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## Wnt-5a signaling in breast cancer metastasis

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# Wnt-5a Signaling in Breast Cancer Metastasis

Annette Säfholm



FACULTY OF MEDICINE  
Lund University

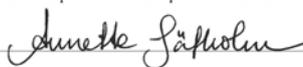
## Academic dissertation

By due permission of the Faculty of Medicine, Lund University, to be defended at the main lecture hall, Pathology Building, Entrance 78, Malmö University Hospital, 9.15 a.m., Friday November 23, 2007.

**Faculty opponent:** Professor Randall Moon, Howard Hughes Medical Institute, Department of Pharmacology, Institute for Stem Cell and Regenerative Medicine, University of Washington School of Medicine, Seattle, WA, USA

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Abstract <p>Breast cancer is the most common form of malignancy affecting women in the Western world. Today, anticancer treatment can control primary breast tumors, but treatment and prevention of metastatic disease still represents a major challenge in the management of breast cancer patients. It has been shown that Wnt-5a plays an important role in breast cancer, since loss of that protein is associated with a higher frequency of metastasis.</p> <p>The findings presented in this thesis show that Wnt-5a inhibits the metastatic behaviour of breast cancer cells by blocking Ca<sup>2+</sup>-induced NFAT activation. This inhibition is mediated through activation of Yes, Cdc42 and CK1<math>\alpha</math>. Furthermore, based on sequence analysis of the Wnt-5a protein, I identified two peptides with Wnt-5a mimicking ability. One of the peptides was shortened and modified to yield the formylated hexapeptide, Foxy-5, which was able to copy the effects of Wnt-5a on the intracellular signaling, adhesion and migration of breast epithelial cells.</p> <p>Experiments were performed in which the mammary fat pads of athymic (immunodeficient) BALB/c mice and normal BALB/c mice were inoculated with 4T1 breast cancer cells. Administration of Foxy-5 every fourth day did not lead to any difference in primary tumor growth, whereas it did radically reduce the level of metastasis to lung and liver compared to controls. The mechanism behind the inhibited metastasis formation seemed to be impaired migration and invasion in the breast cancer cells. In addition, the Wnt-5a coreceptor Ror2 was found to be involved in regulation of breast cancer cell migration and to associate with DDR1, a tyrosine kinase receptor that participates in Wnt-5a signaling. These results further emphasize the importance of Wnt-5a in breast cancer metastasis, and they identify a novel potential treatment strategy.</p>		
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## *Till min familj*

*Vi glömmet höst, och vi glömmet vår  
Vi glömmet allt och tror att tiden är svår  
Men man kommer på, sen efteråt  
att vi hade rätt kul emellanåt*

Lars Winnerbäck

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Cover: Migrating breast cancer cell stained with crystal violet

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## LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I** J Dejmek<sup>§</sup>, **A Säfholm**<sup>§</sup>, C Kamp Nielsen, T Andersson and K Leandersson. Wnt-5a/Ca<sup>2+</sup>-Induced NFAT Activity is Counteracted by Wnt-5a/Yes-Cdc42-Casein Kinase 1 $\alpha$  Signaling in Human Mammary Epithelial Cells. *Mol. Cell. Biol.*, 2006; 26, 16: 6024-6036  
<sup>§</sup>J.D. and A.S. contributed equally to this study
- II** **A Säfholm**, K Leandersson, J Dejmek, C Kamp Nielsen, B O Villoutreix and T Andersson. A Formylated Hexapeptide Ligand Mimics the Ability of Wnt-5a to Impair Migration of Human Breast Epithelial Cells. *J. Biol. Chem.*, 2006; 281: 2740-2749
- III** **A Säfholm**, J Tuomela, J Rosenkvist, J Dejmek, P Härkönen and T Andersson. A Wnt-5a-Derived Hexapeptide Radically Impairs 4T1 Breast Cancer Metastasis in Mice. *Submitted manuscript*
- IV** **A Säfholm** and T Andersson. The Receptor Tyrosine Kinase Ror2 Associates with Discoidin Domain Receptor 1 and Participates in the Regulation of Human Breast Cancer Cell Migration. *Manuscript*

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Publication not included in the thesis but referred to in the text:

J Dejmek, A Dejmek, **A Säfholm**, A Sjölander and T Andersson. Wnt-5a protein expression in primary Dukes B colon cancers identifies a subgroup of patients with good prognosis. *Cancer Res.*, 2005; 65, 20: 9142-9146

## **ABBREVIATIONS**

ALT	alternative lengthening of the telomeres
AP-1	activator protein 1
APC	adenomatous polyposis coli
Bcl-2	human B-cell lymphoma 2
BRCA	breast cancer susceptibility gene
$\beta$ -TrCP	$\beta$ -transducin-repeat-containing protein
CAM	cell adhesion molecule
CamKII	Ca <sup>2+</sup> /calmodulin-dependent protein kinase II
Caspase	cysteine aspartyl-specific protease
CDK	cyclin-dependent kinase
CEA	carcinoembryonic antigen
CK	casein kinase
COX-2	cyclooxygenase 2
CRD	cysteine-rich domain
CRIB	Cdc42/Rac interactive binding region
CSF	colony-stimulating factor
CtBP	C-terminal binding protein
DAG	diacylglycerol
DCC	deleted in colorectal carcinoma
DDR	discoidin domain receptor
Dkk	dickkopf
Dvl	dishevelled
ECM	extracellular matrix
EGF	epidermal growth factor
EpCAM	epithelial cell adhesion molecule
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FLIP	FLICE inhibitory protein
Frz	Frizzled receptor
GAP	GTPase-activating protein
GDI	GDP dissociation inhibitor
GEF	guanine nucleotide exchange factor
GSK-3 $\beta$	glycogen synthase kinase 3 $\beta$
GST	glutathione S-transferase
HER	human epidermal growth factor receptor
HIF1 $\alpha$	hypoxia-inducible factor 1 $\alpha$
ICAM-1	intercellular adhesion molecule-1
Ig	Immunoglobulin family

---

*Abbreviations*

IGF-I	insulin-like growth factor I
IL	interleukin
IP <sub>3</sub>	inositol trisphosphate
JNK	Jun N-terminal kinase
LEF	lymphocyte-enhancing factor
67LR	67-kDa laminin receptor
LRP	low density lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteases
NCAM	neural cell adhesion molecule
NES	nuclear export signal
NFAT	nuclear factor of activated T-cells
NLK	nemo-like kinase
NLS	nuclear localization sequence
NPI	Nottingham prognostic index
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PI3K	phosphatidyl inositol 3 kinase
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PR	progesterone receptor
pRB	retinoblastoma protein
PTI	Photon Technology International
ROCK	Rho-associated kinase
Ror	receptor tyrosine kinase-like orphan receptor
Ryk	receptor related to tyrosine kinase
sFRP	secreted Frizzled-related protein
SH	Src homology
sLe <sup>x</sup>	sialyl Lewis-x
SV40	simian virus 40 large T antigen
TAM	tumor-associated macrophages
TCF	T-cell factor
TGF-β	transforming growth factor β
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotide transferase dUTP nick-end labeling
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
Wg	wingless
WIF-1	Wnt inhibitory factor-1



## **INTRODUCTION**

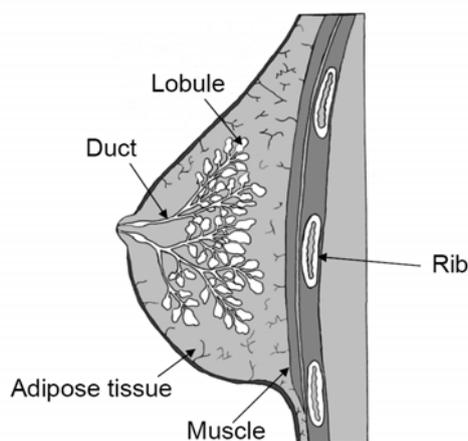
Cancer is not a novel phenomenon. Indeed, it has probably affected humans, animals, and plants since the beginning of time, and efforts to treat human cancer were already made in our ancient past. Tumorigenesis involves the transformation of normal cells into cancer cells, and it is a very slow process that seems to be due to the accumulation of gene mutations. It is believed that several mutations must occur to initiate tumorigenesis, which is why most cancers occur late in life. Cancer cells are characteristically self-sufficient with regard to self propagation of growth signals, evasion of apoptosis (programmed cell death), limitless replication, and the ability to stimulate angiogenesis (formation of new blood vessels). Many tumor cells also acquire the capacity to invade and metastasize (i.e., form new tumors at other sites in the body) and this constitutes the primary cause of cancer-related deaths.

Previous studies in our research group have shown that expression of a protein called Wnt-5a in breast cancer tissue correlated with reduced incidence of metastasis and a longer disease-free patient survival. The present thesis explored these findings in greater detail. In particular I examined the ability of Wnt-5a to inhibit breast cancer migration and characterized a synthetic peptide, Foxy-5, that could mimic the functions of Wnt-5a and impair breast cancer metastasis *in vivo*.

## **BACKGROUND**

### **DEVELOPMENT OF THE BREAST**

Until the onset of puberty, development of the human breast follows similar paths in males and females. During puberty in females, the amounts of luteinizing hormone and follicle-stimulating hormone increase, which causes the ovaries to produce estrogens. Under the influence of estrogens and progesterone, the female breasts begin to develop. The duct system matures, and there is deposition of fat. The onset of ovulation and formation of the corpus luteum lead to rising levels of estrogens and progesterone, which results in further development of the mammary glands and establishment of the overall breast anatomy, which is shown in Figure 1.



**Figure 1.** Schematic illustration of breast anatomy.

The female breasts undergo a proliferative phase during each menstrual cycle, but they do not become fully developed until a pregnancy occurs. Throughout pregnancy the breast tissue goes through profound proliferative and secretory alterations, and connective and adipose tissues are gradually replaced by large, densely packed lobules. During lactation, the alveoli become distended with milk. After pregnancy and lactation, the mammary glands involute to resemble their previous appearance. At menopause, the ovaries become less sensitive to the gonadotropic hormones secreted by the pituitary gland, which results in decreased secretion of estrogens and progesterone. The connective tissue is gradually replaced with adipose tissue, and the lobules diminish in size and eventually disappear, whereas the ducts remain essentially intact.

## BREAST ANATOMY

The female breast contains between 15 and 25 lobes embedded in adipose tissue. The lobes are radially distributed around the nipple and are interspersed with fibrous septa. The lobes are subdivided into smaller compartments known as lobules, which contain the alveolar ducts. During pregnancy, those ducts develop into a large number of grape-like milk-secreting glands called the alveoli. Myoepithelial cells surround the alveoli and oxytocin-stimulated contractions of those cells aids the transportation of milk towards the nipple. The milk passes from the alveoli into secondary tubules, and from there it enters the mammary ducts.

## BREAST CANCER

Breast cancer is the most common form of malignancy affecting women in the Western world and accounts for almost 30% of all female cancers. One in ten Swedish women will develop breast cancer during their lifetime, and about 7,000 new patients (of which around 40 are men) are diagnosed with this disease every year [1]. The incidence of breast cancer has been rising since the 1960s, although the number of deaths has remained constant at around 1,500 annually [2]. The five-year survival rate in Sweden is approximately 80% [3].

The incidence is very low in women under the age of 35 and the average age at detection is 65. The chances of cure are good if the breast cancer is detected at an early stage, and thus mammography is of great importance, because it can detect a malignancy before it is palpable. Accordingly, research has shown that mammography can reduce breast cancer mortality [4].

Risk factors for developing breast cancer include a family history of the disease, (especially if the mother or a sister has been affected) [5], age, precancerous breast changes [6], exposure to ionizing radiation such as x-rays early in life [7], high intake of alcohol [8] as well as obesity [9]. There is also evidence that the risk of breast cancer is increased by the following: prolonged exposure to ovarian hormones due to early onset of menses and late menopause; having no children or the first child born after 34 years of age; extended use of postmenopausal hormone therapy [7]. In addition, oral contraceptives have long been regarded as a risk factor, although a meta-analysis that included over 50,000 breast cancer patients and 100,000 controls, indicated that the increase in risk with such agents is very modest and is essentially eliminated five years after discontinuing the use of those drugs [10].

## ***Pathology and prognostic factors***

The majority of breast cancers are adenocarcinomas originating from the glandular epithelium of the terminal duct lobular unit, and they can be either invasive or noninvasive. The term noninvasive refers to the cancers that have not penetrated the basement membrane, and they are classified as ductal carcinoma *in situ*, lobular carcinoma *in situ*, or intraductal papillary carcinoma. Most cases of carcinoma *in situ* are of the ductal form. Depending on the cell type they resemble, the invasive carcinomas are designated ductal or lobular. Invasive ductal carcinoma is undoubtedly the most widespread form of breast cancer, constituting 75–85% of all cases [11]. Besides these common variants there are less frequent types of breast cancer, such as medullary, mucinous, tubular, apocrine, and metaplastic carcinomas [12].

To be able to arrive at a prognosis, breast cancers are categorized by use of various classification systems. The most widely applied method is TNM classification [13], which evaluates the size of the primary tumor (T; graded from 1 to 4), the presence of lymph node metastases (N; graded from 0 to 3), and the occurrence of distant metastases (M; graded as 0 or 1). Another classification system was developed by Bloom and Richardson and was later refined by Elston and Ellis [14]. This strategy is based on histological grade, and it involves microscopic examination of sections of the primary breast tumor to analyze the mitotic index, nuclear polymorphism and tubule formation. Evaluation of these three variables identifies the tumors as being well differentiated, moderately differentiated, or poorly differentiated, and the patients with well differentiated breast cancer have the best prognosis. In the Nottingham prognostic index (NPI), assessments of tumor size, lymph node status, and histological grade are combined to determine the odds that a patient will benefit from adjuvant therapy.

Besides evaluating the stage and grade of the breast tumor, the expression of the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) in the primary tumor is evaluated in order to decide on a treatment strategy [15].

## ***Hereditary breast cancer***

The majority of breast cancers arise sporadically, i.e., the patients lack a genetic predisposition for the disease, although approximately 10% of all cases are associated with heredity [5, 16]. Several gene mutations have been found to be responsible for familial breast cancers. The first of these to be detected, and also the most strongly related to hereditary breast malignancies (responsible for about 17% of all such cases [17]), were breast cancer susceptibility genes 1 and 2 (designated BRCA1 and BRCA2), which give rise to two proteins that are

involved in DNA damage repair. In recent years, additional genes underlying such disease have been found, and these include mutations in the CHEK2, p53, pTEN, and ATM genes, most of which are very rare. Taken together, all the gene mutations that are known to predict greater susceptibility to breast cancer account for around 20–25% of the risk of developing a hereditary form of this disease [18].

### ***Molecular subtypes***

Breast cancer is a very heterogeneous disease with respect to both morphology and histology. Introduction of the microarray technique has made it possible to investigate gene expression and copy number levels of 20,000 to 25,000 genes in only a small amount of tissue, which in turn has provided detailed information about the diversity of breast cancers. For example, microarray analyses have revealed that breast tumors cluster into different molecular profiles depending on their gene expression pattern, and, based on those findings, new tumor subtypes have been identified, that may be of clinical relevance, although not yet clinically used.

Tumors that are negative for ER have been crystallized into three subtypes: a normal breast-like group, a basal epithelial-like group, and a group overexpressing HER2. Substantial expression of basal epithelial genes was found in the normal breast-like group, whereas the gene expression pattern of the basal epithelial-like subtype was equivalent to that exhibited by the myoepithelial duct cells, which included extensive p53 mutation [19] and expression of HER1 and c-KIT [20]. Most basal epithelial-like tumors are triple-negative, which means that they lack expression of ER, PR, and HER2 [21]. This subtype accounts for 17–37% of all breast cancers [22], and it is especially common in premenopausal African and African-American women [23]. Moreover, carriers of the BRCA1 mutation often fall into the basal like-group [21]. The triple-negative breast cancer patients have a very unfavorable prognosis, because they lack targets for the most important and most effective treatment strategies (i.e., hormonal therapy and HER2 antibody treatment).

In one study [19], gene expression in ER-positive tumors was found to be similar to expression in luminal epithelial cells, and it could be further subdivided into luminal subtypes A and B, and possibly also C, with the highest expression of ER $\alpha$  in group A followed by B and C.

### ***Treatment***

If by the time of detection the cancer is limited to the breast tissue, the primary treatment alternative is surgical removal of the tumor. In addition a sentinel

## *Background*

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lymph node biopsy is performed to determine if the cancer has spread to the lymph ducts or lymph nodes in the axilla. In cases involving lymph node metastasis, the prognosis and choice of treatment are different. Rapidly proliferating cells, such as those seen in malignancies, are more sensitive to irradiation than nonproliferating cells are, and therefore surgery is usually combined with radiotherapy of the remaining breast tissue to eliminate possible residual cancer cells and thereby minimize the risk of recurrence. In addition, adjuvant therapy (i.e., treatment given to patients with no detectable remaining tumor) is often used to target possible micrometastases.

Around 70% of breast tumors express hormone receptors such as ER and PR [24], and thus, usually for five years after surgery, patients in that category are given anti-hormone therapy, with drugs like the selective ER modulator tamoxifen or aromatase inhibitors, since that approach has been shown to decrease recurrence [24, 25]. A relatively novel treatment strategy includes the use of monoclonal antibodies, and an example of this is trastuzumab, which blocks the activity of HER2 and has proven to be an effective form of treatment for the approximately 25–30% of breast tumors that overexpress the HER2 receptor [26, 27].

In contrast to radiation, chemotherapy represents a systemic treatment, and hence it is used for breast cancers that are more aggressive or have already spread to distant locations. The types of chemotherapy most commonly given to patients with such disease are alkylating agents, antimetabolites, anthracyclines, and mitotic inhibitors, all of which block cell division, although by different mechanisms. Alkylating agents (e.g., cisplatin) cause DNA damage and tumor cell apoptosis due to DNA crosslinking; antimetabolites (e.g., methotrexate) inhibit synthesis of DNA nucleotides; anthracyclins (e.g., doxorubicin) interfere with enzymes that are necessary for DNA replication; mitotic inhibitors (e.g., docetaxel) hinder the functions of the proteins responsible for segregating chromosomes in proliferating cells. A combination of two or three different chemotherapies is often used.

## ***Metastatic breast cancer***

Metastases are the main cause of death in breast cancer patients and they most often occur in the skeleton, the lungs, the liver, and the brain, as well as the adrenal glands and ovaries. Several techniques are employed to detect metastases, chiefly skeletal scintigraphy using radiolabeled phosphates or diphosphonates, x-rays, computed tomography, ultrasound, and magnetic resonance imaging [28].

In a study of breast cancer in the United States [29], it was found that in 6% of the cases the disease had already spread to distant sites at the time of

diagnosis, and the five-year survival rate was 26% for patients with distant metastases. Furthermore, in a study conducted in Canada in 1999–2001 [30], the median survival time after relapse was noted to be slightly less than two years. Metastatic breast cancer and primary breast cancer entail the same treatment alternatives: surgery, radiation therapy, hormonal treatment, monoclonal antibodies, and chemotherapy. There is no curative therapy available for metastatic breast cancer, and therefore the main aim of treating such patients is to increase survival time and improve life quality [31].

### ***Breast cancer stem cells***

The mammary epithelium contains multipotent stem cells, which have been isolated based on their expression of cell surface proteins. These stem cells are not differentiated, and thus they have the capacity for self-renewal and can perpetually produce differentiated cells. This is illustrated by a study in which it was found that single mammary epithelial cells gave rise to an entire mammary structure in the cleared fat pads of nude mice [32].

The idea that cancer stem cells might be able to initiate and sustain tumors, was proposed long ago, although that notion was subsequently abandoned because it could not be substantiated. However, interest in the theory has recently been revived, and supporting evidence is now being gathered. The primary argument in favour of such a role for cancer stem cells is that they are long-lived and divide slowly, and thus they are more likely to accumulate the multiple mutations that are thought to be necessary for transformation than is the case in somatic cells, which are constantly replaced [33].

In further corroboration of this theory, it has been found that only a small proportion of human breast tumor cells that were inoculated into the mammary fat pads of nude mice could induce tumor formation in the animals [34]. More precisely, the breast tumor cells were sorted by their cell surface markers, and it was observed that as few as 100 cells expressing the cancer stem cell markers were sufficient to generate tumors in the mice, whereas inoculation of the animals with up to 50,000 cells that did not express those markers failed to induce tumors. The tumors that arose from the cancer stem cells displayed cell type diversity similar to that of the original tumors, and the new tumors could be passaged several times.

The stem cell theory has also provided the following explanation for why full-term pregnancies result in a long-term decrease in the risk of developing breast cancer: the highly vulnerable multipotent breast stem cells in the terminal end buds differentiate during the pregnancy and thereby become less vulnerable to carcinogenic agents [35].

## TUMOR CHARACTERISTICS

Tumor formation requires several different changes in cell behavior, which explains why this malignant process is relatively rare considered from the perspective of a human lifetime. The changes in behavior can take place at different time points during tumorigenesis, and yet they are all required. The transformed cells exhibit self-sufficiency in growth-promoting signals, insensitivity to growth-inhibitory signals, limitless replication, and avoidance of apoptosis, as well as the ability to induce angiogenesis and also to invade and metastasize [36].

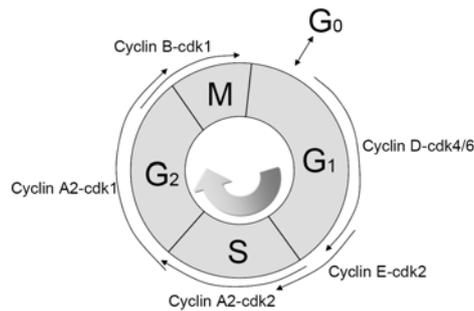
Furthermore, it seems that carcinoma formation depends on the ability of transformed epithelial cells to recruit stromal cells; in some tumors, up to 90% of the malignant tissue is composed of stromal cells, the majority of which are myofibroblasts [37]. Stromal cells have an important role in tumorigenesis, since they can mediate transformation of epithelial cells by altering angiogenesis, proliferation, and cell death [38, 39]. Tumor cells express a protein called transforming growth factor  $\beta$  (TGF- $\beta$ ), which promotes differentiation of the fibroblasts and facilitates their production of extracellular matrix (ECM) proteins such as collagen, fibronectin, and elastin [37]. TGF- $\beta$  is often overexpressed in breast cancer, even though a large proportion of such growths are themselves resistant to TGF- $\beta$  signaling [37].

It is believed that a number of mutations must occur to allow all of the mentioned changes in cell behavior that are required for tumorigenesis. At the mutational frequency seen in normal cells, it is highly unlikely that the number of mutations needed for tumor formation can occur in a single cell during its lifetime. However, tumor cells often have mutations in the protective systems that are intended to detect and repair alterations in DNA, and that situation leads to chromosome instability and accumulation of mutations [36]. Another more recent hypothesis claims that epigenetic alterations in aspects such as the methylation status of genes involved in tumorigenesis can change gene expression and direct the mutations to the hypermethylated genes [40].

### ***Proliferation***

As mentioned above, cell proliferation is essential for tumorigenesis, and it involves progression through the cell cycle (shown in Figure 2). Most cells are quiescent and maintained in the resting phase ( $G_0$ ), but when stimulated to growth, they enter the  $G_1$  phase and begin to prepare for division. Thereafter, the DNA is duplicated during what is called the synthesis phase (S), and preparation for division proceeds in the subsequent  $G_2$  phase. Finally the cell enters the mitotic phase (M), which includes the actual process of division. The most important checkpoints in the cell cycle occur in the late  $G_1$  phase and the

late G<sub>2</sub> phase, and they involve ascertaining the accuracy of the genome. Once the late G<sub>1</sub> checkpoint has been passed, the cell no longer depends on growth signals to fulfill the cell cycle. The cell cycle is regulated mainly by cyclins and cyclin-dependent kinases (CDKs), which play an important role in phosphorylating, and thereby inactivating, the growth-inhibitory retinoblastoma protein (pRB) [41].



**Figure 2.** Cell proliferation occurs by progressing through the cell cycle, which is tightly regulated by cyclins and CDKs. From the resting state (G<sub>0</sub>) the cells enter the G<sub>1</sub> phase, the DNA synthesis phase (S), the G<sub>2</sub> phase and eventually, the mitotic phase (M), where the actual division occurs.

In normal tissues, growth signals are usually emitted by neighboring cells or are generated via systemic signals. There is growing evidence that tumor-associated myofibroblasts function in a similar way to provide the epithelial tumor cells with growth factors [37]. However, many cancer cells have gained the ability to produce growth factors, activate mitogenic signaling cascades, and inactivate growth-inhibiting signals as a means of stimulating their own proliferation. Examples of this include the previously mentioned overexpression of the growth factor receptor HER2 in many breast tumors [27], as well as the frequent upregulation of the mitogenic Ras/Raf/mitogen-activated protein kinase (MAPK) pathway [42]. Another mitogenic protein is *c-myc*, which is upregulated in up to 60–100% of all breast cancers [43–46] and is known to induce proliferation by regulating many of the cell cycle proteins and suppressing transcription of growth-inhibiting genes [47].

Common growth-inhibitory signals that are frequently lost in tumors include pRB and TGF- $\beta$ , both of which prevent progression through the G<sub>1</sub> phase of the cell cycle [48, 49]. Most of the signals that block growth converge at pRB activation. Notably, transfection with the viral oncogene simian virus 40 large T antigen (SV40), which inactivates pRB, is a widely used method to establish cell-lines [50].

### ***Limitless replication***

Normal cells can progress through the cell cycle a limited number of times, (around 50), and thereafter they begin to senesce. Each time a normal cell goes through the cycle, the ends of the chromosomes are shortened by about 50–100bp, and, eventually, this truncation prevents further replication of the chromosomes. However, in approximately 90% of tumors this obstacle is overcome by upregulation of telomerases, which prolong the chromosomes; in the remaining part of the tumors, the telomeres are protected by an alternative lengthening of the telomeres (ALT), which induces recombination of those structures [51]. Expression of the human telomerase transcriptase gene is regulated by c-myc, which in that may contribute to transformation [52].

### ***Apoptosis***

The rate of tumor expansion is determined not only by the proliferative capacity, but also by the rate of tumor cell death. The main route of death for cancer cells is apoptosis, which is an intrinsic protection system that exists in virtually all cells and is aimed at eradicating unneeded or potentially dangerous cells such as those that are damaged, mutated, or infected with viruses. Interestingly, many growth-inducing proteins, including c-myc and Ras, can induce both proliferation and apoptosis [50]. Nevertheless, most tumors have acquired one or several ways of escaping apoptosis [53].

Apoptosis involves programmed cell death that is characterized by condensation and fragmentation of DNA, followed by cell shrinkage, membrane blebbing, and finally phagocytosis of the remaining apoptotic bodies, and occur without inducing inflammation. This is in contrast to necrotic cell death, which entails increased permeability of the plasma membrane and subsequent cell swelling [54]. Apoptosis can proceed via a pathway that depends on cleavage-provoked activation of several different cysteine aspartyl-specific proteases (called caspases); these include initiator caspases (designated 2, 8, 9, and 10), executor caspases (designated 3, 6, and 7), and other caspases [55]. However, apoptosis can also occur in a caspase-independent manner, and in that case involves activation of proteases like cathepsins and calpains [56].

Apoptosis can be induced by several stimuli, for example chemotherapeutic drugs, hypoxia, and activation of the death receptors Fas and tumor necrosis factor (TNF) [57, 58]. In response to such cellular stress, the tumor suppressor p53 is activated and accumulated in the nucleus, and it can trigger irreversible growth arrest and apoptosis [58]. It seems that the main mechanism by which p53 inhibits tumorigenesis is to trigger apoptosis, and it has been found that p53 mutations occur in 20–35% of breast tumors [58, 59]. Another way for tumor cells to evade apoptosis is to modulate the expression of proteins

involved in that terminal process, such as caspases, FLICE inhibitory protein (FLIP), and the human B-cell lymphoma 2 (Bcl-2) family [60], or, alternatively, to upregulate survival signals like the phosphatidylinositol 3 kinase (PI3K) pathway [50].

## **Angiogenesis**

Angiogenesis involves the formation of new blood vessels from the existing vascular system, which is necessary for solid tumors to be able to grow larger than approximately 0.5 mm<sup>3</sup> [61]. The new vessels supply the growing tumor with oxygen and nutrients, and also remove metabolic waste products. In addition, the vascular endothelial cells produce growth factors, such as fibroblast growth factor (FGF), granulocyte colony-stimulating factor (CSF), and platelet-derived growth factor (PDGF), which further stimulate tumor proliferation [62].

Induction of angiogenesis, or “the angiogenic switch”, is initiated by several factors, including low levels of oxygen and glucose [63, 64], and it also requires a change in the balance between the inducers and inhibitors of angiogenesis. It has been claimed that vascular endothelial growth factor (VEGF) and FGF-2 are the most important angiogenesis inducers, and elevated levels of those two molecules can be detected in serum from tumor patients with tumors and are correlated with prognosis [65]. However, there are also naturally occurring substances that inhibit angiogenesis, for example angiostatin [66], interferon  $\alpha$  [67], and thrombospondin [68].

A low vessel density has been shown to indicate a favorable prognosis in invasive breast cancer [69] and it has also been observed that the amount of angiogenesis is related to metastasis of that disease [70]. Administration of angiogenesis inhibitors, mainly those targeting VEGF and the VEGF receptors and their tyrosine kinase activity, has provided promising results when combined with conventional cancer treatments [71]. Notwithstanding, it is also possible that an opposite treatment strategy could be effective, as indicated by a recent study in which measures taken to promote angiogenesis led to inhibition of tumor growth due to the inefficiency of the vessels that were formed [72].

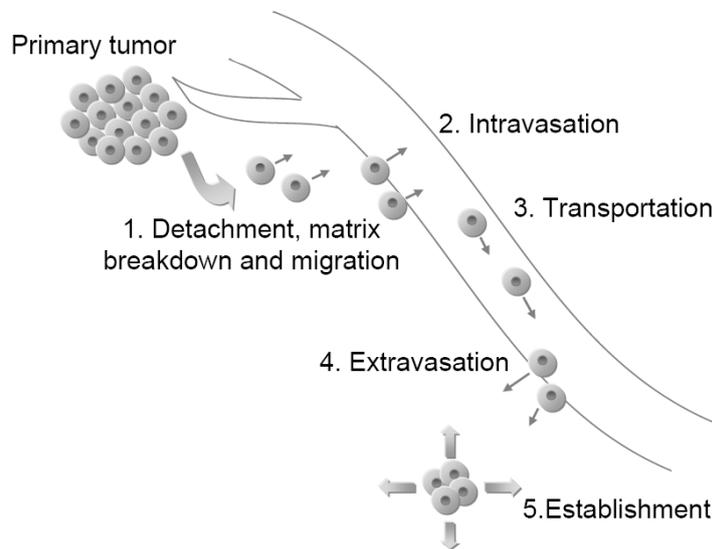
## **Tumor metabolic microenvironment**

Most solid tumors have a lower extracellular pH than occurs in normal tissues [73-75], and, this acidity increases the propensity for invasiveness [76, 77], and contributes to the metastatic process, for example by inducing release of cathepsins [78] and upregulating matrix metalloproteases (MMP) in the tumor

cells [79]. The acidic environment arises for several reasons, one of which is reduced clearance of metabolic acids provoked by poor vascular perfusion and an elevated rate of glycolysis [80]. Inefficient blood vessels and poor lymphatic drainage cause hypoxia and upregulation of hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ), which leads to increased glycolysis [81]. However, experiments using glycolysis-deficient cell lines have shown that the acidity of tumors is not solely the result of excessive production of lactate [82]. An additional factor in that context is activation of the Na<sup>+</sup>/H<sup>+</sup> exchange system [83, 84], and in particular the vacuolar type of H<sup>+</sup>-ATPase exporters, which has been shown to be overexpressed in drug-resistant tumor cells [85]. Stimulation of the activated Na<sup>+</sup>/H<sup>+</sup> exchange system causes a slight rise in intracellular pH in many solid tumors [75, 83, 84], which is beneficial to the tumor cells because it promotes growth [83], tumorigenicity [86], and invasiveness [87, 88].

## METASTASIS

Figure 3 presents a schematic diagram of the metastasis of solid tumors. The process is initiated by loss of adhesion, breakdown of the ECM and basement membrane. Thereafter, the tumor cells migrate into the blood or lymphatic vessels (i.e., undergo intravasation) and are transported with the fluid in question. They eventually adhere to the vessel wall, and, at the site of



**Figure 3.** The metastasis of solid tumors is initiated by loss of adhesion, matrix breakdown, and migration. This is followed by intravasation, transportation within the vascular system, extravasation, and establishment at a new site.

anchoring, they undergo extravasation (migrate out of the blood or lymphatics). Finally, the cells can establish a tumor in a new environment, if they are able to survive, proliferate, and stimulate angiogenesis in that distant location. The inability to perform any of the steps in the metastatic process leads either to formation of micrometastases or to metastatic failure [89].

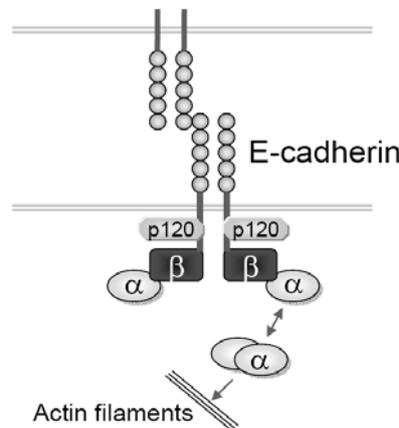
Even small tumors can send large number of cells into the circulation [90], although studies have indicated that less than 0.01% of the circulating tumor cells actually led to formation of distant tumors [91]. The organ distribution of metastases is not random, and that issue has been a matter of debate ever since Paget launched his theory claiming “the dependence of the seed upon the soil” [92]. Studies have shown that the organ-specific distribution of the tumor cells both occur through specific adhesion of the malignant cells to the vessel wall and as a consequence of mechanical trapping in the microvasculature [93]. Thus, the destination of the tumor cells is determined by factors such as the expression of adhesion receptors by the tumor cells and the vascular endothelial cells, but also by the microenvironment (e.g., expression of growth factors and chemokines) in the target organs [94, 95].

## **Adhesion**

Carcinomas, including breast cancers, arise from epithelial cells, which are normally tightly connected to each other by several structures, such as tight junctions, adherens junctions and desmosomes. For these cells to be able to exhibit invasive behavior, their cell-cell adhesion receptors have to be downregulated, and they must detach from the ECM. In addition, their capacity for adhesion must be altered to allow both migration and adherence to vascular endothelial cells during intravasation and extravasation [96]. In line with this, it seems that the repertoire of cell adhesion receptors in invading tumor cells undergoes changes that facilitate a more invasive phenotype [97]. More specifically, these modifications affect cell-cell adhesion receptors (e.g., the cadherins and the selectins), and cell-matrix adhesion receptors (e.g., the integrins).

### ***E-cadherin***

E-cadherin is an adhesion receptor that mediates  $\text{Ca}^{2+}$ -dependent interactions with cadherins on adjacent cells.  $\beta$ -catenin or  $\gamma$ -catenin bind to the distal part of the cytoplasmic domain of E-cadherin with high affinity [98], and  $\beta$ -catenin also interacts with  $\alpha$ -catenin with low affinity [99], as shown in Figure 4. Studies have shown that  $\alpha$ -catenin switches between binding  $\beta$ -catenin and interacting with the actin cytoskeleton [100], and hence the complex is dynamic. Furthermore, the p120 protein binds to the juxtamembrane part of



**Figure 4.** E-cadherin forms homotypic cell-cell adhesion complexes and p120 and  $\beta$ -catenin bind to its cytoplasmic tail, whereas  $\alpha$ -catenin switches between binding  $\beta$ -catenin and binding the actin cytoskeleton.

the cytoplasmic tail of E-cadherin without the involvement of the catenins and regulates the stability of the cadherins [101].

Downregulation or loss of E-cadherin often occurs in invasive and metastatic carcinomas, and it is correlated with poor survival [102, 103]. In a majority of carcinomas, the absence of E-cadherin expression is not caused by mutations, but is instead due to dysregulation of transcription factors, hypermethylation of the promoter, or cleavage of the extracellular domain by proteases [104-107]. E-cadherin is considered to be a strong invasion inhibitor [108], and that assumption is supported by the finding that re-expression of E-cadherin in cells that had lost that protein, prevented the invasive behavior of those cells [109, 110]. However, it is not clear whether the impeded invasiveness was due to the adhesion effect of E-cadherin or to participation of that protein in cell signaling, although it is likely that both effects are important [109, 111].

Through its interaction with  $\beta$ -catenin, E-cadherin has been implicated in the Wnt signaling pathway. Studies have shown that the same region of  $\beta$ -catenin is responsible for interactions with E-cadherin, adenomatous polyposis coli (APC), and T-cell factor (TCF)/lymphocyte-enhancing factor (LEF), which indicates that association with the different binding partners is competitive [112, 113]. Accordingly, upon tyrosine phosphorylation of  $\beta$ -catenin, for example by Src, the association of  $\beta$ -catenin with the cadherin-catenin complex is lost [114, 115], and  $\beta$ -catenin instead forms a complex with BCL-9, whereby the function of  $\beta$ -catenin is switched from adhesion to signaling [114]. However, recent findings show that a conformational change of  $\beta$ -catenin, induced by tyrosine phosphorylation of N-terminal residues, generates a monomeric form of  $\beta$ -catenin that selectively functions in Wnt signaling [116]. Results presented

in the same study show that cadherin-binding was mainly accomplished by a  $\beta$ -catenin- $\alpha$ -catenin dimer, suggesting that different forms of  $\beta$ -catenin are targeted to signaling and E-cadherin-binding respectively. It has also been shown that cadherins can activate PI3K and Rho GTPases, and the Rho GTPases can in turn influence the cadherin-actin complex [117-119].

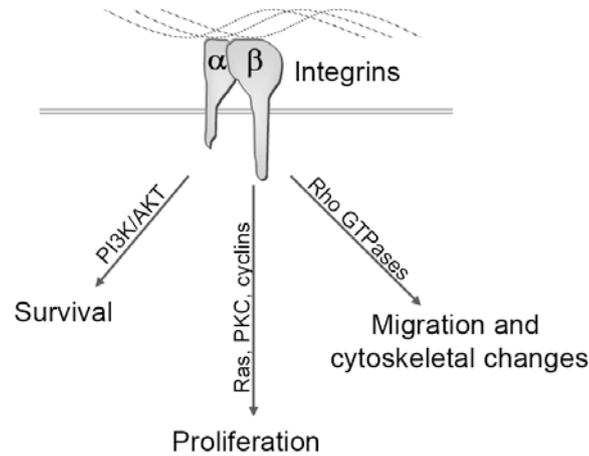
### *H- and N-cadherin*

Besides E-cadherin, two other members of the cadherin family of cell-cell adhesion molecules, the H-cadherin and the N-cadherin, have been implicated in the progression of breast cancer. H-cadherin is expressed by ductal epithelial cells, and was shown to be lost early during the development of breast cancer [120]. Furthermore, it appears that expression of N-cadherin is often upregulated in breast carcinomas, which leads to increased invasiveness through interaction of this protein with the FGF receptor, and subsequent induction of MMP-9 activity [121, 122]. Studies have indicated that N-cadherin can display its invasion-inducing behavior even in the presence of E-cadherin [122, 123]. It is likely that expression of N-cadherin improves interactions with stromal and epithelial cells and thereby aids the establishment of a tumor and the metastatic process [124]. In support of that possibility, N-cadherin has also been shown to facilitate transmigration of melanoma cell through vessel walls [125].

### *The integrins*

Integrins are by far the most well-known and well-studied of the cell-matrix adhesion receptors, and the integrins consist of heterodimers of  $\alpha$  and  $\beta$  subunits, which are known to be combined in at least 24 different ways in mammals [126]. Ligands for the integrins include ECM proteins such as fibronectin, vitronectin, collagens, laminins, and fibrinogen, as well as some members of the Ig superfamily, such as vascular cell adhesion molecule (VCAM) [126]. The integrin-combinations differ with regard to substrate specificity, and some combinations bind several of the ECM proteins, whereas others bind only one.

In addition to cell adhesion, the integrins are involved in several intracellular signaling events, as well as formation of focal adhesions, i.e., multi-protein complexes that connects integrins with the cytoskeleton. As shown in Figure 5, integrin signaling plays several roles that are related to tumor progression. For example, they have been reported to inhibit caspase activation [127] and induce anti-apoptotic signaling [128], and thereby reduce apoptosis. It has also been observed that, upon binding to its ligands, integrins can stimulate cell migration by activating Rho GTPases [129] and also promote proliferation by stimulating cyclin expression [130]. The effects of integrins that support



**Figure 5.** Integrin signaling promotes tumor progression, by affecting cellular processes such as proliferation, survival, and migration.

migration, proliferation, and survival can also be exerted on endothelial cells, which means that they are pro-angiogenic in nature [127, 131].

Association of carcinoma cell integrins with the ECM proteins has an important role in organ-specific metastasis, and for example the  $\alpha_v\beta_3$  integrins have been implicated in the specific spreading of breast tumor cells to the lung and bone [132]. In normal breast tissue, the most common variants are the  $\alpha_2\beta_1$ ,  $\alpha_3\beta_1$ , and the  $\alpha_6\beta_4$  integrins [97]. The expression of the integrins that are normally present in the epithelial cells is generally decreased in carcinoma cells [133], and loss of these proteins can be correlated with tumorigenesis and reduced differentiation of the tumor [134].

On the other hand, tumor-specific integrins are often upregulated in breast cancer cells. Overexpression of the  $\alpha_v\beta_3$  integrin is the most pronounced in connection with invasive behavior and metastasis [135], although, upregulation of the  $\alpha_6\beta_4$  integrin has also been linked to increased invasion and metastatic progression of breast cancer [136]. Notably, studies have demonstrated that inhibitors of  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  integrins can block tumor growth, angiogenesis and metastasis [137-139].

### *The 67-kDa laminin receptor*

The 67-kDa laminin receptor (67LR) is a glycoprotein whose main function is to stabilize laminin-integrin binding. It has been found in normal breast epithelium as well as breast cancer samples [140], and it is associated with increased invasion and poor prognosis [141-143]. In addition, it has been shown that expression of 67LR covaries with the expression of  $\alpha_6\beta_4$  integrin, and also

that those two proteins associate physically with each other, findings that suggest mutual involvement in laminin binding [144]. Furthermore, 67LR has been observed to induce expression of proteases [145], and it has also been proposed to contribute to increasing proliferation [146], which might explain why this molecule is associated with invasiveness.

### *The discoidin domain receptors*

The discoidin domain receptors 1 and 2 (DDR1 and DDR2) are transmembrane tyrosine kinase receptors that are activated by binding to various types of collagen [147], and in that acts as cell-matrix adhesion receptors. Expression of DDR1 and DDR2 has been detected in many kinds of tumors, including those of the breast. In studies of breast tumors, it was observed that the expression of DDR1 was upregulated in cancer cells as compared to the normal surrounding cells [148, 149], although there was no evidence of a correlation between the protein levels of DDR1 and patient survival [150]. By comparison, Ford *et al.* [151] detected upregulation of DDR1 expression and also noted that that was correlated with a better overall prognosis as well as improved overall survival in non-small cell lung carcinoma, whereas they found that expression of DDR2 was downregulated and had no impact on prognosis. Interestingly, it seems that DDR1 is expressed primarily by the epithelial cells in a tumor, whereas DDR2 is expressed mainly by the stromal cells [152].

Considering DDR1, there is definite evidence that it is involved in enhancing adhesion to collagen [153, 154], and it has also been shown that this protein is upregulated by p53, and it induces survival via activation of a MAPK pathway [155, 156]. Even though findings are inconsistent with regard to the involvement of DDRs in proliferation and migration, there are results indicating that DDR1 causes reduced proliferation in breast epithelial cells [157], kidney cells [153], and macrophages [158], whereas it induces increased proliferation in smooth muscle cells [159]. Similarly, DDR1 has been shown to be connected with diminished migration in breast epithelial [157] and kidney cells [155]. Moreover, expression of DDR1b has been reported to decrease migration towards collagen in leukocytes, whereas expression of DDR1 and DDR1a appears to mediate increased migration in smooth muscle cells and leukocytes (the DDR1 isoforms are described in Figure 13) [154, 159, 160]. On the other hand, it has been shown that expression of DDR2 augments proliferation in fibroblasts and hepatic stellate cells [161-163], but causes decreased migration in adipocytes [164]. Several studies have indicated that the DDRs regulate expression of MMP, which may also affect the invasion process [147, 160-162]. In conclusion, although the research findings concerning the effects of the DDRs on tumor progression are somewhat contradictory, they do clearly underline the involvement of these receptors in tumor progression.

### *The selectins*

To undergo intravasation or extravasation, tumor cells must adhere to the vascular endothelial cells, unless it is a question of extravasation in an organ that has an incomplete endothelial cell lining, for example the liver [165]. Studies have shown that anchoring to the vascular endothelial cells is brought about mainly by selectins and occurs in a manner similar to leukocyte extravasation [166]. Selectins constitute a small group (E-, L-, and P-selectin) of  $\text{Ca}^{2+}$ -dependent cell adhesion receptors that bind primarily to the ligand sialyl Lewis-x (sLe-x). The expression of selectins show distinct cell specificity; lymphocytes have constitutive expression of L-selectins, whereas inflammatory stimuli induce expression of P-selectin on platelets, and E-selectin and P-selectin on endothelial cells [94]. The selectin-mediated adhesion is very weak, albeit sufficient to retard the flow of the tumor cells within vessels enough to allow those cells to establish firmer interactions with other adhesion receptors (e.g., integrins and CAMs) and to penetrate the vessel wall.

Noteworthy in this context is that the presence of the sLe-x ligand in carcinomas has been correlated with progressive disease, in particular liver metastasis [167]. Moreover, in different investigations it was found that the interaction of tumor cells with E-selectins on endothelial cells was necessary for colorectal carcinoma cells to form liver metastases [168], for melanoma cells to form lung metastases [169], and for breast cancer cells to adhere to a TNF-stimulated endothelial cell layer [170]. The importance of expression of selectins and their ligands in formation of distant metastases has also been shown in several other cancers [171, 172]. Interestingly, studies have indicated that cytokines released by the tumor cells may induce expression of selectins on vascular endothelial cells [173].

### *The immunoglobulin-like adhesion receptors*

The majority of cell adhesion molecules (CAMs) belong to the immunoglobulin (Ig) superfamily and form chiefly homophilic or heterophilic cell-cell associations with other members of this group. *In vitro* experiments have shown that the glycoprotein MUC-1, that is expressed by breast cancer cells, can bind to intercellular adhesion molecule-1 (ICAM-1) expressed by the endothelial vascular cells, and thereby enhance transendothelial migration of the breast tumor cells [174]. Interestingly, aberrant expression of MUC-1 [175] and ICAM-1 [176] were correlated with a worse prognosis in breast cancer patients and metastatic potential of breast cancer cell lines, respectively. Furthermore, the epithelial cell adhesion molecule (EpCAM) was overexpressed in primary and metastatic breast cancers [177] and results presented in the same study show that downregulation of EpCAM dramatically decreased invasion and migration of breast cancer cells *in vitro*. The CAM family member L1, that is otherwise restricted to neural tissues, was expressed

in several cancer cell lines, including breast, and disrupted E-cadherin mediated cell-cell adhesion and promoted tumor cell migration [178]. These data indicate that several CAMs participate in the progression of breast tumors.

In addition, research has indicated that invasiveness of other types of tumors is related to changes in expression of neural CAM (NCAM) [179], the Ig-member deleted in colorectal carcinoma (DCC), and the CAM-members carcinoembryonic antigen (CEA), and MUC18 [180].

### **Matrix degradation**

It is assumed that degradation of both the basement membrane and ECM proteins is a prerequisite for tumor cell invasion and metastasis. Nevertheless, researchers have also described the occurrence of amoeboid-like tumor cell movement, that does not depend on protease activity [181]. Furthermore, many epithelial tumor cells do not produce ECM proteases themselves, but instead receive a supply of those proteins from stromal cells such as fibroblasts and immune cells [182, 183]. Expression of the ECM proteases is localized to tumor cells in the invasive front [183], and it is regulated by, for example, the growth factors epidermal growth factor (EGF) and PDGF and the cytokines TNF- $\alpha$  and interleukin-1 (IL-1) [184]. Increased expression of all types of ECM proteases has been associated with tumor cell invasion [185]. The MMPs are ECM proteases that play a key role because they are essential for angiogenesis [186], tumor growth, and macrophage infiltration into tumors [187], and they also cooperate with integrins to induce migration [188].

### **Migration**

Once the tumor cells have lost adhesion and have broken through the basement membrane, they have to migrate towards vessels in order to metastasize. In lymphatic vessels, the basement membrane is rudimentary or absent [189] and accordingly, it is easier for the tumor cells to penetrate these vessels, which is why formation of malignancies in lymph nodes close to the primary tumor is an early event in metastatic disease. Tumor cell migration requires changes in the cytoskeleton that involve actin polymerization and lead to cell polarization and formation of membrane protrusions that bind to ECM proteins [190]. The strength of the adhesion determines the velocity of the migration: if it is either too strong or too weak, it will slow the movement of the cells [191, 192]. Focal adhesions form and attach to the actin fibers, leading to activation of signaling molecules such as focal adhesion kinase (FAK), Src, PI3K, and the Rho GTPases [190]. In turn, these signaling pathways induce interaction of myosin II with the actin filament, which generates the

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mechanical force needed for both forward movement and detachment of the rear end of the cell [191].

### *The cytoskeleton*

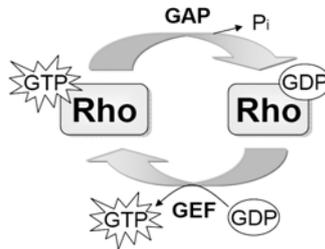
The cytoskeleton consists of three types of filaments – microtubules, intermediate filaments, and actin filaments – the functions of which overlap. The microtubules are considered to be essential for transportation of proteins and organelles throughout the cell, the intermediate filaments are important for maintaining the overall shape of the cell, and the actin filaments control cell movement [193].

Actin polymerization is necessary for creation of protrusions in the leading edge, which cause the front of the cell to move forward and for formation of the contractile stress fibers, which are required for contraction of the trailing edge. Half of the actin in nonmuscle cells exists as short filaments that enable rapid elongation [194]. The subunits of an actin filament point in the same direction, and hence the filament is polar. The actin filament is lengthened by addition of monomers (most bound to profilin) at the plus end, and this continues until capping proteins such as gelsolin bind at that end and block further elongation. Furthermore, binding of the Arp2/3 complex along the actin filament results in formation of branches and lowers the concentration of actin monomer needed for polymerization. Actin monomers dissociate mainly from the minus end of the filament, and this process is accelerated by cofilin [195]. Actin-binding proteins like gelsolin also induce disassembly of actin monomers in response to a  $\text{Ca}^{2+}$  signal or Src activation [194].

### *Rho GTPases*

The Rho GTPases are key signaling molecules responsible for cytoskeletal changes induced by membrane receptors. This group includes Rho (isoforms A, B, and C), Rac (isoforms 1, 2, and 3) and Cdc42, and the main function is to regulate cell motility. Activation of Rac and Cdc42 in the front of a migrating cell is necessary for actin polymerization and formation of lamellipodia and filopodia, whereas activation of Rho in the rear end of the cell is required for contraction of the trailing edge [196].

The Rho GTPases cycle between an inactive GDP-bound form and an active GTP-bound form, as shown in Figure 6. The activation status is controlled by three types of regulators: the guanine nucleotide exchange factors (GEFs) that facilitate activation (i.e., the GDP-to-GTP exchange), the GTPase-activating proteins (GAPs) that promote inactivation (i.e., enable hydrolyzation of GTP to GDP), and the GDP dissociation inhibitors (GDIs) that bind the inactive conformation of Rho GTPases and inhibit the GDP-to-GTP exchange [196].



**Figure 6.** The activation status of Rho GTPases is controlled by exchange of GTP and GDP, which is in turn regulated by GAPs and GEFs.

### ***Tumor-associated macrophages***

Solid tumors often contain infiltrates of inflammatory cells, and in the past, these infiltrating cells were regarded as proof of a host-mediated immune response towards such lesions. However, over the last few decades this view has been revised, because there is increasing evidence that inflammation can in fact promote tumorigenesis [197, 198] and that chronic inflammation can cause cancer [197].

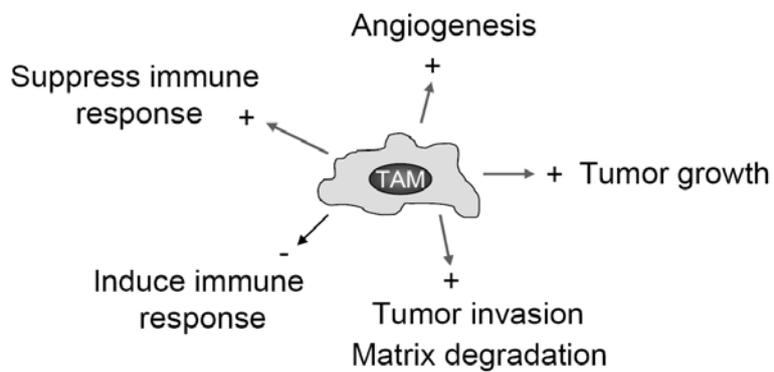
Tumor-associated macrophages (TAMs) constitute a significant proportion of the infiltrated inflammatory cells in a solid tumor [199]. TAMs are recruited to the tumor environment by molecules such as TGF- $\beta$  and CSF, which are secreted by the tumor cells [200, 201]. Both high density of TAMs [202] and substantial expression of the macrophage growth factor and chemoattractant CSF-1 [203] in tumors, have been correlated with a poor outcome of the patient. Furthermore, elegant studies performed by Lin *et al.* [204] showed that eradication of CSF-1 production markedly delayed the onset of metastasis, whereas induction of CSF-1 accelerated tumor progression.

TAMs have a dual function in tumor progression. First of all, they can produce angiogenesis inducers and growth factors (e.g., VEGF and EGF), cytokines (e.g., TNF- $\alpha$ ), proteases, and MMPs and thereby facilitate angiogenesis, growth and invasion of the tumors, and matrix breakdown [202], as shown in Figure 7. Secondly, TAMs can also aid tumor cell migration, invasion, and intravasation by secreting EGF, which acts as a chemotactic factor for the tumor cells [201]. However, upon activation by interferon or IL-12, TAMs may eradicate the tumor cells or present the tumor-associated antigens to T-cells and thereby induce an immune response [200]. Nonetheless, it is believed that TAMs are primarily protumorigenic, since both tumor cells and TAMs have the ability to suppress the immune response by secreting factors such as IL-4, IL-6, and IL-10, and it is also known that TAMs are poor antigen-presenting cells [200]. In addition, infiltrations of other myeloid cells, including neutrophils, dendritic

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cells, and mast cells have been found in various tumors, although their involvement in tumor progression is still controversial [205].

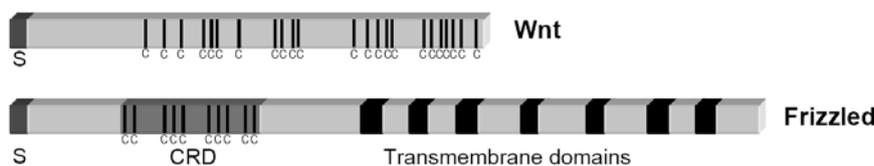


**Figure 7.** Tumor-associated macrophages secrete an abundance of factors that affect tumor progression both positively and negatively, although it seems that the protumorigenic functions predominate.

## ESSENTIALS OF WNT SIGNALING

### *Wnt proteins*

Wnt-5a belongs to the Wnt family of proteins, which includes at least 19 secreted cysteine-rich glycoproteins found in vertebrates (structure illustrated in Figure 8). The name of this family originates from a combination of the designations of the two highly homologous genes *int-1* and *wg* (wingless), which were independently discovered in mice and *Drosophila*, respectively. It has been shown that the Wnt proteins are glycosylated on several residues by the *porcupine* gene product, a modification that is important for secretion [206-208]. In addition, a palmitate residue that is essential for the function of Wnts (at least Wnt-3a and Wnt-5a) is added post-translationally [208, 209].



**Figure 8.** Structure of the Wnt and Frizzled proteins. Designations: **S**, the signal sequence; **C**, a conserved cysteine; **CRD**, the cysteine-rich domain of the Frizzled protein. Modified from [210].

To function properly, Wnt family members also require sulfated proteoglycans, which bind the proteins tightly to the cell surface and probably also aid receptor ligation [211, 212]. Studies have demonstrated that the heparan sulfate proteoglycan glypican-3 binds to Wnt-5a and thereby enhances the signaling of that protein [213], and that syndecan-1 is essential for the activity of Wnt-1 [214]. Wnt proteins are important for cellular functions such as differentiation, proliferation, apoptosis, migration and stem cell renewal [215-219]. Through these and other actions, Wnt proteins are involved in diseases such as cancer, Alzheimer's and pulmonary fibrosis [220].

### *Wnt receptors and coreceptors*

#### *Frizzled and LRP*

Wnt proteins bind to seven-transmembrane G-protein-coupled receptors of the Frizzled (Frz) family [221-224], of which 10 human members are currently known [225]. It is believed that this interaction is accomplished by the highly glycosylated cysteine-rich domain (CRD) of the Frz receptors (shown in Figure 8) [223]. A recent study indicated that expression of Frz on the cell membrane can be regulated by Shisa, a protein that binds to the Frzs and retains them in the endoplasmic reticulum [226]. Both Wnt-3a and Wnt-5a have also been shown to induce endocytosis of Frz receptors [208, 227, 228], and it seems that

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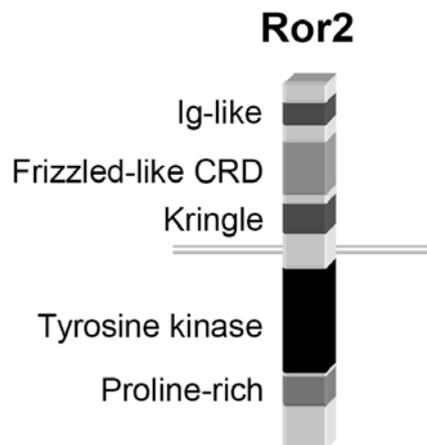
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an intact endocytotic process is essential for, at the least,  $\beta$ -catenin signaling [229]. Furthermore, it has been suggested that the low density lipoprotein receptor-related protein 5/6 (LRP5/6) has a coreceptor function involving transduction of the Wnt signals [230-232]. In addition, studies have shown that LRP6 can form a complex together with Frz and Wnt [230]. Notably, it has been indicated that only the  $\beta$ -catenin signaling pathway actually requires LRP5/6 [233].

The Wnt proteins induce different signaling pathways, and reports indicate that the type of pathway that is activated is determined by the Wnt protein in question, the receptor involved, and coreceptor expression, and probably also on the intracellular signaling proteins that are present [232, 234, 235]. For example, some investigations have shown that Wnt-5a activates  $\beta$ -catenin signaling in the presence of Frz-5 [236] or Frz-4 in combination with LRP5 [237], whereas other studies have found that Wnt-5a inhibits  $\beta$ -catenin signaling in the presence of Ror2 [237] or endogenous Frzs [208, 237, 238].

### *Ror2*

There is evidence that receptor tyrosine kinase-like orphan receptor 2 (Ror2) acts as a coreceptor in Wnt signaling. Ror2 belongs to the same tyrosine kinase receptor family as the structurally related protein Ror1, both of which contain many interaction domains, such as the following (see Figure 9): an Ig-like domain, a Frizzled-like CRD [239], a Kringle domain, a tyrosine kinase domain, and a proline rich domain [240]. Ror2 also contains a YALM motif that probably interacts with Src homology 2 (SH2) domains of Csk, Shc, and the p85 subunit of PI3K upon tyrosine phosphorylation [241].



**Figure 9.** Structure of the Ror2 receptor, with the different conserved domains indicated. Modified from [240].

In humans, mutations in the Ror2 gene are responsible for the autosomal recessive Robinow syndrome [242] and the autosomal dominant brachydactyly type B [243], which are two hereditary skeletal disorders characterized by, among other things, shortened limbs. In agreement with that, Ror2 knockout mice have been found to exhibit abnormalities of the tail, face, limbs, and heart that are very similar to those displayed by patients with Robinow syndrome [244-246]. Interestingly, it has also been found that Wnt-5a knockout mice exhibit deformities that are very similar to those seen in Ror2 knockout mice [244, 247], and Ror2 has indeed been shown to form complexes with Wnt-1, -2, -3, -3a, -4, -5a, and -5b [237, 244, 248], as well as rat Frz-2 and human Frz-5 [244]. Moreover, it has been reported that the ectodomain of *Xenopus* Ror2 associates with *Xenopus* Wnt-5a, Wnt-8, and Wnt-11 [249].

Considering functional aspects, studies have shown that Ror2 is required for the following Wnt-5a induced effects: activation of XPAPC in *Xenopus* [250], inhibition of convergent extension movements in *Xenopus* [244], and migration of human cells [251] and inhibition of TCF/LEF reporter activation in mouse cells [237]. Ror2 alone has also been implicated in reduced migration, as indicated by the findings that, in neurons, downregulation of Ror2 resulted in enhanced extension of axons [252], and loss of CAM-1, the *C. elegans* ortholog of Ror2, caused the cells to migrate beyond their normal destinations [253]. In addition, a role as a survival kinase has been proposed for Ror2 based on the observation that treatment with siRNA targeting Ror2 resulted in increased drug-induced apoptosis in cervical carcinoma cells [254].

Studies have indicated that some of the functions of Ror2 are dependent on the tyrosine kinase domain [237, 251, 255], whereas others are not [248, 249, 253]. Furthermore, in two of the cited investigations it was noted that the tyrosine phosphorylation of exogenously expressed Ror2 was not affected by overexpression of Wnt-1 or Wnt-3 [248], or by stimulation with Wnt-5a [255]. However, Ror2 was in other experiments found to both associate with and be serine/threonine phosphorylated by casein kinase (CK) 1 $\epsilon$ , and that interaction induced an autophosphorylation of tyrosine residues in Ror2 [255].

### *Ryk*

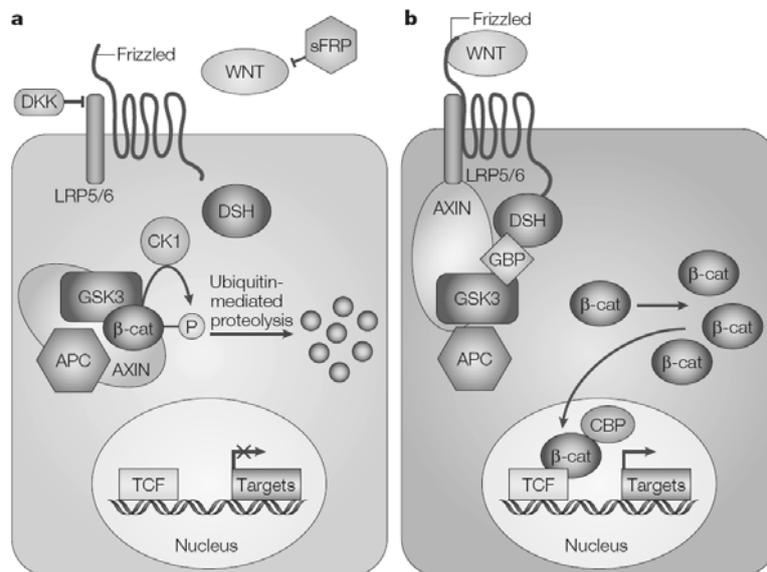
The subfamily called receptor related to tyrosine kinase (Ryk) has also been implicated in Wnt signaling and its members include Derailed [256] and Doughnut [257] in *Drosophila*, Lin-18 in *C. elegans* [258, 259], and Ryk in vertebrates [260]. Ryk proteins are not structurally related to Frzs, but they bind Wnts through a Wnt inhibitory factor-1 (WIF-1)-like domain [261]. Furthermore, Ryk has been suggested to serve as a receptor for Wnt-5a [256, 262], but it has also been found to mediate Wnt-1-induced activation of TCF [263]. In addition, Ryk has been shown to interact with the CRD of Frz-8 [263], which implies that a Wnt-Frz-Ryk complex is formed. There are also results

## Background

indicating that Ryk proteins lack catalytic activity [264], which is supported by the finding that a mutation in the active site of Derailed had no effect on the function of the protein [265]. In studies of human tissues [266], Ryk protein was detected in most normal tissues and was also found to be upregulated in ovarian cancer. However, the level of expression of the protein was not related to the grade of the tumors, although Ryk was found to exhibit a capacity for transformation [266]. The importance of the Ryk proteins is largely unknown, although it has been suggested that they are involved in cellular recognition [267].

## The Wnt/ $\beta$ -catenin signaling pathway

Considering all types of Wnt signaling, the most thoroughly studied is the  $\beta$ -catenin pathway, which is also referred to as canonical. In the absence of a Wnt signal,  $\beta$ -catenin is bound by Axin and APC, and it is subsequently phosphorylated on serine and threonine residues, first by CK1, and possibly also CK2 [268], and thereafter by the constitutively active glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) [269], as seen to the left in Figure 10. The phosphorylated  $\beta$ -catenin is ubiquitinated by  $\beta$ -transducin-repeat-containing protein ( $\beta$ -TrCP) and degraded by the proteasome [270]. The DNA-binding proteins TCF and



**Figure 10.** Intracellular signaling in the absence (a) and presence (b) of Wnt stimulation. Adapted by permission from Macmillan Publishers Ltd: [220].

LEF act in cooperation with the C-terminal binding protein (CtBP) and the groucho family of proteins to actively repress gene transcription in the nucleus [271, 272].

Transduction of a Wnt signal mediates phosphorylation and activation of Dishevelled (Dvl), which in turn binds and inhibits GSK-3 $\beta$ . Dvl also recruits Axin to the cell membrane, where it binds to the phosphorylated LRP5/6 and is then degraded [273, 274]. Thereby, the phosphorylation and inactivation of  $\beta$ -catenin is impaired, as shown to the right in Figure 10.  $\beta$ -catenin accumulates in the cell, binds to TCF/LEF, and is translocated to the nucleus, where it mediates transcription of genes like cyclooxygenase 2 (COX-2), MMP-7, cyclin D1, and c-myc, as well as the recently discovered targets VEGF and amphiregulin (a complete list of target genes is available on the Wnt homepage: <http://www.stanford.edu/~rnusse/wntwindow.html>).

### ***Wnt-inhibitory factors***

Wnt signaling is also regulated by secreted molecules called WIF-1, Cereberus, the eight vertebrate secreted Frizzled related proteins (sFRP), and the dickkopf (Dkk) family members, as seen in Figure 10. WIF-1, Cereberus, and sFRPs block Wnt signaling by binding to the ligand and thereby preventing it from interacting with the Frz receptor [275, 276]. However, low levels of sFRPs have also been shown to augment Wnt signaling [277]. Since the mentioned factors can bind the Wnt proteins, theoretically, they should be able to inhibit all intracellular signals induced by the different Wnt proteins and their receptors and coreceptors. Indeed, the sFRP called Crescent has been shown to bind Wnt-5a and to participate in regulation of signals that are discrete from the Wnt/ $\beta$ -catenin pathway [278].

In contrast to the sFRPs, Dkk has been shown to inhibit Wnt signaling by binding the coreceptor LRP5/6, and it is therefore likely to specifically block the Wnt/ $\beta$ -catenin pathway [276]. Besides engaging LRP5/6, Dkk also binds the single-transmembrane receptor Kremens and thereby mediates endocytosis of LRP5/6 [279].

### ***Wnt-5a signaling***

Wnt-5a has been shown to signal through the receptors Frz-2 [280-283], Frz-4 [237, 284], and Frz-5 [285-287]. In different studies, it was observed that expression of Wnt-5a RNA in breast epithelial cells was upregulated by activation of protein kinase C (PKC) [288] and by growing the cells to confluence [289], whereas it was downregulated by growing the cells on [290] or in [289, 290] collagen, or by activation of Ras [290] or inhibition of PKC [288].

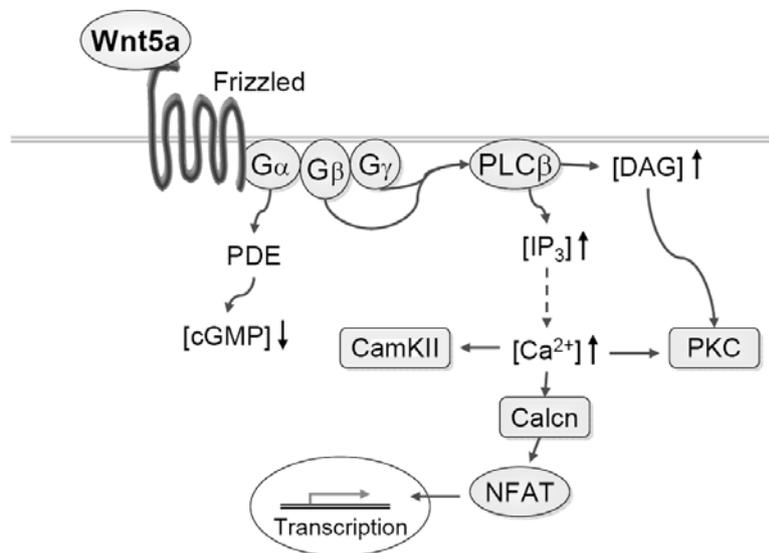
## Background

Furthermore, in another investigation [291], it was observed that expression of Wnt-5a mRNA was upregulated in gastric cancer cells that were stimulated with TNF- $\alpha$ . However, the importance of these findings is debatable, since it has also been shown that Wnt-5a is regulated on a post-transcriptional level [150, 292], by, for example, the RNA-binding protein HuR, which reportedly binds to and inhibits translation of Wnt-5a mRNA [293].

### $Ca^{2+}$ signaling activation

It has been demonstrated that Wnt-5a can induce an intracellular  $Ca^{2+}$  signal in zebrafish embryos [281, 294, 295], mouse cells [296, 297], and in thyroid carcinoma cells [292]. The receptor that has been studied most extensively in this context is Frz-2 [281, 282, 294, 296, 297], although Frz-3, Frz-4, Frz-5, and Frz-6 have also been shown to mediate PKC activation [282, 286], which indicates that they too can induce activation of a  $Ca^{2+}$  signaling pathway. By comparison, it seems that other receptors in the Frz family (e.g., Frz-1, and Frz-8), can only induce  $\beta$ -catenin signaling [282, 298].

It has been proposed that the intracellular release of  $Ca^{2+}$  occurs through stimulation of  $G_{\alpha o}$  and  $G_{\alpha t2}$  [283, 296], as a result of which phospholipase C $\beta$  (PLC $\beta$ ) is activated and translocated to the cell membrane, where it



**Figure 11.** Wnt-5a induces release of  $Ca^{2+}$  by activating G-protein-coupled Frz receptors, which leads to activation of CamKII, calcineurin, and PKC. Modified from [299].

hydrolyzes phospholipids to generate the second messengers inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) (see Figure 11) [281, 300]. IP<sub>3</sub> subsequently interacts with the SERCA-ATPase pump at the membrane of the endoplasmic reticulum and induces the release of Ca<sup>2+</sup> [301], whereas DAG activates PKC [302]. Research has shown that several Ca<sup>2+</sup>-sensitive enzymes, including Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CamKII), calcineurin, and PKC are activated in the Wnt/Ca<sup>2+</sup> signaling pathway [282, 286, 298, 303].

It has also been reported that stimulation of Wnt-5a/Frz-2 causes activation of protein kinase G (PKG) and phosphodiesterases (PDEs), which in turn leads to a sharp decrease in cGMP levels [296, 304]. Notably, suppression of cGMP activity has been shown to be essential for the Ca<sup>2+</sup> signaling induced by Wnt-5a/Frz-2 [296]. Moreover, Wnt-5a-mediated Ca<sup>2+</sup> signaling has been observed to provoke nuclear factor of activated T-cells (NFAT)-dependent transcriptional activity in *Xenopus* and mouse cells [296, 297, 305].

### **NFAT**

The NFAT family consists of the four cytosolic proteins NFAT1–4 and one constitutively nuclear protein designated NFAT5, which does not depend on calcineurin for its activation [306]. NFAT1–4 are transcription factors that are dephosphorylated and thereby activated by the phosphatase calcineurin, which in turn is activated by Ca<sup>2+</sup> signaling [307]. Calcineurin also prevents nuclear export of NFAT1–4 by masking nuclear export signals and by keeping them dephosphorylated [308]. On the other hand, nuclear import of NFAT1–4 is inhibited by phosphorylation caused by kinases such as GSK-3 $\beta$ , CK1, and Jun N-terminal kinase (JNK) [309-312], plausibly achieved through a mechanism involving phosphorylation of serines, which conceals important nuclear localization sequences [308]. The transcription factor activator protein 1 (AP-1) is considered to be the main interaction partner of NFATs, and activation of AP-1 is regulated chiefly by PKC/Ras and MAPK pathways, which include JNK and extracellular signal-regulated kinase (ERK) [313]. NFAT proteins have been implicated in regulating a large number of genes in the immune system (e.g., IL-2, IL-8, and COX-2) [313].

Studies have shown that constitutive activation of NFAT2 induces transformation of fibroblasts [314], and activation of NFAT1 enhances invasion and migration in breast cancer cells [315], which may, at least in part be mediated through COX-2 upregulation [316]. NFAT5 apparently also has tumor-promoting characteristics, since it has been found to provoke migration, and it is activated downstream of  $\alpha_6\beta_4$  integrins [315], which, as previously mentioned, are often upregulated in breast cancer. In contrast, loss of NFAT4 has been shown to result in increased predisposition to mammary adenocarcinoma in mice (albeit in a very small number of animals) [317], which

## Background

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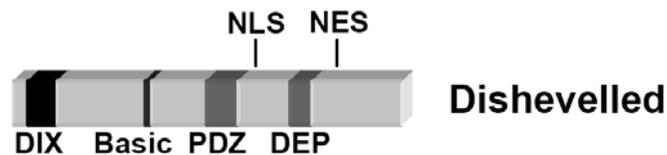
implies a role for NFAT4 in the prevention rather than the promotion of cancer.

### Activation of additional intracellular signals

Several studies have shown that Wnt stimulation leads to phosphorylation of members of the Dvl family, proteins that have been shown to mediate activation of  $\beta$ -catenin signaling [318],  $\text{Ca}^{2+}$  signaling [294], and activation of JNK [319, 320]. Nonetheless, Wnts have also been reported to mediate Dvl-independent functions [238, 297, 320].

Considering that all these signals can converge in Dvl activation, it is not yet completely known how they achieve their specificity. Notwithstanding, the kinase Par-1 and Naked proteins have been shown to bind to different Dvl domains and to potentiate  $\beta$ -catenin signaling and JNK activation, respectively [321]. It seems that the signaling specificity is also regulated by the conserved Dvl domains DIX, PDZ, and DEP, which are shown in Figure 12. Experiments have demonstrated that DIX and PDZ, are essential for  $\beta$ -catenin signaling [322], whereas only the DEP domain is necessary for activation of JNK [319, 322, 323]. Furthermore, Dvl-constructs lacking the DIX domain have been shown to induce both  $\text{Ca}^{2+}$  release and JNK activation, even though such a construct is incapable of inducing  $\beta$ -catenin signaling [294, 320].

Interestingly it was recently discovered that Dvl translocates to the nucleus, a process that is regulated by a nuclear localization sequence (NLS) and a nuclear export signal (NES) [324]. This study further revealed that the translocation of Dvl seems to be critical for its function in  $\beta$ -catenin signaling, but not for JNK activation.



**Figure 12.** Structure of Dishevelled, showing the conserved domains DIX, PDZ, and DEP. The positions of the recently suggested nuclear localization sequence (NLS) and nuclear export signal (NES) are also indicated. Modified from [211, 324].

It is known that Wnt-5a stimulation and Dvl engagement can lead to activation of JNK [320, 325], a subgroup of MAPKs which is primarily activated by environmental stress and cytokines [321]. Studies have also shown that these Wnt signals involve activity of Rho GTPases and Rho-associated kinase (ROCK) [250, 326, 327], which in turn provides a link between Wnt signaling and regulation of the cytoskeleton. Even though Dvl and Rho GTPases have

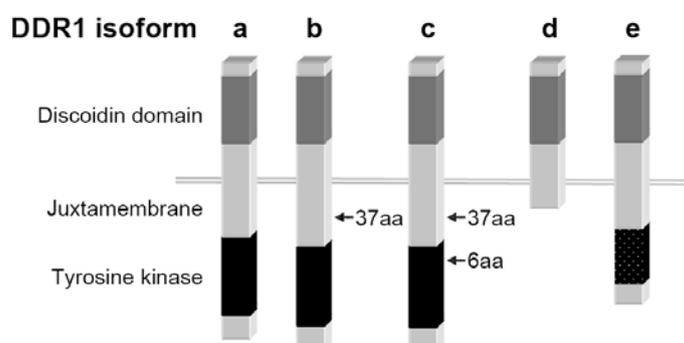
been shown to activate JNK [319, 326], it is not completely clear whether the Wnt-induced activation of JNK do require Dvl and Rho GTPases [320]. Furthermore, in light of results demonstrating that similar Dvl constructs can induce activation of both JNK and  $\text{Ca}^{2+}$  signaling [294, 320] and that both JNK and Cdc42 can be activated by  $\text{Ca}^{2+}$  release [328, 329], it seems possible that the Wnt-induced  $\text{Ca}^{2+}$  and JNK/Rho GTPase signals converge.

Wnt-5a has also been reported to induce activation of the MAPK p38 [297], FAK [208, 219], and HER1 [330]. Furthermore, there is evidence that the activity of Src family members [215], and also PI3K [215, 250], Akt [215], and ERK [215, 284] are necessary for the functions of Wnt-5a.

### DDR1 signaling

Wnt-5a is also an essential component of collagen-induced activation of the DDR1 receptor [331]. DDR1 and DDR2 contain a characteristic discoidin homology domain, which is responsible for their interaction with collagens [332, 333]. Five different splice forms of DDR1 (designated a–e) have been described (Figure 13). The b and c isoforms have a 37-amino-acid insert in the juxtamembrane region that encodes a Shc-binding site, and DDR1b has actually been observed to bind Shc upon exposure to collagen [147]. It is also known that DDR1c has a six-amino-acid insert in the tyrosine kinase domain, the DDR1d isoform lacks the tyrosine kinase domain, and DDR1e is kinase dead [334].

In experiments conducted by Alves and coworkers, it was found that most of the colon cancer cell lines tested coexpressed the a, b, d, and e isoforms of DDR1, and expression of the d-isoform did not prevent activation of the a and b variants [334]. Other investigators have reported that DDR1b is the predominant isoform expressed during embryogenesis, whereas the DDR1a



**Figure 13.** Five different splice forms have been shown of DDR1, of which the d and e isoform lacks kinase activity. Modified from [334].

## *Background*

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isoform is upregulated in breast cancer cell lines [335]. Furthermore, DDR1 seems to be a crucial factor in breast development, as indicated by the finding that DDR1 knockout mice exhibited both hyperproliferation and abnormally branched mammary glands, and they were unable to lactate [157].

Regarding intracellular signaling, studies have shown that activated DDR1 interacts with the p85 domain of PI3K [336, 337], and, by creating a chimeric DDR1 receptor, Foehr *et al.* [338] discovered that participation of PLC $\gamma$  is important for the full response. The chimeric DDR1 receptor also mediated strong activation of ERK, which, together with activation of p38, had previously been detected downstream of the wild-type DDR1b [156, 158, 338]. In other experiments [337], the presence of Wnt-5a was found to be essential for collagen-induced activation of DDR1 in breast epithelial cells, and such activation also required G $_{i/o}$ -proteins and Src activity.

### *Wnt-5a antagonization of the Wnt/ $\beta$ -catenin pathway*

Several studies have shown that Wnt-5a can counteract  $\beta$ -catenin signaling [208, 237, 339]. For instance, it has been found to inhibit the functions of Wnt/ $\beta$ -catenin [340, 341] and downregulation of Wnt-5a induces transformation in approximately the same way as activation of Wnt/ $\beta$ -catenin signaling does [342]. The mechanism underlying these effects is not yet known, although several suggestions have been made.

Wnt-5a has been found to upregulate expression of Siah2, which can mediate  $\beta$ -catenin degradation without the help of GSK-3 $\beta$  [238]. Wnt-5a can also activate nemo-like kinase (NLK) via CamKII [303], and NLK suppresses the transcriptional activity of TCF/LEF by inducing phosphorylation, and in that way inhibits the DNA-binding ability of that protein complex [343]. Competition for signaling molecules involved in both pathways has also been suggested as a possible mechanism [344]. In addition, a study has demonstrated that  $\beta$ -catenin signaling can inhibit some of the effects of Wnt-5a [345].

### *Effects of Wnt-5a on cell behavior*

Wnt-5a is a non-transforming protein [217, 346, 347], and in fact it is known to reverse transformation [348, 349] and has been found to suppress telomerase activity in renal carcinoma cells [342]. Nevertheless, results concerning the effects of Wnt-5a on proliferation are contradictory. A tumor suppressor function is implied by studies showing that Wnt-5a restrains cell growth [342, 347, 350, 351], possibly by inhibiting FGF, Shh, and BMP signals [351, 352] or by inhibiting expression of cyclin D1 [350]. However, it has also been suggested that Wnt-5a mediates increased proliferation of progenitor cells [247, 353, 354],

pancreatic cancer cells [355], glioma cells [356], and vascular endothelial cells [284], and in two studies it inhibited apoptosis in vascular endothelial cells [284], and in an osteoblastic cell line [215]. In contrast, other investigations have indicated that Wnt-5a has no effect on either apoptosis or proliferation [345, 357].

Wnt-5a has also been observed to play a role in regulation of cell attachment and movement [358], although the effects reported regarding migration vary considerably. Regarding adhesion it has for example been reported that Wnt-5a increases adhesion of breast epithelial cells [331], fibroblasts [208], and melanoma cells [286], and also augments N-cadherin-mediated cell-cell adhesion in cardiac myocytes [359]. Furthermore, Wnt-5a has been found to mediate decreased migration or invasion in breast epithelial cells [331], colon cancer cells [360], and in a thyroid cell line [292], whereas other investigations have indicated that it gives rise to increased migration or invasion in gastric cancer cells [219], fibroblasts [208, 251], vascular endothelial cells [284], pancreatic cancer cells [355], breast epithelial cells [361], and melanoma cells [251, 286]. The enhanced migration in melanoma cells might be explained by results showing that expression of Wnt-5a induced PKC-dependent expression of Snail, which led to loss of E-cadherin [362].

### ***Wnt-5a in breast cancer***

In humans, Wnt-5a protein is expressed in normal breast epithelium [363] and has also been reported to be important for a proper development of the mammary gland [364]. In breast cancer tissue, the Wnt-5a protein expression is often at much lower levels, and such a loss of expression has been correlated with a higher incidence of early relapse and death [150, 363], which indicates that Wnt-5a is involved in metastasis formation. Several other studies have examined the expression of Wnt-5a RNA in breast cancer cell lines and tissues, but the results are contradictory [365-368]. In any case, as previously discussed, it seems that expression of Wnt-5a is regulated at the translational level, since in two investigations it was found that Wnt-5a mRNA was present in all breast and thyroid tumors examined, whereas Wnt-5a protein was detected only in a minority of the same tumors [150, 292], which might explain the divergent findings regarding expression of Wnt-5a mRNA in breast cancer. Notably, cytoplasmic expression of HuR, a protein known to negatively regulate translation of Wnt-5a mRNA [293], is also correlated with poor outcome in ER-positive breast cancers [369], which supports the observation that lost expression of Wnt-5a protein predicts a negative outcome in breast cancer patients.

### ***Wnt-5a in other types of cancer***

Dysregulation of Wnt-5a expression has been shown not only in breast cancers, but in other types of malignancies as well. Liang *et al.* [350] found that mice heterozygous for Wnt-5a developed chronic B cell lymphomas and spontaneous B cell lymphomas of clonal origin. These authors also noted that expression of both Wnt-5a mRNA and protein was lost in the malignant tissues, which, together with anti-proliferative effects observed *in vitro*, suggests that Wnt-5a possesses a tumor-suppressor function in B cells. Similarly, in other investigations it was found that decreased expression of Wnt-5a protein in Dukes' B colon cancers was correlated with a poor prognosis [360], and that Wnt-5a also had a tumor-suppressing effect in thyroid carcinomas [292].

The opposite has also been reported. For example, a high level of Wnt-5a protein expression was noted to be correlated with advanced stage and poor prognosis in gastric cancer [219] and melanoma [286, 370], and in another study Wnt-5a protein was found to be upregulated in pancreatic cancer tissue compared to adjacent normal tissue [355]. Considering Wnt-5a RNA, expression has been shown to be lower in endometrial carcinoma cells than in normal endometrial cells [371], downregulated in pancreatic carcinoma [372], and lost in high-risk neuroblastomas [373, 374], although in the latter experiments expression was regained after differentiation was induced by exposure to retinoic acid. In addition, Wnt-5a RNA was upregulated in astrocytoma and glioma [356], and Wang and coworkers [375] found that the Wnt-5a gene was hypomethylated and overexpressed in 65% of the prostate cancer samples they studied. However, it should be kept in mind that, as previously mentioned, the expression of Wnt-5a mRNA does not necessarily correlate with the level of the protein.

### ***Wnt/ $\beta$ -catenin in breast cancer***

Mutations activating the  $\beta$ -catenin signaling pathway are found in most types of cancers, and this is especially frequent in tumors located in the colon [376]. By comparison, mutations in either  $\beta$ -catenin or APC rarely occur in breast carcinomas [377-380], although such defects have been reported in breast fibromatosis [381]. However, in two studies [382, 383], elevated levels of  $\beta$ -catenin were detected in 60% of breast cancers and were correlated with expression of cyclin D1 and with poor prognosis of the patients. Furthermore, researchers have observed that Wnt-stimulation was provided by stromal cells associated with breast tumors [384]. Reduced expression of the Wnt-inhibiting proteins WIF-1 and sFRP has also been reported in breast carcinomas [385-387], and was shown to contribute to activation of Wnt/ $\beta$ -catenin signaling [388].

Very little is known about expression of the Frz receptors in breast cancer. However, upregulated protein expression of Frz-1 and/or Frz-2 in breast cancer has been demonstrated by use of an antibody that detects both those proteins [365], and studies conducted by another research group found that both the normal and the cancerous breast epithelial cell lines that were tested expressed all Frz receptors, except Frz-9 and Frz-10 [389].



## **THE PRESENT INVESTIGATIONS**

### **AIMS**

The overall aim of the present investigations was to study the impact of Wnt-5a on breast cancer metastasis, and in particular to achieve the following:

- I. To further elucidate the intracellular signaling pathways downstream of Wnt-5a protein that lead to inhibited migration of breast cancer cells.
- II. To find a synthetic peptide that could mimic the functions of Wnt-5a, such as the adhesion-increasing and migration-decreasing effects on breast epithelial cells, and that could also activate key intracellular signals in a manner similar to Wnt-5a.
- III. To determine whether such a synthetic peptide could influence breast cancer metastasis *in vivo* by means of its effect on migration, which is an important function in metastasis formation.
- IV. To study the proposed Wnt-5a coreceptor Ror2 in order to ascertain its effects on breast cancer cell migration and its involvement in Wnt-5a signaling.

## METHODOLOGY

### **Cell lines**

A variety of cell types were used in the present investigations, among them the breast epithelial cell line HB2, which is a subclone of the MTSV-1.7 line originating from a normal breast epithelial cell immortalized with SV40 [390]. In three of the studies (Papers I, II and IV), I used HB2 cells stably transfected with a pLNCX vector exhibiting the Wnt-5a cDNA in an antisense direction (Wnt-5a antisense), a pLNCX Wnt-5a HA vector (Wnt-5a overexpressing), or an empty pLNCX vector (Wnt-5a neo), which had previously been produced in our laboratory [331].

I also used the four human mammary epithelial cell lines MCF-7, T47D, MDA-MB-468, and MDA-MB-231, all of which were derived from pleural effusions from patients with metastatic breast cancer. It has been shown that the MDA-MB-468 and MDA-MB-231 lines are more invasive than the MCF-7 and T47D cells [391]. In the experiments described in Paper III, I employed the mouse mammary epithelial cell line 4T1, which originates from spontaneous breast cancer in a BALB/c mouse [392]. The 4T1 cells are widely used to study breast cancer metastasis, because, when inoculated into the mammary fat pad of mice, they spontaneously metastasize to liver and lung within four weeks [393]. Furthermore, in the study reported in Paper I, we also utilized human embryonic kidney epithelial cells of the HEK293 line, and SYF cells, which are mouse embryonic fibroblasts deficient in Src, Yes, and Fyn.

### **Western blot and immunoprecipitation**

Protein expression was evaluated in either whole cell lysates (boiled in 2x Laemmli buffer) or cell lysates from which the cytoskeletal fraction had been removed (cells lysed in a lysis buffer containing protease inhibitors and then centrifuged at 15'000 rpm). Protein concentration was determined using Coomassie dye reagent. Lysates intended for immunoprecipitation were prepared using lysis buffer and were pre-cleared with either Protein A or Protein G Sepharose, and thereafter incubated with the antibody and Protein A or Protein G Sepharose.

Protein lysates and immunoprecipitates were separated on 6–12% SDS gels by electrophoresis and then transferred to PVDF membranes. The membranes were blocked in either 5% non-fat milk or 3% bovine serum albumin (for phospho-antibodies), and were subsequently incubated with the primary antibody and a horseradish peroxidase-conjugated secondary antibody, which were detected using an enhanced chemiluminescence kit. A Reblot Strong solution was used for reprobing of the membranes.

### ***Luciferase reporter assay***

A dual luciferase reporter assay was employed to evaluate the transcriptional activity of NFAT and TCF (Paper I). Cells were transiently transfected with luciferase vectors containing DNA-binding elements recognized by NFAT (NFAT-pGL2) or TCF (TOPflash). In this assay, binding of the transcription factor to the site of interest results in expression of the firefly luciferase enzyme, which can catalyze a bioluminescence reaction; the amount of light emission detected is proportional to the binding activity of the targeted transcription factor. As background controls, we utilized a TATA-pGL3 containing a TATA box and a FOPflash vector with a mutated TCF binding site, and the samples were normalized by *Renilla* reporter gene transcription.

### ***Immunofluorescence and F-actin staining***

To evaluate the subcellular localization of NFAT (Paper I), cells grown on coverslips were fixed with paraformaldehyde and then permeabilized and incubated with a NFAT antibody and a fluorescently labeled secondary antibody. To label F-actin (Paper II), cells were grown on cover glass coverslips and then fixed with paraformaldehyde and incubated with the fluorescently labeled mushroom toxin phalloidin, which binds F-actin with high affinity. The samples were examined and photographed in a fluorescence microscope.

### ***Transfection***

Using oligofectamine, cells were transiently transfected with c-myc-tagged Cdc42 or Rac1 dominant negative vectors, the NFAT-inhibiting pVIVIT-GFP vector, or empty vector (Paper I). The expression levels were analyzed by Western blotting with a c-myc antibody or by examination in an immunofluorescence microscope. In one set of experiments (Paper IV), lipofectamine was used to transfect cells with Ror2 siRNA or a negative control siRNA. Western blotting for Ror2 was done to ensure the efficiency of the transfections.

### ***Cytosolic free calcium***

To evaluate cytosolic free calcium (Papers I and II), cells grown on glass coverslips were loaded with Fura-2/AM and placed in a microscope connected to a Photon Technology International (PTI) imaging system. Fura-2 fluorescence was recorded before and after stimulation with recombinant Wnt-3a, Wnt-5a, or the Foxy-5 peptide. The ratio of Ca<sup>2+</sup>-saturated to Ca<sup>2+</sup>-free Fura-2 was calculated using measurements obtained at excitation wavelengths rapidly

### *The present investigation*

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alternating between 340 and 380 nm, and emission recorded at 510 nm. Fluorescence intensity ratio (340/380 nm) was calculated using PTI image master software.

### **GST pulldown**

The Cdc42/Rac interactive binding region (CRIB) of PAK1B, which recognizes only active GTP-bound Cdc42 and Rac1, was expressed in *E. coli* as a fusion protein with glutathione S-transferase (GST). To determine amounts of active Cdc42 and Rac1, breast epithelial cells were lysed in a lysis buffer containing protease inhibitors, and the GST-PAK-CRIB fusion protein was added together with GST beads. The amounts of Cdc42 and Rac1 bound to CRIB were quantified by Western blotting, and blots used to determine the total levels of Cdc42 and Rac1 in the original lysates were run in parallel (Paper I).

### **Migration and invasion**

Cells were detached from culture dishes with Versene, and their capacity to migrate was determined in a Transwell®-system consisting of an upper and a lower chamber separated by a membrane with 8- $\mu$ m pores. The cells were placed in the upper chamber, and the lower chamber was filled with serum-free medium containing insulin-like growth factor I (IGF-I) (Papers II and III) or 10% serum (Paper IV) as a chemoattractant. In the indicated experiments in Paper II, the membranes were precoated with collagen I.

The invading capacity of the cells was investigated in a similar manner in Matrigel™ invasion chambers. Medium containing 10% serum was used as chemoattractant, and the recombinant Wnt-5a and the peptides were (in all cases, except where indicated in Paper III) placed together with the cells in the upper chamber. Cells that migrated or invaded were fixed with paraformaldehyde, stained with crystal violet, and counted in a light microscope.

### **PHD prediction and peptide synthesis**

The 3D structure of a protein sequence can be predicted if the 3D structure of a similar protein (> 25% identical) is known. Inasmuch as such information was lacking for the Wnt-5a protein, we predicted the secondary structure and/or relative solvent accessibility according to the PHD method [394] in order to identify potentially solvent-exposed loop segments (Paper II). We used the Wnt-5a protein sequence from six different species for the multiple sequence

alignment. The predicted accuracy of the PHD prediction is approximately 75% for globular water-soluble proteins [394].

The peptides were synthesized by Eurogentec, Pepscan Systems or Sigma Aldrich on several occasions, and they were subjected to quality control by RP-HPLC and mass spectrometry. The purity of the peptides was 60–70% in the initial screening (Paper II), but was > 95% in all subsequent experiments (Papers II–IV).

## **Adhesion**

To investigate adherence ability, cells were detached from culture dishes with Versene and allowed to adhere to uncoated or collagen-I-coated wells for 60 to 90 min. Thereafter, non-adherent cells were washed away, and the remaining cells were treated in either of two ways: (1) incubated in MTS solution, which is reduced to a colored formazan product in the presence of living cells, and then subjected to absorbance measurements at 490 nm (Paper II); (2) fixed and then stained with crystal violet, after which the stained cells were dissolved in 50% acetic acid and absorbance was measured at 595 nm (Paper IV).

## **RT-PCR**

To evaluate expression of Ror2 (Papers III and IV) RNA was obtained using TRIzol® Reagent and then treated with DNase and reversely transcribed with Superscript™ II RNase H-reverse transcriptase. Thereafter, the reaction products were subjected to PCR amplification with Taq DNA polymerase and primers towards human and mouse Ror2 and  $\beta$ -actin. To control for DNA contamination, reverse transcription was also performed in the absence of the reverse transcriptase enzyme. PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

## **Apoptosis**

Apoptosis was investigated *in vitro* by analyzing caspase 3 activity, poly (ADP-ribose) polymerase (PARP) cleavage, and Hoechst staining, and also *in vivo* by staining tissue sections using TUNEL (terminal deoxynucleotide transferase dUTP nick-end labeling) (Paper III). Caspase 3 activity was studied in both adherent and floating cells using the fluorogenic substrate peptide Ac-DEVD-AMC, which, in the presence of caspase 3, is cleaved and generates free fluorescent AMC. The amount of free AMC was measured at an excitation wavelength of 390 nm and emission of 460 nm. The ubiquitously expressed PARP is cleaved and inactivated by caspase 3 during apoptosis. We performed

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Western blotting with a PARP-specific antibody to detect the intact PARP protein and the cleavage product in cell lysates.

Apoptosis can be both a caspase-dependent and a caspase-independent process. Therefore, to analyze such cell death, I also used blue-fluorescent Hoechst dye, which recognizes late-apoptotic chromatin condensation. Cells grown on coverslips were fixed with paraformaldehyde and stained with Hoechst dye, after which the apoptotic cells were counted in a fluorescence microscope. In all experiments *in vitro*, cells treated with the chemotherapeutic drug Taxol were used as a positive control for apoptosis.

To evaluate the proportion of apoptotic cells in metastatic tissues, lung and liver sections were stained in a TUNEL assay, which labels late-apoptotic DNA breaks. The slides were counterstained with the nuclear DAPI stain, and the percentage of TUNEL-positive cells in the lung and liver metastases were counted in a fluorescence microscope.

### **Proliferation**

Proliferation was evaluated *in vitro* by cell counting and analysis of thymidine incorporation, and also *in vivo* by staining for the proliferation marker Ki67. Cell counting was performed by detaching the cells with Versene, incubating them in trypan blue dye (which is excluded by viable cells), and counting the unstained cells before and after the incubation time (Papers III and IV). To estimate the extent of cell proliferation, cells were incubated with tritium-labeled thymidine, which is incorporated into DNA as it is synthesized. Trichloroacetic acid was added to precipitate the nucleotides, after which the cells were solubilized in NaOH, and the radioactivity was measured in a beta-counter (Paper III). To evaluate the rate of proliferation in experiments *in vivo*, lung and liver sections were immunohistochemically stained for Ki67, a nuclear protein that is expressed only during cell proliferation. The percentage of Ki67-positive cells in lung and liver metastases was determined in a light microscope (Paper III).

### **Breast cancer metastasis *in vivo***

The 4T1 mouse breast cancer cell line was used to evaluate breast cancer metastasis in athymic BALB/c mice and in normal BALB/c mice. Animals of the athymic BALB/c strain lack a functional thymus, and thus they cannot produce mature T lymphocytes. Due to this defect in immune response, these mice are often used in cancer studies, because inoculated tumor cells are not rejected, even if they originate from other species, such as humans. However, a defective immune system might influence the tumor progression, and therefore

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we also performed the experiments on normal immunocompetent BALB/c mice. The major advantage of using the 4T1 cell line is that it originates from BALB/c mice, and hence it is not rejected by normal BALB/c mice.

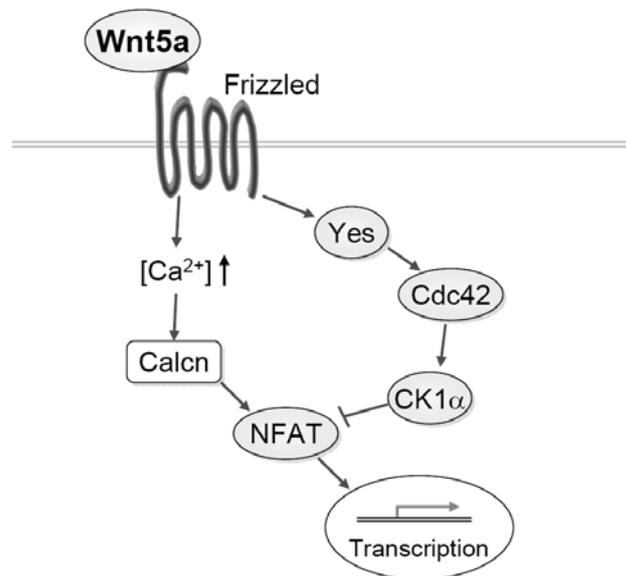
The 4T1 cells were inoculated into the mammary fat pad of mice under anesthesia, and peptides or phosphate-buffered saline (PBS) was injected intraperitoneally every fourth day. The mice were weighed and the volume of the primary tumor was calculated throughout the study, which was concluded after 25 days. Lung and liver sections were stained with hematoxylin and eosin and then evaluated for metastases in a light microscope with blinded samples. A computer program was used to calculate the metastatic area in the tissue sections. The experiments using mice were carried out at the Central Animal Laboratory of the University of Turku, and the experimental procedures performed on the animals were reviewed by the local ethics committee on animal experimentation at the same university and were approved by the local Provincial State Office of Western Finland.

## RESULTS

### **Paper I**

Wnt-5a has been shown to induce an intracellular  $\text{Ca}^{2+}$  signal in zebra fish embryos [281, 294, 295], mouse embryonic cells [296], and in a thyroid carcinoma cell line [292]. If Wnt-5a also triggers such a signal in breast cancer cells, it could activate NFAT, a transcription factor implicated in promoting breast cancer invasion [315]. However, it is not known whether Wnt-5a can induce a  $\text{Ca}^{2+}$  signal in breast cancer cells, and it is a matter of debate if such an effect of Wnt-5a actually leads to NFAT activation [238, 296, 297, 305]. Therefore, we decided to perform experiments to address these questions.

Our results showed that Wnt-5a, but not Wnt-3a, did induce a significant  $\text{Ca}^{2+}$  signal in breast epithelial cells, and it elicited a limited NFAT1 activation. In a previous study in our laboratory [337], it was found that certain effects of Wnt-5a are sensitive to a Src tyrosine kinase inhibitor. Therefore, we conducted experiments to determine whether a Src kinase was involved in the Wnt-5a-induced activation of NFAT1 (Paper I). Surprisingly, inhibition of the activity of Src family kinases significantly potentiated the Wnt-5a-mediated NFAT activation, which indicates that a Src kinase does block NFAT activity in breast epithelial cells. Hence, we investigated this observation in greater detail and found that the Src family kinase Yes and the Rho GTPase Cdc42



**Figure 14.** Wnt-5a induces  $\text{Ca}^{2+}$ -mediated NFAT activation, which is simultaneously counteracted by a  $\text{CK1}\alpha$ -mediated NFAT phosphorylation, and  $\text{CK1}\alpha$  is in turn activated downstream of Wnt-5a-induced activation of Yes and Cdc42.

were activated downstream of Wnt-5a stimulation, and they proved to be involved in NFAT inhibition (see Figure 14). We also examined whether JNK played a role in this context, since it is known that JNK is activated by Rho GTPases, and there is evidence both that it is [244] and that it is not [238] activated by Wnt-5a. Our results indicated that JNK is not a component of the Wnt-5a signaling pathways that involves NFAT in breast epithelial cells.

We also found that ionomycin-induced NFAT activation was prolonged when the Yes/Cdc42 pathway was blocked by exposure to a Src family kinase inhibitor. This observation shows that Wnt-5a does inhibit NFAT activity via a Yes/Cdc42 signaling pathway, and this was also the case when NFAT activation was achieved by Wnt-5a-independent means. Next, we wanted to pinpoint the kinase responsible for phosphorylating and thereby inhibiting NFAT. GSK-3 $\beta$  is involved in Wnt signaling [269] and is also a known NFAT kinase [309] and therefore a likely candidate, but our data showed that GSK-3 $\beta$  did not cause the NFAT phosphorylation. Instead, we found that Wnt-5a stimulation led to a Src-kinase-dependent association between CK1 $\alpha$  and NFAT1, indicating that CK1 $\alpha$  could induce NFAT1 phosphorylation downstream of Yes activation. The final goal was to evaluate the importance of this pathway for breast epithelial cell invasion. In agreement with our previous findings [331, 360], Wnt-5a stimulation inhibited invasion. However, in cells lacking all Src family kinases, Wnt-5a caused an NFAT-dependent increase in invasion, once again showing that Wnt-5a-mediated activation of Yes/Cdc42/CK1 $\alpha$  does actually inhibit NFAT activity and thereby also impedes the invasive behavior of breast epithelial cells.

The results presented in this paper show that Wnt-5a induces Ca<sup>2+</sup>-mediated NFAT activation, which is simultaneously inhibited by CK1 $\alpha$  phosphorylation downstream of Wnt-5a-induced activation of Yes and Cdc42. These counteracting signals offer an explanation for the previously reported inconsistent conclusions regarding the effects of Wnt-5a on NFAT, and, more importantly, they represent a possible mechanism by which Wnt-5a can inhibit breast cancer invasion.

## ***Paper II***

Wnt-5a is abundantly expressed in normal mammary epithelial cells, and loss of such expression in primary breast cancer cells is correlated with early relapse and a poor prognosis for the patient [363], which indicates that Wnt-5a plays a role in suppressing breast cancer metastasis. Based on our present knowledge regarding the ability of Wnt-5a to increase adhesion and decrease migration in breast epithelial cells [331], it can be presumed that Wnt-5a achieves its metastasis-suppressing effect early in the metastatic process by preventing cancer cells from leaving the primary tumor. Perhaps,

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reconstitution of the impact of Wnt-5a on breast cancer cells could inhibit the metastatic process. Unfortunately, purification of active Wnt ligands has proven to be a difficult task [208, 237], and another problem is that Wnt ligands bind heparan sulfate proteoglycans [213], which greatly limits their distribution. Therefore, my aim in this study (Paper II) was to find a peptide that mimics the effects of Wnt-5a on migration in order to determine whether such a ligand can inhibit breast cancer metastasis.

Based on prediction of secondary structure we identified and synthesized fourteen 8–15-amino-acid peptides in regions of the Wnt-5a protein likely to be solvent-exposed. The peptides were screened to determine whether they possessed the same ability as Wnt-5a to mediate collagen-induced activation of DDR1 [337] in a breast epithelial cell line lacking endogenous Wnt-5a expression. Four of the peptides were found to induce DDR1 phosphorylation, and two of those that also increased cell adhesion in a concentration-dependent manner and inhibited migration, possibly due to an increase in the amount of actin stress fibers in the cells.

My next objective was to ascertain whether the activity could be retained in a shorter peptide, and thus I deleted two amino acids at a time from the shorter of the two peptides. When the originally 12-amino-acid peptide was truncated to six amino acids, the ability to induce adhesion was completely lost. Interestingly, this six-amino-acid peptide had a methionine in its N-terminus, and according to previous studies [395], adding a formyl group to the N-terminal methionine of another peptide had resulted in much greater affinity in interaction with a G-protein-coupled receptor. Consequently, I again synthesized the six-amino-acid peptide, but this time with a formylated N-terminal methionine, and, surprisingly, the peptide (which we call Foxy-5) regained its full adhesion-increasing activity. Foxy-5 also inhibited migration of breast cancer cells in a manner similar to Wnt-5a, and accordingly, its effect was also hindered by the previously described Frz-5-blocking antibody [286, 287]. Solid tumors are known to have an acidic microenvironment *in vivo* for several different reasons, including poor perfusion [80], and thus it is important that Foxy-5 was able to block cancer cell migration even at a lower pH. Furthermore, I discovered that Foxy-5 had the same ability as Wnt-5a to trigger a Ca<sup>2+</sup> signal, but it lacked effects on JNK activation or  $\beta$ -catenin phosphorylation. In conclusion, in this study I identified a small peptide that can mimic the influence of Wnt-5a on adhesion, migration and Ca<sup>2+</sup> signaling in breast cancer cells.

### **Paper III**

Having identified a peptide that can mimic the effects of Wnt-5a on migration, my next goal was to find out whether this peptide would have an impact on

breast cancer metastasis *in vivo* (Paper III). Cells of the mouse breast cancer line 4T1 are often used in this type of investigation, because they form spontaneous metastases in lung and liver within four weeks of inoculation into the mammary fat pad [393]. I initially studied the effects of Foxy-5 and Wnt-5a on proliferation, apoptosis, and migration in 4T1 cells *in vitro*, since those processes are important in metastasis formation. Both Wnt-5a and Foxy-5 inhibited migration and invasion, whereas neither induced proliferation or apoptosis. The absence of an effect on proliferation agrees with the earlier finding in our laboratory that expression of Wnt-5a protein was not correlated with expression of the proliferation-related protein Ki-67 in breast cancer tissue [150].

In subsequent experiments using athymic BALB/c mice, 4T1 cells were inoculated into the mammary fat pad, and the animals were given PBS, the Foxy-5 peptide (5 µg), or a control peptide (5 µg) intraperitoneally every fourth day (the first injections four hours before cell inoculation). Primary tumor growth and the weight of the mice were monitored throughout the study, without detecting any significant differences between the groups. On the other hand, evaluation of histological sections revealed that the mice treated with Foxy-5 had 70% less liver and lung metastases compared those given PBS or the control peptide. There were no differences between the groups with regard to proliferation and apoptosis of the metastatic lung and liver cells, with the exception of a minute decrease in proliferation in the lung metastases of mice treated with Foxy-5. These findings, together with the results obtained *in vitro*, suggest that the underlying cause of the Foxy-5-mediated inhibition of metastasis formation involves decreased cell migration rather than effects on proliferation and apoptosis.

The athymic mouse model is frequently used in cancer research. However, it is possible that the defective immune response in such animals can influence tumor progression and consequently also the outcome of treatment with anti-metastatic agents. Therefore, we repeated our experiments using normal BALB/c mice, although in this case we inoculated a smaller number of 4T1 cells and used a fourfold higher concentration (20 µg) of the peptides. This approach was chosen because data in the literature [396] and our own pilot experiments had indicated that normal BALB/c mice develop more abundant metastases compared to athymic BALB/c mice. The animals treated with the Foxy-5 peptide developed fewer lung and liver metastases than those treated with PBS, although the effect was not as pronounced as in the athymic mice. Interestingly, animals given the control peptide showed an increased number of lung and liver metastases, possibly due to macrophage activation. We repeated the experiment using a concentration of the Foxy-5 peptide that had a maximum effect in a limited dose-response experiment (i.e., 40 µg), and found that that amount of Foxy-5 inhibited metastasis formation in liver and lung by 60–90% compared to treatment with PBS.

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In summary, the findings reported in this paper show that in both athymic and normal BALB/c mice, Foxy-5 inhibited formation of breast cancer metastases in liver and lung by 60–90% compared to controls, and I believe this effect is due predominantly to the inhibitory influence of this peptide on 4T1 tumor cell migration.

### **Paper IV**

It has been suggested that the tyrosine kinase receptor Ror2 serves as a receptor for Wnt-5a [237, 244], and a subsequent report described experiments in which Ror2 was found to be essential for the effects of Wnt-5a on migration of fibroblasts and malignant melanoma cells [251]. Furthermore, the migratory capacity of cells is at least partly regulated by the aspect of adhesive strength, and previous findings published by our research group indicate that a large proportion of the Wnt-5a-mediated adhesion of breast epithelial cells occurs through the collagen-binding tyrosine kinase receptor DDR1 [337]. Therefore, I initiated this investigation (Paper IV), to elucidate the interactions between Ror2, DDR1, and Wnt-5a. I found endogenous Ror2 protein in only one of our breast epithelial cell lines (i.e., T47D cells), and HB2 cells also showed weak expression of Ror2 mRNA. Accordingly, I chose to use the T47D cell line in this study, and I the first looked for a physical interaction between the DDR1 and Ror2 receptors. Quite surprisingly, I observed that DDR1 was associated with endogenous Ror2 in unstimulated cells. Considering that other investigators had previously described the phenomena of signaling crosstalk and transactivation of cell surface receptors [397], I activated the DDR1 receptor in T47D cells by plating on collagen, but I did not detect any transactivation of Ror2.

The presence of Wnt-5a is essential for collagen-induced activation of DDR1 [331], whereas the influence of Wnt-5a on the activation status of endogenous Ror2 has not been studied. Therefore, I stimulated T47D cells with recombinant Wnt-5a and with the Foxy-5 peptide, which led to increases in both tyrosine and serine/threonine phosphorylation of Ror2. It seems likely that such phosphorylation of Ror2 is implemented by CK1 $\epsilon$ . Although that hypothesis has not yet been tested, it is supported by the following results obtained in other studies: Wnt-5a was shown to induce CK1 $\epsilon$  activation [398], and CK1 $\epsilon$  was found to be elicit serine/threonine phosphorylation of Ror2, which in turn caused autophosphorylation of tyrosine residues in Ror2 [255].

Next, I examined the effects of Ror2 on adhesion and migration. Upon siRNA-mediated downregulation of Ror2, the adhesion of T47D cells to both uncoated and collagen-I-coated surfaces was decreased, whereas migration was strongly increased. These observations fit nicely with the theory launched by DiMilla *et al.* [156], which states that cells that are firmly adherent, for example breast

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epithelial cells, must decrease the strength of the adhesion in order to augment migration [192]. I also found that downregulation of Ror2 led to an increase in cell number, which might reflect a survival-effect caused by parallel upregulation of DDR1 [156].

In conclusion, in this study I have found that Ror2 interacted with DDR1, and it underwent both tyrosine and serine/threonine phosphorylation by stimulation with either Wnt-5a or Foxy-5. In addition, breast epithelial cell adhesion was decreased and migration was increased by downregulation of Ror2. These findings suggest that a hitherto unknown interaction between DDR1 and Ror2 is to be added to the Wnt-5a signaling network.

## DISCUSSION

The findings of the present studies show that stimulation with Wnt-5a can induce a  $\text{Ca}^{2+}$  signal that leads to NFAT1 activation, although they also demonstrate that Wnt-5a provokes activation of Yes/Cdc42/CK1 $\alpha$  that results in phosphorylation, and thereby inhibition, of NFAT1. Furthermore, it was found that the Wnt-5a-mediated activation of Yes/Cdc42/CK1 $\alpha$  has the potential to counteract the NFAT activation induced by other agents that trigger an intracellular  $\text{Ca}^{2+}$  signal. The  $\alpha_6\beta_4$  integrin is known to be associated with poor prognosis in breast cancer patients [136], and other investigators have observed that that integrin induces invasiveness of breast cancer cells through NFAT activation [315]. That finding, together with the current data showing that Wnt-5a can inhibit NFAT activity, offers at least a partial explanation for how Wnt-5a can suppress migration and invasion in breast epithelial cells, effects of Wnt-5a that were described here and that have also been reported in previous publications by our research group [331].

Notably, we found that stimulation with Wnt-5a induced a  $\text{Ca}^{2+}$  signal, which refutes results recently published by other investigators [237]. This discrepancy could very well be due to technical aspects, such as the sensitivity or the settings of the instruments used, although it might also reflect cellular differences, particularly with regard to the expression of receptors and coreceptors.

It seems that partial clarification of the ability of Wnt-5a to inhibit migration is also provided by my observation that levels of actin stress fibers were elevated in breast epithelial cells treated with one of the peptides that mimicked the effects Wnt-5a on migration. This can be compared with earlier results from our laboratory, which revealed increased amounts of polymerized actin in colon cancer cells that were treated with recombinant Wnt-5a [360]. Just as the adhesive strength of the cells determines the migratory ability, the stiffness of the cytoskeleton may also be involved in regulation of that faculty. A balance between actin polymerization and depolymerization is a prerequisite for an efficient migratory response, consequently an elevated level of polymerized actin can very well impair cell migration [399-401]. Presumably, the increased stress fiber formation could be mediated by activation of Rho GTPases, some of which are known to be activated by Wnt-5a (Paper I and [219]).

Besides inhibiting NFAT activity and migration, Wnt-5a has also been shown to increase cell adhesion, both in the present studies as well as in previously published work [208, 286, 331]. Since migration is strictly regulated by the adhesive strength of the cells in question, it is plausible that increased adhesion in cells that are firmly adherent, for example in breast epithelium, may completely impair the ability to migrate [192]. Strongly adherent cells must instead reduce their attachment in order to be able to move, which agrees

with the present finding that downregulation of Ror2 in breast epithelial cells led to decreased adhesion and also dramatically augmented the migratory capacity of the cells.

If the main effect of Wnt-5a is to increase adhesion, and the impact on migration is a secondary feature of the amplified adhesive strength, this would give rise to different effects on migration depending on the basal adhesive strength of the cells. Such a scenario might explain why other investigators have found that Wnt-5a increased cell migration in melanoma cells and fibroblasts [251, 286], because melanoma cells are normally loosely adherent, and some of the fibroblasts used in the cited experiments were even non-adherent. In support of this theory, it was also noted that Wnt-5a stimulation increased the adhesion of the melanoma cells [286] and the non-adherent fibroblasts [208].

It should be pointed out that in many of the studies in which Wnt-5a was found to increase migration and invasion, the Wnt-5a was used as a chemoattractant (i.e., placed in the lower chamber of the invasion/migration system) [219, 251, 361]. In contrast, in investigations that demonstrate a decrease in migration or invasion upon stimulation with Wnt-5a (Paper II and III, and [360]), the protein was placed in the upper chamber together with the cells. Results shown in Paper III, reveal that Wnt-5a had no effect on the invasional capacity of the breast cancer cells when placed in the lower chamber of the invasion system. However, when placed in the upper chamber of the same system, Wnt-5a effectively inhibited invasion of the cells. These findings clearly indicate that these methodological differences are highly unlikely to explain the contradictory results in the literature concerning the effect of Wnt-5a on cell migration. A more plausible explanation could be related to variation in the expression of receptors, coreceptors, or signaling molecules, or differences in intracellular signaling response or other thus far unknown features. In a recent study of pancreatic cancer cells [355], it was found that Wnt-5a induced invasion and migration, and it also elicited Wnt/ $\beta$ -catenin signaling, but not PKC activation. Those observations indicate that the detected differences in migratory response discussed here may in fact be caused by the induction of dissimilar intracellular signals.

Incongruities regarding the ability of Wnt-5a to, among other things, induce  $\text{Ca}^{2+}$  signaling, activate Dvl, PKC, JNK, or NFAT proteins, or provoke TCF/LEF-mediated transcription are ubiquitous in the literature, including the present papers. For example, in the breast epithelial cell lines we used, Wnt-5a did not cause TCF/LEF-mediated transcription, unless LRP6 was overexpressed. These results agree with a previous study in which it was found that Wnt-5a inhibited Wnt-3a-induced TCF/LEF-mediated transcription, but when Frz-4 and LRP5 were overexpressed, Wnt-5a was instead able to provoke TCF/LEF-mediated transcription [237]. These observations suggest that

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receptor expression determines signaling specificity, and they also demonstrate that overexpression of LRP5/6 does lead to activation of the Wnt/ $\beta$ -catenin pathway.

My work has also identified a Wnt-5a-derived formylated hexapeptide that we named Foxy-5. This peptide can mimic the abilities of Wnt-5a to induce a  $\text{Ca}^{2+}$  response and to increase adhesion and decrease migration in breast epithelial cells. Interestingly, the effects of both Wnt-5a and Foxy-5 on migration were inhibited when incubating the cells with a Frz-5 antibody that had previously been shown to block the Wnt-5a/Frz-5 response [286, 287]. Most studies investigating the Wnt-5a-induced  $\text{Ca}^{2+}$  response have used Frz-2 as a model of this signal, implying either that the migratory inhibition is not mediated through  $\text{Ca}^{2+}$  activation or that Frz-5 is also capable of inducing a  $\text{Ca}^{2+}$  response. The latter of those ideas is supported by the previous finding that Wnt-5a induces PKC activation through the Frz-5 receptor [286].

We also found that the Foxy-5 peptide markedly inhibited 4T1 breast cancer metastasis to liver and lung in two different types of mice. This concurs with previous data showing that the expression of Wnt-5a protein in human breast tumors [150, 363] and colon cancer tumors [360] is associated with a good prognosis, longer disease-free survival, and increased overall survival. The inhibitory effect of Foxy-5 on metastasis formation is probably due to the ability of the peptide to decrease migration of breast cancer cells. That conclusion is based on my results showing that Foxy-5 caused no major changes in apoptosis or proliferation in experiments *in vitro* or metastatic cells *in vivo*, which agrees with a study of breast tumors performed in our research group in which expression of the Wnt-5a protein was not related to expression of the proliferation marker Ki-67 [150]. These observations are part of the above-mentioned discrepancies between experiments performed *in vitro*, the results of which show that Wnt-5a both inhibited [342, 347, 350, 351] and induced [247, 284, 353, 355] proliferation, and it both blocked apoptosis [215, 284] and had no effect on either apoptosis or proliferation [345, 357].

I also detected expression of the proposed Wnt-5a receptor Ror2 in the less invasive breast epithelial cell lines T47D and HB2. Despite the small number of samples used in those experiments, it can be speculated that the absence of Ror2 expression in the invasive cell lines, along with the inhibitory impact on breast epithelial cell migration, indicates that one of the functions of Ror2 is to prevent tumor progression. In support of that suggestion, Ror2 has been shown to participate in the ability of Wnt-5a to block the transforming Wnt/ $\beta$ -catenin pathway [237, 402]. Both Wnt-5a and Foxy-5 induced phosphorylation of Ror2, and such a tumor-suppressive function might contribute to the restraining influence of Wnt-5a on breast cancer metastasis. Arguing against this assumption is the recent finding showing that the presence of Ror2 is required for Wnt-5a migration in fibroblasts and malignant melanoma cells [251].

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Clearly, much additional work is needed to explain the mechanisms underlying all these contradictory findings.

## FUTURE PERSPECTIVES

Obviously, I have not investigated all potential effects of Foxy-5 on breast cancer metastasis. It is plausible that the inhibitory impact of this peptide on metastasis formation also involves other mechanisms that are not related to tumor cell migration. An example of this might be the capacity to counteract the Wnt/ $\beta$ -catenin pathway, which is known to promote tumorigenesis in several ways, such as by inducing cell transformation, angiogenesis and expression of MMPs and thereby increasing ECM breakdown and cell invasion [217, 349, 376]. The ability of Wnt-5a to inhibit the Wnt/ $\beta$ -catenin pathway has been studied extensively [237, 238, 340, 349], but it was not addressed in the present studies, and hence logical continuation of the current research would be to ascertain whether Foxy-5 can also exert this effect.

Importantly, Foxy-5 was able to inhibit formation of breast cancer metastases, presumably explained by its influence on tumor cell migration, which introduces the possibility of novel treatment strategies. While current treatment options mainly aim at inhibiting tumor cell growth or inducing apoptosis, the Foxy-5 peptide targets a specific step in the metastatic process, namely the tumor cell migration. However it must be noted that in my study, Foxy-5 was given at a much earlier stage than would be possible in patients. The next step would thus be to investigate whether Foxy-5 can impede metastasis formation even if it is administered at a later stage, that is, when the tumor is already established and potentially has started to seed cells into the lymphatics and the vascular system. In addition it would be interesting to investigate whether a cooperative metastasis inhibition could be achieved by simultaneous administration of Foxy-5 and an agent that has already been established in clinical treatment, such as the estrogen receptor modifier tamoxifen, the HER2 antibody or a chemotherapeutic drug.

## SUMMARY

The main findings of the present studies can be summarized as follows:

- ★ Wnt-5a induced a Ca<sup>2+</sup> signal, which led to NFAT activation. However, Wnt-5a also induced activation of Yes and Cdc42, resulting in CK1 $\alpha$ -mediated NFAT phosphorylation and inactivation.
- ★ Wnt-5a inhibited NFAT activation induced by other Ca<sup>2+</sup> signaling agents, and it also blocked breast epithelial cell invasion by inhibiting NFAT activity.
- ★ A formylated hexapeptide, Foxy-5, was identified based on secondary structure prediction of Wnt-5a. Foxy-5 could mimic the abilities of Wnt-5a to induce Ca<sup>2+</sup> release and to increase cell adhesion and decrease cell migration.
- ★ Wnt-5a and Foxy-5 inhibited breast epithelial cell migration and invasion via the Frz-5 receptor, but they did not affect proliferation or apoptosis.
- ★ Intraperitoneal injection of Foxy-5 every fourth day inhibited metastasis of 4T1 breast cancer cells to lung and liver in both athymic and normal BALB/c mice.
- ★ Ror2 was expressed in less invasive breast epithelial cell lines, and siRNA-mediated downregulation of Ror2 led to decreased adhesion and increased migration.
- ★ Ror2 associated with, but was not transactivated by, DDR1.
- ★ Both Foxy-5 and Wnt-5a induced tyrosine and serine/threonine phosphorylation of Ror2.

## **POPULÄRVETENSKAPLIG SAMMANFATTNING**

Bröstcancer är den vanligaste tumörformen hos kvinnor i Sverige och västvärlden. Varje år drabbas cirka 7000 kvinnor och antalet som får bröstcancer har ökat sedan flera årtionden tillbaka. Överlevnaden hos bröstcancerpatienter är hög jämfört med många andra cancertyper, ungefär 80% överlever mer än fem år efter diagnos. Om tumören är liten och inte spridit sig till andra delar av kroppen är chanserna stora att bota bröstcancer. Om brösttumören däremot redan spridit sig när den upptäcks eller kommer tillbaka en tid senare i form av dottertumörer i lever, lungor eller skelett, är cancer svårare att bekämpa och överlevnadstiden i genomsnitt kort. Mycket kraft riktas därför på att försöka förhindra att tumören återkommer, genom att t.ex. tilläggsbehandla med strålning och läkemedel efter att brösttumören opererats bort, för att på det viset utplåna eventuellt kvarvarande tumörceller.

Bildandet av spridda tumörer är en flerstegsprocess som kräver flera förmågor hos cancercellerna. Förutom de förmågor cellerna behöver ha för att bli cancerceller, som t.ex. att de kan dela sig oändligt antal gånger, att de växer fort, att de bildar sina egna tillväxtproteiner och att de kan få blodkärl att växa in till sig så att de får näring, behöver de ytterligare några förmågor för att kunna sprida sig till en annan plats i kroppen. En av de förmågorna är att reglera hur hårt de sitter fast i omgivande vävnad. För att bilda tumörer på andra ställen i kroppen behöver cancercellerna kunna vandra iväg mot blodkärl eller lymfkärl, ta sig in i kärlet och transporteras med blodet eller lymfan till en ny omgivning där de kan vandra in, fortsätta dela sig och på så sätt bilda en ny tumör. Om de däremot sitter hårt fästa vid andra celler och den omgivande bindväven i bröstkörteln har de svårare för att förflytta sig och därmed mindre möjlighet att bilda dottertumörer på andra platser i kroppen.

Ett protein som verkar vara viktigt för att förhindra spridning av bröstcancer är Wnt-5a. Wnt-5a tillverkas inne i cellerna, släpps ut så fort det är tillverkat, för att kunna binda till mottagarämnen, som sitter på cellernas yta. Då Wnt-5a bundit till mottagarämnet skickas signaler in i cellen som berättar hur cellen ska bete sig. Wnt-5a finns normalt sett i bröstvävnaden och får cellerna att fästa hårdare till den omgivande bindväven och att minska sin rörelseförmåga. Bröstceller som har Wnt-5a stannar alltså på sin plats i bröstkörteln i högre utsträckning. När vår forskargrupp undersökte om Wnt-5a också finns i brösttumörer, upptäcktes det att bara cirka hälften av brösttumörerna hade normal mängd Wnt-5a. I de andra brösttumörerna fanns det för lite Wnt-5a-protein och de patienterna fick fortare och i högre utsträckning tumörer på andra ställen i kroppen. Det verkade alltså som att låg nivå av Wnt-5a hade samband med tumörernas spridning.

Mitt projekt började med att vi undersökte vilka signaler som aktiveras inne i cellen då Wnt-5a binder till sitt mottagarämne. I den första artikeln studerade

vi hur Wnt-5a påverkar ett protein som heter NFAT. Aktivering av NFAT är sedan tidigare kopplat till en ökad rörelseförmåga hos bröstcancer celler och till spridning av bröstcancer. Vi visar att behandling av cellerna med Wnt-5a leder till frisättning av kalcium inuti cellerna och den ökade nivån av kalcium leder i sin tur till en svag aktivering av NFAT. Men vi såg också att Wnt-5a samtidigt hade möjlighet att minska aktiviteten hos NFAT i cellerna genom att aktivera andra signalproteiner; Src, Cdc42 och CK1 $\alpha$ . Detta betyder alltså att om NFAT aktiveras kraftigt på något annat sätt i cellerna, kan Wnt-5a behandling hämma denna aktivering. Wnt-5a verkar alltså kunna minska rörelseförmågan hos bröstcancer cellerna och därmed spridningen av bröstcancer genom att reglera aktiviteten hos NFAT.

Därefter funderade vi på om det finns möjlighet att förhindra bröstcancer från att sprida sig genom att behandla med Wnt-5a. Att behandla med hela Wnt-5a proteinet skulle troligen inte fungera, eftersom Wnt-5a dels fastnar på de första cellerna som träffas på och därför inte skulle nå bröstcellerna och dels är väldigt svårt och dyrt att framställa. En bättre metod verkade vara att istället försöka hitta en mindre proteindel som härmar Wnt-5a. I andra artikeln fick vi därför hjälp av en forskare i Paris att analysera Wnt-5a proteinets troliga struktur och fick av honom förslag på olika delar av proteinet som sannolikt var exponerade på proteinets utsida och därför kan ha möjlighet att interagera med mottagarämnet. Vi testade 14 olika proteindelar, var och en likadan som en liten bit av Wnt-5a proteinet, för att se om någon av proteindelarna kunde få cellerna att fästa bättre till underlaget och därigenom få dem att röra sig mindre. Vi hittade två proteindelar som fick cellerna att både fästa bättre och röra sig mindre. Vi upptäckte också att en trolig anledning till att cellerna rörde sig mindre var att de bildade mycket nytt cellskelett när vi gav dem proteindelen, vilket antagligen gjorde cellerna stelare och mindre rörliga. Därefter kortade vi ner proteindelen successivt och upptäckte att en hälften så stor proteindel, som vi kallar Foxy-5, fungerade lika effektivt som den längre som vi först upptäckte.

I den tredje artikel undersökte vi om Foxy-5 och Wnt-5a påverkade några av de andra förmågor som är viktiga för att cancer celler ska kunna bilda tumörer på annan plats i kroppen, nämligen tillväxten och förmågan att undvika programmerad självdöd, s.k. apoptos, hos cellerna. Wnt-5a och Foxy-5 verkade dock varken påverka tillväxt eller apoptos hos cellerna, utan bara hindra rörelseförmågan. Vi undersökte därefter om behandling med Foxy-5 kunde påverka hur mycket bröstcancer som spred sig till andra ställen i kroppen, vilket gjordes i två olika typer av möss, en mustyp som saknar immunförsvar och en mustyp med normalt immunförsvar. Mustypen som saknar immunförsvar är vanlig vid cancer forskning, därför började vi med den. Men avsaknad av immunförsvar kan påverka tumörernas tillväxt och spridning, så vi valde att göra försöket även i en mustyp som har normalt immunförsvar.

### *Populärvetenskaplig sammanfattning*

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När vi gav Foxy-5 till mössen var fjärde dag fick mössen väldigt mycket färre och mindre tumörer i både lungorna och levern än de kontrollmöss som behandlats med saltlösning eller kontrollproteindelar. Foxy-5 hämmade bildandet av spridda tumörer i lika stor utsträckning i båda mustyperna.

I den fjärde artikeln gick vi djupare in på Ror2, ett protein som sitter på ytan av cellerna och tidigare visats vara nödvändig för att Wnt-5a ska ha effekt. Vi såg att Ror2, liksom Wnt-5a har betydelse för rörelseförmågan hos cellerna och såg också att Ror2 kan binda till DDR1, ett mottagarämne som fäster till bindvävsproteinet kollagen och ökar bröstcellernas vidhäftningsförmåga i närvaro av Wnt-5a. Aktivering av DDR1 påverkade inte aktiviteten hos Ror2, däremot aktiverades Ror2 av Wnt-5a.

Sammanfattningsvis har vi funnit att Wnt-5a hämmar rörelseförmågan hos bröstcancerceller. Orsaken till att Wnt-5a minskar rörelseförmågan ligger bland annat i att det reglerar aktiviteten hos NFAT och förändrar cellens inre skelett. Vi har också hittat en interaktion mellan två mottagarämnen som tidigare visats vara inblandade i Wnt-5as effekter var för sig, DDR1 och Ror2, och sett att Ror2 påverkar rörelseförmågan hos bröstcancercellerna. Vi har tagit fram en proteindel, Foxy-5, som ökar vidhäftningsförmågan till bindväven i bröstkörteln och minskar rörelseförmågan hos bröstcancerceller. Slutligen har vi också sett att Foxy-5 kan hindra spridningen av bröstcancer till lever och lunga i två olika mustyper, både i en som saknar immunförsvar och i en som har normalt immunförsvar. Dessa resultat stärker kunskapen kring Wnt-5a och dess effekter på bröstcancerceller och visar på en ny möjlighet för att specifikt behandla spridningen av bröstcancer.

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