keratinocyte culture model. These cells formed a morphologically well-organized *in vitro* epidermis in the absence of feeder cells when grown for 2 weeks on type

I collagen gel in culture inserts at the air-liquid interface. Organotypic REK cultures expressed the suprabasal differentiation markers keratin 10, involucrin and filaggrin. Furthermore, granular cells contained keratohyalin granules and lamellar bodies, and cornified envelopes and tightly packed keratin filaments were present in the corneocytes, indicating that REKs differentiate properly. In this work, it was noted that vitamin C supplementation of the culture medium further improved epidermal morphology, in other words it contributed to keratinocyte differentiation. Vitamin C enhanced the number and the size of keratohyalin granules, and the expression of profilaggrin and filaggrin mRNA and protein. These results show for the first time that vitamin C has direct effects on keratinocyte gene expression. Moreover, vitamin C increased the quantity and

organization of the stratum corneum intercellular lipid lamellae, the main determinants of the epidermal permeability barrier.

An effective barrier in stratum corneum is a result of proper keratinocyte differentiation and an indicator of fully functional epidermis. In spite of a lot of effort, the permeability has remained high in *in vitro* skin models. The formation of epidermal permeability barrier in organotypic REK cultures was investigated by measuring the permeation rates for many test compounds (e.g. mannitol, corticosterone), and by measuring trans-epidermal water loss (TEWL). The morphological improvements obtained with vitamin C were associated with enhanced barrier function. The permeability results with many different test compounds indicated



Fig. 1. Sanna Pasonen-Seppänen (middle), Department of Anatomy, University of Kuopio, defended her thesis on November 18, 2005. Docent Raija Tammi (right) from the Department of Anatomy, University of Kuopio, was Chairman, and Docent Paraskevi Heldin (left) from Ludwig Institute for Cancer Research, Uppsala, Sweden was the Opponent.

that organotypic REK culture (with vitamin C) provides a close estimate of human epidermal permeabilities, and thus offers an excellent model for permeability tests of topical drugs.

In the second part of this work, the effects of some potent modulators of keratinocyte growth (EGF, KGF, all-trans retinoic acid, TGF- $\beta$ ) on keratinocyte differentiation and hyaluronan (HA) metabolism were investigated utilizing the organotypic REK culture. HA is the main extracellular matrix molecule in the vital cell layers of normal skin epidermis, and has been suggested to contribute to keratinocyte proliferation, migration and differentiation by signalling through its cell surface receptor, CD44. This large glycosaminoglycan is synthesized by three different plasma membrane HA synthase enzymes (HAS1, 2, 3). The amount of HA is elevated in several pathological processes, as in psoriasis, in injured skin and in well-differentiated squamous cell cancers. Thus, understanding the regulatory mechanisms controlling its synthesis is important.

The mitogenic growth factors EGF, KGF and all-trans retinoic acid (RA) stimulated keratinocyte HA synthesis by upregulating Has2 and Has3 mRNA. Elevated HA production was associated with increased epidermal thickness (proliferation) and disturbed epidermal differentiation, as indicated by diminished keratin 10 expression, defective profilaggrin maturation and barrier formation. In EGF, KGF and all-trans RA-treated

cultures HA was located especially in the upper spinous and granular cell layers, the site that is almost devoid of HA in normal human skin. Furthermore, in these cultures, HA also resided intracellularly, contrary to control cultures. An anti-proliferative growth factor, TGF- $\beta$ , induced epidermal atrophy and had an opposite effect on keratinocyte hyaluronan production by down-regulating Has2 and Has3.

In monolayer REK cultures, treated with KGF and EGF, increased HA synthesis correlated with keratinocyte migration. Our results suggest that HA mediates the KGF- and EGF-stimulated keratinocyte migration. During wound healing, HA provides a favourable matrix for keratinocyte migration. It has also been shown to stimulate migration by activating signalling cascades that influence the intracellular locomotory system.

In the last part of this work, we studied the mechanisms by which retinoids control cell proliferation and

differentiation. Although all-trans RA is widely used in the therapy of several skin disorders, such as psoriasis and acne, its exact regulatory mechanisms on keratinocyte growth are largely unknown. All-trans RA-induced epidermal hyperplasia and hyaluronan production were blocked with (i) AG1478, an inhibitor of the EGF-receptor (EGFR); (ii) UO126, an inhibitor of the MEK kinase; and (iii) GM6001, an inhibitor of the matrix metalloproteinases. These effects were consistent with the findings that all-trans RA upregulated HB-EGF mRNA and increased the phosphorylation of EGFR, ERK1/2, JNK and c-JUN, suggesting that all-trans RA regulates keratinocyte proliferation and hyaluronan synthesis at least partly through EGFR-signalling. Furthermore, studies with protein synthesis inhibitor cycloheximide showed that the activation of ERK1/2 after all-trans RA treatment does not require de novo protein synthesis, suggesting that the activation of this signalling pathway is a primary response to all-trans RA.

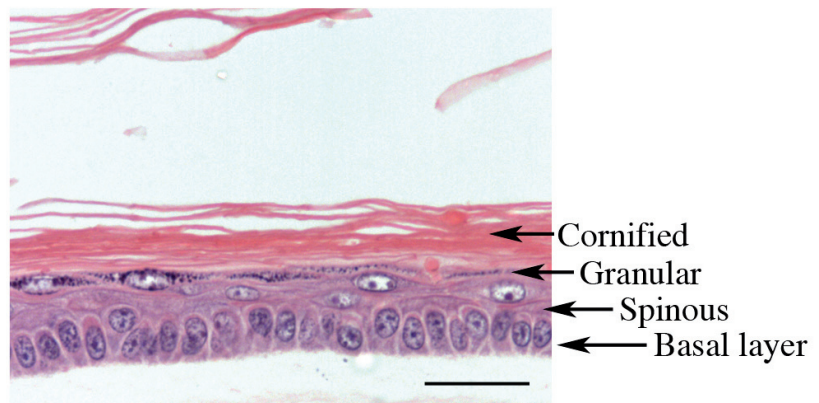


Fig. 2. The morphology of organotypic rat epidermal keratinocyte culture. Scale bar 50  $\mu$ m.

In summary, an in vitro epidermal model was established in this work. This organotypic REK culture model, currently used in drug transport, toxicity and basic keratinocyte biology research in many laboratories, can reduce the need for animal tests. Furthermore, this work provides new insights into the regulation of keratinocyte hyaluronan metabolism. Alterations in hyaluronan synthesis regulate epidermal keratinocyte behaviour - hyaluronan appears to keep keratinocytes in an undifferentiated state able to proliferate and migrate. Thus, the upregulation of hyaluronan synthesis is probably favourable for wound healing.

### List of original publications

1. Pasonen-Seppänen S, Suhonen M, Kirjavainen M, Urtti A, Miettinen M, Tammi M, Tammi R. Formation of permeability barrier in epidermal organotypic culture for studies on drug transport. *J Invest Dermatol* 2001; 117: 1322-1323.
2. Pasonen-Seppänen S, Suhonen M, Kirjavainen M, Suihko E, Urtti A, Miettinen M, et al. Vitamin C enhances differentiation of a continuous keratinocyte cell line (REK) into epidermis with normal stratum corneum ultrastructure and functional permeability barrier. *Histochem Cell Biol* 2001; 116: 287-297.
3. Pasonen-Seppänen S, Karvinen S, Törrönen K, Hyttinen JMT, Jokela T, Lammi M, et al. EGF upregulates, whereas TGF- $\beta$  downregulates, the hyaluronan synthases Has2 and Has3 in organotypic keratinocyte cultures: correlations with epidermal proliferation and differentiation. *J Invest Dermatol* 2003; 120: 1038-1044.
4. Karvinen S, Pasonen-Seppänen S, Hyttinen JMT, Pienimäki JP, Törrönen K, Jokela TA, et al. Keratinocyte growth factor stimulates migration and hyaluronan synthesis in the epidermis by activation of keratinocyte hyaluronan synthases 2 and 3. *J Biol Chem* 2003; 278: 49495-49504.
5. Pasonen-Seppänen S, Maytin E, Törrönen K, Hyttinen JMT, Hascall V, MacCallum D, et al. All-trans retinoic acid induces hyaluronan production in epidermal keratinocytes by increased Has2 and Has3 expression partly mediated by EGFR-Erk signaling (manuscript in preparation).