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STUDIES ON THE ROLE OF THE CATHELICIDIN PEPTIDE LL-37 IN EPITHELIAL BIOLOGY

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To my family

ABSTRACT

The biology of epithelial immunity as well as of tissue repair is complex and highly regulated. A variety of molecules, cell types, and biological processes such as, cell differentiation, proliferation and migration, programmed cell death and antimicrobial mechanisms contribute to maintaining a balance between tissue damage and tissue repair. Different sources of evidence indicated that the cathelicidin hCAP18/LL-37 might, not only act as an antibacterial molecule, but as a key regulator of the above mentioned processes. We therefore tested this concept through *in vivo* and *in vitro* studies aimed at determining whether and how hCAP18/LL-37 could be such a multifunctional molecule.

The goal of this thesis was to investigate the role of LL-37 in epithelial cell biology by addressing the following questions:

How is the expression of the human cathelicidin gene (CAMP) controlled in primary keratinocytes? Does LL-37 contribute to tissue repair through either, regulation of cell proliferation, differentiation and/or programmed cell death? Which genes become expressed in the presence of LL-37 and what potential biological processes do they control? And does LL-37 contribute to defects in cell proliferation, differentiation and/or migration during pathological states such as cancer?

We found that the active form of Vitamin D (VD) and its metabolites induced the expression of CAMP. As the biological effects of VD are mediated by the vitamin D receptor (VDR), which, once activated binds to response elements in the promoter region of target genes (VDRE), we tested the CAMP promoter for the presence of VDR binding sites. We identified one active VDRE binding site at about ~500 bp from the transcription start site. I*n vivo* stimulation of human skin with the vitamin D analog calcipotriol resulted in high expression of hCAP18 at the mRNA and protein levels, compared to control skin samples from the same individuals (Paper I).

Tissue homeostasis is maintained by, among others, the selective removal of cells through mechanisms such as apoptosis or programmed cell death. Because cell proliferation and differentiation are central to skin biology, we tested the role of LL-37 in the apoptosis of human keratinocytes. We found that LL-37 prevented camptothecin-induced apoptosis, which was associated with decreased caspase-3 activity and increased expression of PGE2 and IAP2, through a mechanism dependent on COX2 activity (Paper II).

By using a microarray approach we found that LL-37 affected the gene expression profile of human keratinocytes with a signifficant effect on STC2. We investigated the mechanism of LL-37-induced STC2 upregulation and found evidence (paper III, manuscript) suggesting that unfolded protein response in keratinocytes might be triggered by expossure to LL-37.

We measured the expression of the hCAP18/LL-37 in a panel of 104 breast cancer tumors and compared it to the levels found in control samples, as well as analyzed its relationship to clinical data. We also studied, *in vitro*, the effect of LL-37 on breast cancer cell migration and in anchorage independent colony formation. In addition, we explored the molecular mechanisms responsible for LL-37 effects on breast cancer cells. To evaluate the relevance of our findings *in vivo*, a xenograft model using severely compromised immunodeficient (SCID) mice was designed to test the effect of the transgenic expression of the hCAP18/LL-37 in tumor formation. The results from these experiments indicate that 1) hCAP18/LL-37 is functionally connected with ErbB2; 2) LL-37 alters breast cancer cell phenotype *in vitro* 3) LL-37 stimulates the migration of breast cancer cells and 4) LL-37 stimulates metastasis formation in SCDI mice *in vivo* (paper IV).

Increasing evidence shows that besides its immune function, LL-37 has tissue-repair-like effects, promotes cell proliferation, migration and angiogenesis. Furthermore LL-37 has been implicated in the pathogenesis of inflammatory skin disease such as psoriasis. Based on our findings, we propose that LL-37 is a key regulator of epithelial homeostasis by influencing, tissue defense, tissue repair and maintenance through control of programmed cell death, in association with vitamin D and likely acting as an alarmin by activating processes such as the unfolded protein response. Thus, these effects become relevant for the study of pathological states such as chronic inflammation and cancer.

“With his hands in his front pockets
he is rising on the tips of his toes
and looking through his eye-lashes
he is satisfied,
by seeing the world in colours”.

A.C

LIST OF PUBLICATIONS

This thesis is based in the following papers, which will be referred to in the text by their Roman numerals

I. Weber G, Heilborn JD, **Chamorro Jimenez CI**, HammarsjoHammarsjö A, Törmä H, Stahle M. Vitamin D induces the antimicrobial protein hCAP18 in human skin.J Invest Dermatol. 2005 May;124(5):1080-2.

II. **Chamorro CI**, Weber G, Grönberg A, Pivarcsi A, Ståhle M. The human antimicrobial peptide LL-37 suppresses apoptosis in keratinocytes. J Invest Dermatol. 2009 Apr;129(4):937-44. Epub 2008 Oct 16

III. **Chamorro CI**, Dieterich C, Wei T , Zettergren L, Flores A, Merino R, Gradin K,Poellinger L, Grönberg A, Pivarcsi A., Ståhle M. The human cathelicidin LL-37 induces expression of stanniocalcin 2 in primary keratinocytes. *Manuscript*

IV. Weber G, **Chamorro CI,** Granath F, Liljegren A, Zreika S, Saidak Z, Sandstedt B, Rotstein S, Mentaverri R, Sánchez F, Pivarcsi A, Ståhle M. Human antimicrobial protein hCAP18/LL-37 promotes a metastatic phenotype in breast cancer. Breast Cancer Res. 2009;11(1):R6. Epub 2009 Jan 30

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Heilborn JD, Nilsson MF, Chamorro J. CI, Sandstedt B, Borregaard N, Tham E, Sørensen OE, Weber G, Ståhle M. Antimicrobial protein hCAP18/LL-37 is highly expressed in breast cancer and is a putative growth factor for epithelial cells. Int J Cancer. 2005 May 1;114(5):713-9

CONTENTS

1 Introduction ... 1
1.1 How do Antimicrobial peptides eliminate microorganisms? ... 1
1.2 Antimicrobial peptides in epithelia ... 3
1.3 The cathelicidins ... 4
1.4 The human cathelicidin hCAP18/LL-37 ... 4
1.5 LL-37 in host defense ... 8
1.5.1 LL-37, a link between innate and adaptive immunity ... 8
1.5.2 LL-37 in wound healing ... 8
1.6 LL-37 and apoptosis ... 9
1.7 How is the cathelicidin expression regulated? ... 10
1.8 LL-37 in cancer biology ... 13
1.9 Does LL-37 induce the unfolded protein response? ... 14
2 AIMS OF THE STUDY ... 17
3 RESEARCH STRATEGY ... 18
3.1 methodology Overview ... 18
3.1.1 Materials ... 20
3.1.2 Methods ... 21
3.1.3 Expression analysis ... 21
3.1.4 *In silico* analysis: ... 22
Vitamin D receptor biding sites in hCAP18 gene ... 22
3.1.5 Analysis of promoter activity by luciferase activity ... 22
3.1.6 Apoptosis assays ... 22
4 Results and discussion ... 26
4.1 Vitamin D as a regulator of CAM gene expression in human keratinocytes ... 26
4.2 LL-37 protects human keratinocytes from apoptosis ... 28
4.3 Treatment with LL-37 resulted in altered gene expression in human KC ... 29
4.3.1 Could LL- 37 activate the unfolded protein response? Clues from STC2 ... 31
4.4 LL-37 promotes a metastatic phenotype in breast cancer ... 33
5 Conclusions ... 37
6 Acknowledgements ... 39
7 References ... 41

LIST OF ABBREVIATIONS

AMP	Antimicrobial Peptides
ATF	Activating Transcription Factor
C/EBP epsilon	CCAAT/Enhancer Binding Protein- epsilon
CAM	Camtothecin
CAMP	Cathelicidin Antimicrobial Protein gene
CHOP	C/EBP Homologous Protein
COX-2	Cyclooxygenase-2 (prostaglandin endoperoxide synthetase)
CYP27B1	25(OH) D_3-1α-hydroxylase
CYP2R1	25-hydroxylase
DC	Dendritic cell
ERBB2	Erythroblastic leukemia viral oncogene homolog 2
EGF	Epidermal Growth Factor
eIF 2α	Eukaryotic translation Initiation Factor 2alpha
ERK1	Extracellular signal Regulated Kinase 1
FGF	Fibroblast Growth Factor
FPRL1	Formyl Peptide Receptor Like-1
GRP78	Glucose Regulated Protein 78
GT box	Regulatory elements in target genes.
HIF-1α,	Hypoxia Inducible Factor-1 alpha
IAP2	Inhibitor of Apoptosis Protein 2
IGF-I	Insulin–Like Growth Factor-I
IRE1	Inositol-Requiring Enzyme 1
MMP	Matrix MetalloProteinase
MSC	Mesenchymal Stromal Cells
NFIL-6	Nuclear Factor for IL-6 expression
P2X7	P2X Purinoreceptor 7
PERK1	PKR-Like Endoplasmic Reticulum Kinase 1
qRT-PCR	Quantitative reverse transcriptase PCR
PGE-2	Prostaglandin E2
PMN	Polymorphonuclear
ROS	Reactive Oxygen Species
SOD2	Superoxide Dismutase 2
STAT3	Signal Transducer and Activator of Transcription 3
STC2	Stanniocalcin 2
TGF-B	Transforming Transforming Growth Factor -beta
TLR2s,	Toll-Like Receptor 2
TR	Thioredoxin Reductase 1
UTR	Untranslated Region
VD	Vitamin D
VDR	Vitamin D Receptor
XBP1	X Box-binding protein Protein 1

1 INTRODUCTION

Antimicrobial peptides (AMPs) are important components of the innate immune system to control microbial threats. These molecules evolved about 600 million years ago, approximately at the time when the first multicellular organisms emerged (A. Linde, 2009) and at present they are broadly distributed in nature. Despite the fundamental role of antimicrobial peptides in host defense, the antimicrobial peptide research field waited long time to be explored. The initial clues about antimicrobial proteins of eukaryotic origin came with the discovery of Lysozyme in 1922 by Alexander Fleming (Fleming, 1932) but only in the middle of the 20th century, exploration of the killing mechanisms of leukocytes lead to the identification of extracts with antimicrobial activity, which were named leukins and phagocytins (Hirsch, 1956). However, the study of antimicrobial peptides took off only in the 1980s with the discovery of the antimicrobial peptides of insects, called cecropins, that were isolated from the silk moth (*Hylaphora cecropia*) by Hans Boman's group in 1981(Steiner et al., 1981) Few years later, the first mammalian antimicrobial peptides were discovered, alpha-defensins derived from human PMNs (Selsted et al., 1983). Another important discovery represents Michael Zasloff's identification of the amphibian-derived AMP (the magainins) from skin of the African clawed frog (*Xenopus laevis*) (Zasloff, 1987). In the beginning of the 90s, PR39, a cathelicidin peptide, was isolated from pigs (Agerberth et al., 1991). At present, the number of known and predicted AMPs found in antimicrobial host defense peptide data bases comprise more than 1500 entries (Brahmachary et al., 2004; Wang, Li, and Wang, 2009).

Structurally, AMPs could be grouped into several categories including α-helical, β-sheets, extended helices and loops. However, there are three common features in their structure that account for their direct antimicrobial activity: (1) they are small, less than 100 amino acid residues in length; (2) they are positively charged, and (3) they are amphipatic (Zasloff, 2002).

1.1 HOW DO ANTIMICROBIAL PEPTIDES ELIMINATE MICROORGANISMS?

The ability of AMPs to destroy microorganisms rests on direct and indirect mechanisms (see Figure 1). These peptides can directly disrupt membranes, interfere with metabolism and modify cytoplasmic components (Bierbaum and Sahl, 1987;

Brogden, 2005; Brotz et al., 1998) The disruption of microbial membranes caused by antimicrobial peptides in is due to electrostatic interactions between the small cationic peptides with the negatively charged microbial membranes (Zasloff, 2002). Upon binding, AMPs cause elastic strain on the lipid bilayer of microbial membranes,

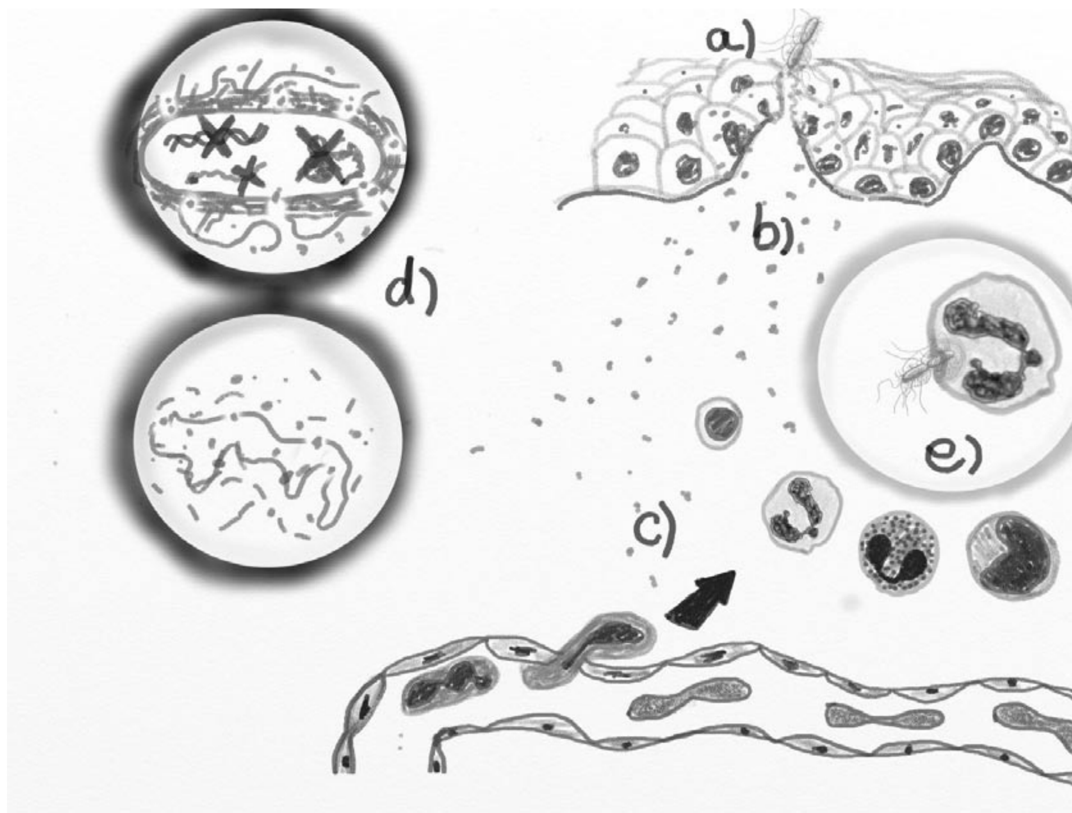


Figure 1: **Events related with antimicrobial peptide release. a)** A microbial challenge triggers antimicrobial peptide production and release from affected cells. **b)** Antimicrobial peptides diffuse in the surrounding tissues, **c)** and contribute to immune cell recruitment from the blood stream. **d)** Direct contact between peptides and microorganisms lead to damage of microbial membranes and to blockage of nucleic acid, protein and membrane synthesis. **e)** Antimicrobial peptides contribute to inducing cell-dependent defense mechanisms.

resulting in the formation of pores (Papo and Shai, 2003; Qian et al., 2008) and microbial cell death. In addition to membrane disruption, antimicrobial peptides can kill microorganism by targeting intracellular processes that are critical for the survival and proliferation of microbes. For example, α -helical peptides (pleurocidin and dermaseptin), proline- and arginine-rich peptides (PR-39 and indolicidin) and defensins (HNP-1) block thymidine, uridine and leucine uptake in *E. coli*, leading to inhibition of DNA, RNA and protein synthesis (Boman, Agerberth, and Boman, 1993). The indirect biological actions of AMPs are even more fascinating; these molecules are able to modulate inflammatory responses by influencing the host's innate and adaptive immune cells. In particular, they show endotoxin-binding and -neutralizing capacity, chemotactic- activities and induction of cytokines and chemokines. Thus, by killing

microbes directly, by stimulating the production of cytokines in neighboring cells and by recruiting leukocytes to the site of infection, AMPs play critical roles against infections (Sorensen, Borregaard, and Cole, 2008).

1.2 ANTIMICROBIAL PEPTIDES IN EPITHELIA

Epithelia function as a front line of defense against microbes by providing a physical barrier and through production and storage of antimicrobial peptides. The production of AMPs by epithelia were likely preserved during evolution because they might have offered a survival advantage in a world teeming with microorganisms. It is well established that the gastrointestinal and skin epithelia, constitutively produce and secrete antimicrobial peptides in many different organisms including humans, frogs (Zasloff, 1992) invertebrates (Dimopoulos et al., 1997) and fish (Rakers et al.).

The human genome contains codes for two main families of AMPs, the defensins and cathelicidins with several genes for defensins while, interestingly, only one gene representing the cathelicidin family and designated CAMP coding for hCAP18/ LL-37(Zanetti, 2004).

Most antimicrobial peptides expressed by human epithelial cells are stored as pro-proteins in specific granules that are released into the extracellular compartment at the interface with the external environment, where they become activated (Selsted and Ouellette, 1995; Zasloff, 2002). Some antimicrobial peptides have a tissue specific distribution. For example, human α-defensins are mainly expressed or secreted by neutrophils or by intestinal Paneth cells (Pazgier et al., 2006) while β-defensins are produced in epithelial cells (Zhao, Wang, and Lehrer, 1996). The expression patterns of AMPs can also vary between tissues. As an example, the reproductive and the colonic epithelium constitutively release AMPs of the defensin and cathelicidin family. In contrast, the skin has low constitutive expression levels of these AMPs. Furthermore, the local distribution of a particular peptide can show a specific pattern; as in colon epithelium, where the expression of cathelicidin is low in the deeper crypts and more abundant in the superficial highly differentiated colon (Hase et al., 2002). In addition, the processing and the activities of the AMPs in epithelia are modulated by the local environments in which they are released (the skin, the gastrointestinal, respiratory, and genitourinary tracts) for example, the presence of specific microorganisms affects the expression of alpha-defensins both *in vitro* and *in vivo* (Bals et al., 1999; Kaiser and Diamond, 2000; Ogushi et al., 2001).

1.3 THE CATHELICIDINS

The cathelicidins belong to a highly heterogeneous family of antimicrobial proteins found in fish (Chang et al., 2006), birds (Xiao et al., 2006), amphibians (Wang et al., 2008) and mammals (Zanetti, Gennaro, and Romeo, 1995). All cathelicidins are produced as a single protein with two domains: a highly conserved cathelin domain composed of about 100 amino acid residues, flanked by a signal peptide fragment on its N-terminus; and by a structurally variable cationic antimicrobial peptide on its C-terminus (Agerberth et al., 2000; Zanetti, Gennaro, and Romeo, 1995). Upon activation, the cathelicidin precursor is proteolytically cleaved by specific proteases. The proteases excise the C-terminus sequence containing the mature antimicrobial peptide from the cathelicidin precursor. Thus in humans the cathelicidin precursor release a peptide with the name LL-37 indicating that a specific protease, such as proteinase-3 in neutrophil and kallikrein 5 in skin cleaves the cathelicidin proprotein at the amino acid sequence alanyl and leucyl, located 37 amino acids before the end of the precursor molecule. After being released from the precursor, LL-37 can suffer additional proteolysis in the skin, generating peptides with alternative antimicrobial and immunostimulatory activities (Yamasaki et al., 2006).

Cathelicidin derived peptides are heterogeneous and fit into different structures such as linear peptides, alpha-helical and amphipathic structure; flat β sheet structure stabilized by disulfide bounds and proline, arginine or tryptophan-rich peptides (Zanetti et al., 2000) (Figure 2).

1.4 THE HUMAN CATHELICIDIN hCAP18/LL-37

In humans, only one cathelicidin member has been identified so far. It is encoded by a single gene (CAMP) and translated into an inactive precursor protein with an approximate mass of 18 kDa, named hCAP-18 (Agerberth et al., 1995; Cowland, Johnsen, and Borregaard, 1995; Larrick et al., 1995). The antimicrobial peptide LL-37 derives from the C-terminal part of hCAP-18, which is mainly expressed by neutrophils and epithelial cells, but also by other leukocytes and mast cells (Zanetti, 2004). Additionally, the N-terminal cathelin domain was also found to vary in antimicrobial activity depending on proteolytic processing or maturation (Zaiou, Nizet, and Gallo,

2003). LL-37 is a positively charged molecule (+6 at physiological pH) with a high content of basic and hydrophobic amino acids. It adopts an α-helical structure under physiological conditions and upon contact with lipid membranes (Henzler Wildman, Lee, and Ramamoorthy, 2003). Being a single representative of the cathelicidin family in humans, one could speculate that CAP18 may not tolerate genetic variation;

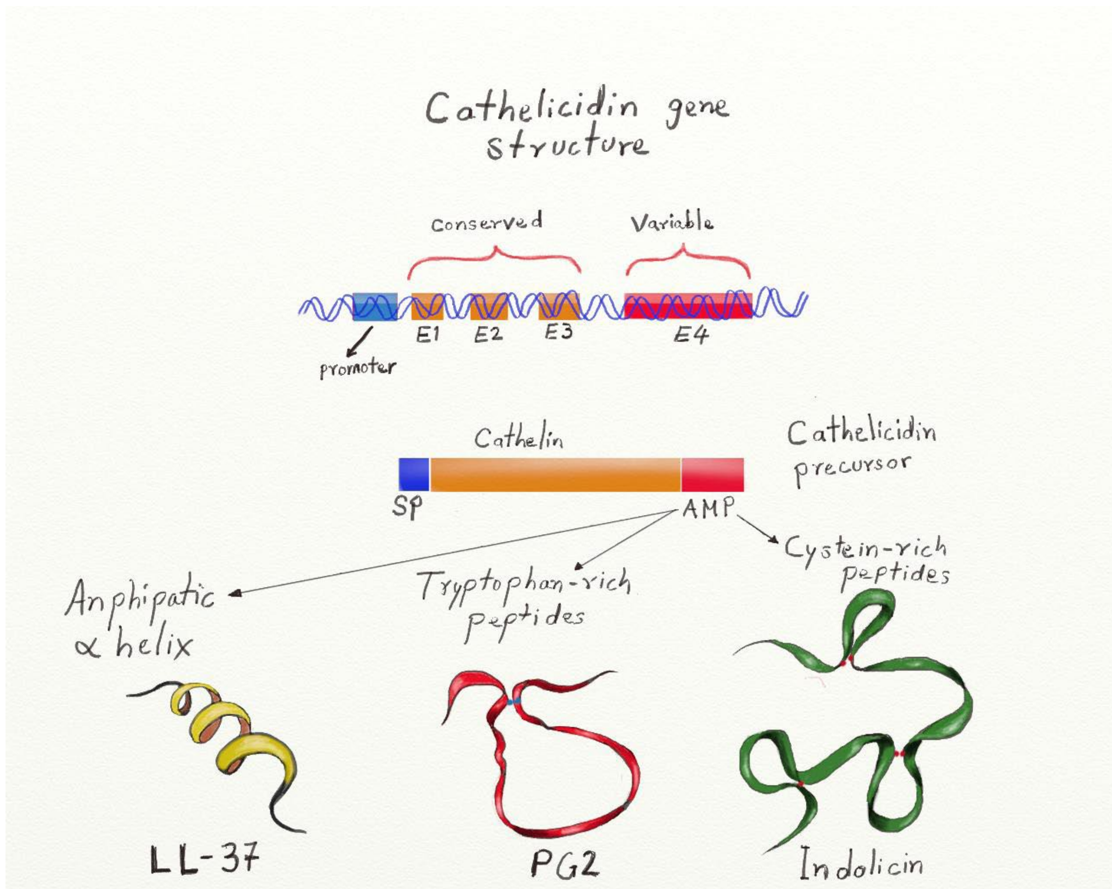


Figure 2: The cathelicidin gene structure. The cathelicidin genes from different species have 4 exons. The three first exons, coding for the signal peptide and the cathelin peptide, are highly conserved sequences, but exon 4 varies widely depending on the species. The antimicrobial peptides are coded in the last exon (Exon 4 in humans) and are released upon enzymatic processing. The cathelicidin-derived AMPs from different species vary widely structurally, some adopt α-helical conformation, others, are enriched in certain aminoacid such as proline and cystein. This figure was modified with permission from Zanetti and Tomasinsing, *current protein and peptide sciences, 2005, 6, 23-24*

however, as described above, after protein processing hCAP18 can generate peptides of different size that may have functional diversity. LL-37 has antimicrobial activity against a variety of microorganisms including Gram/negative and Gram/positive

bacteria (Braff et al., 2005b), vaccina virus (Howell et al., 2004) and *Candida albicans* (Lopez-Garcia et al., 2005). The proteinases that process hCAP18 and release peptides

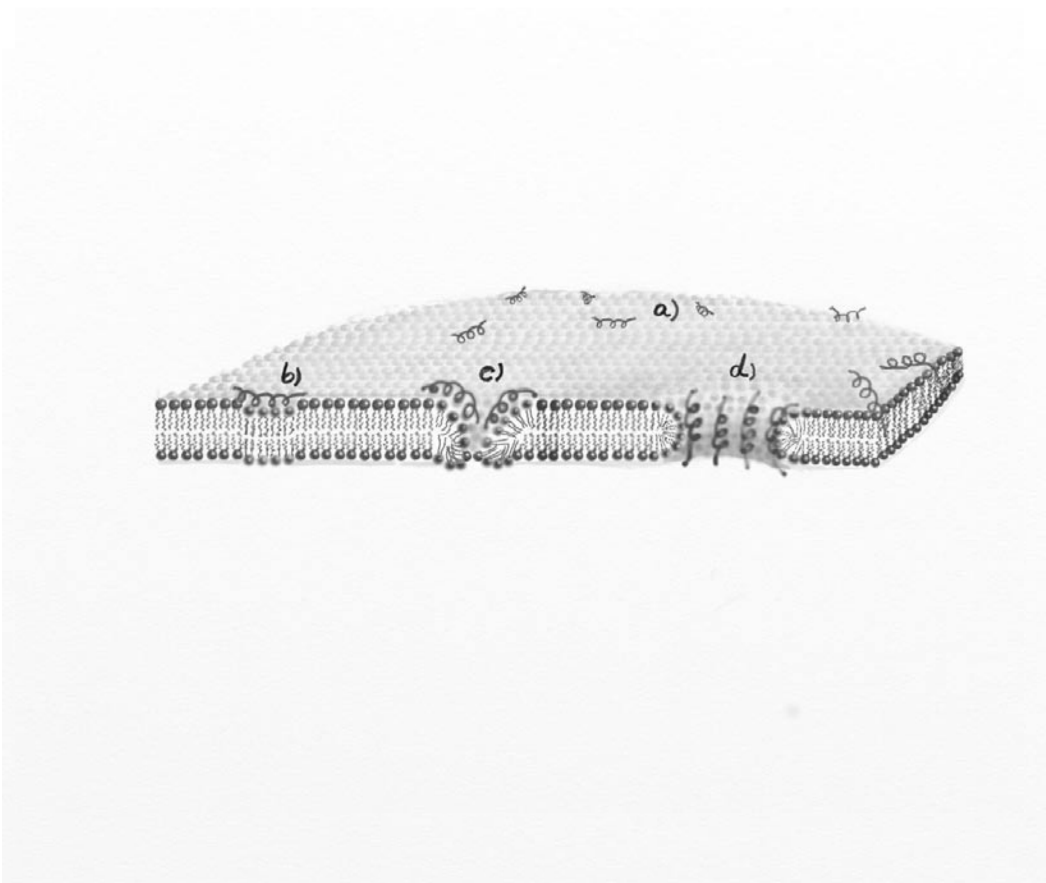


Figure 3 The toroidal model to explain the mechanism of LL-37 induced membrane destruction in microorganisms. a) LL-37 peptides lie parallel to the cell membrane, b) interacts with the lipid polar heads, c) intrusion into the lipid bilayer, d) toroid formation (inspired by Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol. 3**,** 238-50) (Brogden, 2005).

from the precursor belong to the serine protease family. The specific type of proteinase depends on cell type, thus, in keratinocytes, kallikreins are the main proteolytic enzymes for hCAP-18 (Yamasaki et al., 2006) while in neutrophils and in seminal plasma, proteinase 3 (Sorensen et al., 2001) and gastricin (Sorensen et al., 2003b) are the main proteases, respectively.

One of the most accepted model of LL-37 interaction with membranes, is the toroidal-pore formation (Gable et al., 2009; Henzler Wildman, Lee, and Ramamoorthy, 2003). This model assumes that electrostatic and hydrophobic interactions mediate contact between LL-37 and the microbial membrane. First, LL-37 lays over the heads of the phospholipid chains, and then it gets absorbed into the head group region penetrating partially into the hydrophobic core of the bilayer. The

insertion of LL-37 into the membrane induces a local membrane curvature in the shape of a torus, and causes disruption in the lipid-lipid contacts leading to the formation of pores (see Figure 3 for a graphic representation). At high concentrations, in the range of 13 to 50 μM, LL-37 can disrupt the membrane of host cells (e.g. human peripheral leukocytes, T-cells and red blood cells) (Johansson et al., 1998), However, LL-37 can affect host cells also at physiological concentrations. LL-37 can activate receptor mediated signaling (such as MAPK, PI3K, and JNK) (Gallo, 2008; Niyonsaba et al., ; Yu et al., 2007) and is able to pass through cell membrane via the activation of endocytic pathways (Lau et al., 2005; Mookherjee et al., 2009b; Sandgren et al., 2004; Zhang et al., 2009) (Figure 4). The ability to pass through the cell membrane has been implicated in psoriasis pathogenesis where LL-37 was hypothesized to bind endogenous DNA or RNA and transport the complex into plasmacytoid dendritic cells resulting in production INF-α. (Lande et al., 2007). In macrophages, LL-37 binds in the cytoplasm to GADPH and also activate cytokine production (Mookherjee et al., 2009b).

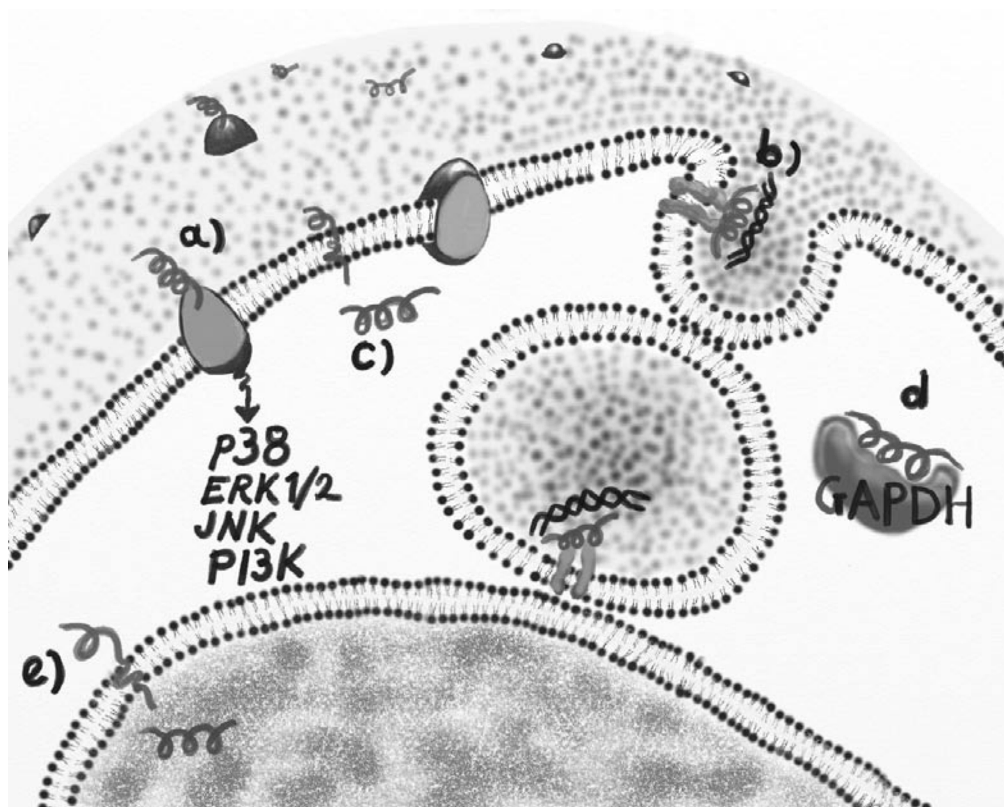


Figure 4 LL-37 interactions with eukaryotic cells. a) Intracellular signaling molecules activated by LL-37 (p38, ERk1/2, JNK, and PI3K) through specific cell surface receptors b). LL-37 mediates nucleic acid delivery to the cells through endocytic pathways c) Once inside the cells LL-37-DNA complexes bind with toll like receptors and activates the production of cytokines. d) LL-37 is also able to bind the metabolic enzyme Glyceraldehyde-3 phospahte dehydrogenase (GAPDH) modulating the expression of cytokines and/or growth factors in macropahes .e) At present ,it is not know if LL-37 is able to translocate inside the nucleus through endocytic- independent mechanisms

1.5 LL-37 IN HOST DEFENSE

1.5.1 LL-37, a link between innate and adaptive immunity

Several studies suggest that LL-37 acts as a link between innate and adaptive immunity (Auvynet and Rosenstein, 2009). Indeed, LL-37 has been shown to regulate inflammatory and immune responses (Mookherjee et al., 2009a). It is chemotactic for neutrophils, monocytes, eosinophils and T cells through FPRL1 (De et al., 2000). Additionally, LL-37 interacts with the purinergic receptor, P2X7 in different cells (Elssner et al., 2004) resulting in the processing and release of IL-1β by monocytes (Elssner et al., 2004) and the inhibition of apoptosis in neutrophils (Nagaoka, Tamura, and Hirata, 2006). LL-37 neutralizes endotoxin, thus limiting inflammatory responses by blocking TNF- α and IL-6 production (Larrick et al., 1995). Furthermore, LL-37 stimulates histamine release from mast cells, which increases vascular permeability (Chen et al., 2006). LL-37-DNA complexes can activate DCs via toll like receptors (TLR), which may contribute to inflammation in psoriasis (Ganguly et al., 2009; Lande et al., 2007). Together, these activities suggest that LL-37 plays a multifunctional role in host defense and homeostasis.

1.5.2 LL-37 in wound healing

Wound healing is a normal dynamic and highly coordinated process that aims at repairing tissues upon injury. This process is achieved through four highly integrated and overlapping phases: hemostasis, inflammation, tissue formation and tissue remodeling (Velnar, Bailey, and Smrkolj, 2009).

The first phase, hemostasis, begins immediately after wounding and results in clotting; it depends on vascular constriction and activation of the coagulation cascade. Several proinflammatory cytokines and growth factors are released during this early phase activating the next phase, inflammation.

Inflammation is characterized by the sequential infiltration of neutrophils, macrophages and lymphocytes into the wound, which in turn, form a barrier against infection. Neutrophils, the first cells to appear (24-36h after injury), remove bacteria, foreign particles and damaged tissue through phagocytosis. Macrophages (appearing 48-72h after injury) eliminate apoptotic cells (including neutrophils) and release cytokines that amplify the inflammatory response by recruiting additional leukocytes. Furthermore, macrophages in the late state of inflammation produce several growth

factors such as TGF-B, EGF, FGF, thus promoting tissue repair by-β. The last cells to enter the wound during the inflammatory phase of wound healing are lymphocytes (about 72h after injury). The function of these cells in wound healing is not yet clear but there are indications that they may modulate scar formation, keratinocyte proliferation, and cell survival (Gawronska-Kozak et al., 2006).

The proliferative or tissue formation phase is characterized by epithelial and dermal proliferation and migration. This is a very intense phase where keratinocytes go through morphological changes and remodel their cytoskeleton to increase cohesion with neighboring cells. In the dermis, fibroblasts and endothelial cells support the production of new blood vessels and collagen deposition that contribute to forming granulation tissue. The complete wound healing involves tissue remodeling and it may take one to four years to complete.

The LL-37 peptide has been implicated in several activities related with wound healing. LL-37 is induced upon wounding (Dorschner et al., 2001) and modulates keratinocyte (Heilborn et al., 2003) and fibroblast (Oudhoff et al.) proliferation epithelial cell migration (Carretero et al., 2008; Shaykhiev et al., 2005; Tokumaru et al., 2005) suppression of collagen synthesis (Park et al., 2009) and angiogenesis (Koczulla et al., 2003). These effects, are mediated through at least 3 different receptors: (1) The epidermal growth factor receptor; (2) the formyl peptide receptor-like 1 (FPRL1, a member of the G-protein coupled receptors) and (3) the purinergic receptor P2X7 (a member of the P2X family of nucleotide-gated channels) (Carretero et al., 2008; Koczulla et al., 2003; Niyonsaba et al., 2007; Shaykhiev et al., 2005; Tokumaru et al., 2005).

1.6 LL-37 AND APOPTOSIS

Apoptosis is the mechanism that leads to controlled cell death, which under normal conditions, causes the selective removal of cells, thus maintaining tissue homeostasis.

Apoptosis is important during wound healing, especially during the removal of granulation tissue after inflammation. As mentioned, high concentrations of LL-37 are cytotoxic to eukaryotic cells because, it can compromise membrane integrity. However, at low concentrations LL-37 has been shown to modulate contrasting effects on apoptosis in different mammalian cells types (Barlow et al., 2006). The role of LL-37

in apoptosis of human keratinocytes will be presented in more detail below, as this was a topic in this thesis.

1.7 HOW IS THE CATHELICIDIN EXPRESSION REGULATED?

Production of proteins is a multistage process regulated at different levels The major steps of the biogenesis of any protein are: 1) transcriptional activation/deactivation mediated by methylation/acetylation. 2) Transcription mediated by transcription factors binding to promoters, enhancers and repressors. 3) Translation dependent on RNA decoding within ribosomes. 4).Translational regulation affecting the rate of peptide production. 5). Post-transcriptional modifications contributing to protein maturation or to alternative peptide structures. 6).) Protein folding affecting the 3D structure of the processed protein. 7). Intracellular protein fate or localization in organelles or cell membrane. 8). Export to the extracellular milieu and protein recycling (Read, 2006).

The expression of CAP18/LL-37 relies on these very same processes; however, the overall picture of its regulation is not complete. Some of the pieces of information we have to date are discussed in the following sections.

1.7.1.1 Transcriptional activation

Many cells and tissues in the human body express hCAP18/LL-37 (Table 1 for some of the cells and tissue expressing CAMP) but its expression can be regulated in a stimulus dependent manner resulting in different levels of the peptides according to body location. Wounding, infection or certain inflammatory diseases may contribute to wide differences in LL-37 expression.

In order for CAP18/LL-37 to become expressed, its sequence must be available for the factors that initiate transcription. Histone methylation reduces transcription while acetylation facilitates it by allowing transcription factor binding to the promoter region of the gene (Rice and Allis, 2001). In the case of hCAP18/LL37, histone acetylation is actively induced by substances such as butyrate, which is a histone deacetylase inhibitor. Butyrate induced hCAP18 expression is dependent of MEK/ERK signalling (Schauber et al., 2003), of the activation of cofactors involved in epigenetic modification of DNA such as SRC3 and P300 (Kida, Shimizu, and Kuwano, 2006; Schauber et al., 2008).

Cell type or tissue	Reference
Bone marrow	(Agerberth et al., 1995; Cowland, Johnsen, and Borregaard, 1995)
Neutrophils	(Sorensen et al., 1997a)
Lymphocytes and monocytes	(Agerberth et al., 2000)
Squamous epithelia	(Frohm Nilsson et al., 1999)
Epithelium of the epididymis and seminal plasma	(Malm et al., 2000)
Lung epithelia	(Agerberth et al., 1999; Bals et al., 1998)
Plasma	(Sorensen et al., 1999; Sorensen et al., 1997b)
Skin	(Dorschner et al., 2003; Dorschner et al., 2001; Frohm et al., 1997)
Eccrine gland and sweat ductal epithelial cells	(Murakami et al., 2002)
Colon epithelium and mucousa	(Hase et al., 2002; Tollin et al., 2003)
Amniotic fluid	(Yoshio et al., 2003)

Table 1. Some tissues and cell types which express hCAP18/LL-37

1.8.1.2 CAMP-related transcription factors

The analysis of the putative promoter region of CAMP indicates that a variety of transcription factors may be involved in its expression. Studies and convincing effects on CAMP expression have been performed for CREB (Chakraborty et al., 2009), C/EBP-ε (Verbeek et al., 1999), AP-1 (Kida, Shimizu, and Kuwano, 2006) and PU.1 (Termen et al., 2008). Patients with a rare congenital disorder called neutrophil-specific granule deficiency have a mutation in the gene coding for C/EBP-ε and fail to express hCAP18/LL-37 and other antimicrobial peptides (Gombart et al., 2003; Gombart et al., 2001).

Additional regulatory elements were found, including a potent repressor (still not characterized) within the 5' sequence, a hypoxic response element and two GT boxes (Elloumi and Holland, 2008). As well as binding sites for the coactivator p300. Furthermore, multiple transcription start sites as well additional splicing variants of

hCAP18 (possessing a 10 to 26amino acid insertion in the cathelin domain) have been reported in bone marrow cells (Elloumi and Holland, 2008)

One of the most important regulators of CAMP expression is the hormonal form of vitamin D (VD), $1.25(OH)_2D_{3.,}$ a hormone that regulates diverse physiological processes such as mineral homeostasis, skeletal integrity and cell growth and differentiation (Verstuyf et al.). The synthesis of the biologically active VD (also known as calcitriol) is complex and involves hydroxylation steps on sterol-like precursor molecules present in the diet or formed in the skin under the influence of UV-B. The hydroxylation steps are catalyzed by two different enzymes called 25-hydroxylase (CYP2R1) and 25(OH) D_3-1α-hydroxylase (CYP27B1). These enzymes are produced in the liver and kidney as well as in the skin by keratinocytes. The biological effects of calcitriol are mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily. Upon VD binding to this receptor in the cytoplasm, conformational changes activate VDR, which is then translocated into the nucleus where it binds, with high affinity, VD response elements (VDREs) in the promoters of target genes (Miller and Gallo).

Interestingly, the colon epithelium constitutively produces hCAP18 (Hase et al., 2002; Schauber et al., 2003). Butyrate originates from the bacterial metabolism within the digestive system; its presence in a tissue that constitutively expresses hCAP18 supports a role of epigenetic regulation in the transcription of this gene. (Schauber et al., 2003). Butyrate also induces hCAP18 expression in other gastrointestinal cell types and in a lung cell line (Kida, Shimizu, and Kuwano, 2006). The effect of butyrate on CAMP expression was recently linked to the presence of the transcription factors PU1 (Termen et al., 2008) in colon. and AP-1 (Kida, Shimizu, and Kuwano, 2006) in lung epithelia, both with putative binding sites in the CAMP promoter.

1.7.1.2 Factors known to trigger transcription of CAMP

hCAP18 levels in cultured keratinocytes increase dramatically upon infection with *Staphylococcus aureus* (Li et al., 2008; Schaller-Bals, Schulze, and Bals, 2002) or stimulation with insulin–like growth factor (IGF-I) (Sorensen et al., 2003a), stimulation with IL1-alpha and LPS (Erdag and Morgan, 2002; Nell et al., 2004), wounding (Heilborn et al., 2003; Sorensen et al., 2003a; Sorensen et al., 2006) and inflammation (Frohm et al., 1997). In contrast, bacteria such as *Neisseria gonorrheae*

and *Shigella spp.* evolved mechanisms to down regulate the expression of LL-37 (Bergman et al., 2005; Islam et al., 2001)

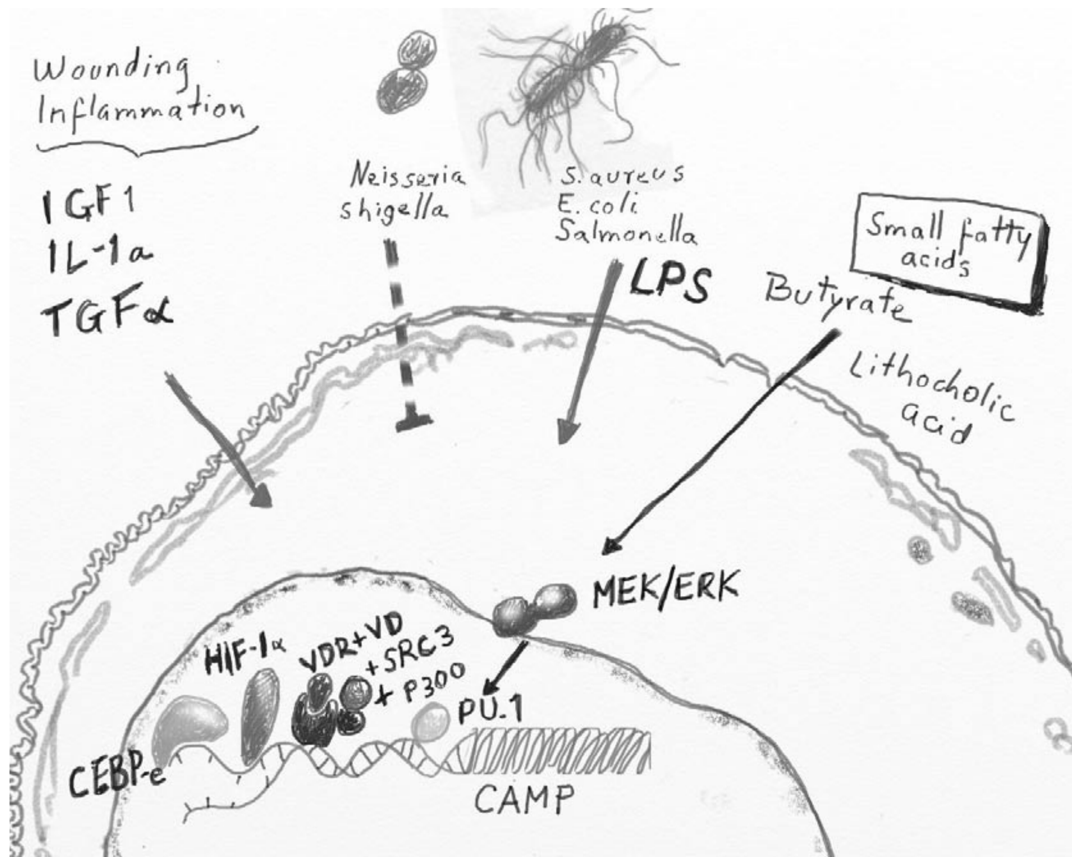


Figure 4. Factors known to modulate hCAP/18 (CAMP) expression. This cartoon shows some of the intracellular mediators of CAMP transcription as well as some external triggering substances. Activating pathways are indicated with solid arrows, factors that cause inhibition of CAMP expression are indicated with a red segmented line. Binding of transcription factors to promoter binding sites is described in an arbitrary manner.

1.8 LL-37 IN CANCER BIOLOGY

So far, I have described CAMP18/LL-37 as a molecule that is involved in diverse processes such as, antimicrobial defense, communication between the innate and the adaptive immune responses, regulation of relevant processes for tissue repair such as inflammation, apoptosis and keratinocyte physiology, etc. However, these very properties suggest that LL-37 may also be of paramount importance during pathological processes such as cancer development and/or progression. Because antimicrobial peptides have immunomodulatory and cytotoxic effects at high concentrations, they have been proposed to have, primarily, anti tumor effects (Ohtake et al., 1999; Winder et al., 1998). Why would LL-37's effects on proliferation, migration and angiogenesis be exclusively relevant in the context of

wound healing and tissue repair? In principle any regulator of such important events is a good candidate for study of in relation to cancer biology, thus LL-37 is a plausible cancer related factor. Addressing this question in practical terms is difficult, considering the great complexity of cancer biology where many players under different conditions are involved.

Early work in our research group linked hCAP18/LL-37 and breast cancer biology in humans. A set of breast cancer tumors was observed to express more LL-37 than normal breast tissue, with an apparent relationship between levels of LL-37 and tumor histological grade. This work led to the hypothesis that hCAP18/LL-37 may be involved in tumor growth (Heilborn et al., 2005). Our group also found that treatment with LL-37 stimulated the proliferation of epithelial cells, suggesting that LL-37 may act as a growth factor (Heilborn et al., 2005). High expression of the hCAP18/ LL-37 was also observed by other research groups who studied lung and ovarian cancer (Coffelt et al., 2009b; Coffelt et al., 2008; von Haussen et al., 2008). Furthermore, stimulation of various cancer cell lines *in vitro* caused proliferation, migration, invasion and matrix metalloproteinase activation (Coffelt et al., 2009a; Coffelt et al., 2009b; Coffelt et al., 2008; von Haussen et al., 2008). We therefore investigated the biological significance of the hCAP18/LL-37 overexpression in breast cancer cells.

Summarizing, LL-37 is involved in the biology of different types of cancer. In this thesis, I will discuss our findings on LL-37 in human breast cancer.

1.9 DOES LL-37 INDUCE THE UNFOLDED PROTEIN RESPONSE?

LL-37 is a multifunctional peptide in eukaryotic cells with stimulatory responses occurring via several receptors and signaling pathways in the low micromolar range in vitro. However, at only slightly higher concentrations (>10 μM), LL-37 is able to cause cell death (Johansson et al., 1998). It is suggested that LL-37 has an important role in tissue responses against injury and infection and it is therefore assumed that cytotoxic effects are not in play at physiological concentrations. One possibility is that LL-37 induces responses that make more resistant to cellular stress that may lead to death; one example with keratinocytes is described in this thesis. I will

discuss the possibility that LL-37 induces a specific program by which a cell copes with stress, the unfolded protein response (UPR).

1.9.1.1 What is UPR?

Recycling of wrongly folded proteins, which in most cases can be up to 30% of newly produced proteins (Schubert et al., 2000) is a basic task for the cells in order to maintain internal homeostasis. The detection, degradation and recycling of miss-folded proteins occurs in the endoplasmic reticulum (ER), and is facilitated by number of proteins known as chaperones; including the glucose-regulated proteins BiP and GRP78; and by a variety of folding enzymes such as protein disulfide isomerase (PDI) (Schroder and Kaufman, 2005). Conditions that trigger ER stress can cause the accumulation of unfolded proteins in the ER lumen. To deal with this stress, the cells activate a process called the Unfolded Protein Response (UPR) (Merksamer and Papa) which activates transcriptional and translational programs aiming to facilitate the cell response to stress.

The Master proteins regulating this process are designated PKR-like ER kinase (PERK), activated transcription factor 6 (ATF6), and Inositol-requiring enzyme 1 (IRE1) (Figure 5). In a cell, different factors lead to the activation of UPR, including nutrient deprivation; oxidative stress or inflammation (Ghosh et al.).

Several genes are target of the UPR response (Schroder, 2008). These UPR genes are involved in cell metabolism, anti-oxidative response, and protective or apoptotic signals. The overall idea with the UPR is for the cell to recover or to die (Malhotra and Kaufman, 2007). There are genes that directly control the protein loading into the ER, others mediate cell cycle arrest that allows cells to repair themselves, and other UPR genes prevent apoptosis although apoptosis can also be promoted under some circumstances (Malhotra and Kaufman, 2007). In this context, UPR may rescue or execute; depending on the intensity of the stress signals and how they modulate the UPR, the affected cells will either recover or die. UPR is also involved in cell differentiation, such as differentiation of B cells into plasma cells (Iwakoshi et al., 2003); and may be involved in differentiation of epidermal keratinocytes (Sugiura et al., 2009). Furthermore, UPR plays an important role in cancer development (Wang, Yang, and Zhang).

During our investigations of LL-37 effects on keratinocytes, several facts hinted at interactions with members of the UPR system and LL-37, we therefore wanted to ask if LL-37 may be involved in alarming the cell about stressful events.

1.9.1.2 1.10.2 UPR induces the stanniocalcin gene expression

Stanniocalcins (STC) is a family of secreted glycoprotein hormones first identified in fish having calcium and phosphate regulating functions. In humans two members have been identified so far: STC1 and STC2. STC2 is expressed by a wide variety of tissues (Chang, Jellinek, and Reddel, 2003; Wagner et al., 1986). The function of STC2 is not clear but it has been implicated in several processes including ovarian development (Luo, Pisarska, and Hsueh, 2005) body growth (Gagliardi et al., 2005), pancreatic α-cell function (Moore et al., 1999) and cancer (Bouras et al., 2002).

Additionally, STC2 was proposed to be a target of UPR in different cells having protective functions during oxidative stress and hypoxia (Ito et al., 2004). I will describe our data linking LL-37 with STC2 regulation in the results and discussion section of this thesis.

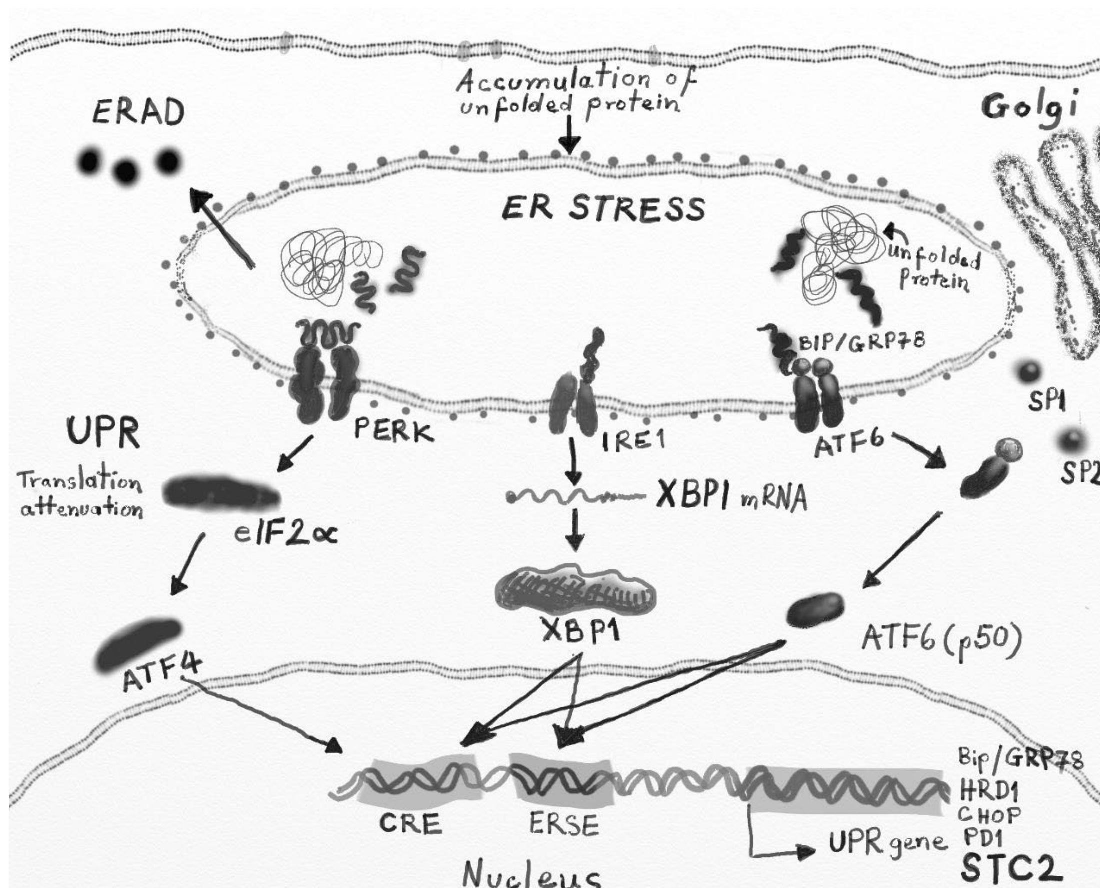


Figure 5: Signal transduction pathways of the unfolded protein response Accumulation of unfolded proteins in the ER, induce the activation of the unfolded protein response (UPR). This process is mediated by three molecules, PKR-like ER kinase (PERK), activated transcription factor 6 (ATF6), and Inositol-requiring enzyme 1 (IRE1). These three molecules interact with the chaperones GRP78 (glucose regulated protein 78). Upon UPR activation, GRP78 dissociates from ATF6, PERK, and IRE1, leading to their activation. Active IRE1 triggers the splicing X box-binding protein 1(XBP1) mRNA. Spliced XBP1 protein is a highly active transcription factor. Active PERK phosphorylates eIF2α, which promotes the translation of the activation transcription factor 4 (CREB). Activated ATF6 acts in concert with XBP1 and ATF4 proteins to induce expression of genes related with the UPR including GRP78, CHOP, PD1. STC2 has been proposed as a target of the UPR through the activation of ATF4. This figure was modified with permission from Sugiora, et al; Journal of Investigative Dermatology (2009) 129, 2126-2145.

2 AIMS OF THE STUDY

The aim of this work was to explore how the human cathelicidin hCAP18/LL-37 affects epithelial cell biology. In particular, we explored the involvement of LL-37 in apoptosis and in breast cancer development. Moreover, we explored the molecular mechanisms controlling the expression of LL-37 in the skin.

Specific aims

- To study the effect of vitamin D on hCAP18 (CAMP) expression in keratinocytes
- To explore the role of LL-37 in apoptosis of keratinocytes
- To study the genetic expression profiles of human keratinocytes exposed to LL-37
- To investigate the mechanisms of LL-37 induced STC2 expression
- To explore the role of LL-37 in breast cancer

3 RESEARCH STRATEGY

We studied the regulation of hCAP18 expression by VD in an experimental system consisting of primary keratinocytes. Repeated measures of hCAP18 mRNA and protein expression were performed and an observational study was attempted in order to explore the potential effects of VD *in vivo*.

To explore a causal relationship between LL-37 and induction or prevention of apoptosis different methods were used to assess cell death in keratinocytes treated with LL-37 and a known pro-apoptotic substance, separate or in combination.

We compared the global expression of genes in culture keratinocytes exposed to LL-37 for 6 hours.

The expression of LL-37 in human breast cancer tumors performed was studied by comparing the mRNA and protein levels in the tumors with those of control normal mammary tissue. These studies were complemented by cell culture experiments with breast cancer cells cultured with and without LL-37 to study intracellular signaling, ability to migrate in artificial systems and to produce colonies in semisolid culture medium. An *in vivo* study of human breast cancer cells with or without transgenic overexpression of hCAP18 was performed to assess the effect of LL-37 on growth and metastatis.

3.1 METHODOLOGY OVERVIEW

Paper I

Exploration of molecular mechanisms controlling the expression of CAMP

We investigated whether the expression of hCAP18 could be influenced by agents that affect the proliferation and differentiation of skin keratinocytes. As skin is the major source of VD, we hypothesized that this hormone might be involved in skin protection, possibly by affecting the production of antimicrobial peptides. Thus, we evaluated the expression of the hCAP18 at the mRNA and protein levels in human keratinocytes upon stimulation with various VD metabolites. To identify the functional vitamin D responsive elements in the hCAP18 gene promoter, we used *in silico*

analysis and sub-cloned about 1 kb genomic region upstream of the transcription start site, containing putative binding sites for VDR. We evaluated the promoter activity of this region by using luciferase constructs to transfect the human keratinocyte line HaCaT. To assay whether vitamin D stimulated hCAP18 expression *in vivo*, we applied vitamin D to the skin of four healthy probands and assayed hCAP18 expression by quantitative Real-Time PCR analysis.

Paper II

Investigation of the role LL-37 in keratinocyte apoptosis

To investigate if LL-37 would affect apoptosis pathways in human keratinocytes, we examined the effect of LL-37 on cells exposed to camptothecin (CAM), a topoisomerase I inhibitor. After treatment with CAM, membrane integrity and DNA fragmentation, as well as the activation of caspase-3, were analyzed. The effect of LL-37 on the expression of the antiapoptotic protein IAP2 was studied at the transcriptional level. To gain a more complete understanding of how LL-37 affects apoptosis in human keratinocytes we used gene array technology to profile gene expression patterns in keratinocytes, 6 hours after treatment with 2 μM LL-37. To validate the results obtained by the microarray analysis, we next performed quantitative real-time PCR analysis in LL-37-treated cells and indentified COX-2 as one of LL-37 induced genes. Since COX-2 mediates its biological effects trough prostaglandin production, we next investigated whether the COX-2 expression correlated with prostaglandin–2 (PGE-2) production by keratinocytes using ELISA technique. Based on our experimental observations, we hypothesized that the anti-apoptotic role of LL-37 is mediated *via* the COX-2 pathway. In order to test this hypothesis, we evaluated the effects of a COX-2 inhibitor on the changes mediated by LL-37 on IAP2 mRNA levels, PGE-2 production and caspase-3 activation.

Paper III

Investigation of the role of LL-37 in breast cancer

To explore the role of hCAP18/LL-37 in breast cancer, we investigated hCAP18 mRNA levels by quantitative RT-PCR in a panel of primary human breast cancer samples (n = 109). In order to understand the biological significance of our findings, the levels of hCAP18 were compared to lymph node and estrogen receptor (ESR) status by means of analysis of variance (ANOVA). Since LL-37 has been linked to EGFR signaling, we decided to investigate whether increased hCAP18 expression was associated with changes in ERBB2 (Human Epidermal Growth Factor Receptor 2), a member of the epidermal growth factor receptor family associated with tumor growth

(Alvarado et al., 2009). To investigate whether hCAP18 regulates the expression of ERBB2 we performed signaling experiments involving the mitogen-activated protein kinase activation (MAPK) through ERBB2 on the ESR-positive breast cancer cell lines MJ1105. Next, to investigate whether LL-37 influences tumor cell behavior, we studied the *in vitro* effects of LL-37 on colony formation and migration of breast cancer cell lines. Finally, to extend our *in vitro* studies to an *in vivo* tumor model, we investigated the effects of hCAP18/LL-37 in a xenograft model by establishing primary tumors with hCAP18 transgenic and control derivatives of MJ1105 cells in severe combined immunodeficiency (SCID) mice and monitored tumor growth and metastasis formation.

Paper IV

Investigation of the effect LL-37 in keratinocyte gene expression: focusing on the STC2 gene

In this work, we aimed to investigate potential genes regulated by LL-37 in human keratinocytes by using a microarray approach. The expression of selected genes was validated by qPCR. We focused on the most upregulated gene which was stanniocalcin 2 (STC2). We evaluated the LL-37-induced STC2 production both at the mRNA and protein level. In order to evaluate the molecular mechanisms behind LL-37 induced STC2, we explored the involvement of different molecules previously associated with LL-37 biological functions by qRT-PCR, including EGFR and FPRL1 signaling. As STC2 is a target of both HIF-1α and ATF4, we explored the involvement of these transcription factors by western blot.

3.1.1 Materials

Patients and samples

Breast cancer samples included in this study were collected consecutively from patients treated at Danderyd´s hospital during the period 1994 and 1998.
The ER status was assessed on routinely processed paraffin sections.

Established cell lines and primary cells

The stable keratinocytes cell line HaCaT was used in the papers I and II. The breast cancer cell lines ZR75-1 MCF7 and derivatives MJ1105 were used in paper III. Human keratinocytes from skin were used in the studies I, II and IV.

3.1.2 Methods

A variety of routine methodologies was applied for the analysis of LL-37 effects in epithelial cell biology, including western blot, qRT-PCR and immunohistochemistry.

3.1.3 Expression analysis

The expression of a gene can be studied with different methods and at different levels; in the studies presented here we used the following methods:

3.1.3.1 Microarray

Microarray is a screening tool to analyse the gene expression in a biological sample. It consists of different nucleic acid probes that are chemically attached to a fluorescent marker. Upon binding to labelled cDNA samples, the expression pattern is analysed using careful computational analysis. The intensity of the fluorescent signals on each probe corresponds to the RNA expression of a particular gene. This technique permits the simultaneous analysis of thousands of sequences of DNA and therefore represents a good screening possibility. In Papers II and III we have used a microarray approach in order to analyse how LL-37 influences gene expression in keratinocytes.

3.1.3.2 Quantitative real time–RT-PCR (qRT-PCR)

RT-PCR is a method used to study the gene expression on the RNA level. Once the RNA is extracted from the cells; it is converted to cDNA (or Reverse transcription). This cDNA is used as a template for a further amplification using specific primers to the gene of interest.

Fluorescent probes are normally used to detect in real time and quantitatively the presence of the cDNA. The quantification is based on the time point during cycling when the exponential amplification of a PCR product is first detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

3.1.3.3 Western Blot

Western blot is used to study gene expression in a tissue or cell extract at the protein level. Proteins are first separated by electrophoresis and transferred onto protein

binding membranes (often nitrocellulose). Specific antibodies are used to detect the presence of the proteins of interest. Once the protein-specific antibody complex has formed different detection systems can be used to visualize the presence of the proteins. These methods use secondary antibodies conjugated with a reporter molecules, which can be easily detected upon a chemical reaction. The visualization system we used was based on a chemoluminiscent substrate activated by an enzyme-conjugated secondary antibody.

3.1.4 *In silico* analysis: Vitamin D receptor biding sites in hCAP18 gene

DNA biding sites are sequences in the DNA where a specific transcription factor can specifically bind (Stormo, 2000). There are bioinformatics tools useful to predict the presence of these sequences in a specific gene or promoter of a gene. In paper I, we used an online bioinformatics tool called matinspector, available at www.genomatix.de, in order to predict the presence of VDR response elements in the promoter region of the hCAP18 gene.

3.1.5 Analysis of promoter activity by luciferase activity

The luciferase vector reporter system was used to the identification of DNA sequences required for gene expression. We used this system in order to determine the activity of the putative VDR binding sites. The basic design of this vector allows the cloning nd insertion of specific promoter regions upstream of the *luc* gene which encode for the enzyme luciferase. After the reporter vector is transfected into culture cells, the intensity of luciferase reflects the transcriptional activity of the inserted promoter region (in our case the region containing the VDR response elements).

3.1.6 Apoptosis assays

3.1.6.1 Induction of apoptosis with Camptothecin

CAM is a drug derivate from the Chinese tree campotheca acuminate; it is an inhibitor of topoisomerase I, a molecule required for DNA synthesis. CAM has been shown to induce apoptosis in a dose dependent manner in vitro. Some of its derivates have been used in clinical trials to treat different types of cancer. The mechanism of apoptosis induction by CAM is not completely clear but it is cytotoxic to breast cancer cell and generates ROS (Reactive Oxygen Species) (Hiraoka et al., 1998).

3.1.6.2 Evaluation of apoptosis by flow cytometry

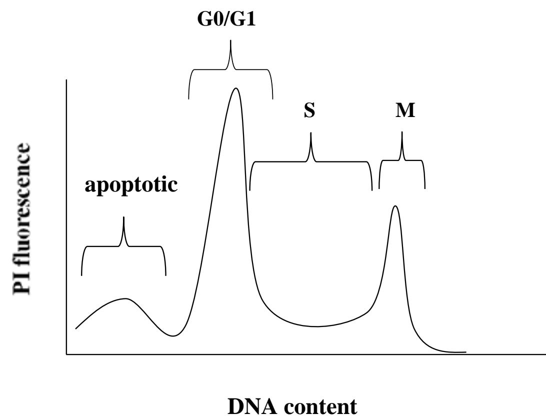
To evaluate the impact of LL-37 in apoptosis of keratinocytes, we used flow cytometry methods that detect apoptotic changes, such as membrane integrity and DNA fragmentation.

General principle of flow cytometry:

Flow cytometry is a laser-based method for optical quantification of components or structural features of cells. Flow cytometers scan single particles or cells as they pass through an excitation light source. Once each particle (or cell) reaches the excitation source the light scatters in different directions. Light scatter provides information regarding the morphology of cells such as size and granularity. Additionally, in combination with specific fluorescent dyes or antibodies, it is possible to obtain information from the cells regarding viability, metabolic state and expression of specific molecules or enzymes.

Propidium iodide (PI)

PI is an intercalating fluorescent dye that can be used to stain DNA. It is a valuable tool for determining the DNA content of a cell. From PI stained- population of ethanol fixed cells, a typical flow cytometry DNA-histogram is produced; representing cells with different DNA content thus representing the different phases of the cell cycle: G0/G1 (2n), S (between 2n and 4n) and M (4n). Since apoptotic cells are characterized by extensive damage to chromatin and cleavage of DNA into oligonucleosomal length fragments, it is possible to detect apoptotic cells in the subG0/G1 histogram (see cartoon below)



YO-PRO assay

In this assay, cells are labeled with two different dyes: YO-PRO® (green-fluorescent) and PI (red fluorescent) in order to discriminate between alive, apoptotic and dead cells. Cells with an intact membrane incorporate any or little amount of dye. Apoptotic cells, due to a change in membrane permeability, show an increased up-take of the vital dye YO-PRO but not to PI, dead cells show fluorescence for both dyes.

Measuring apoptosis by Caspase-3 activity

Caspases is a family of cysteine proteases, which play a central role in the initiation and execution phases of apoptosis. Upon activation, these enzymes cleave specific substrates and thereby mediate many of the typical biochemical and morphological changes in apoptotic cells, such as cell shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing (Kohler, Orrenius, and Zhivotovsky, 2002). Hence, the detection of activated caspases can be used as a biochemical marker for apoptosis. The method used here is based on the cleavage of a synthetic caspase substrate upon incubation with lysates from apoptotic cells. We used the VDVAD-AMC substrate, which contains tetra peptide sequences mimicking the cleavage sites of caspase-3. The tetra peptide is conjugated to a fluorochrome, 7-amino-4-methylcoumarin (AMC). Upon cleavage of the substrate, the liberated chromophore is detected spectrophotometrically.

In vitro tumorogenesis assay

In order to assay the capacity of LL-37 to influence tumor biology (paper IV) we use *in vitro* and *in vivo* models of tumorigenesis. *In vitro* we assayed the capacity of LL-37 to influence the anchorage independent growth and migration of a breast cancer cell line by using a colony formation assay and the Boyden chamber migration assay, respectively.

Colony formation:

In this assay, the cell are suspended in agar medium and plated over a surface containing agar. After incubation for a certain period of time, the colonies are counted and cathegorized morphologically.

The Boyden chamber

This assay is currently the most commonly used to evaluate migration. It involves the migration of cells through a microporous filter toward a chemoattractant placed in a well below the filter. After migration, the filter is stained and the cells on the lower side of filter are counted.

In vivo tumorigenic assays

There are several murine models to study the factors involved in malignant transformation, invasion and metastasis. One of them is the human tumor xenograft model. In this model, human tumor cells are injected into immunocompromised mice that do not reject the human cells (Morton and Houghton, 2007; Richmond and Su, 2008). The formation of tumors and development of metastases can be monitored in a relatively short time (1 to 4 months).

A xenograft model with the severely compromised immunodeficient (SCID) mice was used in order to test the effect of the transgenic expression of the hCAP18/LL-37 in tumor formation of the breast cancer cell line MJ1105.

4 RESULTS AND DISCUSSION

4.1 VITAMIN D AS A REGULATOR OF CAMP GENE EXPRESSION IN HUMAN KERATINOCYTES

In paper I we reported our findings about molecular mechanisms controlling hCAP18 expression. We found that the active form of VD, $1,25(OH)_2D_3$, induced the expression of the cathelicidin gene in human keratinocytes. This effect was also observed using VD metabolites, as well as the VD analog MC 903 (Calcipotriol) (Kragballe, 1995), used in the treatment of psoriasis but not with the VD precursor, 7-DHC. In order to understand the molecular biology behind the induction of LL-37 expression through VD, we studied the CAMP promoter region and found three potential vitamin D response elements (VDRE) upstream of the transcription start site. These were tested for their activity on transcription in a luciferase-based reporter system and through targeted sequence modifications. One VDRE, ~500 bp from the first coding nucleotide, increased transcription about 3-fold compared to control constructs. This finding explains the connection between VD and hCAP18 as a consequence of direct interaction that can eventually be manipulated for therapeutic purposes.

As an initial attempt to explore this concept, calcipotriol was applied onto the skin of four probands, which resulted in high expression of hCAP18 at the mRNA and protein levels, compared to control skin samples from the same individuals. This result together with those of other studies, including the early work by Wang and collaborators (Wang et al., 2004), provided strong initial evidence that the interaction between $1,25(OH)_2D_3$ and CAMP is part of fundamental homeostatic and defense mechanisms.

Since our findings were published, many other research groups have verified that vitamin D is a regulator of antimicrobial protein expression and innate immunity. In fact, the control of cathelicidin expression by vitamin D is shared by different cell types and tissues in humans (Gombart et al., 2007; Schauber et al., 2006) and may play an important role in antimicrobial defense (Schauber et al., 2007) against organisms such as *S. aureus* (Schauber et al., 2006) *Pseudomona aeruginosa* (Wang et al., 2004) and *Mycobacterium tuberculosis* (Liu and Modlin, 2008; Liu et al., 2006; Liu et al., 2007). $1,25(OH)_2D_3$ also induces the expression of molecules involved in

cell recognition and pathogen recognition such as TLR2 and CD14 , thus amplifying antimicrobial responses (Schauber et al., 2007).

In the skin, Vitamin D_3 (VD3) is synthesized by keratinocytes from the precursor 7-DHC upon UVB irradiation of UV-B light. VD3 is sequentially hydroxylated by specific enzymes in different organs such as the kidney and liver, which yields 25(OH)D_3 (calcidiol) and finally the metabolically active form, 1,25$(OH)_2D_3$ (calcitriol). Despite keratinocytes being able to express the complete enzymatic machinery required to produce metabolically active vitamin D (Schauber et al., 2007) we found that *in vitro,* keratinocytes treated with the VD precursor were unable to induce LL-37 expression, which indirectly suggests that no active VD was available in this experimental setting. It needs further investigation whether the lack of UV-B exposure in this artificial cell culture setting could explain this finding. Our research group found that *in vivo* stimulation of human skin with UV-B light, stimulates expression of hCAP18, suggesting that this effect is mediated *via* VD (Mallbris et al., 2009). Furthermore, topical treatment with the vitamin D analog calcipotriol enhances hCAP18/LL-37 mRNA and protein expression during acute wounding (Heilborn et al., 2009) but only mRNA in chronic ulcers (Heilborn et al., 2003). Regulation of hCAP18 by VD seems a recent event in evolutionary terms because VDREs in the promoter region of the cathelicidin gene have only been found in primates (humans and chimpanzee) (Gombart, Borregaard, and Koeffler, 2005).

Injury and toll like receptor signaling modulate the production of CYP27B1, the enzyme that converts calcidiol into calcitriol (Schauber et al., 2007), leading to local production of hCAP18. Histone acetylation is an important epigenetic mechanism that also influences the expression of hCAP18 *via* vitamin D in different cells (Gombart et al., 2007; Peric et al., 2009; Schauber et al., 2006; Schauber et al., 2008), as indicated by experiments showing that butyrate or tricostatin A (two histone deacetylase inhibitors) potentiate vitamin D-induced cathelicidin expression.

Vitamin D is a multifunctional hormone with profound effects in different systems. VD coordinates the activity of several pathways that interestingly link VD TLR2s, CYP27B1 (Heilborn et al., 2009; Schauber et al., 2007) and hCAP18 to both wound healing and antimicrobial defense. Studying VD in relation to hCAP18 could further link hCAP18/LL-37 to other VD-regulated effects such as immunomodulation (Baeke et al.) and cancer (King et al.). It would also be important to explore the

possibility that improper regulation of these molecules could be a component of inflammatory diseases such as psoriasis, rosacea and others.

4.2 LL-37 PROTECTS HUMAN KERATINOCYTES FROM APOPTOSIS

LL-37 is able to induce cell death at high concentrations (above of 13 μM) by damaging cell membranes, but at low concentrations it promotes or prevents apoptosis in different cell types (Barlow et al., 2006) although no information on apoptosis was available regarding keratinocytes. We therefore aimed, in paper II, at studying the role of LL-37 on keratinocyte apoptosis.

We found that, at a low concentration (2 μM), LL-37 protected human keratinocytes from CAM-induced apoptosis (DNA damaging agent). The protection was evident by a decreased number of apoptotic cells detected by flow cytometry, and by a decrease in caspase-3 activity. In addition, we used gene array methodology to study the gene expression profile induced by LL-37 and found that the expression of some apoptosis-related genes were significantly increased. COX-2 was one such gene and we included it in our apoptosis studies because it has been shown to prevent apoptosis through the production of PGE2 (Liou et al., 2007; Papadimitriou et al., 2007). We found that keratinocyte cell cultures treated with CAM had increased caspase-3 activity, which was reduced about 2 fold if the cultures received LL-37 simultaneously. But, if these cultures received LL-37 plus a COX-2 enzyme inhibitor, the caspase-3 activity went up again to a similar level as in cultures treated with CAM alone. We therefore hypothesize that LL-37 prevents apoptosis through a COX-2-dependent mechanism. As PGE-2 can be induced by the COX-2 enzyme, we measured this prostaglandin in a similar experimental setting and found that the use of a COX-2 inhibitor reduced the amount of PGE-2 compared to cultures treated with LL-37 alone.

PGE-2 can modulate the expression of members of the inhibitors of apoptosis protein family (IAPs) (Nishihara et al., 2003; Roy et al., 1997) which in turn can modulate the activity of caspase-3 (Roy et al., 1997). As we found that LL-37 induced a decrease in aspase-3 activity via COX-2 and likely PEG-2, we targeted IAP members as plausible mediators of LL-37's protective effect on apoptosis. Moreover, the porcine cathelicidin PR-39 has antiapoptotic functions through the activation of IAP2 (a family member of the IAP family). In our experiments, IAP-2 expression increased two-fold after treatment with LL-37 and COX-2 was necessary for the induction of IAP-2 by

LL-37. Altogether our results suggest that LL-37 protects human keratinocytes from apoptosis by a COX-2 dependent mechanism involving the production of PGE-2 and the expression of IAP2.

In agreement with our findings, LL-37 also prevents apoptosis in neutrophil through antiapoptotic proteins (bcl-2 family) and the inhibition of caspase-3 through FPRL1 and P2X7 receptors and ERK-1/2 signalling (Barlow et al., 2006; Nagaoka, Tamura, and Hirata, 2006). The involvement of these or other receptors and signaling molecules in the LL-37 antiapoptotic functions in human keratinocytes remains to be investigated. So far, we are the only group who has studied the role of LL-37 in apoptosis of human keratinocytes.

Our findings could have *in vivo* relevance, as both LL-37 and IAP2 were overexpressed in psoriatic skin (Tovar-Castillo et al., 2007). We found that LL-37 increased *in vitro* IAP2 expression in human keratinocytes. It is possible that LL-37 which is highly expressed in psoriatic skin may be involved in reducing keratinocyte apoptosis in psoriasis.

4.3 TREATMENT WITH LL-37 RESULTED IN ALTERED GENE EXPRESSION IN HUMAN KERATINOCYTES

As an exploratory tool, we performed one microarray study with a small number of keratinocytes samples treated *in vitro* with low concentrations (2μM) of LL-37. By using a FDR of 10% we found that LL-37 induced the expression of about 140 genes. After functional classification by using the Gene Ontology mapping we found significant association with genes involved with stress response; including inflammatory cytokines (IL-1α and IL-1β) and antioxidant enzymes (TR and SOD2) Other categories included response to wounding, cell cycle regulation and inflammatory responses (Table2 and figure 6).

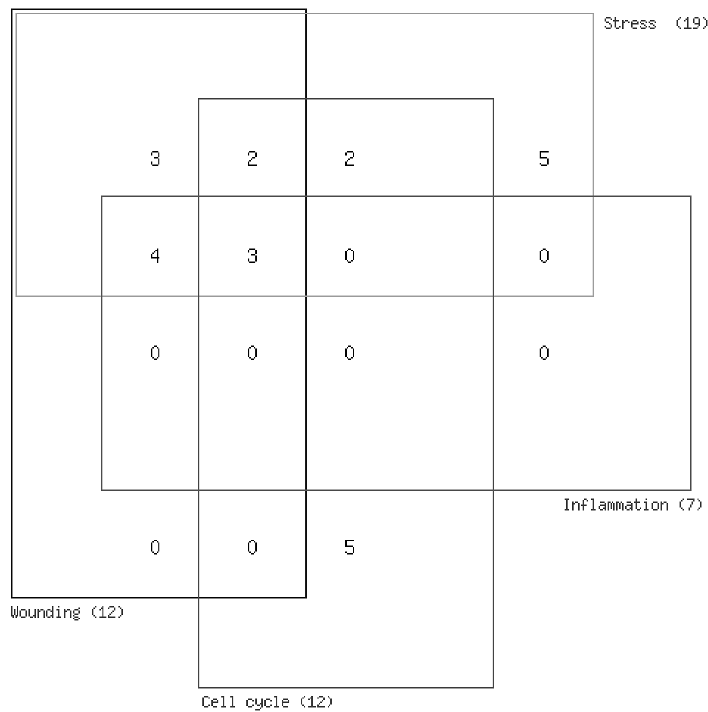


Figure 6 Venn diagram illustrating the intersections between a list of LL-37-regulated genesclustered under the category of wounding, stress, cell cycle and inflammation. This diagram was generated on line using a Chris Seidel Venn Diagram Generator (www.pangloss.com/seidel/protocols/venn.cgi.).

The results of this microarray were confirmed for a small set of selected genes by using qRT-PCR and in some cases western blot.

Today there is one reported microarray experiment performed on LL-37 (10µM) treated keratinocytes (Braff et al., 2005a). The published data from that microarray study indicates a clear induction of genes related with pro-inflammatory responses. Which make physiological sense, since the LL-37 concentrations used there were in the range of concentration reached upon wounding and inflammation (Ong et al., 2002; Schaller-Bals, Schulze, and Bals, 2002).

Our microarray data indicates that LL-37 is able to modulate gene expression at low concentrations, including genes involved in wounding, keratinocyte differentiation and cell cycle, although many of this data needs to be validated, it suggest a number of potential pathways to be explored.

The most upregulated gene by LL-37 was STC2, a gene with unknown functions in the skin biology so far. We found that LL-37 induced mRNA and protein STC2 expression level in a time- and concentration-dependent manner. These effects were dependent on new protein synthesis indicating that an unknown

protein/transcription factor is required for the LL-37 dependent increase in STC2 transcription.

A plausible candidate is the transcription factor HIF-1α, since STC2 has an active biding site in its promoter region (Law et al., 2008; Law and Wong) and LL-37 has been shown to induce the expression of HIF-1α (Rodriguez-Martinez et al., 2008). To test this hypothesis, we treated keratinocytes with LL-37 under hypoxic (as a positive control) or normoxic conditions. Although hypoxia induced HIF-1α expression, LL-37 did not induce the activation of this transcription factor. Excluding the involvement of this factor in LL-37 induced STC2.

In order to explore upstream mechanism of LL-37 induced STC-2 expression, we treated these cells with specific inhibitors targeting FPRL1 and EGFR signaling. We found that LL-37-induced STC2 expression independent of FPRL1 and EGFR signalling.

4.3.1 Could LL- 37 activate the unfolded protein response? Clues from STC2

STC2 is part of the unfolded protein response (UPR) as it has been shown to be activated after oxidative stress and hypoxia through a mechanism that involves the activation of a protein controlling the UPR; the transcription factor ATF4/CREB2 (Ito et al., 2004). Interestingly, ATF4 was one of the genes upregulated by LL-37 in our microarray suggesting that the LL-37-induced STC2 may be mediated through ATF4/CREB2. To validate the microarray data and to obtain more information about the temporal regulation of ATF4/CREB2, we treated keratinocytes with LL-37 for 6, 12 and 24 hours and measured ATF4/CREB2 protein levels. Already 6 hours after LL-37 treatment we observed a marked increase in ATF4/CREB2 levels, which was sustained for up to 24 hours post-treatment. Although these preliminary results suggest that LL-37 might induce STC2 through induction of ATF4/CREB2 in keratinocytes, functional studies testing this possibility are necessary to clarify the role of ATF4/CREB in LL-37 induced STC-2.

The UPR is a mechanism activated by cells to cope with conditions that causes ER stress, such as nutrient deprivation, oxidative stress and inflammation. In this regard, is possible that LL-37 may activate stress response programs in the cells by activating the production of reactive oxygen species (ROS) leading to oxidative stress. Supporting this possibility, LL-37 induces the production of reactive oxygen species

(ROS) in neutrophils, enhancing their microbial killing capacity (Alalwani et al., ; Zheng et al., 2007; Zughaier, Shafer, and Stephens, 2005) and in a fibroblasts (Iaccio et al., 2009). Although the production of ROS plays important roles as second messengers and agents controlling infections (Harrison, 2009), within a certain concentration range, ROS induce stress responses triggering specific changes in the expression of antioxidant enzymes (Black et al., ; Imlay, 2008). Interestingly, our expression analysis showed that LL-37 induced the expression of superoxide dismutase 2 (SOD2) and thioredoxin reductase 1 (TR); two ROS scavenging and antioxidative response genes (Landis and Tower, 2005; Mustacich and Powis, 2000).

ROS can target ER-based calcium channels causing a release of calcium into the cytoplasm (Zhang). The increase in the levels of calcium in the cytoplasm can in turn signal the mitochondria and activate the formation of additional ROS. This new ROS in turn can further target more of the ER calcium channels. This creates a vicious ROS-calcium production circle between ER and mitochondria exacerbating ER stress. The result is an accumulation of unfolded proteins in the ER and the activation of the UPR(Malhotra and Kaufman, 2007).

LL-37 is able to induce a rapid increase in intracellular calcium concentrations in epithelial cells (De Yang et al., 2000) and increased ROS production in fibroblast. The question that needs to be resolved is if LL-37 ROS production is involved in the observed responses in keratinocytes.

The term alarmin is used to describe endogens molecules that give alarm signal to the adaptative immune system, normally through its interactions with cell receptors (Harris and Raucci, 2006; Oppenheim et al., 2007). LL-37 fit into this category. But what if LL-37 is also "alarming" to normal epithelial cells?

LL-37 may represent an alarm signal when injurious stimuli are present in the tissues. In the epidermis for example, infections and/or trauma provoke a rapid release of LL-37 from intracellular storage vesicles, as well as rapid mRNA expression. The released peptide can then protect epithelia by damaging microorganisms, by modulating the cutaneous immune system and by promoting tissue repair.

We have shown that LL-37 protects human keratinocytes from apoptosis by mechanisms that include the induction of COX-2 and production of prostaglandins. However the upstream mechanism behind the induction of COX-2 and prostaglandins were not explored. We consider now the hypothesis that LL-37 is activating the UPR response in human keratinocytes. This may induce genes with protective function such

as STC2 and antioxidant proteins, which could protect keratinocytes during unfavorable circumstances.

Gene ID	**name**	**Fold increase**
3028036	Vascular Endothelial Growth Factor	1.6
3025023	Interleukin 1α	3.4
3029531	Protein Phosphatase 1	2.1
2873857	Interleukin 1β	3.4
2878460	Cd55 Antigen	1.5
2875314	Activating Transcription Factor 4	2.1
2923156	Eukaryotic Translation Initiation Factor 1	2-0
2864556	Superoxide Dismutase 2	2.6
3027183	ER Degradation Enhancer	2.1
2877911	Epiregulin	2.1
2878298	Interleukin 1 Receptor Antagonist	2.0
2877757	CCAATT/Enhancer Binding Protein (C/Ebp)	2.0
3027036	Plasminogen Activator	2.0
3024434	S100 Calcium Binding Protein A9 (Calgranulin B)	2.0
2872035	Histone Deacetylase 9	2.0
2858750	Prostaglandin-Endoperoxide Synthase 2, COX2)	2.0
3026997	Phospholipase A2	2.0
2863214	Stanniocalcin 2	14
2865791	X-Box Binding Protein 1 (XBP1)	2.0
2874306	Small proline-rich protein 2f	1,8
2972244	Small proline-rich protein 2a	2.0
2856612	Involucrin	2.0
3024525	Thioredoxin reductase 1	2.3

Table 2: Selected LL-37-induces genes.

4.4 LL-37 PROMOTES A METASTATIC PHENOTYPE IN BREAST CANCER

The role of hCAP18/LL-37, in cancer development and progression is poorly understood. We previously found that hCAP18/LL-37 is highly expressed at the protein level in breast cancer samples and that the treatment of epithelial cells with hCAP18/LL-37 stimulated cell proliferation (Heilborn et al., 2005). To further explore the role of hCAP18/LL-37 in breast cancer we examined hCAP18 mRNA expression in 109 breast cancer samples and found that the average level of hCAP18 mRNA expression was at least one order of magnitude higher in breast cancer tissues in comparison with normal breast tissue.

In order to explore the clinical relevance of hCAP18/LL-37 overexpression, we compared hCAP18/LL-37 levels with that of estrogen receptor (ER) and the presence of lymph node metastasis. We found that expression of hCAP18/LL-37 showed a significant correlation with the presence of lymph node metastases in estrogen receptor-positive samples.

Amplification and overexpression of the tyrosine kinase receptor gene ErbB2 is a hallmark of metastasis development in breast cancer. We investigated whether increased hCAP18 expression was associated with changes in ErbB2 levels. Our data demonstrate a highly significant correlation between the expression of hCAP18 and ErbB2 genes both in ER-positive and ER-negative tumours.

To investigate whether hCAP18 regulates the expression of ErbB2 we explored the effects of exogenous LL-37 and transgenic overexpression of hCAP18 on ErbB2 signaling using breast cancer cell lines ZR75-1 and derivatives of MCF7 (MJ1105). We found that hCAP18/LL-37 amplified Heregulin-induced mitogen-activated protein kinase (MAPK) signaling through ErbB2, thus identifying a functional association between hCAP18/LL-37 and ErbB2 in breast cancer.

To further explore the molecular mechanisms of how hCAP18/LL-37 cooperates with ErbB2, we performed inhibition experiments targeting previously reported mechanism of LL-37 activity. Inhibition of metalloproteinase release of EGF-liked ligands, and GPCR, did not modulate the effect of LL-37 on ErbB2 phosphorylation or MAPK activation.

We investigated the effect of *in vitro* stimulation of human breast cancer cell lines with LL-37 peptide on anchorage-independent colony formation. We found that LL-37 did not affect the number of colonies, but profoundly affected their morphology in all the cell lines studied. In the presence of LL-37, colonies became less compact and were surrounded by satellites. This observation suggested that LL-37 might promote a migratory and invasive cell phenotype. In order to test this possibility we use a Boyden chamber assay to evaluate the LL-37 capability of induce MCF7 breast cancer cell migration. We found that LL-37 stimulated the migration of these cells.

To further investigate hCAP18/LL-37 *in vivo*, we established primary tumors with hCAP18 transgenic and control derivatives of MJ1105 (MJ1105 containing a hCAP18/LL-37 expression vector or an empty vector) cells in severe combined immunodeficiency (SCID) mice and monitored tumor growth and metastasis formation. We found a significant increase in metastases in the mice injected with the hCAP18 transgenic cell line and analysis of hCAP18 transgenic tumors showed enhanced activation of MAPK signaling.

All together, our results indicate that 1) hCAP18/LL-37 is functionally connected with ErbB2; 2) LL-37 alters breast cancer cell phenotype *in vitro*
3) LL-37 stimulates the migration of breast cancer cells and 4) LL-37 stimulates metastasis formation in SCDI mice *in vivo*.

Previous reports showed that LL-37 affects ovarian (Coffelt et al., 2008) and lung tumor formation (von Haussen et al., 2008) through the activation of multiple mechanisms including cell proliferation, production of proangiogenic factors, migration and matrix metalloproteinase secretion (Coffelt et al., 2009b; Coffelt et al., 2008; von Haussen et al., 2008); Our *in vitro* agar colony formation assay and *in vivo* mice tumorogenesis model indicated that LL-37 is promoting a metastatic phenotype, which is a conceivable property for LL-37, since this peptide induces the expression of the transcription factors SNAIL and SLUG and the expression of matrix proteases in keratinocytes. SLUG and SNAIL are involved in the transformation of epithelial cells into mesenchymal cells, characterized by a more motile, invasive and aggressive phenotype (Medici, Hay, and Olsen, 2008). Whether LL-37 is promoting the expression of these molecules in other cells including breast cancer cells, remains to be investigated.

Strengthening the possibility of LL-37 acting as a pro-metastatic factor, are our clinical and experimental data showing a correlation between expression of hCAP18 and the tyrosine kinase receptor ERbB2 expression and activation. This receptor is often present in aggressive breast cancers and is an important element controlling metastasis formation (Freudenberg et al., 2009). In ovarian cancer cells, LL-37 potentiates an aggressive behavior by stimulating cancer cell migration, invasion and MMP secretion through its engagement with the FPRL1 receptor (Coffelt et al., 2008).

However, the mechanisms mediating the activation of ERbB2 by LL-37 and the induction of a metastatic phenotype in breast cancer, requires further investigation because our initial attempts using chemical inhibitors against FPRL1, EGFR and G_i proteins did not affect the ERBb2 activation mediated by LL-37.

It has been proposed that LL-37 stimulates the recruitment and activation of multipotent mesenchymal stromal cells (MSC) into the microenvironment of the ovarian tumor (Coffelt et al., 2009a). Once activated, these cells release proangiogenic and inflammatory molecules that contribute to tumor progression. In breast cancer, cytokines from MSC origin are associated with breast cancer metastasis(Karnoub et al., 2007; Soria and Ben-Baruch, 2008), thus further linking LL-37 to tumor progression.

Thus, recognizing the importance of inflammation in the development and progression of cancer (Porta et al., 2009), LL-37 a highly versatile molecule, with both protective and repair functions, could contribute to cancer trough its proinflammatory and repair-like functions

5 CONCLUSIONS

In this thesis we have studied some of the non antimicrobial properties of LL-37 in human keratinocytes and in breast cancer cells. The main findings and implications of our work are:

1. **Vitamin D induces the expression of hCAP18 through binding to VDRE in the cathelicidin gene promoter**. These findings became a starting point in our understanding of cathelicidin expression in skin and revealed a ground field with potential therapeutic applications connecting vitamin D, UVB light and antimicrobial protein expression.

2. **LL-37 protects human keratinocytes from apoptosis through the activation of the COX-2, PGE2 and IAP-2 gene expression**. These are exciting results as LL-37 is an important element of skin biology participating in wound healing and immune responses and eventually in disease. For example, high LL-37 levels in psoriasis could alter the turnover of keratynocytes by preventing apoptosis thus contributing to the thickening of the skin epithelium. Additionally, during tissue injury and wound healing, keratinocytes and infiltrating inflammatory cells, might produce a gradient of LL-37 peptide concentrations conducive to preventing apoptosis while keeping the antimicrobial function and shielding the tissues from its own cytotoxic effects. A relevant is whether LL-37 may also participate in antiapoptotic mechanism in other cell types present in the skin, such as fibroblasts.

3. **Treatment with LL-37 results in altered gene expression in human keratinocytes**. We have identified LL-37-regulated genes in primary human keratinocytes. Several of the LL-37-regulated genes were related to inflammation, stress response; including inflammatory cytokines (IL-1α and IL-1β) and antioxidant enzymes (TR and SOD2), cell differentiation program in keratinocytes, including involucrin and members of the SPRR family of keratinocyte differentiation markers. LL-37 also induced the expression of growth factors and cell cycle regulators such as VEGF and epiregulin.

4. **LL-37 induces the expression of STC2 mRNA and protein** Our preliminary findings suggest that LL-37 induces the expression of STC2 trough the UPR-related transcription factor ATF4. These findings could link many of the LL-37 related properties with the stress response program in the cells. Future studies should address the possible involvement of the P2X7 receptor, ROS production or other specific LL-37 interactions with intracellular components, mitochondria or ER for example, in this response.

5. **hCAP18/LL-37 promotes a metastatic phenotype in breast cancer**. We observed that breast cancer tumors highly express hCAP18/LL-37. Clinically, this expression was positively associated with the presence of estrogen receptor and of metastasis into lymph nodes. At the transcriptional level, hCAP18 levels correlated with the expression of tyrosine kinase receptor ErbB2. *In vitro*, LL-37 altered the cell growth phenotype during colony formation and stimulated the migration of breast cancer cells. Furthermore, overexpression of hCAP18 in

a breast cancer cell line with low malignancy phenotype, promoted metastatic disease in a SCID mice model. All together, our data indicate that hCAP18/LL-37 may be important for human breast cancer metastasis, and therefore represents a potential therapeutic target.

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7 REFERENCES

A. Linde, B. W., O. P. Höner, L. Dib, C. Ross, A. R. Tamayo, F. Blecha and T. Melgarejo (2009). Natural History of Innate Host Defense Peptides *Probiotics and Antimicrobial Proteins* **Volume 1,** (Number 2)**,** 97-112.

Agerberth, B., Charo, J., Werr, J., Olsson, B., Idali, F., Lindbom, L., Kiessling, R., Jornvall, H., Wigzell, H., and Gudmundsson, G. H. (2000). The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood* **96**(9)**,** 3086-93.

Agerberth, B., Grunewald, J., Castanos-Velez, E., Olsson, B., Jornvall, H., Wigzell, H., Eklund, A., and Gudmundsson, G. H. (1999). Antibacterial components in bronchoalveolar lavage fluid from healthy individuals and sarcoidosis patients. *Am J Respir Crit Care Med* **160**(1)**,** 283-90.

Agerberth, B., Gunne, H., Odeberg, J., Kogner, P., Boman, H. G., and Gudmundsson, G. H. (1995). FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. *Proc Natl Acad Sci U S A* **92**(1)**,** 195-9.

Agerberth, B., Lee, J. Y., Bergman, T., Carlquist, M., Boman, H. G., Mutt, V., and Jornvall, H. (1991). Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. *Eur J Biochem* **202**(3)**,** 849-54.

Alalwani, S. M., Sierigk, J., Herr, C., Pinkenburg, O., Gallo, R., Vogelmeier, C., and Bals, R. The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils. *Eur J Immunol* **40**(4)**,** 1118-26.

Auvynet, C., and Rosenstein, Y. (2009). Multifunctional host defense peptides: antimicrobial peptides, the small yet big players in innate and adaptive immunity. *Febs J* **276**(22)**,** 6497-508.

Baeke, F., Takiishi, T., Korf, H., Gysemans, C., and Mathieu, C. Vitamin D: modulator of the immune system. *Curr Opin Pharmacol*.

Bals, R., Wang, X., Meegalla, R. L., Wattler, S., Weiner, D. J., Nehls, M. C., and Wilson, J. M. (1999). Mouse beta-defensin 3 is an inducible antimicrobial peptide expressed in the epithelia of multiple organs. *Infect Immun* **67**(7)**,** 3542-7.

Bals, R., Wang, X., Zasloff, M., and Wilson, J. M. (1998). The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc Natl Acad Sci U S A* **95**(16)**,** 9541-6.

Barlow, P. G., Li, Y., Wilkinson, T. S., Bowdish, D. M., Lau, Y. E., Cosseau, C., Haslett, C., Simpson, A. J., Hancock, R. E., and Davidson, D. J. (2006). The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system. *J Leukoc Biol* **80**(3)**,** 509-20.

Bergman, P., Johansson, L., Asp, V., Plant, L., Gudmundsson, G. H., Jonsson, A. B., and Agerberth, B. (2005). Neisseria gonorrhoeae downregulates expression of the human antimicrobial peptide LL-37. *Cell Microbiol* **7**(7)**,** 1009-17.

Bierbaum, G., and Sahl, H. G. (1987). Autolytic system of Staphylococcus simulans 22: influence of cationic peptides on activity of N-acetylmuramoyl-L-alanine amidase. *J Bacteriol* **169**(12)**,** 5452-8.

Black, A. T., Joseph, L. B., Casillas, R. P., Heck, D. E., Gerecke, D. R., Sinko, P. J., Laskin, D. L., and Laskin, J. D. Role of MAP kinases in regulating expression of antioxidants and inflammatory mediators in mouse keratinocytes following exposure to the half mustard, 2-chloroethyl ethyl sulfide. *Toxicol Appl Pharmacol*.

Boman, H. G., Agerberth, B., and Boman, A. (1993). Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. *Infect Immun* **61**(7)**,** 2978-84.

Bouras, T., Southey, M. C., Chang, A. C., Reddel, R. R., Willhite, D., Glynne, R., Henderson, M. A., Armes, J. E., and Venter, D. J. (2002). Stanniocalcin 2 is an estrogen-responsive gene coexpressed with the estrogen receptor in human breast cancer. *Cancer Res* **62**(5)**,** 1289-95.

Braff, M. H., Hawkins, M. A., Di Nardo, A., Lopez-Garcia, B., Howell, M. D., Wong, C., Lin, K., Streib, J. E., Dorschner, R., Leung, D. Y., and Gallo, R. L. (2005a). Structure-function relationships among human cathelicidin peptides: dissociation of antimicrobial properties from host immunostimulatory activities. *J Immunol* **174**(7)**,** 4271-8.

Braff, M. H., Zaiou, M., Fierer, J., Nizet, V., and Gallo, R. L. (2005b). Keratinocyte production of cathelicidin provides direct activity against bacterial skin pathogens. *Infect Immun* **73**(10)**,** 6771-81.

Brahmachary, M., Krishnan, S. P., Koh, J. L., Khan, A. M., Seah, S. H., Tan, T. W., Brusic, V., and Bajic, V. B. (2004). ANTIMIC: a database of antimicrobial sequences. *Nucleic Acids Res* **32**(Database issue)**,** D586-9.

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3**(3)**,** 238-50.

Brotz, H., Bierbaum, G., Leopold, K., Reynolds, P. E., and Sahl, H. G. (1998). The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. *Antimicrob Agents Chemother* **42**(1)**,** 154-60.

Carretero, M., Escamez, M. J., Garcia, M., Duarte, B., Holguin, A., Retamosa, L., Jorcano, J. L., Rio, M. D., and Larcher, F. (2008). In vitro and in vivo wound healing-promoting activities of human cathelicidin LL-37. *J Invest Dermatol* **128**(1)**,** 223-36.

Chakraborty, K., Maity, P. C., Sil, A. K., Takeda, Y., and Das, S. (2009). cAMP stringently regulates human cathelicidin antimicrobial peptide expression in the mucosal epithelial cells by activating cAMP-response element-binding protein, AP-1, and inducible cAMP early repressor. *J Biol Chem* **284**(33)**,** 21810-27.

Chang, A. C., Jellinek, D. A., and Reddel, R. R. (2003). Mammalian stanniocalcins and cancer. *Endocr Relat Cancer* **10**(3)**,** 359-73.

Chang, C. I., Zhang, Y. A., Zou, J., Nie, P., and Secombes, C. J. (2006). Two cathelicidin genes are present in both rainbow trout (Oncorhynchus mykiss) and atlantic salmon (Salmo salar). *Antimicrob Agents Chemother* **50**(1)**,** 185-95.

Chen, X., Niyonsaba, F., Ushio, H., Nagaoka, I., Ikeda, S., Okumura, K., and Ogawa, H. (2006). Human cathelicidin LL-37 increases vascular permeability in the skin via mast cell activation, and phosphorylates MAP kinases p38 and ERK in mast cells. *J Dermatol Sci* **43**(1)**,** 63-6.

Coffelt, S. B., Marini, F. C., Watson, K., Zwezdaryk, K. J., Dembinski, J. L., LaMarca, H. L., Tomchuck, S. L., Honer zu Bentrup, K., Danka, E. S., Henkle, S. L., and Scandurro, A. B. (2009a). The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through recruitment of multipotent mesenchymal stromal cells. *Proc Natl Acad Sci U S A* **106**(10)**,** 3806-11.

Coffelt, S. B., Tomchuck, S. L., Zwezdaryk, K. J., Danka, E. S., and Scandurro, A. B. (2009b). Leucine leucine-37 uses formyl peptide receptor-like 1 to activate signal transduction pathways, stimulate oncogenic gene expression, and enhance the invasiveness of ovarian cancer cells. *Mol Cancer Res* **7**(6)**,** 907-15.

Coffelt, S. B., Waterman, R. S., Florez, L., Honer zu Bentrup, K., Zwezdaryk, K. J., Tomchuck, S. L., LaMarca, H. L., Danka, E. S., Morris, C. A., and Scandurro, A. B. (2008). Ovarian cancers overexpress the antimicrobial protein hCAP-18 and its derivative LL-37 increases ovarian cancer cell proliferation and invasion. *Int J Cancer* **122**(5)**,** 1030-9.

Cowland, J. B., Johnsen, A. H., and Borregaard, N. (1995). hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. *FEBS Lett* **368**(1)**,** 173-6.

De, Y., Chen, Q., Schmidt, A. P., Anderson, G. M., Wang, J. M., Wooters, J., Oppenheim, J. J., and Chertov, O. (2000). LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* **192**(7)**,** 1069-74.

Dimopoulos, G., Richman, A., Muller, H. M., and Kafatos, F. C. (1997). Molecular immune responses of the mosquito Anopheles gambiae to bacteria and malaria parasites. *Proc Natl Acad Sci U S A* **94**(21)**,** 11508-13.

Dorschner, R. A., Lin, K. H., Murakami, M., and Gallo, R. L. (2003). Neonatal skin in mice and humans expresses increased levels of antimicrobial peptides: innate immunity during development of the adaptive response. *Pediatr Res* **53**(4)**,** 566-72.

Dorschner, R. A., Pestonjamasp, V. K., Tamakuwala, S., Ohtake, T., Rudisill, J., Nizet, V., Agerberth, B., Gudmundsson, G. H., and Gallo, R. L. (2001). Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus. *J Invest Dermatol* **117**(1)**,** 91-7.

Elloumi, H. Z., and Holland, S. M. (2008). Complex regulation of human cathelicidin gene expression: novel splice variants and 5'UTR negative regulatory element. *Mol Immunol* **45**(1)**,** 204-17.

Elssner, A., Duncan, M., Gavrilin, M., and Wewers, M. D. (2004). A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J Immunol* **172**(8)**,** 4987-94.

Erdag, G., and Morgan, J. R. (2002). Interleukin-1alpha and interleukin-6 enhance the antibacterial properties of cultured composite keratinocyte grafts. *Ann Surg* **235**(1)**,** 113-24.

Fleming, A. (1932). Lysozyme: President's Address. *Proc R Soc Med* **26**(2)**,** 71-84.

Freudenberg, J. A., Wang, Q., Katsumata, M., Drebin, J., Nagatomo, I., and Greene, M. I. (2009). The role of HER2 in early breast cancer metastasis and the origins of resistance to HER2-targeted therapies. *Exp Mol Pathol* **87**(1)**,** 1-11.

Frohm, M., Agerberth, B., Ahangari, G., Stahle-Backdahl, M., Liden, S., Wigzell, H., and Gudmundsson, G. H. (1997). The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J Biol Chem* **272**(24)**,** 15258-63.

Frohm Nilsson, M., Sandstedt, B., Sorensen, O., Weber, G., Borregaard, N., and Stahle-Backdahl, M. (1999). The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. *Infect Immun* **67**(5)**,** 2561-6.

Gable, J. E., Schlamadinger, D. E., Cogen, A. L., Gallo, R. L., and Kim, J. E. (2009). Fluorescence and UV resonance Raman study of peptide-vesicle interactions of human cathelicidin LL-37 and its F6W and F17W mutants. *Biochemistry* **48**(47)**,** 11264-72.

Gagliardi, A. D., Kuo, E. Y., Raulic, S., Wagner, G. F., and DiMattia, G. E. (2005). Human stanniocalcin-2 exhibits potent growth-suppressive properties in transgenic mice independently of growth hormone and IGFs. *Am J Physiol Endocrinol Metab* **288**(1)**,** E92-105.

Gallo, R. L. (2008). Sounding the alarm: multiple functions of host defense peptides. *J Invest Dermatol* **128**(1)**,** 5-6.

Ganguly, D., Chamilos, G., Lande, R., Gregorio, J., Meller, S., Facchinetti, V., Homey, B., Barrat, F. J., Zal, T., and Gilliet, M. (2009). Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med* **206**(9)**,** 1983-94.

Gawronska-Kozak, B., Bogacki, M., Rim, J. S., Monroe, W. T., and Manuel, J. A. (2006). Scarless skin repair in immunodeficient mice. *Wound Repair Regen* **14**(3)**,** 265-76.

Ghosh, R., Lipson, K. L., Sargent, K. E., Mercurio, A. M., Hunt, J. S., Ron, D., and Urano, F. Transcriptional regulation of VEGF-A by the unfolded protein response pathway. *PLoS One* **5**(3)**,** e9575.

Gombart, A. F., Borregaard, N., and Koeffler, H. P. (2005). Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *Faseb J* **19**(9)**,** 1067-77.

Gombart, A. F., Kwok, S. H., Anderson, K. L., Yamaguchi, Y., Torbett, B. E., and Koeffler, H. P. (2003). Regulation of neutrophil and eosinophil secondary granule gene expression by transcription factors C/EBP epsilon and PU.1. *Blood* **101**(8)**,** 3265-73.

Gombart, A. F., O'Kelly, J., Saito, T., and Koeffler, H. P. (2007). Regulation of the CAMP gene by 1,25(OH)2D3 in various tissues. *J Steroid Biochem Mol Biol* **103**(3-5)**,** 552-7.
Gombart, A. F., Shiohara, M., Kwok, S. H., Agematsu, K., Komiyama, A., and Koeffler, H. P. (2001). Neutrophil-specific granule deficiency: homozygous recessive inheritance of a frameshift mutation in the gene encoding transcription factor CCAAT/enhancer binding protein--epsilon. *Blood* **97**(9)**,** 2561-7.
Harris, H. E., and Raucci, A. (2006). Alarmin(g) news about danger: workshop on innate danger signals and HMGB1. *EMBO Rep* **7**(8)**,** 774-8.
Harrison, C. J. (2009). Innate immunity as a key element in host defense against methicillin resistant Staphylococcus aureus. *Minerva Pediatr* **61**(5)**,** 503-14.
Hase, K., Eckmann, L., Leopard, J. D., Varki, N., and Kagnoff, M. F. (2002). Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. *Infect Immun* **70**(2)**,** 953-63.
Heilborn, J. D., Nilsson, M. F., Jimenez, C. I., Sandstedt, B., Borregaard, N., Tham, E., Sorensen, O. E., Weber, G., and Stahle, M. (2005). Antimicrobial protein hCAP18/LL-37 is highly expressed in breast cancer and is a putative growth factor for epithelial cells. *Int J Cancer* **114**(5)**,** 713-9.
Heilborn, J. D., Nilsson, M. F., Kratz, G., Weber, G., Sorensen, O., Borregaard, N., and Stahle-Backdahl, M. (2003). The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. *J Invest Dermatol* **120**(3)**,** 379-89.
Heilborn, J. D., Weber, G., Gronberg, A., Dieterich, C., and Stahle, M. (2009). Topical treatment with the vitamin D analogue calcipotriol enhances the upregulation of the antimicrobial protein hCAP18/LL-37 during wounding in human skin in vivo. *Exp Dermatol*.
Henzler Wildman, K. A., Lee, D. K., and Ramamoorthy, A. (2003). Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37. *Biochemistry* **42**(21)**,** 6545-58.
Hirsch, J. G. (1956). Phagocytin: a bactericidal substance from polymorphonuclear leucocytes. *J Exp Med* **103**(5)**,** 589-611.
Howell, M. D., Jones, J. F., Kisich, K. O., Streib, J. E., Gallo, R. L., and Leung, D. Y. (2004). Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. *J Immunol* **172**(3)**,** 1763-7.
Iaccio, A., Cattaneo, F., Mauro, M., and Ammendola, R. (2009). FPRL1-mediated induction of superoxide in LL-37-stimulated IMR90 human fibroblast. *Arch Biochem Biophys* **481**(1)**,** 94-100.
Imlay, J. A. (2008). Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* **77,** 755-76.
Islam, D., Bandholtz, L., Nilsson, J., Wigzell, H., Christensson, B., Agerberth, B., and Gudmundsson, G. (2001). Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. *Nat Med* **7**(2)**,** 180-5.
Ito, D., Walker, J. R., Thompson, C. S., Moroz, I., Lin, W., Veselits, M. L., Hakim, A. M., Fienberg, A. A., and Thinakaran, G. (2004). Characterization of stanniocalcin 2, a novel target of the mammalian unfolded protein response with cytoprotective properties. *Mol Cell Biol* **24**(21)**,** 9456-69.
Iwakoshi, N. N., Lee, A. H., Vallabhajosyula, P., Otipoby, K. L., Rajewsky, K., and Glimcher, L. H. (2003). Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat Immunol* **4**(4)**,** 321-9.
Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. D., and Agerberth, B. (1998). Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J Biol Chem* **273**(6)**,** 3718-24.
Kaiser, V., and Diamond, G. (2000). Expression of mammalian defensin genes. *J Leukoc Biol* **68**(6)**,** 779-84.
Karnoub, A. E., Dash, A. B., Vo, A. P., Sullivan, A., Brooks, M. W., Bell, G. W., Richardson, A. L., Polyak, K., Tubo, R., and Weinberg, R. A. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* **449**(7162)**,** 557-63.

Kida, Y., Shimizu, T., and Kuwano, K. (2006). Sodium butyrate up-regulates cathelicidin gene expression via activator protein-1 and histone acetylation at the promoter region in a human lung epithelial cell line, EBC-1. *Mol Immunol* **43**(12)**,** 1972-81.
King, A. N., Beer, D. G., Christensen, P. J., Simpson, R. U., and Ramnath, N. The vitamin D/CYP24A1 story in cancer. *Anticancer Agents Med Chem* **10**(3)**,** 213-24.
Koczulla, R., von Degenfeld, G., Kupatt, C., Krotz, F., Zahler, S., Gloe, T., Issbrucker, K., Unterberger, P., Zaiou, M., Lebherz, C., Karl, A., Raake, P., Pfosser, A., Boekstegers, P., Welsch, U., Hiemstra, P. S., Vogelmeier, C., Gallo, R. L., Clauss, M., and Bals, R. (2003). An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* **111**(11)**,** 1665-72.
Kohler, C., Orrenius, S., and Zhivotovsky, B. (2002). Evaluation of caspase activity in apoptotic cells. *J Immunol Methods* **265**(1-2)**,** 97-110.
Kragballe, K. (1995). Calcipotriol: a new drug for topical psoriasis treatment. *Pharmacol Toxicol* **77**(4)**,** 241-6.
Lande, R., Gregorio, J., Facchinetti, V., Chatterjee, B., Wang, Y. H., Homey, B., Cao, W., Wang, Y. H., Su, B., Nestle, F. O., Zal, T., Mellman, I., Schroder, J. M., Liu, Y. J., and Gilliet, M. (2007). Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* **449**(7162)**,** 564-9.
Landis, G. N., and Tower, J. (2005). Superoxide dismutase evolution and life span regulation. *Mech Ageing Dev* **126**(3)**,** 365-79.
Larrick, J. W., Hirata, M., Balint, R. F., Lee, J., Zhong, J., and Wright, S. C. (1995). Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect Immun* **63**(4)**,** 1291-7.
Lau, Y. E., Rozek, A., Scott, M. G., Goosney, D. L., Davidson, D. J., and Hancock, R. E. (2005). Interaction and cellular localization of the human host defense peptide LL-37 with lung epithelial cells. *Infect Immun* **73**(1)**,** 583-91.
Law, A. Y., Lai, K. P., Ip, C. K., Wong, A. S., Wagner, G. F., and Wong, C. K. (2008). Epigenetic and HIF-1 regulation of stanniocalcin-2 expression in human cancer cells. *Exp Cell Res* **314**(8)**,** 1823-30.
Law, A. Y., and Wong, C. K. Stanniocalcin-2 is a HIF-1 target gene that promotes cell proliferation in hypoxia. *Exp Cell Res* **316**(3)**,** 466-76.
Li, Q., Kumar, A., Gui, J. F., and Yu, F. S. (2008). Staphylococcus aureus lipoproteins trigger human corneal epithelial innate response through toll-like receptor-2. *Microb Pathog* **44**(5)**,** 426-34.
Liou, J. Y., Ellent, D. P., Lee, S., Goldsby, J., Ko, B. S., Matijevic, N., Huang, J. C., and Wu, K. K. (2007). Cyclooxygenase-2-derived prostaglandin e2 protects mouse embryonic stem cells from apoptosis. *Stem Cells* **25**(5)**,** 1096-103.
Liu, P. T., and Modlin, R. L. (2008). Human macrophage host defense against Mycobacterium tuberculosis. *Curr Opin Immunol* **20**(4)**,** 371-6.
Liu, P. T., Stenger, S., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., Ochoa, M. T., Schauber, J., Wu, K., Meinken, C., Kamen, D. L., Wagner, M., Bals, R., Steinmeyer, A., Zugel, U., Gallo, R. L., Eisenberg, D., Hewison, M., Hollis, B. W., Adams, J. S., Bloom, B. R., and Modlin, R. L. (2006). Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* **311**(5768)**,** 1770-3.
Liu, P. T., Stenger, S., Tang, D. H., and Modlin, R. L. (2007). Cutting edge: vitamin D-mediated human antimicrobial activity against Mycobacterium tuberculosis is dependent on the induction of cathelicidin. *J Immunol* **179**(4)**,** 2060-3.
Lopez-Garcia, B., Lee, P. H., Yamasaki, K., and Gallo, R. L. (2005). Anti-fungal activity of cathelicidins and their potential role in Candida albicans skin infection. *J Invest Dermatol* **125**(1)**,** 108-15.
Luo, C. W., Pisarska, M. D., and Hsueh, A. J. (2005). Identification of a stanniocalcin paralog, stanniocalcin-2, in fish and the paracrine actions of stanniocalcin-2 in the mammalian ovary. *Endocrinology* **146**(1)**,** 469-76.
Malhotra, J. D., and Kaufman, R. J. (2007). Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal* **9**(12)**,** 2277-93.

Mallbris, L., Carlen, L., Wei, T., Heilborn, J., Nilsson, M. F., Granath, F., and Stahle, M. (2009). Injury downregulates the expression of the human cathelicidin protein hCAP18/LL-37 in atopic dermatitis. *Exp Dermatol*.
Malm, J., Sorensen, O., Persson, T., Frohm-Nilsson, M., Johansson, B., Bjartell, A., Lilja, H., Stahle-Backdahl, M., Borregaard, N., and Egesten, A. (2000). The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa. *Infect Immun* **68**(7)**,** 4297-302.
Medici, D., Hay, E. D., and Olsen, B. R. (2008). Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. *Mol Biol Cell* **19**(11)**,** 4875-87.
Merksamer, P. I., and Papa, F. R. The UPR and cell fate at a glance. *J Cell Sci* **123**(Pt 7)**,** 1003-6.
Miller, J., and Gallo, R. L. Vitamin D and innate immunity. *Dermatol Ther* **23**(1)**,** 13-22.
Mookherjee, N., Hamill, P., Gardy, J., Blimkie, D., Falsafi, R., Chikatamarla, A., Arenillas, D. J., Doria, S., Kollmann, T. R., and Hancock, R. E. (2009a). Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. *Mol Biosyst* **5**(5)**,** 483-96.
Mookherjee, N., Lippert, D. N., Hamill, P., Falsafi, R., Nijnik, A., Kindrachuk, J., Pistolic, J., Gardy, J., Miri, P., Naseer, M., Foster, L. J., and Hancock, R. E. (2009b). Intracellular receptor for human host defense peptide LL-37 in monocytes. *J Immunol* **183**(4)**,** 2688-96.
Moore, E. E., Kuestner, R. E., Conklin, D. C., Whitmore, T. E., Downey, W., Buddle, M. M., Adams, R. L., Bell, L. A., Thompson, D. L., Wolf, A., Chen, L., Stamm, M. R., Grant, F. J., Lok, S., Ren, H., and De Jongh, K. S. (1999). Stanniocalcin 2: characterization of the protein and its localization to human pancreatic alpha cells. *Horm Metab Res* **31**(7)**,** 406-14.
Morton, C. L., and Houghton, P. J. (2007). Establishment of human tumor xenografts in immunodeficient mice. *Nat Protoc* **2**(2)**,** 247-50.
Murakami, M., Ohtake, T., Dorschner, R. A., Schittek, B., Garbe, C., and Gallo, R. L. (2002). Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. *J Invest Dermatol* **119**(5)**,** 1090-5.
Mustacich, D., and Powis, G. (2000). Thioredoxin reductase. *Biochem J* **346 Pt 1,** 1-8.
Nagaoka, I., Tamura, H., and Hirata, M. (2006). An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X7. *J Immunol* **176**(5)**,** 3044-52.
Nell, M. J., Tjabringa, G. S., Vonk, M. J., Hiemstra, P. S., and Grote, J. J. (2004). Bacterial products increase expression of the human cathelicidin hCAP-18/LL-37 in cultured human sinus epithelial cells. *FEMS Immunol Med Microbiol* **42**(2)**,** 225-31.
Nishihara, H., Kizaka-Kondoh, S., Insel, P. A., and Eckmann, L. (2003). Inhibition of apoptosis in normal and transformed intestinal epithelial cells by cAMP through induction of inhibitor of apoptosis protein (IAP)-2. *Proc Natl Acad Sci U S A* **100**(15)**,** 8921-6.
Niyonsaba, F., Ushio, H., Hara, M., Yokoi, H., Tominaga, M., Takamori, K., Kajiwara, N., Saito, H., Nagaoka, I., Ogawa, H., and Okumura, K. Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. *J Immunol* **184**(7)**,** 3526-34.
Ogushi, K., Wada, A., Niidome, T., Mori, N., Oishi, K., Nagatake, T., Takahashi, A., Asakura, H., Makino, S., Hojo, H., Nakahara, Y., Ohsaki, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Moss, J., and Hirayama, T. (2001). Salmonella enteritidis FliC (flagella filament protein) induces human beta-defensin-2 mRNA production by Caco-2 cells. *J Biol Chem* **276**(32)**,** 30521-6.
Ohtake, T., Fujimoto, Y., Ikuta, K., Saito, H., Ohhira, M., Ono, M., and Kohgo, Y. (1999). Proline-rich antimicrobial peptide, PR-39 gene transduction altered invasive activity and actin structure in human hepatocellular carcinoma cells. *Br J Cancer* **81**(3)**,** 393-403.

Ong, P. Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R. L., and Leung, D. Y. (2002). Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* **347**(15)**,** 1151-60.
Oppenheim, J. J., Tewary, P., de la Rosa, G., and Yang, D. (2007). Alarmins initiate host defense. *Adv Exp Med Biol* **601,** 185-94.
Oudhoff, M. J., Blaauboer, M. E., Nazmi, K., Scheres, N., Bolscher, J. G., and Veerman, E. C. The role of salivary histatin and the human cathelicidin LL-37 in wound healing and innate immunity. *Biol Chem.*
Papadimitriou, A., King, A. J., Jones, P. M., and Persaud, S. J. (2007). Anti-apoptotic effects of arachidonic acid and prostaglandin E2 in pancreatic beta-cells. *Cell Physiol Biochem* **20**(5)**,** 607-16.
Papo, N., and Shai, Y. (2003). Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes? *Peptides* **24**(11)**,** 1693-703.
Park, H. J., Cho, D. H., Kim, H. J., Lee, J. Y., Cho, B. K., Bang, S. I., Song, S. Y., Yamasaki, K., Di Nardo, A., and Gallo, R. L. (2009). Collagen synthesis is suppressed in dermal fibroblasts by the human antimicrobial peptide LL-37. *J Invest Dermatol* **129**(4)**,** 843-50.
Pazgier, M., Hoover, D. M., Yang, D., Lu, W., and Lubkowski, J. (2006). Human beta-defensins. *Cell Mol Life Sci* **63**(11)**,** 1294-313.
Peric, M., Koglin, S., Dombrowski, Y., Gross, K., Bradac, E., Buchau, A., Steinmeyer, A., Zugel, U., Ruzicka, T., and Schauber, J. (2009). Vitamin D analogs differentially control antimicrobial peptide/"alarmin" expression in psoriasis. *PLoS One* **4**(7)**,** e6340.
Porta, C., Larghi, P., Rimoldi, M., Totaro, M. G., Allavena, P., Mantovani, A., and Sica, A. (2009). Cellular and molecular pathways linking inflammation and cancer. *Immunobiology* **214**(9-10)**,** 761-77.
Qian, S., Wang, W., Yang, L., and Huang, H. W. (2008). Structure of transmembrane pore induced by Bax-derived peptide: evidence for lipidic pores. *Proc Natl Acad Sci U S A* **105**(45)**,** 17379-83.
Rakers, S., Gebert, M., Uppalapati, S., Meyer, W., Maderson, P., Sell, A. F., Kruse, C., and Paus, R. 'Fish matters': the relevance of fish skin biology to investigative dermatology. *Exp Dermatol.*
Read, T. S. a. A. P. (2006). "HUMAN MOLECULAR GENETICS 3."
Rice, J. C., and Allis, C. D. (2001). Histone methylation versus histone acetylation: new insights into epigenetic regulation. *Curr Opin Cell Biol* **13**(3)**,** 263-73.
Richmond, A., and Su, Y. (2008). Mouse xenograft models vs GEM models for human cancer therapeutics. *Dis Model Mech* **1**(2-3)**,** 78-82.
Rodriguez-Martinez, S., Cancino-Diaz, J. C., Vargas-Zuniga, L. M., and Cancino-Diaz, M. E. (2008). LL-37 regulates the overexpression of vascular endothelial growth factor (VEGF) and c-IAP-2 in human keratinocytes. *Int J Dermatol* **47**(5)**,** 457-62.
Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *Embo J* **16**(23)**,** 6914-25.
Sandgren, S., Wittrup, A., Cheng, F., Jonsson, M., Eklund, E., Busch, S., and Belting, M. (2004). The human antimicrobial peptide LL-37 transfers extracellular DNA plasmid to the nuclear compartment of mammalian cells via lipid rafts and proteoglycan-dependent endocytosis. *J Biol Chem* **279**(17)**,** 17951-6.
Schaller-Bals, S., Schulze, A., and Bals, R. (2002). Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. *Am J Respir Crit Care Med* **165**(7)**,** 992-5.
Schauber, J., Dorschner, R. A., Coda, A. B., Buchau, A. S., Liu, P. T., Kiken, D., Helfrich, Y. R., Kang, S., Elalieh, H. Z., Steinmeyer, A., Zugel, U., Bikle, D. D., Modlin, R. L., and Gallo, R. L. (2007). Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J Clin Invest* **117**(3)**,** 803-11.
Schauber, J., Dorschner, R. A., Yamasaki, K., Brouha, B., and Gallo, R. L. (2006). Control of the innate epithelial antimicrobial response is cell-type specific and

dependent on relevant microenvironmental stimuli. *Immunology* **118**(4)**,** 509-19.
Schauber, J., Oda, Y., Buchau, A. S., Yun, Q. C., Steinmeyer, A., Zugel, U., Bikle, D. D., and Gallo, R. L. (2008). Histone acetylation in keratinocytes enables control of the expression of cathelicidin and CD14 by 1,25-dihydroxyvitamin D3. *J Invest Dermatol* **128**(4)**,** 816-24.
Schauber, J., Svanholm, C., Termen, S., Iffland, K., Menzel, T., Scheppach, W., Melcher, R., Agerberth, B., Luhrs, H., and Gudmundsson, G. H. (2003). Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways. *Gut* **52**(5)**,** 735-41.
Schroder, M. (2008). Endoplasmic reticulum stress responses. *Cell Mol Life Sci* **65**(6)**,** 862-94.
Schroder, M., and Kaufman, R. J. (2005). ER stress and the unfolded protein response. *Mutat Res* **569**(1-2)**,** 29-63.
Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W., and Bennink, J. R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404**(6779)**,** 770-4.
Selsted, M. E., Brown, D. M., DeLange, R. J., and Lehrer, R. I. (1983). Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. *J Biol Chem* **258**(23)**,** 14485-9.
Selsted, M. E., and Ouellette, A. J. (1995). Defensins in granules of phagocytic and non-phagocytic cells. *Trends Cell Biol* **5**(3)**,** 114-9.
Shaykhiev, R., Beisswenger, C., Kandler, K., Senske, J., Puchner, A., Damm, T., Behr, J., and Bals, R. (2005). Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. *Am J Physiol Lung Cell Mol Physiol* **289**(5)**,** L842-8.
Sorensen, O., Arnljots, K., Cowland, J. B., Bainton, D. F., and Borregaard, N. (1997a). The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood* **90**(7)**,** 2796-803.
Sorensen, O., Bratt, T., Johnsen, A. H., Madsen, M. T., and Borregaard, N. (1999). The human antibacterial cathelicidin, hCAP-18, is bound to lipoproteins in plasma. *J Biol Chem* **274**(32)**,** 22445-51.
Sorensen, O., Cowland, J. B., Askaa, J., and Borregaard, N. (1997b).
Sorensen, O. E., Borregaard, N., and Cole, A. M. (2008). Antimicrobial peptides in innate immune responses. *Contrib Microbiol* **15,** 61-77.
Sorensen, O. E., Cowland, J. B., Theilgaard-Monch, K., Liu, L., Ganz, T., and Borregaard, N. (2003a). Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol* **170**(11)**,** 5583-9.
Sorensen, O. E., Follin, P., Johnsen, A. H., Calafat, J., Tjabringa, G. S., Hiemstra, P. S., and Borregaard, N. (2001). Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* **97**(12)**,** 3951-9.
Sorensen, O. E., Gram, L., Johnsen, A. H., Andersson, E., Bangsboll, S., Tjabringa, G. S., Hiemstra, P. S., Malm, J., Egesten, A., and Borregaard, N. (2003b). Processing of seminal plasma hCAP-18 to ALL-38 by gastricsin: a novel mechanism of generating antimicrobial peptides in vagina. *J Biol Chem* **278**(31)**,** 28540-6.
Sorensen, O. E., Thapa, D. R., Roupe, K. M., Valore, E. V., Sjobring, U., Roberts, A. A., Schmidtchen, A., and Ganz, T. (2006). Injury-induced innate immune response in human skin mediated by transactivation of the epidermal growth factor receptor. *J Clin Invest* **116**(7)**,** 1878-85.
Soria, G., and Ben-Baruch, A. (2008). The inflammatory chemokines CCL2 and CCL5 in breast cancer. *Cancer Lett* **267**(2)**,** 271-85.
Steiner, H., Hultmark, D., Engstrom, A., Bennich, H., and Boman, H. G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **292**(5820)**,** 246-8.
Stormo, G. D. (2000). DNA binding sites: representation and discovery. *Bioinformatics* **16**(1)**,** 16-23.

Sugiura, K., Muro, Y., Futamura, K., Matsumoto, K., Hashimoto, N., Nishizawa, Y., Nagasaka, T., Saito, H., Tomita, Y., and Usukura, J. (2009). The unfolded protein response is activated in differentiating epidermal keratinocytes. *J Invest Dermatol* **129**(9)**,** 2126-35.

Termen, S., Tollin, M., Rodriguez, E., Sveinsdottir, S. H., Johannesson, B., Cederlund, A., Sjovall, J., Agerberth, B., and Gudmundsson, G. H. (2008). PU.1 and bacterial metabolites regulate the human gene CAMP encoding antimicrobial peptide LL-37 in colon epithelial cells. *Mol Immunol* **45**(15)**,** 3947-55.

Tokumaru, S., Sayama, K., Shirakata, Y., Komatsuzawa, H., Ouhara, K., Hanakawa, Y., Yahata, Y., Dai, X., Tohyama, M., Nagai, H., Yang, L., Higashiyama, S., Yoshimura, A., Sugai, M., and Hashimoto, K. (2005). Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *J Immunol* **175**(7)**,** 4662-8.

Tollin, M., Bergman, P., Svenberg, T., Jornvall, H., Gudmundsson, G. H., and Agerberth, B. (2003). Antimicrobial peptides in the first line defence of human colon mucosa. *Peptides* **24**(4)**,** 523-30.

Tovar-Castillo, L. E., Cancino-Diaz, J. C., Garcia-Vazquez, F., Cancino-Gomez, F. G., Leon-Dorantes, G., Blancas-Gonzalez, F., Jimenez-Zamudio, L., Garcia-Latorre, E., and Cancino-Diaz, M. E. (2007). Under-expression of VHL and over-expression of HDAC-1, HIF-1alpha, LL-37, and IAP-2 in affected skin biopsies of patients with psoriasis. *Int J Dermatol* **46**(3)**,** 239-46.

Wagner, G. F., Hampong, M., Park, C. M., and Copp, D. H. (1986). Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. *Gen Comp Endocrinol* **63**(3)**,** 481-91.

Wang, G., Li, X., and Wang, Z. (2009). APD2: the updated antimicrobial peptide database and its application in peptide design. *Nucleic Acids Res* **37**(Database issue)**,** D933-7.

Wang, G., Yang, Z. Q., and Zhang, K. Endoplasmic reticulum stress response in cancer: molecular mechanism and therapeutic potential. *Am J Transl Res* **2**(1)**,** 65-74.

Wang, T. T., Nestel, F. P., Bourdeau, V., Nagai, Y., Wang, Q., Liao, J., Tavera-Mendoza, L., Lin, R., Hanrahan, J. W., Mader, S., and White, J. H. (2004). Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol* **173**(5)**,** 2909-12.

Wang, Y., Hong, J., Liu, X., Yang, H., Liu, R., Wu, J., Wang, A., Lin, D., and Lai, R. (2008). Snake cathelicidin from Bungarus fasciatus is a potent peptide antibiotics. *PLoS One* **3**(9)**,** e3217.

Velnar, T., Bailey, T., and Smrkolj, V. (2009). The wound healing process: an overview of the cellular and molecular mechanisms. *J Int Med Res* **37**(5)**,** 1528-42.

Verbeek, W., Lekstrom-Himes, J., Park, D. J., Dang, P. M., Vuong, P. T., Kawano, S., Babior, B. M., Xanthopoulos, K., and Koeffler, H. P. (1999). Myeloid transcription factor C/EBPepsilon is involved in the positive regulation of lactoferrin gene expression in neutrophils. *Blood* **94**(9)**,** 3141-50.

Verstuyf, A., Carmeliet, G., Bouillon, R., and Mathieu, C. Vitamin D: a pleiotropic hormone. *Kidney Int*.

Winder, D., Gunzburg, W. H., Erfle, V., and Salmons, B. (1998). Expression of antimicrobial peptides has an antitumour effect in human cells. *Biochem Biophys Res Commun* **242**(3)**,** 608-12.

von Haussen, J., Koczulla, R., Shaykhiev, R., Herr, C., Pinkenburg, O., Reimer, D., Wiewrodt, R., Biesterfeld, S., Aigner, A., Czubayko, F., and Bals, R. (2008). The host defence peptide LL-37/hCAP-18 is a growth factor for lung cancer cells. *Lung Cancer* **59**(1)**,** 12-23.

Xiao, Y., Cai, Y., Bommineni, Y. R., Fernando, S. C., Prakash, O., Gilliland, S. E., and Zhang, G. (2006). Identification and functional characterization of three chicken cathelicidins with potent antimicrobial activity. *J Biol Chem* **281**(5)**,** 2858-67.

Yamasaki, K., Schauber, J., Coda, A., Lin, H., Dorschner, R. A., Schechter, N. M., Bonnart, C., Descargues, P., Hovnanian, A., and Gallo, R. L. (2006). Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *Faseb J* **20**(12)**,** 2068-80.

Yoshio, H., Tollin, M., Gudmundsson, G. H., Lagercrantz, H., Jornvall, H., Marchini, G., and Agerberth, B. (2003). Antimicrobial polypeptides of human vernix caseosa and amniotic fluid: implications for newborn innate defense. *Pediatr Res* **53**(2)**,** 211-6.
Yu, J., Mookherjee, N., Wee, K., Bowdish, D. M., Pistolic, J., Li, Y., Rehaume, L., and Hancock, R. E. (2007). Host defense peptide LL-37, in synergy with inflammatory mediator IL-1beta, augments immune responses by multiple pathways. *J Immunol* **179**(11)**,** 7684-91.
Zaiou, M., Nizet, V., and Gallo, R. L. (2003). Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence. *J Invest Dermatol* **120**(5)**,** 810-6.
Zanetti, M. (2004). Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* **75**(1)**,** 39-48.
Zanetti, M., Gennaro, R., and Romeo, D. (1995). Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett* **374**(1)**,** 1-5.
Zanetti, M., Gennaro, R., Scocchi, M., and Skerlavaj, B. (2000). Structure and biology of cathelicidins. *Adv Exp Med Biol* **479,** 203-18.
Zasloff, M. (1987). Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci U S A* **84**(15)**,** 5449-53.
Zasloff, M. (1992). Antibiotic peptides as mediators of innate immunity. *Curr Opin Immunol* **4**(1)**,** 3-7.
Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**(6870)**,** 389-95.
Zhang, K. Integration of ER stress, oxidative stress and the inflammatory response in health and disease. *Int J Clin Exp Med* **3**(1)**,** 33-40.
Zhang, X., Oglecka, K., Sandgren, S., Belting, M., Esbjorner, E. K., Norden, B., and Graslund, A. (2009). Dual functions of the human antimicrobial peptide LL-37-Target membrane perturbation and host cell cargo delivery. *Biochim Biophys Acta*.
Zhao, C., Wang, I., and Lehrer, R. I. (1996). Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett* **396**(2-3)**,** 319-22.
Zheng, Y., Niyonsaba, F., Ushio, H., Nagaoka, I., Ikeda, S., Okumura, K., and Ogawa, H. (2007). Cathelicidin LL-37 induces the generation of reactive oxygen species and release of human alpha-defensins from neutrophils. *Br J Dermatol* **157**(6)**,** 1124-31.
Zughaier, S. M., Shafer, W. M., and Stephens, D. S. (2005). Antimicrobial peptides and endotoxin inhibit cytokine and nitric oxide release but amplify respiratory burst response in human and murine macrophages. *Cell Microbiol* **7**(9)**,** 1251-62.