Linköping University Medical Dissertations
No. 1412

PSORIASIN

FOR BETTER OR FOR WORSE IN SICKNESS AND IN HEALTH

THE ROLE OF PSORIASIN IN ANGIOGENESIS
AND DIFFERENTIATION OF EPITHELIAL CELLS

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Linköping 2014

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ISBN: 978-91-7519-283-3

ISSN 0345-0082

Cover: Microscopic image of human keratinocytes.

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Printed in Sweden by LiU-tryck, Linköping 2014

“OUR GREATEST WEAKNESS LIES IN GIVING UP.
THE MOST CERTAIN WAY TO SUCCEED IS TO TRY JUST ONE MORE TIME”
— THOMAS A EDISON

ABSTRACT

Psoriasin (S100A7), a member of the S100 family of calcium-binding proteins, is highly expressed in high-grade ductal carcinoma in situ (DCIS) and in the benign hyperproliferative skin disorder psoriasis. Both breast cancer and psoriasis are diseases which are characterized by hyperproliferation and a disturbed differentiation of the epithelial cells as well as a pronounced angiogenesis. The potential role of psoriasin in angiogenesis and the epithelial cell differentiation remain unclear.

The aim of this thesis was to investigate the cellular effects of psoriasin in angiogenesis and the differentiation processes, with special emphasis on breast cancer and psoriasis.

We found that psoriasin expression was induced in mammary epithelial cells and keratinocytes by oxidative stress. Psoriasin expression was shown to induce vascular endothelial growth factor (VEGF) expression and several other pro-angiogenic factors in epithelial cells. Upon down-regulation of psoriasin, H₂O₂-induced expression of VEGF was decreased as well as the pro-angiogenic factors heparin-binding EGF-like growth factor (HB-EGF) and matrix metalloproteinase (MMP)-1.

Extracellular psoriasin contributed to the subsequent induction of proliferation, migration and tube formation of endothelial cells. The proliferative effect of psoriasin was shown to be mediated by the receptor for advanced glycation end products (RAGE).

Furthermore, psoriasin induced reactive oxygen species (ROS) in both endothelial and epithelial cells through the action of RAGE, and contributed to the expression of the pro-angiogenic factors in endothelial cells.

The expression of psoriasin was up-regulated in mammary epithelial cells and keratinocytes in response to differentiation-inducing stimuli and was shown to be regulated by pathways involved in epithelial cell differentiation. Upon psoriasin down-regulation the shift towards a more differentiated CD24⁺-phenotype of mammary epithelial cells was abolished. Furthermore, the expression of the differentiation markers involucrin, desmoglein 1, transglutaminase 1 and CD24 was decreased in keratinocytes upon down-regulation of psoriasin expression.

In vivo we demonstrated a gradient of psoriasin expression in the psoriatic epidermis, with intense expression in the suprabasal differentiated layers, and a similar staining pattern between psoriasin and the differentiation marker CD24 in DCIS tumors.

In conclusion, our findings describe psoriasin as a mediator in the angiogenic process and a contributor of epithelial cell differentiation. Consequently, psoriasin is possibly a contributor to the development and progression of breast cancer and psoriasis and a potential target in the treatment of these diseases.

POPULÄRVETENSKAPLIG SAMMANFATTNING

BETYDELSEN AV PROTEINET PSORIASIN FÖR NYBILDNINGEN AV BLODKÄRL OCH UTMOGNADEN AV CELLER FÖR UTVECKLINGEN AV BRÖSTCANCER OCH PSORIASIS

Bröstcancer är den vanligaste cancerformen bland kvinnor. I likhet med andra cancersjukdomar uppstår bröstcancer genom okontrollerad celldelning och invadering av närliggande vävnad. Beroende på cancercellernas mognadsgrad växer de olika snabbt och cancer har ett mer eller mindre aggressivt förlopp.

Psoriasis är en kronisk inflammatorisk hudsjukdom som kännetecknas av kraftigt fjällande röda hudförändringar, så kallade plack. Den sjukliga processen i psoriasis utgörs av inflammation i huden samt kombinationen av kraftig celldelning och onormal cellutmognad, vilka är egenskaper som psoriasis delar med cancer. Till skillnad från cancer är den ökade celldelningen vid psoriasis strikt kontrollerad.

Den ökade celldelningen i en brösttumör eller i psoriasisplacken bidrar till ett ökat behov av syre och näring. Detta behov tillgodoses genom bildningen av nya blodkärl. Ett ökat antal nya blodkärl samt en onormal mognadsprocess av celler i den växande tumören samt i psoriasisplacken i huden karaktäriserar både bröstcancer och psoriasis.

Psorasin är ett kalciumbindande protein som förekommer i mycket rikliga mängder i vissa tidiga former av bröstcancer samt i psoriasisplacken i huden.

Syftet med detta avhandlingsarbete var att undersöka psoriasins roll i nybildningen av blodkärl samt för cellers utmognad.

Vi studerade samspelet mellan psorasin, fria syreradikaler och tillväxtfaktorer och fann att mängden av psorasin och tillväxtfaktorer ökade i celler som utsattes för fria syreradikaler. När vi minskade mängden psorasin i cellerna minskade även mängden av tillväxtfaktorer. Vi fann även att celler som behandlades direkt med psorasin delar sig snabbare, rör sig snabbare samt börjar bilda blodkärlsliknande strukturer. Dessa effekter visade sig vara beroende av en receptormolekyl på cellernas yta, kallad RAGE, vilken binder psorasin och på så sätt förmedlar dess effekt. Vidare visade vi att psorasin i sig själv bidrar till ökade nivåer av fria syreradikaler genom att binda till RAGE.

Vi har även studerat kopplingen mellan psoriasin och cellers utmognad. Vi visade att mängden av psoriasin och ämnen som bidrar till cellers mognad ökade i celler vilka utsattes för behandlingar för avsikt att påskynda utmognaden. När vi minskade mängden psoriasin i cellerna minskade nivåer av dessa ämnen och utmognaden hindrades. I brösttumörer och psoriasishud från patienter fann vi en gradient av psoriasin där mängden av psoriasin kunde kopplas till cellernas mognadsgrad.

Ju fler nya blodkärl som bildas desto mer syre och näring kan cancercellerna och psoriasishuden få tillgång till och då gynnas celldelningen. Vidare påverkar den onormala utmognaden av celler utvecklingen av bröstcancer och psoriasis genom att celler uppvisar ett onormalt beteende. Våra resultat visar att psoriasin främjar nybildningen av blodkärl samt är betydelsefull för cellers utmognad och skulle därmed kunna utnyttjas i utvecklingen av nya behandlingsmetoder för bröstcancer och psoriasis. Genom att hindra effekten av psoriasin skulle blodkärlsnybildningen, vilken är livsnödvändig för celldelning, minska och cellutmognaden regleras och därmed skulle sjukdomarnas utveckling kunna hindras.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV):

- I. Shubbar E, **Vegfors J**, Carlström M, Petersson S and Enerbäck C. 2012. Psoriasin (S100A7) increases the expression of ROS and VEGF and acts through RAGE to promote endothelial cell proliferation. *Breast Cancer Res Treat.* Jul;134(1):71-80.
- II. **Vegfors J**, Bivik C, Ekman A-K and Enerbäck C. Psoriasin (S100A7) contributes to stress-induced angiogenesis in psoriasis by the regulation of angiogenic factors in keratinocytes and promotion of angiogenic properties of dermal endothelial cells. *In manuscript.*
- III. **Vegfors J**, Petersson S, Kovács A, Polyak K and Enerbäck C. 2012. The expression of psoriasin (S100A7) and CD24 is linked and related to the differentiation of mammary epithelial cells. *PLoS One.* 7(12):e53119.
- IV. **Vegfors J**, Ekman A-K, Bivik C and Enerbäck C. Psoriasin (S100A7) is regulated by protein kinase C (PKC) and contributes to keratinocyte differentiation by regulating the expression of epidermal differentiation markers. *In manuscript.*

ABBREVIATIONS

AMP	Antimicrobial peptide
CaCl ₂	Calcium chloride
CAPE	Caffeic acid phenethyl ester
CoCl ₂	Cobalt chloride
DCIS	Ductal carcinoma <i>in situ</i>
dnIKKB	Dominant negative I kappa B kinase-beta
ECM	Extracellular matrix
EDC	Epidermal differentiation complex
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
H ₂ O ₂	Hydrogen peroxide
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
HEKn	Human epidermal keratinocytes, neonatal
HMVEC-d	Human dermal microvascular endothelial cell
HUVEC	Human umbilical vein endothelial cell
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-17	Interleukin 17
Jab1	c-Jun activation domain-binding protein 1
K1	Keratin 1
K10	Keratin 10
MAPK	Mitogen-activated protein kinase
MMP-1	Matrix metalloproteinase 1
MMP-9	Matrix metalloproteinase 9
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MUC1	Mucin 1
NAC	N-acetyl-cystein

ABBREVIATIONS

NBT	NitroBlue tetrazolium
NF- κ B	Nuclear factor-kappa beta
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
polyHEMA	Poly-2-hydroxy-ethylmethacrylate
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
RPLP0	Ribosomal protein, large, P0
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
sRAGE	Soluble receptor for advanced glycation end products
THBS-1	Thrombospondin 1
TNF- α	Tumor necrosis factor alpha
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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INTRODUCTION

PSORIASIN

Psoriasin (S100A7) belongs to the S100 protein family of small calcium binding proteins. Psoriasin was first identified in 1991 as highly upregulated in the psoriatic epidermis [1].

THE S100 FAMILY

Throughout evolution S100 gene duplications have led to an increase in the gene number and diversity within the S100 family. Today, more than 20 different S100 proteins have been identified [2]. The proteins differ from one another in the length and sequence which specify the biological activity of the individual protein [3]. S100 proteins are produced as monomers and spontaneously form dimers. The dimerization seems important for the biological function. Upon binding of calcium, conformation changes enable the binding of target proteins.

S100 proteins have a wide range of intracellular functions, including regulation of calcium homeostasis, protein phosphorylations, enzyme activities, cytoskeleton rearrangement, transcriptional activities, cell growth and differentiation, and regulation of the inflammatory response [4-7]. S100 proteins are regulated at the transcriptional level, although, their intra- and extracellular roles can be altered by post-translational modifications [3, 8-9]. Extracellular S100 proteins act in an autocrine and paracrine manner via activation of surface receptors. The extracellular functions include regulation of cell proliferation and activation, apoptosis and chemotaxis [3].

THE GENE

Psoriasin is encoded by a gene located on chromosome 1 (1q21), within the epidermal differentiation complex (EDC) [10]. The psoriasin gene comprises three exons and two introns, encoding a protein of 101 amino acids. The first exon encodes most of the 5' untranslated region of the transcript, while exon two and three encode the start codon and the N-terminal EF-hand and the C-terminal EF-hand, respectively (Figure 1). Mutational analysis of the coding sequence of psoriasin in psoriatic patients has not revealed any nucleotide variants compared to healthy controls [11].

Sequencing of the human S100 gene cluster has identified five copies of S100A7-like genes (S100A7a-S100A7e) [12]. Two out of the five S100A7-like genes, S100A7a and S100A7c, express highly similar protein sequences. Sequence variation in the regulatory and promoter regions of the S100A7a and S100A7c genes suggest that the genes are expressed in a different way by the action of regulatory transcription factors [12]. S100A7a is also known as S100A15 or koebnersin.

Figure 1. The gene encoding the psoriasin protein comprises three exons and two introns. Exon two encodes the N-terminal EF-hand while exon three encodes the C-terminal EF-hand.

THE PROTEIN

Psoriasin is a low molecular weight protein of 11.4 kDa. S100 proteins are highly homologous and psoriasin share conserved structural motifs consisting of two EF-hands with other S100 proteins. EF-hands are calcium binding motifs composed of two helices joined together by a loop (helix-loop-helix) which is the calcium binding part.

Psoriasin contains a C-terminal canonical calcium-binding EF-hand motif and an N-terminal non-canonical EF-hand motif with lower affinity for calcium binding (Figure 2). The C-terminal EF-hand is connected to a stretch of amino acids referred to as the C-terminal extension. Between the two EF-hand motifs is the area known as the hinge region. It is the C-terminal extension and the hinge regions that are the most variable parts between the different S100 proteins and hence are responsible for the specific biological properties of the proteins. The calcium binding properties of S100 proteins are thought to be important for their functional activity [4]. In addition, psoriasin contains zinc binding sites [13].

Figure 2. The psoriasin protein contains two calcium-binding EF-hands which are separated by the hinge region. Psoriasin forms anti-parallel homodimers and upon calcium binding a hydrophobic region is exposed which facilitates the interaction of target proteins.

Psoriasin forms non-covalent anti-parallel homodimers with one calcium ion being bound by the canonical EF-hand motif in each monomer [14] (Figure 2). The binding of calcium is essential for the formation of the accurate structure [14]. In response to calcium binding, the protein undergoes a conformational change that exposes a hydrophobic region [15-16]. The hydrophobic region is required for interaction with target proteins and the subsequent biological effects [15]. Contrary to other S100 proteins, binding of calcium does not result in a large conformation change of psoriasin [17].

EXPRESSION AND LOCALIZATION

Psoriasin has been reported to be secreted from epithelial cells [18]. In addition, psoriasin has also been shown to be located within the nucleus and in the cytoplasm of keratinocytes and breast epithelial cells [19]. The expression of psoriasin is restricted to epithelial cells and cannot be detected in fibroblasts, endothelial cells or lymphocytes [1].

Psoriasin demonstrates a restricted expression in normal tissues. In healthy skin, psoriasin is present at low levels while other epithelial tissues do not display detectable levels [1]. The protein is abundant in psoriatic keratinocytes and has also been demonstrated to be up-regulated in other skin diseases like atopic dermatitis that display hyperproliferation and inflammation [20]. In various skin cancers psoriasin expression is frequently observed in abnormal keratinocytes in squamous cell carcinoma *in situ* and in invasive squamous cell carcinoma but rarely in non-invasive or invasive basal cell carcinoma [21]. In addition to skin pathologies, psoriasin expression is also demonstrated in non-squamous carcinomas including gastric cancer [22], melanoma [23] and breast cancer [24]. In healthy breast epithelial cells psoriasin is expressed at low or undetectable levels. However, in breast cancer cells psoriasin is up-regulated and can be detected in the nucleus and cytoplasm and is also secreted [19, 25].

ROLE AND FUNCTION

Psoriasin has been shown to function as a chemotactic agent stimulating the infiltration of CD4⁺ T cells and neutrophils into the epidermis [26]. The binding of psoriasin to the receptor for advanced glycation end products (RAGE) has been confirmed [27]. By binding to RAGE psoriasin mediates chemotaxis [28]. RAGE is a member of the immunoglobulin (Ig) superfamily of cell surface receptors [29-30]. It is considered to be a pattern recognition receptor (PRR) that does not recognize a specific ligand but a class of ligands. RAGE is thought to recognize spatial structures rather than amino acid sequences [31].

RAGE consists of an extracellular domain comprising three Ig domains, a single transmembrane spanning helix, and a short, highly acidic C-terminal cytosolic domain which is required for the signal transduction [32]. RAGE can be expressed as both a full-length membrane-bound form, and as various soluble forms lacking the transmembrane domain [33]. The activation of RAGE transmits signals through various intracellular pathways including the nuclear factor-kappa beta (NF-κB) pathway, which is responsible for the production of pro-inflammatory cytokines [34]. In addition to the NF-κB pathway, RAGE activates the phosphatidylinositol 3-kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK) pathways [35-36]. These signaling pathways often interact to regulate the overall cellular responses to various stimuli, stresses and environmental conditions [37]. The expression of RAGE itself is controlled by NF-κB transcription factors [34]. The activation of the RAGE-NF-κB signaling pathway triggers a potent positive-feedback loop, which result in an increased cell surface expression of RAGE and an amplification of the signal [38-39].

RAGE is expressed in low levels in a wide range of differentiated adult cells and is associated with inflammation-related pathological conditions. Endothelial cells up-regulate RAGE upon inflammation and injury [40]. Furthermore, RAGE expression has been detected in a variety of human tumors including breast tumors [41].

Psoriasin is secreted by tumor cells. After being released into the extracellular space psoriasin can interact with cell surface receptors on tumor cells such as RAGE, promoting carcinogenesis [42]. Extracellular psoriasin binds to RAGE and activates NF- κ B which controls the activation of several genes involved in immune responses including interleukin (IL)-8, as well as cell proliferation [28]. Psoriasin expression is induced by pro-inflammatory cytokines in keratinocytes and breast cancer cells [43-44].

Psoriasin has been shown to interact with the multifunctional signaling protein c-Jun activation domain-binding protein 1 (Jab1) [45]. Several of the tumorigenic effects of psoriasin on pro-survival and invasive pathways including NF- κ B, PI3K/Akt and activator protein 1 (AP-1), are mediated by the interaction of psoriasin with Jab1 [45-46]. In the nucleus, psoriasin binds to and initiates Jab1 [47].

Psoriasin is one of the dominating antimicrobial peptides (AMPs) of healthy skin, showing antimicrobial activity *in vitro* preferentially against *E. coli* and other Gram negative bacteria [48]. In contrast to the antimicrobial function of other known AMPs, no signs of perforation of the bacteria indicate that psoriasin kills *E. coli* in a different mode of action. The antimicrobial activity of psoriasin was found to be Zn²⁺ sensitive suggesting that psoriasin kills *E. coli* by Zn²⁺ sequestration [48-49].

BREAST CANCER

Breast cancer is the most common form of cancer in women world-wide, representing a variety of tumors with different cell origin. There is a high degree of heterogeneity among breast tumors, which can be divided into subtypes depending on gene expression patterns and clinical outcomes.

THE BREAST TISSUE

The breast is composed of a progressive branching system of ducts that originate in one of many terminal duct lobular units, which are the smallest functional units of the breast, and ends at the nipple. The terminal duct lobular unit, the structure from which the majority of breast cancers arise, is composed of two types of epithelial cells (Figure 3). The inner layer consists of luminal epithelial cells, which are potential milk secreting cells, and the outer layer consists of contractile myoepithelial cells, which produce the basement membrane. The distinction of cells of the lobular subtype from the ductal subtype is based upon differences in cell morphology. The terminal duct lobular units are surrounded by a microenvironment composed of the extracellular matrix (ECM) and various stromal cells.

Figure 3. The terminal duct lobular unit is composed of an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells, surrounded by the basement membrane.

BREAST CANCER DEVELOPMENT AND PROGRESSION

The malignant transformation of a normal cell into a cancer cell, and the progression into a solid tumor, is a complex multi-step process. Cancer cells have defects in the regulatory pathways involved in proliferation and homeostasis. They differ from normal cells in many important characteristics, including uncontrolled growth, immortalization, loss of contact inhibition, loss of differentiation, increased invasive capacity, evasion of the host immune surveillance processes, evasion of apoptosis, induction of angiogenesis and tissue invasion and metastasis [50-51].

Breast tumorigenesis is a sequential progression through defined clinical and pathological stages starting with hyperproliferation, progressing into *in situ* carcinoma, followed by invasive carcinoma and eventually culminating in metastatic breast cancer [52] (Figure 4).

Figure 4. The progression of breast cancer is a stepwise process from ductal hyperplasia via ductal carcinoma *in situ* to invasive carcinoma and eventually metastasis. The transition from the pre-invasive stage of ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC) occurs as the tumor cells break the basement membrane and invade the surrounding tissue.

GENETICS

Both genetic and environmental factors correlate with an increased risk of breast cancer development. The classical model for cancer development, which presumes that a series of mutations occurring in a cell can lead to cell transformation, is also true for breast cancer development. Like other cancers, amplifications of oncogenes or deletions of tumor suppressor genes underlie mammary tumorigenesis. A large number of genes are differentially expressed at different stages and in different types of lesions during the progression of breast cancer [53].

INFLAMMATORY FEATURES

An inflammatory component is present in the microenvironment of most tumorigenic tissues [54]. The mammary tissue microenvironment contributes to the outcome of tumor cells. The release and activation of growth factors and cytokines from the ECM or cell surface influence

the tumor cell survival. Cancer-associated inflammation involves the infiltration of immune cells, the presence of inflammatory cytokines and chemokines, and the tissue remodeling and angiogenesis. Cancer-associated stromal cells such as fibroblasts and immune cells affect the tumorigenic potential of the mammary stroma. In breast cancer, epidemiological data suggests that inflammation is associated with poor prognosis.

DUCTAL CARCINOMA *IN SITU* (DCIS)

Ductal carcinoma *in situ* (DCIS) is a heterogeneous type of cancer characterized by proliferation of malignant epithelial cells that are confined by the basement membrane of the mammary ducts. DCIS can be considered as an early breast cancer lesion.

The major issue in breast cancer progression is the transition of tumor cells of the pre-invasive stage of DCIS to invasive ductal carcinoma (IDC) (Figure 4). The majority of invasive carcinomas are referred to as ductal. DCIS is believed to be the precursor of IDC, based on its frequent coexistence with invasive lesions and on its high rate of recurrence as an invasive tumor at its original site [55]. Some DCIS, if untreated, will rapidly progress to invasive cancer, while others will change very little during several years [56].

PSORIASIN IN DCIS

In breast cancer psoriasin was first identified as one of the most abundantly expressed genes in epithelial cells of pre-invasive DCIS [25, 57]. Although the most frequent cytogenetic abnormalities in breast carcinomas involves chromosome 1 the high expression of psoriasin was confirmed not caused by amplification of the psoriasin locus on chromosome 1q21 [25]. The expression of psoriasin is low in normal breast and benign pathologies [57], but among the most highly expressed genes in high-grade DCIS [25, 53]. Whereas the expression is often reduced in invasive breast carcinoma, persistent high expression is associated with markers of poor prognosis, such as lack of the estrogen and progesterone receptors [19, 25, 58]. A high level of psoriasin expression within invasive tumors also correlates with indicators of increased metastatic potential. Psoriasin expression within breast tumor cells is associated with inflammatory infiltrates, important for cancer progression [19].

PSORIASIS

Psoriasis is a chronic inflammatory skin disease. Psoriasis has three principal histological features: epidermal hyperproliferation and abnormal keratinocyte differentiation, dilated and increased growth of blood vessels, and an infiltrate of inflammatory cells [59].

THE EPIDERMIS

The epidermis, the outermost layer of the skin, is a stratified squamous epithelium, which acts as the major physical and chemical barrier. The majority of cells in the epidermis are keratinocytes, which are organized into four histological distinct layers, the basal layer (stratum basale), the spinous layer (stratum spinosum), the granular layer (stratum granulosum) and the cornified layer (stratum corneum) [60] (Figure 5). In the basal layer, epidermal stem cells are responsible for the constant renewal of cells. The keratinocytes start to differentiate as they migrate through the cell layers [60]. During the differentiation process, the cells exit the cell cycle and begin to express epidermal differentiation markers. Finally, the cells lose their nuclei and organelles and become corneocytes, which are shed from the surface of the skin [60]. The differentiation process, from cell division in the basal

layer to the shedding of the corneocyte, normally takes roughly one month. Beneath the epidermal layers is the dermis located, which contains collagen and elastic tissue and thin arterial capillaries that carry nutrition and oxygen to the skin.

Figure 5. In the basal layer (stratum basale) stem cell division account for the constant renewal of cells. As the keratinocytes migrate through the spinous layer (stratum spinosum) and the granular layer (stratum granulosum) they differentiate and lose their nuclei and organelles and eventually become corneocytes. In the cornified layer (stratum corneum) the corneocytes are shed from the surface of the skin. Beneath the epidermis is the dermis located.

THE PSORIATIC EPIDERMIS

In psoriatic skin, the epidermis becomes thickened due to hyperproliferation of the keratinocytes (Figure 6). The keratinocyte differentiation is altered with an aberrant expression of differentiation markers and a reduced or missing granular layer. The characteristic adherent silvery scales of the psoriatic plaque are a result of hyperproliferative epidermal keratinocytes with a premature process of maturation and incomplete cornification. Parakeratosis, seen in the psoriatic epidermis, is characterized by the preservation of nuclei in the cornified layer and is associated with the thinning or loss of the granular layer. The migration of keratinocytes from the basal layer to the cornified layer is dramatically shortened from approximately one month in normal skin to only one week in psoriatic skin.

Figure 6. In psoriatic skin compared with normal skin the epidermis is thickened due to the hyperproliferation of keratinocytes. In the upper dermis of the psoriatic skin an increased number of tortuous and leaky capillaries are found.

In contrast to the normal epidermis, in psoriatic epidermis active inflammation occurs and is accompanied by a massive increase in the number of inflammatory cells. The inflammatory cells produce cytokines, which increase the inflammation. The redness of the psoriatic lesions is a consequence of the increased number of tortuous and leaky capillaries and increased blood circulation due to dilated capillaries in the upper dermis.

GENETICS

Psoriasis is a multi-factorial disease in which several genes and environmental stimuli interact. Although psoriasis has a strong genetic susceptibility a specific psoriasis gene or gene combination has not been identified. Genetic studies suggest several psoriasis susceptibility loci. At least nine chromosomal loci (*PSORS1–9*) have been identified for which evidence of linkage to psoriasis has been observed. The major chromosomal locus for linkage is *PSORS1*, which accounts for 35–50% of the heritability of the disease. Certain major histocompatibility complex antigens are associated with psoriasis and the strongest association appears to be with the HLA-Cw6 locus in *PSORS1* [61].

INFLAMMATORY FEATURES

Psoriasis is a complex disease with many underlying mechanisms, which involve the interplay between epidermal keratinocytes, leukocytes and vascular endothelium [62-63].

Psoriasis was initially believed to be a disease primarily of epidermal keratinocyte proliferation and differentiation [64]. However, after the discovery that immunosuppressive agents are effective in psoriasis therapy, it is now considered to be an immune-mediated disease [65]. Inflammatory infiltrates appear early in the psoriatic lesions, even before the epidermal changes can be observed [66]. The over-expression of pro-inflammatory cytokines is considered to be responsible for the initiation, maintenance and recurrence of psoriatic skin lesions.

Pro-inflammatory cytokines from T helper 1 (Th1) cells predominate in the psoriatic lesion and psoriasis was initially believed to be a Th1-mediated disease, driven by interferon gamma (IFN- γ) [62-63]. Interleukin (IL)-23, a cytokine involved in the development of Th17 cells, as well as IL-17 and IL-22, Th17 derived cytokines, have also been found to play major roles in psoriasis [67], suggesting psoriasis to be Th17-mediated.

It has been shown that psoriasis is caused by an interaction between epidermal keratinocytes and the immune system. The keratinocytes within the psoriatic epidermis are abnormal in many aspects and likely influences immune cells by producing inflammatory cytokines and chemokines. Although, the pathogenesis of psoriasis is still unclear, the role of the keratinocytes remains an important feature.

PSORIASIN IN PSORIASIS

Psoriasin is highly expressed in psoriatic keratinocytes. Psoriasin is proposed to have an important role in keratinocyte differentiation and in the pathogenesis in psoriasis [26]. Furthermore, psoriasin has been suggested to contribute to cutaneous inflammation in psoriasis [28, 68]. The Th17 cytokines IL-17 and IL-22 secreted by psoriatic T cells are increased in the psoriatic skin and are important players in psoriasis pathogenesis. Psoriasin is pronounced in the epidermis by these Th17 cytokines [69].

ANGIOGENESIS

Angiogenesis is the formation of new blood vessels from the existing vasculature. All cells and tissues are dependent on a regular supply of oxygen and nutrients. No metabolically active tissue is located more than a few micrometers from a capillary. Angiogenesis is a key process during embryogenesis when new vessels are formed, whereas during adulthood vessels remain quiescent [70]. Endothelial cells retain the ability of dividing in response to different stimulus, including hypoxia and inflammation. Angiogenesis is tightly regulated by the balance between pro- and anti-angiogenic factors. In conditions of tumor growth and chronic inflammation, such as psoriasis, the pro-angiogenic stimuli become excessive and the balance is tilted, resulting in angiogenesis. These pathological conditions induce an angiogenic response in order to cope with the increased oxygen and nutrient demand, important for disease development [71].

Angiogenesis is initiated by the activation of vascular endothelial cells through several factors including the major pro-angiogenic factor VEGF. It is a multistep process that includes vasodilatation, increased vascular permeability, destabilization of existing blood vessels, degradation of the ECM, endothelial cell proliferation and migration, lumen formation and vessel maturation [71-72]. Proteolysis of the basement membrane is a crucial requirement for the formation of new vessels. Matrix metalloproteinases (MMPs) are enzymes that break down and remodel the ECM. The remodeled ECM permits the migration of endothelial cells. Furthermore, ECM degradation leads to the release of pro-angiogenic factors stored in the ECM, promoting angiogenesis. In activated endothelial cells MMPs are strongly induced and subsequently activated in contrast to quiescent endothelial cells which produce little or no active proteases like MMPs [73].

ANGIOGENESIS IN BREAST CANCER

Angiogenesis is a crucial requirement for the growth, progression and metastatic spread of a tumor [50, 74-75]. Microscopic tumors that fail to induce angiogenesis result in quiescent tumors without the ability to progress in size. Tumors may persist for long periods of time as microscopic lesions that are in an inactive state [76-77]. Conversely, in growing tumors the insufficiency of vascular support results in necrosis or apoptosis.

Dysregulated signaling and hypoxic conditions, which are common in solid tumors, lead to sustained and uncontrolled angiogenesis. Chronic inflammation mechanisms, such as the production of reactive oxygen species (ROS) and secretion of pro-inflammatory cytokines, can also promote angiogenesis in tumor progression. The malignant cells undergo an angiogenic switch in response to hypoxia, leading to secretion of angiogenic factors and proteolytic enzymes. The angiogenic switch will culminate in the activation of endothelial cell proliferation, migration and establishment of a capillary network, providing the growing tumor mass with all the required metabolites. Tumor angiogenesis also provides tumor cells with the opportunity to enter the circulation with the possibility to form distant metastases.

VEGF is a potent and specific mitogen for vascular endothelial cells and the major angiogenic factor in physiological and pathological angiogenesis, stimulating the cascade of events required for angiogenesis. VEGF is over-expressed in a variety of tumors [78-79]. Binding of VEGF to vascular endothelial growth factor receptor (VEGFR)-2 on endothelial cells promotes differentiation, proliferation and migration [80]. VEGFR-1 has been shown to be expressed on breast cancer cells [81], and to be implicated in tumor growth and progression [82-83]. Furthermore, VEGFR-2 has been found to be expressed in breast cancer [82-84].

ANGIOGENESIS IN PSORIASIS

Psoriasis was early suggested to be an angiogenesis-dependent disease [85]. The increased number of blood vessels is required to meet the great nutritional need of the hyperproliferative psoriatic epidermis. In psoriasis the microvasculature undergoes morphological alterations such as increased permeability and dilation as well as tortuosity and elongation of dermal capillaries [86-87]. These alterations are among the earliest detectable histological feature during the development of psoriatic lesions.

A number of angiogenic factors are produced by psoriatic keratinocytes, including VEGF, HIF-1, IL-8 and angiopoietins [88-89]. Keratinocyte-derived VEGF is a potent mitogen for endothelial cells via VEGFR [90]. In psoriasis an over-expression of VEGF in epidermal keratinocytes and an enhanced expression of VEGFRs on endothelial cells have been demonstrated [91]. Until recently, VEGFRs were thought to be absent in the epidermis, but epidermal keratinocytes have now been found to express both VEGFR-1 and VEGFR-2 [92]. VEGF has autocrine effects on the behavior of epidermal cells and may contribute to keratinocyte proliferation and epidermal barrier homeostasis [93].

An initial trigger of angiogenesis, such as the presence of activated T cells or tissue hypoxia, induces the secretion of angiogenic factors by pre-lesional keratinocytes [88]. A pro-angiogenic role has been attributed to the Th17 cytokine IL-17 [94-95]. IL-17 triggers the production of chemokines, growth factors and adhesion molecules by epithelial cells, fibroblasts and endothelial cells. Furthermore, IL-17 induces keratinocyte production of VEGF.

DIFFERENTIATION

Differentiation is the process wherein cells gradually become more specialized by the change in phenotype. The general view of the differentiation process is that it is unidirectional, but in conditions like cancer observations show that the differentiation process can reverse and that cancer cells can dedifferentiate [96].

BREAST EPITHELIAL CELL DIFFERENTIATION AND BREAST CANCER

The breast is not fully developed at birth but continues to develop in adulthood. The development is initiated during embryogenesis and continues during fetal development, puberty and pregnancy [97]. Complete differentiation is not attained until the first fulltime pregnancy [97].

All tumors show abnormalities in differentiation. The presence of immature and the lack of mature terminally differentiated cells is a feature of cancer. Highly differentiated tumors have a better prognosis than poorly differentiated tumors [98]. In tumors which display a high degree of differentiation the cells are morphologically similar to the native tissue, whereas the opposite is true for a low degree of differentiation, where cells have lost the structural organization and similarity with the surrounding tissue. The degree of differentiation within the DCIS lesion has a large impact on the outcome. Cases displaying low differentiation most often progress to become invasive carcinoma, whereas cases of high differentiation are less likely to progress. In DCIS with comedo lesions a central necrotic core is the result of intra-lesional hypoxia. Tumor cells close to the necrotic core within the hypoxic region, acquire a less mature, dedifferentiated phenotype [99]. Hypoxia may contribute to the conversion of DCIS cells into invading tumor cells. Hypoxia has been shown

to induce epithelial to mesenchymal transition (EMT) in tumors. This process describes how epithelial cells phenotypically transdifferentiate towards a more mesenchymal spindle-shaped cell, simultaneously gaining increased capacity for invasiveness and motility.

KERATINOCYTE DIFFERENTIATION AND PSORIASIS

The differentiation process of epidermal keratinocytes progresses through multiple, strongly regulated steps. Keratinocytes begin as stem cells in the basal layer providing a continuous supply of cells that replenish the epidermis [60]. Once committed to differentiation, basal keratinocytes lose their proliferative potential and undergo morphological changes and alters the gene expression as they migrate towards the epidermal surface and form the spinous, granular and cornified layers (Figure 5).

Cells in the spinous layer are characterized by numerous intracellular desmosomal connections around the cell periphery. As keratinocytes enter the granular layer, major differentiation events become evident and markers of differentiation are expressed. The most prominent morphological feature of cells in the granular layer is the protein rich granules. These granules contain products of keratinocyte differentiation, such as involucrin. Involucrin becomes cross-linked by the enzyme transglutaminase, resulting in the formation of the cornified envelope [100]. Involucrin is cross-linked early in the cornified envelope formation and forms a scaffold for incorporation of other proteins. As keratinocytes progress from the granular layer to the cornified layer they lose their nuclei and organelles. Keratinocytes in the cornified layer, which are called the corneocytes, are surrounded by the cornified envelope.

In the psoriatic skin, the onset of epidermal differentiation is delayed and abnormal and the formation of the cornified envelope is initiated prematurely [101]. Transglutaminase 1 and involucrin are up-regulated in psoriasis [102-103]. Keratin expression is disrupted in psoriasis. Keratin 1 (K1) and K10, markers of terminal differentiation, are down-regulated whereas K6 and K16, markers of abnormal hyperproliferative conditions, are up-regulated [104]. Filaggrin, normally found in the granular layer of the epidermis, is absent in psoriatic lesions. The loss of the granular layer in psoriasis accounts for the absence of filaggrin.

AIMS OF THESIS

GENERAL AIM

The general aim of this thesis was to investigate the cellular effects of the S100 protein psoriasin in angiogenesis and differentiation, with special emphasis on breast cancer and psoriasis.

SPECIFIC AIMS

The specific aims of the included papers were:

- to explore the role of psoriasin in angiogenesis and the direct effects on endothelial cells, subsequently promoting increased angiogenesis important for breast tumor growth (**Paper I**).
- to define the angiogenic properties of psoriasin in the epidermis and to investigate the effects on dermal endothelial cells, that may promote angiogenesis in psoriasis (**Paper II**).
- to evaluate whether psoriasin has a role in the differentiation process of mammary epithelial cells, potentially affecting the progression of breast cancer (**Paper III**).
- to investigate the involvement of psoriasin in keratinocyte differentiation, possibly contributing to the development of psoriasis (**Paper IV**).

MATERIAL AND METHODS

CELL LINES

In paper I and III, the normal immortalized human mammary epithelial cell line, MCF10A, and the human breast cancer cell line, MDA-MB-468, both obtained from American Type Culture Collection (ATCC), were maintained in 10% fetal bovine serum in McCoy's medium, or in 5% horse serum in Dulbecco's Modified Eagle medium (DMEM)/F12 medium supplemented with 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone.

The human epidermal keratinocytes of neonatal origin (HEKn) used in paper II and IV, were cultured in Epilife medium supplemented with 1% Epilife Defined Growth Supplement (EDGS) and 0.1% calcium chloride (CaCl_2) (0.6 μM).

In paper I, the human umbilical vein endothelial cells (HUVEC) were grown in Medium 200 supplemented with Low Serum Growth Supplement (LSGS). The dermal-derived human microvascular endothelial cells (HMVEC-d) used in paper I and II, were cultured in endothelial basal medium (EBM) supplemented with microvascular endothelial cell growth medium-2 (EGM-2-MV).

All cell culture media were supplemented with 1% penicillin/streptomycin and the cultures were grown at 37 °C in a humidified atmosphere containing 5% CO_2 .

CULTURE CONDITIONS AND TREATMENTS

To examine the role of intracellular psoriasin, cells were infected with adenoviral- or retroviral vectors expressing psoriasin protein, or stimulated by hydrogen peroxide (H_2O_2) (75-500 μM) or $\text{TNF-}\alpha$ (5-10 ng/ml) to induce the endogenous expression of psoriasin. The effect of hypoxia was studied by culturing cells in approximately 1% oxygen or treatment with the hypoxia-mimicking agent cobalt chloride (CoCl_2) (500 μM).

MCF10A cells were maintained in confluence for 5-10 days or in suspension for 3 days in order to investigate the endogenous expression of psoriasin. For the suspension cultures,

poly-2-hydroxy-ethylmethacrylate (polyHEMA) coated plates (10 mg/cm² in 95% ethanol) were used. To induce differentiation of HEK293, the extracellular calcium concentration was increased to 2 mM during 48 hours, using CaCl₂, or cells were treated with 1 μ M 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

To evaluate the extracellular role of psoriasin, the recombinant psoriasin protein, made in the laboratory or purchased, was used in the concentration of 0.15-10 μ g/ml. VEGF, in the concentration of 10 ng/ml, was used as a positive control for stimulating endothelial cells in a mitogenic and pro-angiogenic manner. With the purpose to study the role of psoriasin as ligand for RAGE, the soluble form of RAGE (sRAGE) (50 ng/ml for 30 minutes) or the antibody directed against RAGE (anti-RAGE) (20 μ g/ml for 3 hours) was used.

N-acetyl-cysteine (NAC) (10 mM) or the adenoviral vector expressing Bcl-2 protein was used for their anti-oxidative effects to study the involvement of psoriasin in ROS-signaling and ROS generation. To evaluate the involvement of signaling pathways, cells were treated with the inhibitors caffeic acid phenethyl ester (CAPE) (50 μ M), Tyrphostin (10 mM), U73122 (10 mM), Wortmannin (50 nM), LY294002 (5 μ M), or were infected with dominant negative I kappa B kinase-beta (dnIKKB) adenoviral vector. Dimethyl sulfoxide (DMSO) was used as the diluents control.

TISSUE SAMPLES

Tissue samples from ductal carcinoma *in situ* (DCIS) tumors (Paper III) and psoriatic skin (Paper IV) were obtained from the files of the Departments of Dermatology and Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden. Informed consent was requested from the patients whenever this was feasible and was obtained. The tissue samples were de-identified before further study. The studies were approved by the ethics committee of the University of Gothenburg.

UP-REGULATION OF PSORIASIN EXPRESSION

Transduction is the process whereby genetic materials are introduced into another cell via a viral vector. We have used adenoviral- and retroviral vectors for the up-regulation of psoriasin expression.

ADENOVIRAL PRODUCTION

Adenoviruses are naked viruses with a linear double stranded DNA (dsDNA) genome. This virus is able to infect both dividing and non-dividing cells. Adenoviruses are able to replicate in the nucleus of the target cell using the cell's own replication machinery. Gene-transfer using adenoviral vectors results in a transient expression as adenoviral DNA does not integrate into the host genome and is not replicated during cell division.

For the transient over-expression of genes, adenovirus were produced using the AdEasy protocol [105]. cDNA of the gene of interest (GOI) was amplified and subcloned into the shuttle vector, Ad-Track-CMV. Homologous recombination between the pAd-Track-GOI and the backbone vector, pAdEasy-1, was made by linearization of pAd-Track-GOI and transformation into competent AdEasy cells, derivatives of *E. coli* BJ5183, containing the adenoviral backbone plasmid pAdEasy-1. For viral production and amplification the recombinant adenoviral plasmid was transfected into the packaging cell line, HEK-293.

Purification of recombinant adenoviruses was made by density gradient centrifugation in a cesium chloride (CsCl) gradient.

Transient over-expression of psoriasin, Bcl-2, phosphorylation defective dnIKKB and GFP, used as the control, was achieved by transduction of target cells with the produced adenoviral vector.

RETROVIRAL PRODUCTION

Retroviruses are single stranded RNA (ssRNA) enveloped viruses that only infect dividing cells. Gene-transfer using retroviral vectors will result in a long lasting, stable expression as retroviruses contain a reverse transcriptase that allows integration into the host genome.

For the stable over-expression of psoriasin, retroviral transduction was used. Recombinant retroviral vectors were produced by cotransfection of HEK-293T cells with the retroviral expressing vector pBABE-puro and the pCL-Ampho packaging vector. Transfection was performed with Lipofectamine reagent according to manufacturer's instructions. Viral supernatants containing fully packaged retroviruses were collected and filtered through a 0.45 µm filter. Target cells were transduced with retrovirus in the presence of polybrene. Cells were maintained in ordinary culture medium and 48 hours post-transduction puromycin was added to culture medium for selection of stable cell lines.

DOWN-REGULATION OF PSORIASIN EXPRESSION

RNA interference (RNAi) is a post-transcriptional gene regulatory mechanism that is mediated by small interfering RNA (siRNAs) in the cytoplasm of the cell, leading to the degradation of the corresponding mRNA molecules. The RNAi process is initiated when double stranded RNA (dsRNA) molecules enter the cytoplasm. The dsRNA may either be long molecules of siRNA, or ssRNA containing two complementary sequences that form a short hairpin RNA (shRNA). siRNA is formed in the cytoplasm by the cleavage of these long dsRNA or shRNA molecules by the enzyme Dicer. The two strands of the double stranded molecule bind to the RNA Induced Silencing Complex (RISC) which separates the two strands. One of the strands is degraded while the other strand binds to the target mRNA and cleaves it, thus preventing the protein from being made.

We have used both siRNA and shRNA for the down-regulation of psoriasin expression.

SMALL INTERFERING RNA (siRNA)

For the transient down-regulation of psoriasin expression, transfection with siRNA directed against psoriasin (Pso-siRNA) was performed, according to manufacturer's instructions. As a control for siRNA experiments control-siRNA (C-siRNA) was used, containing a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA. siRNA at the concentration of 0.5 µM was mixed with transfection reagent and incubated 45 minutes at room temperature. The siRNA-transfection reagent mixture was diluted in transfection medium before it was added to cells. Cells were incubated with siRNA-mixture for 7 hours before shifting to ordinary culture medium. Cells were further cultured for 18 hours followed by treatment with the intention to induce psoriasin expression.

For the transient down-regulation of psoriasin in HEKn for the PrestoBlue proliferation assay, cells were transfected in suspension, according to manufacturer's instructions with minor modifications. The siRNA-transfection reagent mixture was added to cells in suspension when they were seeded.

SHORT HAIRPIN RNA (shRNA)

shRNA is produced in the target cell from a DNA construct that is delivered to the nucleus by a viral vector and becomes part of the target cell's genome. This construct encodes sequences of complementary ssRNA creating a dsRNA molecule with a hairpin loop. The gene silencing effect remains stable as the cell continues to produce the shRNA, which enters the RNAi pathway. shRNAs, as opposed to siRNAs, are synthesized in the nucleus of cells, further processed and transported to the cytoplasm, and then incorporated into the RISC.

For the generation of a cell line with a stable down-regulation of psoriasin expression shRNA clones were established. For the expression of psoriasin-shRNA (Pso-shRNA) and control-shRNA (C-shRNA) oligos were subcloned into the lentiviral pLKO-puro vector. Cells were transfected with the pLKO-puro constructs using Lipofectamine LTX with plus reagent. Stable clones were selected for two weeks in medium containing puromycin.

RECOMBINANT PSORIASIN PROTEIN PRODUCTION

Recombinant protein is a protein encoded by a gene that has been cloned into a system that supports the expression and has been generated to produce large quantities of proteins.

E. coli M15, maintained in LB medium, was used as the host strain for the pQE30-psoriasin 6xHis-tag coding expression vector, provided by Dr. Kornelia Polyak, at the Dana-Farber Cancer Institute. The construct, pQE30-psoriasin, was transformed into *E. coli* and transformants were selected on plates in LB medium containing 200 µg/ml ampicillin and 25 µg/ml kanamycin. Selected transformants were propagated and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added to induce the expression of the 6xHis-tag recombinant psoriasin protein. The expression of the 6xHis-tag recombinant psoriasin protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads according to the instructions of the Ni-NTA affinity chromatography purification kit. Psoriasin was identified by SDS-PAGE and Coomassie blue staining using FlourChem 8000 camera according to the manufacturer's instructions.

Commercially available recombinant psoriasin protein was primarily used in experiments.

RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA was extracted from cells using RNeasy Mini Kit with DNase-I or RNeasy Plus Mini Kit, according to the manufacturer's instructions. The concentration and purity of each RNA sample were measured using a Nanodrop ND-1000 Spectrophotometer.

cDNA was synthesized from 0.3-1 µg of the total RNA with oligo(dT)18 and random hexamer primers using Maxima First Strand cDNA Synthesis Kit or by SuperScript II RNase H-reverse Transcriptase, according to the manufacturer's instructions.

QUANTITATIVE REAL-TIME PCR (qPCR)

Quantitative real-time PCR (qPCR) was performed using the SYBR Green PCR Master Mix. SYBR Green is a DNA binding dye that emits fluorescence when bound to dsDNA. Due to the non-specific nature of the SYBR Green detection system any dsDNA will be detected. To validate the method, non-specific products formation was verified by a dissociation curve.

PCR amplification was performed with specific primer pairs of target genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin and ribosomal protein, large, P0 (RPLP0) were used as the reference genes. The primers for psoriasin, VEGF, RAGE and GAPDH have been previously described [106-110]. The primers for the remaining target genes were designed using the Primer Express Software version 3.0. Amplification and detection of mRNA were performed on the 7500 or 7900 real-time PCR System. The PCR reaction comprised 40 cycles. For comparison of mRNA expression the comparative C_t ($2^{-\Delta\Delta C_t}$) method was used [111], a mathematical method that calculates the change in gene expression as a relative fold difference between an experimental sample and a reference sample. The reference genes, GAPDH, β -actin or RPLP0, used for normalization were evaluated and controlled.

MAGNETIC ACTIVATED CELL SORTING (MACS)

For the enrichment of the CD24⁺ cell population from confluent cultures of MCF10A, cells were magnetically isolated based on the specific surface antigen, using the magnetic activating cell sorting (MACS). Cells were labeled with a mouse anti-human CD24 antibody (1:20). For separation, cells were magnetically labeled with goat anti-mouse IgG MACS MicroBeads and applied to a LS column placed in a strong magnetic field in the QuadroMACS Separator. Separation was performed according to the manufacturer's instructions. During separation, the unlabeled cells (negative selection) pass through the column while the magnetically labeled CD24⁺ cells (positive selection) are retained within the column. After a washing step, the column was removed from the magnetic field of the separator, and the target cells were eluted from the column.

FLOW CYTOMETRY

Flow cytometry analyses were performed to measure the expression of CD24, CD44, and mucin1 (MUC1) on the surface of MCF10A cells. Cells were incubated with antibodies for 30 minutes at 4°C. The antibodies used were FITC-conjugated mouse anti-human CD24 (1:20), PE-conjugated mouse anti-human CD44 (1:50) and FITC-conjugated mouse anti-human CD227 (MUC1) (1:30). Flow cytometry analyses were performed using the FACSaria and the results were analyzed using the FACSDiva Software v.6.1.3.

WESTERN BLOTTING

Western blotting, or immunoblotting, is a method for the detection of separated denatured proteins, based on the molecular mass, using antibodies.

Cell pellets were thawed on ice in twice the volume hypotonic buffer (EDTA 10 mM, Tris 50 mM) containing DTT (1 mM). Disruption of cells was performed by sonication and supernatants containing proteins were collected after centrifugation (10 minutes, 10 000 × g, 4°C). The protein concentration was determined using Bio-Rad protein assay. Sample buffer (SDS 2%, glycerol 10%, Tris pH 6.8) containing β -mercaptoethanol was added to each sample of equal amounts of protein. Denaturation was performed before loading of samples on a NuPAGE 4-12% Bis-Tris Gel together with Novex sharp pre-stained protein standard. Following electrophoresis, proteins were transferred by blotting at 30 V for 1.5 hours to a Protran nitrocellulose transfer membrane. The membrane was blocked in 5% skimmed milk

powder in Tris-buffered saline-0.05% Tween for at least 1 hour. The membrane was incubated with primary psoriasin mouse monoclonal antibody (1:500) or GAPDH rabbit polyclonal IgG antibody (1:500), respectively, for at least 1 hour in gentle shaking. The membrane was incubated with horse radish peroxidase (HRP)-conjugated goat anti-mouse (1:2000) or goat anti-rabbit (1:10000) secondary antibodies for 1 hour, followed by incubation in SuperSignal West Pico Chemiluminescent Substrate. HRP conjugated to the secondary antibodies reacts with the substrate and the chemiluminescence signal was registered using Fuji film LAS-1000 camera. Protein expression was quantified using the Multi Gauge v 3.0 software and correlated to the internal control GAPDH.

IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemistry (IHC) is a method for the detection of proteins in tissue sections, using antibodies.

Formalin-fixed 4 μm paraffin-embedded sections from tissue blocks were mounted onto frost-coated glass slides, deparaffinized in xylene, dehydrated in a gradient of alcohol and blocked for endogenous peroxidase activity in 3% H_2O_2 . Sections were pre-treated in 10 mM citrate buffer in a pressure cooker for antigen retrieval. Primary antibodies, anti-S100A7 (1:200 and 1:400) and anti-CD24 (1:50) were allowed to bind for 25 minutes at room temperature or at 4°C over night. Antigen-antibody complexes were visualized using an ABC detection system with 3,3'-diaminobenzidine (DAB) as the chromogen. Cell nuclei were counterstained with hematoxylin.

APOPTOSIS DETECTION ASSAY

Apoptosis is the process of programmed cell death.

Apoptosis was analyzed by the FITC Annexin V Apoptosis Detection Kit I, according to the manufacturer's instruction. During early stages of apoptosis, phosphatidylserines are translocated from the inner side of the cell membrane to the outer side. Annexin V is a phosphobinding protein that binds phosphatidylserines. To distinguish apoptotic cells from necrotic cells staining with propidium iodide (PI) is necessary, as translocation of phosphatidylserines also occurs during necrosis. The cell membrane of necrotic cells is leaky and thus these cells are stained by PI. Cells were stained with Annexin V-FITC and PI and analyzed using the Gallios flow cytometer and the Kaluza analysis software. Annexin V⁻PI⁻ cells were considered to be viable, Annexin V⁺PI⁻ cells apoptotic and Annexin V⁺PI⁺ cells dead or necrotic.

NITROBLUE TETRAZOLIUM (NBT) ASSAY

The NitroBlue Tetrazolium (NBT) assay was performed to measure the generation of ROS, according to manufacturer's instructions [112-113]. The membrane permeable yellow-colored NBT at the final concentration of 1 mg/ml was added to the culture media at the end of the period of cell stimulation. Cells were incubated and fixed with ice-cold methanol. NBT absorbed by the cells is reduced into water-insoluble blue formazan particles by intracellular superoxide anion (O_2^-). The intracellular blue formazan particles were dissolved with 2 M

potassium hydroxide (KOH) and DMSO. The absorbance was measured spectrophotometrically at 630 nm.

CELL VIABILITY AND PROLIFERATION ASSAY

Cell viability and proliferation was determined using CellTiter 96[®] AQueous One Solution Cell proliferation assay (MTS) or PrestoBlue cell viability reagent, according to the manufacturer's instructions.

The MTS assay contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS). The activity of cellular dehydrogenases in viable cells reduces the tetrazolium salt into a soluble formazan product. The quantity of formazan was measured by the amount of absorbance at 490 nm.

The PrestoBlue cell viability reagent is a resazurin-based solution containing a cell-permeable, non-fluorescent compound that is blue in color. This compound is modified by the reducing environment within the cytosol of viable cells to turn red in color. This change was detected using absorbance measurements. The absorbance was read at 570 nm, with reading at 600 nm as the reference wavelength for normalization. The absorbance is directly proportional to the number of viable cells in proliferation. The fold change of the absorbance was calculated for treated cells against untreated cells to demonstrate the proliferation.

Moreover, cell proliferation was confirmed by trypan blue exclusion.

CELL MIGRATION ASSAY

The scratch assay is a commonly used assay to measure basic cell migration, and parameters such as speed, persistence and polarity. Cells were allowed to grow to confluence. After that the cultures were scratched with a pipette tip and washed with PBS to remove debris. Cells at the edge of the scratch migrate to fill the empty space. Cells were allowed to migrate in reduced serum media containing treatments for 18 hours. Each condition was performed in triplicate. Complete medium, containing serum and supplements, or VEGF (10 ng/ml) were used as positive controls. Following incubation, culture medium was removed, and cells were washed with PBS. Cell migration was visualized using an Olympus IX51 inverted microscope and a PC-connected Olympus DP70 camera.

TUBE FORMATION ASSAY

The formation of endothelial cells in capillary-like structures was studied on Geltrex reduced growth factor basement membrane matrix. Geltrex was polymerized for 30 minutes at 37°C in a 24-well plate, according to the manufacturer's instructions. Cells were suspended in culture medium, without growth factors, containing 1% serum and added to wells coated with polymerized Geltrex. Each condition was performed in triplicate. Complete medium or VEGF (10 ng/ml) were used as positive controls and medium without growth factors, supplemented with 1% serum or PBS, was used as negative controls. Following incubation, culture medium was removed, and cells were washed with PBS. The capillary-like network structures of endothelial cells were visualized using an Olympus IX51 inverted microscope and a PC-connected Olympus DP70 camera after 18 hours of incubation.

STATISTICAL ANALYSES

Statistical analyses in paper I and III were performed using paired and non-paired Student's t-test. In paper II and IV statistical analyses were performed with paired Student's t-test or the Wilcoxon signed rank test. Correlations in paper III were analyzed using Spearman's rank correlation test. A value of $p < 0.05$ was considered statistically significant. The results are presented as the mean $\pm$ standard deviation (SD) (Paper I and Paper III) or standard error of mean (SEM) (Paper II and Paper IV) for at least three independent experiments.

RESULTS AND DISCUSSION

PAPER I

Neovascularization is essential for tumor growth and subsequent metastasis [75]. In normal tissue the vasculature is tightly regulated by the balance between pro- and anti-angiogenic signals. In cancer, this process is often disturbed, which is important for the neoplastic progression [114]. Angiogenesis is a complex, multistep process involving extracellular matrix remodeling, endothelial cell migration and proliferation, capillary differentiation and finally lumen development [71].

DCIS is one of the earliest forms of breast cancer and is considered to be a precursor of invasive ductal carcinoma [115]. High-grade comedo DCIS is associated with increased VEGF levels and blood vessel density [116]. Psoriasin has been reported to be highly expressed in high-grade DCIS [25, 56-57, 117-118]. Down-regulation of psoriasin in a highly psoriasin-expressing breast cancer cell line decreases the VEGF levels and the blood vessel density, and inhibits tumor growth *in vivo* [106]. These findings raised the question whether psoriasin may promote tumor growth by angiogenesis.

The vector-mediated induction of psoriasin expression in the normal breast epithelial cell line MCF10A significantly increased the VEGF expression. Similarly, suspension culture of MCF10A cells, which induces the endogenous psoriasin expression, also caused a significantly increased expression of VEGF. VEGF is known to be a potent growth factor and a multifunctional cytokine, and is involved in tumor angiogenesis [80, 119]. The over-expression of VEGF is considered to be the major factor underlying pathological angiogenesis in cancer, as well as in chronic inflammation such as psoriasis [120]. VEGF has been shown to be up-regulated in DCIS and invasive breast carcinoma compared with normal breast tissue [121-122]. High-grade comedo DCIS, over-expressing psoriasin, is associated with increased angiogenesis [116].

Like VEGF, psoriasin is induced by ROS [123]. We found that induction of the psoriasin expression by H₂O₂ led to an up-regulation of the VEGF expression. Moreover, knock-down of psoriasin significantly reduced the H₂O₂-induced expression of VEGF. These

results suggest that psoriasin mediates the increased expression of VEGF in mammary epithelial cells.

In contrast to the findings in epithelial cells, we found that psoriasin was neither expressed nor inducible in endothelial cells by different treatments. As psoriasin may be secreted by the cells that express it [1, 25] we hypothesized that extracellular psoriasin can affect endothelial cells.

HUVECs that were treated with various concentrations of recombinant psoriasin showed significantly induced proliferation, comparable to the proliferation seen by VEGF stimulation. The proliferative effect of psoriasin was confirmed in HMVEC-d. Vector-mediated intracellular up-regulation of psoriasin in HUVECs caused no change in proliferation (Figure 7a).

The lack of induced endothelial cell proliferation by intracellular psoriasin led to the hypothesis that the proliferative effect may be mediated by the binding of extracellular psoriasin to a specific receptor. The multi-ligand RAGE is able to bind several ligands, including the S100 proteins [28, 32, 124-126] and is expressed in endothelial cells [127]. RAGE transduces inflammatory responses and plays a role in the pathogenesis of several diseases including cancer and inflammatory diseases [128-129]. Recently, S100A8 and S100A9 were shown to promote tumor cell growth via RAGE ligation [126]. The common structural features and sequence homology among the S100 proteins raised the question of whether psoriasin is a ligand to RAGE in endothelial cells. HUVECs treated with a soluble truncated form of the receptor (sRAGE) or an antibody directed against the RAGE immunoglobulin domains (anti-RAGE) displayed an abrogated proliferation in response to psoriasin treatment (Figure 7b). Treatment with sRAGE also reduced psoriasin-induced HUVEC tube formation (Figure 7c). sRAGE acts on the ligands and not on the receptor itself, meaning that treatment with sRAGE reduces the bioavailability of the ligand in contrast to preventing RAGE signal transduction [130]. RAGE expression and positioning on the plasma membrane tend to co-localize with the presence of molecules most likely binding to the receptor. Correspondingly, we found that extracellular psoriasin induced RAGE expression in endothelial cells (Figure 7d). Our data suggest that RAGE-mediated signaling is involved in psoriasin-induced endothelial cell proliferation and capillary-like tube formation. Thus, psoriasin secreted from epithelial cells may interact with RAGE on the surface of endothelial cells, which may in turn induce angiogenesis.

Figure 7. Inhibition of RAGE diminishes endothelial cell proliferation and capillary-like tube formation in response to recombinant psoriasin protein. **a)** Proliferation of HUVECs infected with psoriasin-expressing adenovirus (Ad-Psoriasin-GFP) was comparable to the proliferation of untreated control cells. **b)** HUVECs treated with sRAGE showed a diminished proliferation in response to psoriasin. **c)** The capillary-like tube formation of HUVECs was induced by psoriasin. HUVECs treated with sRAGE displayed a reduction in tube formation. **d)** Treatment with psoriasin induced RAGE expression at the mRNA and protein level in HUVECs. [110]

Low levels of ROS induce proliferation of a variety of cells, among them the endothelial cells [131]. ROS also induces VEGF expression [132]. Stress stimuli, such as ROS, have been found to induce psoriasin [123]. Keratinocytes over-expressing S100A8 and S100A9 have been shown to promote NADPH oxidase activation followed by ROS generation [133]. We hypothesized that psoriasin may induce low levels of ROS, which in turn may lead to a further increase in ROS levels, VEGF expression and cell proliferation. HUVECs exposed to extracellular recombinant psoriasin displayed a significant induction of ROS generation (Figure 8a). The demonstrated increase in intracellular ROS levels upon treatment was in the same range as previously demonstrated for S100B [35]. We also found that recombinant VEGF induced ROS in HUVECs. It has previously been shown that the interaction of RAGE with its ligands may generate ROS [34]. We found that sRAGE significantly eliminated the ROS generation in endothelial cells after treatment with psoriasin (Figure 8b). Furthermore, HUVECs pretreated with adenoviral Bcl-2 protein, an antioxidant protein, followed by treatment with psoriasin, showed a decreased proliferation (Figure 8c). Altogether, these findings suggest that psoriasin induces proliferation of endothelial cells through the interaction with RAGE and increased levels of ROS.

Figure 8. Psoriasin induces endothelial cell proliferation through the interaction with RAGE and increased levels of ROS. **a)** Treatment with psoriasin and VEGF increased the ROS generation in HUVECs. **b)** HUVECs treated with sRAGE followed by treatment with recombinant psoriasin displayed a reduced ROS formation. **c)** HUVECs infected with Bcl-2-expressing adenovirus (Ad-Bcl2-GFP), prior to the treatment with recombinant psoriasin, led to a reduction in HUVEC proliferation. [110]

ROS affects a wide range of cellular functions, from cell proliferation to cell death, depending on differences in the amount and duration of ROS production. Exposure of endothelial cells to low concentrations of H_2O_2 stimulates cell proliferation and angiogenesis, whereas severe oxidative stress causes cell death [131, 134-135]. Cancer cells produce ROS which could be one of the triggers of the angiogenic process in the tumor associated endothelial cells [135]. Small amounts of ROS are produced by a variety of hormones and growth factors, including VEGF, following binding to cell membrane receptors [136]. In addition, ROS may even mimic the action of growth factors [137].

We show that treatment with H₂O₂ significantly increased the ROS generation in mammary epithelial cells. Moreover, in H₂O₂-treated MCF10A with down-regulated psoriasin the level of ROS was reduced, further demonstrating a role of psoriasin in ROS generation. Consequently, the regulation of ROS levels by psoriasin demonstrates a pro-angiogenic effect.

Psoriasin is induced by H₂O₂ in mammary epithelial cells and functions through RAGE to promote endothelial cell proliferation and tube formation and an increased generation of ROS. The identification of RAGE as a receptor for psoriasin raises the possibility of targeting psoriasin-mediated effects.

PAPER II

Psoriasis is characterized by an infiltration of inflammatory cells and extensive new blood vessel formation [138]. Angiogenesis is considered to be one of the key features in psoriasis pathogenesis. Modifications in the microvasculature represent some of the earliest detectable changes in the development of the psoriatic lesion [88, 139]. The primary mediators of angiogenesis in psoriasis are derived from the epidermis, where keratinocytes are the major source of pro-angiogenic cytokines. VEGF is the main epidermis-derived growth factor and is strongly up-regulated in psoriatic skin lesions [91].

Psoriasin, which is highly expressed in the psoriatic epidermis, is secreted by keratinocytes and breast epithelial cells suggesting that psoriasin has an extracellular function [1, 25, 140]. To understand the role in the regulation of angiogenesis in psoriasis, we investigated the effect of psoriasin in keratinocytes and dermal derived endothelial cells.

Cells respond to stress and changes in their environment by altered transcription of specific genes [141]. ROS play an important role in signaling transduction and induce the transcription of cytokines, chemokines and growth factors, which play crucial roles in many biological processes, including angiogenesis [142].

We found that treatment with H₂O₂ induced the expression of psoriasin in keratinocytes. In the psoriatic epidermis enhanced cellular proliferation and increased metabolic demand results in hypoxia. Hypoxia is a stress factor that has been shown to induce ROS [143]. In accordance with this, the expression of psoriasin was significantly induced by hypoxia. Likewise, the hypoxia-mimicking agent CoCl₂ induced psoriasin.

In addition to psoriasin, the pro-angiogenic factors VEGF, heparin-binding epidermal growth factor-like growth factor (HB-EGF), MMP-1, MMP-9 and IL-8, were significantly induced by H₂O₂-treatment, whereas the anti-angiogenic factor thrombospondin (THBS)-1 was decreased (Figure 9a-g). Upon down-regulation of psoriasin expression we found a significant decrease in the expression of VEGF, HB-EGF and MMP-1 in keratinocytes (Figure 9b-d). The expression of MMP-9 and IL-8 was decreased but did not reach statistical significance (Figure 9e-f). Furthermore, the expression of THBS-1 was significantly increased in H₂O₂-treated cells with down-regulated psoriasin expression (Figure 9g). These results indicate that psoriasin amplifies the angiogenic response to H₂O₂ in keratinocytes.

Figure 9. Down-regulation of psoriasis expression in H₂O₂-treated keratinocytes regulates the expression of angiogenic factors. HEK293T treated with H₂O₂ displayed an increased mRNA expression of psoriasis (a) and angiogenic factors. Down-regulation of psoriasis suppressed the H₂O₂-induced mRNA expression of VEGF (b), HB-EGF (c), MMP-1 (d), MMP-9 (e) and IL-8 (f) and increased the mRNA expression of THBS-1 (g).

Treatment of HMVEC-d with psoriasis caused a significant increase in the expression of the pro-angiogenic factors VEGF and IL-8. Although it was not statistically significant, we also detected an increase in MMP-1 and IL-6 expression. The expression of THBS-1 was found to be significantly reduced by psoriasis.

The key steps in the angiogenic process involve proliferation, migration and differentiation of endothelial cells. Extracellularly administered psoriasis was shown to promote HMVEC-d proliferation (Figure 10a-b). Furthermore, psoriasis was found to induce endothelial cell migration and capillary-like tube formation (Figure 10c-d).

Our results indicate that extracellular psoriasis induce pro-angiogenic cytokines in dermal derived endothelial cells and increases the angiogenic properties to an extent comparable to that of VEGF.

Figure 10. Psoriasis induces cell proliferation, migration and capillary-like tube formation of dermal derived endothelial cells. HMVEC-d showed increased proliferation when treated with psoriasis or VEGF (a-b). HMVEC-d treated with psoriasis or VEGF demonstrated an induced migration (c) and capillary-like tube formation (d).

We demonstrated that psoriasis promote several aspects of the angiogenic process *in vitro*, including the induction of factors for proteolysis of the basement membrane, endothelial cell migration, proliferation and vessel formation.

Proteolysis of the basement membrane is a critical step during the very early stages of angiogenesis and MMPs are important mediators in these early events. H₂O₂-induced expression of MMP-1 and MMP-9 in keratinocytes was shown to be regulated by psoriasin. Moreover, MMP-1 demonstrated a tendency of up-regulation in dermal derived endothelial cells treated with psoriasin.

VEGF is the main angiogenic factor and one of the most important growth factor leading to vascular remodeling in psoriasis [91, 144]. VEGF can act both in a paracrine manner, after having been released by nearby cells, and in an autocrine manner in VEGF-producing endothelial cells [145]. VEGF and IL-8 are mitogenic factors for endothelial cells. The finding that psoriasin induces the expression of both VEGF and IL-8 in dermal endothelial cells, in parallel with the findings of induced cell proliferation, support the notion of VEGF and IL-8 as mitogenic factors for endothelial cells and illustrates the importance of psoriasin.

Previous results describe a negative correlation between THBS-1 and psoriasin expression in normal and DCIS specimens [146]. In agreement with those findings, we detected a significant increase in THBS-1 expression in H₂O₂-treated keratinocytes where the psoriasin expression was down-regulated. In addition, psoriasin-treated dermal endothelial cells showed a decreased THBS-1 expression.

We found an augmented HMVEC-d migration after psoriasin-treatment, comparable to the migration upon treatment with VEGF and IL-17. IL-17 is a key cytokine in psoriasis that has recently been shown to have a role in mediating angiogenesis. This cytokine strongly induces psoriasin expression in keratinocytes. In addition to being able to increase the expression of psoriasin, IL-17 has been shown to induce ROS and VEGF expression [147-148].

Several angiogenic growth factors, including VEGF and IL-17, activate the PI3K and NF- κ B signaling pathways in endothelial cells and regulate downstream target molecules involved in blood vessel growth and homeostasis [149-151]. Activation of NF- κ B mediates angiogenesis by the regulation of gene expression. The production of the pro-angiogenic growth factor VEGF and the chemokine IL-8 has been shown to be regulated by NF- κ B activation [152]. Furthermore, the expression of the pro-angiogenic MMP-9 is regulated through NF- κ B elements within the MMP-9 gene [153]. The PI3K signaling pathway is known to mediate VEGF expression and VEGF-induced endothelial migration [154-155]. Numerous endothelial cell stimuli, including ROS, activate PI3K signaling and NF- κ B is known to be induced by stress factors such as hypoxia and ROS [156-157].

The promoting effect of psoriasin on HMVEC-d migration was effectively blocked by the inhibition of the PI3K and NF- κ B pathways. These results demonstrate the involvement of the PI3K and the NF- κ B signaling pathways in the psoriasin-induced dermal endothelial cell migration. Thus, our data show that psoriasin, like IL-17, induces endothelial cell migration mediated through the PI3K and NF- κ B pathways.

In paper I we report that psoriasin is induced by H₂O₂ in mammary epithelial cells, promotes ROS generation, and induces endothelial cell proliferation and tube formation, thus likely to be involved in angiogenesis. The present paper further provides the aspect that psoriasin is involved in angiogenesis, by regulation of the H₂O₂-induced expression of angiogenic factors in keratinocytes. Psoriasin also has a role in angiogenesis by the induction of proliferation, migration and capillary-like tube formation, and induced expression of pro-angiogenic factors in dermal endothelial cells.

PAPER III

Psoriasin is highly expressed in high-grade DCIS [25, 56-57, 117-118], an early form of breast cancer considered to be a precursor of invasive ductal carcinoma [115]. Whereas the expression is often reduced in the transition from *in situ* to invasive carcinoma, persistent high levels of psoriasin in invasive breast carcinomas are associated with markers of poor prognosis and poor clinical outcome [19]. The mechanism that underlies the altered psoriasin expression is unknown, but may reflect changes in cellular differentiation or response to extracellular stress signals [25].

The gene that encodes the psoriasin protein is located within the EDC [158]. This gene cluster also contains genes for several differentiation markers that play important roles in the terminal differentiation of the human epidermis. Psoriasin expression has previously been associated with well differentiated squamous cell carcinoma as compared to moderate and poorly differentiated squamous cell carcinoma [159-160]. Observations have also been reported in oral, bladder, breast and skin cancer [25, 58, 161-162].

The high level of psoriasin in DCIS, the correlation with epithelial differentiation and the gene localization within the EDC propose that psoriasin might be involved in the epithelial differentiation process.

Psoriasin has previously been demonstrated to be up-regulated by the loss of attachment to the ECM and prolonged confluence in mammary epithelial cells [25, 123, 163]. Conditions like suspension and confluence mimic the *in vivo* situation that is likely to occur in a high-grade comedo DCIS tumor environment, suggesting a specific role for psoriasin in the initiation of these breast tumors.

MCF10A cultured in suspension and in confluence demonstrated a shift towards the more differentiated CD24⁺ phenotype, in parallel with the endogenous induction of psoriasin. Moreover, we demonstrated an increase in the luminal differentiation marker, MUC1, and a decrease in the breast stem cell marker, CD44. The demonstrated positive correlation between psoriasin and CD24 expression indicates that the degree of shift is dependent on the expression level of psoriasin. In humans, CD24 has been identified as a molecular marker for distinction between differentiated luminal and myoepithelial cells [164]. Breast cancer stem cells are characterized by the low or negative expression of CD24 in conjunction with the high expression of CD44 [164].

Our results demonstrate an increased psoriasin expression by *in vivo* mimicking conditions, accompanied by a shift towards a more differentiated CD24⁺ phenotype. The positive correlation between psoriasin and the CD24 expression may imply a causal relationship or be the result of differentiation.

The expression of psoriasin and MUC1 was confined to the CD24⁺ population of mammary epithelial cells. In the CD24⁺ fraction of confluence-cultured MCF10A cells a higher psoriasin expression was verified (Figure 11a), while the expression of CD44 was reduced (Figure 11b). The level of MUC1 expression in the CD24⁺ cell fraction was comparable to the CD24 level. These data suggest that psoriasin, as well as MUC1 is predominately expressed in CD24⁺ cells, supporting the notion that psoriasin is expressed in more differentiated mammary epithelial cells.

Figure 11. Psoriasin and MUC1 expression is elevated in CD24⁺ mammary epithelial cells. **a)** Psoriasin expression was confined to the CD24⁺ cell fraction of MCF10A cultured in confluence. **b)** Separated CD24⁺ cells displayed an increase in the expression of CD24 and a decrease in the expression of CD44, compared with negative selection. The expression of MUC1 was increased in the same level as CD24 expression.

We have previously shown that psoriasin expression is induced by ROS and down-regulated by treatment with the antioxidants Bcl-2 and NAC [123]. In this paper, confluence cultured MCF10A cells, treated with NAC showed no induction of psoriasin and CD24 expression, which suggests that psoriasin and CD24 expression is regulated by ROS. Psoriasin expression in mammary epithelial cells correlates with NF- κ B signaling and increased cellular survival [46, 123]. NF- κ B is involved in many biological processes, including proliferation, survival and differentiation. Likewise, cells treated with the inhibitors of NF- κ B, CAPE and dnIKK β , did not increase the expression of psoriasin or CD24 following confluence culture.

Psoriasin is induced by EGF signaling [165]. EGFR-inhibition by Tyrphostin demonstrated a trend towards a decreased expression of both psoriasin and CD24, suggesting that EGFR signaling may be implicated in their regulation. Treatment with phospholipase C (PLC)-inhibitor (U73122) or PI3K inhibitor (Wortmannin) did not affect the expression of CD24. The reduced psoriasin expression in response to treatment with U73122 and Tyrphostin may depend on the high dose of DMSO used. Overall, treatment with inhibitors had no effect on CD44 expression. These findings suggest that the expression of both psoriasin and CD24 is regulated by ROS and the NF- κ B pathway.

The role of psoriasin in the differentiation of mammary epithelial cells was further supported by the reduced shift towards a CD24⁺ phenotype upon down-regulation of psoriasin. Confluence- and suspension-cultured cells with down-regulated psoriasin expression demonstrated a reduced expression of CD24, in conjunction with an increased expression of CD44 (Figure 12). No significant difference in MUC1 expression was observed. Treatment of MCF10A cells with recombinant psoriasin protein did not affect the expression of CD24, CD44 or MUC1 compared with untreated MCF10A cells. These results suggest that induced endogenous psoriasin expression contributes to the shift towards a CD24⁺ phenotype and that psoriasin is important in the differentiation process of mammary epithelial cells.

Figure 12. Endogenous psoriasin causes increased CD24 expression. a) No induction of psoriasin expression was observed in Pso-shRNA treated cells, compared with C-shRNA treated cells, in confluence or suspension cultured MCF10A. **b)** Pso-shRNA treated cells showed a decrease in the expression of CD24 and an increase in the expression of CD44, compared with C-shRNA. **c)** The expression of psoriasin in Pso-siRNA treated cells, in suspension cultured MCF10A, was dramatically downregulated, compared with C-siRNA. **d)** Pso-siRNA treated cells showed a reduced CD24 expression, compared with C-siRNA treated cells.

The expression of CD24 has been shown to be significantly higher in DCIS compared with normal tissue [166-167]. We showed an intense staining and a similar staining pattern of psoriasin and CD24 in DCIS *in vivo* (Figure 13). Psoriasin expression (Figure 13a) was observed in the cytoplasm and the nucleus, whereas CD24 expression (Figure 13b) was observed in the cytoplasm, the nucleus and in the plasma membranes of the mammary epithelial cells of the DCIS tumors. Psoriasin and CD24 staining was detected in a weak to strong expression among the cases. Although psoriasin and CD24 expression showed heterogeneity both between and within cases a similar staining pattern was demonstrated. In 61.9% (13/21) of cases a concordant expression was seen, of which ten cases showed double-positive and three cases double-negative expression. In two out of the 21 investigated cases, normal breast tissue showed a weak psoriasin staining, while none of the analyzed cases showed positive CD24 staining. These findings confirm the association between psoriasin and CD24 expression observed *in vitro*.

Figure 13. Psoriasin and CD24 demonstrate a similar staining pattern in DCIS. Psoriasin and CD24 showed an extremely intense staining and a similar staining pattern in DCIS. a) Psoriasin staining was observed in the cytoplasm and the nucleus of the cells. **b)** CD24 staining was observed in the cytoplasm, the nucleus and in the plasma membranes of the cells.

In normal epithelial cells the MUC1 expression is weak to moderate. In DCIS, the expression is strong and corresponds to the expression of psoriasin [146]. CD24 has been shown to be a marker of tumor aggressiveness and the expression of CD24 promotes breast cancer development [168-170]. The expression of both psoriasin and CD24 in breast cancer has been associated with a poor prognosis [19, 45, 47, 170-172]. Likewise, MUC1 is frequently

over-expressed in many cancers, including breast cancer, and correlates with poor outcome [173]. Furthermore, the gene encoding MUC1 is, like psoriasin, located within the EDC.

Psoriasin is induced by differentiation-inducing conditions and correlates with CD24 expression in mammary epithelial cells. Furthermore, psoriasin is regulated by ROS and NF- κ B and contributes to the shift towards a more differentiated phenotype.

PAPER IV

The skin barrier and epidermal structure is created by the proliferation of basal keratinocytes followed by a tightly regulated process of terminal differentiation [60]. As the keratinocytes migrate from the basal layer, several differentiation markers, such as K1, K10, involucrin, transglutaminase, loricrin and filaggrin, are produced and become apparent in the spinous and granular layers. Psoriasin expression has been described in the differentiated layers of the epidermis and correlates with the degree of keratinocyte differentiation [161].

Psoriasis is characterized by keratinocyte hyperproliferation and a disturbed differentiation process with epidermal thickening and the absence of the granular layer. The sequence of differentiation marker expression in the psoriatic epidermis differs from that of the normal skin [174]. Involucrin and transglutaminase appear in lower layers, while filaggrin is either absent or found in the upper layer of the psoriatic epidermis.

The high expression level in the psoriatic epidermis, in conjunction with the disturbed differentiation process, suggests that psoriasin is involved in keratinocyte differentiation.

Psoriasin expression is increased in psoriasis but sparse in normal skin [175]. We detected a markedly elevated psoriasin expression in the psoriatic epidermis where psoriasin formed a gradient from a weak expression in the basal cell layer to an intense expression in the more differentiated suprabasal layers (Figure 14). The gradient of psoriasin expression in the psoriatic epidermis corresponds with the previously reported calcium gradient. This pattern of psoriasin expression suggests an involvement of psoriasin in the keratinocyte differentiation process.

Figure 14. Psoriasin is expressed in a gradient in the psoriatic skin. Psoriasin displays a weak expression in the basal cells, whereas the expression is substantially higher and more intense in the upper, more differentiated, suprabasal layers, composing a gradient of psoriasin expression in the psoriatic skin.

Elevated calcium concentrations induce keratinocyte differentiation by increasing the expression of differentiation-responsive epidermal genes and morphological changes [176-177]. Here, we found that differentiation-inducing treatment, consisting of CaCl_2 and TPA markedly increased the psoriasin expression, indicating that stimuli that induce differentiation also induce the psoriasin expression in keratinocytes.

PKC is an important pathway in epidermal differentiation [178-179]. PKC-inhibition effectively block the calcium-induced differentiation of keratinocytes [180]. We found that CaCl_2 treatment significantly induced the expression of psoriasin, involucrin, K1 and K10 (Figure 15a-d). Previous studies have shown that S100A8 and S100A9 expression is up-regulated in response to PKC activation [181]. Following inhibition of PKC-signaling the expression of psoriasin was significantly reduced (Figure 15a). Treatment with PKC inhibitor also reduced the expression of involucrin, as has been previously demonstrated (Figure 15b). However, PKC-inhibition did not affect the expression of K1 and K10 (Figure 15c-d). These results demonstrate that psoriasin, in conjunction with the late differentiation marker involucrin, is regulated by the PKC-signaling pathway in differentiated keratinocytes, suggesting an association between psoriasin and the late differentiation marker involucrin.

Figure 15. Psoriasin expression induced by calcium is regulated by PKC. Calcium treatment induced the mRNA expression of psoriasin (a), involucrin (b), K1 (c) and K10 (d). The inhibition of PKC led to decreased psoriasin (a) and involucrin (b) mRNA expression.

Apoptosis is rare in cultures of keratinocytes and is not stimulated by removal of growth factors [182]. In agreement, we did not find any apoptotic population in differentiated keratinocytes in culture. The down-regulation of psoriasin expression in keratinocytes did not cause any difference either in cellular morphology or in proliferation. Likewise, we did not see any difference in apoptosis frequency in keratinocytes with down-regulated psoriasin expression following CaCl_2 -treatment. Thus, the down-regulation of endogenous psoriasin expression does not affect the morphology, proliferation or apoptosis of keratinocytes.

In agreement with previous studies [176-177], we show that calcium treatment triggered a morphological change and the induction of psoriasin as well as the differentiation markers, K1, K10, involucrin, filaggrin, desmoglein 1, desmocollin 1, transglutaminase 1 and CD24 (Figure 16a-g).

Psoriasin knock-down experiments demonstrated the role of psoriasin in terminal differentiation of keratinocytes, as down-regulation of psoriasin modulated the expression of differentiation markers specifically expressed in the suprabasal layers of the epidermis. Down-regulation of psoriasin resulted in a significantly diminished expression of involucrin, desmoglein 1, transglutaminase 1 and CD24 in keratinocytes treated with CaCl_2 (Figure 16).

The expression of filaggrin was decreased but did not reach statistical significance, while the down-regulation of psoriasin showed no effect on desmocollin 1, K1 or K10. These data reveal that psoriasin expression is important for calcium-induced differentiation of keratinocytes.

Figure 16. Down-regulation of psoriasin expression suppresses calcium-induced keratinocyte differentiation. The mRNA expression of psoriasin, involucrin, filaggrin, desmoglein 1, desmocollin 1, transglutaminase 1 and CD24 was induced by calcium treatment (a-g). Down-regulation of psoriasin expression (a) resulted in decreased mRNA expression of involucrin (b), desmoglein 1 (d), transglutaminase 1 (f) and CD24 (g). The reduced expression of filaggrin did not reach statistical significance (c). No reduction of desmocollin 1 mRNA expression was demonstrated (e).

Differentiation markers specific for the granular layer in normal skin, including involucrin and transglutaminase, appear in the lower layers of the psoriatic epidermis, whereas markers such as filaggrin are either absent or found in the cornified layer [102-103, 183]. Late differentiation markers, including involucrin and transglutaminase, have been demonstrated to be over-expressed in the psoriatic skin [102, 174, 184], while the early differentiation markers, K1 and K10, have been reported to be down-regulated [104]. In agreement, we demonstrated that psoriasin, which is highly up-regulated in psoriasis, regulates several late differentiation markers whereas it did not affect the early differentiation markers, K1 and K10, which is known to be down-regulated or absent in the psoriatic epidermis.

The formation of cell-cell interactions through desmosome completion is triggered by high calcium levels [185-186]. The desmosomal cadherins, desmoglein and desmocollin, tightly link cells together in the extracellular space [187]. We found desmoglein 1 expression to be increased by calcium treatment. This expression was reduced when the psoriasin expression was down-regulated.

CD24, a differentiation marker in mammary epithelium [164], has previously been detected in the upper, more differentiated layers of human epidermis [188]. In Paper III, we showed that CD24 expression is regulated by psoriasin in mammary epithelial cells [189]. Likewise, in this paper, CD24 expression was found to be regulated by psoriasin in differentiated keratinocytes.

Without the presence of psoriasin, calcium-induced differentiation of keratinocytes is absent, as demonstrated by the lack of induction of several late differentiation markers. The gene that encodes psoriasin is located within the EDC [190-192], which contains differentiation-responsive genes important for the terminal differentiation of the human epidermis [190-191]. This further proposes the involvement of psoriasin in the epidermal differentiation. The fact that PKC-inhibition and psoriasin down-regulation did not affect the expression of K1 and K10, demonstrates that psoriasin is associated with late rather than early differentiation markers.

Psoriasin is induced in keratinocytes by calcium-induced differentiation. Furthermore, psoriasin is controlled by PKC and contributes to terminal differentiation of keratinocytes by the regulation of differentiation markers.

CONCLUSIONS

OVERALL CONCLUSION

Psoriasin is highly expressed in DCIS and psoriasis, conditions which are characterized by hyperproliferation, a pronounced angiogenesis and a disturbed cellular differentiation. We conclude that psoriasin has the potential to promote angiogenesis through the regulation of angiogenic factors in epithelial cells. Psoriasin also contributes, possibly in a paracrine manner, to the subsequent induction of proliferation, migration and tube formation of endothelial cells. Furthermore, we conclude that psoriasin promote epithelial cell differentiation through the regulation of differentiation markers, and is regulated by pathways involved in epithelial cell differentiation. The findings are summarized in figure 17. Altogether, the findings of this thesis suggest that psoriasin may be a contributor to the development and progression of breast cancer and psoriasis. Furthermore, findings not only describe psoriasin as a mediator in the angiogenic and differentiation processes, but also suggest that it constitutes a potential target in the treatment of breast cancer and psoriasis.

SPECIFIC CONCLUSIONS

The specific conclusions of the included papers are as follows:

PAPER I

Psoriasin contributes to ROS-generation in mammary epithelial and endothelial cells, and the subsequent VEGF expression in epithelial cells. Psoriasin functions through RAGE to promote endothelial cell proliferation and capillary-like tube formation. Consequently, psoriasin secreted from epithelial cells may play a role in breast cancer progression by promoting oxidative stress response and angiogenesis. The over-expression of psoriasin in DCIS in combination with the elevated level of ROS and VEGF expression makes psoriasin a potentially marker for angiogenesis. The identification of RAGE as a receptor for psoriasin raises the possibility of targeting psoriasin-mediated angiogenic effects relevant for breast cancer progression.

PAPER II

Psoriasin is induced by cellular stress and amplifies a stress-induced angiogenic process in keratinocytes by the regulation of angiogenic factors. Furthermore, psoriasin has a potent angiogenic activity, verified *in vitro* by the induction of cell proliferation, migration and tube formation along with the induction of pro-angiogenic factors in dermal endothelial cell. Psoriasin promoted by oxidative stress and the high expression of psoriasin in the psoriatic epidermis may contribute to the increased vascularization and the progression of the disease. Our findings raise the possibility to use psoriasin as a potential target for anti-angiogenic treatment in psoriasis.

PAPER III

Psoriasin is linked to the differentiation marker CD24 in mammary epithelial cells. In culture conditions mimicking the *in vivo* conditions seen in DCIS, psoriasin plays a crucial role in the shift towards a more differentiated CD24⁺ phenotype. The contribution to a more differentiated epithelial phenotype and the high expression in DCIS *in vivo*, suggest that psoriasin is essentially involved in the differentiation process of mammary epithelial cells and potentially the progression of breast cancer.

PAPER IV

Psoriasin is highly expressed in the abnormal differentiated psoriatic keratinocytes *in vivo* and up-regulated in response to differentiation-inducing stimuli *in vitro*. The psoriasin-induced expression of differentiation markers demonstrates that psoriasin contributes to keratinocyte differentiation. Psoriasin expression, in conjunction with keratinocyte differentiation, is mediated by the PKC signaling pathway. The high level and expression pattern of psoriasin in the psoriatic epidermis and the demonstrated impact on the expression of differentiation markers suggest that psoriasin contributes to keratinocyte differentiation and possibly involved in the development and progression of psoriasis.

Figure17. Summarizing figure of the findings in this thesis. Psoriasin expression is induced in mammary epithelial cells and keratinocytes by cellular stress and in response to differentiation-inducing stimuli. In **paper I** we found that psoriasin increased VEGF expression in mammary epithelial cells. The treatment of endothelial cells with psoriasin increased cell proliferation and tube formation mediated by RAGE. Psoriasin was shown to induce ROS in both endothelial and epithelial cells through the action of RAGE. In **paper II** psoriasin expression was shown to increase the expression of VEGF and several other pro-angiogenic factors in keratinocytes. Extracellular psoriasin contributed to the subsequent induction of proliferation, migration and tube formation and contributed to the expression of the pro-angiogenic factors VEGF and IL-8 in dermal endothelial cells. The psoriasin-induced migration was shown to be mediated by the PI3K and NF-κB signaling pathways. In **paper III**, confluence- and suspension culture demonstrated an increased expression of psoriasin and CD24 in mammary epithelial cells regulated by ROS and the NF-κB signaling pathway. In **paper IV** we found that the expression of the differentiation markers involucrin, desmoglein 1, transglutaminase 1 and CD24 in keratinocytes was induced by psoriasin. The induction of psoriasin and involucrin expression was regulated by the PKC signaling pathway.

ACKNOWLEDGEMENTS

Jag vill tacka alla som på ett eller annat sätt har bidragit till denna avhandling. Speciellt vill jag tacka några av er...

Min handledare, **Charlotta Enerbäck**, som tog sig an mig från dag ett av mitt examensarbete och som gav mig möjligheten att utföra intressanta projekt och slutligen skriva denna bok. Tack för det förtroende och frihet du gett mig och för att du alltid haft tid för att diskutera såväl experimentupplägg som resultat som inte blivit som förväntat.

Mina biträdande handledare, **Anna-Karin Ekman**, **Maria Carlström** och **Ingemar Rundquist** som framför allt hjälp mig inför disputationen genom kommentarer och korrekturläsning. Anna-Karin, med dina råd och kommentarer vad gäller språk och grammatik har avhandlingen blivit lite bättre och språkmässigt mer begriplig.

Min inofficiella handledare, diskussionspartner och reseledare **Cecilia Bivik Eding**, **Stina Lassesson** som stått för en stor del av den upplärning jag inte gjort på egen hand. **Gunna Sigurdardottir** och **Maria Yhr** för trevliga Vadstenakonferenser.

”Min student” **Emilia Lindblad** som jag handledde på labb och vars resultat jag kunde ta del av till avhandlingen. Tack till medförfattare Emman Shubbar, Anikó Kovács och Kornelia Polyak för era bidrag till denna avhandling.

”Söderkvists grupp”. **Peter Söderkvist**, **Deepti Verma**, **Jonas Ungerbäck**, **Lena Thunell**, **Jenny Welanders**, **Ravi Kumar Dutta**, **Mohamed Ali Mosrati**, **Naomi Yamada**, **Annika Stjärnström**, och alla studenter som kommit och gått. Tack för god stämning, trevligt sällskap och fika i alla möjliga former!

Annette Molbaek och **Åsa Schippert**, för att ni alltid tar er tid för mer eller mindre dumma frågor. Utan er hade motgångarna varit så många fler.

Maria Turkina och **Elin Karlsson** mina kompanjoner i handledning av studenter i basgrupp.

Florens Sjögren och **Uno Johansson** på flödesenheten för all hjälp när jag brottades med flödescytometrin. **Håkan Wiktander** för all praktisk hjälp och snaps på julfesterna. Medarbetarna på **Klinisk genetik** som stod ut med oss innan ni så småningom flyttade till nya lokaler och vi kunde använda ert gamla labb för julfest och därefter inflyttning och invigning av Ingrid Asp Psoriasis Research Center.

Ingrid Asp som gjorde forskningen inom psoriasis möjlig och för ditt gedigna intresse.

Mina vänner och kollegor med respektive, **Caroline Bivik** och **Niklas Stadler**, **Anna Eskilsson**, **Anna Forsberg**, **Lina Wirestam** och **Martin Berggram** samt **Angelika Holm** och **Ulf Dånmark**. Ni får mig att inse att jag inte är ensam om motgångar och om att "aldrig" komma någonstans i forskningen. Jag ser fram emot era disputationer! Tack för alla trevliga fester, middagar och fikastunder!

F&S Linköping "Vet inte vad du måste orka. Bara hur!" stämmer verkligen. Utan all svett, mjölksyra och träningsvärk hade denna avhandling inte varit möjlig! Så otroligt mycket energi jag fått genom åren och alla tankar på jobb har stundvis varit som bortblåsta. Ett särskilt stort tack till stationsgänget!

Sist men inte minst, mina närmaste! Även om ni kanske inte alltid förstått vad jag egentligen gör på "jobbet" har ni varit ett enormt stort stöd.

Familjen Vegfors, jag känner mig verkligen som en av er. **Eva** och **Magnus**, **Carl**, **Anna**, **Vincent** och **William**, **Johan** och **Madeleine** för många trevliga och varma stunder. Tack för den stående inbjudan till Lilla Vänstern där lugnet kan infinna sig.

Leif Prejer för ditt engagemang och intresse för forskning.

Mamma och **Pappa** för er kärlek och stöttning, nu är jag inte student längre!

Syster **Hanna**, **Erik** och **Hugo** för alla skratt, renoveringsarbeten och mysiga stunder. Min stora lillebror, **Henning**. Du är min förebild! Med din vilja, envishet och humor kommer du gå långt i livet!

Christian, min trygghet, mitt liv, mitt allt! För ditt stora tålamod och uppmuntran. Utan din tekniska hjälp hade inte artikel III blivit godkänd för publicering! Jag älskar dig! ♥

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