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The Role of the δ-Opioid Receptor in Skin Homeostasis and Wound Healing

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THE ROLE OF THE δ-OPIOID RECEPTOR IN SKIN HOMEOSTASIS AND WOUND HEALING

Thèse de doctorat ès science de la vie (PhD)

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Table of Contents

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ACh	Acethycholine
ACTH	Adrenocorticotropic hormone
ACTN1	Actinin, alpha-1
ADP	Adenosine di-phosphate
AHPs	Adult hippocampal progenitors
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AP-1	Activator protein-1
AP-2	Activator protein-2
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine tri-phosphate
BDNF	Brain-derived neurotrophic factor
BM	Basal membrane
BPE	Bovine pituitary extract
BSA	Bovine serum albumine
C/EBP	CCAAT/enhancer-binding protein
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CCR5	CC chemokine receptor 5
Cdc 42	Cell division cycle 42
CDH2	Cadherin 2, N-Cadherin
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene related peptide
CHO	Chinese hamster ovary
CI	Cell index
CISH	Chromogenic in situ hybridisation
CO_2	Carbon dioxide
Cot	Mitogen-activated protein kinase kinase kinase 8
CREB	cAMP response element-binding
CTPS	Cytidine 5-prime triphosphate synthetase
CXCR2	Chemokine, CXC motif, receptor 2
CXCR4	Chemokine, CXC motif, receptor 4
Cys	Cysteine
DAMGO	[D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin
Dlx3	Distal-less homeobox 3
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOR	δ - opioid receptor
DPDPE	[D-$Pen^{2,5}$]-enkephalin
DTT	Dithio-1,4-threitol
DUSP6	Dual-specificity phosphatase
ECM	Extracellular matrix
EDTA	Ethylen-diamine-N,N,N',N'-tetraacetate
EEA 1	Early endosome antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	1,2-bis-(2-aminoethoxyethane)-N,N,N',N'-tetraacetic acid
EL	Extracellular loop
ER	Endoplasmatic reticulum
ERK	Extracellular-signal regulated kinase
ETV4	Ets variant gene 4
ETV5	Ets variant gene 5
FBS	Fetal bovine serum
FBXO32	F-box only protein 32
FCS	Fetal calf serum
FLG	Filaggrin
FOXD1	Forkhead box D1
FOXD3	Forkhead box D3
FOXN1	Forkhead box N1
Fra1	Fos-like antigen 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBP2	Guanylate-binding protein 2, interferon-inducible
GDP	Guanosine di-phosphat
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GR	Glucocorticoid receptor
GRHL 3	Grainyhead-like 3
GRKs	G protein-coupled receptor kinases
GTP	Guanosine tri-phosphate
HDAC	Histone deacetylase
HIVEP3	Human immunodeficiency virus type 1 enhancer-binding protein 3
HMG-14	High mobility group nucleosomal binding protein 1
HPRT-1	Hypoxanthine guanine phosphoribosyltransferase 1
HPV	Human papillomavirus
HUAEC	Human umbilical arterial endothelial cells
Id	Inhibitor of DNA binding
IF	Intermediate Filament

IKKa	Inhibitor of NF - κB kinase-alpha
IL	Intracellular loop
IL-1	Interleukin 1
IL-8	Interleukin 8
IP_3	Inositol tri-phosphate
IRF 6	Interferon regulatory fatcor 6
IVL	Involucrin
JNK 1-3	c-Jun N-terminal kinase 1 to 3
K^+	Potassium
KCNJ13	Potassium channel, inwardly rectifying, subfamily J, member 13
KLF 4	Kruppel-like factor 4
KO	Knock-out
KOR	κ - opioid receptor
KRT 1	Keratin 1
KRT 10	Keratin 10
KRT 14	Keratin 14
KRT 16	Keratin 16
KRT 5	Keratin 5
K-SFM	Keratinocyte serum-free medium
LAMP 1	Lysosome-associated membrane protein 1
LB	Luria broth
Leu	Leucine
LIPG	Lipase, endothelial
LOR	Loricrin
LPS	Lipopolysaccaride
LRP8	Low density lipoprotein receptor-related protein 8
MAP3K	Mitogen-activated protein kinase kinase kinase
MAP4K	Mitogen-activated protein kinase kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MAPKAPK	Mitogen-activated protein kinase-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MCM6	Minichromosome maintenance, S. pombe, homolog of, 6
MEK	MAP/ERK kinase kinase
MEKK1	MAP/ERK kinase kinase kinase 1
Met	Methionine
miRNAs	Micro ribonucleic acid
MIZ 1	Myc-interacting zinc finger protein 1
MMP	Matrix metalloproteinase
MNKs	MAP kinase-interacting kinases
MOR	μ - opioid receptor
Mos	v-Mos moloney murine sarcoma viral oncogene homolog
mOvol1	Mouse Ovo, drosophila, homolog-like 1
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormones
MSKs	Mitogen- and stress-activated kinases
NA	Numerical aperture
Na^+	Sodium
NES	Nuclear export signal
NF-κB	Nuclear Factor - κB
NGF	Nerve growth factor
NHEK	Normal human epidermal keratincytes
NICS	Neuro-immuno-cutaneous system
NO	Nitric oxide
NOLC1	Nucleolar and coiled-body phosphoprotein 1
NPC	Nuclear pore complex
NPY	Neuropeptide Y
Oct	Octamer binding transcription factor
OD	Optical density
p.a.	pro analysis
p120ctn	p120-catenin
PAGE	Polyacrylamide gel electrophoresis
PARP	PolyADP-ribose polymerase
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDYN	Pro-dynorphin
PENK	Pro-enkephalin
Phe	Phenylalanine
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Pointed-like domain
PMSF	Phenylmethylsulfonylfuoride
POMC	Pro-opiomelanocortin
POU2F3	POU domain, class 3, transcription factor 2
POU-HD	POU-homeodomain
POU-S	POU-specific domain
PPAR	Peroxisome proliferator-activated receptor
Rac	Ras-related C3 botulinum toxin substrate 1
Rb	Retinoblastoma
RNA	Ribonucleic acid
RPL13a	60S ribosomal protein L13a
rpm	Revolutions per minute
RSKs	90 kDa ribosomal S6 kinases
RTK	Receptor tyrosine kinases

RT-PCR	Reverse transcriptase – polymerase chain reaction
RXRα	Retinoic X receptor alpha
SCIP	POU domain, class 3, transcription factor 1
SDS	Sodium dodecylsulfate
SEM	Standard error of the mean
Skn-1	POU domain, class 3, transcription factor 2
SLC7A11	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11
SP	Substance P
SPR-1	Psoriasis susceptibility 1 candidate gene 2
SPRR2A	Small prolin-rich proteins 2A
SPRs	Small prolin-rich proteins
STAT3	Signal transducer and activator of transcription 3
TA	Transactivation domain
TBS	Tris-buffered saline
TF	Transcription factor
TG1	Transglutaminase 1
TGFβ	Transforming growth factor β
TM	Transmembrane domaine
TNFα	Tumor necrosis factor α
TRPV	Transient receptor potential cation channel subfamily V
USF 1	Upstream stimulatory factor 1
UTR	Untranslated region
UV	Utraviolet
UVB	Ultraviolet B
VASP	Vasodilator-stimulated phosphoprotein
VIP	Vasoactive intestinal polypeptide
WT	Wild type
ZNF367	Zinc finger protein 367

Summary

In view of the constant environmental assaults that the skin must endure, the delicate balance of an eloquent sequence of epidermal gene expression and repression, that is required for appropriate differentiation and proliferation of keratinocytes, might easily become derailed in the absence of robust stabilizing mechanisms. The presence of a local neuroendocrine system is thereby important to coordinate a response towards irritations. In fact, the expression of several neurohormones, neurotransmitters, and neuropeptides, including pro-opiomelanocortin derivatives, such as β-endorphin and [Met^5]-Enkephalin has been shown in skin, as well as expression of the δ-opioid receptor (DOR). However, there is currently a lack of understanding of the molecular mechanisms by which their signalling modulates keratinocyte function.

The present work demonstrates that DOR signalling specifically activates the ERK 1/2 MAPK pathway in human keratinocyte cell lines. This activation inhibits cell proliferation, resulting in decreased epidermal thickness in an organotypic skin model. Furthermore, DOR expression markedly delays induction of keratin intermediate filament Keratin 10 (KRT 10) and KRT 1 during *in vitro* differentiation, and abolishes the induction of KRT 10 in the organotypic skin model. This is accompanied by deregulation of involucrin (IVL), loricrin (LOR), and filaggrin (FLG), illustrated by a markedly reduced induction of their expression upon initiation of differentiation *in vitro*.
Additionally, POU2F3 was identified as a transcription factor mediating the DOR induced regulation of keratinocyte differentiation related genes. It was revealed that DOR-mediated ERK-dependent downregulation of this factor affects key aspects of keratinocyte function.

However, it is evident that additional triggers influence the functionality of the DOR itself. Calcium at concentrations above 0.1 mM and cell-cell contact both enhance the presence of receptor molecules on the keratinocytes cell surface. Keratinocytes with internalized receptor do not respond to DOR ligands in the same way as keratinocytes with a functional membrane localized receptor.

This work suggests that upon specific extrinsic or intrinsic impulses, keratinocytes are able to respond via the neuro-epidermal opioidergic system. This response must be spatially and temporarily controlled in order to avoid an imbalance of epidermal homeostasis and delayed wound healing, as observed in the DOR knock-out mouse model of Bigliardi-Qi *et al*., 2006. Understanding this highly complex process substantiates the development of better treatments for pathological skin conditions. Complementing previous studies in DOR-deficient mice, these data suggest that DOR activation in human keratinocytes significantly influences epidermal morphogenesis and homeostasis, and might have a major impact during the wound healing process.

Résumé

Au regard des agressions environnementales constantes que la peau doit endurer, l'équilibre fragile entre l'expression et la répression des gènes épidermiques, nécessaire à la différentiation et la prolifération des kératinocytes, pourrait facilement être perturbé en l'absence des mécanismes de stabilisation robustes. La présence d'un système neuroendocrinien local est donc importante afin de coordonner une réponse aux éventuelles irritations. En effet, l'expression de plusieurs neurohormones, des neurotransmetteurs et des neuropeptides, y compris des dérivés pro-opiomélanocortine comme la β-endorphine et [Met5]-enképhaline, ainsi que l'expression du récepteur δ-opioïde (DOR) a été démontré dans la peau. Cependant, les mécanismes moléculaires par lesquels ils modulent la fonction des kératinocytes sont mal connus.

Le présent travail démontre que la voie de signalisation DOR active spécifiquement la voie ERK 1/2 MAPK dans les lignées cellulaires de kératinocytes humains, inhibant la prolifération des cellules et entraîne une diminution de l'épaisseur épidermique dans un modèle organotypique de peau. De plus, l'expression de DOR retarde nettement l'induction de la kératine 10 (KRT 10) et la kératine 1 (KRT 1) dans une modèle 2D de différentiation *in vitro*, et supprime l'induction de KRT 10 dans un modèle organotypique de peau. Ceci est accompagné de la dérégulation de l'involucrine (IVL), la loricrine (LOR) et la filaggrin (FLG), résultant en une induction nettement réduite de leur expression lors de l'initiation de la différentiation *in vitro*.

De plus, POU2F3 a été identifié comme un facteur de transcription régulant les gènes de différentiation des kératinocytes modulés par DOR. Il a été démontré que la régulation négative de POU2F3 via la voie DOR-ERK affecte les principaux aspects de la fonction des kératinocytes.

Toutefois, il est évident que des facteurs supplémentaires influencent la fonctionnalité de la voie DOR elle-même. Le calcium et le contact cellule-cellule augmentent la quantité des récepteurs à la surface cellulaire des kératinocytes. Les kératinocytes dont les récepteurs sont internalisés ne répondent pas de la même manière que ceux possédant des récepteurs fonctionnels localisée à la membrane.

Ce travail suggère que lors de signaux intrinsèques ou extrinsèques spécifiques, les kératinocytes sont capable de répondre via le système opioïdergique neuro-epidermique. Cette réponse doit être spatialement et temporairement contrôlée afin d'éviter un déséquilibre de l'homéostasie épidermique et un retard de cicatrisation. La compréhension de ce processus très complexe pourrait permettre à terme le développement de meilleurs traitements des affections cutanées pathologiques. En complément des études précédentes sur des souris DOR-déficientes, ces données suggèrent que l'activation de DOR dans les kératinocytes humains influence la morphogenèse et l'homéostasie de l'épiderme, et pourrait jouer un rôle lors du processus de cicatrisation.

CHAPTER I

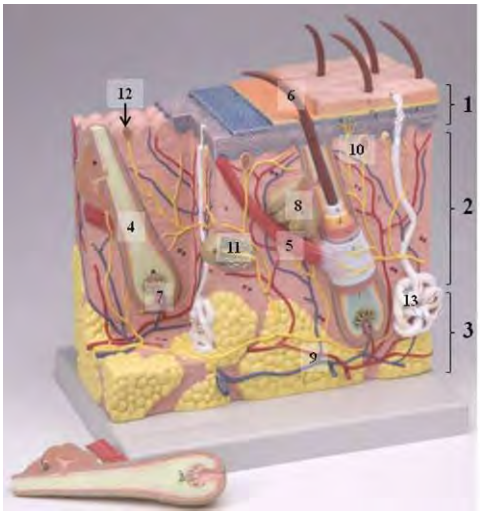
GENERAL INTRODUCTION

I.1. THE HUMAN SKIN

I.1.1. The Structure and Function of the Human Skin

The skin is the largest and one of the most diverse organs of the human body. It helps us sensing external effects while at the same time protecting us from them. Pathogens, mechanical, chemical, osmotic, and thermal assaults as well as ultraviolet (UV) radiation need to be controlled. Serving as a protective barrier keeping stressors out and essential body fluids in, the skin has its major function in the interface between the organism and the environment (Baroni et al., 2012; Elias, 2005; Menon and Kligman, 2009; Tsuruta et al., 2002; Valacchi et al., 2012). Additionally, skin carries out many biological synthetic processes, including formation of vitamin D under the influence of UVB radiation and synthesis of cytokines and growth factors. But skin is equally the target of a variety of hormones. Its complex structure and biological functions are associated with various ailments, including developmental defects, autoimmune disorders, allergies, and cancer (Gray's Anatomy - 39th Edition 2005).

The skin is organized in three layers, the uppermost epidermis, underneath the dermis, followed by the hypodermis. Several specialized structures such as nerve fibres, free nerve endings, mechanoreceptors, hair follicles, sebaceous or sweat glands, blood and lymphatic vessels are embedded within, as illustrated in Figure 1.



1) Epidermis
2) Dermis
3) Hypodermis
4) Hair follicle
5) Erector muscle of hair
6) Hair shaft
7) Dermal papilla
8) Sebaceous gland
9) Blood vessels
10) Nerve fibers (yellow)
11) Pacinian corpuscle (mechanoreceptor: touch and vibration)
12) Meissner's corpuscles or tactile corpuscles (mechanoreceptor: touch)
13) Sweat gland

Figure 1 - Structure of human skin

Adapted from Human Anatomy Models, IDS. Ltd

I.1.1.1. The Epidermis

The epidermis is a continuously self-renewing, stratified squamous epithelium and the main cell type present are keratinocytes. They are equipped with numerous receptors receiving signals from cytokines like tumour necrosis factor alpha (TNFα) (Coffey, Jr. et al., 1987; Pillai et al., 1989; Staiano-Coico et al., 1990; Trefzer et al., 1991), transforming growth factor beta (TGFβ) (Choi and Fuchs, 1990; Kane et al., 1991; Mansbridge and Hanawalt, 1988; Partridge et al., 1989; Pelton et al., 1989), interleukin 1 (IL-1) (Blanton et al., 1989; Hauser et al., 1986; Kupper et al., 1986), neuromediators like calcitonin-gene related peptide (CGRP) (Takahashi et al., 1993) or opioids (Bigliardi et al., 1998; Bigliardi-Qi et al., 1999; Cheng et al., 2008; Nissen and Kragballe, 1997; Schauer et al., 1994), and growth factors like epidermal growth factor (EGF) (Barrandon and Green, 1987; Green et al., 1987; Green and Couchman, 1985; Pittelkow et al., 1993; Rheinwald and Green, 1977). Keratinocytes undergo a highly reproducible terminal differentiation program resulting in the formation of four distinct layers, which provide the protective barrier, crucial for survival. The innermost layer is the basal layer, followed by the spinous, granular and the outermost cornified layer (McGrath et al., 2004).

The result of keratinocyte differentiation is the expression of a unique array of keratin intermediate filament (IF) differentiation markers in each compartment (Figure 2), which may reflect the different structure and metabolic requirements of cells in each layer. Keratins represent the largest group of intermediate filament proteins and are differentially expressed as pairs of type I and type II intermediate filaments. They mainly function to maintain cell and tissue integrity (Koster and Roop, 2004a).

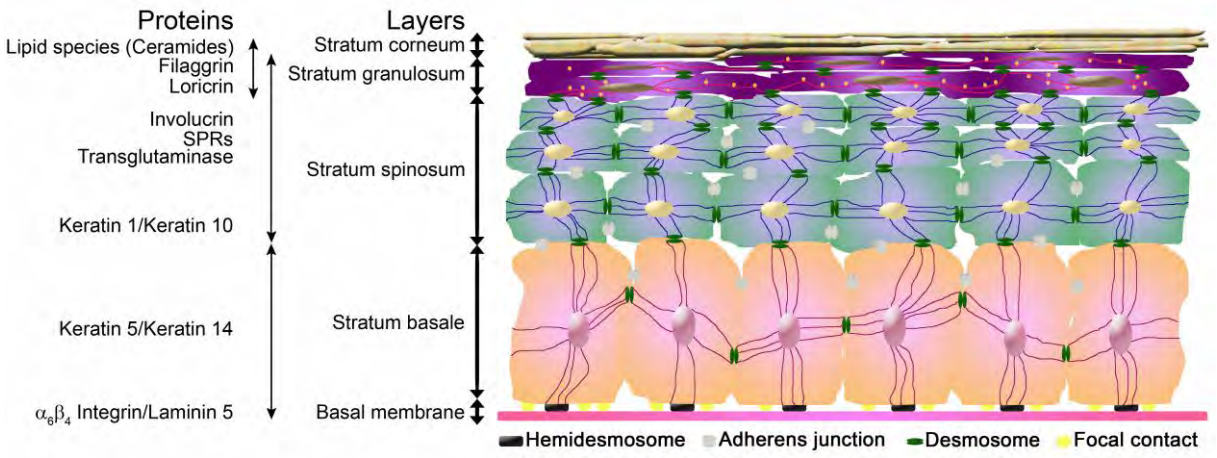


Figure 2 - Structure of the epidermis

The epidermis is composed of several cell layers. Resting on the basement membrane is the stratum basale, consisting of proliferating, transient-amplifying cells. The basal layer stratifies to give rise to differentiated cell layers of the stratum spinosum, stratum granulosum and the stratum corneum. SPRs, small proline-rich proteins Adapted from (Fuchs and Raghavan, 2002).

Epidermal growth and proliferation need to be balanced carefully and are regulated in time and space by the process of homeostasis. This process assures that the number of epidermal cells remains constant. Therefore, new cells generated by each cell division has to exactly compensate the number of cells lost by differentiation and desquamation (Blanpain and Fuchs, 2009). Basal cells attach to an underlying basement membrane that is rich in extracellular matrix (ECM) proteins, including collagen type IV, nidogen, perlecan, and large laminins like laminin 5, the main ECM ligand of the epidermal basement membrane. The attachment depends on two types of cell-junction adhesion complexes, hemidesmosomes and focal adhesions (Watt, 2002). Hemidesmosomes contain a transmembrane core, including $\alpha_6\beta_4$ integrins and type XVII collagen that connect intracellular to the keratin IF network, via the linker proteins plectin and dystonin (BP230). The integrins and collagen XVII connect to extracellular matrix proteins laminin-332 and laminin-331. Further, anchoring fibrils, mainly constituted of collagen VII, extend from the basal membrane zone into the dermis and by forming a loop, they reattach back onto the basal membrane. This structure provides the mechanical strength needed for the epidermal-dermal anchoring. On the other side, focal

adhesions contain $\alpha_3\beta_1$ integrins, which connect to the actin and microtubule networks of the keratinocytes. During wound healing theses proteins regulate migration and attachment of the keratinocytes to the underlying extracellular matrix to enable the re-epithelialisation. Genetic alterations of hemidesmosomal components result in several types of blistering disorders of the skin (Christiano and Uitto, 1996; Nagy and McGrath, 2010) and are likely to be involved in the development and progression of certain cancers (Hashmi and Marinkovich, 2011).

Basal cells as well as keratinocytes from the upper layers also have to adhere to one another. Intercellular adhesion is mediated by three types of intercellular junctions: the desmosome, adherens junctions, and tight junctions (Yuki et al., 2007). All types of intercellular junctions are essential for epithelial sheet formation and tissue integrity. During terminal differentiation, as the sheets migrate upwards towards the skin surface, epidermal cells must constantly change their intercellular interactions. On the other side, during wound healing, epidermal cells transiently downregulate intercellular adhesion, accompanied with an increase in cell proliferation to facilitate the re-epithelialisation process (Garrod et al., 2005; Thomason et al., 2012).

Adherens junctions are composed of a transmembrane core of E-cadherin, whose ectodomain binds Calcium (Ca^{2+}) to mediate transcadherin interactions between neighbouring cells. Intracellular E-cadherin binds to β-catenin and p120-catenin (p120ctn). These molecules then associate with an array of actin regulatory proteins, including Rho-GTPase and α-catenin that in turn interacts with vasodilator-stimulated phosphoprotein (VASP), formins, ajuba, and α-actinin. This cascade of interacting regulatory proteins influences the actin-myosin network to coordinate cell-cell adhesion and migration (Perez-Moreno et al., 2003) (Figure 3A).

Desmosome structures consist mainly of heterophilic interactions between transmembrane glycoproteins that belong to the desmocollin and desmoglein subfamily of cadherins. As illustrated in Figure 3B, the desmosomal cadherins desmocolin and desmoglein are connected

to intermediate filaments through a protein complex consisting of plakoglobin, desmoplakin and plakophilin (Schmidt and Koch, 2007). This connection provides a cohesive integrated mechanical framework across and within stacks of keratinocytes. Cell-cell junction-associated proteins connect structure and signalling molecules, so that intercellular junctions function to integrate a number of cellular processes, ranging from cytoskeletal dynamics to proliferation, transcription, and differentiation (Garrod, 1996; Muller et al., 2008). The cadherins may thereby act as a membrane trap for β-catenin, p120ctn or plakoglobin, increasing their degradation at the membrane and augmenting the turnover of cytosolic molecules. This process prevents their nuclear translocation, therefore regulating the transcriptional activities of β-catenin, p120ctn and plakoglobin (Daniel, 2007; Maher et al., 2009; McCrea et al., 2009; Zhurinsky et al., 2000).

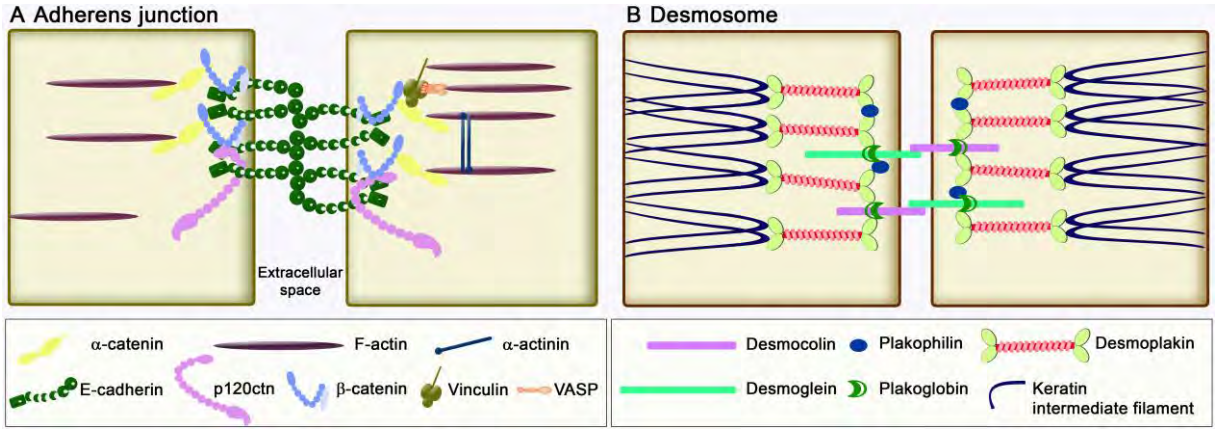


Figure 3 - Adherens junctions and desmosomes in the epidermis

Simplified models of (A) an adherens junction and (B) a desmosome, which highlights some of the main protein - protein interactions found in these structures. Adherens junctions and desmosomes mediate cell - cell contact between all cells of the epidermis and are present in all metabolically active cell layers. The adherens junctions form a bridge between the actin cytoskeleton of neighbouring cells. By contrast, desmosomes associate with the keratin filament cytoskeleton of cells. p120ctn, adherens junction protein p120, p120-catenin; VASP, vasodilator-stimulated phosphoprotein. Adapted from (Fuchs and Raghavan, 2002).

In addition to keratinocytes, the epidermis contains melanocytes, Langerhans cells and Merkel cells. Melanocytes produce melanin, the main natural pigment of the skin that is then transferred to keratinocytes. Melanin not only influences the colour of skin, but also protects the deeper layers from UV radiation (Bessou-Touya et al., 1998) and acts as scavenger of free

radicals (Menter et al., 1998). Langerhans cells are mobile dendritic, antigen presenting cells, present in all stratified epithelia. They become mature after contact with antigens (Romani et al., 2012). Finally, Merkel cells are pressure-detecting mechanoreceptors (Boulais and Misery, 2008).

I.1.1.2. The Dermis

The dermis is a supportive and elastic connective tissue, principally constituted by fibroblasts and fibrous molecules that compose the ECM. Embedded into this matrix are blood vessels, nerves, lymphatics, and skin appendages. As the epidermis is non-vascularized, it receives its nutrients from the underlying dermis and this epithelial-mesenchymal interaction is essential for normal tissue maintenance and during wound healing.

The dermis undergoes continuous turnover involving ECM production and degradation, synthesized and organized by fibroblasts. Fibroblasts are mesenchymal cells of varied origins and comprise a heterogeneous population of cells defined according to their location within the dermis. Two subpopulations of fibroblasts reside in the two distinct dermal layers: the papillary and reticular dermis (Sorrell and Caplan, 2004). Different populations of fibroblasts are associated with hair follicles but additional subpopulations of dermal fibroblasts might exist as well (Gharzi et al., 2003; Jahoda et al., 1991; Millar, 2002; Nolte et al., 2008; Sorrell and Caplan, 2009).

An important characteristic of fibroblasts is that, through mechanical stress, inflammation or TGFβ1 stimulation, they acquire a new differentiation state and become myofibroblasts (Vaughan et al., 2000). Myofibroblasts, in response to monocyte/macrophage derived factors, produce a provisional wound matrix, that is enriched in foetal-like fibronectin and hyaluronan (Gailit and Clark, 1994; Juhlin, 1997; Singer and Clark, 1999). These cells also provide the motive force to contract the wound (Sappino et al., 1990). At present, it is well accepted that

the differentiation of fibroblasts into myofibroblasts represents a key event during skin wound repair (Li and Wang, 2011).

I.1.1.3. The Hypodermis

The hypodermis is a layer of loosely arranged connective tissue of variable thickness representing the deepest part of the skin. It is often composed of adipocytes, organised in lobules, separated by connective tissue. It plays an important role in thermoregulation, acts as shock absorber, and constitutes a store of metabolic energy (Hayward and Keatinge, 1981).

I.1.2. Epidermal Homeostasis and Differentiation

The physiological process that maintains a constant number of cells in self-renewing organs is called tissue homeostasis. Skin stem cells have an unlimited capacity to self-renew and are the main components of the epidermal homeostasis. They reside in different compartments, the bulge region of the hair follicle, sebaceous glands, and in the basal layer of interfollicular epidermis. One model for epidermal homeostasis suggests that stem cells from the basal layer divide asymmetrically with the mitotic spindle positioned parallel to the basement membrane, produce cells with either transient proliferative capacity and cells that remain stem cells (Figure 4) (Blanpain and Fuchs, 2009). Transient amplifying cells might then undergo a number of divisions residing in the basal layer, before they execute the program of terminal differentiation, migrate upwards, and slough from the surface, so that throughout life in humans the epidermis replenishes itself every four weeks (Fuchs, 2007; Potten, 1981). Another model proposed that stem cells in the basal layer give rise directly to differentiating cells without the step of a transient amplifying cell (Clayton et al., 2007). The cell division is hereby the crucial step. Oriented, asymmetric cell divisions control the polarity of the cells and the modification of cellular adhesion with its neighbouring cells, as well as attachment to

the underlying basement membrane (Lechler and Fuchs, 2005; Poulson and Lechler, 2010).

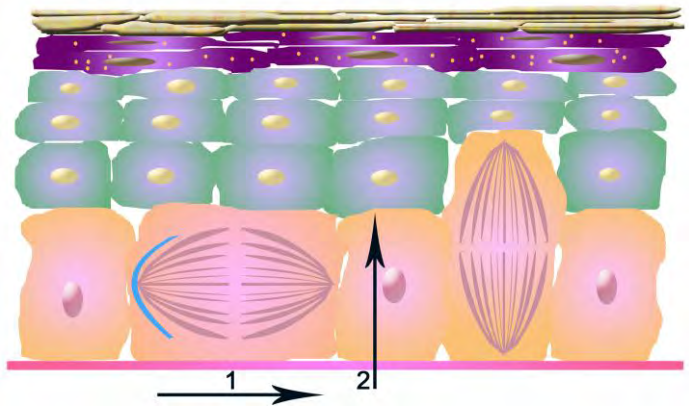


Figure 4 - Stem Cell division during adult homeostasis in mouse tail skin.

During epidermal homeostasis in the adult mouse tail skin, asymmetric cell divisions occur with the plane of division parallel to the basal membrane, such that only one daughter cell inherits a cell fate determinant, such as Numb, and remains a stem cell (step 1), whereas the other becomes transient amplifying, committed to terminal differentiation, and probably undergoes delamination to reach the spinous layers (step 2). Combined lineage tracing experiments and mathematical modelling suggest that epidermal tail homeostasis in mice does not require the existence of transient amplifying cells. Therefore, the second model proposes perpendicular cell division giving rise to a stem cell and a directly differentiating keratinocyte through lost of contact with the basement membrane (Blanpain and Fuchs, 2009; Clayton et al., 2007). Figure adapted from (Blanpain and Fuchs, 2009).

Any imbalance between proliferation and differentiation might result in a thinning of the skin and loss of protection, in case of too little proliferation, whereas too much proliferation is a characteristic of hyperproliferative disorders like psoriasis and cancer (Bernard et al., 1985; Cangkrama et al., 2013; Demehri et al., 2009; Descargues et al., 2008; Ferone et al., 2012; Guttman-Yassky et al., 2009; Li et al., 2012; Mansbridge et al., 1984; Marinari et al., 2009; Neumüller and Knoblich, 2009). Additionally, during a wounding situation the epidermis must sense when to activate keratinocytes for proliferation and migration and to stop, once the wound is healed (Arwert et al., 2012; Chong et al., 2009; Florin et al., 2006; Frank et al., 1996; Li et al., 2010; Natarajan et al., 2006; Thelu et al., 2002).

When keratinocytes withdraw from the cell cycle and start to differentiate they exit the basal layer and switch off the expression of genes encoding the IF keratin 5 (KRT 5) and KRT 14. Entering the spinous layer, cells switch on the expression of KRT 10 and KRT 1 to form a more robust IF network. This change in keratin expression was discovered more than 30 years

ago and is widely accepted as most reliable indication that a keratinocyte has undergone a commitment to terminal differentiation (Fuchs and Green, 1980).

As keratinocytes differentiate further, they start to express involucrin (IVL). This protein is one of the key players in early-cornified envelope assembly and its expression initiates in the upper spinous layer. Intrachain head-to-head and head-to-tail cross-linking of IVL and additional structure proteins is enabled by the activity of transglutaminase 1 (TG1). Active TG1 generates γ-glutamyl ε-lysine crosslinks, to create an indestructible proteinaceous scaffold along the entire inner cell membrane, to hold keratin microfibriles. As spinous cells further progress towards the granular layer, they start producing keratohyalin granules, packed with the histidine-rich protein profilaggrin or cystine-rich loricrin (LOR), which are then further processed to bundle keratin IFs, in order to generate keratin macrofibrillar cables. The process is accompanied by the synthesis of keratinisation-specific lipids, which are synthesised and accumulated in the trans-Golgi apparatus and bud off as cytoplasmic lamellar bodies. Continuing the differentiation processes, keratinocytes start to express proteins such as small proline-rich proteins (SPRs), late cornified envelope proteins, hornerin, repetin, and cornulin, resulting in a reinforcement of the cornified envelope structure. During the late steps of differentiation, ceramides from the lamellar bodies are attached to the cross-linked protein scaffold by ester-bond formation, and cellular organelles, including the nucleus, are destroyed. Once terminal differentiation is completed, keratinocytes exist as dead corneocytes that are sandwiched by lipid lamellae on the outside and filled with an indestructible fibrous mass of keratins that is encased by the cornified envelope (Kalinin et al., 2002).

The morphological changes associated with differentiation and stratification have been studied extensively, but the molecular mechanisms that regulate these processes are only partially understood. Mouse genetic studies have identified multiple signalling pathways involved (Blanpain and Fuchs, 2009). These pathways include Notch, mitogen-activated

protein kinases (MAPK), and nuclear factor-κB (NF-κB). Transcription factors such as p63, the AP-1 and AP-2 family of proteins, the CCAAT/enhancer-binding protein (C/EBP), interferon regulatory factor 6 (IRF6), grainyhead-like 3 (GRHL3), Kruppel-like factor 4 (KLF4), and peroxisome proliferator-activated receptor (PPAR) proteins have been shown to be involved as well (Dai and Segre, 2004). The interplay between these pathways is complex and new members are constantly joining the network.

One of the key regulatory transcription factors in epidermis is p63, playing an important role during embryonic development, but is also, together with the Notch pathway, controlling the switch from the basal to the spinous cell type in adults. Gain- and loss-of-function studies have shown that p63 is required for initiation of the stratification process and to maintain the renewal potential of different epithelial stem cells (Candi et al., 2006; Koster et al., 2004; Koster and Roop, 2004b). The canonical Notch pathway is crucial during the early switch from basal to spinous cells (Rangarajan et al., 2001). The Notch signalling interferes with the expression of transcription factors like C/EBP, which in turn associate with the AP-2 family of transcription factors influencing the commitment to terminal differentiation (Wang et al., 2008). An additional important signalling mechanism involved in the commitment to terminal differentiation is Ca^{2+} signalling. In the mature epidermis an increasing gradient of extracellular Ca^{2+} concentration is present from the basal to the cornified layer (Menon et al., 1985; Menon et al., 1992). Studies have demonstrated that the increase in Ca^{2+} is required for keratinocyte terminal differentiation and active barrier formation (Elias et al., 2002; Yuspa et al., 1989). When terminal differentiation of keratinocytes is induced *in vitro*, other than by increasing extracellular Ca^{2+} concentration, an increase of intracellular Ca^{2+} is still observed. Signalling mechanisms activating the release of Ca^{2+} from intracellular stores are suggested to be involved in this process (Li et al., 1996; Sharpe et al., 1989; Sharpe et al., 1993) and one of the signalling pathways being induced by an increase of Ca^{2+} concentration in keratinocytes is

Notch (Okuyama et al., 2004).

Another influential factor on epidermal stratification and differentiation is inhibitor of NF-κB kinase-alpha (IKKα). Acting independently of NF-κB, IKKα influences the commitment to terminal differentiation, as mice lacking this gene have a hyperplastic epidermis with proliferating cells not only in the basal but also in the spinous layer, while the granular and cornified layers are absent (Hu et al., 2001; Takeda et al., 1999).

Additionally, it has been shown that retinoblastoma protein (Rb) deficient keratinocytes re-enter the cell cycle after commitment to differentiation *in vitro* and mice deficient of epidermal Rb expression show an aberrant co-expression of KRT 5 and KRT 10. This indicates the importance of Rb in epidermal proliferation and differentiation (Balsitis et al., 2003; Ruiz et al., 2004).

Yet another important transcription factor, c-Myc, has been shown to be expressed in basal proliferating keratinocytes, indicating a function in the regulation of proliferation of basal cells (Bull et al., 2001). But, an increased expression in cultured keratinocytes *in vitro* promotes terminal differentiation and causes a reduction in growth (Gandarillas and Watt, 1997). Myc induces differentiation and loss of cell polarization in a Miz1-dependent manner. Miz1 (Myc-interacting zinc finger protein 1; Zbtb17) is a zinc-finger transcription factor and has been shown to be bound and regulated by Myc (Peukert et al., 1997; Schneider et al., 1997). Miz1 is involved in the upregulation of cell cycle inhibitor p21cip1 in response to UV irradiation (Herold et al., 2002) and is critical for its repression during skin tumorgenesis (Honnemann et al., 2012). In addition, regulation of cell adhesion is a major function of the Myc-Miz1 complex in skin and suggests that this may contribute to Myc-induced exit from the epidermal stem cell compartment towards a transient-amplifying and afterwards differentiating keratinocyte stage (Gebhardt et al., 2006). Miz1 binds and stimulates the expression of genes in the absence of Myc, therefore activation of Myc and its interaction

with Miz1 indirectly leads to the repression of these genes.

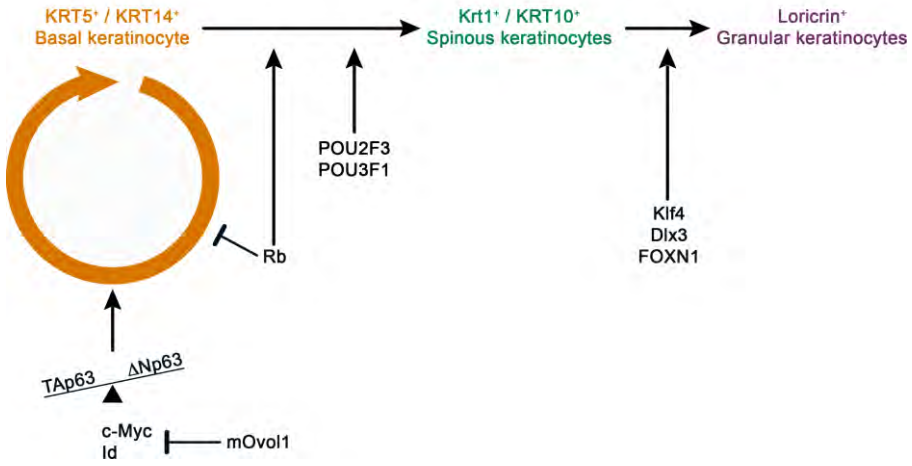


Figure 5 – Transcription factors regulating keratinocyte differentiation

A range of transcription factors (TFs) regulating stem cell maintenance in basal layer keratinocytes and differentiation of interfollicular epidermis are represented in this figure. Basal keratinocytes need to maintain a stem cell pool as well as proliferate in order to give rise to transient amplifying cells and initiate progression towards terminal differentiation. The balance between the two splice variants of p63, TAp63 and ΔNp63, determines the proliferative state of the keratinocyte. c-Myc drives stem cells towards transient amplifying cells and the Id family of proteins influences proliferation, by interfering with the DNA binding of transcription factors that lead cells towards differentiation. mOvol1 is required for the exit from proliferation and direct commitment to differentiation. POU domain TFs are involved in the repression of basal-specific keratins and activation of early and late stage differentiation genes. Klf4 has been shown to be involved in the expression of structural protein components of the cornified envelope, Dlx3 influences the transcription of terminal differentiation markers, and FOXN1 stimulates the expression of early differentiation markers but suppresses late stage markers. Adapted from (Dai and Segre, 2004).

Furthermore, POU2F1 (Oct-1), POU3F1 (Tst-1/Oct-6/SCIP), and POU2F3 (Oct-11/Skn-1/Epoc-1), members of the octamer-binding POU transcription factor family of proteins, have been shown to be involved in epidermal differentiation. Overexpression of POU2F3 in keratinocytes facilitates differentiation and ablation of both POU2F3 and POU3F1 in mice leads to ectopic expression of KRT 14 in spinous cells (Andersen et al., 1997b).

Complexity is further added to the signalling network by the action of microRNAs (miRNAs) that fine-tune the signalling (Hildebrand et al., 2011; Yi et al., 2006) as well as by epigenetic mechanisms (Mulder et al., 2012).

I.1.3. The Neuro – Endocrine – Cutaneous Interaction

Neurocutaneous interactions are capable of influencing a variety of physiological and pathophysiological functions including wound healing, cell growth, inflammation, immunity, and pruritus. All epidermal cells (keratinocytes, melanocytes, Langerhans cells, and Merkel cells) express sensor proteins and neuropeptides regulating the neuro-immuno-cutaneous system (Boulais and Misery, 2008). Skin cells secrete numerous neuropeptides such as opioids, substance P (SP), CGRP, vasoactive intestinal polypeptide (VIP), acetylcholine (ACh), catecholamines, endocannabinoids, endothelin (ET) or nerve growth factor (NGF) and are therefore capable of modulating, activating or inhibiting sensory neurons located within epidermal cells. Corresponding receptors can be found either directly on skin cells, on embedded immune cells or sensory and autonomic nerves, what makes it difficult to distinguish whether the receptors facilitate inter-keratinocyte signalling, immune cell-keratinocyte interaction or keratinocyte-neuron signalling or all at once (Lumpkin and Caterina, 2007).

Sensory nerves send many primary afferent fibres to all layers of the skin and build up a three-dimensional network (Roosterman et al., 2006). They pass through the dermis and penetrate the basement membrane to innervate epidermal cells or remain as free endings. Unmyelinated C fibres, with free-branching endings in close contact to keratinocytes, can be found up to the granular layer. In μ-opioid receptor (MOR) knock-out (KO) mice and chronic pruritic skin, changes in the structure of these fibres could be observed (Bigliardi-Qi et al., 2005; Bigliardi-Qi et al., 2007). These nerve fibres are clearly involved in somatosensation however, the terminals are relatively sparse. The skin can detect patterns at a very fine and smaller scale, which suggests that nerve terminals receive help from epidermal sensors (Denda et al., 2007; Gopinath et al., 2005; Johansson et al., 1999; Krimm et al., 2006; Provitera et al., 2007). Therefore, the epidermis can be considered as a sensory organ.

In addition, after different stimuli including heat, lipopolysaccharides (LPS), thermal mud baths, and UVB radiation (Slominski et al., 2000) human skin can produce pro-opiomelanocortin (POMC), the precursor of endorphins, which is able to modify the pain threshold and modulate different activities of the immune system (Brazzini et al., 2003). Conversely, the brain can affect cutaneous functions in an efferent manner to stimulate the target tissues; for example during neurogenic inflammation (Saraceno et al., 2006). Hence, the brain-epidermis connection is multi-directional and leads, according to Boulais and Misery, to the integrated neuro-immuno-cutaneous system (NICS), a common language shared by sensory neurones, keratinocytes, melanocytes, Langerhans cells, and Merkel cells, with the neuromediators as their words (Boulais and Misery, 2008).

It is currently understood that SP plays a key role in pain sensitisation (Misery et al., 1999) and leads to mast cell degranulation (Toyoda et al., 2002). POMC and derivatives are immunomodulators and neurotrophins like NGF are mitogenic proteins, which also stimulate nerve fibre sprouting, regulate neuropeptide synthesis, and probably contribute to psoriasis (Pincelli et al., 1994). The catecholamines act as inflammatory factors. ACh, CGRP, VIP, and neuropeptide Y (NPY) seem to have different functions, depending on the skin microenvironment. Therefore, the NICS acts locally, at the level of the neurogenic inflammation, but it is also considered to affect the whole organism via endocrine and neurocrine pathways.

Furthermore, the transient receptor potential cation channel subfamily V (TRPV) family and purinergic receptors are also thought to participate in many cutaneous phenomena. They have been shown to be involved in cell growth, differentiation, neuronal regeneration, wound healing, inflammation, and yet more (Boulais and Misery, 2008; Burnstock, 2006). Keratinocytes express receptors like TRPV1, TRPV3, and TRPV4 (Dhaka et al., 2006). TRPV channels enable them to sense thermal and noxious stimuli and perhaps osmotic

variation. The stimulation of these receptors is followed by the release of neuropeptides like SP, which then act as neurotransmitters on target cells or modulators of epidermal functions as mentioned before.

The ability of keratinocytes to interact with neurones has been demonstrated *in vitro*. In co-culture models, keratinocytes exhibit a strong trophic effect towards sensory neurones and close contact could be detected between the two cell types (Chateau et al., 2007; Chateau and Misery, 2004; Ulmann et al., 2009). The mechanism involved in signal transduction from keratinocytes to sensory neurones remains unclear. One hypothesis is that the signal goes through a paracrine adenosine tri-phosphate (ATP) signal, activating the purinergic receptors P2X2, P2X3, and P2Y2. It has been shown that ATP-activated cells can increase their intracellular Ca^{2+} concentration, producing a Ca^{2+} wave that is able to propagate to neighbouring cells. The ATP-dependent Ca^{2+} waves, so produced by keratinocytes, can induce an increase in intracellular Ca^{2+} concentration, not only in adjacent keratinocytes but also in sensory neurones (Koizumi et al., 2004).

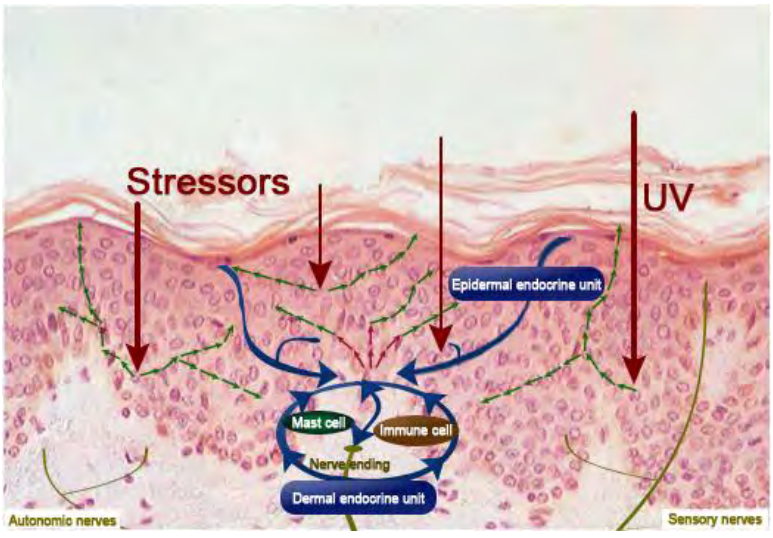


Figure 6 - The skin neuro-endocrine system

The skin neuro-endocrine system follows the algorithms of classical neuro-endocrine or endocrine systems of the human body. It serves as a mediator for signal exchange between body internal processes and the environment. Skin cells are subjected to neurohormonal regulation and produce and secrete neuro-peptides, biogenic amines, melatonin, opioids, cannabinoids, acetylcholine, steroids, secosteroids as well as growth factors and cytokines themselve. Immune cells are as well part of the skin neuro-endocrine system. Histological image from (Gray's Anatomy - 39th Edition 2005) and signalling scheme adapted from (Slominski et al., 2012).

Another putative pathway of communication from keratinocytes to neurones is implicated by the presence of substances like NGF or the inflammatory cytokine interleukins, IL-1α and IL-

8, subsequent to receptor activation. These mediators are released upon activation of the keratinocytes by neuropeptides like SP, CGRP, VIP, galanin, and probably other proteins expressed by keratinocytes themselves (Dallos et al., 2006). Hence, the activation of one keratinocyte can lead to the activation of neighbouring cells in a paracrine manner, and finally to the depolarisation of nerve terminals.

Moreover the opioid system is present and functional in mouse and human skin. The expression of δ-opioid (DOR), μ-opioid (MOR), and κ-opioid receptors (KOR) on mRNA level has been shown in human epidermal keratinocytes, fibroblasts and melanocytes (Bigliardi et al., 1998; Bigliardi et al., 2009; Bigliardi-Qi et al., 2006; Kauser et al., 2003; Kauser et al., 2004; Tominaga et al., 2007; Wintzen et al., 2001). Compared to human brain tissue the amount of both MOR and DOR is low in skin cells. Keratinocytes express 1000 times less MOR and 2000 times less DOR, fibroblasts approximately 20 000 times less MOR and 1500 times less DOR mRNA than brain (Bigliardi et al., 2009).

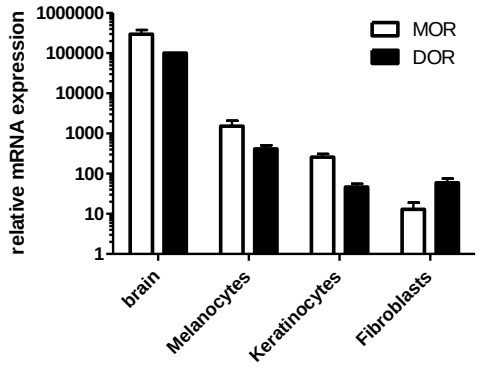


Figure 7 - relative MOR and DOR mRNA expression in primary cultured skin cells

qRT-PCR of mRNA isolated from fibroblasts, melanocytes and keratinocytes, compared to human brain mRNA extracts. Value of brain DOR is normalized to 100 000. Adapted from (Bigliardi et al., 2009)

Nevertheless, the functional activity of the β-endorphin/MOR system in skin melanocytes was demonstrated by Kauser *et al.(Kauser et al., 2003; Kauser et al., 2004; Kauser et al., 2005; Tobin and Kauser, 2005)* and alterations of β-endorphin expression were observed in pathological human skin by Slominski *et al.* (Slominski et al., 1993; Slominski et al., 2004). The treatment of epidermal melanocytes with β-endorphin results in increased melanogenesis and proliferation. Yet, skin organ culture experiments showed functional activity of the

endogenous β-endorphin/MOR system in epidermal keratinocytes as well through downregulation of MOR protein expression, upregulation of TGF-β receptor type II and KRT16 expression upon prolonged incubation with β-endorphin (Bigliardi-Qi et al., 2000). KRT16 is not expressed in normal human skin but appears in regenerating epithelial cells of the epidermis during wound healing, which led to first hypothesis of opioid receptor involvement in wound healing. *In vitro* a stimulatory effect on migration of keratinocytes by addition of morphine could be demonstrated (Bigliardi et al., 2002), indicating as well the functionality of the opioid receptor system in skin cells. Possibly activated by opioids to regulate migration could be the mitogen-activated protein kinase (MAPKs) extracellular signal-regulated kinase 1 and 2 (ERK 1/2) pathway, the protein kinase C (PKC) or the phosphoinositide 3-kinase (PI3K) pathway (Chernyavsky et al., 2005; Eisinger and Ammer, 2008a; Fitsialos et al., 2007; Heiss et al., 2009; Matsubayashi et al., 2004; Ranzato et al., 2009; Sharma et al., 2003a). These pathways were shown to be involved in either primary keratinocyte migration *in vitro* or migration and proliferation during wound healing (Sharma et al., 2003b; Watson et al., 2009). Any alteration in the rate of migration of these cells would change the time available for cell maturation and might be regulated by both synthesis and processing of the keratin filaments, which in turn influence the re-epithelialisation and remodelling process in wound healing.

One phenotype of DOR and MOR KO mice is a significant atrophy of the epidermis (Bigliardi-Qi et al., 2007). Additionally, DOR KO mice show a higher expression of KRT 10 than wild type mice and a significant wound healing delay of about two days as well. The wound margin of these mice exhibits epidermal hypertrophy at day three (Bigliardi-Qi et al., 2006). These observed phenotypes in DOR KO mice indicate a role for DOR in keratinocyte migration and differentiation and therefore an involvement in skin homeostasis and wound healing.

I.2. THE OPIOID RECEPTOR SYSTEM

I.2.1. The Opioid Ligands – Endogenous and Exogenous

The term "opioid" describes all compounds with morphine-like action. It does not distinguish between different chemical structures, that include alkaloids as well as peptides, nor if the molecule originates from endogenous or exogenous sources. Morphine itself has a long history of application for therapeutic purposes and the pleasurable subjective effects. Researchers were always eager to understand its mechanisms of action in human.

In the 1960s and '70s the brain was recognized as the main target for morphine induced analgesia (Mayer and Liebeskind, 1974; Pert and Yaksh, 1974; Pert and Yaksh, 1975; Tsou and Jang, 1964) and further analysis lead then to the identification of several endogenously produced opioid compounds. Until now, ten endogenous opioids have been identified, which can be grouped into six different families of compounds, the endorphins (Doneen et al., 1977; Li et al., 1976), enkephalins (Hughes et al., 1975), dynorphins (Goldstein et al., 1979), nociceptin (Meunier et al., 1995; Mollereau et al., 1994; Reinscheid et al., 1995), endomorphins (Hackler et al., 1997; Zadina et al., 1997), and morphiceptin (Chang et al., 1981), according to their receptor selectivity and structure. They are transcribed from only three different genes and generated by subsequent processing of the common precursor proteins pro-opiomelanocortin (POMC), pro-enkephalin (PENK), and pro-dynorphin (PDYN) (Feng et al., 2012). Cloning of these genes revealed that PENK contains both [Met^5]-enkephalin and [Leu^5]-enkephalin transcripts (Comb et al., 1982; Gubler et al., 1982; Noda et al., 1982), the second precursor PDYN the dynorphin peptides (Goldstein et al., 1979; Kakidani et al., 1982), and POMC contains β-endorphin as well as adrenocorticotropin (ACTH) and melanocyte-stimulating hormones (MSH) (Chretien et al., 1979; Nakanishi et al., 1979). The sequence of [Met^5]-enkephalin is also embedded in POMC but the protein is not transcribed from this gene (Feng et al., 2012; Li and Chung, 1976). For endomorphin a

precursor has not yet been identified (Terskiy et al., 2007). Other peptides with morphine-like activity have been generated by proteolytic fragmentation of the milk protein casein and were termed β-casomorphins (Brantl et al., 1979; Chang et al., 1981) or from the blood protein haemoglobin, called hemorphins (Brantl et al., 1986; Nyberg et al., 1997). Additionally, MOR (dermorphin) and DOR (deltorphin) selective peptides have been isolated from frog skin (Broccardo et al., 1981; Erspamer et al., 1989).

The knowledge of the structure and the unique properties of each endogenous opioid gave rise to the development of numerous exogenous opioids, in order to enhance the analgesic potencies and to reduce the side effects in clinical applications (Ballet et al., 2008). One of the most potent DOR agonists developed is SNC80, a highly selective non-peptide δ-opioid agonist, 2000 fold more selective over μ-opioid receptors than δ-opioid receptors. Because SNC80 is producing convulsions at high doses, it is not used medically but it is a useful drug in scientific research. Furthermore naltrindole, a highly selective non-peptide δ-opioid antagonist, has been developed. Naltrindole has a 223 and 346 fold greater activity at DOR than at MOR and KOR. With the discovery of additional functions of the opioid receptors, other clinical applications than analgesia came into focus, so that now the development of highly receptor specific agonists is more important, in order to tap the full potential of opioids in clinic.

I.2.2. General Introduction into the Family of Opioid Receptors

In 1973, the laboratories of Simon, Snyder, and Terenius independently reported the biochemical discovery of opioid receptors, by utilizing radioligand binding assays. They were found to be integral membrane proteins primarily located in neurons (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). A few years later, due to their heterogeneous properties, the three “classical” types of opioid receptors were named μ (MOR), κ (KOR) and δ (DOR)

(Martin et al., 1976). Molecular cloning confirmed the three receptors in 1992 and 1993 (Chen et al., 1993; Evans et al., 1992; Kieffer et al., 1992; Yasuda et al., 1993). The forth receptor, the nociceptin receptors, was discovered in 1994 using a degenerate PCR based on sequences of the known opioid receptors, to clone a novel receptor from a human brainstem cDNA library (Mollereau et al., 1994). All four opioid receptors differ in their affinity for various endogenous opioid ligands and in their cellular distribution. Their signalling regulates a variety of physiological functions, including pain control, locomotion, emotional tone, diuresis, thermoregulation, and reward (Kieffer and Gavériaux-Ruff, 2002). MOR also mediates the pain-relieving effects of some of the clinically most effective drugs. However, drugs acting on DOR also exhibit strong analgesic activity without abuse liability (Rapaka and Porreca, 1991), a property considered as a hallmark of MOR agonists (Contet et al., 2004).

Opioid receptors are mainly found in the central and peripheral nervous systems and the functional and biochemical characterization focused extensively on the neuronal system in the last three decades. However, in peripheral tissue, such as skin (Salemi et al., 2005), cornea, and the cochlea (Wenk and Honda, 1999), the presence of opioid receptors was identified and additionally in T, B, and monocyte cell lines (Gavériaux et al., 1995). Functional activity of DOR could be demonstrated in peripheral tissue such as heart, gastrointestinal tract, and the immune system. Involvement of opioids in cell differentiation and proliferation (Caballero-Hernandez et al., 2005; Hahn et al., 2010) as well as cytokine production (Bessler et al., 1990) makes them an interesting target in skin research and a good study model for the neuro-cutaneous system.

As the field of opioid receptor research develops and the functional network of opioid mediated signalling is deciphered, a major goal of the opioid research remains the rational design of more efficient therapeutic drugs targeting specific functions with minimal

deleterious side-effects.

I.2.3. The Opioid Receptor Functions in Human

I.2.3.1. Ionic Homeostasis

Opioids were shown to be involved in the regulation of ionic homeostasis under normoxic and hypoxic/ischemic conditions. The maintenance of ionic gradients is essential to the function of neurons and other cell types, such as keratinocytes. Opioids can for example interfere with ionic homeostasis, by influencing the Ca^{2+} ion flux in cells in two different ways. They can either induce an elevation of intracellular Ca^{2+} (Spencer et al., 1997; Thorlin et al., 1998; Wang et al., 1993) or lead to an inhibition of Ca^{2+} entry (Chao et al., 2007a; Komai and McDowell, 2007; Piros et al., 1996; Rola et al., 2008). The classical coupling of the receptors to $G_{i/o}$ subtype of G proteins leads to an inhibition of voltage-operated Ca^{2+} channels in the plasma membrane and adenylyl cyclase activity, and therefore prevents an elevation of Ca^{2+} in the cell. Nevertheless, elevation of intracellular Ca^{2+} due to mobilisation of Ca^{2+} from intracellular stores has been shown in different neuronal and other cell systems like SH-SY5Y human neuroblastoma (Connor and Henderson, 1996), NG108-15 mouse neuroglial (Jin et al., 1994; Okajima et al., 1993), neuro2a (Spencer et al., 1997), and CHO-DOR (Yeo et al., 2001) cells. This is often a result of the synergy of opioid receptor signalling with other Ca^{2+} release signalling pathways, mainly the G_q-coupled receptor-activated inositol phosphate (IP_3) pathway (Samways and Henderson, 2006). The direct cellular environment is therefore a deciding force for the opioid mediated effect on Ca^{2+} homeostasis.

Potassium (K^+) and Sodium (Na^+) ion homeostasis across the plasma membrane is also regulated by opioid signalling. The activation of inwardly rectifying K^+ channels by the classical $G_{i/o}$ signalling enhances the electrochemical gradient by increasing the intracellular concentration of K^+, inhibiting membrane depolarization and therefore neuronal excitation.

Additionally, the work from D. Chao *et al.* demonstrated how important this mechanism could be under hypoxia/ischemia conditions for example during a stroke, cardiac ischemia or hypoxic encephalopathy. The activation of DOR greatly attenuates hypoxia/ischemia induced increase in extracellular K^+ and the decrease in extracellular Na^+. DOR maintains the ionic homeostasis by regulating Na^+ and K^+ ionic flux during such severe environmental stress and therefore protects the neuronal cells from its deleterious effect (Chao et al., 2007b; Chao et al., 2012; Kang et al., 2009).

I.2.3.2. Opioid mediated impact on cell proliferation and differentiation

That opioids influence cell proliferation is accepted but depending on the cell system examined, the results can be different. For example, the activation of DOR but not MOR in CHO cells resulted in a potentiation of foetal calf serum- (FCS) or growth-factor-stimulated growth. Inability of MOR to potentiate CHO proliferation suggested a divergence in effector molecules regulated by MOR and DOR and a difference in their ability to activate the MAPK or similar protein kinase signalling pathways, which promote proliferation (Law et al., 1997). A.I. Persson *et al.* showed that β-endorphin and selective MOR and DOR agonists stimulate proliferation of isolated rat adult hippocampal progenitors (AHPs). Incubation with β-endorphin for 48 hours increased the number of AHPs found in mitosis, the total DNA content, and the expression of proliferating cell nuclear antigen (PCNA), and could be antagonized by naloxone. Proliferation was mediated through phosphorylation of ERK 1/2 and depended on phosphatidylinositol 3-kinase and both intra- and extracellular Ca^{2+} (Persson et al., 2003). A stimulation of proliferation by β-endorphin has as well been shown for human melanocytes (Kauser et al., 2003; Kauser et al., 2004). An inhibition of astrocyte proliferation by MOR signalling has been shown by M. Miyatake *et al.*, who reported that morphine and [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) inhibit EGF-stimulated ERK activation

and cell proliferation (Miyatake et al., 2009). In contrast, KOR activation resulted in activated ERK and proliferation via an EGF receptor transactivation-mediated mechanism (McLennan et al., 2008). At clinically relevant concentrations, morphine stimulated human umbilical arterial endothelial cells (HUAEC) proliferation in a MAPK dependent manner (Leo et al., 2009). Furthermore, it was shown that rat lymphocytes induce proliferation after exposure to non-peptidic synthetic opioids (Caballero-Hernandez et al., 2005). In contrast, multipotent neural stem cells from embryonic mouse brain were promoted to differentiate upon activation of DOR by the synthetic agonist SNC 80 (Narita et al., 2006).

The opioid mediated influence on cell proliferation and differentiation needs to be validated in additional cell systems and the molecular mechanisms need to be further analysed in order to understand the capability of opioids in these processes.

I.2.3.3. Analgesia

The reaction to acute traumas results in the release of endogenous opioids and an increase of opioid blood levels. This counteracts the deleterious stimulus and pain. In animal models it has been shown that muscle injury, fixed-pressure haemorrhagic shock, and LPS administration elevates the circulating levels of β-endorphin and that the MOR is the opioid receptor mediating this stress-induced analgesia (Molina, 2002). In human, clinical studies demonstrated an increase of β-endorphin levels after oral (Troullos et al., 1997), gynaecological, and abdominal surgeries (Kho et al., 1993; Ozarda et al., 2002). Opioids regulate the nociceptive information by blocking neurotransmitter release and signal transmission in nerve fibres and are therefore widely used in treatment of severe pain conditions. Using KO mouse models, the MOR was identified as central mediator of analgesia. In MOR KO mice morphine analgesia was abolished or strongly reduced in models for thermal (Kieffer and Gavériaux-Ruff, 2002; Sora et al., 1997), mechanical (Fuchs et al.,

1999), chemical (Sora et al., 1999), and inflammatory (Qiu et al., 2000) pain (Gavériaux-Ruff and Kieffer, 2002). Heat pain behaviour is produced by the activity of the TRPV1 positive nociceptors and can be blocked by MOR selective agonists. In contrast, mechanical pain is generated by the activity of different populations of neurons (myelinated and unmyelinated nonpeptidergic nociceptors) and can selectively be blocked by DOR agonists (Scherrer et al., 2009). In DOR KO mice, DOR agonist mediated analgesia was either abolished, reduced or unchanged, depending on the nociceptive assay and route of administration applied (Zhu et al., 1999). KOR receptors are involved in the regulation of chemical visceral pain (Simonin et al., 1998). The distinct opioid receptors are expressed in different subsets of peripheral nerves and facilitate the discriminative regulation of different pain stimuli. Additionally, it was reported that DOR agonists enhance the analgesic potency and efficacy of MOR agonists (Ananthan, 2006) and so the interaction of the receptors adds another facet of pain modulation by opioid receptors.

An important molecule to mediate the downstream effects of opioid induced analgesia is β-arrestin. Several studies using β-arrestin KO mice confirmed the involvement in opioid-receptor mediated analgesia and tolerance/dependence (Bohn et al., 1999; Bohn et al., 2000; Bohn et al., 2002; Bohn et al., 2003).

I.2.3.4. Addiction

The improper use of opioids can lead to a drug addiction. The drugs directly or indirectly affect various transmitter systems, notably dopaminergic and glutamatergic neurons (Cunha-Oliveira et al., 2008; Lalumiere and Kalivas, 2008), which results in neuronal dysfunction and neurotoxicity. The behavioural pattern of addiction includes compulsive drug-seeking, persistent abuse of substances despite the often irreparable consequences for physical health and social exclusion, and the high probability of relapse even after a prolonged drug-free

period (Ivanov et al., 2006). The activation of endogenous opioid peptides, in particular the MOR and KOR agonists, has been attributed to be relevant in the neurochemical mechanisms of opioid reward, dependence, and vulnerability to addiction. Opioid abuse leads to opioid tolerance in the nervous system. Receptor tolerance and adaptation involve complex mechanisms of receptor regulation, including desensitisation and internalisation. The upregualtion of cAMP/protein kinase A (PKA) and cAMP response element-binding (CREB) as well as MAPK signalling in opioid-sensitive neurons are involved in the processes of tolerance and withdrawal. Further, these signalling processes modify the gene expression profile of the affected neurons, possibly changing the synaptical plasticity during the cycles of intoxication and withdrawal and might be responsible for long-lasting alterations (Christie, 2008; Przewlocki, 2004). Also, opioid addiction may negatively influence immune function, so that it predisposes an individual to opportunistic infections, such as hepatitis, bacterial pneumonia, tuberculosis, abscesses, central nervous system infections, endocarditis, and AIDS (Feng et al., 2012; Korolenko et al., 2007; Ordaz-Sanchez et al., 2003; Quaglio et al., 2003).

I.2.3.5. Immune function

Acute and chronic opioid administration is known to have inhibitory effects on the humoral and cellular immune response. Opioids act thereby like cytokines directly on the immune cells, but their actions are complicated and often indirect. The presence of DOR, MOR, KOR, and non-classical opioid-like receptors on cells of the immune system has been shown pharmacologically. *In vitro*, the phagocytic activity of non-elicited macrophages is inhibited by agonists of DOR, MOR, and KOR and this can be reversed with the appropriate selective antagonist (Szabo et al., 1993). Opioid signalling suppresses multiple components of the immune defence response, including the activity of natural killer cells (Carr et al., 1993),

neutrophil complement and immunoglobulin receptor expression (Menzebach et al., 2003; Welters et al., 2000), chemokine-induced chemotaxis (Grimm et al., 1998), and phagocytosis (Welters et al., 2000). Studies in mice models reported the decrease of natural killer cell cytotoxicity and interferon-gamma mRNA levels after acute morphine administration accompanied by an increase of the mortality rate of mice with herpes simplex virus infections (Jamali et al., 2007; Jamali et al., 2012). DOR agonist DPDPE triggers monocyte adhesion and the agonist SNC 80 stimulated rat thymic and human leucocyte chemotaxis. Furthermore, it was suggested that KOR activation induces an anti-inflammatory response through the downregulation of chemokine, cytokine and chemokine receptor expression, while MOR promotes a proinflammatory response (Finley et al., 2008). The overall response *in vivo* is likely to be result of the central nervous system and hypothalamic-pituitary-adrenal axis, which illustrates the complexity of the opioid mediated modification of the immune function (McCarthy et al., 2001).

I.2.3.6. Additional opioid receptor functions

In hibernating animals the circulating levels of opioids increase dramatically, which is considered as a “hibernation induction trigger” (Bruce et al., 1987; Bruce et al., 1996; Cui et al., 1996; Feng et al., 2012; Nurnberger et al., 1991; Ye and Cai, 1995). During hibernation the energy stores of the animals are depleted and intracellular acidosis and hypoxia can be observed similar to ischemic conditions. The activity of DOR could thereby have a protective role for the peripheral and central nervous system to prevent stress induced cell damage as well as to mediate analgesia and respiratory depression during the hibernation state (Borlongan et al., 2004; Borlongan et al., 2009).

Respiratory depression is one of the negative side effects of opioid receptor activation. MOR stimulation thereby strongly affects the rate and depth of breathing and the respiratory responsiveness towards CO_2 and hypoxia. It increases the upper airway resistance, and reduces pulmonary compliance, which can result in a complete apnoea. DOR has less pronounced effects and KOR activation has no effect on respiration. The regulation of respiratory functions is mediated by direct action on respiratory control centres in the brain (Feng et al., 2012; Mutolo et al., 2007; Pattinson, 2008).

Opioid signalling is as well involved in emotional response. DOR receptors act as natural inhibitors of stress and anxiety. Rodent models showed that DOR can produce antidepressant and anxiolytic effects (Saitoh et al., 2005). An increased expression of brain-derived neurotrophic factor (BDNF) mRNA was reported after DOR agonist administration. Also, central acting KOR was shown to upregulate BDNF mRNA, an effect seen with most clinically used anti-depressants. This indicates an antidepressant-like effect for both DOR and KOR signalling (Zhang et al., 2006a; Zhang et al., 2007).

In accord, opioid agonists showed stimulatory effects on feeding in rodents, while antagonists inhibit food intake and weight gain in ob/ob mice (Marczak et al., 2009). The stimulation of MOR preferentially increases the intake of high fat diet. KOR KO mice fed with high-energy diet gain less body weight and fat mass compared to the wild type mice and show an attenuation of triglyceride synthesis in the liver (Czyzyk et al., 2010).

The opioid receptor system is present in the heart and in sympathetic nerve fibres and ganglion cells (Steele et al., 1996; Weihe et al., 1983). Endogenous opioid ligands like β-endorphin (Forman et al., 1989), enkephalins (Barron et al., 1992; Lang et al., 1983; Weihe et

al., 1983; Weihe et al., 1985), and dynorphins (Spampinato and Goldstein, 1983; Weihe et al., 1985) are produced, stored, and released from myocardial cells. Upon stress, such as ischemia, the myocardial opioid levels are increased (Eliasson et al., 1998; Peart et al., 2005). Both DOR and KOR have been shown to mediate cardioprotection. KOR mediates the ameliorating effects of myocardial ischemia preconditioning on infarct and arrhythmia, whereas DOR mediates the effects only on infarct (Wang et al., 2001). Thereby, the opioid mediated regulation of PKC, NO synthesis, and ATP-sensitive K^+ channels participates in the cardioprotective mechanisms (Lishmanov et al., 2007; Maslov et al., 2009).

I.2.4. The Delta Opioid Receptor

The existence of multiple DOR subtypes was proposed since the early 1970s. The molecular basis of its pharmacological diversity has therefore long remained a matter of debate (Zaki et al., 1996). In 1992, gene cloning led to the isolation of a single mouse *oprd1* gene, encoding for only one DOR protein (Evans et al., 1992; Kieffer et al., 1992). The human homolog was identified two years later (Knapp et al., 1994). The coding region of *oprd1* consists of three exons separated by two introns and generates only one mRNA variant (NM_000911.3). Nevertheless, transcript variants have been described in conjunction with malignancy, where due to atypical mRNA processing in human melanoma and neuroblastoma cells, a receptor lacking the third cytoplasmic loop is generated. A possible consequence might be an effect on signal-response coupling and that could be involved in tumour genesis (Mayer et al., 2000).

The primary amino acid structure allocates the receptor into the family of rhodopsin-like G protein-coupled receptors (GPCR). The three-dimensional (3D) structure, as seen in Figure 8, is characterised by seven hydrophobic transmembrane domains (TM) connected by alternating intracellular (IL) and extracellular (EL) loops.

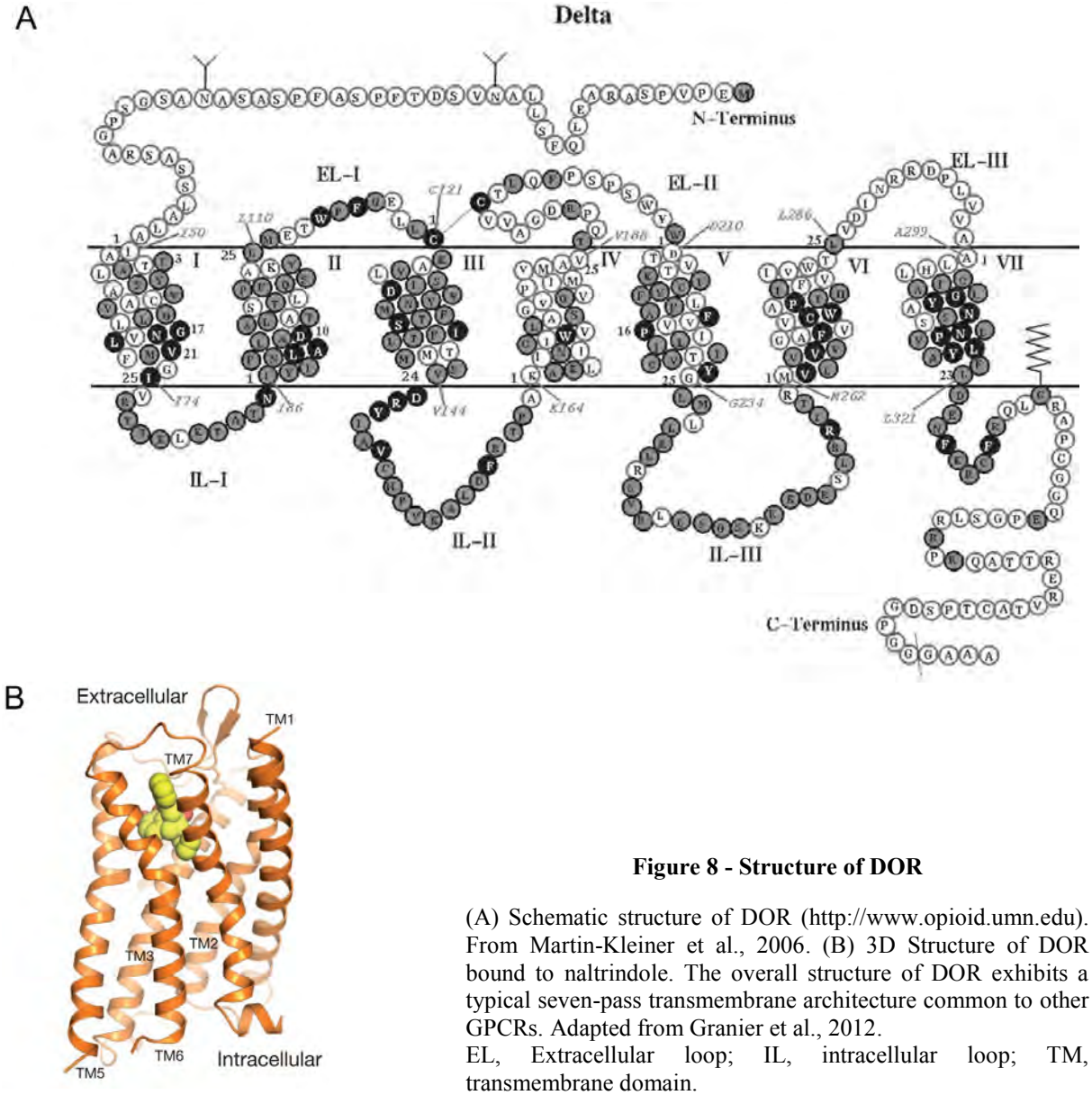


Figure 8 - Structure of DOR

(A) Schematic structure of DOR (http://www.opioid.umn.edu). From Martin-Kleiner et al., 2006. (B) 3D Structure of DOR bound to naltrindole. The overall structure of DOR exhibits a typical seven-pass transmembrane architecture common to other GPCRs. Adapted from Granier et al., 2012.
EL, Extracellular loop; IL, intracellular loop; TM, transmembrane domain.

The amino (N) terminal part is located at the extracellular side, whereas the carboxyl (C) tail is located in the cytoplasm. Asparagine residues in the N-terminal part are glycosylated and mutations of these residues decrease the steady-state level of the receptor at the cell surface (Markkanen and Petäjä-Repo, 2008). The regions spanning TM domains 5-7 are responsible for DOR-selective binding of ligands (Chaturvedi et al., 2001). The first and the third IL and TM domain 5, together with the C-terminal end of the receptor, are responsible for the specific G protein-coupled intracellular signalling (Figure 8). In contrast to MOR, DOR can

efficiently stimulate phospholipase C (PLC) by interaction with Gα16 protein (Chan et al., 2003). DOR is a highly dynamic ligand recognition protein. It has multiple active conformations, reflecting different binding modes in the ligand binding pocket (Befort et al., 1996). The ligand-receptor-complex determines a multitude of physiological cellular responses. Receptor activation and subsequent regulations are strongly drug dependent and show that the receptor by itself does not determine the final response (Kieffer and Evans, 2009).

Previously, the expression of the DOR in skin has been demonstrated in cultured skin cells and animal models only on mRNA level by Bigliardi-Qi *et al.* and others (Salemi et al., 2005; Weihe et al., 1983; Zagon et al., 1996). Our laboratory now confirmed the presence of DOR mRNA in human skin by *in situ* hybridization using paraffin-embedded normal human corporal skin sections. The positive chromogenic *in situ* hybridization (CISH) signal was found in the epidermis and displays a gradually expression of DOR mRNA. Most prominently, the signal was detected in the upper part of stratum granulosum and to a lesser extends in stratum spinosum of human epidermis (Mei Bigliardi-Qi, unpublished data). However, it was evident that not all keratinocytes express the same amount of DOR, so that some areas in the granular layer showed a more intense staining pattern than other areas.

I.2.5. The Delta Opioid Receptor Mediated Signalling

As mentioned previously, DOR-deficient mice show alterations in skin differentiation, a delay in wound healing (Bigliardi-Qi et al., 2006), and exhibit enhanced inflammatory pain (Gavériaux-Ruff et al., 2008). Studies suggested similar signalling pathways induced by opioids in neuronal cells and in non-neuronal systems (Lasukova et al., 2009; Wang et al., 2003). Therefore, second messengers like cyclic adenosine monophosphate (cAMP), intracellular Ca^{2+}, and second messenger-induced kinases are likely to be involved in opioid

mediated skin cell regulation.

Upon activation of DOR the physiological response is predominantly mediated through the $G_{i/o}$ subtype of G proteins. Agonist binding modifies the helical packing of the receptor, and a rearrangement in the positioning of TM domains 3, 6, and 7 has been proposed to drive the transition between inactive and active conformations of the receptor (Decaillot et al., 2003). Modification of the intracellular structure leads to its interaction with the heterotrimeric G protein (composed of α–, β– and γ–subunit). The GPCR acts as a guanine nucleotide exchange factor, catalysing the exchange of guanosine-diphosphate (GDP) for guanosine-triphosphate (GTP) on Gα and induces the dissociation of Gα and Gβ/γ subunits from each other. Subsequently, intracellular effectors and pathways are modulated (Ritter and Hall, 2009), including inhibition of some types of adenylyl cyclases and Ca^{2+} channels, and stimulation of PLC and K^+ channels (Law et al., 2000). In addition, activation of DOR has been linked to the regulation of MAPKs ERK 1/2 and p38, as well as to signalling cascades stimulated by Akt, Cdc 42, Rac, receptor tyrosine kinases (RTKs), signal transducer and activator of transcription 3 (STAT3), and Src (Archer-Lahlou et al., 2009; Eisinger and Ammer, 2008a; Eisinger and Ammer, 2008b; Kam et al., 2003; Lo and Wong, 2004; Shahabi et al., 2006). The inhibition of adenylyl cyclases directly downregulates the cAMP level and therefore influences PKA activity and downstream factors, like Rap1. This can result in an interaction of Rap1 and B-Raf, stimulating the activation of ERK (Dugan et al., 1999). At the same time, the coupling of the Gβ/γ subunit with PLC initiates phosphoinositide hydrolysis and IP_3 receptor activation, which in turn leads to a release of Ca^{2+} from intracellular stores and a subsequent stimulation of Ca^{2+}/calmodulin-dependent kinase. Following, Rap1 is activated and stimulates integrin function (Belcheva and Coscia, 2002; Kinashi, 2005; Sahyoun et al., 1991). This in turn transactivates, via a PKC dependent mechanism, RTKs like the EGFR, initiating the ERK signalling pathway (Belcheva et al., 2001; Eisinger and Ammer,

2008a; Eisinger and Ammer, 2008b; Wang et al., 2009). These mechanisms are simplified illustrated in Figure 9A.

The initial step terminating G protein-mediated signalling is phosphorylation of Serine 363, Threonine 361 and Threonine 358 through association of GPCR kinases (GRKs) with agonist-bound receptor (Claing et al., 2002; Guo et al., 2000). The recruitment of β-arrestin 1 and 2 to the C-terminal tail and third intracellular loop of phosphorylated receptors is then facilitated (Cen et al., 2001), which results in uncoupling from the G protein and desensitization. β-arrestins interact with clathrin and the clathrin adaptor complex AP2 to drive receptor internalization into endosomes. Once internalized, receptors are targeted to specialized intracellular compartments, where they are dephosphorylated and recycled back to the plasma membrane or processed towards the lysosomal degradation pathway (Claing et al., 2002)

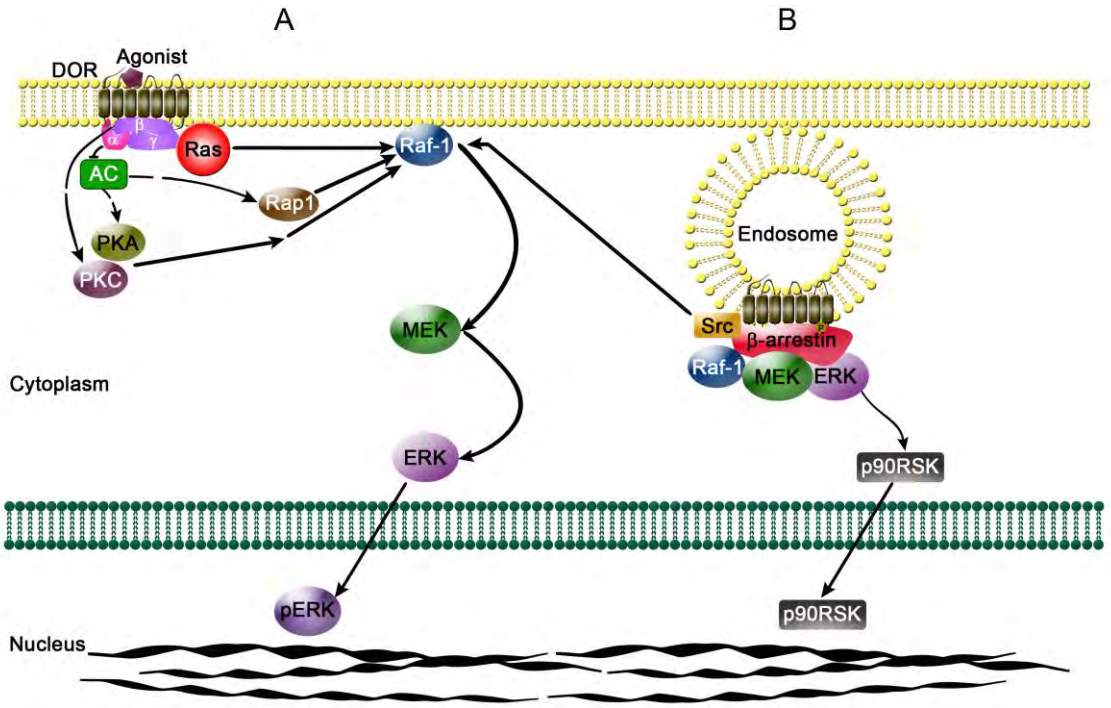


Figure 9 - Crosstalk between opioid receptor and ERK cascade

Crosstalk between opioid receptor and ERK cascade through G protein and β-arrestin pathway. Irrespective of the specificity of different cell lines (for example, different cells may have different kinase isoforms), opioid receptor, can crosstalk with ERK 1/2 through two pathways, the (A) G protein dependent and the (B) receptor internalization induced by β-arrestin dependent. In the former pathway, ERK 1/2 activation may be variable because of the cell type specific different responses to PKA activation. Adapted from (Bian et al., 2012).

Additionally, recruitment of arrestins to activated receptors can also lead to the initiation of arrestin-mediated signalling pathways, for example the MAPK ERK 1/2 signalling cascade. β-arrestin 2 acts as a scaffold that binds all the component kinases of the module, which activates ERK 1 and ERK 2 (Raf, MEK, ERK) (Figure 9B) (Lefkowitz and Shenoy, 2005).
Signalling studies showed that receptor activation and subsequent regulation like phosphorylation and internalisation are strongly agonist-dependent (Marie et al., 2008; Okura et al., 2003), leading to a high heterogeneity of the signalling as a consequence of the divers cellular environment of the receptor. Many G proteins exist and their expression differs between cell types. The variable combination of G protein subunits and the high number of possible G protein-associated signalling pathways has expanded dramatically (Marinissen and Gutkind, 2001). Each cell has highly interconnected cytoplasmatic signalling routes that might lead to temporally distinct patterns. Altogether, this shows that DOR mediated signalling is very complex and that the receptors are highly dynamic proteins. The final cellular response to DOR activation will most probably be a result after the integration of a complex network of biochemical responses.

I.2.6. The Receptor Oligomerisation

DOR is able to form both homo- and heterodimers. Hetero-oligomers with MOR show altered pharmacological behaviour to highly selective agonists for each receptor as well as higher affinity and efficiency of interaction with G_z over G_i (Fan et al., 2005; George et al., 2000). Heterodimerisation of MOR and DOR only occurs at the cell surface and requires receptor-G protein interaction (Law et al., 2005). Furthermore, it leads to a constitutive recruitment of β-arrestin 2 to the receptor complex, influencing the dynamics of ERK 1/2 signalling (Rozenfeld and Devi, 2007).
In addition, DOR can also form heterodimers with other GPCRs. Coexpression with α_{2A}-

adrenergic receptors promotes DOR-mediated neurite outgrowth, suggesting that the presence of inactive α_{2A}-adrenergic receptors can enhance DOR-mediated signalling (Rios et al., 2004). On the other side, interaction with the sensory neuron-specific receptor-4 (SNSR-4) inhibits the signalling of DOR (Breit et al., 2006). Finally, oligomerisation with different chemokine receptors like CCR5 (Suzuki et al., 2002) could be demonstrated. The CXCR2 antagonist SB225002 enhances the function of DOR agonists only in the presence of CXCR2 (Parenty et al., 2008) and interaction of CXCR4 appears to lock both receptors in an inactive conformation (Pello et al., 2008).

Formation of homo- or heterodimers may result in a new receptor conformation especially in the intracellular domains of the receptor. The consequence is that regulatory patterns of DOR trafficking and signalling become more subtle, including receptor localization, internalization, ligand-binding properties, and downstream signalling (Bian et al., 2012). Oligomerisation adds a level of complexity to DOR function and is a mechanism by which the products of a limited number of receptor genes may give rise to a greater diversity of signalling units with unique properties. Numerous questions surrounding opioid receptor homo-oligomerisation and hetero-oligomerisation remain, and their answers hold the potential to diversify the understanding of opioid system biology.

I.3. THE ERK MAP KINASE SIGNALLING PATHWAY

The family of mitogen-activated protein kinases (MAPK) signalling pathways consist of four distinct cascades named after their MAPK component: the extracellular signal-regulated kinase 1 and 2 (ERK 1/2); c-Jun N-terminal kinase 1 to 3 (JNK 1-3); p38 MAPK α, β, γ, and δ (p38 α-δ); and ERK 5. The MAPK signalling cascades are evolutionary conserved, intracellular signal transduction pathways activated by various extracellular stimuli and intracellular processes regulating cellular functions such as growth, proliferation, differentiation, mobility, survival, and apoptosis. Each of the cascades is composed of three to five levels of MAP4K, MAP3K, MAPKK, MAPK, and MAPKAPK. The MAP3K, MAPKK, and MAPK constitute the core cascade.

The ERK signalling pathway was the first one of the MAPK cascades to be defined (Ray and Sturgill, 1988; Sturgill et al., 1988). This pathway is commonly initiated at membrane receptors (RTKs; GPCRs; ion channels, and others). Upon activation through recruitment of adaptor proteins and exchange factors the activation of a small GTP-binding protein (e.g. Ras, Rap) is induced. The signal is then transmitted to the MAP3K tier of the cascade (mainly Raf1 and B-Raf) and further to the MAPKKs MAPK/ERK kinase 1 (MEK 1) and MEK 2. These two kinases are composed of a large regulatory N-terminal domain containing a nuclear export signal (NES), followed by a catalytic kinase domain and a shorter C-terminal region. MEK 1/2 are activated through serine phosphorylation and then further phosphorylate and activate their only known substrate, native ERK 1 and 2 at Threonine 202 and Tyrosine 204. With that step the cascade has reached the MAPK level and will then spread to numerous target molecules including the MAPKAPK components (90 kDa ribosomal S6 kinases, RSKs; MAPK-interacting kinases, MNKs; mitogen- and stress-activated kinases, MSKs) and many more substrates. Most of these substrates are phosphorylated in the nucleus by ERK molecules that translocate upon stimulation. In addition, ERK interacts with substrates of

cytoskeletal elements (Klemke et al., 1997) and cellular organelles such as mitochondria (Alonso et al., 2004; Poderoso et al., 2008; Tamura et al., 2004), and the Golgi apparatus (Shaul and Seger, 2006) (Figure 10).

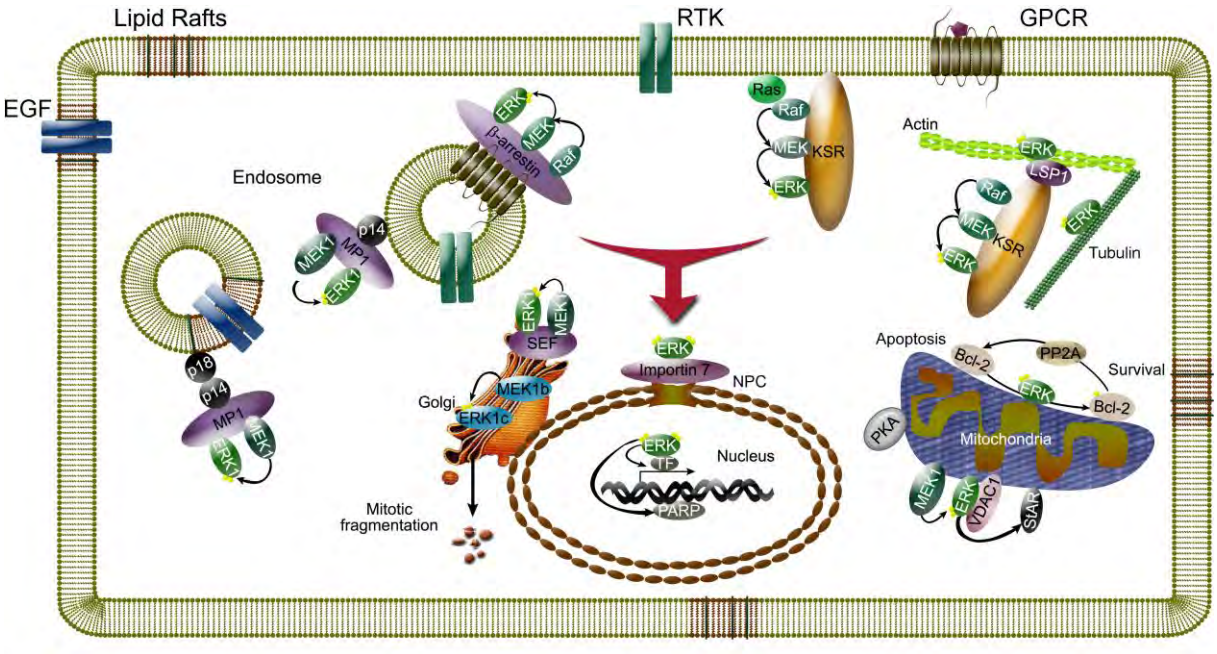


Figure 10 - ERK distribution within the various compartments of the cell

The activation of the ERK 1/2 cascade results in a significant translocation of ERK molecules into the nucleus, which is mediated by importin 7. In addition, due to interaction with specific scaffold proteins ERK is located to cellular organelles such as Golgi apparatus, endosomes, mitochondria, cell membrane, and cytoskeletal elements. In each of these organelles, ERK 1/2 can either regulate intrinsic activities or direct ERK signals to nearby cytoplasmic substrates. NPC, nuclear pore complex; RTK, receptor tyrosine kinase; Adapted from (Wortzel and Seger, 2011).

With over 200 possible targets the signal specificity has to be fine tuned in order to induce distinct physiological processes. The currently known mechanisms determining the signal specificity can be categorized into five types:

1) Duration and strength of the signal

This model is mainly based on the observed EGF and NGF mediated signalling in PC12 cells. Both factors induce strong ERK 1/2 activation but result in distinct cellular responses. EGF causes a transient activation peaking at 15 minutes and decreasing back to basal level after 40 min, which enhances proliferation of the cells. On the other side NGF induces a sustained activation resulting in neuronal differentiation of these cells. Dependent on the signal length

the activity of regulatory proteins could be altered and different cellular processes might be induced by immediate early genes (Mansour et al., 1994; Nguyen et al., 1993; Traverse et al., 1994).

2) Interaction with scaffold proteins

Scaffolding proteins facilitate the interaction of components of different levels of the signalling cascade by forming a multi-protein complex and bringing them into close proximity. They can protect components from inactivating phosphatases (Perlson et al., 2005), recruit substrates or direct the signal to specific upstream or downstream targets. Like that, scaffolds influence the kinetics of induction of the signalling cascade, modify the strength and duration of the ERK activation, allow better interaction between signalling components, regulate the localization of the cascade, and modify crosstalk with other signalling pathways (Shaul and Seger, 2007; Wortzel and Seger, 2011). The most important scaffold protein during ERK signalling upon GPCR stimulation is β-arrestin. It interacts with ERK, as well as MEK 1 and Raf1 in the membrane by irreversible binding and prevents ERK from translocation to the nucleus, resulting in preferential phosphorylation of cytoplasmic substrates (Luttrell et al., 2001; Pierce et al., 2001; Tohgo et al., 2002). During GPCR mediated ERK activation most ERK molecules will still translocate to the nucleus, suggesting that β-arrestin interacts only with a small portion of ERK molecules and other cascades for ERK activation are acting in parallel (Chuderland and Seger, 2005).

3) Compartmentalization of the signalling components in cell organelles or cellular regions

The localization of components of the ERK signalling cascade depends largely on their interaction with regulatory proteins. In resting cells molecules are localized to the cytoplasm mainly because of interaction with anchor and the above mentioned scaffold proteins. Upon stimulation, Rafs interact with the activated Ras and are therefore recruited to the plasma

membrane, and to membranes of other compartments. Most of the proteins of the following MAPK signalling levels, MEK 1/2, ERK 1/2, and RSKs are released from their cytoplasmic anchor allowing translocation into the nucleus or other organelles. This translocation of activated ERK enables regulation of transcription in the nucleus or mitochondria as well as regulation of mitotic Golgi fragmentation (Shaul and Seger, 2006; Wortzel and Seger, 2011). Localization to the outer surface of organelles or cytoskeleton molecules directs the phosphorylation of specialized substrates without significant nuclear translocation of ERK resulting in distinct cellular responses under varying conditions. Therefore, the signalling in one cellular compartment may have a different outcome from a similar signalling event in another localisation.

4) Cross-talk and interplay with other signalling pathways

The ERK signalling cascade is a central signal transduction pathway but other signalling pathways active at the same time might modulate the outcome. Phosphorylation and dephosphorylation processes by enzymes that are not part of the ERK cascade can affect the kinetics, strength, and the localization of the signal. In addition many of the signals from other pathways are likely to converge at downstream targets of the ERK cascade, such as transcription factors and inhibitors (Raman and Cobb, 2003).

5) Multiple components and isoforms in each level of the cascade

On the level of the MAP3Ks different proteins, including Rafs, MEKK1, Cot, and Mos, may be involved in the activation of the cascade under different conditions. The MAPKK level is less diverse but it has been demonstrated that MEK 1 and MEK 2 have distinct functions during cell cycle progression (Liu et al., 2004) and an alternative splice form MEK1b is specifically active during regulation of mitotic Golgi fragmentation (Shaul et al., 2009). Further downstream, the MAPKs ERK 1 and ERK 2 are expressed in essentially all cells and tissues, whereby ERK 2 is the predominant isoform. Knock-out mice models showed that

ERK 2 can substitute most functions of ERK 1 but ERK 2 knock-out mice die early in development indicating that ERK 1 is not able to compensate ERK 2 (Shaul and Seger, 2007). Additionally, ERK 1 has an alternative splice form termed ERK 1c that possesses unique functions that are not observed with any other ERK (Aebersold et al., 2004; Yung et al., 2000).

About half of the identified ERK 1/2 substrates are nuclear proteins, of which the main group consisting of transcription factors, mostly regulating immediate early genes. For example, the nuclear ETS domain-containing Elk1 transcription factor induces the expression c-Fos, which is an important immediate early gene involved in proper progression of proliferation and differentiation (Eferl and Wagner, 2003; Treisman, 1996). Other immediate early genes such as c-Myc and Fra1 (Murphy et al., 2004) are activated as well. Further, immediate or late genes like members of the nuclear receptor family, including the oestrogen receptor (Kato et al., 1995), PPARγ (Hu et al., 1996), retinoic X receptor alpha (RXRα) (Solomon et al., 2001), and the glucocorticoid receptor (GR) (Krstic et al., 1997) have to be mentioned as ERK signalling target genes as well, although mostly suppression of transcription has been reported for these genes. One of the main transcription repressors regulated by ERK 1/2 is Ets2 repressor factor (Erf1), which suppresses transcription in many resting cells and is exported from the nucleus after phosphorylation by ERK which thereby alleviates its suppression of transcription (Plotnikov et al., 2011). For keratinocytes Gazel *et al.* identified Elk1, Elk4, and forkhead box D3 (FOXD3) as the principle targets of the ERK pathway while sharing the signalling targets forkhead box D1 (FOXD1), aryl hydrocarbon receptor nuclear translocator (ARNT), and upstream stimulatory factor 1 (USF1) with the p38 pathway (Gazel et al., 2008). Beside its well-known regulation of transcription factors, it was shown that ERK 2 can directly regulate gene expression by activity-independent binding to promoter regions of

certain genes (Hu et al., 2009).

Another important effect, even if ERK has only an accessory role (Plotnikov et al., 2011), is the influence of the ERK cascade on chromatin remodelling by regulating for example histone deacetylase 4 (HDAC4) (Zhou et al., 2000), the chromatin-arranging proteins histone H3 and HMG-14 (Brami-Cherrier et al., 2009; Soloaga et al., 2003), or the PolyADP-ribose polymerase 1 (PARP1) (Cohen-Armon et al., 2007).

Despite many other signalling pathways the ERK 1/2 MAPK pathway has been demonstrated to be active in epidermis and cultured keratinocytes *in vitro*. It plays a key role in regulating keratinocyte proliferation, differentiation, and survival (Eckert et al., 2002; Johansen et al., 2003). ERKs are activated in Psoriasis and are involved in epidermal hyperproliferation and skin inflammation (Hobbs et al., 2004; Takahashi et al., 2002) and can regulate the migration of epithelial sheets during wound healing (Matsubayashi et al., 2004) as well as the response to mechanical stretching in the epidermis (Yano et al., 2004). The study by Gazel *et al.* identified the ERK pathway as positive regulator of the RNA splicing machinery and nuclear envelope components, while it suppresses the steroid synthesis and mitochondrial energy production in keratinocytes. Along with the p38 pathway it is involved in promotion of epidermal differentiation and the repression of genes that antagonize wound repair, inflammation, and immune response (Gazel et al., 2008).

I.4. THE TRANSCRIPTION FACTOR POU2F3

POU domain transcription factors belong to a family of transcription factors with a homeodomain. Most of the POU proteins bind to a specific octamer DNA motif, 5'-ATGCAAAT-3' regulating promoter-dependent and cell type-specific pathways during development and cellular differentiation (Ryan and Rosenfeld, 1997). POU domain proteins contain a bipartite DNA-binding domain consisting of a POU-specific segment, which is characterized by four α-helices surrounding a hydrophobic core and a POU homeodomain consisting of a helix-turn-helix structure with three α-helices. A flexible linker that enables the protein to adopt various monomer configurations on DNA divides the two domains. The regions outside the POU domain are divergent and contain the transactivation domains. A region with significant similarity to the canonical sterile α motif/pointed domain was suggested to function essentially as a protein-protein interaction interface (Cabral et al., 2003) (Figure 11).

POU interacting proteins can be classified into four classes: DNA-binding transcriptional activators, coregulators, basal factors, and replication factors (Andersen and Rosenfeld, 2001; Herr and Cleary, 1995). Despite their DNA-binding ability POU domain proteins have also been shown to regulate transcription through protein-protein contacts, in a DNA binding-independent manner (Sugihara et al., 2001). POU factors can be modified by phosphorylation leading to modification of binding to the DNA (Segil et al., 1991). The expression of several POU domain genes has been described in epidermis, namely *oct-1*, *oct-6*, and *pou2f3*.

POU2F3, also known as Oct-11, Epoc-1 or Skn-1, is highly homologous to the POU domain of Oct-1 (POU2F1) and Oct-2 (POU2F2) and therefore belongs to the class-II POU domain proteins as classified by He *et al.* (He et al., 1989) and Spaniol *et al.* (Spaniol et al., 1996). It was first isolated in 1990 in a screen for novel POU domain genes expressed during spermatogenesis (Goldsborough et al., 1990) and three years later characterized in the mouse

by Goldsborough *et al.* (Goldsborough et al., 1993). In the same year Andersen *et al.* (Andersen et al., 1993) and Yukawa *et al.* (Yukawa et al., 1993) independently cloned the cDNA of POU2F3 and characterized its expression pattern in rat and mouse. Analysis of the expression during mouse development by Andersen *et al.* revealed a biphasic pattern of expression with a signal at embryonic day 7.5 that disappeared between day 9.5 and 12.5 and reappeared on day 14.5. *In situ* hybridisation of rat embryos gave an intense signal only at embryonic day 17 in epidermal structures throughout the embryo, with no specific detection in any other region. In adult rat skin POU2F3 is expressed in suprabasal cells of the epidermis and in cortex cells of the hair follicle. In contrast to these results Yukawa *et al.* showed the expression in basal epidermal cells and in some cellular components of the hair follicle but not in suprabasal layers of the epidermis. Additionally, they found not only POU2F3 expression in mouse skin but also in thymus, stomach, and testis. Furthermore, Faus *et al.* (Faus et al., 1994) detected as well POU2F3 RNA hybridisation in the basal rather than suprabasal epidermal layer of human neonatal skin, while in 2003, a fourth group investigating human POU2F3 function, detected mRNA presence only in the suprabasal epidermal layers (Cabral et al., 2003). The expression pattern remained controversial although immunohistochemical analysis of mouse tissue showed the expression of the protein throughout all epidermal layers with the highest expression in the suprabasal layers (Andersen et al., 1997b) so that Andersen *et al.* suggested in their final conclusion, that the expression is not restricted to a specific epidermal layer but clearly overlaps with the expression of specific markers of differentiating keratinocytes.

Alternatively spliced transcript variants encoding multiple isoforms have been observed for the *pou2f3* gene. Variant 2, Skn-1i differs in the 5' UTR, lacking a portion of the 5' coding region, and uses an alternate start codon, compared to variant 1, Skn-1a. The encoded isoform 2 is longer and has a distinct N-terminus.

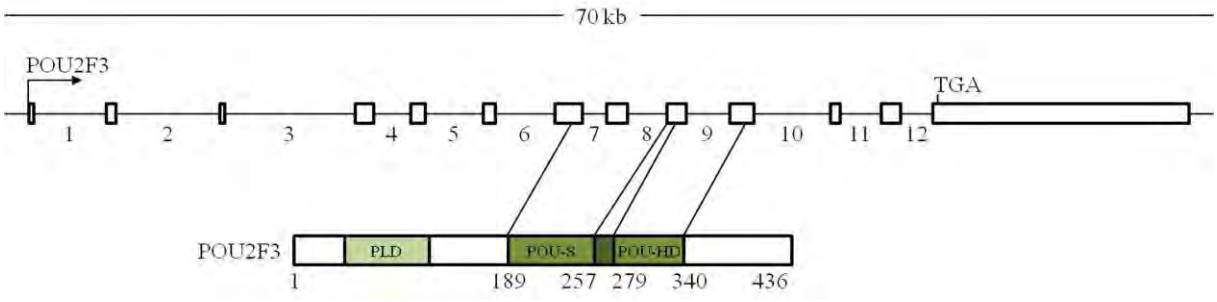


Figure 11 - Genomic organization of the human POU2F3 gene

The transcription start site for POU2F3 isoform 1 is indicated by an arrow. Exons are represented as boxes, introns are numbered, and the stop codon is labelled as TGA. The POU-specific domain (POU-S), the POU-homeodomain (POU-HD), and the pointed-like domain (PLD) and the corresponding amino acid residues are represented.

Skn-1a is predicted to encode a 54 kD protein that binds the promoter region and regulates the expression of KRT 10 (Andersen et al., 1993), SPRR2A (Fischer et al., 1996), IVL (Welter et al., 1996), human papillomavirus (HPV) 1A (Andersen et al., 1997a), HPV 16, and HPV 18 (Yukawa et al., 1996). The divergent N-terminus of Skn-1i modifies the transactivation ability of POU2F3. Its N-terminus might alternate the conformation, possibly through the formation of intramolecular complexes and blockage of the function of the helices involved in protein-DNA interaction. Skn-1i transcript and protein are expressed at much lower level than Skn-1a (Andersen et al., 1997a). It has been speculated that the isoforms have different activating and inhibiting functions in keratinocyte differentiation and proliferation. Another splice variant induced specifically in normal human epidermal keratinocytes by high Ca^{2+}, a trigger for keratinocyte differentiation, supports this hypothesis (Nakajima et al., 2008).

A correlation of POU2F3 activity, increased KRT 10 expression, and PKC activation was seen and a reporter gene assay indicated direct binding of POU2F3 on the KRT 10 promoter (Andersen et al., 1993; Andersen et al., 1997b). A second differentiation related gene, SPRR2A a member of the small proline-rich family of cornified envelope precursor proteins, was shown to be upregulated by direct binding of POU2F3 to the regulatory promoter sequence (Fischer et al., 1996). On the other side, the keratinocyte differentiation related

genes encoding IVL and profilaggrin are downregulated by POU2F3. Competition experiments with ΔNp63, an essential factor for epidermal morphogenesis, showed antagonizing effects of POU2F3 and ΔNp63 on KRT 14 and KRT 10 expression (Lena et al., 2010). The interaction with protein complexes like AP-1 (Jang et al., 2000; Welter et al., 1996), Ets (Kahle et al., 2005), Ese-1 (Cabral et al., 2003), and CBP/p300 (Sugihara et al., 2001) enables to target several epidermal genes at once and multiple mechanism of regulation. POU2F3 knock-out mice did not reveal a distinct phenotype. Mice did not exhibit any failure in epidermal terminal differentiation after analysing the morphology and the expression of KRT 14, KRT 1, KRT 10, and filaggrin (FLG). LOR expression was slightly changed with POU2F3 KO mice having more superficial expression of LOR and the layer of LOR positive cells appearing thinner, while the mRNA levels are unchanged. These results indicate that POU2F3 is not essential for terminal differentiation of epidermal keratinocytes and that there are compensatory mechanisms and molecules that orchestrate epidermal stratification (Andersen et al., 1997b). Another study by Hildesheim *et al.* (Hildesheim et al., 2001) concluded that POU2F3 primarily promotes keratinocyte proliferation and subsequently influences stratification by increasing the number of cells inducing differentiation genes and committing to terminal differentiation.

Nevertheless, the POU2F3 KO mouse model revealed faster wound closure of KO mice compared to wild type mice using meshed skin graft or simple incisional wounding techniques. POU2F3 is expressed at low level at the suprabasal cells of the migratory wound front. Therefore, Andersen *et al.* hypothesized that this downregulation facilitates the phenotype change of wound front keratinocytes to a KRT14 and SPR-1 positive post-mitotic activated cell phenotype (Andersen et al., 1997b). In POU2F3 KO mice this upregulation is enhanced and might explain the faster wound closure.

Additionally, POU2F3 is a candidate tumour suppressor protein. Aberrant promoter

methylation of this gene may play a role in cervical cancer (Abercrombie et al., 1960; Zhang et al., 2006b).

CHAPTER II

AIM OF THE THESIS

Determining the molecular mechanism of δ-opioid receptor (DOR) mediated influence on skin homeostasis and wound healing

The skin is our largest body organ and must bear a burden of constant environmental assaults while maintaining epidermal integrity and homeostasis. Tissue injury is one of the most severe disruptive incidents skin encounters and requires an orchestrated interaction between immune cells, keratinocytes, fibroblasts, and endothelial cells in order to restore the homeostasis. The local neuroendocrine system will thereby act to activate cellular messengers, to coordinate responses to the changing environment. The opioidergic system is part of this cutaneous communication network and could thereby influence cellular functions. Currently, there is a lack of knowledge how the opioid receptor signalling accomplishes these tasks.

Furthermore, local applications of opioids are used for treatment of inflammatory pain conditions such as burns, skin grafts, and chronic wounds. In order to tap the full potential as well as for safe and effective clinical applications of such treatments, we need to understand the molecular mechanisms through which they alter keratinocyte functions.

The present work is based on previous *in vivo* observations in DOR-deficient mice (Bigliardi-Qi *et al.*, 2006). These mice exhibit markedly increased expression of KRT 10, alongside an atrophic epidermis, suggesting to a role of the DOR during stratification and skin homeostasis. Accompanying a delay in re-epithelisation during the wound healing process, the wound margins showed an epidermal hypertrophy of activated, KRT 6 positive keratinocytes.

The present work was aiming to investigate the underlying mechanisms that could explain the aberrant epidermal phenotypes and delayed wound healing in mice lacking the DOR. The study describes the molecular consequences of DOR activation in keratinocytes and uncovers downstream effectors influencing the expression of keratinocyte differentiation related genes. Using standard *in vitro*

overexpression systems and keratinocyte differentiation models, as well as organotypic skin models, we addressed the role of DOR in gene regulation and its influence on epidermal morphogenesis.

CHAPTER III

RESULTS

III.1. The cell membrane localisation of the DOR in cultured primary, HaCaT, and N/TERT-1 keratinocytes is Calcium dependent

Although functionality of opioid receptors under standard keratinocyte culture conditions has been shown before (Bigliardi et al., 2002; Nissen et al., 1997), the expression pattern *in vivo* suggests a refinement of these conditions in order to perform suitable *in vitro* studies.

Under standard keratinocyte culture conditions in keratinocyte growth medium (K-SFM), supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF), at a basal Calcium (Ca^{2+}) concentration of 0.09 mM, cells (primary keratinocytes, HaCaT, N/TERT-1) are round and more separated from each other than in medium with a higher Ca^{2+} concentration of 1.2 mM. Colonies spread out more and cells adhere less tight to each other in low Ca^{2+}. The formation of Ca^{2+} dependent desmosomal cell-cell junctions reduces dramatically, in order to achieve culture conditions for fast expansion of keratinocytes, without induction of differentiation due to contact inhibition in tightly adherent colonies. The overexpression of a green fluorescent protein (GFP) tagged DOR in HaCaT and primary keratinocytes revealed distinct localisation of the receptor under these two different culture conditions (Figure 12). In high Ca^{2+}, DOR favours membrane localisation, whereas under low Ca^{2+}, the receptor is internalised and often accumulates at the perinuclear region (Figure 12). This effect is solely due to the Ca^{2+} concentration and not differentiation dependent. Although primary keratinocytes start to induce differentiation markers like KRT 10 under 1.2 mM Ca^{2+} culture conditions, however, HaCaT cells are not differentiating under these conditions, but show the same distribution pattern.

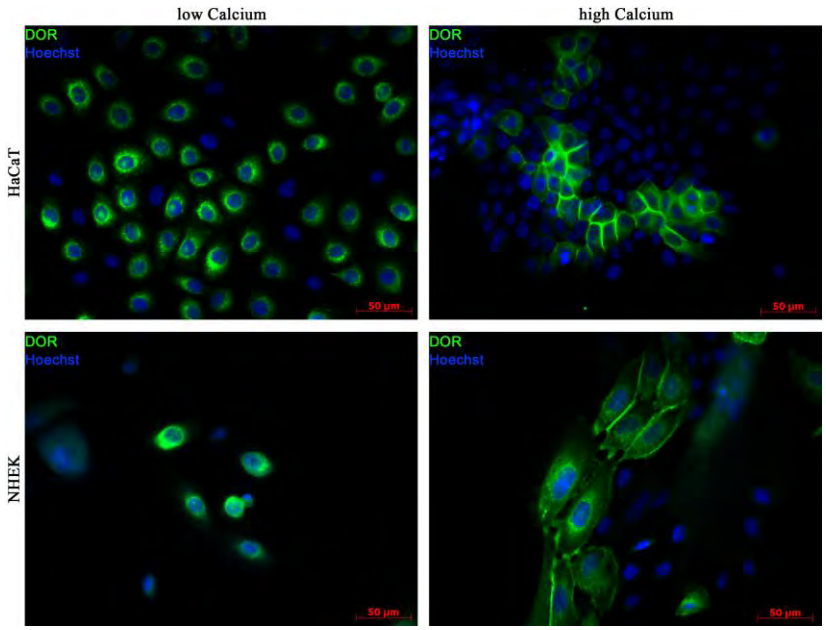


Figure 12 - DOR-GFP expression in primary keratinocytes and the HaCaT cell line

HaCaT cells and primary keratinocytes (NHEK) were grown for 5 days in 0.09 mM or in 1.2 mM Ca^{2+} medium (K-SFM, BPE, EGF) and then fixed in 4% formaldehyde. Cells were subjected to immunocytochemistry using anti-GFP in order to visualize DOR. Nuclei were counterstained with Hoechst dye. Fluorescence images were taken with a Zeiss AxioImager Z1 microscope equipped with a Plan-Neofluar 40x/1.30 NA oil-immersion lens.

The localisation changes are reversible in both directions. The keratinocyte cell line N/TERT-1 is routinely cultured under medium Ca^{2+} concentrations of 0.4 mM, which leads to a membrane localisation of DOR-GFP. If the cells are then shifted to 0.09 mM low Ca^{2+} medium overnight a loss of Ca^{2+} dependent desmosomal structures and the internalisation of DOR-GFP can be observed (Column 1 of Figure 13). Re-addition of Ca^{2+} to a concentration of 1.2 mM, results in gradually re-establishment of desmosomal structures and prior to that, in DOR membrane re-localisation. Already after 15 min in high Ca^{2+}, the DOR distribution pattern indicates that DOR is undergoing intracellular redistribution. One hour after addition of Ca^{2+} (Column 3 of Figure 13), DOR has mainly located back into the membrane, whereas desmoplakin, used as the marker for desmosomal structure formation, only partially re-localised. Only after eight hours of incubation, a re-formation of mature desmosomes can be observed (Column 4 of Figure 13). Cells constantly cultured in high Ca^{2+} without Ca^{2+} switch display fully mature desmosomes and stable DOR membrane expression (Column 5 of Figure 13) and might reflect more closely the situation *in vivo*. These findings suggest that Ca^{2+} dependent cell-cell adhesion influences the DOR concentration in the membrane and through that, possibly the functionality of DOR in cultured keratinocytes.

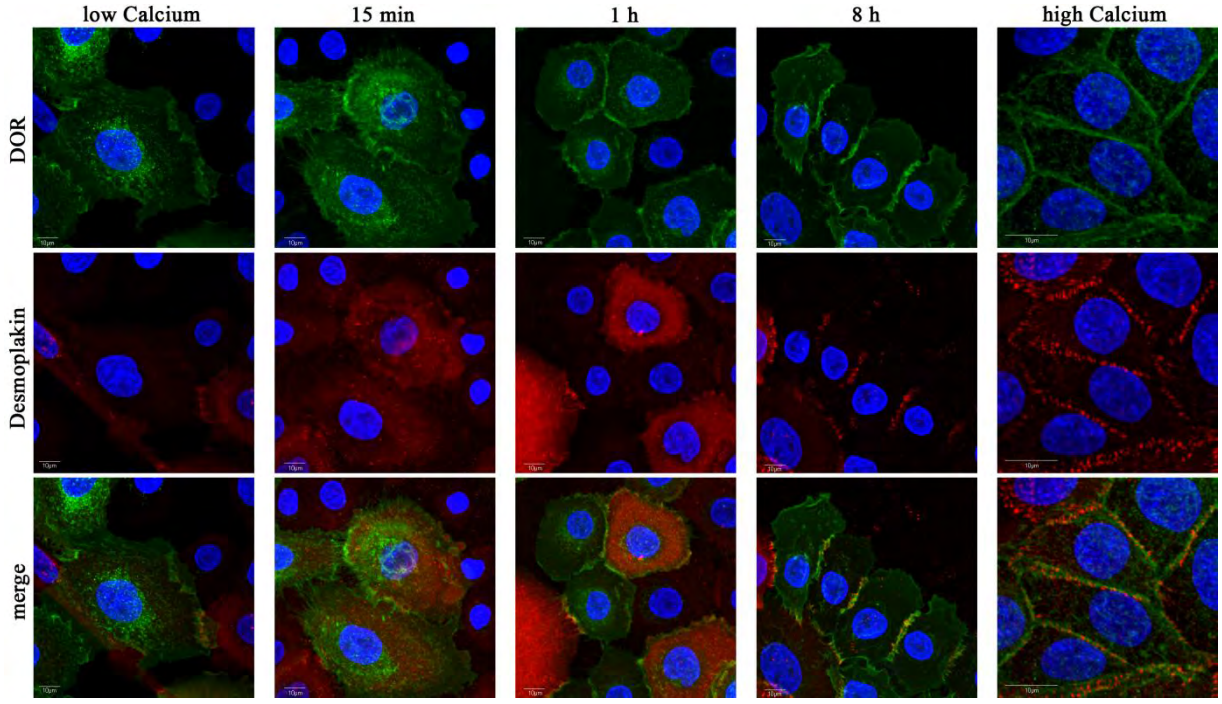


Figure 13 - Calcium influences DOR subcellular localisation

N/TERT-1 cells overexpressing DOR-GFP were cultured until 90% confluence in 0.4 mM Ca^{2+} medium. Overnight they were incubated in 0.09 mM Ca^{2+} medium. Morphology change and nearly complete loss of desmosomal junctions can be observed while DOR is internalised. Medium was then changed to 1.2 mM Ca^{2+} medium and cells were fixed after indicated time points in 4% paraformaldehyde. In order to visualize cell-cell-junction assembly and DOR localisation immunocytochemistry was performed using anti-GFP and anti-desmoplakin antibodies. Nuclei were visualized using Hoechst dye. Fluorescence image stacks were obtained at 0.1 µm intervals in Z-section with an Olympus FV1000 microscope and an UPLFLN 40x/1.3 NA oil-immersion lens (Olympus, Tokyo, Japan).

III.2. Internalised DOR-GFP protein is localised in different subcellular compartments

Further, it was of interest in which subcellular compartment the receptor is localised upon low Ca^{2+} mediated internalisation. Several studies from the group of Ulla E. Petäjä-Repo and M. Bouvier suggested, that the endoplasmic reticulum (ER) plays an important role in receptor maturation and cell-surface localisation (Markkanen et al., 2008; Petäjä-Repo et al., 2000b; Petäjä-Repo et al., 2001b; Tuusa et al., 2007; Tuusa et al., 2010b). Petäjä-Repo *et al.* estimated that only 40% of the DOR precursor proteins are converted to the mature form and eventually reach the cell surface. The other fraction is retained in the ER and subjected to proteasomal degradation (Petäjä-Repo et al., 2000a; Petäjä-Repo et al., 2001a). Therefore, immunocytochemical staining of DOR-GFP overexpressing keratinocytes with a calnexin

antibody was performed and the localisation of DOR-GFP and calnexin signals analysed using confocal microscopy. Calnexin together with calreticulin promotes the folding of glycosylated proteins in the ER and interacts with DOR as shown by Tuusa et al. (Tuusa et al., 2010a). Indeed, a partial co-localisation of DOR and calnexin can be observed, yet the majority of DOR is not localised in the ER under low Ca^{2+} culture conditions of keratinocytes. Under high Ca^{2+}, a similar amount of DOR co-localises with calnexin, indicating a similar maturation rate of DOR under both conditions (Figure 14). Therefore, the internalisation under low Ca^{2+} culture conditions might not reflect a lower maturation rate, accompanied with less cell surface exposure of DOR, so that other mechanisms might be involved.

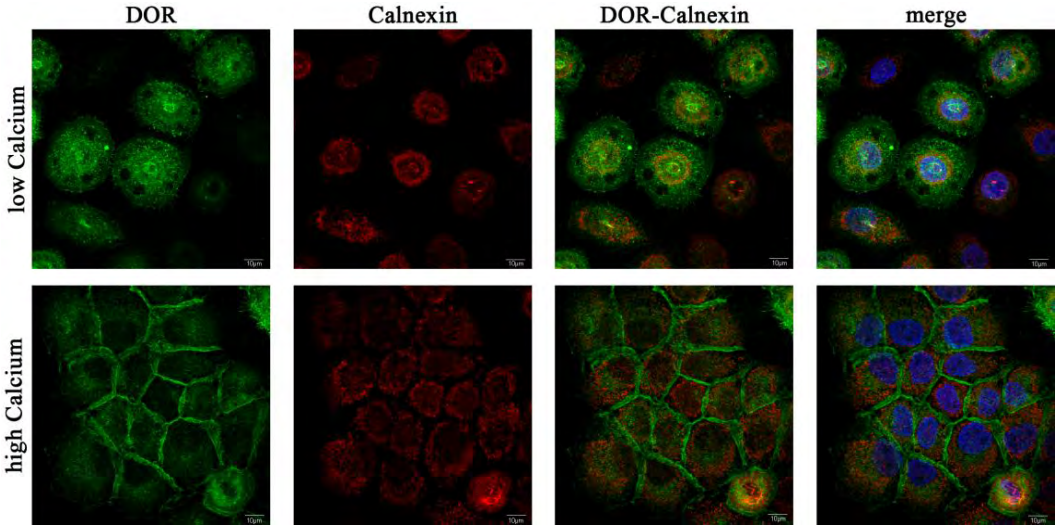


Figure 14 - DOR co-localisation study with ER marker Calnexin

HaCaT cells overexpressing DOR-GFP were cultured for 5 days in either 0.09 mM or 1.2 mM Ca^{2+} medium (K-SFM, BPE, EGF) to 80% confluence. Cells were fixed in 4% paraformaldehyde and subjected to immunocytochemistry for DOR and ER localisation, using anti-GFP and anti-calnexin antibodies. Nuclei were visualized using Hoechst dye. Fluorescence image stacks were obtained at 0.45 µm intervals in Z-section with an Olympus FV1000 microscope and an UPLSAPO 100X/1.4 NA oil-immersion lens (Olympus, Tokyo, Japan).

It was further reported that the half-life of DOR in the membrane is about 20 h (Petäjä-Repo et al., 2000c). To exclude that inefficient membrane localisation, due to high membrane turnover with rapid receptor endocytosis, is the reason for internalized receptor in low Ca^{2+}, the co-localization of DOR with endosome marker early endosome antigen 1 (EEA1) was analysed (Figure 15). A partial co-localisation of DOR with the endosome marker was

detected in low Ca^{2+} cultures. The amount of DOR-GFP positive particles exceeds that of the early endosomes strongly. Therefore, co-localisation might represent random events.

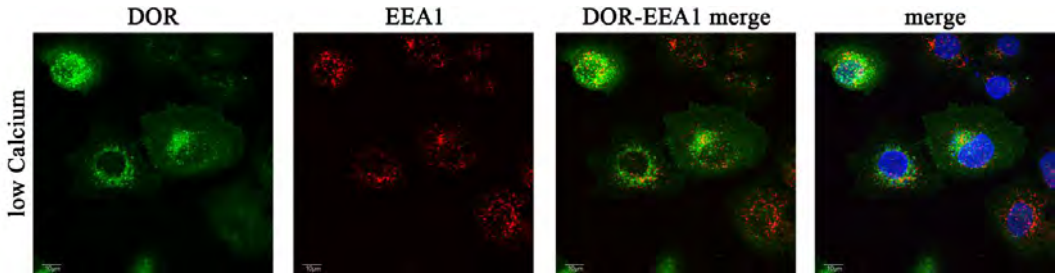


Figure 15 - DOR co-localisation study with early endosome marker EEA1

HaCaT cells overexpressing DOR-GFP were cultured for 5 days in 0.09 mM (K-SFM, BPE, EGF) to 80% confluence. Cells were fixed in 4% paraformaldehyde and subjected to immunocytochemistry for DOR and early endosomes, using anti-GFP and anti-EEA1 antibodies. Nuclei were visualized using Hoechst dye. Fluorescence image stacks were obtained at 0.4 µm intervals in Z-section with an Olympus FV1000 microscope and an UPLSAPO 100X/1.4 NA oil-immersion lens (Olympus, Tokyo, Japan).

To fully exclude the lysosomal degradation pathway as target of internalised receptor under low Ca^{2+} conditions, further analysis of the associated of DOR-GFP with the lysosome-associated membrane protein 1 (LAMP1) were carried out. Immunocytochemical staining confirmed again only a partial co-localisation. Under both low and high Ca^{2+} culture conditions, similar amounts of DOR and LAMP1 (Figure 16) co-localise as seen before for the ER protein calnexin (Figure 14).

In conclusion, the staining for subcellular DOR distribution confirmed partial localisation of DOR with ER and lysosomes, as well as early endosomes. No significant difference of co-localisation signal could be observed between low and high Ca^{2+} cultured cells after analysis with the Olympus FluoView software. Additional studies are necessary to explore the subcellular localisation of overexpressed DOR-GFP in keratinocytes under low Ca^{2+} culture conditions, in order to understand the functionality of the DOR especially in primary keratinocytes in culture.

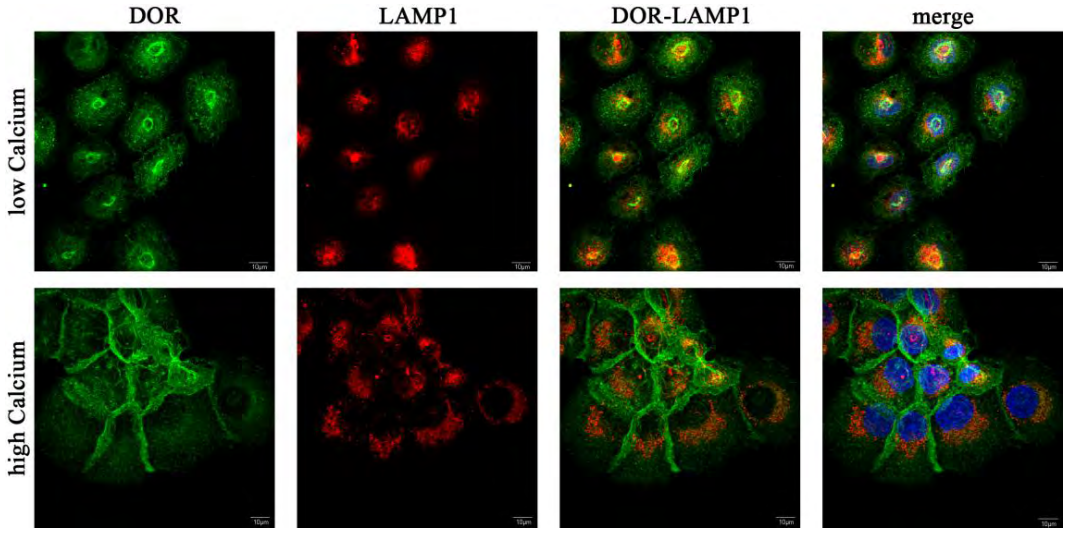


Figure 16 - DOR co-localisation study with lysosome marker LAMP1

HaCaT cells overexpressing DOR-GFP were cultured for 5 days in either 0.09 mM or 1.2 mM Ca^{2+} medium (K-SFM, BPE, EGF) to 80% confluence. Cells were fixed in 4% paraformaldehyde and subjected to immunocytochemistry for DOR and lysosomes, using anti-GFP and anti-LAMP1 antibodies. Nuclei were visualized using Hoechst dye. Fluorescence image stacks were obtained at 0.45 µm intervals in Z-section with an Olympus FV1000 microscope and an UPLSAPO 100X/1.4 NA oil-immersion lens (Olympus, Tokyo, Japan).

III.3. Immediate morphological changes in response to DOR activation measured by impedance recording

The classical G protein dependent signalling pathway, activated by DOR, can result in morphological changes following second messenger activation. These changes can be detected by cellular impedance recording using the xCELLigence System (Roche). HaCaT cells were cultured on the surface of integrated microelectrode sensors in the bottom of the wells in electronic plates (E-Plates). The presence or absence of cells sensitively and precisely affects the electronic and ionic exchange between cell culture media and the microelectrodes. Thus, the electrode impedance provides information about the biological status of the cells and can monitor morphological changes. On order to test the potential of several DOR ligands cells were treated with SNC80, a highly DOR specific agonists, [Met^5]-enkephalin**,** the DOR specific endogenous peptide agonist, and naltrindole, the higly DOR selective antagonistThe assay system expresses impedance in arbitrary Cell Index (CI) units. The CI at each time point is defined as $(R_n-R_b)/15$; where R_n is the cell-electrode impedance of the well when it contains

cells and R_b is the background impedance of the well with the media alone (Abassi et al., 2004; Fitzsimons et al., 2004; Solly et al., 2004). After HaCaT cells did adhere to the plate and the recording stabilized, cells were treated at final concentrations of 100 nM SNC80, 100 nM [Met^5]-enkephalin, 10 μM naltrindole or the equivalent concentration of the buffer vehicle DMSO. Cell responses were monitored in real-time at one-minute intervals. Initial cellular responses were detected within minutes of ligand addition. Maximal responses towards SNC80 (Figure 17A) and [Met^5]-enkephalin (Figure 17B) were observed during the first 35-45 minutes after agonist addition. The addition of agonist resulted in a robust response in DOR-overexpressing cells and a slight increase of the CI in control cells in comparison to vehicle treated cells. The addition of medium itself and the manipulation of the plate outside the incubator affected the CI, but the ligand-mediated effect in DOR-overexpressing cells was still significantly above the control. In DOR-overexpressing cells the SNC80 induced increase of CI can be reduced by co-administration of naltrindole, but naltrindole itself results in a significant increase of the CI, which is most likely as well an effect on the morphology of the HaCaT cells. The peak for naltrindole appears after 27 minutes, clearly before the SNC80 caused maximal response at 40 minutes (Figure 17C). Naltrindole efficiently blocks the [Met^5]-enkephalin mediated CI changes as well (Figure 17D). The shape of the curve for both the GFP control and DOR-overexpressing cells indicates that naltrindole alone influences two different signalling mechanisms. After the first peak, a second increase of the CI is observed, which starts around 30 minutes after ligand addition and reaches the maximum around 40-45 minutes. In control cells both the agonists and antagonist change the CI stronger than the vehicle control, while naltrindole results in a higher response than SNC80 and [Met^5]-enkephalin (Figure 17E and F). This indicates that both agonist and antagonist influence intracellular signalling with an effect on morphology in control cells, possibly through endogenously expressed receptors that are present at low levels.

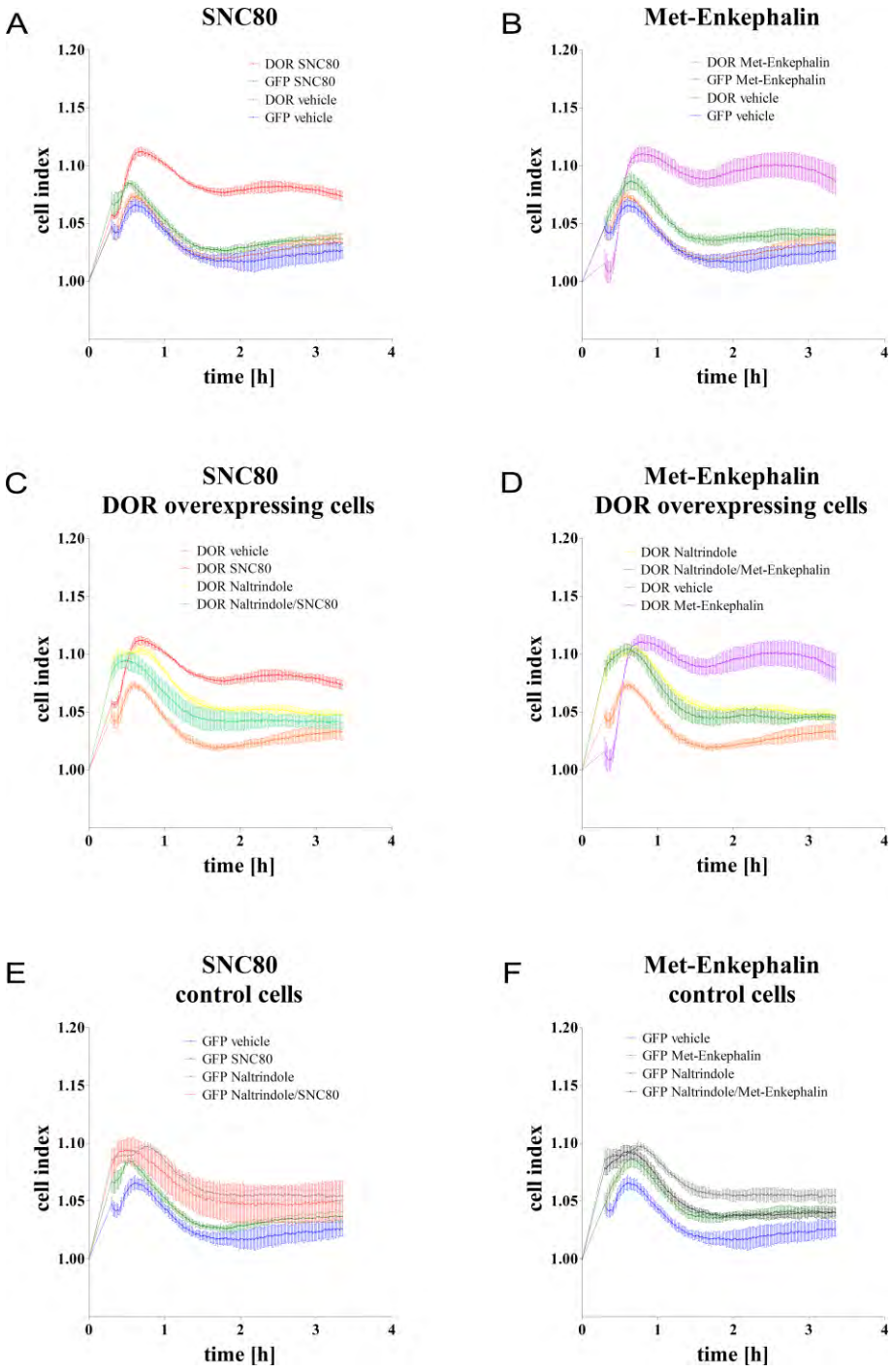
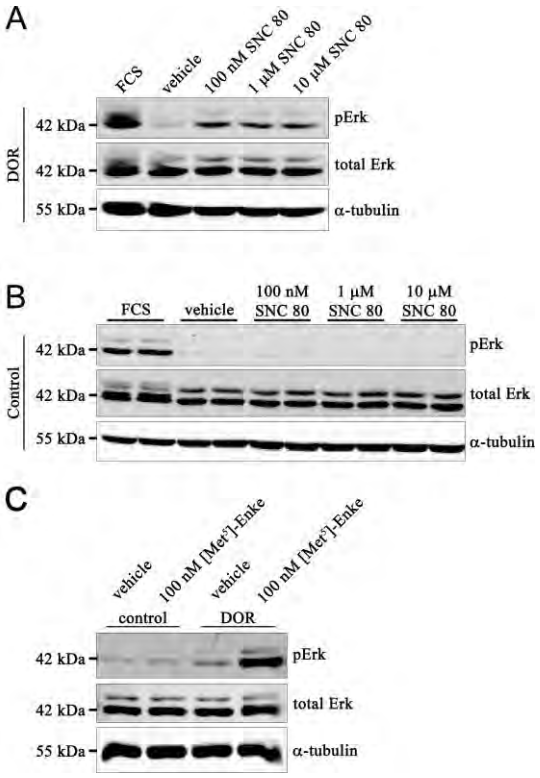


Figure 17 - Impedance changes indicate morphological responses to DOR ligand administration

HaCaT cells overexpressing DOR or control cells were seeded at 4000 cells per well on E-Plates, and the cells were continuously monitored for two days to ensure a stable CI measurement. After two days a final concentration 100 nM of SNC80, 100 nM [Met^5]-enkephalin, 10 µM naltrindole or 0.04 % DMSO vehicle control were added to the cells without further medium change. Cell response was monitored every 1 min for 3 h. All curves are normalized to the time point after the two days of culture exactly before removing the plate for addition of ligands. (A) To control or DOR-overexpressing cells SNC80 or vehicle control medium were added. In (B) [Met^5]-enkephalin was added to control cells or overexpressing cells. (C) The effect of DOR inhibition by naltrindole on the SNC80 mediated change of CI in DOR-overexpressing cells was measured and in (D) the same inhibitory effect by naltrindole on [Met^5]-enkephalin was monitored in DOR-overexpressing cells only. (E) and (F) represent the control cells treated with both agonists and the antagonist naltrindole. The graphs represent the means of quadruplicates (n = 4) from one experiment (n = 1). Error bars indicate standard deviation.

III.4. DOR activation triggers MAPK pathway signalling in keratinocytes

That extracellular signal-regulated kinase 1 and 2 (ERK 1/2) are downstream signalling targets of several GPCRs, including DOR, is widely accepted in this field of research. The majority of studies used CHO (Xu et al., 2010) or HEK293 (Eisinger and Ammer, 2008a; Eisinger and Ammer, 2009) cells overexpressing a tagged DOR or neuronal cell lines such as NG108-15 (Eisinger and Ammer, 2008b), endogenously expressing high levels of DOR, to investigate the intracellular signalling mechanisms. These models were applied to keratinocytes by overexpressing the GFP-tagged receptor in two different keratinocyte cell lines. HaCaT cells (Boukamp et al., 1988; Schoop et al., 1999) are spontaneously immortalised cells, whereas N/TERT-1 cells are immortalized by introducing the expression of telomerase reverse transcriptase (TERT) (Dickson et al., 2000b; Natarajan et al., 2006; Utikal et al., 2009). Both cell lines have the potential to induce keratinocyte differentiation, but N/TERT-1 cells resemble more closely the behaviour of primary keratinocytes in culture. Experiments, addressing the question whether the endogenous DOR agonist [Met^5]-enkephalin and the highly DOR specific synthetic alkaloid agonist SNC 80 were able to trigger ERK 1/2 signalling, were performed. HaCaT cells overexpressing DOR and control-transduced cells were treated at different concentrations from 100 nM to 10 μM of SNC 80 for 15 minutes, cells lysed, and subjected to Western blot analysis for phosphorylated ERK 1/2 as indicator for activation. A response towards the ligand can only be detected in DOR overexpressing cells. Figure 18A shows a solid response of the ERK signal 15 minutes after addition of SNC 80, regardless of the concentration applied. This indicates that a concentration of 100 nM is sufficient to induce a maximal response in these cells. No phosphorylation of ERK 2 can be detected in control cells as seen in Figure 18B. [Met^5]-enkephalin applied only at one concentration of 100 nM induces as well a strong



phosphorylation of ERK 2, specifically in DOR overexpressing cells but not in control cells (Figure 18A).

Figure 18 - Endogenous and synthetic DOR agonists mediate ERK signalling pathway activation in keratinocytes

HaCaT cells stably overexpressing DOR-GFP or control transduced cells were cultured until 80% confluence and then starved in serum free medium for 6 h. For (A) and (B) the diluted ligand was added at concentrations of 100 nM, 1 µM, and 10 µM for 15 min. Cells were lysed in modified RIPA buffer and lysates underwent protein quantification. 20 µg of protein lysate were subjected to Western blotting for detection of phospho ERK, total ERK and tubulin by fluorography using the Odyssey infrared imager. Panel (A) shows ERK 2 phosphorylation in DOR expressing cells after SNC 80 mediated activation of the receptor whereas control cells in panel (B) display no ERK 2 activation. Samples in panel (C) were treated with 100 nM [Met^5]-enkephalin for 10 min, then lysed, and subjected to Western blot analysis as for panel A) and B). Only DOR cells treated with agonist display specific ERK 2 phosphorylation.

Comparing the magnitude of ERK 2 activation, a difference between both ligands could be observed, while the duration was similar (Figure 20C). In HaCaT cells, [Met^5]-enkephalin is more potent in inducing an ERK 2 phosphorylation than SNC 80. A time course study of ERK activation over two hours for SNC 80 and [Met^5]-enkephalin shows the maximal response for [Met^5]-enkephalin five minutes after addition of ligand and for SNC 80 ten minutes after addition (Figure 19). The semi-quantitative analysis of the Western blot signal from six samples per time point in each group, shows an increase of ERK 2 phosphorylation after [Met^5]-enkephalin addition of about 16 fold, while SNC 80 leads to only six fold increase compared to vehicle controls at time zero. A two-way ANOVA analysis revealed a significant difference between the SNC 80 and [Met^5]-enkephalin groups at five and ten minutes. In both groups, the phosphorylation of ERK 2 decreases fast within 30 minutes, but stays slightly above the basal level for at least 60 minutes (Figure 19C). The process of DOR mediated ERK activation can be described as transient in HaCaT keratinocyte cells.

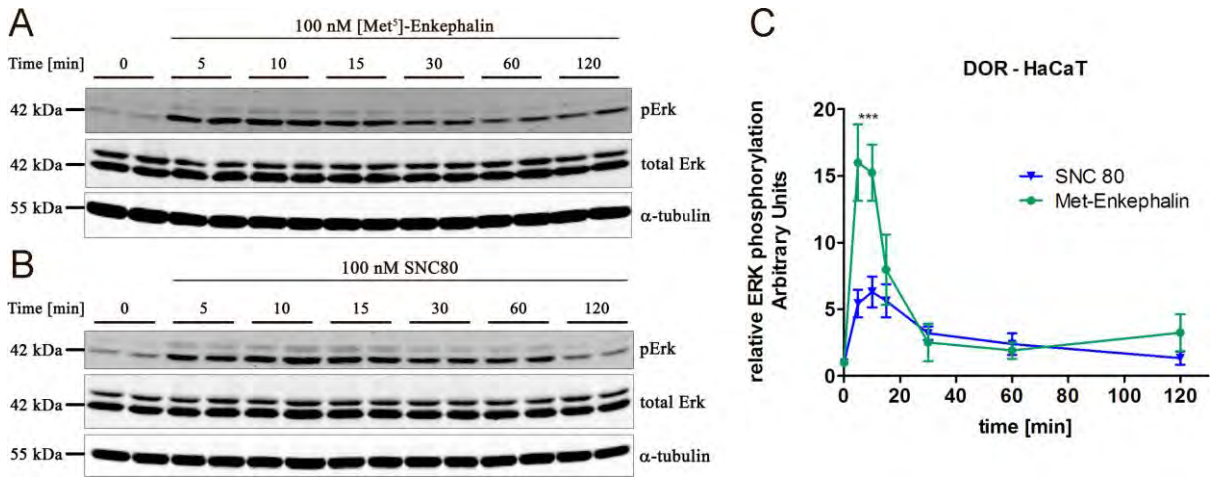


Figure 19 - DOR activation mediates transient ERK 1/2 signalling in HaCaT keratinocytes with a different maximal response for [Met^5]-enkephalin and SNC 80

HaCaT cells stably overexpressing DOR-GFP cells were cultured until 80% confluence and then starved in serum free medium for 6 h. Ligands were added at concentrations of 100 nM for indicated times, cells lysed in modified RIPA buffer, and lysates underwent protein quantification. 20 µg of protein lysate were subjected to Western blotting for detection of phospho ERK, total ERK and tubulin by fluorography using the Odyssey infrared imager. Panel (A) shows ERK 2 phosphorylation in DOR expressing cells after [Met^5]-enkephalin mediated activation of the receptor and panel (B) displays ERK 2 phosphorylation in DOR expressing cells after SNC 80 treatment. (C) shows the graph of the semi-quantification of ERK phosphorylation over time. Each value represents mean of six replicates from two independent experiments. Error bars indicate standard deviation. Two-way ANOVA: *** $p < 0.001$ for time point five and ten minutes.

The second keratinocyte cell line N/TERT-1 shows similar responsiveness towards DOR agonists [Met^5]-enkephalin and SNC 80. The difference observed, is the magnitude of the response, which is lower in N/TERT-1 than in HaCaT cells. A three to four fold increase in ERK phosphorylation five minutes after addition of ligand, compared to the six to 15 fold increase in HaCaT cells, was observed. In addition, [Met^5]-enkephalin has the same potency as SNC 80 and the phosphorylation level of ERK decreases fast, within 15 minutes to a basal level. Therefore, DOR-overexpressing N/TERT-1 cells show a less pronounced and more transient activation of ERK than HaCaT cells (Figure 20).

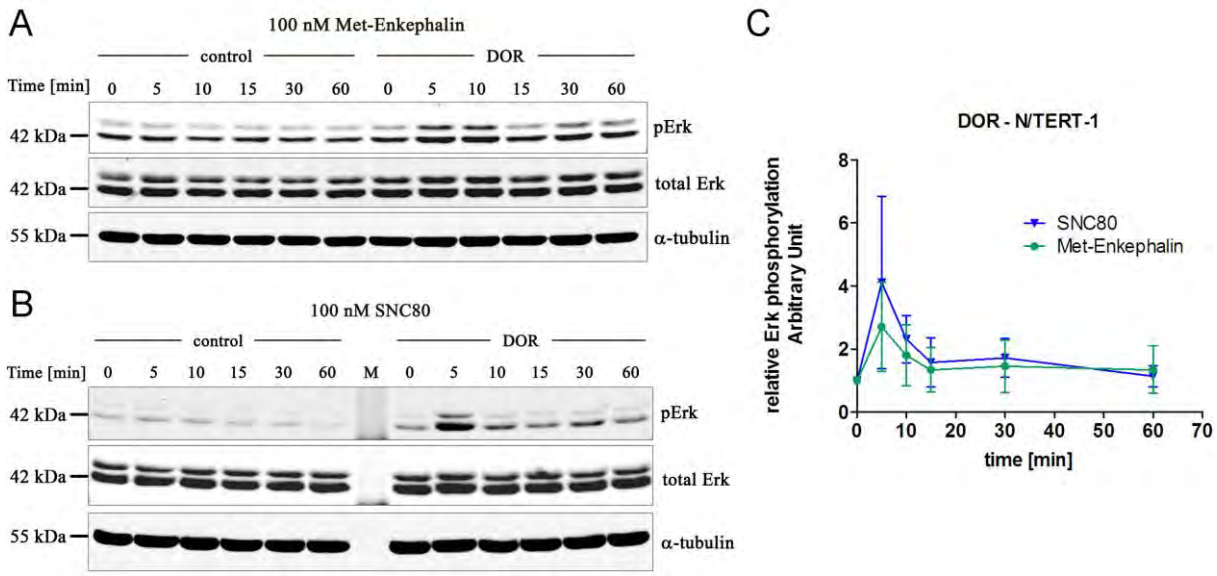


Figure 20 - DOR activation mediates transient ERK 1/2 signalling in N/TERT-1 keratinocytes with a similar response for [Met^5]-enkephalin and SNC 80

N/TERT-1 cells stably overexpressing DOR-GFP or control transduced cells were cultured until 80% confluence and then starved in serum free medium over night. Ligands were added at concentrations of 100 nM for indicated times, cells lysed in modified RIPA buffer, and lysates underwent protein quantification. 20 µg of protein lysate were subjected to Western blotting for detection of phospho ERK, total ERK and tubulin by fluorography using the Odyssey infrared imager. Panel (A) shows ERK 2 phosphorylation in DOR expressing cells after [Met^5]-enkephalin mediated activation of the receptor and panel (B) displays ERK 2 phosphorylation in DOR expressing cells after SNC 80 treatment. (C) shows the graph of the semi-quantification of ERK phosphorylation over time comparing the [Met^5]-enkephalin and SNC 80 mediated response in DOR-overexpressing N/TERT-1. Each value represents the mean of three independent experiments. Error bars represent standard deviation.

For further confirmation of a DOR specific ERK activation in keratincoytes, the DOR inhibitor naltrindole was used to block DOR activation in the presence of agonists. In both, HaCaT and N/TERT-1 cells, a naltrindole concentration of 10 µM is sufficient to fully block the SNC80 mediated activation of ERK 2. Antagonist was added five minutes prior to agonist, in order to effectively block the receptors. Figure 21A, the immunoblot for phosphorylated ERK, shows the increase in phosphorylation ten minutes after addition of SNC 80 and reduced phosphorylation signal when naltrindole was co-applied. The statistical analysis after quantification of the relative ERK phosphorylation reveals a significant induction by application of SNC80 alone and its inhibition by naltrindole co-application (Figure 21B).

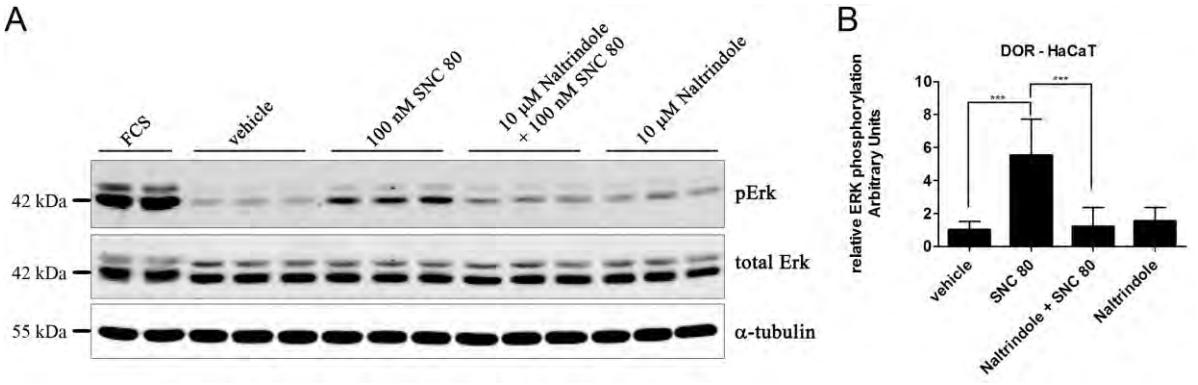


Figure 21 - DOR mediated ERK signalling in HaCaT cells can be blocked by DOR specific antagonist naltrindole

HaCaT cells stably overexpressing DOR-GFP were cultured until 80% confluence. Cells were starved in serum free medium for 6 h. Foetal calf serum (FCS), as positive control, was added for 30 min, naltrindole 5 min prior to SNC80 for co-administration, naltrindole alone for 15 min, and SNC 80 for 10 min. Cells were lysed in modified RIPA buffer and lysates underwent protein quantification. 20 µg of protein lysate were subjected to Western blotting for detection of phospho ERK, total ERK and tubulin by fluorography using the Odyssey infrared imager. Panel (A) shows the immnunoblot of the ERK 2 phosphorylation in DOR expressing cells and panel (B) the graph of the semi-quantification of ERK phosphorylation. Each value represents the mean of nine replicates from three independent experiments. Error bars indicate standard deviation.
One-way ANOVA: *** $p < 0.001$.

A similar experiment with N/TERT-1 cells gave the same results (Figure 22). Application of SNC80 for ten minutes induced ERK phosphorylation in DOR-overexpressing cells, which could successfully be blocked by pre-incubation with naltrindole. Additionally, PD98059 was included in this experiment. This inhibitor blocks MEK 1 function and therefore inhibits ERK activation further downstream in the DOR signalling cascade. With this inhibitor, the DOR mediated signalling cascade is blocked to the same degree as with naltrindole, if DOR agonist SNC80 was co-applied in DOR-overexpressing cells. In control cells and if only inhibitor was added all residual ERK phosphorylation disappeared. This indicates in the likewise a DOR specific ERK activation by the DOR agonist SNC80.

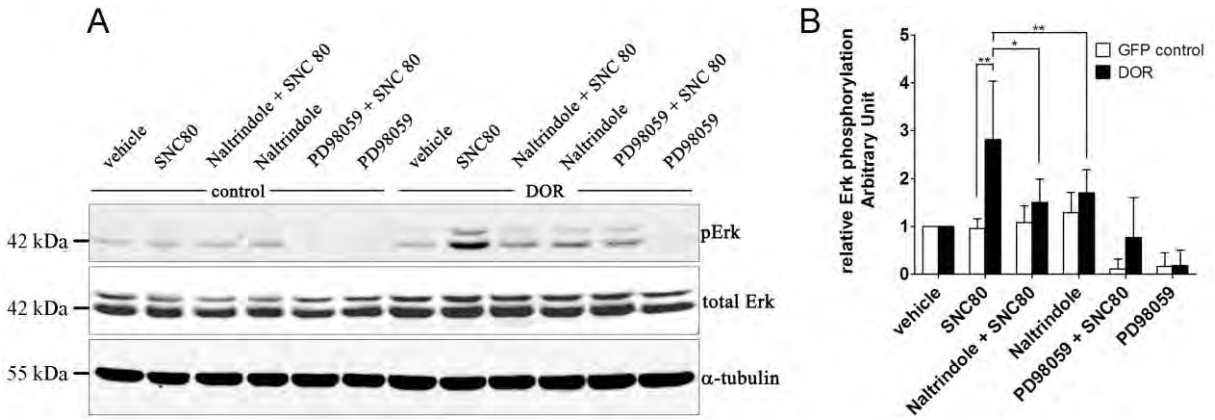


Figure 22 - DOR mediated ERK signalling in N/TERT-1 cells can be blocked by DOR specific antagonist naltrindole and ERK inhibitor PD98059

N/TERT-1 cells stably overexpressing DOR-GFP or control-transduced cells were cultured until 80% confluence. Cells were starved in serum free medium overnight. Vehicle control DMSO was added for 10 min, SNC 80 was added for 10 min, naltrindole 5 min prior to SNC80 for co-administration, naltrindole alone for 15 min, PD98059 30 min prior to SNC80, and PD98059 alone for 40 min. Cells were lysed in modified RIPA buffer and lysates underwent protein quantification. 20 µg of protein lysate were subjected to Western blotting for detection of phospho ERK, total ERK and tubulin by fluorography using the Odyssey infrared imager. Panel (A) shows the immnunoblot of the ERK 1/2 phosphorylation in DOR expressing and control cells and panel (B) the graph of the semi-quantification of ERK phosphorylation. Each value represents mean from three independent experiments. Error bars indicate standard deviation. One-way ANOVA: ** $p < 0.01$, * $p < 0.05$.

III.5. DOR signalling in keratinocytes inhibits proliferation *in vitro*

DOR overexpression dramatically changes the phenotype of N/TERT-1 keratinocytes. Colonies of DOR-overexpressing N/TERTs are more spread out than control cell colonies (Figure 23), and exhibit drastically reduced cell proliferation rates.

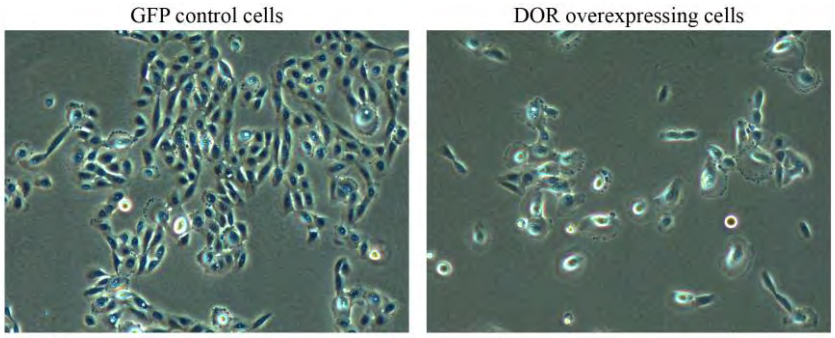
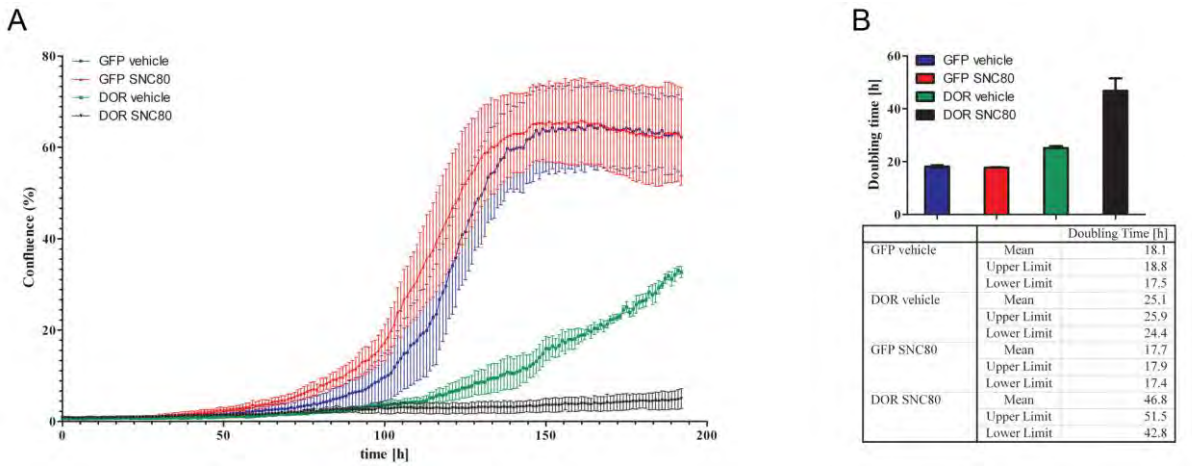


Figure 23 - DOR overexpression changes keratinocyte morphology

A Phase contrast images of control and DOR N/TERT-1 cultures were captured at 20x magnification using a Nikon Eclipse TS100 Microscope. 5 days after plating the same amount of N/TERT-1 cells into standard culture vessels, DOR-overexpressing cells formed markedly fewer and smaller colonies, consistent with less proliferation and an increase in cell size.

Cells display more ruffling at the edges, which might, together with the colony spreading, reflects an increased migratory activity. Compared to the control cells the size of the keratinocytes increases, which is often seen in keratinocytes with reduced proliferation capacity.

Using the INCUCYTE™ Kinetic Imaging System proliferation was monitored over eight days, in order to quantify our observations. The same numbers of N/TERT-1 control and DOR-overexpressing cells were plated into a 96-well plate. Phase-contrast images were automatically captured every hour and processed by the INCUCYTE software, which calculated the percentage confluence per sample.



		Doubling Time [h]
GFP vehicle	Mean	18.1
	Upper Limit	18.8
	Lower Limit	17.5
DOR vehicle	Mean	25.1
	Upper Limit	25.9
	Lower Limit	24.4
GFP SNC80	Mean	17.7
	Upper Limit	17.9
	Lower Limit	17.4
DOR SNC80	Mean	46.8
	Upper Limit	51.5
	Lower Limit	42.8

Figure 24 - The DOR inhibits keratinocyte proliferation

(A) The proliferation curves of DOR-overexpressing and control cells, in either vehicle control medium or 100 nM SNC80-containing medium were obtained from the images captured hourly with a 10x objective lens by the Incucyte machine. The graph depicts the mean ± SEM percentage confluence per field of view of one representative experiment run in triplicates, per culture condition. (B) Doubling time was calculated in GraphPad Prism from the growth curve in A) using a non-linear regression model and is displayed as mean with upper and lower limit. The doubling time for DOR-overexpressing cells increases drastically as compared to control cells.

Figure 24A shows the growth curves of both, control cells and DOR-overexpressing cells, cultured in the presence of 100 nM SNC80 or vehicle control. While control cells entered an exponential growth phase before plateauing after about six days in culture, DOR cells had a markedly reduced proliferative capacity. The addition of the DOR ligand SNC80 into the culture medium further decreased cell proliferation to almost undetectable levels.

Accordingly, the doubling time of DOR-overexpressing cells (24.4 - 25.9 hours) under basal conditions was about 1.4 times higher than that of control cells (17.5 - 18.8 hours), and further increased to 42.8 - 51.5 hours under SNC80, while control cells were unaffected by this treatment (doubling time 17.4 – 17.9 hours) (Figure 24B).

III.6. DOR delays *in vitro* keratinocyte differentiation

In order to investigate if DOR is involved in epidermal homeostasis the capability of control and DOR-overexpressing keratinocytes to differentiate was investigated. The transition from basal to spinous epidermal layer keratinocytes is accompanied by suppression of KRT 5 and KRT 14 transcripts and the upregulation of KRT 1 and KRT 10 intermediate filaments (Fuchs et al., 1980). The changes in expression of KRT 1 and KRT 10 in an *in vitro* model for keratinocyte differentiation using HaCaT and N/TERT-1 cells were analysed. Both cell types were grown to confluence before differentiation was induced by growth factor withdrawal in the presence of DOR agonists. HaCaT cells were differentiated for up to 48 hours in the presence of SNC80. Within six hours, control cells start to induce KRT 1 and KRT 10 expression and continuously increase the expression level during further differentiation (Figure 25A and B) as observed by quantitative real-time PCR. 12 hours after induction of differentiation in the presence of SNC80 for both genes, a significant difference between control and DOR cells is observed. DOR cells induce KRT 1 with a factor of 1.8, while control cells express 3.5 times more KRT 1. A similar trend is observed for KRT 10. This difference is lost after additional 12 hours of differentiation when both cells express the same amount of KRT 1 and 10. This expression pattern indicates a markedly delayed induction of KRT 1 and KRT 10 intermediate filaments upon keratinocyte differentiation in DOR-overexpressing cells.

A different pattern is observed for the differentiation marker IVL (Figure 25C). This early-cornified envelope gene is normally induced after prolonged differentiation. Indeed an induction of about three fold within 48 hours is observed in both control and DOR-overexpressing cells. Along the way, DOR cells display a first rapid increase of IVL expression four hours after induction of differentiation in the presence of SNC80 that is not observed in control cells. These changes indicate the involvement of the DOR in the differentiation process but the limited differentiation capacity of HaCaT cells does not allow further conclusions.

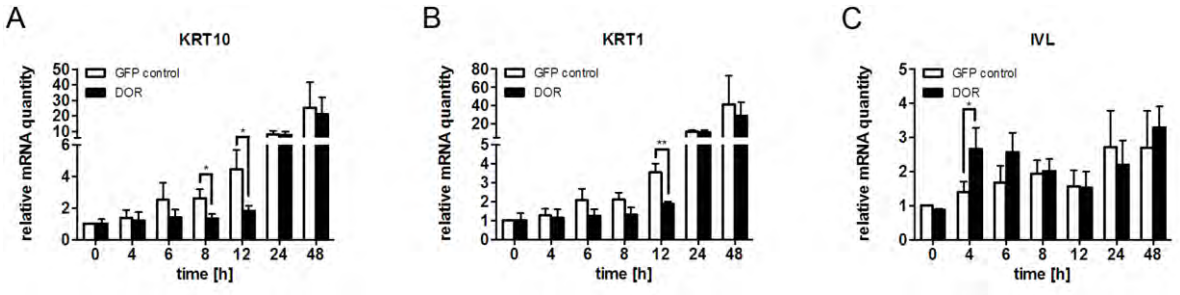


Figure 25 - The DOR alters expression of keratinocyte differentiation related genes in HaCaT keratinocytes

HaCaT cells were grown to confluence and subjected to growth factor withdrawal induced differentiation. RNA was extracted at indicated time points and subjected to real-time PCR quantification. (A) In the presence of 100 nM SNC80 GFP control cells rapidly induce KRT 10 expression while DOR-overexpressing cells do not induce the expression within 12 h of incubation. 24 h after addition of ligand and induction of differentiation, no difference in KRT 10 expression level between DOR and control cells can be detected. (B) Similar to KRT 10 expression, in the presence of 100 nM SNC80 GFP control cells rapidly induce KRT 1 expression while DOR-overexpressing cells start the expression after about 12 h of incubation. 24 h after addition of ligand and induced differentiation no difference in KRT 1 expression level between DOR and control cells can be detected. (C) The analysis of mRNA expression of IVL in the presence of SNC80 reveals a rapid induction in DOR-overexpressing cells after 4 h of incubation before the expression level is equalised with that of the control cells, which show a delayed expression of IVL in this experiment. The graphs represent the mean of three independent experiments. Error bars indicate standard deviation. t-test results: ** $p < 0.01$, * $p < 0.05$.

N/TERT-1 cells, in contrast to HaCaT cells, have a higher competence to undergo the complete differentiation process (Dickson et al., 2000a). The changes of KRT 10 and KRT 1 expression take different courses in N/TERT-1 cells than in HaCaT cells. Therefore, the protocol was adjusted accordingly. Additionally, due to the previously observed reduced proliferation rate of N/TERT-1 DOR-overexpressing cells, 1.25 times more DOR cells than

control cells were plated initially, to start at the same confluence as control cells when inducing differentiation.

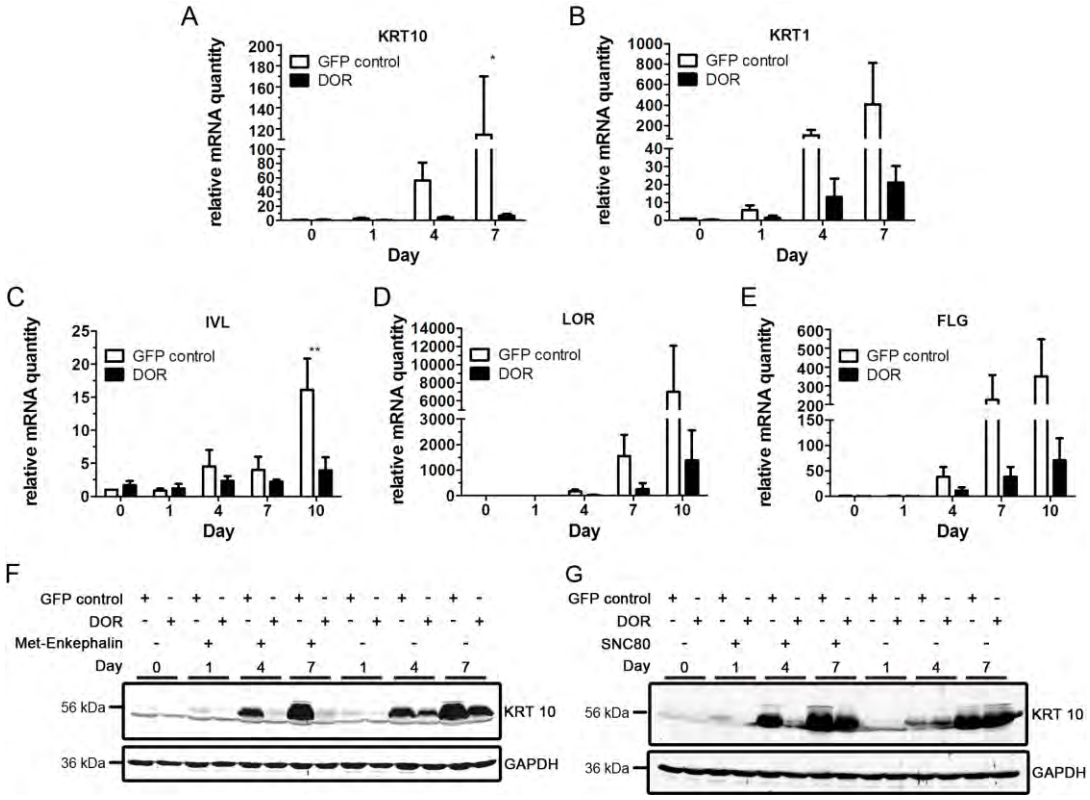


Figure 26 - The DOR impairs keratinocyte differentiation in N/TERT-1 cells

N-TERT-1 cells overexpressing DOR, or control cells, were grown to confluence before differentiation was induced by removal of growth factors. Cultures were kept under the influence of [Met^5]-enkephalin or SNC80 during further cultivation for up to 10 days. The time course of differentiation marker genes was analysed by quantitative real-time PCR and Western blotting. The expression level of (A) KRT 10, (B) KRT 1, and (C) IVL, strongly increase from day 4 onwards and for (D) LOR, and (E) FLG from day 7 onwards in control cells but not in DOR-overexpressing cells. Data are represented after normalization to RPL13a expression as mean values ± SEM of three independent experiments. Vehicle-treated control cells were used as reference. Two-way ANOVA analysis in A) and C), ** $p < 0.01$ and * $p < 0.05$ (F) Western blot analysis confirms the delayed induction of KRT 10 protein expression in DOR-overexpressing cells compared to control cells. Whole cell lysates from confluent cultures (day 0) and day 1, day 4, and day 7 of growth withdrawal-induced differentiation with and without addition of 100 nM DOR agonist [Met^5]-enkephalin were prepared and analysed by immunoblot for KRT 10 expression, with equal loading verified by GAPDH labelling. In DOR-overexpressing cells only minimal KRT 10 induction is observed for the duration of the whole time course. (G) N/TERT-1 cells with and without DOR overexpression were subjected to differentiation for 7 days under the influence of 100 nM DOR agonist SNC80 or vehicle control. Whole cell lysates from confluent cultures analysed for KRT 10 expression by immunoblot show delayed KRT 10 expression in DOR-overexpressing cultures under the influence of SNC80 at day 4. Equal loading was confirmed by GAPDH labelling

Analysis of the mRNA levels of KRT 10 and KRT 1 by quantitative real-time PCR indicated an induction of both transcripts from day one of differentiation. In control cells, continued differentiation was associated with further strong upregulation of these transcripts, while

DOR-overexpressing cells severely lagged behind in the induction process (Figure 26A and B). With advancing differentiation, the mRNA levels of IVL and LOR, as well as FLG increased markedly at day seven after induction of differentiation in control cells, but in DOR cells only at day 10 was a comparable increase in expression of these genes detected (Figure 26C – E).

A delay in KRT 10 induction during early differentiation under the influence of DOR activation was also seen at the protein level. The increase in KRT 10 observed in control cells at day four and seven of differentiation was not detected in DOR-overexpressing cells, indicating that early differentiation is markedly delayed in the presence of the DOR and the endogenous agonist [Met^5]-enkephalin (Figure 26F). If DOR-overexpressing cells were differentiated without addition of agonist, only a minor reduction in KRT 10 protein expression compared to control cells was detected (Figure 26F, lane 11-14). Similar results were obtained with the exogenous agonist SNC80. Until day four of differentiation, protein expression of KRT 10 could substantially be prevented under the influence of SNC80, but at day seven only a minor difference in protein level could be detected (Figure 26G, lane 5-8). These altered expression patterns, in comparison to control cells, suggest a strong involvement of DOR-mediated signalling in the regulation of keratinocyte differentiation.

III.7. DOR signalling changes the gene expression profile in keratinocytes

Two different gene expression studies were performed. In order to find early response genes after activated DOR signalling through SNC80, control and DOR-overexpressing cells were treated with 100 nM agonist for four hours. The collected RNA was subjected to microarray analysis. A total number of 234 genes were significantly altered, after the interaction of SNC80 with the DOR in overexpressing cells, in correlation to control cells. Of these genes, only 59 displayed a fold change bigger than 1.5 and only cytidine triphosphate (CTP) synthase had a fold change above two.

Application of the GeneGo software tool indicated, that cell adhesion, motility, and polarity pathways, as well as RNA processing networks are strongly linked to our observed expression changes. Including all 234 significantly changed genes, POU2F3, with a 1.31 fold decrease of expression observed in the microarray, was finally identified as keratin IF regulating factor, linked to the modulation of keratinocyte differentiation, and chosen to be further validated.

In a second gene expression study HaCaT control and DOR-overexpressing cells were subjected to 12 hours of [Met^5]-enkephalin treatment.

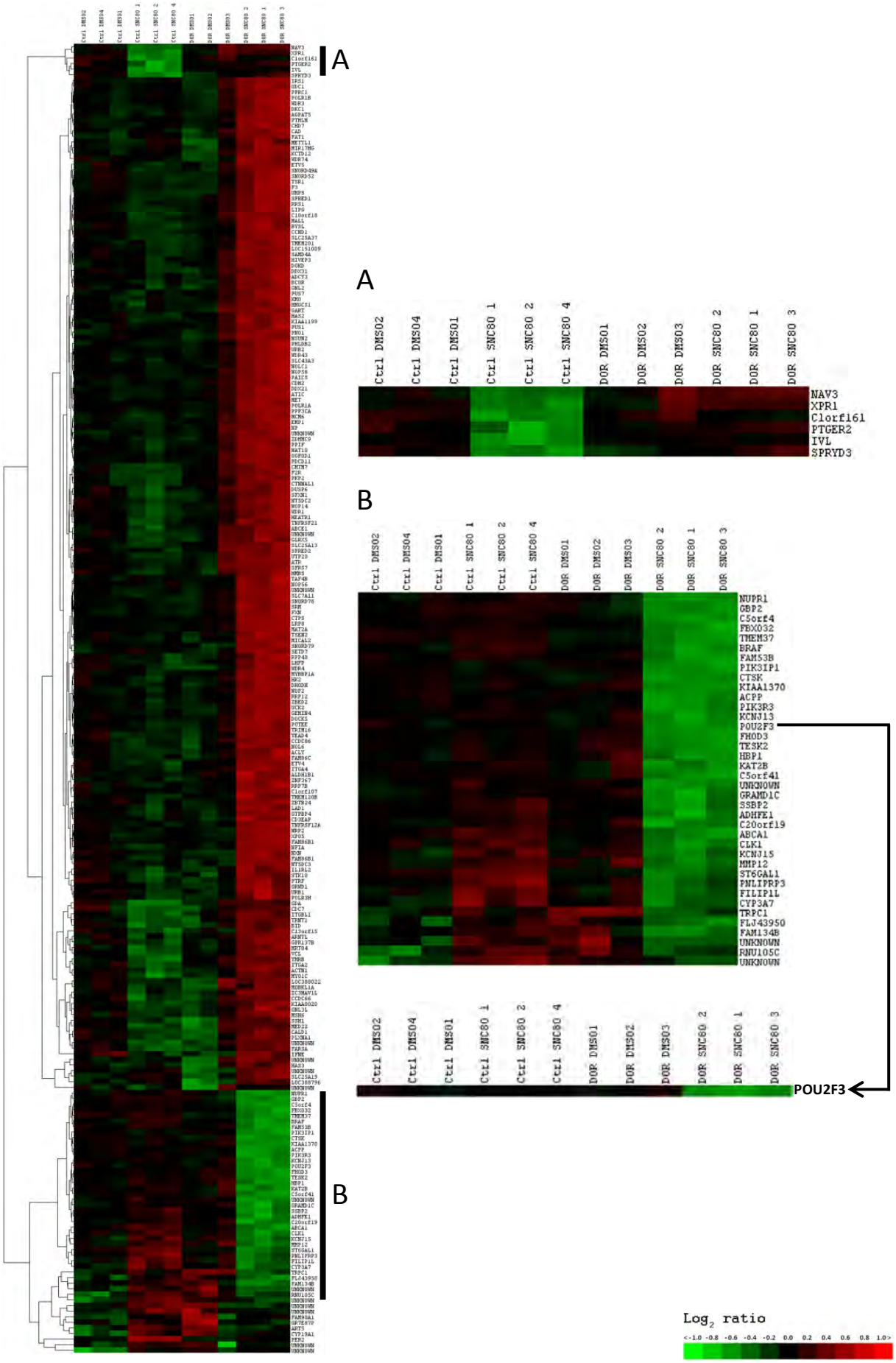

A
B
Ctrl DMS02
Ctrl DMS04
Ctrl DMS01
Ctrl SMC80 1
Ctrl SMC80 2
Ctrl SMC80 4
DOR DMS01
DOR DMS02
DOR DMS03
DOR SMC80 2
DOR SMC80 1
DOR SMC80 3
NAV3
XPR1
C1orf161
PTGER2
IVL
SPRYD3
NUPR1
GBP2
C5orf4
FBXO32
TMEM37
BRAF
FAM53B
PIK3IP1
CTSK
KIAA1370
ACPP
PIK3R3
KCNJ13
POU2F3
FHOD3
TESK2
HBP1
KAT2B
C5orf41
UNKNOWN
GRAMD1C
SSBP2
ADHFE1
C20orf19
ABCA1
CLK1
KCNJ15
MMP12
ST6GAL1
PNLIPRP3
FILIP1L
CYP3A7
TRPC1
FLJ43950
FAM134B
UNKNOWN
RNU105C
UNKNOWN
POU2F3
Log2 ratio
<-1.0 -0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6 0.8 1.0>

Figure 27 - Overview of transcriptome changes after 4h treatment with DOR agonist SNC80

Hierarchical clustering of 234 genes altered by the DOR-SNC80 interaction using a linkage algorithm was performed and visualized in a heat map. Horizontal stripes represent genes, and columns show experimental samples. Clustering was performed only on genes, whereas samples were ordered by treatment and cell line. Logarithmic values are shown in the heat map using red and green colour codes for up- and downregulation, respectively. Black indicates no change. Two groups of genes were extracted. (A) shows the genes that are upregulated by the SNC80 effect, which means that they are downregulated in control cells treated with SNC80 in comparison to vehicle treatment but that DOR-overexpressing cells prevents this downregulation in the presence of SNC80. (B) represents genes that are specifically downregulated by SNC80 in DOR-overexpressing cells. POU2F3 belongs to this group of genes.

The same analysis looking at the DOR-SNC80 interaction effect as performed for the first microarray, was applied to the second one. This time a total number of 117 genes were statistically significant altered in their expression pattern. Similar to the first array the majority of genes displayed a fold change below two. Seventeen altered genes were common in both datasets. This suggests that these genes are true targets of DOR mediated signalling in keratinocytes.

gene	Fold change 4 h SNC80	Fold change 12 h [Met^5]-enkephalin
ACTN1	1.381	1.284
CDH2	1.409	1.352
CTPS	2.154	1.597
DUSP6	1.577	1.821
ETV4	1.874	2.765
ETV5	1.469	2.462
FBXO32	-1.386	-1.429
GBP2	-1.605	-1.609
HIVEP3	1.341	1.35
KCNJ13	-1.577	-1.963
LIPG	1.545	1.478
LRP8	1.802	1.655
MCM6	1.635	1.583
MMP12	-1.292	-2.205
NOLC1	1.539	1.413
SLC7A11	1.822	1.723
ZNF367	1.322	1.382

Table 1 - Common genes significantly modulated by DOR agonists in two independent gene expression studies

The 17 genes reflect different functional categories including structural proteins, a MAPK signalling cascade regulator, metabolic enzymes, transcription factors, ion channel, cell surface receptors, DNA regulatory factors, a matrix-degrading enzyme, and a membrane transporter and are not well characterized in keratinocyte function.

The enrichment analysis of the 12 hour dataset indicated negative regulation of proliferation as the most significant cellular signalling network affected. ECM remodelling and cell adhesion networks were, similar to the first array, highly enriched in our dataset. Canonical pathways influenced by DOR-SNC80 interaction include mainly nucleoside triphosphate metabolic pathways, that cannot be directly connected to specific keratinocyte functions. The strongest, by DOR mediated signalling regulated transcription factors, are Ets variant gene 4

(ETV4) and Ets variant gene 5 (ETV5). The regulation of these factors could be validated by quantitative real-time PCR but the correlation to functional changes in keratinocytes was not yet established. Both gene expression datasets hold the potential for further investigations.

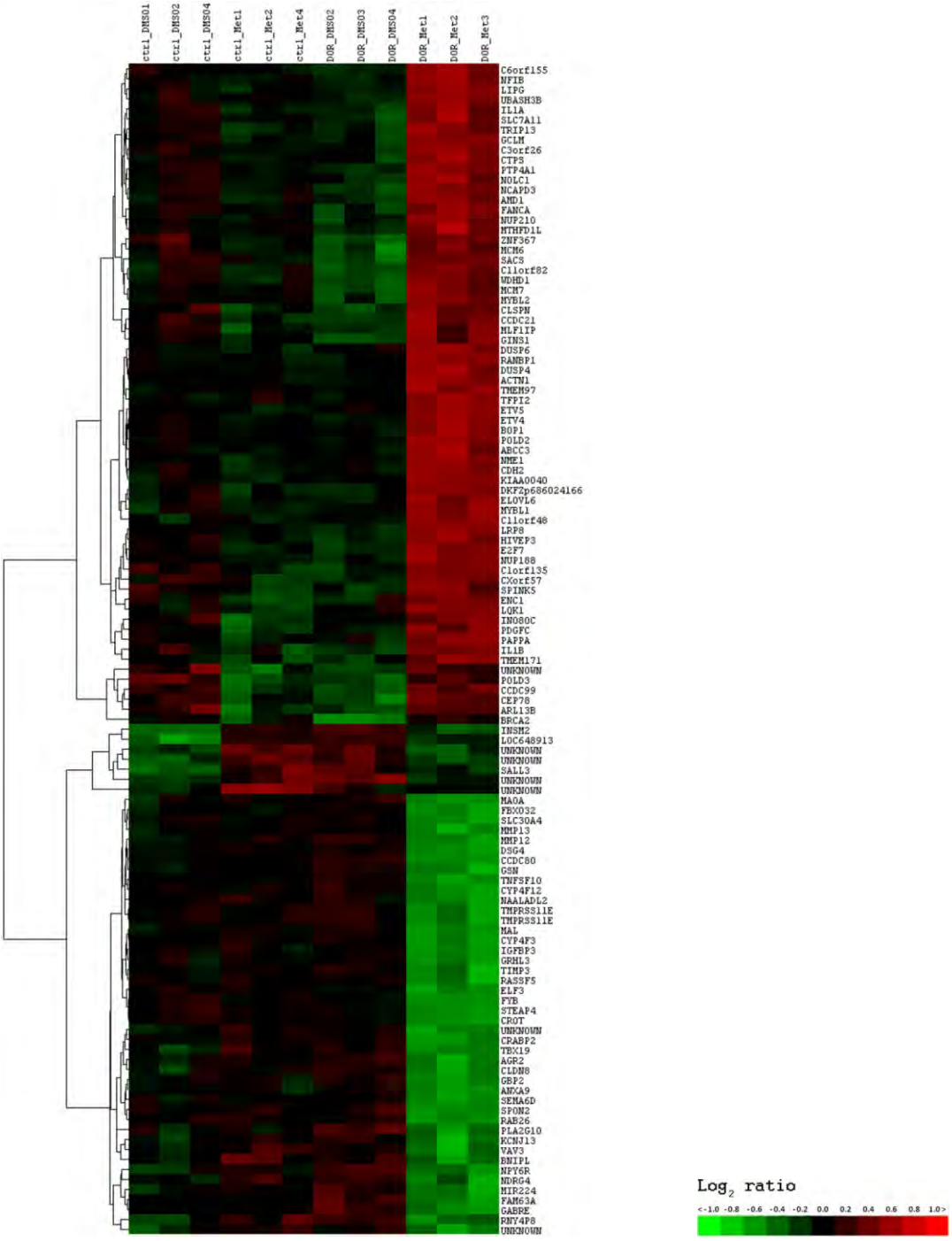


Figure 28 - Overview of transcriptome changes after 12h treatment with DOR agonist [Met^5]-enkephalin

Hierarchical clustering of 117 genes altered by the DOR-SNC80 interaction using a linkage algorithm was performed and visualized in a heat map. Horizontal stripes represent genes, and columns show experimental samples. Clustering was performed only on genes, whereas samples were ordered by treatment and cell line. Logarithmic values are shown in the heat map using red and green colour codes for up- and downregulation, respectively. Black indicates no change.

III.8. The transcription factor POU2F3 is involved in DOR-mediated regulation of keratinocyte differentiation

In order to resolve the involvement of DOR mediated signalling in epidermal homeostasis and especially KRT 10 regulation, the analysis of the transcription factor POU2F3 was advanced. This protein POU2F3 is involved in the transition from the basal to the spinous keratinocyte phenotype, as well as in the regulation of KRT 10 and IVL expression in accord with the observed results for the early phase of differentiation in HaCaT cells (Figure 25) (Andersen et al., 1993b; Andersen et al., 1997; Welter et al., 1996b). Indeed, in comparison to control cells DOR-overexpressing HaCaT (Figure 29A) and N/TERT-1 (Figure 30A) cells showed aberrant POU2F3 expression during the process of differentiation. Cells were grown to confluence in normal growth medium and differentiation induced by growth factor withdrawal with concurrent addition of SNC80 in HaCaT and [Met^5]-enkephalin in N/TERT-1 cultures to the culture medium. mRNA expression of POU2F3 was analysed by quantitative real-time PCR. In HaCaT cells, a significant repression of POU2F3 expression after four hours until eight hours after ligand addition can be detected. With advancing differentiation, the repression is abrogated and POU2F3 is slightly induced in both control and DOR-overexpressing cells (Figure 29A). In HaCaT cells, the observed POU2F3 expression pattern was in accordance with the previous observed regulation of KRT 10 and IVL (Figure 25). The literature reported a positive effect of POU2F3 on KRT 10 and a negative effect on IVL expression (Andersen et al., 1993a; Welter et al., 1996a). The repression of POU2F3 in the HaCaT model system therefore showed a decreased KRT 10 and increased IVL expression at the analysed four hour time point.

If the DOR antagonist naltrindole was applied at the same time, the significant 50% reduction of POU2F3 mRNA level at four hours after addition of SNC80 could be reversed. This shows a clear correlation of DOR activation and POU2F3 mRNA repression (Figure 29B). Further, it was of interest if DOR mediated ERK signalling is involved in POU2F3 regulation. For this

purpose, the MEK 1 inhibitor PD98059 was added to the culture. In the absence of ERK activation SNC80 mediated DOR signalling was no longer able to repress POU2F3 expression. Furthermore, inhibition of ERK resulted in an induction of POU2F3 expression indicating a direct correlation of ERK activity and POU2F3 expression (Figure 29C).

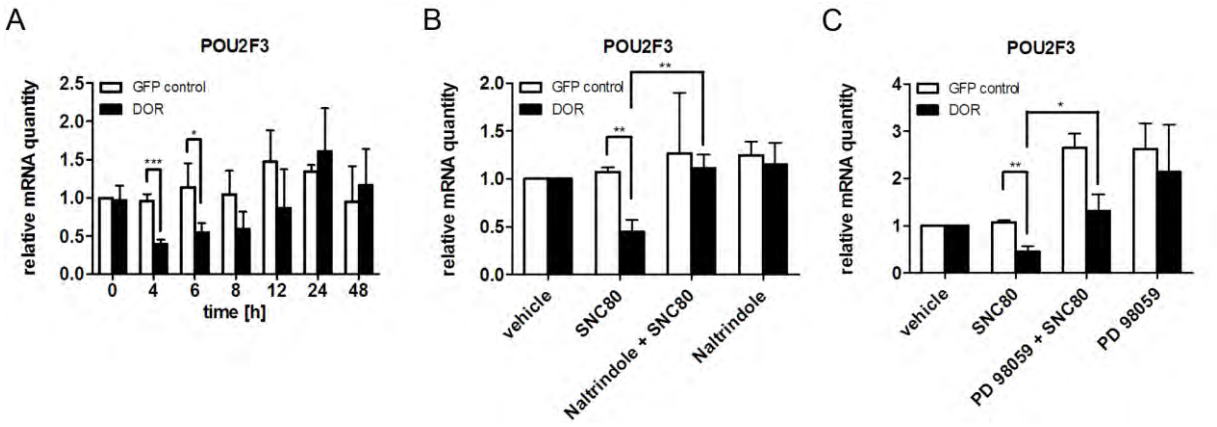


Figure 29 - POU2F3 is an early target gene of DOR mediated ERK activation in HaCaT cells

(A) HaCaT cells were grown to confluence and subjected to growth factor withdrawal induced differentiation. RNA was extracted at indicated time points and subjected to real-time PCR quantification. Within 4 h after addition of DOR specific ligand SNC80 to the cultures a strong downregulation of POU2F3 can be observed in DOR-overexpressing HaCaT cells while expression in control cells remains unchanged. Within 12 h of incubation the expression of POU2F3 goes back to the initial state and further increases while differentiation progresses. (B) HaCaT cells were subjected to differentiation in the presence of 100 nM DOR agonist SNC80, 5 min preincubation with 10 μM of the antagonist naltrindole prior to SNC80 addition, the vehicle control or antagonist alone. Cells were incubated for 4 h before RNA was extracted and subjected to quantitative real-time PCR. Naltrindole could effectively block the SNC80 mediated downregulation of POU2F3. This confirms that the regulation of POU2F3 by SNC80 is DOR specific. (C) HaCaT cells were subjected to differentiation in the presence of 100 nM DOR agonist SNC80, 30 min preincubation with 20 μM of the MEK1 inhibitor PD98059 prior to SNC80 addition, the vehicle control, or PD98059 alone. Cells were incubated for 4 h before RNA was extracted and subjected to quantitative real-time PCR. The inhibition of ERK could effectively reverse DOR mediated POU2F3 downregulation, indicating an ERK-dependent, DOR mediated mechanism. At the same time control cells subjected to double treatment and DOR cells with inhibitor treatment alone show a 2.5 fold induction of POU2F3 mRNA expression. The graphs represent the mean of three independent experiments. Error bars indicate standard deviation. t-test results: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

In N/TERT-1 cells, no effect on POU2F3 expression after four hours of SNC80 treatment in DOR-overexpressing cells was observed. This indicates that POU2F3 is not an early target gene in N/TERT-1 cells as compared to HaCaT cells. Hence, the protocol for N/TERT-1 cells was modified in conjunction with the previously observed time course of KRT 10 expression upon induction of differentiation. At day one after induction of differentiation, DOR activation was associated with a downregulation of POU2F3 mRNA, while control cells

induced POU2F3 1.7 fold compared to day zero expression. The relative mRNA expression level of POU2F3 at day seven was only seven fold greater in DOR-overexpressing cells, but 21 fold increased in control cells (Figure 30A).

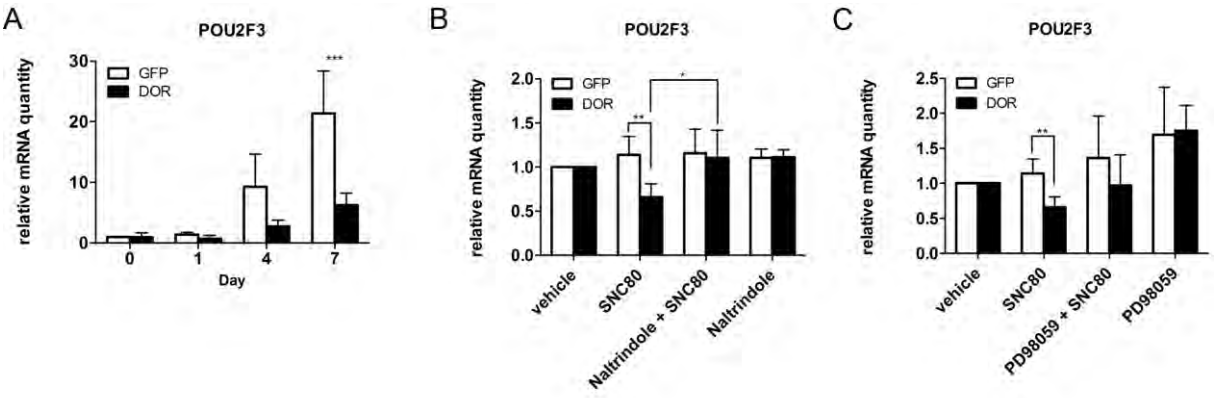


Figure 30 - POU2F3 is a late response target gene of DOR-mediated signalling in N/TERT-1 cells

(A) Keratinocytes were grown to confluence and differentiation was induced by removal of growth factors. Cultures were kept under the influence of [Met^5]-enkephalin during further cultivation for 10 days. During the time course of differentiation, POU2F3 expression was analysed by quantitative real-time PCR. Strong differentiation associated induction in control cells can be observed, in contrast to DOR-overexpressing cells. Expression is represented after normalization to RPL13a as mean values ± SEM of three independent experiments. Vehicle-treated control cells were used as reference. Two-way ANOVA analysis, *** $p < 0.001$. (B) Keratinocytes were differentiated for one day in the presence of 100 nM SNC80, 10 µM of the DOR-specific inhibitor naltrindole, or vehicle control. Expression of POU2F3 was analysed by quantitative real-time PCR and relative quantity is represented after normalization to RPL13a expression using the respective vehicle control as reference. A successful reversion of DOR mediated repression of POU2F3 by inhibition of DOR signalling through naltrindole can be observed. The graph represents the mean ± SEM of five independent experiments. t-test reveals ** $p < 0.01$ and * $p < 0,05$. (C) Keratinocytes were differentiated for one day in the presence of 100 nM SNC80, 20 µM of the ERK inhibitor PD98059, or vehicle control. Expression of POU2F3 was analysed by quantitative real-time PCR and relative quantity is represented after normalization to RPL13a expression using the respective vehicle control as reference. ERK inhibition can partially reverse DOR mediated POU2F3 repression. ERK inhibition alone leads to an increase of POU2F3 expression. The graph represents the mean ± SEM of four independent experiments. t-test reveals ** $p < 0.01$.

POU2F3 regulation in N/TERT-1 cells was as well specifically DOR-mediated and could be blocked by the DOR antagonist naltrindole. Cells were pre-incubated with 10 µM naltrindole for five minutes prior to 100 nM SNC80 addition to efficiently block DOR activation. During 24 hours of incubation, POU2F3 inhibition could be reverted by naltrindole, resulting in similar expression levels in control and DOR-overexpressing cells after one day of differentiation (Figure 30B). To test whether the regulation of POU2F3 was mediated by ERK activation in the same way as in HaCaT cells, we blocked the activation of ERK and analysed

again the expression level changes of POU2F3. The prevention of ERK 1/2 activation by pre-incubation with the MEK 1 inhibitor PD98059 revealed a strong correlation between POU2F3 expression and ERK 1/2 activity. In DOR-overexpressing cells, PD98059 abolished SNC80-mediated POU2F3 repression (Figure 30C). The complete inhibition of the ERK 1/2 MAPK signalling resulted again in the induction of POU2F3 mRNA expression in both control and DOR-overexpressing cells. This indicated, in both keratinocyte cell lines, the positive regulation of a POU2F3 repressor by DOR-mediated activation of ERK 1/2, which is released upon ERK 1/2 deactivation.

III.9. DOR signalling impairs stratification and homeostasis in a reconstructed-epidermis model

To further investigate the influence of the DOR on epidermal regeneration and homeostasis, a reconstructed tissue with regular features of epidermal differentiation and morphogenesis was generated using organotypic skin cultures (Bell et al., 1983; Stark et al., 1999). Contracted collagen lattices with incorporated dermal fibroblasts were used as dermal equivalents and either control N/TERT-1 or DOR-overexpressing cells were seeded on top for culture at the air-liquid interface, in order to induce stratification, for 14 days. DOR-overexpressing cells, in contrast to control cells, exhibited a poor capacity to form a fully-differentiated epidermis (Figure 31A). The previously observed slow proliferation rate of DOR-overexpressing cells (Figure 24) correlated with an atrophic (thinner) epidermis in organotypic cultures with DOR-overexpressing N/TERT-1 keratinocytes.

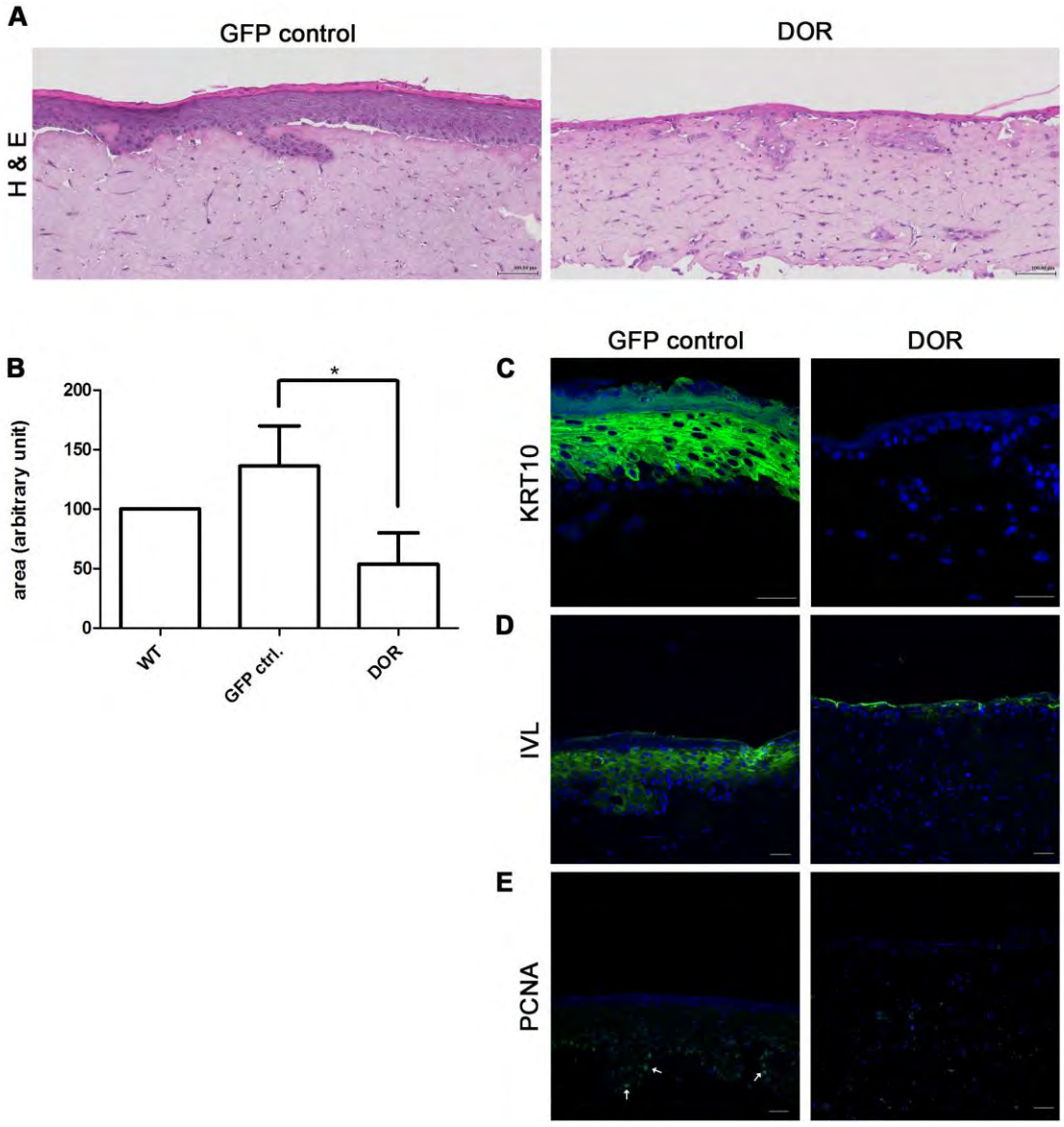


Figure 31 - DOR activity results in decreased epidermal thickness and atypical stratification

Organotypic cultures of N/TERT-1 cells transduced with control or DOR-GFP viruses were generated. (A) Haematoxylin and eosin (H&E) staining of organotypic raft cultures showed decreased epidermal thickness when DOR was overexpressed. (B) shows the quantification of the epidermal area generated by the different cell lines. The graph is generated from three independent experiments with at least two duplicates per experiment. Each experiment was normalised to the respective WT control. Error bars represent standard deviation. One-way ANOVA revelas * $p < 0.05$. (C) Immunofluorescence analysis of the differentiation marker KRT 10 shows no expression of KRT 10 in the epidermis with two to three layers of DOR-overexpressing keratinocytes but strong induction in the suprabasel layers of the control. Scale bars represent 50 µm. (D) IVL labelling revealed expression in DOR cultures and a strong expression in control cells. (E) Corresponding to the proper stratification, PCNA as a marker for proliferating cells was expressed in control cultures mainly in the basal layer. On the other side, no expression could be detected in DOR organotypic cultures in correlation with the atrophic phenotype. Scale bars represent 50 µm.

Accordingly, proliferating cell nuclear antigen (PCNA) could not be detected in DOR cultures, but was present in control cultures (Figure 31D). Immunofluorescence analysis of KRT 10 revealed the absence of this early differentiation marker in DOR-overexpressing cells (Figure 31B), indicating an impairment of epidermal differentiation, as observed in the keratinocyte monolayer differentiation experiments (Figure 26). Although only two to three cell layers were present, and despite the absence of KRT 10, a slight expression of IVL was detected in DOR-overexpressing organotypic cultures (Figure 31C). This indicates a deregulation of epidermal differentiation by DOR signalling if the DOR is active in basal keratinocytes. During normal skin homeostasis, the DOR might possibly interfere at the transition from spinous to granular layer keratinocytes where KRT 10 expression is switched off and IVL expression switched on, which would correlate well with the expression pattern detected by in situ hybridisation (unpublished data Mei Bigliardi-Qi).

CHAPTER IV

DISCUSSION AND PERSPECTIVES

IV.1. Discussion

The human skin is constantly exposed to numerous pathological processes, agents, and events. The response to these environmental assaults requires local as well as systemic mechanisms, in order to maintain skin and body homeostasis. This is accomplished by the neuro-endocrine system in the skin. Numerous studies highlighted the different protagonists of this system, for example the catecholaminergic, histaminergic, cannabinoid, cholinergic or steroidergic system, and others (Slominski et al., 2012). In the present study, the involvement of the opioidergic system in skin homeostasis was confirmed.

DOR functionality in keratinocytes dependents on the state of the cell

The presence of opioid receptors in human skin has been known for several years, but we are only just beginning to understand their function. The tools for detection of endogenous opioid receptor proteins have historically been insufficient, leading to a reliance on detection of mRNA expression for tissue analysis. *In situ* hybridisation shows that DOR is predominantly expressed in the more differentiated layers of the human epidermis, which correlates well with the expression of the endogenous ligand enkephalin. Slominski *et al*. (Slominski et al., 2011) demonstrated an *in vivo* compartmentalization of [Met^5]- and [Leu^5]-enkephalin antigens. They observed strong expression in differentiating epidermal and follicular keratinocytes in the outer root sheath. Additionally, they saw a selective expression in specialized fibroblasts of the perifollicular dermal sheath and follicular papilla, as well as myoepithelial cells of the eccrine glands. Further analysis of the regulation of enkephalin expression in cultured skin cells upon physical and biological stimuli, revealed an upregulation in response to UVB and lipopolysaccharide (LPS) exposure.

The localisation studies of the present manuscript illustrate, that the presence of ligand is not sufficient to induce the activation of the DOR in keratinocytes. Under standard keratinocyte culture conditions in low Ca^{2+} medium the majority of receptor is internalised and therefore not available for ligand induced cellular signalling. If the receptor was overexpressed in primary keratinocytes, no

response towards exogenous ligand SNC80 could be detected, despite the confirmation of solid receptor overexpression on mRNA level. Only after reliable localisation of the receptor by fusion to a GFP tag and the switch to the keratinocyte cell line HaCaT, which is routinely cultured in high Ca^{2+} medium, the influence of Ca^{2+} on receptor localisation was detected. The Ca^{2+} switch experiment in Figure 13 revealed that the receptor distribution, either to the cell surface or intracellular compartments, is a dynamic process in keratinocytes. The compartmentalisation is reversible by changing the Ca^{2+} concentration and shows a membrane localisation in high Ca^{2+} medium and an intracellular distribution in low Ca^{2+} medium. The accumulation in areas of the cell membranes with cell-cell contact, rather than at free edges of a colony, strongly suggests a function of the DOR in paracrine cell-cell communication in keratinocytes. Close proximity to desmosomal or other cellular adhesion structures might facilitate the recruitment of DOR interacting proteins and subsequent target molecules to modify cytoskeletal dynamics in order to promote cellular migration (Charbaji et al., 2012; Eisinger and Ammer, 2008a; Huang et al., 2004; Pullikuth et al., 2007; Scott et al., 2006; Thomason et al., 2012).

The exact localisation upon internalisation of the DOR in low Ca^{2+} medium has not yet been clarified. Several studies about the receptor maturation demonstrated the involvement of the endoplasmatic reticulum (ER) in quality control, post-translational modification, and subsequent membrane localisation (Leskelä et al., 2009; Leskela et al., 2012; Markkanen et al., 2008; Petäjä-Repo et al., 2000; Petäjä-Repo et al., 2001; Petäjä-Repo et al., 2006). A nonsynonymous single nucleotide polymorphism (SNP) (c.80T>G) has been described for the DOR (Gelernter et al., 2000), causing a Phenylalanine substitution at position 27 of the amino acid sequence to a Cystein (p.Phe27Cys) in the receptor N-terminus. Both lentiviral constructs used for our overexpression studies encode for the Cys-27 variant, which was reported to show inherent compromised maturation. The Cys-27 variant accumulates in pre-Golgi compartments of the secretory pathway and has impaired targeting to the ER-associated degradation following long-term expression. In addition, the cell surface receptors of the Cys-27 variant internalized constitutively. However, the pharmacological characteristics and the maturation kinetics of this variant, in comparison to the Phe-27 variant, were identical and the current database reference sequence encodes for the Cys-27 variant (NM_000911.3: c.80G>T; NP_000902.3:

p.Cys27Phe; rs1042114) (Gelernter et al., 2000; Leskelä et al., 2009; Leskela et al., 2012). Based on the studies from U.E. Petäjä-Repo's group, the analysis of the distribution of the DOR-GFP fusion protein in keratinocytes in the present manuscript focused on the localisation to ER, endosomes, and lysosomes. Immnuofluorescence staining could neither confirm the accumulation of the DOR under low Ca^{2+} conditions in the ER nor in the lysosomal degradation pathway. Co-localisation signal with the ER marker calnexin did show an interaction of these two proteins, but the co-localisation signal was similar to the high Ca^{2+} condition, which does not indicate a difference in maturation (Figure 14). Furthermore, co-localisation with the early endosome marker EEA1 indicated a rather random co-localisation of DOR with endosomes, than an accumulation due to constitutive internalisation. A lysosomal distribution of DOR-GFP could be demonstrated as well, but only explained the localisation of a subpopulation of receptor. In order to convincingly characterize the subcellular localisation in low Ca^{2+} cultured keratinocytes, further studies with cellular organelle marker proteins are necessary. Additionally, the exact redistribution behaviour over time and in correlation to the cell morphology changes need to be characterised, to explain the differences for DOR-GFP localisation under low Ca^{2+} observed.

Another point, concerning cellular localisation of the DOR, has to be taken into account as well. The localisation of the DOR into large dense-core vesicles (LDCV), which allows stimulus-triggered exocytosis of the receptor, has been reported. This could contribute to control the receptor functionality (Guan et al., 2005; Wang et al., 2010). In low Ca^{2+} medium cell-cell contact through adherence junctions is reduced, therefore direct communication between cells might be less advanced than in high Ca^{2+} medium, with strong intracellular connections. Cell surface localisation of DOR might not be beneficial for the cell under low Ca^{2+} conditions and is therefore abolished, but could be established within an hour, as demonstrated by the Ca^{2+} shift experiment (Figure 13). Additionally, it has been shown, that the GFP tag might interfere with recognition of sorting signals for the LDCV localisation of DOR (Wang et al., 2010). This does not explain the differences observed under low and high Ca^{2+} culture conditions. Though, the interaction of DOR and the sarcoplasmatic reticulum Ca^{2+}-ATPase 2 (SERCA2b) has been shown to influence receptor membrane localisation. Thereby, a low local luminal ER Ca^{2+} concentration might trigger the interaction with SERCA2b and would

delay the folding, until the whole polypeptide has been translated. The DOR precursors associate simultaneously with SERCA2b and calnexin in a dynamic manner, that is regulated by Ca^{2+} (Burdakov et al., 2005; Pitonzo et al., 2009; Tuusa et al., 2007; Tuusa et al., 2010). The Ca^{2+} dependent redistribution of the DOR might not be observed for the endogenous receptor because the impact of the GFP tag might trigger different maturation mechanisms. This question will be addressed further, once the appropriate tools for detection of endogenous opioid receptor proteins are available.

The DOR triggers immediate cellular responses and activation of ERK 1/2 signalling

The wound healing process is characterized by an acute inflammatory phase, a proliferation phase, and a remodelling phase. Several studies have described stimulatory effects of opioid receptors on the re-epithelisation process, whereby enhancement of keratinocyte migration was supposed to be a crucial function of opioid activity. *In vitro* both, the endogenous ligand β-endorphin and the exogenous ligand morphine, induce keratinocyte migration (Bigliardi et al., 2002; Kuchler et al., 2010; Wolf et al., 2009). The major second messenger pathways, coupled to the DOR-associated signalling, are known to activate some combinations of Ca^{2+}/PLC, β-arrestin/MAPK, and Rho family GTPases (Bian et al., 2012; Kam et al., 2003), which subsequently results in cell morphology changes. Using the xCELLigence system, variations of the cellular impedance, reflecting morphological changes, can be detected in real-time. This system allows the measurement of the cumulative effect of multiple signalling pathways (Atienza et al., 2005; Yu et al., 2006). In order to confirm the impact of the DOR agonists on keratinocyte morphology and intracellular signalling, this technique was applied. The sensitivity of the assay shows clearly, that keratinocytes are very responsive to mechanical manipulations, which partially masks the DOR mediated response. However both, the endogenous opioid [Met^5]-enkephalin and the exogenous opioid SNC80, are able to produce a solid change of the cell index (CI). The DOR specific antagonist naltrindole significantly reduces these changes. On the other side, naltrindole itself has a positive impact on the measurements of the CI and might therefore act as partial agonist in certain aspects of the intracellular signalling mechanisms of DOR, to influence cell morphology. The xCELLigence system clearly demonstrates that DOR activation has a short-term effect on keratinocytes, if they express sufficient amount of

receptor on the cell surface. The observed response was maximal after about 40 minutes. The system enables to define the time points for selective analysis and could be used to test the properties of new substances. However, it does not allow conclusions about the exact mechanisms behind the response.

Therefore, a major part of the present work was to elucidate the molecular signalling mechanisms of activated DOR in human keratinocytes. From previous studies in other overexpression systems, for example with HEK293 cells, it was known, that DOR activation is capable of causing downstream MAPK signalling, and especially ERK 1/2 phosphorylation and activation (Audet et al., 2005; Eisinger and Ammer, 2008a; Eisinger and Ammer, 2008b; Eisinger and Ammer, 2009; Fryer et al., 2001). But different cell types may vary in the composition of downstream molecules and might then experience a different response following DOR activation (Chen et al., 2006; Gross et al., 2006; Shahabi et al., 2006). The current study reveals, that DOR-mediated signalling in keratinocytes does involve the ERK 1/2 MAPK pathway. A transient signal of ERK 1/2 activation was seen both with the endogenous DOR peptide agonist [Met^5]-enkephalin, and the synthetic DOR-specific alkaloid ligand SNC80 in two different keratinocyte cell lines. In HaCaT cells [Met^5]-enkephalin was a stronger activator of the ERK 1/2. Because [Met^5]-enkephalin is a less-selective endogenous peptide agonist compared to the highly DOR-selective agonist SNC80, additional activation of μ-opioid receptor subtypes present at low level in HaCaT cells might enhance ERK signalling (Lecoq et al., 2004). N/TERT-1 cells show a higher basal ERK activity that might mask the DOR-mediated activation in the Western blot analysis, which results in a differently observed ERK response towards the DOR agonists. The activation of ERK in DOR-overexpressing cells is clearly receptor mediated, as shown by the antagonisation with naltrindole. The activation of other signalling pathways, namely p38 and AKT, was excluded by Western blot. The ERK 1/2 pathway is therefore the major pathway activated by DOR-mediated second messenger signalling in keratinocytes. The exact profile of second messengers involved, has not yet been identified and might include β-arrestin-mediated signalling, as well as transactivation of EGF receptor, via a PKC and integrin dependent mechanism (Eisinger and

Ammer, 2008a). PKC as target molecule of DOR signalling in keratinocytes has been confirmed in preliminary experiments but further studies are necessary to define the exact signalling cascade.

The DOR-mediated signalling affects keratinocyte proliferation and differentiation

A major interest of this project was to investigate the functional consequences of the DOR activation in keratinocytes. The data from an earlier report by Nissen *et al.* suggested an involvement in differentiation, through inhibition of transglutaminase type 1 and KRT10 expression. Additionally, they observed an inhibition of proliferation in the order of 10-15% by application of [Met^5]-enkephalin to primary keratinocytes (Nissen et al., 1997). The current study now confirms and expands these data and enlightens the molecular mechanisms behind this DOR-mediated phenotype.

The observed effect that DOR-mediated signalling has on N/TERT-1 keratinocyte cultures is profound. It not only inhibits proliferation, but also impairs differentiation, leading to a deregulation of the epidermal stratification process. The morphological changes of DOR-overexpressing cultures reflect the phenotype of differentiating keratinocytes. They appear enlarged and flattened, forming fewer colonies in culture. Nevertheless, DOR activated keratinocytes are not inducing differentiation, which was confirmed on mRNA and protein level for the early differentiation marker KRT 10 (Figure 26). Keratinocytes were also not capable of inducing the expression of late differentiation markers IVL, LOR, and FLG *in vitro*. Studies of KRT 10 knock-out mice reported hyperproliferation of basal keratinocytes and an increase in KRT 6/16 and KRT 17 expression (Porter et al., 1996; Reichelt et al., 1997; Reichelt et al., 2002; Reichelt et al., 2004; Wallace et al., 2012). In our *in vitro* two dimensional models KRT 16 was not increasing, which corresponds to their reduced proliferation and correlates with the DOR KO mice phenotype of hyperproliferative KRT 6 positive wound edge keratinocytes (Bigliardi-Qi et al., 2006). The organotypic culture model indicated the induction of IVL during the stratification process and the HaCaT differentiation model clearly showed a short term induction of IVL upon DOR activation, while at the same time KRT 10 expression was repressed. In monolayer N/TERT-1 and HaCaT cultures a tendency to higher IVL expression in DOR-overexpressing cells under basal growth conditions could be observed. This indicates that DOR signalling is involved in KRT 10 repression and IVL induction during keratinocyte differentiation, and this might have an

impact on the transition from the spinous to the granular keratinocyte phenotype. The observed repression of the transcription factor POU2F3 plays a role in this regulation. It has been shown to activate KRT 10 and to inhibit IVL expression (Andersen et al., 1993; Welter et al., 1996). During the transition from spinous to granular layer the reduced expression of POU2F3 might be the trigger to switch off the KRT 10 expression and to induce IVL. Like that DOR regulation can be beneficial to the epidermal homeostasis.

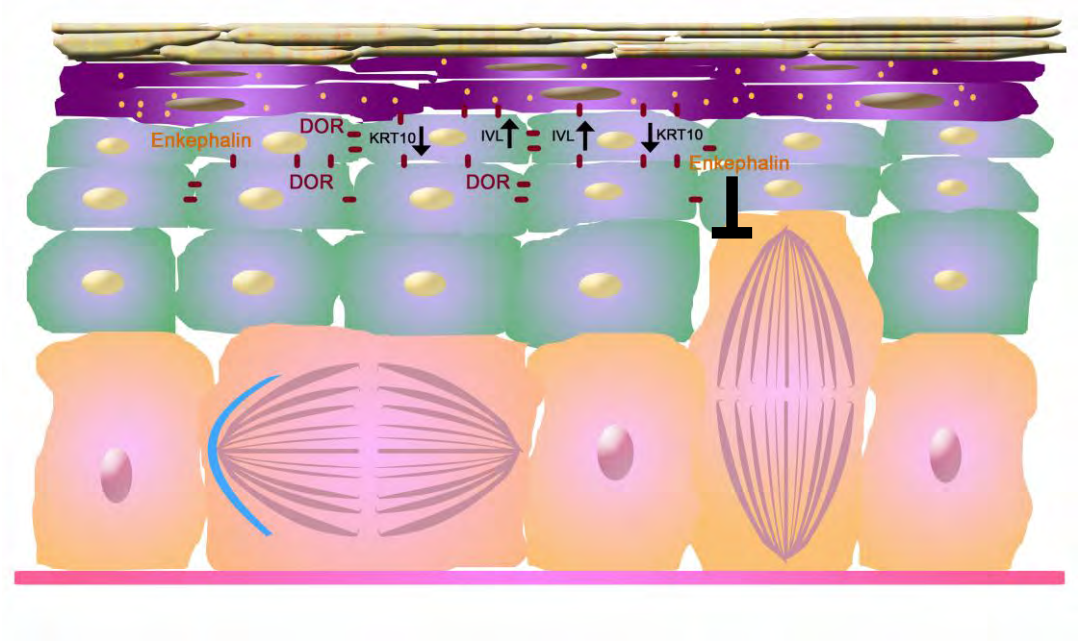


Figure 32 - Model for DOR mediated effect on skin homeostasis

The opioidergic system might be activated in normal skin by environmental factors and might help maintain the epidermal homeostasis. By inhibition of POU2F3 proliferation could be reduced and KRT 10 expression repressed, while at the same time IVL expression is induced in the upper epidermal layer. This might serve as a protection mechanism, to enhance the epidermal barrier and stop proliferation of basal cells until the perturbation is removed.

It is possible, that in our two and three dimensional *in vitro* models, the altered early differentiation phase in DOR-overexpressing N/TERT-1 cells lead to a deregulation of the subsequent differentiation processes, abolishing LOR and FLG expression. According to the DOR expression pattern *in vivo*, the early phase might be executed normally, so that DOR only influences subsequent processes during epidermal differentiation. DOR-deficient mice showed increased expression of KRT10, possibly because of the expression in keratinocytes is not being switched off in their transition to the granular layer, leading to an atrophic epidermis (Bigliardi-Qi et al., 2006).

N/TERT-1 cells have the ability to differentiate *in vitro*, but differentiation of the monolayer does not reflect the exact *in vivo* situation. The loss of contact with the basement membrane was shown to be a strong trigger for a keratinocytes to commit to differentiation (Adams et al., 1989; Gebhardt et al., 2006; Watt et al., 2008; Wu et al., 2012). The applied model of confluence induced *in vitro* differentiation, including growth factor withdrawal, does not include this aspect. Of note, this cannot be mimicked in two dimensional cell cultures. Suspension induced differentiation would be an additional tool, that could help to explain the DOR phenotype (Gandarillas et al., 1999). Our monolayer keratinocyte culture represented more closely basal epidermal conditions and might not reflect the actual state of a keratinocyte expressing functional DOR. The timeframe of DOR expression during differentiation is not necessarily the same as found in the *in vitro* culture model.

It has been shown that the loss of p63 is connected to decreased proliferation and significantly reduced induction of KRT 10/1 in a HaCaT monolayer keratinocyte culture (Wu et al., 2012). The knock-down of p63 triggered an arrest in phase G0/G1 of the cell cycle, without affecting cell death. Furthermore, an upregulation of the cell cycle inhibitors p21 and p15 and a downregulation of c-Myc expression were observed. The p63 isoform ΔNp63 was thereby identified to indirectly effect c-Myc expression, via the Wnt/β-Catenin and Notch pathways. The comparison of genes, altered by either c-Myc knock-down or p63 knock-down, revealed 71 genes antagonistically regulated. These genes were associated with cell migration and cell adhesion and might lead to keratinocyte differentiation, via changes of keratinocyte adhesion to the BM. Of these 71 genes we saw four genes regulated in the same direction as observed in the Myc knock-down cells. This might indicate that upon DOR activation c-Myc activity is down regulated. The bioinformatic tools used for our gene expression study indicated indeed that c-Myc was a key factor in the regulation of DOR signalling target genes in HaCaT, and might be an important factor in the N/TERT-1 cultures as well. The involvement of DOR in keratinocyte migration has been shown in culture systems and direct molecular targets have partially been found (Charbaji et al., 2012). The influence on differentiation has partially been established with this work, but further analyses are necessary. It would further be of interest to investigate, if p63 expression is altered in DOR activated keratinocytes and how other identified factors influence keratinocyte differentiation.

Another important factor detected in the microarray studies was DUSP6. This protein belongs to a class of dual-specificity phosphatases, designated MKPs that dephosphorylate the MAPK protein ERK. DUSP6 blocks both, the phosphorylation and enzymatic activation of the MAP kinase ERK2 by mitogens. We saw an upregulation of this factor, indicating that the ERK pathway might not induce proliferation as a functional consequence of DOR activation. This corresponds as well to the observed phenotype in N/TERT-1 cells.

Relevance of the DOR function during the wound healing process

The work focused further on the transcription factor POU2F3, after it was identified as direct target of DOR activation. This protein is a critical factor in the complex regulatory network of differentiation. The POU2F3 protein is expressed in two isoforms. The full length isoform, Skn-1a, functions as a transcriptional activator, while the other, Skn-1i, is capable of repressing transcription (Andersen et al., 1993; Cabral et al., 2003; Hildesheim et al., 1999; Takemoto et al., 2010). POU2F3 has been shown to regulate KRT 10 as well as IVL *in vitro*, two molecules the expression of which is altered in DOR-overexpressing cells. It was further suggested that the full length isoform enhances epidermal stratification by promoting keratinocyte proliferation (Hildesheim et al., 2001). In correlation with Hildesheim et al. we observed low proliferation and reduced POU2F3 expression, accompanied by poor stratification in DOR activated keratinocytes.

Andersen et al. reported that POU2F3-deficient mice showed no obvious deviation from normal skin phenotype, probably due to compensatory mechanisms, and so its function was proposed to be more important for wound healing (Andersen et al., 1997). Wound edge keratinocytes are more migratory and flexible than homeostatic keratinocytes in order to efficiently close the wound. POU2F3 was suggested to be repressed in these cells, to enable this phenotype change, and the DOR might be involved in this process. DOR-deficient mice experienced delayed wound healing and showed hypertrophic wound edges. The wound edge keratinocytes in these mice might not have undergone the necessary molecular changes, such as DOR-mediated downregulation of POU2F3, in order to migrate properly and close the wound efficiently (Bigliardi-Qi et al., 2006). Further experiments are necessary to clarify this connection with DOR activity as well as POU2F3 regulation during wound

healing. Further, the relevance of the two different POU2F3 isoforms in the function of DOR mediated KRT10 regulation remains to be elucidated.

During the wound healing process the exchange of soluble mediators between the epidermis and dermis compartments is crucial for appropriate wound healing. The cytokine IL-1 has been shown to be involved in this interaction. IL-1 produced by keratinocytes increases the PPAR β/γ expression in dermal fibroblasts. This activates sIL-1Ra production, a natural antagonist of IL-1 signalling. The secreted antagonist will repress the IL-1 effect on keratinocytes and therefore attenuate their proliferation, to avoid hyperproliferation at the wound side (Chong et al., 2009). The signalling of IL-1 was also shown to be involved in MMP regulation, important during the ECM remodelling phase of the wound healing process. The induction of the antagonist sIL-1Ra modulates MMP 8 and 13 expression in fibroblasts and our gene expression studies indicated as well a downregulation of MMP12 and MMP13 in HaCaT keratinocytes upon DOR activation. This suggests, that DOR downregulates these MMPs and might therefore explain the delayed wound healing in DOR-deficient mice, that displayed an increased expression of MMP2 at the wound side. Increased MMP activity might alter ECM remodelling and lead to an aberrant migration of the wound edge keratinocytes. MMP12 has mainly been described as macrophage elastase and is involved in bacterial clearance (Houghton et al., 2009; Lanone et al., 2002). Though, the catalytic domain can recognize and cleave skin collagen type I and III (Taddese et al., 2010) and its expression has been described in psoriatic lesions (Suomela et al., 2001). MMP13 has been shown to regulate wound granulation tissue and is involved in keratinocyte migration, angiogenesis and contraction in mouse skin wounds (Hattori et al., 2009; Toriseva et al., 2012). If DOR alters the ECM remodelling process and influences the crosstalk between keratinocytes and fibroblasts during wound healing, it might help to reduce scar formation by fine tuning the wound healing process. The spatio-temporal pattern of DOR activation during wound healing might herby play an important role. Rook *et al*. reported that topical morphine inhibited wound closure. They found delayed recruitment of macrophages and myofibroblasts, a prolonged inflammatory phase, delayed progression into the proliferative state and increased residual scar areas. The initial delay was transient and was made up by an accelerated re-epithelisation (Rook et al., 2007; Rook et al., 2008; Rook et al., 2009). Accordingly, in the current study, some organotypic cultures

with DOR-overexpressing cells showed reduced contraction of the dermal collagen lattice, which indicates an altered dermal-epidermal interaction and less myofibroblast formation. Together with the previously described DOR-mediated alterations of keratinocytes, this might indicate that DOR should not be activated in the early phases of wound healing, but its activity might be beneficial in the remodelling phase of the wound healing. The present study suggests that the DOR is expressed and activated in a spatially and temporally controlled manner in human skin cells. Deregulation will lead to alterations in epidermal differentiation and wound healing. The application of exogenous opioidergic drugs might therefore be helpful if applied to the right target cells and during the correct stages of wound healing, but importantly, this study highlights the fact that they may also be ineffective or harmful. Wounds are heterogeneous and the knowledge of the exact expression pattern of DOR during the normal wound healing process in different models would help to define the right moment for opioid treatments in the clinic. These experiments using human keratinocyte cell lines prove that opioids are not only involved in pain regulation during wounding but also in processes of wound healing and skin homeostasis.

Relevance of the DOR function in skin photoaging

The functions of DOR suggest that its activity could protect from photoaging. UV radiation modulates the release of MMPs and could change the structural integrity of the dermis (Kossodo et al., 2004). Destruction of the dermal collagen results in the formation of wrinkles. Slominski *et al.* described the release of the endogenous DOR agonist enkephalin upon UV radiation in cultured keratinocytes (Slominski et al., 2011). If at the same time the receptor is functionally active on the keratinocytes, this might start a signalling cascade, resulting in reduced MMP activity. This suggests a protective mechanism of DOR activity against UV induced aging. At the same time, DOR mediated inhibition of differentiation and the growth arrest of keratinocytes might protect from cancer formation. The activation of the ERK MAPK pathway can modulate different anti-proliferative events, such as apoptosis, autophagy, and senescence, depending on the stimulus and cell type. ERK mediated apoptosis and autophagy have been shown in keratinocytes by Lee *et al.* (Lee et al., 2005; Lee et al., 2006) in apoptosis models using DNA-damaging agents. Most likely, the intrinsic apoptotic pathway,

characterized by the release of cytochrom c from mitochondria and subsequent activation of the initiator caspase 9, is involved in this mechanism. Thereby a sustained ERK activity was important, which might not be initiated by DOR itself, but by the UV induced DNA damage. DOR signalling might modulate the signalling pathway to enhance apoptosis after UV radiation and therefore protect from carcinogenic transformations.

Additionally, ERK kinase activity has been related to induction of senescence. In response to oncogenic hyperproliferative signals, primary cells can undergo cell cycle arrest, leading to premature oncogene-induced senescence (Collado et al., 2007). This process is often triggered by constitutivly active forms of Ras, PAK4, Raf or MEK (Cagnol et al., 2010). Our four hour genexpression study indicated a downregulation of B-Raf in DOR activated HaCaT cells, and it would be of interest, if this could also be detected in N/TERT-1 cells. N/TERT-1 cells are closer to primary keratinocytes and might be more sensitive to oncogene-induced senescence. However, they are p16/INK4a-deficient, express TERT, and are not expected to induce senescence (Rheinwald et al., 2002). Keratinocytes have been shown to induce senescence independent of the telomere status but this process was p16/INK4a- and p53 dependent. However, in human fibroblasts inhibition of p16 or p53 was not sufficient to reverse senescence and might indicate that cells do not depend on these factors to induce senescence (Kuilman et al., 2008; Michaloglou et al., 2005; Zhuang et al., 2008).

Conclusion and Perspectives

The DOR is an important regulator of keratinocyte function, controlling the proliferation-differentiation state, adhesion and migration of the cells. In the present work, the molecular mechanism driving inhibition of keratinocyte differentiation, by stimulation of the DOR, were demonstrated. Thereby, the ERK 1/2 MAPK signalling pathway, activated by the DOR, inhibits the transcription factor POU2F3. This impairs epidermal stratification by inhibiting proliferation and the expression of differentiation-related genes.

It was hypothesised, that this signalling cascade is involved in spinous-to-granular layer transition of keratinocytes during the process of stratification. Furthermore, the spatio-temporal morphological change of wound edge keratinocytes could be regulated by this pathway. This process is accompanied by further regulatory mechanisms, possibly influencing dermal-epidermal communication and ECM remodelling. A combination of several factors will determine the overall effect of the DOR during the wound healing process. Other functions, for example during photoaging, could also be assumed. Both, the exact involvement in wound healing and photoaging remain to be explored.

DOR cells are more motile and display altered expression of cellular adhesion molecules *in vitro*. These phenotype changes positively contribute to the wound healing process. Wound models could help to further explore these changes *in vivo*. Because the epidermal-dermal interaction is important during wound healing and photoaging, this has to be taken into account for future studies. The MMPs altered by the DOR on mRNA level, are supposed to target collagen type I or elastin fibres. It would be of interest to investigate alterations of these fibres, during the wound healing process or after UV radiation and to establish the correlation to DOR activation.

The limit of tools for endogenous DOR detection complicates the detailed characterisation of its expression pattern and cellular localisation. It is important to understand the *in vivo* triggers for DOR membrane localisation, in order to predict if cells will be responsive to opioid drugs. In certain pathological conditions DOR might be deregulated, leading to an enhancement of the symptoms.

The present manuscript revealed a Ca^{2+} dependent cell surface localisation. It was evident that N/TERT-1 cultures under low Ca^{2+} had less pronounced phenotypes. Proliferation was not different than in control cells and only a mild delay in keratinocyte differentiation could be detected. There are indications that other factors influence DOR function in keratinocytes as well. Our current culture conditions involve many unknown factors. In order to move away from the overexpression system, towards the detection of endogenous DOR functions in keratinocytes, we need to better understand the DOR. Primary keratinocytes in culture are very heterogeneous. Soluble factors and cell-cell contacts strongly influence the expression pattern of cell surface receptors, cytokines or hormones. Even *in vitro*, these factors are difficult to control and need therefore to be characterized. *In vivo*, further interaction levels are added to the system. The peripheral nervous system, immune cells and fibroblast of the dermis secrete many molecules, altering the keratinocytes microenvironment and therefore their state. Fibroblast-keratinocyte co-cultures could help to identify soluble factors. Single cell PCR approaches might be used to characterise subpopulations of keratinocytes. There are still many unknown variables that need to be resolved, before we can address the effective clinical application of opioid treatments in skin therapy.

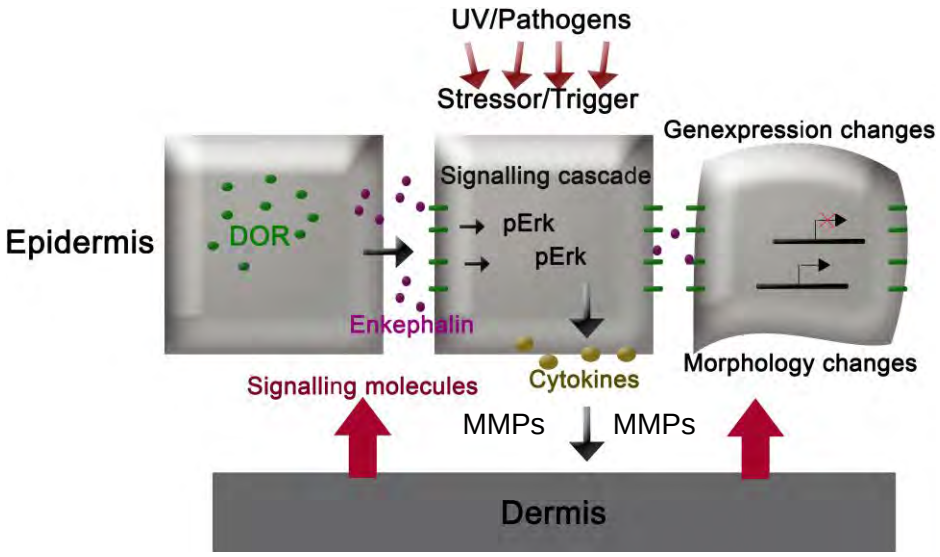


Figure 33 - Model for keratinocyte modulation upon triggered activation of DOR signalling

After an external trigger, DOR will be localised to the cell surface and the endogenous agonist enkephalin will be secreted. In a paracrine and autocrine mechanism DOR signalling will be activated in keratinocytes leading to alterations in gene expression and cell morphological changes. Keratinocytes secrete cytokines, such as IL-1, leading to a crosstalk with the dermal compartment and induction of remodelling processes. At the same time keratinocytes undergo morphological changes in order to migrate and adhere differently, necessary to efficiently respond to the changed environment.

CHAPTER V

MATERIAL AND METHODS

V. MATERIAL AND METHODS

V.1. Reagents

V.1.1. Chemicals

If not otherwise stated, all standard laboratory chemicals were of standard analytical (p.a.) grade and were purchased from Sigma-Aldrich (Switzerland, Singapore), Merck (Switzerland, Singapore), Applichem (Singapore), Promega (Switzerland, Singapore) or 1stBase (Singapore).

Opioid ligands SNC80 and Naltrindole were from Tocris Biosciences and Met-Enkephalin from Sigma Aldrich. The MEK 1 inhibitor PD98059 was purchased from Promega.

V.1.2. Reaction kits

The indicated reaction kits were used according to the manufacturer's instructions. Any changes to the protocols are mentioned in the corresponding methods section. The QIAshredder, RNeasy Mini Kit, RNase-Free DNase Set, QuantiTect SYBR Green PCR Kits, Effectene Transfection Reagent, and the Plasmid Purification Kit were from Qiagen®. The reverse transcription PrimeScript™ RT-PCR Kit was purchased from TaKaRa.

V.1.3. Plasmids and constructs

Plasmids and expression vectors used in this study are listed in Table 2.

Table 2 - Plasmids and constructs used in this work

Plasmid	Description	Source/Reference
hDOR-GFP in TRIP-PGK-IRESNEO-WHV	hDOR with C-terminal fusion to GFP	T. Baumann, M. Bigliardi-Qi NCBI entry NM_000911
psPAX2	Lentiviral packaging	Prof. C. Widmann, Lausanne, Switzerland
pMD2.G	Lentiviral envelope	Prof. C. Widmann, Lausanne, Switzerland
hDOR-GFP in pEZ-Lv122	hDOR with C-terminal fusion to GFP	EX-A1155-Lv122 GeneCopoeia NCBI entry U10504

V.1.4. Antibodies

Primary antibodies used in this work are listed in table 3.

Table 3 - Primary antibodies used in this work

Antibody	Supplier/source	Species	Clone	Reference n°
α-p44/42 MAPK (ERK 1/2)	Cell Signaling	rabbit monoclonal	137F5	4695
α-Phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204)	Cell Signaling	rabbit monoclonal	197G2	4377
α-GFP	abcam	rabbit polyclonal		ab290
α-Keratin 10	abcam	mouse monoclonal	DE-K10	ab9026
α-Keratin 10	Thermo Fisher Scientific (Neomarkers)	mouse monoclonal	DE-K10	MS-611
α-Involucrin	Thermo Fisher Scientific (Neomarkers)	mouse monoclonal	Sy5	MS-126
α-PCNA	Santa Cruz Biotechnology	rabbit polyclonal		sc-7907 (FL-261)
α-Desmoplakin	D. Garrod, University of Manchester, UK, E. Birgit Lane, IMB, Singapore	mouse monoclonal	11-5F	(Parrish et al., 1987);
α-α-Tubulin	Sigma-Aldrich	mouse monoclonal	DM 1A	T9026
α-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Life Technologies	mouse monoclonal	3E8AD9	A21994
α-Calnexin	abcam	mouse monoclonal	AF18	ab31290
α-EEA1	BD Transduction Laboratories; Frederic Bard, IMCB, Singapore	mouse monoclonal	14/EEA1	610457
α-LAMP-1	Developed by J.T. August and J.E.K. Hildreth; Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology; E. Birgit Lane, IMB, Singapore	mouse monoclonal	H4A3	(Hildreth et al., 1985)

Secondary antibodies

Secondary antibodies for immunofluorescence staining were from Life Technologies (Molecular Probes) goat anti-mouse AlexaFluor 594 conjugated, goat anti-rabbit AlexaFluor 488 conjugated, and goat anti-rabbit AlexaFluor 594 conjugated.

Secondary antibodies for immunoblot were goat anti-mouse IRDye 800 and goat anti-rabbit IRDye

700DX purchased from Rockland Immunochemicals as well as goat anti-rabbit AlexaFluor 680 conjugated from Life Technologies (Molecular Probes).

V.2. Methods

V.2.1. Cultivation of primary fibroblast

Human primary fibroblasts were isolated from infant foreskin. Briefly, the foreskin was trimmed of subcutaneous tissue and minced into small pieced using scissors. The tissue was transferred into a 0.25% dispase (Gibco) solution in Dulbecco's modified Eagle's medium (DMEM) and incubated overnight at 4 °C. The following day the epidermis was separated from the dermis. The dermis was then incubated in 0.25% Trypsin-EDTA (Gibco) at 37 °C for at least 40 min. The digested tissue was centrifuged for 10 min at 500 x g and washed once in DMEM medium. The remaining pellet was resuspended in 5 ml fibroblast growth medium containing DMEM (Gibco), 10% (v/v) foetal bovine serum (J R Scientific, PAA), 100 U/ml penicillin, and 100 µg/ml streptomycin (PAA) and platted 1:5 into separate 10 cm culture vessels. After overnight incubation at 37°C and 5% CO_2 medium was changed and remaining dermis pieces removed from the culture. Cultures were kept until 80% confluence and medium changed every second day. To detach adherent cells they were washed with PBS (without Ca^{2+} and Mg^{2+}) and incubated for up to 5 min with trypsin/EDTA or TrypLE™ Express (Gibco) at 37°C. Trypsination was stopped by addition of serum containing culture medium. The primary fibroblasts were used until passage 6.

V.2.2. Cultivation of keratinocytes

Primary keratinocytes

Primary human keratinocytes were isolated from infant foreskin. The foreskin was trimmed of subcutaneous tissue and minced into small pieced using scissors. The tissue was transferred into a 0.25% dispase solution in DMEM and incubated overnight at 4 °C. The following day the epidermis was separated from the dermis. The epidermis was then incubated in 0.25% Trypsin-EDTA at 37 °C for 30 min. The digested tissue was centrifuged for 10 min at 500 x g and washed once in keratinocyte

serum-free medium (K-SFM; Gibco). The remaining pellet was resuspended in 5 ml keratinocyte growth medium containing K-SFM (Gibco; 0.09 mM Ca^{2+}), supplemented with 5 ng/ml epidermal growth factor (EGF) and 50 µg/ml Bovine Pituitary Extract (BPE; Gibco) and platted 1:5 into 10 cm culture vessels. After overnight incubation at 37°C and 5% CO_2 medium was changed and remaining larger pieces removed from the culture. The cells were grown to 70% confluence at 37°C and 5% CO_2 and subcultured using 0.25% Trypsin-EDTA. Trypsinisation was stopped by removing the enzyme solution after centrifugation and approximately 1 x 10^4 per T75 flask were carried as stock. Cells were subcultured every 7 days and medium was changed every 2-3 days. The primary keratinocytes were used until passage 4.

HaCaT cells

HaCaT cells (Boukamp et al., 1988) were maintained in DMEM containing 10% (v/v) foetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. To detach adherent cells they were washed with PBS (without Ca^{2+} and Mg^{2+}) and incubated for up to 8 min with trypsin/EDTA or TrypLE™ Express at 37°C. Trypsination was stopped by addition of serum containing culture medium. Cells were subcultured every 2-3 days.

N/TERT-1 cells

N/TERT-1 (Dickson et al., 2000; Rheinwald et al., 2002) cells, developed at Dr. J. Rheinwald's laboratory (Harvard Medical School, Boston, MA, USA) are derived from neonatal foreskin and immortalized by transfection to express telomerase reverse transcriptase (TERT). They were cultured in K-SFM supplemented with 0.2 ng/ml EGF and 25 µg/ml BPE and an adjusted Ca^{2+} concentration to 0.4 mM, grown to 50% confluence at 37°C and 5% CO_2 and subcultured using TrypLE™ Express. Trypsinisation was stopped by removing the enzyme solution after centrifugation and approximately 1 x 10^4 per T75 flask were carried as stock. Cells were subcultured every 7 days and medium was changed every 2-3 days.

V.2.3. Plasmid Purification, cultivation of HEK 293 cells, lentiviral production, and viral transduction

Plasmid purification

For amplification of plasmid DNA the bacterial strain XL1-blue was transformed. Cultures were grown at 37°C in LB medium containing the appropriate antibiotic. Plasmids were purified from overnight cultures inoculated with a single colony using the Qiagen® plasmid purification kit following the manufacturer's instructions. The DNA concentration was determined by measuring the optical density of the sample at a wavelength of 260 nm (OD 260) using a spectrophotometer.

Cultivation of HEK 293 cells

HEK 293FT and HEK 293Ta cells were cultured in DMEM containing 10% foetal bovine serum, MEM Non-Essential Amino Acids (Gibco), and 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C and 5% CO_2. To detach adherent cells they were washed with PBS (without Ca^{2+} and Mg^{2+}) and incubated for up to 2 min with trypsin/EDTA or TrypLE™ Express at 37°C. Trypsination was stopped by addition of serum containing culture medium. Cells were subcultured every 2-3 days.

Lentiviral particle production

Production of recombinant lentiviral particles was modified from the method described by J.Y. Yang et al. (Yang et al., 2004). In brief, HEK293FT cells were co-transfected using the calcium phosphate DNA precipitation method (Jordan et al., 1996) with 20 µg of the lentiviral vector (TRIP-PGK-IRESNEO-WHV) containing the cDNA of interest (hDOR-GFP), 6 µg of the envelope protein-coding plasmid (pMD2.G), and 15 µg of the packaging construct (psPAX2) in a 10 cm culture dish. HEK293Ta cells were transfected with purified 1.5 µg of DOR plasmid (GeneCopoeia) and 1 µg of human lentiviral packaging and envelope vectors (psPax, pMD2.G) using Qiagen® Effectene Transfection Reagent, according to manufacturer's instructions. 48 h after transfection, the virus-containing medium was harvested and concentrated by ultracentrifugation at 22.000 rpm at 4°C for 2 hours and 16 min in a Beckman Coulter JS-24.38 rotor.

Titration of lentiviral particles

The titer of the viral stock was determined as described by (Barde et al., 2010). Briefly, target cells were transduced with a serial dilution of virus particles in a 12-well plate and incubated for 48 h. Cells were then harvested and fixed in 1% formaldehyde in PBS for 5 min. Fixed cells were washed and GFP expression was analysed using flow cytometry. Dilutions yielding 1% to 20% of GFP positive cells were used for titer calculations.

$$T\ /ml\ \frac{\left(\text{Number of target cells counted at day of plating}\ \frac{\text{P positi e cells}}{}\right)}{\text{olume of iral particles in ml}}$$

Transduction of target cells

Transduction of all keratinocytes (HaCaT and N/TERT-1 cell lines, primary keratinocytes) was performed at 30% confluence with a multiplicity of infection of approximately 10, in the presence of 10 µg/ml Polybrene (Millipore). After 24 hours incubation at 37°C in a 5% CO_2 humidified atmosphere, the viral particle-containing medium was replaced with fresh medium and cells were further cultured for at least 48 hours before being used in specific experiments.

V.2.4. Treatment of cells

For ligand treatment keratinocytes were grown in 6-well culture vessels until 90% confluence. Cells were starved of growth supplement for 6 h (HaCaT) or overnight (N/TERT-1), due to the different media compositions and the corresponding ligand was added for the respective time. Inhibitor PD98059 was added 30 min prior to agonist addition and the DOR antagonist Naltrindole 5 min prior to agonists. Treatment of cells was at a final concentration of 100 nM for SNC80, 100 nM [Met^5]-enkephalin, 10 µM Naltrindole, 20 µM PD98059, or DMSO vehicle control. SNC80 and PD98059 were dissolved in DMSO and [Met^5]-enkephalin and Naltrindole in water. DMSO (0.001 – 0.1% final) was added to control cells.

V.2.5. xCELLigence impedance measurements

A 96-well E-plate was filled with 50 µl of complete DMEM per well and the background was measured in the xCELLigence machine. 4000 HaCaT cells per well were added in a total volume of 100 µl and cell index was continuously measured every 15 min for 1 h followed by hourly measurements for two days. 10 µl of diluted ligand was added to the medium and the impedance was measured every minute for 3 h and then switched to hourly measurement for continues observation. The measurement was normalized to the time point before addition of ligands and the values for the cell index generated by the xCELLigence software extracted and analysed using GraphPad Prism 5.

V.2.6. Gene expression analysis

Extraction of total RNA

After the respective time of the treatment was over, the medium from 6 cm culture vessels containing 90% confluent HaCaT cells was aspirated and cells were once rinsed with PBS. 1 ml TRIZOL (Invitrogen) was added at room temperature and cell lysates collected in an RNase free microtube. Lysates were shortly vortexed, frozen in liquid nitrogen, and stored at -80 °C until further extraction. Samples were then thawed, 200 µl Chloroform added, shaked vigorously by hand for 15 s, and incubated at room temperature for 3 min. Microtubes were centrifuged at full speed for 15 min at 4 °C and the upper aqueous phase transferred into a new tube. 500 µl Chloroform were added to the aqueous phase, samples were vortexed, and centrifuged at full speed for 15 min at 4 °C. The upper phase was transferred into a fresh tube and subjected to another round of chloroform extraction. To the fresh aqueous phase an equal volume of 70% ethanol was added and the solution mixed by pipetting. The samples were loaded onto an RNeasy column (Qiagen®) and centrifuged for 15 s at 10,000 rpm at room temperature. The column was washed with 700 µl buffer RW1 from the RNeasy Mini Kit (Qiagen®) and centrifuged at 10,000 rpm for 15 s. this washing step was repeated a second time. The column was placed into a new collection tube and washed twice with 500 µl buffer RPE from the RNeasy Mini Kit (Qiagen®). After a final centrifugation for 2 min at 10,000 rpm RNA was eluted with 50 µl of water and put on ice. The RNA concentration was determined by the optical

density of the sample at 260 nm (OD260) using a spectrophotometer and chemical purity determined by the ratio of the 260 / 230 nm reading from the spectrophotometer.

RNA was further purified by sodium acetate precipitation. To 100 µl of RNA in water 10 µl of 3 M sodium acetate, pH 5.2 were added. Further, 2 µl Glycogene (5 mg/ml) and 275 µl cold 100% ethanol were added. Solution was mixed well and incubated at -80 °C for 40 min. Samples were centrifuged at 4 °C at full speed for 40 min. Supernatant was removed and pellet washed with 70% ethanol. Samples were centrifuged at full speed for 5 min at 4 °C and washed a second time with 70% ethanol. After centrifugation and removal of supernatant, the pellet was completely air-dried at room temperature, finally resuspended in water, and left on ice for 10 min. Samples were vortexed and quick spun and the RNA concentration determined by measuring the optical density of the sample at 260 nm (OD260) using a spectrophotometer.

Target preparation and hybridisation on microarray

RNA was further processed by the Lausanne Genomic Technologies Facility to prepare the probes for the Affymetrix whole transcriptome microarray analysis. The Ambion® WT Expression Kit was used to generate sense strand cDNA from total RNA for further fragmentation and labelling using the Affymetrix GeneChip® WT Terminal Labeling Kit according to the manufacturer's recommendations. The processed samples were hybridized on a human gene 1.0 ST array and the generated expression profile analysed. Three replicates for each condition (DOR-overexpression vehicle treated, DOR-overexpression agonist treated, GFP control cells vehicle treated, and GFP control cells agonist treated) were scanned, normalized and data transformed to logarithmic scale to the base 2 for statistical calculations in a 2x2 factorial design.

Statistical analysis of the microarray data

A linear model was built with the four experimental groups as factors and the comparisons of interest as contrasts were extracted. The adjusted p-values (controlling for FDR, false discovery rate) were computed separately for each comparison using the Benjamini-Hochberg method. Comparisons were

the DOR effect (DOR.DMSO vs CTL.DMSO), the agonist effect (CTL.agonist vs CTL.DMSO), the DOR effect in presence of agonist (DOR.agonist vs CTL.agonist), the agonist effect in presence of DOR (DOR.agonist vs DOR.DMSO) and the interaction ((DOR.aonist-DOR.DMSO) vs (CTL.agonist-CTL.DMSO)). The adjusted p-value was used to test for significant differential expression ($p < 0.05$). The data received from the interaction comparison were used for subsequent studies. The gene enrichment of functional pathways was performed using the MetaCore GeneGo software. Hierarchical clustering of expression patters was performed using Cluster 3.0 and JTree View programs (Eisen et al., 1998). The interaction ratios were shown by heat map using red and green colour codes for up- and down-regulation, respectively. The experiments were ordered by drug and cell type in columns of the heat map for visualisation of patterns following treatment with agonists.

V.2.7. Extraction of total RNA

Total RNA from cultured cells in a 6-well culture vessel was isolated using the RNeasy Mini Kit (Qiagen®) according to the manufacturer's instructions. The RNA concentration was determined by the optical density of the sample at 260 nm (OD260) using a spectrophotometer. If not used immediately, the RNA was quick frozen on dry ice or in liquid nitrogen and stored at -80 °C.

V.2.8. Reverse transcriptase PCR (RT-PCR)

Reverse transcription was performed according to the provided manual of the PrimeScript RT reagent Kit (TaKaRa). The reaction was performed in a total volume of 20 µl, containing 1 µg RNA, 25 pmol Oligo dT Primer, 50 pmol Random 6 mers, 1X PrimeScript buffer, and PrimeScript RT Enzyme Mix I. The reaction mixture was incubated at 37 °C for 20 min, followed by 85 °C for 5 s and cooling to 4 °C.

V.2.9. Quantitative real-time PCR

Quantitative PCR assays were carried out on a real-time PCR detection system (StepOne™ Real-Time PCR System or 7500 Fast Real-Time PCR System; Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) or QuantiFast SYBR Green PCR Kit (Qiagen®) in a reaction volume of 20 µl, employing approximately 30 ng of cDNA template. To evaluate the expression level of human KRT1, KRT10, IVL, and POU2F3 mRNAs, commercially available QuantiTect Primer Assays (Qiagen®, reference n° QT00014182, QT00017045, QT00082586, and QT00029057) were used. Primers for loricrin (LOR), filaggrin (FLG), Hypoxanthine phosphoribosyl transferase 1 (HPRT1), and Ribosomal protein L13a (RPL13a) are listed in table 4.

The RPL13a expression was used for normalization in all N/TERT-1 experiments and HPRT1 in all HaCaT experiments. Quantification was performed using the comparative 2-ΔΔCT method.

Table 4 - Real-time PCR primer used in this work

Name	Sequence	source
LOR forward	5'-TCA TGA TGC TAC CCG AGG TTT G-3'	(Kovacs et al., 2012)
LOR reverse	5'-CAG AAC TAG ATG CAG CCG GAG A-3	(Kovacs et al., 2012)
FLG forward	5'-GAA GAC AAG GAT CGC ACC AC-3'	(Kovacs et al., 2012)
FLG reverse	5'-ATG GTG TCC TGA CCC TCT TG-3'	(Kovacs et al., 2012)
RPL13a forward	5'-CTC AAG GTC GTG CGT CTG AA-3'	D. Hohl, Lausanne, Switzerland
RPL13a reverse	5'-TGG CTG TCA CTG CCT GGT ACT-3'	D. Hohl, Lausanne, Switzerland
HPRT1 forward	5'-TGA CAC TGG CAA AAC AAT GCA-3'	D. Hohl, Lausanne, Switzerland
HPRT1 reverse	5'-GGT CCT TTT CAC CAG CAA GCT-3'	D. Hohl, Lausanne, Switzerland

V.2.10. Extraction of proteins for Western blot analysis

After the appropriate treatment the reaction was stopped by aspiration of the medium following a two times wash step with ice cold PBS. Plates were either frozen in -80 °C and extracted at a later time point or immediately subjected to protein extraction. Cells were lysed in RIPA-like lysis buffer, comprising 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 1% IGEPAL CA-630, 0.1% SDS, 0.5% Sodium deoxycholic acid at pH 7.4, freshly supplemented with 1 mM DTT, 1 mM PMSF, 10 mM Na_3VO_4, and 1x protease inhibitor (Roche).

Proteins were quantified using the Bradford method (Bradford, 1976) with a colorimetric assay using

Protein Assay (Bio-Rad) according to the manufacturer's instructions.

Protein extracts for keratin detection were prepared by lysis of cells with a buffer containing 10 mM TRIS-HCL, 5 mM EDTA, 5 mM EGTA at pH 7.5, supplemented with 4% SDS, 1% Triton X-100, 160 mM DTT, 0.8 mM PMSF, 0.8 mM Na_3VO_4, and 1x protease inhibitor. Lysates were boiled for 5 min at 95 °C and kept at 4 °C until gel electrophoresis.

V.2.11. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

20 µg of protein per cell extract were separated on vertical, discontinuous SDS-polyacylamide gels (Laemmli, 1970). Samples were prepared by addition of 6x SDS sample buffer and heating at 95°C for 5 min. Depending on the required separation range the acrylamide concentration was adjusted to 10% or 12% in casting gel buffer. The composition of the stacking gel was 5% acrylamide in stacking gel buffer. Electrophoresis was performed with SDS running buffer at 30 mA per gel using Mini-PROTEAN® electrophoresis chambers (Biorad). The prestained molecular weight marker PageRuler Plus (Fermentas) was used as a size reference.

V.2.12. Immunoblot analysis (Western blot)

After protein separation via SDS-PAGE samples were subsequently transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting using the wet technique. Blotting was performed at 100 V for 1 h 32 min with the Mini Trans-Blot® Cell (BioRad). Membranes were next incubated for at least 30 min in TBS (20 mM TRIS, 150 mM NaCl, pH 7.5) blocking solution, containing 0.1% Tween-20 and 5% non-fat milk. The primary antibody was added and incubated either for 45 min at room temperature or overnight at 4 °C.

The ERK specific antibodies were incubated with a 1:1000 dilution in TBS containing 0.1% Tween-20 and 5% BSA overnight at 4°C. or the APDH (: 8) , the α-Tubulin (1:2000), and KRT10 (1:200) antibodies blots were incubated in TBS containing 0.1% Tween-20 and 5% non-fat milk overnight at 4°C (KRT10) or for 45 min at room temperature (APDH, α-Tubulin). Blots were then washed with TBS-Tween 0.1%, incubated with the appropriate secondary antibody (1:5000 dilution)

for 1 hour at room temperature and subsequently visualized with the Odyssey infrared imaging system (LICOR Biosciences). Quantification of the fluorescence signal was done using the Odyssey software and normalized to the background signal. Obtained values of integrated intensity were exported to an EXCEL file, and relative ERK phosphorylation was calculated by normalization to the total ERK signal and the respective vehicle control as reference for each group.

To reprobe a blotting membrane with a different primary antibody, the membranes were stripped off the bound antibodies. They were incubated for 2x 30 min at room temperature in stripping buffer containing 25 mM Glycine, 1.5% SDS at pH 2. Following extensive washing with TBS-T for 1 h, the membranes were blocked for 45 min in blocking solution before addition of the new primary antibody.

V.2.13. Immunocytochemistry and fluorescence microscopy

Primary keratinocytes, HaCaT, or N/TERT-1 cells were seeded into 24-well plates containing glass coverslips and subjected to the treatments indicated in the figures. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, washed twice with PBS, permeabilised and blocked using 0.3% Triton X-100 and 10% goat serum in PBS for 1 h at room temperature. The co erslips were incubated o ernight at 4°C with the corresponding primary antibodies (α-GFP 1:900, α-desmoplakin : , α-calnexin : 8 , α-EEA : 3 , and α-LAMP-1 1:100) diluted in PBS, 5% goat serum, 0.1% Triton X-100. After three washes in PBS, coverslips were incubated for 1 h with a 1:300 dilution of labelled secondary antibodies in PBS. The coverslips were then washed three times in PBS and stained with 10 μg/ml Hoechst 33258 (Sigma Aldrich), washed twice in PBS and mounted onto microscope slides (Fluorescence Mounting Medium, Dako)

HaCaT cells and primary keratinocytes from Figure were examined by conventional widefield fluorescence microscopy using a Zeiss Axiovision Imaging system equipped with a Plan-Neofluar 40x/1.30 NA oil-immersion lens.

Confocal images of stained HaCaT cells were captured as z-stacks and projected to a single image using an Olympus FV1000 microscope system with an UPLSAPO 100X/1.40 NA or UPLFLN

40x/1.3 NA oil-immersion lens.

V.2.14. Immunohistochemistry and confocal microscopy of organotypic cultures

Organotypic skin cultures were fixed in 10% neutral buffered formalin. Paraffin embedding, sectioning, and haematoxylin and eosin staining was provided by the Institute of Molecular and Cell Biology Core Histopathology Laboratory, Singapore. Five micron thick sections were deparaffinised and stained with haematoxylin/eosin for morphological analysis. For fluorescence imaging deparaffinised sections underwent heat-mediated antigen retrieval in citrate buffer at pH 6 following blocking with 10% goat serum in PBS, 0.5% Tween-20 and 1% BSA. Antibodies were subsequently added and incubated overnight at 4 °C. Slides were then washed in PBS/0.5% Tween-20 and incubated for 1 h with a 1:300 dilution of secondary antibody. After further washing steps, nuclei were counterstained with Hoechst dye (see above) for 5 min at room temperature and mounted with Dako Fluorescence Mounting Medium. Confocal images were captured as z-stacks and projected to a single image using an Olympus FV1000 microscope system with an UPLFLN 40x/1.3 NA oil-immersion lens and UPLSAPO 20x/0.75 NA dry lens. Haematoxylin and eosin staining was imaged using a Ariol high resolution fluorescence and brightfield slide scanner as service by the Institute of Molecular and Cell Biology Core Histopathology Laboratory, Singapore.

V.2.15. Incucyte proliferation assay

1000 N/TERT-1 cells per well were plated into a 96-well plate and allowed to adhere overnight. The next day, medium was changed to either vehicle control medium or 100 nM SNC80-containing medium, and the plate was placed into the Incucyte machine. The machine captured images of the cultures every hour with the 10x/1.49 NA dry objective lens. Percentage confluence was calculated from the images using the metrics from the Incucyte software. The doubling time was calculated in GraphPad Prism 5 from the obtained growth curve using a nonlinear regression model, and is displayed as mean with upper and lower limit.

V.2.16. Organotypic culture

A dermal equivalent was generated under sterile conditions by preparing a gel containing rat tail collagen type I (BD, reference n°354236) and dermal fibroblasts (100,000 cells per ml). 2 ml of this suspension were transferred into a cell culture insert (pore size 1 μm, BD) in a 6-well plate and incubated for three hours at 37 °C and 5% CO_2, until solidified. Dermal equivalents were cultured in A D medium: DMEM, and HAM's 2 medium (3:) supplemented with U/ml penicillin, and 100 μg/ml streptomycin (PAA), 10% foetal bovine serum (J R Scientific), 1% Glutamax (Life Technologies), 0.4 μg/ml Hydrocortisone (Sigma-Aldrich), 5 μg/ml Insulin (Sigma-Aldrich), 1.8×10^{-4} M Adenine (Sigma-Aldrich), 10 ng/ml EGF (Sigma-Aldrich), 5 μg/ml Transferrin (Sigma-Aldrich), and 2×10^{-11} M 3,3',5-Triiodo-L-thyronine (T3) (Sigma-Aldrich). After 48 h, N/TERT-1 cells (200,000 per culture) were seeded on top of the dermal equivalent and cultured under submerged conditions in FAD medium until a confluent keratinocyte monolayer had developed. Cultures were then shifted to air-liquid interface conditions by placing the culture insert into a deepwell plate (BD). Medium was changed to FAD medium without EGF supplementation and cultures were grown for 14 days. Subsequently, cultures were cut in half: one part was fixed in 10% neutral buffered formalin, while the other was frozen in Tissue-Tek O.C.T. compound (Sakura, USA) for further analysis.

V.2.17. Quantification of the epidermal area from organotypic cultures

The whole Haematoxylin and eosin stained tissue section was imaged using an Ariol high resolution fluorescence and brightfield slide scanner as service by the Institute of Molecular and Cell Biology Core Histopathology Laboratory, Singapore. The darker Haematoxylin and eosin staining represents the epidermis generated by the respective N/TERT-1 cells. Using ImageJ a threshold was defined to exclude all non epidermal tissue from the image and the remaining area was quantified. The experiment was performed in duplicates and repeated three times. For each experiment the mean of the GFP control and DOR cultures were normalised to the untransduced wild type control and the three independent experiments were combined for statistical analysis. A one-way ANOVA analysis with a Newman-Keuls post-hoc-test was performed in order to determine the p-value.

CHAPTER VI

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