Functional genetic studies of Psoriasin:
A potential biomarker for breast cancer with a poor prognosis

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Cover image: A ribbon diagram of the homodimeric psoriasin molecule. Adapted from Anderson KS *et al.* 2009 [1].

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"Nobody can go back and start a new beginning but
anyone can start today and make a new ending"
- Maria Robinson

Breast cancer is the most common malignancy in women. There is a high degree of heterogeneity in breast tumours and they can be divided into subtypes that have different expression pattern and clinical outcome. Ductal Carcinoma *in situ* (DCIS) is regarded as a precursor of invasive ductal breast cancer. Some DCIS lesions will not change in many years, while other will rapidly progress into invasive cancer. It is therefore important to be able to distinguish clinical subgroups of DCIS with a high risk of progression to invasive disease.

Aim: Psoriasin is one of the most abundant transcripts in high-grade DCIS with higher risk of local recurrence. Psoriasin has been associated with poor clinical outcome, suggesting its potential involvement in tumour progression. To date, several functions of psoriasin have been proposed, but none of these can fully explain its involvement in breast tumour progression. The aim of this thesis was to elucidate the functional relevance of psoriasin for the initiation and progression of DCIS, and to gain insight into regulatory pathways that control the expression.

Results: High-grade DCIS is characterised by a high apoptotic rate and reactive oxidant species (ROS) are known to influence this process. We report the induction of psoriasin by ROS in normal mammary epithelial cells. This induction was repressed by the anti-apoptotic protein Bcl-2 and the antioxidant NAC. Normal mammary epithelial cells with a stable retroviral overexpression of psoriasin were significantly more resistant to ROS-induced cell death. Furthermore, we demonstrate that the NF-κB pathway is potentially involved in the induction of psoriasin expression. (**Paper I**)

IFNγ has been shown to exert anti-tumour action in breast cancer. We report the downregulation of psoriasin by IFNγ in a breast cancer cell line and the downregulation of psoriasin induced by culturing mammary epithelial cells in suspension (loss of contact to extracellular matrix). This effect was shown to be mediated by the activation of the STAT1 signalling pathway. In a mouse mammary epithelial cell line with tetracycline-induced psoriasin expression, we observed the increased viability of psoriasin-expressing cells after IFNγ treatment. (**Paper II**)

The massive induction of psoriasin in suspension culture compared to other stimuli (starvation, confluence and ROS) suggests that changes in adhesion to the extracellular matrix may contribute to the expression of psoriasin. We showed that the downregulation of intercellular adhesion molecule 1 (ICAM-1) (by short hairpin RNA) in mammary epithelial cells increased the expression of psoriasin, through the phospholipase C (PLC)-IP3 pathway, as well as the oncogenic protein mucin1 (MUC1). (**Paper III**)

The interaction between breast epithelial cells and the extracellular matrix contribute to pathological processes and to the normal development of a differentiated structure. Psoriasin has previously been related to epithelial cell differentiation in the skin. We now report that mammary epithelial cells shifted from a CD44$^+$/CD24$^-$ to a

CD44$^-$/CD24$^+$ phenotype (representing differentiated luminal epithelia) when cultured in confluent and suspension conditions. Interestingly, this result was not observed when psoriasin was suppressed using short hairpin RNA. (**Paper IV**)

Conclusions: We have shown data suggesting that the high expression of psoriasin in high-grade DCIS tumours may be dependent on the production of ROS and a change in adhesion to the ECM, involving ICAM-1 and MUC1. The psoriasin expression leads to increased survival of the breast epithelial cells. Our data also reveal that psoriasin is tightly linked to the expression of CD24. Therefore, it is likely that psoriasin play a role in the differentiation of mammary epithelial cells.

Bröstcancer är den vanligaste formen av cancer hos kvinnor. Mellan 15 till 20 kvinnor insjuknar dagligen i bröstcancer i Sverige. Sjukdomen är mycket heterogen och brösttumörer kan delas in i undergrupper baserat på uttrycksmönster och prognos. Duktal Cancer in situ (DCIS) uppfattas som ett förstadium till invasiv duktal bröstcancer, vilken är den vanligaste formen av bröstcancer. Vissa DCIS tumörer förändras inte på många år, medan andra snabbt kan utvecklas till en invasiv cancer. För att utveckla bra behandlingsmetoder är det viktigt att kunna urskilja de mest aggressiva formerna av DCIS och därför är det betydelsefullt att hitta bra markörer för denna grupp av brösttumörer.

Syfte: Psoriasin är mycket kraftigt uttryckt i high-grade DCIS, vilket är den grupp av DCIS som har en hög risk för återfall. Psoriasin har korrelerats med en dålig prognos, vilket tyder på att detta protein kan påverka tumörutvecklingen. Flera funktioner för psoriasin har föreslagits, men ingen kan helt förklara betydelsen av proteinet för tumörtillväxt. Syftet med denna avhandling var att studera psoriasin och karaktärisera den funktionella betydelsen av proteinet för initiering och utveckling av high-grade DCIS och att beskriva mekanismer som reglerar uttrycket.

Resultat: High-grade DCIS kännetecknas av en hög apoptos-aktivitet och denna process vet man påverkas av fria syreradikaler. Vi visar att fria syreradikaler inducerar psoriasin i normalt bröstepitel och att denna induktion hämmas av det anti-apoptotiska proteinet Bcl-2 och antioxidanten NAC. Normalt bröstepitel med ett retroviralt överuttryck av psoriasin uppvisar resistens mot effekterna av fria syreradikaler. Dessutom visar vi att NF-κB, en välkänd överlevnads- signalväg, är involverad i induktionen av psoriasin. **(Paper I)**

IFNγ har visats ge en negativ inverkan på tumörtillväxt. Efter IFNγ behandling ser vi en nedreglering av psoriasin i celler där psoriasin är inducerad genom att odla cellerna i suspension (cellerna förlorar kontakt med extracellulära matrix). Denna nedreglering visades vara beroende av STAT1-signalering. Genom ett tetracyklin-inducerat psoriasinuttryck, demonstrerar vi en ökad överlevnad i psoriasin-uttryckande celler efter IFNγ behandling. **(Paper II)**

Den massiva induktionen av psoriasin vid odling i suspension jämfört med andra tillstånd (svält, konfluens och fria syreradikaler), tyder på att förändringar i adhesion till extracellulära matrix kan bidra till uttrycket av psoriasin i normalt bröstepitel. Vi visar att nedreglering av intercellulär adhesion molekyl 1 (ICAM-1) (med short hairpin RNA) i bröstepitel bidrar både till uttrycket av psoriasin, genom fosfolipas C (PLC)-IP3 signalering, och induktionen av onko-proteinet mucin 1 (MUC1). **(Paper III)**

Interaktionen mellan bröstepitel och extracellulära matrix bidrar till patologiska processer, som exempelvis bröstcancerutveckling, men dessa interaktioner har också en stor betydelse för normal differentiering av bröstepitel. Psoriasin har tidigare korrelerats med differentieringsgraden hos epitelceller i huden. Vi visar nu att bröstepitel, odlade under omständigheter som inducerar psoriasin, förändras från en

CD44$^+$/CD24$^-$ fenotyp till en CD44$^-$/CD24$^+$ fenotyp (vilket motsvarar differentierat luminalt epitel). Detta resultat kunde inte observeras när psoriasinuttrycket nedreglerades med short hairpin RNA. **(Paper IV)**

Slutsatser: Våra resultat tyder på att det höga uttrycket av psoriasin i high-grade DCIS beror på en hög produktion av fria syreradikaler och en förändrad adhesion till extracellulära matrix. Adhesionsmolekyler som kan vara involverade är ICAM-1 och MUC1. Bröstepitel som uttrycker psoriasin visar en ökad överlevnad, vilket gynnar tumörutvecklingen. Våra resultat visar också att psoriasinuttrycket är korrelerat till uttrycket av differentieringsmarkören CD24. Detta fynd tyder på att psoriasin kan ha en funktionell roll i differentiering av bröstepitel.

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

I. Carlsson H, Yhr M, **Petersson S**, Collins N, Pollyak K, Enerbäck C. Psoriasin (S100A7) and Calgranulin-B (S100A9) Induction is Dependent on Reactive Oxygen Species and is Downregulated by Bcl-2 and Antioxidants. *Cancer Biol Ther.* 2005 Sep 23;4(9)

II. **Petersson S**, Bylander A, Yhr M, Enerbäck C. S100A7 (Psoriasin), highly expressed in Ductal Carcinoma *In Situ* (DCIS), is regulated by IFN-gamma in mammary epithelial cells. *BMC Cancer* 2007 Nov 6;7(1):205

III. **Petersson S**, Shubbar E, Yhr M, Kovacs A and Enerbäck C. Loss of ICAM-1 signaling induces psoriasin (S100A7) and MUC1 in mammary epithelial cells. *Under review.*

IV. **Petersson S**, Nilsson J, Shubbar E and Enerbäck C. Psoriasin (S100A7) expression is linked to the differentiation marker CD24. *In manuscript*

*O*THER PUBLICATIONS NOT INCLUDED IN THIS THESIS

Carlsson H, **Petersson S**, Enerbäck C. Cluster analysis of S100 gene expression and genes correlating to psoriasin (S100A7) expression at different stages of breast cancer development. *International journal of oncology* 2005 Dec;27(6):1473-81

Petersson S, Shubbar E, Enerbäck L, Enerbäck C. Expression of S100 proteins in melanocytes and melanocytic lesions. *Melanoma Res.* 2009 Aug;19(4):215-25.

Anderson K, **Petersson S**, Wong J, Shubbar E, Lokko N, Enerbäck C. Elevation of serum EGF, TNF-alpha, and IL-1Ra in active psoriasis vulgaris. *Submitted*

CAPE	Caffeic acid phenethyl ester
cDNA	Complementary DNA
CGAP	Cancer Gene Anatomy Project
CP	Crossing point
DAB	Diaminobenzidine
DCIS	Ductal Carcinoma *in situ*
DNA	Deoxyribonucleic acid
dnIKKβ	Dominant-negative IKKβ
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
ECM	Extracellular matrix
EDC	Epidermal differentiation complex
ER	Estrogen receptor
ERα	Estrogen receptor alpha
HRP	Horseradishperoxidase
ICAM-1	Intracellular adhesion molecule 1
IFNγ	Interferon-gamma
IHC	Immunohistochemistry
IKKβ	Inhibitor-kappaB kinase-β
ISG	Interferon-stimulated genes
Jab1	Jun-activating binding protein 1
MACS	Magnetic activating cell sorting
MMP13	Matrix metalloproteinase 13
MOI	Multiplicity of infection
mRNA	Messenger RNA
MUC1	Mucin 1
NAC	N-acetylcysteine
NBT	Nitro Blue Tetrazolium
NF-κB	Nuclear factor- kappaB
PCR	Polymerase chain reaction
PLC	Phospholipase C
RNA	Ribonucleic acid
RNAi	RNA interference
polyHEMA	Poly-2-hydroxy-ethylmethacrylate
ROS	Reactive oxidant species
RT-PCR	Reverse transcriptase PCR
RTQ-PCR	Real-Time quantitative PCR
SAGE	Serial analysis of gene expression
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
STAT1	Signal transducer and activator of transcription 1
TNFα	Tumour necrosis factor alpha
TSG	Tumour suppressor genes
VEGF	Vascular endothelial growth factor

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Basic genetics

A human body consist of approximately 100 billons cells, and each cell contains information that is necessary to guide the growth to any specific cell type in the body like muscle cells, blood cells and epithelial cells. This information is known as DNA (deoxyribonucleic acid), which is composed of a double-stranded polymer containing four different nucleotides; adenine (A), cytosine (C), guanine (G) and thymine (T). The composition and arrangements of these nucleotides will differ between individuals and this is the key that make us unique. The DNA in a cell is organised into 23 chromosome pairs and together they represent the human genome.

For the generation of a living organism, specific sequences of DNA, known as genes, will be expressed and result in ribonucleic acids (RNA) and proteins (gene products). The translated information from a gene to a functional protein is known as "The central dogma of molecular biology" and was first proposed by Francis Crick in 1957 (Figure 1) [2]. During DNA replication, there is a production of two identical DNA molecules, which is necessary before each cell division. For the production of gene products, the DNA is first transcribed to RNA, followed by translation of the messenger RNA (mRNA) to a protein.
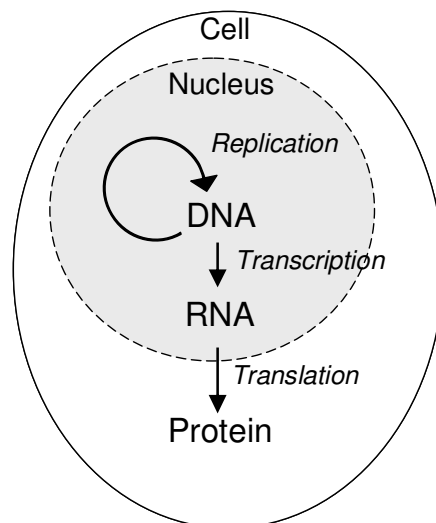
Figure 1. The central dogma of molecular biology.

Several initiating factors are essential for the transcription of a gene; these include a promoter, several transcription factors and RNA polymerase. The promoter, which is a regulatory sequence of the DNA, is most often located immediately upstream to the gene. Other regulatory sequences, located near or within a gene, can also influence the gene transcription. These include enhancers and silencers. Beside the regulatory sequences, the gene expression can be regulated by other mechanisms including epigenetic factors, such as methylation and acetylation. A change in the DNA sequence could also contribute to a modification in gene expression. A gene often consists of non-coding (intron) and coding (exon) regions. The non-coding regions

are spliced out from the pre-mRNA molecules, whereas the coding sequence will be translated into proteins in the translation machinery (ribosomes). The mRNA consist of the same nucleotides as the DNA, with exception that thymine is replaced by uracile (U). The nucleotides form groups of three (a codon) which represent one single amino acid. There are 64 possible triplet combinations of the codons in the mRNA but only 20 different amino acids. This means that several codons represent the same amino acid. The protein, which consists of a polymer of amino acids, undergoes several post-translational modifications before it yields the final active protein. These reactions include; processing to remove a sequence usually at the end of the protein (proteolytic processing), addition of new groups such as phosphate (phosphorylation) or sugars (glycosylation), and modification of existing groups such as disulfide bond formation between cysteine amino acids.

Changes in the nucleotide composition of the DNA can occur and they are often due to spontaneously errors during DNA replication and also DNA repair [3]. The induction of DNA changes can also be caused for example by radiation, viruses and chemicals. Any change in the DNA is referred as a mutation and every day a human cell can achieve about 6000 mutations. Most of them are repaired but some of them become permanent. Small-scale mutations include point mutation, insertions and deletion, whereas large-scale mutations involve translocation, loss or gain of whole chromosomes or larger chromosome regions. Many mutations will pass unnoticed or perhaps cause small effects. These changes can give rise to variants of genes, also known as alleles. Some mutations can affect the phenotype and become pathogenic and they are most likely arisen in or near a gene. In an evolutionary prospective, mutations can also give a benefit for an organism to have a better chance to adapt to environmental changes.

Cancer genetics

Cancer is a genetic disease, but non-genetic factors like the environment clearly play important roles [4]. Carcinogenesis, the process for cancer development, is a multi-step process. First there is an uncontrolled cell growth originating from a specific cell, resulting in a tumour cell mass. Malignant tumours have the ability to invade the surrounding normal tissue, whereas benign tumours don't have this capacity and therefore stay in the place where it started "*in situ*". Tumours can be categorised based on the cell of origin. Carcinomas, the most common, are derived from epithelial cells, sarcomas from connective tissue or mesenchymal cells, and lymphomas from hematopoietic cells.

Cancer is caused by mutations in the genome. Inherited mutations from the parents are presented in all cells from birth, and new mutations may occur by carcinogens like tobacco smoke, radiation and chemicals. It has been argued that between four to eight mutations must occur for the initiation of a cancer [5]. Two major groups of genes are altered in cancer.

Oncogenes
Proto-oncogenes are expressed in normal tissues and control cell growth and differentiation. A gain-of-function mutation or deregulation of these proto-oncogenes will convert them to oncogenes that promote tumour progression. When these genes are overexpressed, the cells are

continuously proliferating. Alteration of one of the alleles is sufficient to affect the phenotype of a cell. In breast cancer, examples of oncogenes are H-RAS, Bcl-1, Bcl-2, erbB2 and c-myc.

Tumour suppressor genes
The tumour suppressor genes (TSG) are the opposite of oncogenes and their expressions protect the normal cells from being transformed to tumour cells. Normally, TSG regulates important cellular activities like cell cycle checkpoints, detection and repair of DNA damage, protein degradation, cell differentiation and apoptosis. Loss-of-function mutation in the TSG could contribute to carcinogenesis, but other mechanisms that interfere with their expression and function may be involved, such as hypermethylation or abnormal protein interaction. According to Knudson *et al.* 1971, both alleles of a TSG must be inactivated in one cell for tumour growth to occur [6]. One example of a TSG in breast cancer is TP53, that code for p53. Most of the TSGs can also be divided into gatekeepers and caretakers. The gatekeepers control that the cell amount is constant and if both alleles are inactivated it will result in uncontrolled cell growth. In contrast, the caretakers, also known as DNA repair genes, promote tumour growth indirectly because inactivation of these genes leads to genetic instability which promotes mutation formation. In breast cancer BRCA1 and BRCA2 may function both as gatekeepers and as caretakers.

Breast cancer

Breast cancer is the leading cause of cancer-related death in women world-wide [7]. Approximately 7000 women were diagnosed with breast cancer in Sweden during 2008 (Cancer incidence in Sweden 2008), and this represent 29% of all cancer cases for women.

To date, several risk factors for the development of breast cancer have been proposed. The life-style has been shown to influence the breast cancer risk. Consequently, when women from low incidence countries move to the west, their risk of breast cancer increases. These modifiable factors can include diet, physical activity, alcohol consumption and radiation exposure. Non-modifiable risk factors include older age, late age of first birth and hormonal exposure by early menstruation and late menopause. The family history of breast cancer has also been shown to increase the risk of developing the disease. About 5-10% of all cases of breast cancer are due to inherited mutations in different breast cancer susceptibility genes [8], but mutations can also be sporadic (non-inherited). BRCA1 and BRCA2 are two high penetrant breast cancer susceptibility genes that account for about 15-20% of all familial breast cancer [9]. Several other breast cancer susceptibility genes have been identified, including TP53, PTEN, E-cadherin, erbB2 and androgen receptors.

Breast cancer heterogeneity

It has become clear that breast cancer is not a single disease. There is a high degree of heterogeneity among breast tumours, which can be divided into subtypes that have different expression patterns and clinical outcomes [10, 11]. Comprehensive

gene expression profiling of a large set of breast tumours has revealed that breast cancer can be categorised into five major molecular subtypes: basal-like, luminal A, luminal B, HER2$^+$/ER$^-$, and normal breast-like [12]. Basal-like tumours have in general a poor prognosis, whereas luminal A tumours have the best prognosis [11]. It is already evident that Ductal Carcinoma *in situ* (DCIS) tumours, a highly heterogeneous type of breast tumours, have a difference in gene expression profile that results in different clinical outcomes. One hypothesis for the high heterogeneity of breast tumours is that all tumours arise from the same cell but the phenotype is determined by differences in genetic and epigenetic events. Another hypothesis is that the high heterogeneity could be explained by distinct cell of origin (Figure 2) [13]. During the normal differentiation of a breast epithelial cell, the bipotential stem cell gradually changes the gene expression profile and will eventually result in myoepithelial or luminal epithelial cells. The tumour initiating cell, "stem cell", could initiate tumour formation from any of these cells and the resulting tumour will have a distinct feature (e.g. luminal or basal- like).
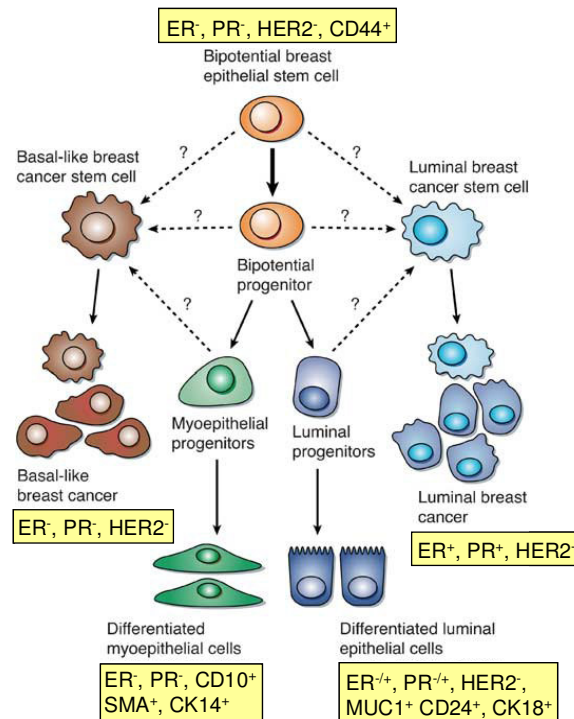

Figure 2. Hypothetical model explaining breast tumour subtypes. The differentiation of normal mammary epithelial cells is illustrated in the middle. Part of this figure is from Shipitsin *et al.* 2008 [14].

Ductal Carcinoma in situ

Most breast cancers originate in the epithelial cells that line the ducts (milk ducts) and some starts in the epithelial cells of the lobules (milk-producing glands). The main types of breast cancers are listed in table 1. Invasive ductal carcinoma is the most common type, accounting for more than 70% of all breast cancers [15]. Ductal Carcinoma *in situ* (DCIS) is regarded as a precursor of invasive ductal breast cancer. Due to the increasing use of mammography, there are an increasing number of patients diagnosed with DCIS today. Some DCIS lesions do not change over many years, while others rapidly progress to invasive cancers. It is therefore important to

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identify potential biomarkers that may distinguish the clinical subgroups of DCIS that represent a high risk of progression to invasive disease.

Table 1. Main types of breast cancer.
In situ carcinoma
        Ductal carcinoma in situ
        Lobular carcinoma in situ
Invasive carcinoma
        Ductal carcinoma
        - Tubular/cribriform carcinoma
        - Colloid (mucinous) carcinoma
        - Medullary carcinoma
        - Papillary carcinoma
        Lobular carcinoma

The initiation and progression of breast cancer from the duct is a multi-step process which is illustrated in figure 3. In normal breast ducts there is one layer of luminal epithelial cells and one layer of myoepithelial cells. This structure is surrounded by a basement membrane and stroma that includes different types of cells (endothelium, leukocytes and fibroblast). In the transition to the *in situ* lesion, luminal epithelial cells starts to hyper proliferate and they will lose contact with the basal membrane, which is intact. At the same time, the number of myoepitelial cells decreases. Characteristic for DCIS are also an inflammatory response with increased number of lymphocytes. More nutrition for the cell mass is needed, so there is also an increase in endothelium. There are two categories of DCIS; non-comedo and comedo. Comedo DCIS, that tends to be more aggressive, also have necrosis in the centre of the breast duct. High-grade DCIS lesions are also more aggressive, as compared to low-grade and intermediate-grade DCIS.
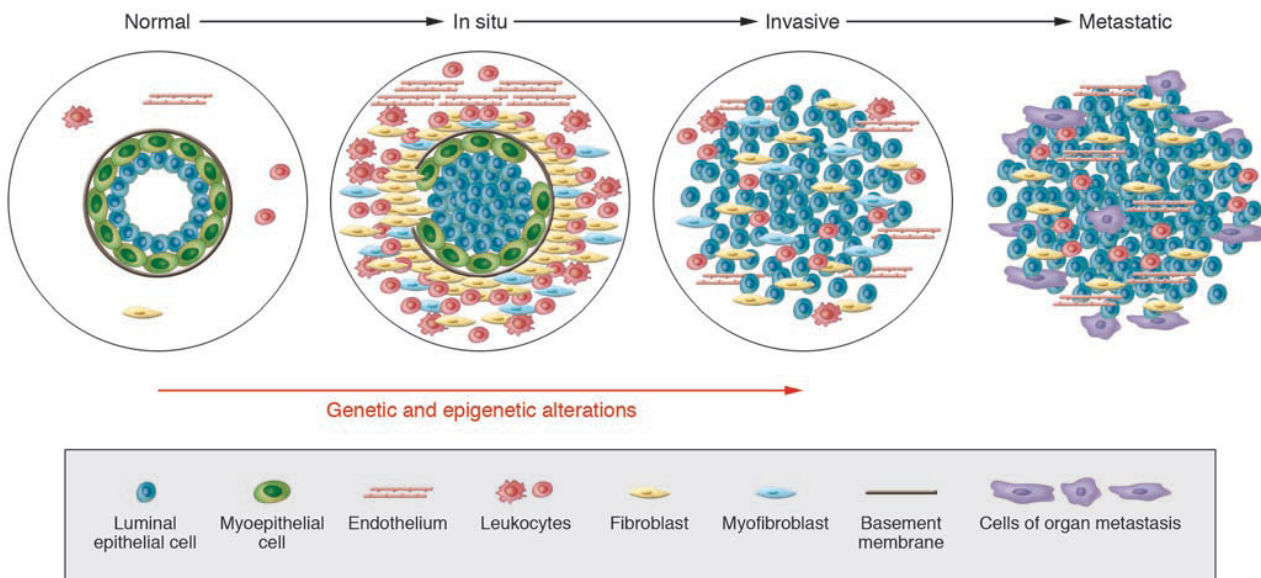

Figure 3. Hypothetical model of breast tumour formation from the duct [13].

The next important step in the breast cancer progression is the transition from DCIS to invasive carcinoma. Both myoepithelial cells and the basal membrane disappear and the proliferating tumour cells can invade the surrounding tissue and eventually

lead to metastasis. During this process (carcinogenesis), there will be a change in gene expression patterns, resulting in phenotypic changes, which is due to accumulation of both genetic and epigenetic alterations.

The progression of breast cancer is now believed to involve epithelial-mesenchymal interactions, which is also known to be critical for terminal differentiation of normal mammary epithelia [16-18]. Dr. Polyak´s laboratory has previously used the serial analysis of gene expression (SAGE) technology to determine the gene expression profiles of particular cell types and tumour progression state [19]. Dramatic gene expression changes were seen in all cell types, and not only in the luminal epithelial cells, during the progression of breast cancer. The aim of Polyak´s study was also to identify genes involved in the initiation and progression of breast tumorigenesis. Using the SAGE technique, psoriasin was identified as one of the most abundant transcript in high-grade DCIS as compared to normal tissue [20].

The S100 family

Psoriasin, also named S100A7, belong to the S100 family, whose members share a high degree of structural and sequence homology. S100 proteins are small, acidic proteins (9-13 kDa) characterised by two Ca^{2+} binding sites of the EF hand type (i.e., helix-loop-helix) (Figure 4). Almost all S100 members exist as homodimers (a few also as heterodimers) and they are connected by non-covalent bonds. Upon Ca^{2+} binding, which is important for its function, a conformational change occur, and each monomer can bind a target protein [21]. Because S100 proteins are dimers they can crosslink two homologous or heterologous target proteins.
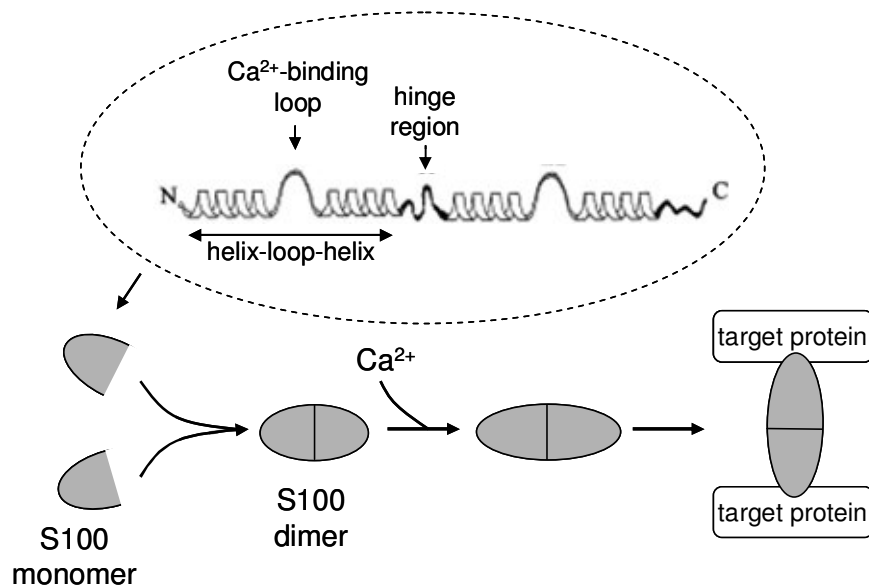
Figure 4. Illustration of the secondary structure of an S100 protein and the interaction of S100 monomers to a functional Ca^{2+} loaded S100 dimer. Part of this figure is from Donato *et al.* [22].

S100 proteins differ from one another mostly in the hinge region and at the C-terminal, which are suggested to give the proteins different biological activities. There is a remarkable diversity of function for the S100 proteins. They have been implicated

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in cellular processes including cell proliferation, apoptosis, differentiation, invasion and metastasis [21]. They have been observed to have both intracellular and extracellular actions and they are expressed in a cell type specific manner. The S100 proteins show altered expression in many cancer and they are in most cases upregulated in different tumours. These findings indicate that the S100 proteins may have potential value as biomarkers or as potential therapeutic targets in many cancers, but the mechanism of action for many of the S100 proteins are still to be discovered.

Several of the S100 proteins have been implicated in breast tumorigenesis [23]. An observation suggesting that the S100 proteins are involved in breast tumour progression is that the most frequent cytogenetic abnormalities in breast carcinomas involve chromosome 1. A gene cluster located on chromosome region 1q21, known as the epidermal differentiation complex (EDC), contains the genes for several differentiation markers but also the genes for many of the S100 proteins [24, 25]. As compared to many of the other S100 proteins, S100A2 expression was observed to be downregulated in breast cancer [26]. Overexpression of S100A4 has been implicated in promoting cell invasion and metastasis, and correlating with this, its expression is associated with unfavourable prognosis in breast cancer [27, 28]. Predominantly psoriasin but also calgranulin-A (S100A8) and calgranulin-B (S100A9), have been shown to be highly expressed in high grade DCIS [20, 29]. These two S100 proteins was shown to form a heterodimer complex [30]. S100A11 was thought to be a tumour promoter, as it was upregulated in breast cancer [31]. Currently, the most studied S100 proteins in breast cancer are S100A4 and psoriasin.

Psoriasin (S100A7)

Psoriasin was originally identified in 1991 as a protein which was highly upregulated in psoriatic keratinocytes but it had limited expression in normal tissues [32]. Upregulated psoriasin expression was also found in squamous cell carcinoma of the skin and bladder [33, 34]. As previously mentioned, psoriasin is one of the S100 proteins that is highly abundant in a subset of DCIS lesions [20, 35]. About 50% of all DCIS lesions are positive for psoriasin expression and these tumours are also associated with higher nuclear grade, estrogen receptor (ER) negative status, necrosis and inflammation [36]. Psoriasin is in most cases downregulated in the transition to the invasive phenotype, but persistent expression of psoriasin in invasive breast cancer has been associated with a worse prognosis [29, 37]. Psoriasin is highly similar in amino acid sequence with S100A15, although it appears that their regulation is highly tissue specific [38].

Psoriasin is secreted and is also present in the cytoplasm and nuclei of breast cancer epithelial cells [20, 37], but the function of psoriasin and regulatory factors controlling the expression in breast carcinogenesis is not fully understood. The group has previously shown that the induction of psoriasin in mammary epithelial cells can be a result of several stress stimuli, including serum depletion and loss of substrate attachment [20]. There is also evidence that loss of the TSG BRCA1 increase the expression of psoriasin [39]. Although psoriasin is correlated to ER-negative tumours, there is one study illustrating an association of psoriasin with ERβ [40]. However, the

high expression of psoriasin in ER-negative tumours may be explained by other factors.

► *Psoriasin- an advantage for breast tumour progression*
Psoriasin is believed to have an advantage for the progression of breast cancer. Psoriasin expression is overexpressed in estrogen receptor-negative breast tumours, both known to be associated with a poor clinical outcome [20, 41]. The expression of this protein is speculated not to be a marker for the recurrence of DCIS, but it may be a potential marker for DCIS with a poor prognosis [36].

Psoriasin has been shown to interact with several proteins, including Jun-activating binding protein 1 (Jab1). This interaction may enhance breast cancer progression, since overexpression of psoriasin in MDA-MB-231 breast cancer cells has been shown to increase nuclear Jab1 activity and enhance tumorigenesis *in vivo* in nude mice. Jab1 is involved in multiple signal transduction pathways, and can for instance increase AP1 activity which downregulate p27^{Kip1}, a protein that inhibit cell proliferation [42]. Another downstream target of Jab1 is nuclear factor-kappa B (NF-κB), a pro-survival pathway. Psoriasin also interact with Ran binding protein M (RanBPM) and this may contribute to breast tumour progression [43].

It has been demonstrated that psoriasin is endogenously expressed in the MDA-MB-468 human breast carcinoma cell line. In collaboration with Dr Polyak, we have previously shown that downregulation of this expression by short hairpin RNA (shRNA) inhibited tumour growth *in vivo*. In accordance with these findings, there was a downregulation of vascular endothelial growth factor (VEGF) in cells with reduced psoriasin levels [44]. This supports an association between high expression of psoriasin and increased angiogenesis.

VEGF↑
Psoriasin ⟶ Tumour growth ⬆
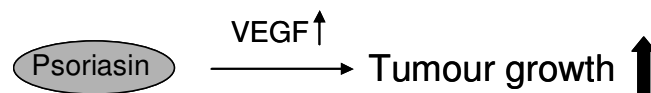
►*Psoriasin- a disadvantage for breast tumour progression*
Our group has shown that endogenous expression of psoriasin in the MDA-MB-468 cell line can reduce invasion. Downregulation of psoriasin by shRNA increased invasion *in vitro* and there was also a corresponding upregulation of matrix metalloproteinase 13 (MMP13).

MMP13↓
Psoriasin ⟶ Invasion ⬇
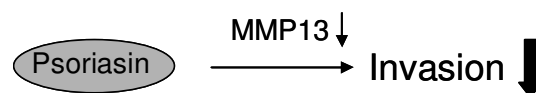
Obviously, there is conflicting results whether psoriasin may have an advantage or disadvantage for breast tumour progression. In this thesis we further evaluated the functional relevance of psoriasin and gained insight into regulatory pathways that control the expression. The main goal of this project was to investigate the role of psoriasin for the initiation and progression of breast cancer, and elucidate psoriasin as a potential biomarker for breast cancer with a poor prognosis.

Psoriasin is one of the most abundant transcripts in high-grade DCIS of the breast with higher risk of local recurrence. The aim of this thesis was to elucidate the functional relevance of psoriasin for the initiation and progression of DCIS, and to characterise important regulatory pathways for psoriasin expression. The goal of this project was to evaluate psoriasin as a potential biomarker for breast cancer with a poor prognosis. The S100-proteins calgranulin-A and calgranulin-B, also elevated in high-grade DCIS but not in the same amount, were also studied. The specific aims for the different papers were as follow:

Paper I

High-grade DCIS is characterised by a high apoptotic rate and reactive oxidant species (ROS) are known to influence this process. The aim of this paper was to investigate if psoriasin and calgranulin-B are expressed due to the production of reactive oxidant species. The function of psoriasin was also investigated during ROS exposure.

Paper II

The aim of this paper was to further explore signal transduction pathways implicated in the regulation of psoriasin, calgranulin-A and calgranulin-B expression. High-grade DCIS is also characterised by an inflammatory respons and IFNγ, a proinflammatory cytokine, was previously shown to exert anti-tumour action in breast cancer. We therefore examined the effect of IFNγ on the psoriasin, calgranulin-A and calgranulin-B expression.

Paper III

High-grade DCIS is characterised by a high proliferation rate and crowded cells consequently lose contact with the extracellular matrix (ECM). The aim of this paper was therefore to investigate whether loss of cell adhesion signalling may contribute to the high psoriasin expression seen in some high-grade DCIS tumours.

Paper IV

The interaction between breast epithelial cells and the ECM contribute both to the normal development of a differentiated structure and pathological processes. The gene that encodes psoriasin and many other S100-genes are located within a gene cluster that also contains the genes for several differentiation markers that play important roles in the terminal differentiation of the human epidermis. The aim of this paper was to evaluate whether psoriasin, calgranulin-A, and calgranulin-B are involved in and are potential markers for the differentiation of mammary epithelial cells.

Cell lines

The following cell lines were used in this thesis:

Normal breast epithelium
- MCF10A (human) (Paper I, II, III and IV)
- Tac2 (mouse) (Paper II)

Mammary breast carcinoma
- MDA-MB-468 (human) (Paper I, II and III)
- SUM190 (human) (Paper II)

Normal skin epithelium
- HEKn (human) (Paper II and III)

Tumours

Tissue specimens for immunohistochemical studies (Paper III) were selected from the files of the Department of Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden. Eleven cases, representing DCIS grade 1, 2 and 3, picked out at random, of paraffin-embedded tissue samples were obtained. The tissue blocks contained tumour tissue and adjacent normal breast tissue. Histological, the tumours showed some degree of structural heterogeneity. The individual tumours contained a single or several components of papillar, cribriform, solid and comedo-type structures.

SAGE libraries were generated from fresh tissue specimens and analysed essentially as previously described as part of the National Cancer Institute Cancer Gene Anatomy Project (CGAP) [45]. The specimens was obtained from the Brigham and Women's Hospital, Massachusetts General Hospital, and Faulkner Hospital (all Boston, MA), Duke University (Durham, NC), University Hospital Zagreb (Zagreb, Croatia), and the National Disease Research Interchange. The SAGE libraries analysed in this thesis (Paper III) were previously reported [46, 47]. These include six normal breast tissues, eight DCIS tumours (five high-grade, comedo DCIS and three intermediate-grade with no necrosis), nine invasive breast tumours and three metastatic breast tumours. Two of the DCIS tumours were pure DCIS, while others were derived from patients with coexisting invasive breast carcinomas.

Methods to influence protein level

In this thesis several methods were used to induce or repress the expression of specific proteins:

Infection with recombinant adenovirus (Paper I and II). The transient overexpression of GFP, Bcl-2 and phosphorylation defective dominant-negative IKKβ (dnIKKβ) was achieved using adenoviral infection. For the

generation of recombinant adenovirus, complementary DNA (cDNA) corresponding to each gene was PCR-amplified and subcloned into the pAd-Track-CMV vector, followed by recombination and adenovirus generation using the AdEasy protocol [48]. The cell lines were infected with the recombinant adenovirus at a multiplicity of infection (MOI) ~100. The adenoviral protein expression, driven by the CMV promoter, was verified using western blotting and fluorescence emission. The adenovirus contains double stranded DNA (dsDNA) and they can not efficiently integrate into the chromosomal DNA.

Infection with recombinant retrovirus (Paper I, III and IV). A stable psoriasin-expressing cell line (MCF10A-HID) was established by infecting normal breast epithelium with a recombinant retrovirus overexpressing psoriasin. The retroviral protein expression was verified using western blotting. The retrovirus contains RNA and relies on the enzyme reverse transcriptase to perform the reverse transcription of its genome from RNA into DNA, which can then be integrated into the host's genome with an integrase enzyme. The virus then replicates as part of the cell's DNA.

Tet-Off system (Paper II). This system regulates psoriasin in normal breast epithelium using tetracycline. For the generation of this system, Tac2 mouse mammary epithelial cells was transfected with two different plasmids, ptTA-IRES-Neo (tTA, tet activator; IRES, internal ribosomal entry; neo, G418 resistance gene) and pBI-EGFP2. The pBI-EGFP2 plasmid was subcloned with psoriasin cDNA. The system induces psoriasin when tetracycline is excluded. An advantage of this system is that the cells are identical and have not adapted to the increased psoriasin expression.

RNA interference (Paper III and IV). RNA interference (RNAi) is a natural process in which the expression of a targeted gene can be knocked down by small interfering RNA (siRNA). siRNA is composed of double stranded RNA (dsRNA) which interfere with and degrade a particular gene transcript. There are two general methods for the production of siRNA in cultured cells. The first method, using transfection or electroporation of synthetic siRNA, will result in a transient downregulation of gene expression. For the generation of a stable downregulation, another method may be applied. Using this technique, cells are introduced with a DNA vector that expresses shRNA and these are processed to siRNAs by enzyme cleavage. The DNA vector can be integrated into the genome and may therefore be transmitted to the daughter cells.

Expression methods

Methods for studying expression can be evaluated both at the RNA level (gene expression) and protein level (protein expression).

Gene expression methods

Gene expression can be investigated by measuring the mRNA transcripts. This is performed by using polymerase chain reaction (PCR), a technique frequently used in molecular research today. The basic of PCR involves three steps: 1) denaturation of dsDNA at 95°C; 2) annealing of primers to single-stranded DNA at primer-specific annealing temperatures, usually between 50 and 65°C; and 3) DNA synthesis by primer elongation at 72°C (Figure 5). These steps, representing one cycle, are normally repeated 30 times. For every cycle (denaturation-annealing-elongation) the amount of new synthesised DNA doubles and it will therefore be an exponential increase in DNA (PCR product).
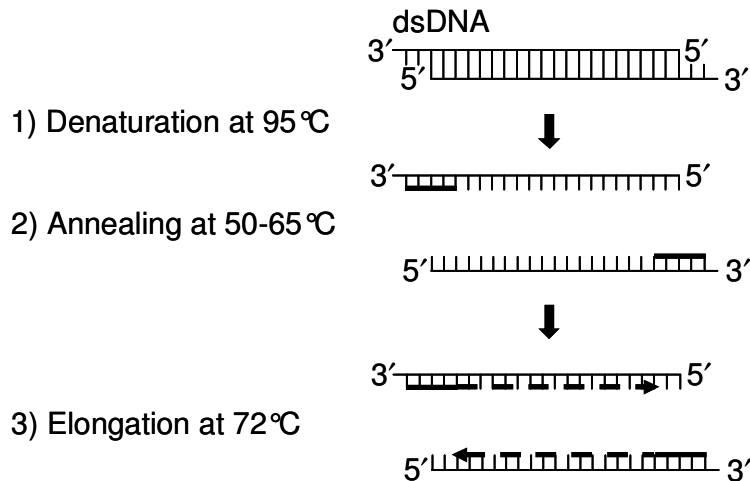

Figure 5. Schematic presentation of the steps in one PCR cycle.

The first step to measure the relative amount of mRNA for a specific gene is to extract the total RNA from cells or tissues. RNA is unstable and is therefore reverse transcribed into complementary DNA (cDNA), which is more stable. This reaction is performed using the enzyme reverse transcriptase, therefore the name reverse transcriptase PCR (RT-PCR). The resulting cDNA is then amplified using PCR with gene-specific primers. The PCR technique has different applications and the following is a description of the different methods used in this thesis.

> **Real-Time quantitative PCR** (Paper II). Real-Time quantitative PCR (RTQ-PCR) is a method for quantification of the mRNA level. Compared to the conventional PCR reaction, this method measures the amount of PCR product after each cycle. In this thesis the relative expression was analysed using RTQ-PCR and the LightCycler instrument, together with the LightCycler FastStart DNA Master SYBR Green I kit. SYBR Green has the ability to bind dsDNA and this DNA-dye complex absorbs blue light and emits green light which can be detected by the instrument. During the PCR reaction the fluorescence emission is measured continuously. To verify the specificity of the amplification reaction the PCR is ended with a melting curve analysis. This process gives a specific temperature when the PCR product is denatured, which is based on the nucleic acid content. The PCR product is also run on an agarose gel to verify one specific band.

Using LightCycler software a crossing point (CP) for each sample is obtained. The CP value is obtained from the exponential phase of the amplification curve and where the reaction reaches a fluorescent activity above background levels. The CP value determined for a gene of interest is normalised to an endogenous control. Using an endogenous control will correct any variations between different samples. We have used β-actin as an endogenous control.

SAGE Genie informatics (Paper III). The SAGE Genie website (http://cgap.nci.nih.gov/SAGE) provides a quantitative view of the gene expression of selected genes in many different tissues from human and mouse. SAGE genie was created by the Cancer Genome Anatomy Project (CGAP) [45] by using SAGE. The basic of SAGE is illustrated in figure 6. 1) First the mRNA from a sample is isolated using magnetic beads and reversed transcribed to cDNA. 2) cDNA is then digested with a restriction enzyme (Nla III). 3) 14 bp tags are released from the 3´ end. These SAGE tags are specific to known genes. 4) The tags are ligated followed by sequencing and bioinformatic analysis. 5) The different tags are then counted to calculate the expression level of each transcript in different samples.
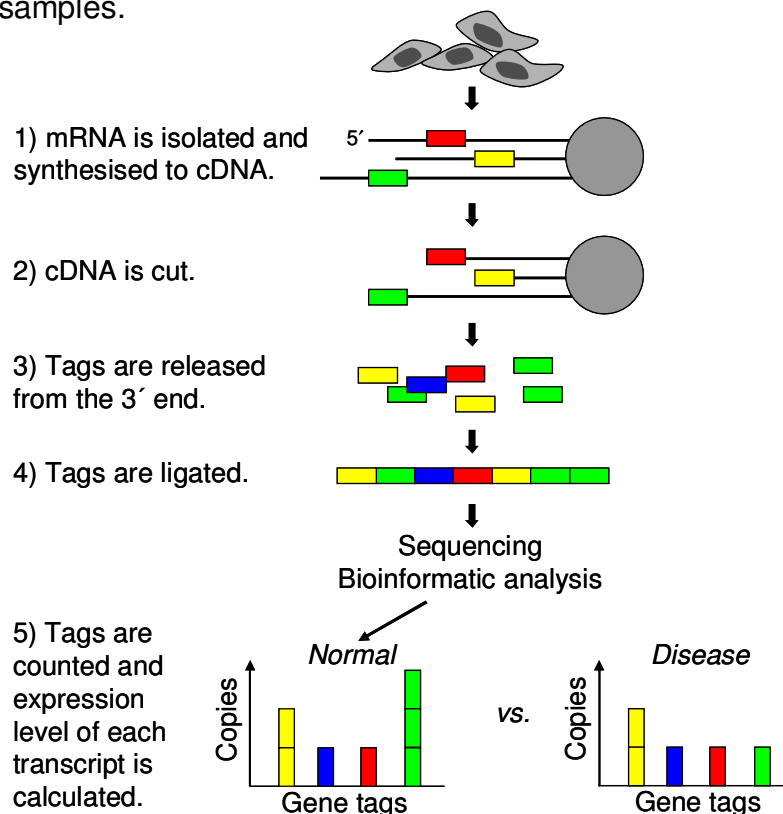
Figure 6. The basic steps in a SAGE protocol.

The SAGE Anatomic Viewer, one of several tools for processing SAGE data in SAGE genie, visualises the relative expression of a specific gene in normal and malignant tissue. To obtain the relative expression of a gene in a single SAGE library (one single tissue piece) Digital Northern can be applied. Dr. Polyak´s laboratory has previously used the SAGE technology to determine the complete gene expression profiles of normal

breast tissue and breast carcinomas at all clinical stages, with the aim of identifying genes involved in the initiation and progression of breast tumorigenesis [46, 47]. Using this technique, psoriasin was identified as one of the most abundant transcript in high-grade DCIS as compared to normal tissue [20].

Protein expression methods

Protein expression can be investigated by measuring the protein level. For identification and localisation of both intracellular and extracellular proteins, antibodies are most commonly used. Antibodies, also known as immunoglobulin's, are produced by B-lymphocytes in the immune system. They consist of two heavy chains and two light chains connected by disulphide bonds (Figure 7). Antibodies are divided into five major classes (IgM, IgG, IgA IgD and IgE) based on their constant region and immune function. On the N-terminal of both the light chain and the heavy chain there is a great variability. These variable regions, with an antigen binding site, have a high specificity to a specific antigen.
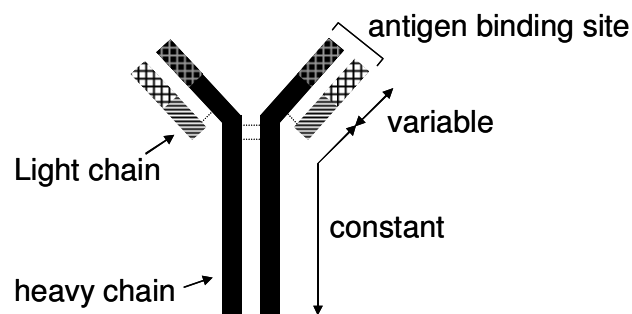

Figure 7. Basic structure of an antibody.

The following is a description of the different protein expression methods used in this thesis.

> **Western blotting** (Paper I, II, III and IV). Western blotting (immunoblotting), introduced by Towbin *et al.* in 1979 [49], is a qualitative and a semiquantitative method to detect specific proteins. The proteins are first separated according to the length of the polypeptide (denatured proteins) or to the 3D structure (native non-denatured proteins) using gel electrophoresis. The proteins are then transferred to a membrane (often nitrocellulose or polyvinylidene difluoride). To prevent any nonspecific binding of antibodies the membrane is then blocked. This can be performed using skimmed milk powder or bovine serum albumin. The detection is then performed using enzyme-labelled antibodies that bind to the protein of interest. In this thesis indirect detection is used, which includes an unlabelled primary antibody that binds to the protein (antigen) followed by a labelled secondary antibody that binds to the primary antibody (Figure 8). With the addition of an appropriate substrate, a detectable product will be produced. Horseradish-peroxidase (HRP)-conjugated secondary antibodies are commonly used and this enzyme cleaves a chemiluminescent substrate that will produce

light. This light (chemiluminescence signal) is then registered with a camera that is designed for chemiluminescent detection.
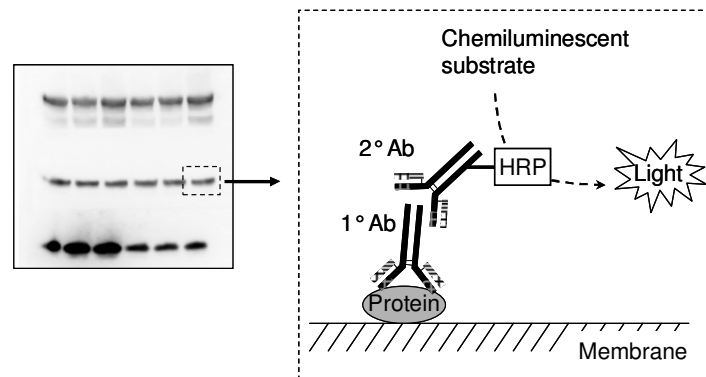

Figure 8. Indirect detection method used in western blotting.

Flow cytometry (Paper III and IV). This is a method that measures and analyse the fluorescence of single cells (and other particles) at high speed. The properties of cells are mostly detected using fluorochrome-labelled antibodies (FITC, PE, APC etc.) that binds to specific antigen. Several cellular parameters can be measured with this method and one application (used in this thesis) is cell surface staining that makes it possible to analyse the expression of specific proteins in the cell membrane. Flow cytometry also measure structural properties like cell size (Forward Scatter) and cytoplasmic granularity (Side Scatter).

Immunohistochemistry (Paper III). Immunohistochemistry (IHC) is a widely used method that visualise the distribution and localisation of specific proteins in a biological tissue section (*in vivo*). The detection of different proteins is most commonly achieved, like in western blotting, with an enzyme-labelled antibody. In this thesis antigen-antibody complexes were visualised with an avidin-biotin complex (ABC) detection system by using diaminobenzidine (DAB) as a chromogen. First a primary antibody binds to an antigen followed by a secondary antibody that is conjugated with biotin. The third step involves avidin that has a very high affinity for biotin. Avidin is also conjugated with a biotin-peroxidase. This peroxidase enzyme can use DAB to produce a colorimetric end product that can be detected.

Functional studies

Cell culture conditions

During the initiation of high-grade DCIS the epithelial cells start to hyper proliferate leading to lost contact with the basal membrane, and an increase in cell density. To determine what happens during this process several cell culture conditions, relevant for the tumour microenvironment, were used *in vitro.* These conditions are valuable model systems that can be used for functional genetic studies that can not be performed *in vivo*. Culture conditions used in this thesis include:

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Confluence (Paper I, III and IV). This condition represents the increase in cell density and this is achieved by maintaining the cells in confluent condition for up to ten days.

Suspension (Paper I, II, III and IV). During suspension culture, normal adherent cells lose contact with the ECM. Cells are plated into poly-2-hydroxy-ethylmethacrylate (polyHEMA) -coated petri dishes which represent when cells lose contact with the basal membrane.

Cell growth studies

In paper I and II the cell growth of mammary epithelium is studied after the exposure of different agents, including H_2O_2 and IFNγ. In this thesis these studies was evaluated using two methods.

MTS assay. This assay is a colorimetric assay that involves a bio reduction of the MTS tetrazolium compound to a coloured formazan product in living cells. Formazan is soluble in the tissue culture medium and the quantity is measured by the absorbance at 490 nm.

Cell counting. Cells growth is counted based on trypan blue exclusion. Using trypan blue, the living cells with an intact cell membrane will not be stained, whereas dead cells have a blue colour under the microscope.

Magnetic activating cell sorting

For the enrichment of specific cell populations, cells can be magnetically isolated based on their surface antigen (CD molecules). Using magnetic activating cell sorting (MACS), which is a trademark name from Miltenyi Biotec, cells are first labelled with antigen-specific antibodies conjugated with magnetic beads. Cells expressing the specific antigen are then isolated by using a column with a strong magnetic force (positive selection), while cells not expressing the antigen flow through (negative selection). This method is performed in paper IV.

Nitro Blue Tetrazolium assay

Nitro Blue Tetrazolium (NBT) assay is a colorimetric analysis used in paper III for detection of ROS. The amount of oxidant radicals is measured through the reduction of a soluble nitroblue tetrazolium dye to insoluble formazan. The production of formazan can be measured at 630 nm.

Statistical methods

Hierarchical clustering (Paper III). In this powerful investigative multivariate method a hierarchical cluster algorithm can be employed to group together samples/tumours displaying similarities in gene expression. The method can be applied with the intention of discovering clinical subgroups of tumours. The output of a cluster analysis can be presented in a dendrogram (tree diagram), which illustrates the grouping

(Figure 9). The dendrogram image demonstrates the degree of relatedness between tissue samples. Each sample is represented by a single branch at the bottom of the dendrogram. Samples displaying similar pattern of expression are grouped together on closely connected branches of the dendrogram. Genes with dissimilar expression are placed further apart.
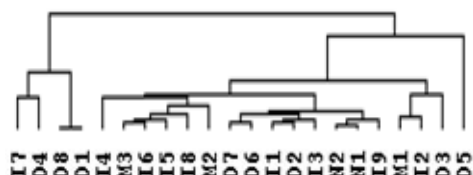

Figure 9. Dendrogram image demonstrates the degree of relatedness of S100-proteins between different breast tissue samples (N= normal, D= DCIS, I= invasive and M= metastatic). Adapted and modified from Carlsson et al., 2005 [50].

Student´s t-test (Paper I, II, III and IV). Student´s t-test can be used to compare two normally distributed populations. This is known as a bivariate statistics, where two variables are involved.

**Wilcoxon`s signed rank test** (Paper I). This test is a non-parametric alternative to the t-test that compares two groups. Wilcoxon`s signed rank test can be used in small populations that are assumed not to be normally distributed.

Spearman's rank correlation test (Paper III). Spearman´s correlation is a non-parametric test for measuring the relationship between two variables. As compared to Pearson´s correlation, that assumes possible relationships (for example linear relationship); this test can be used without making these assumptions of the variables.

Two-sided versus one-sided p-values. Two-sided p-values are calculated when the results may occur in either direction. This is in most cases the correct procedure; however, in some cases the hypothesis is restricted to an effect in one direction only (for example down- or up-regulation). In these cases it is considered to be appropriate to use one-sided p-values.

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In the transition from a normal mammary tissue to high-grade comedo DCIS several observations can be made. The luminal epithelial cells start to hyper proliferate. This will increase the cell density and the cells lose contact with the basal membrane. An increase in the apoptotic rate is also observed [51] and surviving tumour cells are likely to be relatively more resistant to apoptosis. We have previously demonstrated an increase in the expression of psoriasin in response to several stimuli, such as the loss of attachment to the extracellular matrix and confluent conditions, in mammary epithelial cells [20, 52]. These stimuli represent conditions that are relevant for the tumour microenvironment seen in high-grade comedo DCIS. In a SAGE analysis of high-grade DCIS, both psoriasin and calgranulin-B were markedly upregulated, as compared with normal mammary epithelial cells [20]. In this thesis we further evaluate the functional relevance of psoriasin, calgranulin-A and calgranulin-B in high-grade comedo DCIS.

Paper I

Reactive oxidant species (ROS), like hydrogen peroxide (H_2O_2), has been shown to play an important role in the induction of apoptosis [53]. One aim of this paper was to investigate whether psoriasin and calgranulin-B are expressed in high-grade comedo DCIS due to the production of ROS. We treated normal mammary epithelial cells (MCF10A) with H_2O_2 at various concentrations and for different lengths of time. Free radicals can cause both p53-mediated apoptosis [54] and necrotic cell death [55] depending on the level of ROS within the cell. Necrosis occurs if oxidative stress is extremely severe, whereas H_2O_2 induces apoptosis at lower concentrations. Consequently, exposure of normal keratinocytes to 700 µM H_2O_2 for more than one hour leads to irreversible toxicity [56]. We detected significant upregulation of psoriasin and calgranulin-B when MCF10A cells were treated with 50-100 µM H_2O_2 for 1 hour and allowed to recover for up to five days in regular culture medium, but we did not observe any induction after longer treatment periods. This may indicate an association between psoriasin/calgranulin-B induction and apoptosis-inducing concentrations of H_2O_2.

To investigate whether certain genes, known to be involved in the regulation of ROS, have an influence on the induced expression of psoriasin and calgranulin-B, we tested the effect of Bcl-2 on psoriasin and calgranulin-B levels after ROS exposure. Bcl-2 is a well-known anti-apoptotic protein with several properties, including anti-oxidative actions. Bcl-2 protects cells from apoptosis induced by ROS, and it has been suggested that there is a reciprocal relationship between Bcl-2 and ROS [57, 58]. We showed that Bcl-2 overexpression repressed the induction of psoriasin and calgranulin-B after ROS exposure. The same effect was obtained with the anti-oxidant N-acetylcysteine (NAC), which indicates that the suppression of psoriasin and calgranulin-B induction was mediated by the anti-oxidant function of Bcl-2. All these data supports the hypothesis that psoriasin and calgranulin-B are expressed due to the production of ROS.

Due to the fact that psoriasin and calgranulin-B was shown to be upregulated by ROS, we hypothesised that the induction of these proteins by loss of attachment to the extracellular matrix (suspension) and by confluent conditions in vitro (seen in this paper, paper IV and by Enerback et al. 2002), could be explained by increased levels of ROS. To evaluate this, psoriasin and calgranulin-B were induced by culturing MCF10A in suspension and confluence conditions. The expression of Bcl-2 and the treatment with NAC led to a decrease in the induction of the proteins in both conditions. This supports the assumption that psoriasin and calgranulin-B expression in these conditions are due to increased level of ROS. Compared to the elevated expression of psoriasin and calgranulin-B in ROS treated and confluent cultured cells, there was a higher induction of psoriasin in cells grown in suspension [20]. Therefore, other mechanism for the expression of especially psoriasin can not be excluded. We have also seen that the endogenous expression of psoriasin, in the mammary breast carcinoma cell line MDA-MB-468, was not reduced by Bcl-2 overexpression, which indicates that other mechanisms (excluding ROS production) may induce psoriasin expression in high-grade DCIS.

The NF-κB transcription factor is activated by a large number of stimuli, such as oxidative stress and DNA damaging agents [59]. NF-κB is one of the most important regulators of cell survival and is known to repress Bcl-2 expression and is activated by ROS [60]. NF-κB is a known downstream target of Jab1, a transcriptional cofactor previously reported to interact with psoriasin in breast epithelial cells [42, 61]. Therefore, the involvement of the NF-κB pathway in the regulation of psoriasin and calgranulin-B expression was evaluated. Inhibition of the NF-κB pathway was performed using a recombinant adenovirus expressing a dnIKKβ. This expression inhibits the release and translocation of NF-κB to the nucleus, where it activates its target genes. Overexpression of dnIKKβ in suspension culture of MCF10A cells showed that the induction of psoriasin was reduced, whereas calgranulin-B was unaffected. Treatment with caffeic acid phenethyl ester (CAPE), an NF-κB inhibitor, also demonstrated a reduction of psoriasin expression. Since our experiments suggested the involvement of the NF-κB pathway in the induction of psoriasin expression we analysed the promoter area (-3000/+200) of psoriasin using the MatInspector program [62] for potential NF-κB binding sites. Two putative NF-κB binding sites were found at position -1705/ -1691 and at position -1025/ -1011. Therefore, the promoter structure also supports the involvement of NF-κB in the regulation of psoriasin. In line with this, tumour necrosis factor alpha (TNFα), a potent inducer of NF-κB, showed an induction of psoriasin in normal culture of MCF10A.

The functional relevance of psoriasin was also investigated and we analysed if psoriasin expression could influence survival during ROS exposure. From our results we see that MCF10A cells with a stable retroviral overexpression of psoriasin were significantly more resistant to H_2O_2-induced cell death than control cells. Our data was also confirmed by Emberley et al. that demonstrated that psoriasin expression in mammary epithelial cells correlates to increased survival and NF-κB signalling [63].

From our findings in paper I we hypothesised that the expression of psoriasin and calgranulin-B in high-grade comedo DCIS are due to ROS production. The induction of ROS may be the result of increase in apoptosis and cell density, but also the result of lost contact to the basal membrane. Our results also indicate that the function of psoriasin may be to protect epithelial cells from ROS-induced cell death and that this

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effect is potentially mediated by the NF-κB pathway. Consequently, the production of psoriasin may contribute to apoptosis resistance in high-grade comedo DCIS. A summary of the results from paper I is illustrated in figure 10.
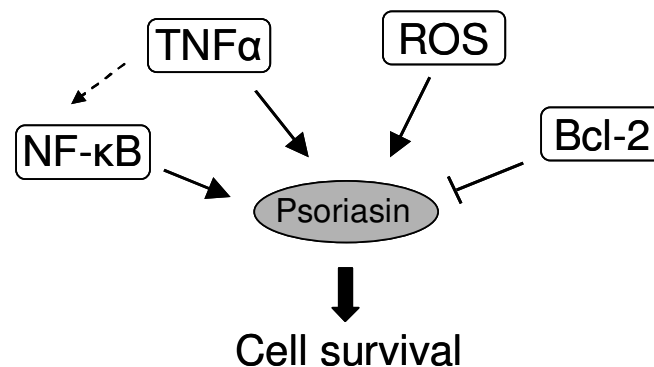

Figure 10. Signals interfering with psoriasin expression.
A summary of the results obtained from paper I.

Paper II

The aim of this paper was to further explore signal transduction pathways used in the regulation of psoriasin, calgranulin-A and calgranulin-B expression. High-grade DCIS is also characterised by an inflammatory response and interferon-gamma (IFNγ), a pro-inflammatory cytokine, has been shown to exert anti-tumour action in breast cancer. This cytokine may have both pro-apoptotic and anti-proliferative effects on a variety of tumour cells [64]. We examined the effect of IFNγ on the psoriasin, calgranulin-A and calgranulin-B expression in mammary epithelium.

We examined the effect of IFNγ in suspension and confluence cultured MCF10A cells, as well as H_2O_2 treated MCF10A cells. From paper I, paper IV and previous studies, these conditions are known to induce psoriasin and calgranulin-B [20]. We recently showed, by the hierarchical clustering of S100 gene expression in 22 breast cancer SAGE libraries, that one group with a distinguishable S100 gene expression profile was characterised by the high concomitant expression of psoriasin, calgranulin-A and calgranulin-B [50]. In this paper we showed that calgranulin-A is also induced by culturing normal epithelium in these different conditions. We therefore focused our interest on all three S100-proteins. We showed that IFNγ treatment suppressed psoriasin induction by suspension, while it had no effect on psoriasin induction by confluent conditions or H_2O_2 treatment. We found no effect of IFNγ on calgranulin-A and calgranulin-B protein levels in none of the conditions tested. In conclusion, we found that IFNγ treatment suppressed psoriasin, induced by suspension, in mammary epithelial cells and that calgranulin-A and calgranulin-B were not modulated by the treatment. In accordance with the effect of IFNγ on psoriasin induction by suspension, we showed that MDA-MB-468 cells treated with IFNγ also downregulate psoriasin. The MDA-MB-468 cells also display a high expression of calgranulin-A and calgranulin-B. However, similar to the findings in suspension, the expression of calgranulin-A and calgranulin-B were not influenced by the IFNγ treatment. The results of western blotting and RTQ-PCR analyses suggest that the effect of IFNγ on psoriasin expression is mediated both at the translational (protein) and transcriptional (mRNA) level in MCF10A and MDA-MB-468 cells. Due to

the anti-tumour action of IFNγ, this cytokine has been utilised for the treatment of breast cancer. Although we see a downregulation of psoriasin by IFNγ in suspension culture of MCF10A and MDA-MB-468 *in vitro*, there is a co-existence of IFNγ and psoriasin in breast tumours *in vivo*. Therefore, treatment with IFNγ on psoriasin-expressing breast tumours may not be sufficient.

IFNγ is an important activator of signal transducer and activator of transcription 1 (STAT1) signalling [65]. To determine whether the downregulation of psoriasin involved the activation of STAT1, we studied the IFNγ/Jak/STAT1 pathway using antibodies against STAT1 and phosphorylated STAT1. The induction of STAT1 confirms the presence of the IFNγ receptor and the phosphorylated form confirms an active signalling pathway. In agreement with this, we demonstrated the upregulation of STAT1 in MDA-MB-468 and MCF10A cells after treatment with IFNγ. IFNγ treatment led to the phosphorylation of the STAT1 protein (Tyr-701) within a few minutes, thus confirming an active signalling pathway. The SUM190 breast carcinoma cell line also has high endogenous psoriasin protein levels. IFNγ did not downregulate psoriasin expression in this cell line, although the presence of the IFNγ receptor could be confirmed. Interestingly, IFNγ treatment did not lead to a marked phosphorylation of the STAT1 protein, as we observed in the MDA-MB-468 cell line. These findings suggest that IFNγ mediates its suppressive effect on psoriasin transcription by activating the STAT1 signalling pathway. An activated and phosphorylated STAT1 has actually been correlated to a good prognosis in breast cancer [66]. This is in line with other studies showing that psoriasin is associated to a poor prognosis; knowing that activated STAT1 can reduce psoriasin expression.

From functional studies of psoriasin in paper I we hypothesised that psoriasin may protect epithelial cells from ROS-induced cell death. Like ROS, we showed in this paper that IFNγ treatment leads to decreased viability in normal mammary epithelial cells. To further evaluate the functional relevance of psoriasin we investigated whether the expression had an effect on cell viability during IFNγ treatment. We established a mammary epithelial Tac2 cell line expressing psoriasin in an inducible manner using the Tet-Off system. We showed that the viability of the psoriasin-expressing Tac2 cells was higher than in the non-expressing Tac2 cells when IFNγ was added at seeding. Interestingly, psoriasin-expressing cells showed a resistant phenotype only when IFNγ was added when cells were plated. Treatment after cell attachment did not confer any difference in cell viability. It should be mentioned that IFNγ may have both pro-apoptotic and anti-proliferative effects and therefore the decreased viability after IFNγ treatment could be the result of reduced proliferation.

The fact that psoriasin, compared to calgranulin-A and calgranulin-B, is more expressed in high-grade DCIS could indicate other mechanisms for the induction of this protein. Our *in vitro* data of suspension cultured MCF10A also showed a massive induction of psoriasin as compared to the other two S100-proteins. The finding that psoriasin was higher when cell lose contact to the extracellular matrix (suspension), led to the hypothesis that the expression in high-grade DCIS is also regulated by changes in adhesion signalling. Interestingly, in this paper we have demonstrated that IFNγ downregulated the massive psoriasin expression in the breast cancer cell line MDA-MB-468 as well as the high psoriasin expression induced by culturing MCF10A in suspension. In contrast, the expression of calgranulin-A and calgranulin-B, in these cell lines, were not affected by the IFNγ treatment. This may indicate that

IFNγ interferes with adhesions signals that specifically modulate psoriasin expression. Identifying these adhesion signals that interfere with psoriasin expression was the focus of paper III.

Paper III

In paper I we hypothesised that the expression of psoriasin and calgranulin-B in high-grade comedo DCIS is due to ROS production, but other mechanism for the expression of especially psoriasin may not be excluded. Specifically, we see a massive induction of psoriasin in suspension culture (when cell lose contact to ECM), compared to the expression of calgranulin-A and calgranulin-B. High-grade DCIS is characterised by a high proliferation rate and crowded cells consequently lose contact with the ECM. These observations suggest that changes in adhesion to extracellular matrix may contribute to psoriasin expression. The aim of this paper was therefore to investigate whether loss of cell adhesion signalling may contribute to the high psoriasin expression seen in high-grade DCIS tumours.

Cell-cell interaction and the anchorage of cells to components of the ECM are mediated primarily by integrins and other adhesion molecules [67]. Integrin receptors bind to their ligands by interacting with short amino acid sequences, such as the RGD sequence (Arg-Gly-Asp). This sequence is recognised by many, but not all of the known integrins. Using RGD-competitive ligand inhibitor (short synthetic peptides containing the RGD sequence) for integrin binding, no induction of psoriasin was observed. Integrins recognizing this sequence do not therefore appear to influence the regulation of psoriasin expression by ECM contact.

We showed in paper II that IFNγ downregulated psoriasin induced by suspension culture of MCF10A cells, but it did not affect psoriasin expression when induced by confluence or ROS. These findings led to the hypothesis that the induction of psoriasin in suspension cultures was due to loss of adhesion signalling, and that IFNγ may interfere this signalling and reduce psoriasin expression (Figure 11).
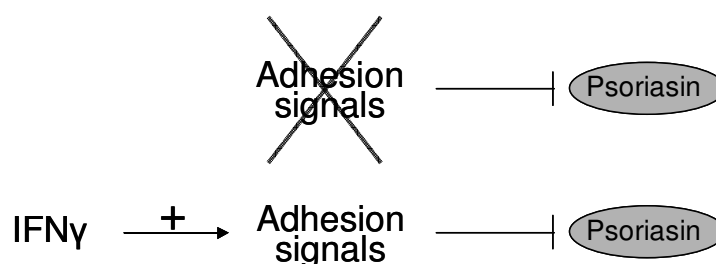
Figure 11. Hypothetical model of signals modulating psoriasin expression in suspension condition.

IFNγ is a multifunctional cytokine that activates the transcription of many genes. Using microarray analysis, de Veer *et al.* identified > 300 interferon-stimulated genes, ISG [68]. Using the ISG database available on their website, 13 adhesion-related molecules stimulated by IFNγ were selected. Utilizing the SAGE database available from the CGAP website, the expression level of these IFNγ-stimulated adhesion molecules and psoriasin were analysed in normal breast tissue and DCIS tumours.

We showed that intracellular adhesion molecule 1 (ICAM-1) and thrombospondin 1 (upregulated by IFNγ) are negatively correlated to psoriasin expression in normal and DCIS specimens. The expression of psoriasin, ICAM-1 and thrombospondin 1 in the different breast cancer stages showed that psoriasin was highly upregulated in DCIS compared with normal breast tissue, while ICAM-1 was downregulated. In contrast, the expression of both psoriasin and ICAM-1 in invasive and metastatic specimens was low. Thrombospondin 1 showed a low expression with minor changes in the different stages. Based on these findings, we focused our interest on ICAM-1 as an adhesion molecules potentially implicated in the regulation of psoriasin. It was actually shown that DNA variants in the ICAM gene region on chromosome 19p13.2 are associated with susceptibility to breast cancer and to breast cancer progression [69].

To further evaluate the role of other adhesion molecules potentially involved in the regulation of psoriasin, and also calgranulin-A and calgranulin-B expression, we analysed 34 adhesion molecules in normal and DCIS SAGE libraries. When analysed the correlation between these three S100 proteins and the expression of the adhesion proteins we found that a few adhesion proteins correlated with only psoriasin, calgranulin-A or calgranulin-B. Interestingly, the expression of the tumour-associated mucin1 (MUC1), which is a ligand for ICAM-1, demonstrated a significant positive correlation to the expression of all three S100 proteins. MUC1 expression is observed to be upregulated in DCIS compared with normal tissue and this upregulation persists in invasive and metastatic specimens. MUC1, which is a transmembrane glycoprotein, is an established tumour marker in breast cancer and is implicated in metastatic spread.

We showed that, in addition to psoriasin, calgranulin-A and calgranulin-B, MUC1 is also induced in suspension cultures of MCF10A cells. We also showed that there is a loss of ICAM-1 expression in suspension. To investigate the direct effect of ICAM-1 gene silencing, we transfected MCF10A cells with shRNA that target ICAM-1 mRNA. We demonstrated that the downregulation of ICAM-1 expression by shRNA led to the induction of psoriasin, calgranulin-A, calgranulin-B and MUC1 expression.

Next, we focused on the mechanism for the upregulation of psoriasin in the MCF10A cells with reduced expression of ICAM-1. We still detected an expression of psoriasin after treatment with the antioxidant NAC. Measurement of ROS production, using NBT assay, showed no difference in intracellular ROS levels by ICAM-1 downregulation. This suggests that signals other than ROS production contribute to the regulation of psoriasin in this context. ICAM-1 is the only known ligand of the MUC1 extracellular domain [70] and binding of MUC1 to ICAM-1 was found to induce intracellular calcium signalling mediated by the phospholipase C (PLC)-IP3 pathway [71]. By blocking the phospholipase C (PLC)-IP3 pathway (using the inhibitors U73122 and 2-APB) in these cells, we showed that the induction of psoriasin diminished. Moreover, m-3M3FBS, a known PLC activator, was demonstrated to induce psoriasin in MCF10A cells. These finding supports the hypothesis that psoriasin is an intracellular calcium-dependent target of the PLC pathway.

The published evidence on the influence of ICAM-1 expression in human breast cancer is controversial. Previous immunohistochemical studies of breast cancer

revealed low expression levels [72]. However, the cytoplasmic levels of ICAM-1 were shown to be significantly higher in breast cancer specimens than in tissue from patients with benign breast diseases and this difference in expression were also found in the corresponding sera of patients [73, 74]. However, it is not clear whether the cytosol expression of ICAM-1 correlates to high expression in the epithelial breast cancer cells. We now report the downregulation of ICAM-1 in breast cancer epithelial cells as compared to normal breast epithelial cells using SAGE databases available from the CGAP website. Interestingly, when examining individual cell types in the tumour microenvironment [19], we observed that the expression of ICAM-1 is actually upregulated in the adjacent stroma cells (unpublished data). The reported upregulation of ICAM-1 in the cytosol of tumours and sera from breast cancer patients may therefore reflect the pronounced expression seen in the surrounding stroma cells in the microenvironment. An interesting hypothesis is that there is an interaction between ICAM-1, supposedly upregulated on the adjacent stroma cells with the elevated MUC1 expression on the epithelial cells in the initial stages of breast cancer development. This interaction could be implicated in the regulation of psoriasin through the PLC-IP3 pathway.

MUC1 is implicated in many physiological processes such as adhesion, development and differentiation. MUC1 is frequently overexpressed in many cancers including breast cancer with a predominantly cytoplasmic expression [75]. Increased levels of MUC1 has also been seen at pregnancy [76]. The intracellular tail of MUC1 can interact with many signalling proteins and transcriptional factors, which leads to many of its oncogenic effects. The overexpression of MUC1 is associated with a poorer prognosis and shorter survival in many cancers, including breast cancer. Consequently, MUC1 has been studied as a potential novel therapeutic target for immunotherapy and promising preclinical data have led to ongoing clinical trials [77]. The co-expression of psoriasin and MUC1 may contribute to the poor clinical outcome characteristic for tumours overexpressing psoriasin. Interestingly, both MUC1 and psoriasin correlate with increased survival in response to oxidative stress (paper I, [78]). Moreover, both proteins have been shown to be regulated by the NF-κB pathway (paper I, [79]). MUC1 affect cancer cell migration by increasing E-cadherin/beta-catenin complex formation [80] and the downregulation of psoriasin has also been linked to increased beta-catenin signalling [81]. We now report their co-expression in response to ICAM-1 downregulation and the positive correlation between their expressions in breast SAGE libraries.

In paper III, we have presented data suggesting that the loss of ICAM-1 expression on normal mammary epithelial cells may contribute to the high expression of psoriasin in high-grade DCIS. The upregulation of psoriasin by ICAM1 shRNA was mediated by the PLC-IP3 pathway. Furthermore, the downregulation of ICAM-1 led to the induction of calgranulin-A, calgranulin-B and MUC1.

Paper IV

As indicated in paper III, psoriasin may be regulated by changes in adhesion to the extracellular matrix (ECM). The interaction between epithelial cells and ECM is providing signals both in pathological processes and in normal development of a differentiated structure [16, 17]. In a previous study, our research group have observed that skin epithelial cells cultured in confluence and in suspension, induced

the expression of psoriasin, calgranulin-B, and the skin differentiation marker keratin I [52]. The expression of keratin 1 indicates that the skin epithelial cells are more differentiated. Psoriasin, calgranulin-A, and calgranulin-B have been demonstrated to be upregulated in normal breast epithelial cells (MCF10A) when cultured in confluent and suspension conditions, as compared to no or very low levels in exponentially growing culture. This has been demonstrated in this paper, paper I, paper II and in previous studies [20]. Both breast and skin epithelial cells have ectodermal origin. Therefore, the induction and functional relevance of these proteins may therefore be similar in these two epithelial cell types. The gene that encodes psoriasin and many other S100-genes are located within a gene cluster that also contains the genes for several differentiation markers that play important roles in the terminal differentiation of the human epidermis. This gene cluster, located on chromosome region 1q21, is known as the epidermal differentiation complex (EDC) [24, 25, 82]. The aim of this paper was to evaluate whether psoriasin, calgranulin-A, and calgranulin-B are involved in and are potential markers for the differentiation of mammary epithelial cells.

In contrast to skin epithelial cells, specific differentiation markers have not been clearly identified for mammary epithelial cells. In humans, CD24 has been suggested as a molecular marker that allows distinction between differentiated luminal epithelial and myoepithelial cells [83]. Recently, the existence of mammary epithelial stem cells was proposed and these are characterised by the presence of CD44 expression [84]. In this paper we demonstrated that confluent- and suspension- cultured MFC10A cells, which produced psoriasin, calgranulin-A and calgranulin-B, showed increased expression of CD24, whereas the level of CD44 was reduced, as compared to normal cultured MCF10A. These results imply that confluent- and suspension- cultured MFC10A cells, with psoriasin, calgranulin-A and calgranulin-B expression, shift from a $CD44^+/CD24^-$ to a $CD44^-/CD24^+$ phenotype and may be more differentiated than normal cultured MCF10A cells. Interestingly, we showed that the degree of shifting could be depending on the expression level of psoriasin.

When analysing the $CD24^+$ cell fraction of the confluence-cultured mammary epithelial cells, we noted that the level of psoriasin was higher than the levels of calgranulin-A and calgranulin-B in this cell fraction. The level of MUC1, known to be expressed on differentiated luminal epithelial cells, was also higher in the $CD24^+$ fraction that showed elevated expression of psoriasin. This supports the notion that psoriasin is expressed in more differentiated mammary epithelial cells, as compared to calgranulin-A and calgranulin-B.

To evaluate the significance of psoriasin in the appearance of the $CD44^-/CD24^+$ phenotype and MUC1 expression, endogenous expression of psoriasin in MCF10A cells, induced during suspension and confluent culturing, was repressed by the transfection of shRNA that blocks psoriasin expression. MCF10A cells with downregulated expression of psoriasin showed significantly reduced induction of CD24 and reduced repression of CD44. No significant difference in MUC1 expression was observed. These results suggest a pivotal role for endogenous psoriasin in the shift from the $CD44^+/CD24^-$ phenotype to the $CD44^-/CD24^+$ phenotype. In contrast, overexpressing exogenous psoriasin in mammary epithelial cells with a retrovirus or treatment with extracellular purified psoriasin protein did not induce a shift in phenotype. However, exogenous psoriasin still upregulated MUC1 expression. These

observations may be due to the fact that there is a difference between endogenous and exogenous expression of psoriasin. Our findings further reveal that endogenous psoriasin, but not the often co-expressed proteins calgranulin-A and calgranulin-B, is linked to the putative differentiation marker CD24 in mammary epithelial cells. Psoriasin have an essential role for the shift from a $CD44^+/CD24^-$ phenotype to a $CD44^-/CD24^+$ phenotype. Therefore, it is likely that psoriasin play a role in the differentiation of mammary epithelial cells.

The hypothesis that psoriasin have a role in the differentiation of normal mammary epithelial cells do need further laboratory analysis. Interestingly, based on our data, we speculate that psorasin may be involved in both differentiation of mammary epithelial cells and breast tumour progression, and that the expression may be induced by the same mechanism. In paper III, we showed that the high-level expression of psoriasin and the expression of calgranulin-A and calgranulin-B in high-grade DCIS tumours could be due to a change in adhesion to the ECM. In this paper, we observed that psoriasin is essential for the expression of the putative differentiation marker CD24 in confluence and suspension cultured normal mammary epithelial cells, conditions known to influence the interaction to ECM. We have also observed in paper III, an increase of psoriasin expression in apocrine metaplasia, which involves the differentiation and transition of a normal mammary epithelium to an apocrine sweat gland. The phenotype of an apocrine sweat gland also includes increased expression of CD24 [85]. These data support our hypothesis that psoriasin expression and CD24 expression are linked, although the mechanism of action and the specific role of psoriasin in the differentiation of mammary epithelial cells remain to be elucidated. We have conflicting result in paper I, showing that neither phorbol ester TPA nor inhibition of PI3K/AKT signalling (both known to stimulate skin epithelial differentiation [86]) induced psoriasin in the MCF10A breast epithelial cell line, as compared to normal skin epithelium. It can be speculated that there are differences in differentiation signal for the different epithelial cell lines.

The different culturing conditions studied in this paper *in vitro*, could mimic the *in vivo* situation likely to occur in high-grade DCIS. Based on our results, we hypothesise that there might be a shift to a $CD44^-/CD24^+$ phenotype, as well as psoriasin expression in the hyper proliferating epithelial cells in high-grade DCIS, due to the increased cell density and the lost contact to the ECM. Supporting the hypothesis that proliferating cells have higher levels of CD24 is demonstrated by Baumann P *et al.* They showed that CD24 expression increased the proliferation of breast cancer cells [87]. In the breast tumour, it is generally believed that there is a small subpopulation of cells, known as the breast cancer stem cells, with higher ability to form new tumours [84]. These are characterised with high CD44 and Bcl-2 expression [88]. We have previously shown that psoriasin is downregulated after treatment with Bcl-2 (paper I), and we hypothesise that the breast cancer stem cells, that express CD44, might not express psoriasin partly due to the expression of Bcl-2. Due to the elevated CD24 expression in combination with psoriasin expression, we hypothesis that psoriasin-expressing tumour cells may exhibit luminal differentiation features. There is some debate as to whether CD24 expression is a prognostic factor for poor outcome in several human cancers. Many studies support the idea that CD24 is a marker for tumour aggressiveness, and that the expression of CD24 promotes breast cancer development [89]. CD24, like psoriasin, has been associated with an unfavourable prognosis for patients with breast cancer [90-92], and the

expression of CD24 has been correlated with estrogen receptor alpha (ERα)-negative tumours [92, 93]. ERα has been shown to downregulate the expression of CD24 [94]. However, other studies have shown the opposite, i.e., that CD24 is expressed at higher levels in ER-positive breast cancer cell lines (MCF7, T47D) than in ER-negative cell lines (MDA-MB-231, HBL-100) [95]. Immunohistochemical studies have shown that the expression of CD24 is significantly higher in DCIS and in invasive breast cancer [96, 97], as compared with normal tissue, whereas Schindelman *et al.* found that CD24 was significantly downregulated in invasive breast cell lines, as compared with non-invasive breast cell lines [98]. These discrepancies might be explained by the fact that the studies were performed both *in vivo* and *in vitro* and this might have influenced the outcomes.

The conclusions for the different papers are as follow:

Paper I

From our data we hypothesise that the expression of psoriasin and calgranulin-B in high-grade comedo DCIS is dependent on ROS production. The induction of ROS could be the result from increase in apoptosis and cell density, but also because of lost contact to the basal membrane. Our results also suggest that the function of psoriasin may be to protect epithelial cells from ROS-induced cell death and that this effect is potentially mediated by the NF-κB pathway. Consequently, the production of psoriasin may contribute to apoptosis resistance in high-grade comedo DCIS.

Paper II

We report the suppression of psoriasin by IFNγ in normal mammary epithelial cells and in the ER-negative MDA-MB-468 breast carcinoma cell line. Our data implies that IFNγ mediates its suppressive effect on psoriasin transcription by activating the STAT1 signalling pathway. Psoriasin-expressing mammary epithelial cells showed increased viability after IFNγ exposure compared with normal cells.

Paper III

Our findings suggest that the downregulation of ICAM-1 in mammary epithelial cells may contribute both to the high expression of psoriasin seen in some high-grade DCIS tumours and to the induction of MUC1.

Paper IV

Our findings reveal that endogenous psoriasin, but not the often co-expressed proteins calgranulin-A and calgranulin-B, is linked to the putative differentiation marker CD24 in mammary epithelial cells. We also demonstrate that psoriasin have an essential role for the shift from a CD44$^+$/CD24$^-$ phenotype to a CD44$^-$/CD24$^+$ phenotype. Therefore, it is likely that psoriasin play a role in the differentiation of mammary epithelial cells.

Psoriasin is previously reported to be a potential biomarker for breast cancer with a poor prognosis. In this thesis, we evaluated the functional relevance of psoriasin and gain insight into regulatory pathways that control the expression in mammary epithelial cells.

We present new data that indicate that psoriasin can increase the cell survival, which could be an advantage for breast tumour formation. Due to increased survival more nutrition is needed for the growing tumour and previous findings from our group showed that psoriasin can promote angiogenesis. It is known that psoriasin can be secreted and ongoing studies suggest that psoriasin may induce the proliferation of endothelial cells. In the future it would be interesting to investigate whether inhibition of this protein could reduce angiogenesis and consequently the growth of breast tumours. However, a disadvantage for the breast cancer patient following the inhibition of psoriasin, is suggested. This finding suggests that loss of psoriasin expression is an advantage for breast tumour progression, due to an increased invasion, at least in the breast cancer cell line this relation was found. There are however some DCIS with persistent expression of psoriasin in the invasive tumours and they are characterised with more blood-vessels due to increased angiogenesis. Maybe an inhibition of psoriasin in combination with other treatment could reduce the growth and progression in a subset of breast tumours.

We also investigated the early molecular steps for the initiation of psoriasin expression. We present data that indicate that the expression of psoriasin in normal mammary epithelial cells can be due to changes in adhesion signalling and that the resulting cells have a more differentiated phenotype. A hypothesis is that psoriasin-expressing tumour cells may exhibit luminal differentiation features. It would be interesting to follow up this hypothesis to evaluate psoriasin as a putative marker for this subset of breast tumours. In paper IV our results is based on observations made in normal mammary epithelial cells, therefore it is important to investigate these findings in breast tumours as well. Today, we have ongoing immunohistochemistry, analysing the co-localisation of psoriasin and the differentiation marker CD24 in breast tumours sections *in vivo*. It would be interesting to analyse the expression patterns of psoriasin and CD24 in different breast cancer cell lines *in vitro*. We also plan to culture cells on different artificial extracellular matrix proteins, since we see that psoriasin is regulated by changes in adhesion. Fibronectin, an extracellular matrix protein, play a major role both in cell adhesion and differentiation and may therefore be investigated to detect changes in psoriasin expression.

We have shown data showing that psoriasin may play a role in the differentiation of normal mammary epithelial cells. To further elucidate the specific function of psoriasin for the differentiation of normal mammary epithelial cells and gain insight into regulatory pathways that control the shift to a $CD44^-/CD24^+$ phenotype, specific inhibitors may be used. The signalling pathway that control psoriasin expression may also control differentiation, including the shift to a $CD44^-/CD24^+$ phenotype. Possible pathways that we are about to investigate are the NF-κB pathway (using CAPE and

dnIKKβ) and the PLC-IP3 pathway (using the 2-APB and U73122 inhibitors). Until today, normal mammary epithelial cells have been observed to upregulate psoriasin only during breast tumour formation. In the future, it would also be of interest to study if the normal development of a terminal differentiated breast could involve an induction of psoriasin and that a persistent expression could result in tumour formation.

Today, the functional role for psoriasin in the initiation and progression of breast cancer is not fully understood. Although there are conflicting results, this thesis present data that support the hypothesis that psoriasin expression may have an advantage for breast tumour progression. Our data support the use of psoriasin as a biomarker for DCIS with a poor prognosis. Interestingly, we also show a role of psoriasin in the differentiation of normal mammary epithelial cells. More research is needed to fully understand the mechanism of action of psoriasin in mammary epithelial cells. We intend to further analyse and increase our knowledge about this protein and its effects, which we hope may ultimately lead to improved breast cancer treatment in the future.

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Ju mer man tänker, ju mer inser man att det inte finns något enkelt svar.
-   Nalle Phu

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