



# LUND UNIVERSITY

## The impact of Wnt5a signaling and tumor associated macrophages in breast cancer

Hagerling, Catharina

2012

[Link to publication](#)

### *Citation for published version (APA):*

Hagerling, C. (2012). *The impact of Wnt5a signaling and tumor associated macrophages in breast cancer*. [Doctoral Thesis (compilation), Department of Laboratory Medicine]. Department of Laboratory Medicine, Lund University.

### *Total number of authors:*

1

### **General rights**

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

### **Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117  
221 00 Lund  
+46 46-222 00 00

Department of Laboratory Medicine, Center for Molecular Pathology,  
Skånes University Hospital Malmö, Lund University, Sweden

# The impact of Wnt5a signaling and tumor associated macrophages in breast cancer

Catharina Medrek, MD



**LUND**  
UNIVERSITY

## **Academic dissertation**

By due permission of the Faculty of Medicine, Lund University, Sweden to be defended at the main lecture hall, Dept of Pathology, Skåne University Hospital, Malmö, on Wednesday 26<sup>th</sup> of September, 2012, at 13.00 for the degree of doctor of Philosophy, Faculty of Medicine

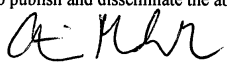
## **Faculty opponent:**

Professor Maréne Landström  
Department of Medical Biosciences,  
Umeå University, Umeå, Sweden

Organization LUND UNIVERSITY		Document name DOCTORAL DISSERTATION	
Department of Laboratory Medicine, Malmö Division of Experimental Pathology		Date of issue 2012-09-26	
Author(s) Catharina Medrek		Sponsoring organization	
Title and subtitle The impact of Wnt5a signaling and tumor associated macrophages in breast cancer			
Abstract <p>Breast cancer is the most common cancer among women worldwide with approximately 1.150.000 new cases each year and accounting for over 400.000 deaths per year. The main cause of death for women with breast cancer is secondary tumors.</p> <p>Downregulation of Wnt5a in primary ductal breast cancer has been correlated with poor outcome and higher tumor grade and found to be an independent predictor of recurrence. The ability of Wnt5a to inhibit tumor progression can partly be explained by Wnt5a induced cell-extracellular adhesion that inhibits cell migration. We found that Wnt5a can further inhibit tumor progression by inducing cell-cell adhesion through CK1<math>\alpha</math>-induced Ser-45 phosphorylation of <math>\beta</math>-catenin promoting <math>\beta</math>-catenin/E-cadherin complex formation, hence in line with prior data indicating a beneficial effect of Wnt5a in breast cancer.</p> <p>Macrophages are part of the innate immune system and they can differentiate into tumoricidal pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages. The anti-inflammatory M2 macrophages will limit pro-inflammatory activity that in abundance would cause additional tissue damage. Tumor associated macrophages (TAMs) have many features in common with M2 macrophages; they are anti-inflammatory and have a weak tumoricidal capacity. We show that Wnt5a induces an anti-inflammatory tolerogenic macrophage phenotype in a pro-inflammatory environment and we could validate our <i>in vitro</i> data by showing the clinical relevance in both breast cancer and sepsis patients.</p> <p>The CD163 marker has been reported to recognize M2 macrophages, while CD68, on the other hand, is a frequently used pan-macrophage marker that recognizes both pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages. We evaluated CD163 as a TAM marker in human breast cancer and compared it to CD68. We revealed and could highlight the clinical importance of analyzing the localization of TAMs in human breast cancer. While TAMs in the tumor nest did not have any correlation with clinicopathological features or patient outcome, we found TAMs in tumor stroma to be highly relevant. CD163<sup>+</sup> TAMs in tumor stroma correlated with unfavorable clinicopathological features, and dense infiltration of CD68<sup>+</sup> TAMs in tumor stroma was an independent risk factor for reduced breast cancer specific survival.</p>			
Key words Breast cancer, Wnt5a, E-cadherin, $\beta$ -catenin, migration, invasion, tumor associated macrophages, CD163, CD68, tumor stroma, Classification system and/or index terms (if any)			
Supplementary bibliographical information		Language	
ISSN and key title 1652-8220		ISBN	
Recipient's notes		70	Price
		Security classification	

Distribution by (name and address)

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature 

Date May 2012

# The impact of Wnt5a signaling and tumor associated macrophages in breast cancer



**LUND**  
UNIVERSITY

Catharina Medrek, MD



Copyright © Catharina Medrek

Department of Laboratory Medicine, Center for Molecular Pathology,  
Skånes University Hospital Malmö, Lund University, Sweden

ISBN 978-91-87189-30-2

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University  
Lund 2011

*In memory of my beloved mother*

“A wise man should consider that health is the  
greatest of human blessings”

Hippocrates (c.460 - 370 BC)



# Contents

List of Papers	9
List of abbreviations	10
Background	13
Cancer	13
Acquired capabilities of cancer	15
E-cadherin/ $\beta$ -catenin complex	19
Regulation of the E-cadherin/ $\beta$ -catenin complex	19
Wnt	21
The canonical, $\beta$ -catenin dependent pathway	21
The non-canonical, $\beta$ -catenin independent pathways	23
Wnt5a	25
Tumor immunology in brief, the 7 <sup>th</sup> hallmark	26
Leukocytes in the tumor microenvironment	26
Tumor associated macrophages	28
Breast anatomy and development	31
Breast cancer	33
Breast cancer pathology and diagnostics	33
Breast cancer treatment	34
The Present Investigation	37
Aims	37
Results and Discussion	38
Paper I	38
Paper II	40
Paper III	42
Conclusions	44
Populärvetenskaplig sammanfattning	45
Acknowledgements	47
References	49



# List of Papers

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

- I. Wnt5a-CK1 $\alpha$  signaling promotes  $\beta$ -catenin/E-cadherin complex formation and intercellular adhesion in human breast epithelial cells  
*Catharina Medrek, Göran Landberg, Tommy Andersson and Karin Leandersson*  
J Biol Chem. 2009 Apr 17;284(16):10968-79
- II. Wnt5a induces a tolerogenic phenotype of macrophages in sepsis and breast cancer patients  
*Caroline Bergenfelz, Catharina Medrek, Elin Ekström, Karin Jirström, Helena Janols, Marlene Wullt, Anders Bredberg, and Karin Leandersson*  
J Immunol. 2012 Jun 1; 188(11):5448-58
- III. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients  
*Catharina Medrek, Fredrik Pontén, Karin Jirström and Karin Leandersson*  
BMC Cancer. 2012 Jul 23; 12:306

Reprints were made with permission from the publishers.

# List of abbreviations

G	gap phase
S	synthesis phase
M	mitotic phase
CDKs	cyclin-dependent kinases
EGFR	epidermal growth factor receptor
HER2	human epidermal growth factor 2 receptor
EGF	epidermal growth factor
TNF $\alpha$	tumor necrosis factor- $\alpha$
TGF $\beta$	transforming growth factor- $\beta$
pRb	retinoblastoma protein
TERT	telomerase reverse transcriptase catalytic subunit
VCAM1	vasculature cell adhesion molecule-1
TAMs	tumor associated macrophages
CAFs	cancer associated fibroblasts
IGF1/2	insulin-like growth factor-1 and 2
IGFR	insulin-like growth receptor
FGF	fibroblast growth factor
VEGF	vascular endothelial growth factor
TSP1	thrombospondin-1
EMT	epithelial to mesenchymal transition
MMPs	metalloproteinases
ECM	extracellular matrix
MET	mesenchymal to epithelial transition
AJ	adherens junctions

Ser	serine residue
CK	casein kinase
GSK3 $\beta$	glycogen synthase kinase 3 $\beta$
Tyr	tyrosine residue
Thr	threonine residue
FZD	frizzled
APC	adenomatous polyposis coli
LRP	low-density-lipoprotein receptor related protein
Dvl	dishevelled
ROCK	Rho-associated kinase
PLC	phospholipase
PIP <sub>2</sub>	phosphatidyl inositol 4,5-biphosphate
IP3	inositol 1,4,5
DAG	1,2 diacylglycerol
PKC	protein kinase C
CaMKII	calmodulin dependent protein kinase II
WIF	Wnt inhibitory protein
sFRPs	secreted Frizzled-related proteins
DKK	dickkopf
DDR1	discoidin domain receptor
DAMPs	damage associated molecular pathways
PAMPs	pathogen associated molecular patterns
T <sub>H</sub> 1	helper 1 cells
T <sub>H</sub> 2	helper 2 cells
IL	interleukin
IFN $\gamma$	interferon $\gamma$
T <sub>C</sub>	cytotoxic T cells
DC	dendritic cells
NK	natural killer
T <sub>reg</sub>	regulatory T cells

MDSC	myeloid-derived suppressor cells
iNOS	nitric oxide synthase
TLR	toll like receptor
CCL2	chemokine CC-motive ligand 2
CSF	colony-stimulating factor
E <sub>2</sub>	estrogen
Pg	progesterone
WHO	world health organization
NHG	Nottingham histological grade
NPI	Nottingham prognostic index
T	size of primary tumor
N	number of infiltrated axillary lymph nodes
M	occurrence of metastases
ER	estrogen receptor
PgR	progesterone receptor
SERM	selective estrogen receptor modulator
AI	aromatase inhibitors
TMA	tissue microarray
TS	tumor stroma
TN	tumor nest
IHC	immunohistochemistry
GRN	granulin

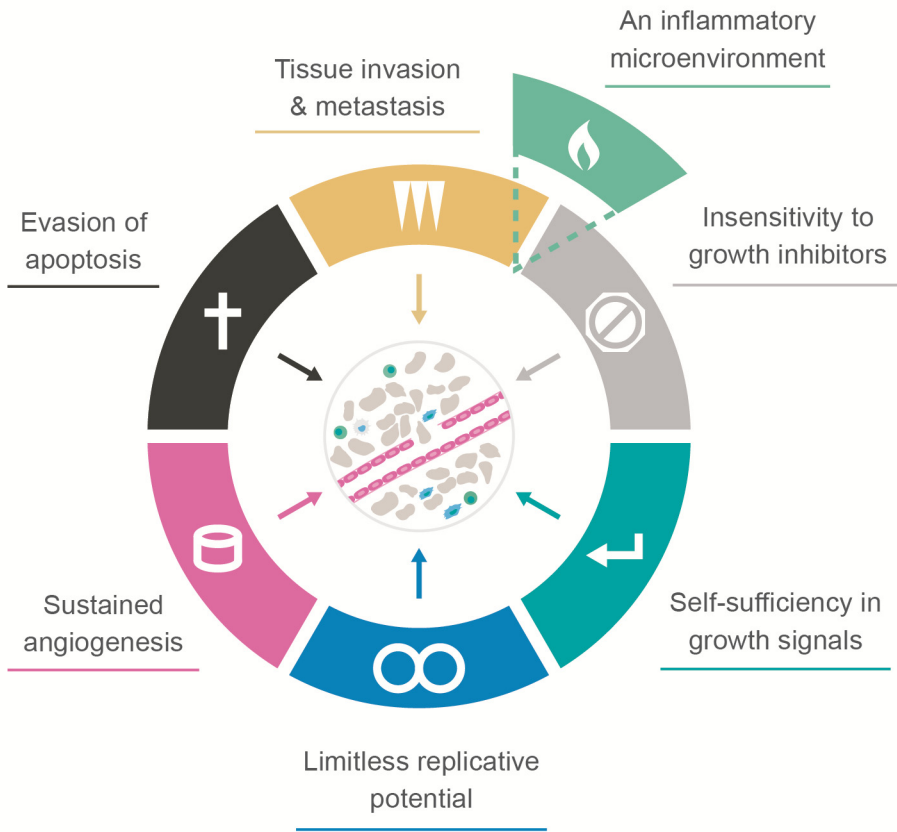
# Background

## Cancer

Hippocrates (460-370 BC) coined the term carcinoma that was later translated from Greek to the Latin word cancer. Cancer, describing a malignant tumor in the body, caused 7,6 million cancer deaths worldwide four years ago and while the incidence of cancer is declining in some parts of the world (western countries) other regions (economically developing countries) have seen increased incidence<sup>1,2</sup>. Tobacco and diet are two major factors behind cancer and additionally infectious agents play a major role in cancer development in economically developing countries<sup>2</sup>. The two most frequently diagnosed cancers in men are lung and prostate cancer, while breast cancer is the leading cancer affecting women worldwide<sup>3,4</sup>. More than 10 million men and women are diagnosed with cancer annually<sup>2,4</sup>.

Tumorigenesis is characterized by progressive changes on the genetic, epigenetic and cellular level. Briefly, for a normal cell to become malignant it will have to become genetically modified (by mutation or through chromosomal abnormality). Genes with an ability to promote cancer, called oncogenes, can be modified in a way that make them constitutively active and affect the expression or structure of proteins that normally promote, for example, cell division. In addition there are other genes normally aiding DNA repair or inhibiting cell proliferation that can be inactivated. These genes have an antitumoral function and are called tumor suppressor genes.

The genetic and epigenetic changes will provide the once normal cell with acquired capabilities, called the hallmarks of cancer, which include sustained proliferative signaling, evasion of growth suppression, resistance to cell death, replicative immortality and induction of angiogenesis, invasion and metastasis, making it malignant (Figure 1)<sup>5</sup>. The acquired capabilities of cancer and the involvement of the tumor microenvironment will be described in brief and exemplified, by typical molecular factors, for each hallmark. In addition, an inflammatory microenvironment, which is suggested to be the 7th hallmark of cancer (Figure 1), will be described in more detail later<sup>6</sup>.

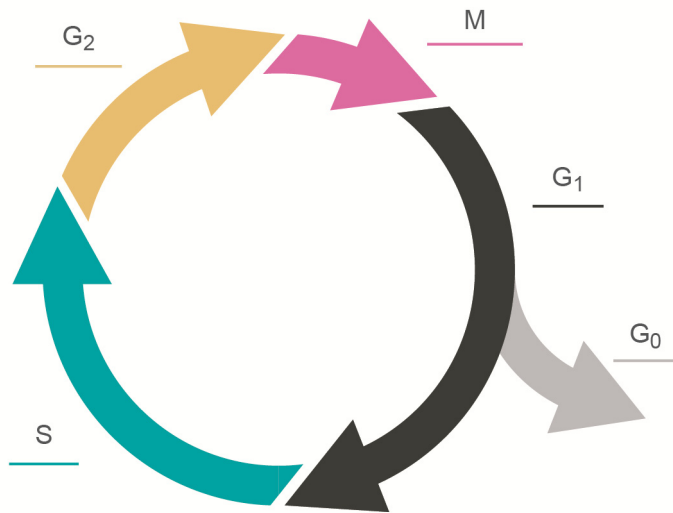


**Figure 1.** The hallmarks of cancer. (Adapted from Hanahan *et al.* and Mantovani<sup>5,6</sup>.)

## *Acquired capabilities of cancer*

### **Control of proliferation**

Cell proliferation, the growth and division of a cell into daughter cells, is achieved when cells advance through the cell cycle. The cell cycle is divided into the gap phase one (G<sub>1</sub>), synthesis phase (S), gap phase two (G<sub>2</sub>) and finally the mitotic phase (M) (Figure 2). The cell is metabolically active during the G<sub>1</sub> phase and eventually enters the S phase, during which the DNA will duplicate. During the G<sub>2</sub> phase the proteins needed for cell division during the M phase are synthesized. The advancement through the cell cycle is controlled by cyclins and cyclin-dependent kinases (CDKs), which monitor checkpoints in the cell cycle. Growth factors stimulate the cell to leave the non-proliferative state (G<sub>0</sub>) and to enter the cell cycle<sup>7,8</sup>.



**Figure 2.** The cell cycle. (Adapted from Weinberg<sup>8</sup>.)

A normal cell depends on the surrounding environment to provide it with proper growth factors. Cell proliferation in a normal tissue can in principle be controlled in a paracrine way where growth factors are produced by nearby cells or by growth factors secreted into the blood circulation by endocrine organs<sup>7</sup>. There are different ways in which cancer cells can initiate proliferation independently of the surrounding environment. To begin with, they can control their proliferation in an autocrine manner by producing their own growth factors. They can further upregulate growth factor receptors, often tyrosine kinase receptors, making the cancer cells react to even low concentrations of growth factors that normally would not initiate proliferation<sup>5,9</sup>. Examples of two tyrosine kinase growth factor receptors upregulated in different cancer types including breast cancer are; the epidermal growth factor receptor (EGFR) and the human epidermal growth factor 2 receptor (HER2/ERBB2)<sup>9,10</sup>. Both EGFR and HER2 can be structurally altered in cancer making them constitutively active and thus capable of emitting proliferation signals independent of growth factors<sup>8</sup>. Examples of growth factors important in cancer are; epidermal growth factor (EGF), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and transforming growth factor- $\beta$  (TGF $\beta$ ). These can be provided by leukocytes and fibroblasts located in the tumor microenvironment<sup>11</sup>. Breast tumor cells have however also been found to produce high levels of TGF $\beta$ <sup>12</sup>. TGF $\beta$  can phosphorylate the retinoblastoma protein (pRb), an important tumor suppressor, and thereby inhibit the antiproliferative function (prevention of advancement through the cell cycle) of pRb<sup>9</sup>. c-Myc, on the contrary, is an important oncogene that promotes proliferation and is upregulated in many cancer types, including breast cancer<sup>9,13</sup>.

### **Unlimited replication**

Normal cells only have a limited proliferative capacity. Telomeres at the end of chromosomes will protect the chromosome ends from fusing together. The telomeres will however shorten for each cell division and inevitably the chromosomes ends will fuse preventing further cell proliferation. Telomerase is a protein complex, including the telomerase reverse transcriptase catalytic subunit (TERT), that elongates telomeric DNA and can prevent telomeres from eroding<sup>14</sup>. Although telomerase is extremely rare in normal cells (only found in germ line and somatic stem cells) 90% of all malignancies, including breast cancer, have functional levels which give cancer cells replicative immortality<sup>9,14,15</sup>. The gene coding for TERT has furthermore been found to be a direct target of c-Myc<sup>16</sup>.

### **Evading cell death**

For a tumor to expand it will not only have to take control of proliferation but, equally important, it will have to evade the apoptotic program that can be triggered by both intra- and extracellular factors. DNA damage, abnormal growth or hypoxia, in a normal cell, will cause upregulation of the tumor suppressor p53, which in turn will initiate production of Bax, a proapoptotic protein belonging to the

Bcl-2 family<sup>9</sup>. Bax provokes leakage of cytochrome c and other proapoptotic proteins from mitochondria, which will cause cell death<sup>5</sup>. More than 50% of human cancers have an inactive p53 pathway, due to a mutation of the p53 tumor suppressor gene, and are hence able to evade the apoptotic program<sup>9,17</sup>. Approximately 35% of all human breast cancers have a mutant p53 gene<sup>18</sup>. Cancer can further evade programmed cell death by having a mutation in the gene encoding for Bax or through upregulation of the antiapoptotic protein Bcl-2 which has the ability to inhibit Bax<sup>5,8</sup>.

The tumor microenvironment can also affect the survival of cancer cells. Interaction between the vasculature cell adhesion molecule-1 (VCAM1) on breast cancer cells and  $\alpha$ 4-integrin on the cell surface of tumor associated macrophages (TAMs) have been found to inhibit the apoptotic program<sup>11</sup>. In addition cancer associated fibroblasts (CAFs) help the cancer cells to evade the apoptotic program by producing insulin-like growth factor-1 and 2 (IGF1/2)<sup>19</sup>. IGF1/2 are survival factors that bind to the insulin-like growth factor receptor (IGF1R) on epithelial cells which will upregulate the antiapoptotic factor Bcl-2 and downregulate Bax<sup>20</sup>.

### **Angiogenesis**

A tumor cannot grow larger than 1-2 mm in diameter without the participation of angiogenesis, the formation of new blood capillaries<sup>21</sup>. Cancer cells can promote angiogenesis by either upregulation of proangiogenic or downregulation of antiangiogenic factors<sup>9</sup>. Fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF) are two proangiogenic factors that are frequently upregulated in cancer and that can promote angiogenesis by binding to tyrosine kinase receptors on endothelial cells<sup>22</sup>. VEGF is upregulated by hypoxia and produced by both fibroblasts and leukocytes, in particular TAMs, in the tumor microenvironment<sup>5,11</sup>. In 2004 Konency *et al* reported that upregulation of VEGF in breast cancer correlated with a more aggressive phenotype and poor outcome<sup>23</sup>. The proangiogenic event induced by VEGF and FGF signaling can however be counteracted by the antiangiogenic factor thrombospondin-1 (TSP1)<sup>5</sup>. Downregulation of TSP1 has been correlated with malignant progression in breast cancer<sup>22</sup>.

### **Invasion and metastasis**

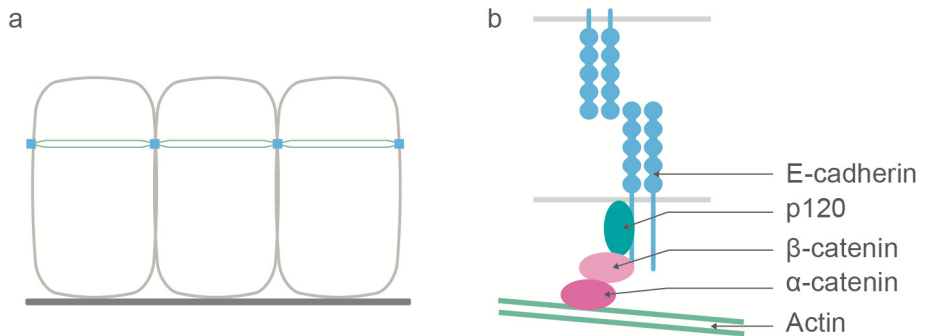
The main cause of death among men and women with cancer will not be the primary tumor but the secondary tumors formed<sup>1</sup>. A cancer cell in a primary carcinoma tumor will have to acquire already mentioned capabilities and in addition gain the ability to move and invade surrounding tissue in order to reach the circulation and form secondary tumors. It has been proposed that invading cells mimic the epithelial to mesenchymal transition (EMT) that normally occurs during embryonic development and wound healing<sup>24</sup>. Epithelial cells are characterized by their polarity, lack of motility and are normally tightly

interconnected by E-cadherin, a homophilic interaction molecule that inhibits proliferation and antagonizes invasion. Loss of E-cadherin function, frequently found in cancer, is regarded as a fundamental feature of EMT and can be caused by deletion or mutation of the E-cadherin gene *CDH1*<sup>25</sup>. The majority of infiltrating lobular breast carcinomas have an E-cadherin mutation<sup>26</sup>. Inadequate E-cadherin function can further be due to inhibition of E-cadherin expression by promoter DNA methylation or by transcriptional repressors such as snail and ZEB, which are both correlated to poor clinical outcome in human breast cancer<sup>25,27</sup>. Snail and ZEB not only affect E-cadherin but are important EMT inducers that alter cell polarity and induce production of metalloproteinases (MMPs)<sup>24</sup>. Leukocytes in the tumor microenvironment further contribute to MMP production which causes extracellular matrix (ECM) degradation and facilitates tumor invasion<sup>11</sup>.

Once a malignant cell with an epithelial origin has detached from the primary tumor and invaded the ECM it might encounter and enter the newly formed permeable blood and lymphatic vasculature. The migration of the malignant cell toward the circulation will be facilitated by TAMs, but the probability of it dying in the circulation will be high<sup>11,28</sup>. Only a small fraction of the cancer cells that reach the circulation survive and have the ability to extravasate and form secondary tumors<sup>28</sup>. The secondary tumors are histopathologically similar to the primary tumors, contrary to what one would expect according to the EMT theory<sup>27</sup>. The reverse mesenchymal to epithelial transition (MET), normally occurring during embryonic development might give an explanation to this EMT contradiction. The E-cadherin gene *CDH1* will for example be silenced in the primary breast cancer tumor, in line with EMT, but activated back again in the secondary tumor via MET<sup>29,30</sup>.

The formation of secondary tumors and their particular location depends on growth factors (e.g. VEGF), produced by the primary tumor, that can upregulate inflammatory chemokines in the pre-metastatic niche and that in turn attract bone marrow-derived haematopoietic progenitor cells of myeloid lineage<sup>31</sup>. The pre-metastatic niches will provide a beneficial environment that recruits circulating cancer cells and allow them to once again take advantage of the acquired capabilities of cancer<sup>31,32</sup>.

# E-cadherin/ $\beta$ -catenin complex



**Figure 3.** a. Polarized epithelium interconnected by homophilic E-cadherin binding in adherens junctions (AJ; in blue). b. The E-cadherin/catenin complex in the cell membrane. (Adapted from Wodarz *et al.* and Baum *et al.*<sup>33,34</sup>.)

Ducts in the normal mammary gland are lined with bilayered epithelial cells around a central lumen<sup>35</sup>. The cell-polarity in the epithelial layer is defined by, and depend on apical adherens junctions (AJ) which bind epithelial cells together via E-cadherin, a calcium ( $\text{Ca}^{2+}$ ) dependent molecule belonging to the cadherin family (Figure 3a)<sup>33</sup>. E-cadherin has an extracellular domain that forms a homophilic connection between adjacent epithelial cells<sup>34</sup>. The juxtamembrane part of E-cadherin binds to p120, which prevents E-cadherin degradation<sup>36</sup>. The cytoplasmic domain of E-cadherin binds to  $\beta$ -catenin<sup>34</sup>.  $\beta$ -catenin in turn interacts with  $\alpha$ -catenin, creating a dynamic link between the E-cadherin/catenin complex and the actin network (Figure 3b)<sup>34,37,38</sup>. The importance of the complex can be illustrated for example, in breast cancer, where low membrane association of  $\beta$ -catenin was reported to correlate with poor outcome<sup>39</sup>.

## *Regulation of the E-cadherin/ $\beta$ -catenin complex*

An intact E-cadherin/ $\beta$ -catenin complex contributes to epithelial integrity and promotes cell-cell adhesion<sup>25</sup>. Decomposition of the complex, on the contrary, favors tumor progression due to loss of adhesion and induced tumor invasion<sup>25</sup>. A reduction in the number of complexes can be caused by TGF $\beta$  and Wnt signaling which upregulate factors (e.g. Snail and Slug) that inhibit E-cadherin transcription. Alternatively it can due to loss of p120 that leads to E-cadherin destabilization and

degradation<sup>40</sup>. However, regulation of the E-cadherin/ $\beta$ -catenin complex is primarily due to changes in phosphorylation of E-cadherin and  $\beta$ -catenin<sup>40</sup>.

The binding affinity of E-cadherin for  $\beta$ -catenin is strengthened through phosphorylation of E-cadherin on serine residue 834 (Ser 834), Ser 836 and Ser 842 by casein kinase 2 (CK2) and glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ). Phosphorylation of E-cadherin on Ser 846 by CK1, on the contrary, inhibits the interaction between E-cadherin and  $\beta$ -catenin<sup>41</sup>. The binding between E-cadherin and  $\beta$ -catenin will further depend on the cleavage of E-cadherin by intra- and extracellular proteases. Upon proteolytic cleavage, E-cadherin is unable to bind to  $\beta$ -catenin and the unattached  $\beta$ -catenin translocate to the nucleus where it can activate canonical Wnt target genes i.e. c-Myc and cyclin D that promote tumor progression<sup>36</sup>. Hence,  $\beta$ -catenin will on the one hand inhibit tumor progression at the cell membrane, but on the other hand promote and trigger tumor progression in the nucleus. The function of  $\beta$ -catenin is highly dependent on the phosphorylation status of the protein and whether or not canonical Wnt signaling can recruit  $\beta$ -catenin from the cell membrane to activate Wnt target genes has long been debated<sup>42</sup>.

Fyn and Fer are two tyrosine kinases that can bind to p120 at the cell membrane and phosphorylate  $\beta$ -catenin on tyrosine residue 142 (Tyr 142) located at the N-terminal<sup>43</sup>. Tyr 142 phosphorylated  $\beta$ -catenin preferentially binds to the co-factor B-cell lymphoma 9-2 (BCL9-2) and to TCF/LEF in the nucleus where the complex enhance activation of canonical Wnt target genes<sup>44,45</sup>.  $\beta$ -catenin with unphosphorylated Tyr 142 will, on the other hand, dimerize with  $\alpha$ -catenin and facilitate binding to E-cadherin at the cell membrane. The phosphorylation status of tyrosine residue 142 hence determines what partner  $\beta$ -catenin preferentially binds and what role  $\beta$ -catenin will have in the cell<sup>45</sup>. In addition, phosphorylation of Tyr 654 on  $\beta$ -catenin will interfere with the ability of  $\beta$ -catenin to bind to the cytoplasmic domain of E-cadherin leading to loss of the complex at the cell membrane, while phosphorylation on threonine residue 112 (Thr 112) and Thr 120 promotes interaction<sup>46,47</sup>.

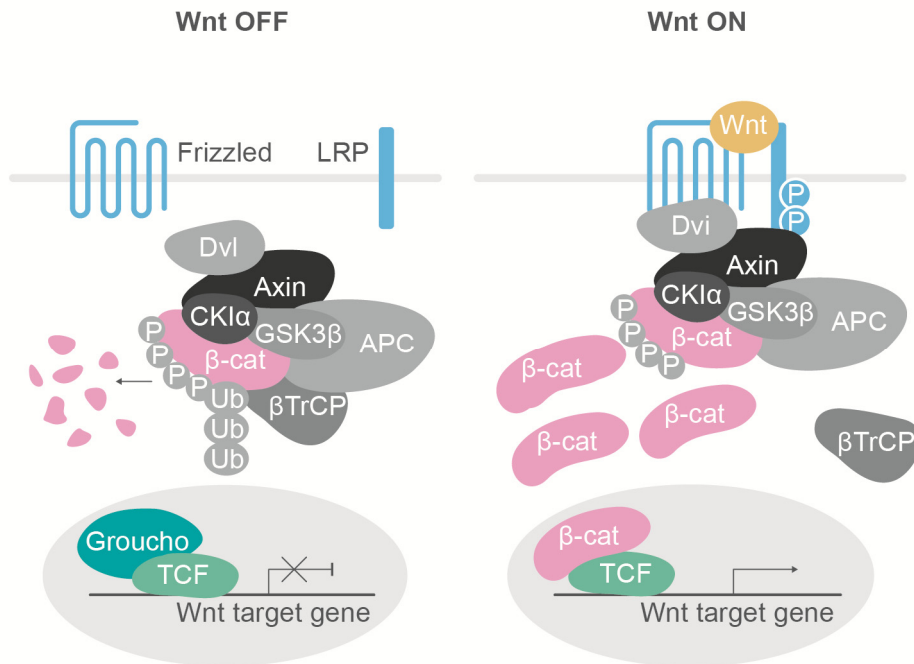
Canonical signaling prevents  $\beta$ -catenin from binding to E-cadherin through the formation of monomeric  $\beta$ -catenin. Upon canonical activation the COOH-terminus of  $\beta$ -catenin folds back and prevents  $\beta$ -catenin and E-cadherin interaction, but maintains the ability to interact with TCF in the nucleus<sup>48</sup>. Canonical signaling can not only activate genes through the formation of monomeric  $\beta$ -catenin but more importantly it can inhibit the  $\beta$ -catenin degradation complex and thereby activate canonical Wnt target genes<sup>49</sup>.

# Wnt

Wnt ligands are highly conserved secreted signaling proteins found in multicellular animals, important for embryonic development and differentiation<sup>50</sup>. Wnt proteins have been divided into the highly transforming group (Wnt1, Wnt3, Wnt3a and Wnt7), the intermediately and the non-transforming group (Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b and Wnt11) according to their transforming capacity of the mouse mammary cell line C57MG<sup>51</sup>. Glycosylation of Wnts, which is important for their secretion, takes place in the endoplasmic reticulum, where the Wnt ligands are further lipid modified with palmitate<sup>52</sup>. The lipid modification (palmitoylation) favors ligand binding to the Frizzled (FZD) family of seven-transmembrane G-protein coupled surface receptors, of which there are ten known members<sup>52,53</sup>. Whether or not all Wnt ligands can bind all FZD receptors remain unknown, however data indicate that Wnt ligands have different affinities for various FZD receptors and their local concentration will influence which pathway will be activated<sup>54</sup>. There are two principle branches of Wnt signaling; the  $\beta$ -catenin dependent canonical signaling pathway and the  $\beta$ -catenin independent non-canonical signaling pathways which have the ability to inhibit canonical signaling<sup>49</sup>.

## *The canonical, $\beta$ -catenin dependent pathway*

The canonical pathway inhibits the  $\beta$ -catenin degradation complex (including Axin, adenomatous polyposis coli (APC), CK1 $\alpha$  and GSK3 $\beta$ ) and thereby stabilizes  $\beta$ -catenin<sup>49</sup>.  $\beta$ -catenin translocate to the nucleus and activates various genes involved in the regulation of proliferation and cell fate determination<sup>36</sup>. In the absence of canonical Wnt signaling  $\beta$ -catenin is targeted for degradation. Axin, a scaffold protein for the degradation complex together with APC, recruits CK1 $\alpha$  to the degradation complex in the cytoplasm<sup>55,56</sup>. CK1 $\alpha$  phosphorylates  $\beta$ -catenin on serine residue 45 which enables further phosphorylation by GSK-3 $\beta$  on serine residues 41, 37 and 33<sup>57</sup>. The phosphorylation on Ser 37 and Ser 33 can be recognized by  $\beta$ -TrCP, part of an E3 ubiquitin ligase complex that induces ubiquitination and degradation of  $\beta$ -catenin<sup>55,57</sup>.

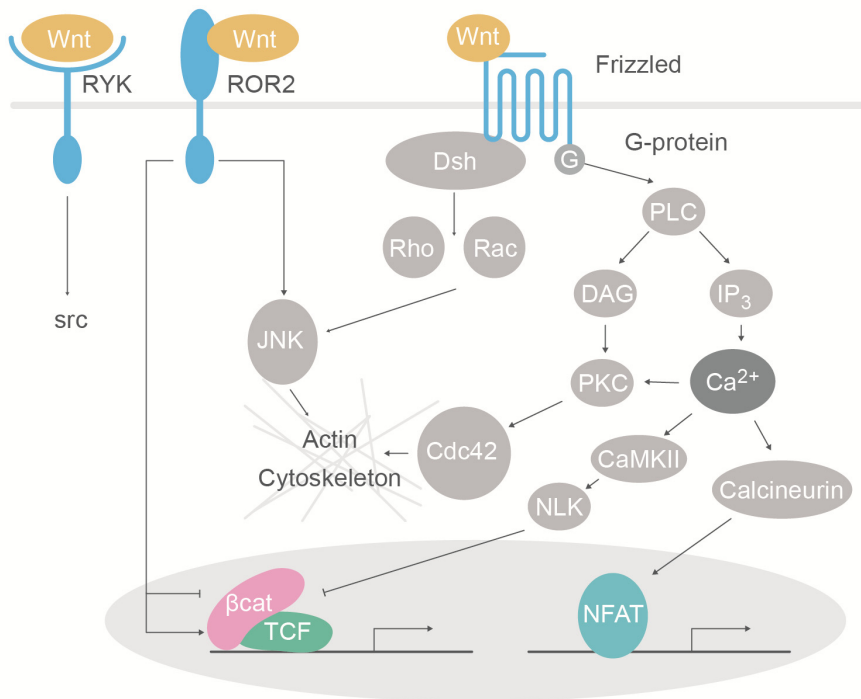


**Figure 4.** Canonical Wnt signaling.  $\beta$ -catenin is targeted for degradation in the absence of canonical Wnt signaling (Wnt OFF). Canonical signaling promotes  $\beta$ -catenin accumulation and translocation to the nucleus where it activates genes (Wnt ON). (Adapted from Li *et al.*<sup>58</sup>.)

Upon canonical signaling (Figure 4), Wnt ligands bind and activate a FZD receptor and the single-membrane co-receptor low-density-lipoprotein receptor related protein 5- (LRP5) or LRP6<sup>56</sup>. The cytoplasmic tail of the LRP receptor is phosphorylated by two serine-threonine kinases, the plasma membrane bound CK1 $\gamma$  and GSK3 $\beta$ , which leads to the recruitment of Axin to the cytoplasmic tail of LRP<sup>59,60</sup>. The GSK3 $\beta$  induced phosphorylation of LRP and the interaction between LRP and Axin is facilitated by Dishevelled (Dvl) which contains a binding motif for Axin<sup>53</sup>. Dvl is recruited to the FZD receptor prior to the phosphorylation of LRP<sup>53</sup>. Until recently it was thought that the recruitment of Axin to LRP at the cell membrane would inactivate the  $\beta$ -catenin degradation complex and make it fall apart. Li *et al.* however showed that an intact  $\beta$ -catenin degradation complex is recruited to LRP at the cell membrane where it continues to recruit and phosphorylate  $\beta$ -catenin. The interaction of  $\beta$ -TrCP with the complex is however disrupted and  $\beta$ -catenin is not targeted for degradation<sup>58</sup>. Eventually the degradation complexes become saturated with  $\beta$ -catenin and newly produced  $\beta$ -catenin can accumulate in the cytoplasm and translocate to the nucleus where it can bind to and displace the transcriptional repressor Groucho from the

DNA-binding transcription factors TCF/LEF and activate genes<sup>55,58</sup>. Canonical Wnt signaling can, for example, upregulate the two proangiogenic factors VEGF and FGF, important for tumor progression, and activate the oncogenes c-Myc and cyclin D, involved in cell proliferation<sup>56</sup>. More than 50% of breast cancers have an upregulation of  $\beta$ -catenin that has been correlated with poor outcome and cyclin D upregulation, indicating activation of the canonical pathway<sup>61,62</sup>. Although N-terminal  $\beta$ -catenin mutations leading to inappropriate stabilization of  $\beta$ -catenin, have been linked to a variety of cancers including colon and prostate cancer, it is uncommon in breast cancer (only found in rare metaplastic breast cancer)<sup>40,55,56</sup>. Deregulation of the canonical pathway in breast cancer can, however, be due to downregulation of APC, found in 36-50% of breast cancers<sup>35</sup>. An additional explanation for  $\beta$ -catenin stabilization in breast cancer is upregulation of the isomerase Pin1, found in 80% of breast cancers. Pin1 inhibits the binding between  $\beta$ -catenin and APC and thereby prevents  $\beta$ -catenin degradation<sup>63,64</sup>.

### *The non-canonical, $\beta$ -catenin independent pathways*



**Figure 5.** Non-canonical Wnt signaling pathways. (Adapted from Angers *et al.* and Sugimura *et al.*<sup>53,65</sup>)

Although nine different non-canonical pathways have been reported they are not fully defined and probably overlap<sup>66,67</sup>. The most studied pathways are the Wnt/JNK pathway and the Wnt/Ca<sup>2+</sup> pathway. The Wnt/Ror2 and Wnt/Ryk pathway will also be covered in brief (Figure 5)<sup>66,67</sup>.

### **Wnt/JNK pathway**

Each epithelial cell in the plane of an epithelial layer has an apical-basolateral polarity which is dependent on the polarized organization of the actin cytoskeleton in the cell<sup>68</sup>. The Wnt/JNK pathway can achieve cell polarity by the modification of the actin cytoskeleton within the cell<sup>65,68</sup>. Wnt ligands, signaling through the JNK pathway, interact with FZD receptors and promote the activation of Dvl<sup>65</sup>. Dvl activates Rho and Rac in two parallel pathways that in turn will mediate regulation of cellular polarity through JNK activity<sup>67,69</sup>.

### **Wnt/Ca<sup>2+</sup> pathway**

The Wnt/Ca<sup>2+</sup> pathway regulates cell polarity and cell migration through the release of intracellular calcium from the endoplasmic reticulum<sup>69</sup>. The binding of Wnt to FZD receptor will lead to the activation of phospholipase C (PLC)<sup>65,68</sup>. PLC is a membrane-bound enzyme that upon activation will cleave phosphatidyl inositol 4,5-bisphosphate (PIP<sub>2</sub>) into two intracellular signaling molecules; inositol 1,4,5 (IP3) and 1,2 diacylglycerol (DAG)<sup>68</sup>. DAG activates protein kinase C (PKC). IP3 binds to calcium channels located in the endoplasmic reticulum membrane, creating a Ca<sup>2+</sup> flux that contributes to the activation of PKC and activates calmodulin dependent protein kinase II (CaMKII) and calcineurin<sup>68,70</sup>. Both PKC and CaMKII can activate the nuclear transcription factors NFκB and CREB<sup>70</sup>. CaMKII can further activate nemo-like kinase (NLK) that will induce phosphorylation of TCF and thereby inhibit the canonical signaling<sup>65,66</sup>. Calcineurin will activate NFAT, a transcription factor inducing production of inflammatory genes<sup>65,66</sup>.

### **Wnt/Ror-2 and Wnt/Ryk pathway**

Wnt can also bind to the membrane-bound tyrosine kinase receptors Ror2 and Ryk<sup>54</sup>. The Wnt/Ror2 pathway can activate JNK via cdc42, affecting polarized cell migration<sup>70</sup>. It can both inhibit the canonical pathway at the level of TCF/LEF transcription and potentiate canonical signaling, depending on which Wnt ligands bind<sup>53,71</sup>. The Wnt/Ryk pathway leads to the activation of src<sup>53</sup>. The importance of the Wnt/Ryk pathway remains unclear, however upregulation of Ryk has been reported in ovarian cancer<sup>72</sup>.

## **Wnt modulation**

The interaction between Wnts and FZD receptors can be antagonized through the binding of Wnt ligands to the Wnt inhibitory protein (WIF) or secreted Frizzled-related proteins (sFRPs)<sup>55</sup>. sFRPs can bind directly to FZD receptors and thereby prevent Wnt ligand and FZD receptor interaction<sup>52</sup>. In addition, Dickkopf (DKK) can bind to the co-receptor LRP and thereby prevent canonical signaling<sup>52,55</sup>.

### *Wnt5a*

Wnt5a belongs to the group of non-transforming Wnt ligands that primarily signal through non-canonical pathways. Wnt5a can hence activate the Wnt/JNK pathway, Wnt/Ca<sup>2+</sup> pathway, Wnt/Ror-2 and Wnt/Ryk pathways which regulate a variety of cellular events including cell migration and differentiation<sup>73</sup>. Wnt5a signaling can further inhibit the canonical pathway (deregulated in variety of cancers) in multiple ways<sup>56,73</sup>. To begin with Wnt5a can inhibit the binding of the canonical Wnt3a ligand to the FZD2 receptor and thereby prevent Wnt3a induced activation of LRP6<sup>74</sup>. In addition, Wnt5a inhibits the recruitment of co-activators (e.g. CREB binding protein) to the promoter region of  $\beta$ -catenin dependent target genes, in a manner dependent on the Ror2 receptor<sup>73,75</sup>. Wnt5a signaling can further interfere with the canonical pathway by promoting degradation of  $\beta$ -catenin<sup>76</sup>. The knowledge that Wnt5a can inhibit the canonical pathway gave rise to the thought that Wnt5a might be a tumor suppressor and indeed downregulation of Wnt5a correlated with higher tumor grade in hepatocellular and thyroid cancer, and with poor outcome in colon and breast cancer<sup>77-80</sup>. Lack of Wnt5a in primary ductal breast cancer has further been correlated with higher tumor grade and found to be an independent predictor of recurrence<sup>81</sup>. One explanation reported, for how Wnt5a counteracts the formation of secondary tumors, is by having an effect on cell migration through the activation of the tyrosine receptor discoidin domain receptor 1 (DDR1)<sup>82</sup>. Activated DDR1 binds to collagen in the ECM and thereby inhibits cell migration<sup>82</sup>.

Contrary to the prior data on Wnt5a in cancer, there are data that indicate that Wnt5a can have a tumor promoting role in cancer<sup>83</sup>. Upregulation of Wnt5a has been correlated to poor outcome in melanoma, gastric and non-small-cell lung cancer<sup>84-86</sup>. The contradicting data that have been reported for Wnt5a indicates that the role of Wnt-5a in different cancers probably depends on the cellular context and the exact machinery behind it will still have to be clarified.

# Tumor immunology in brief, the 7<sup>th</sup> hallmark

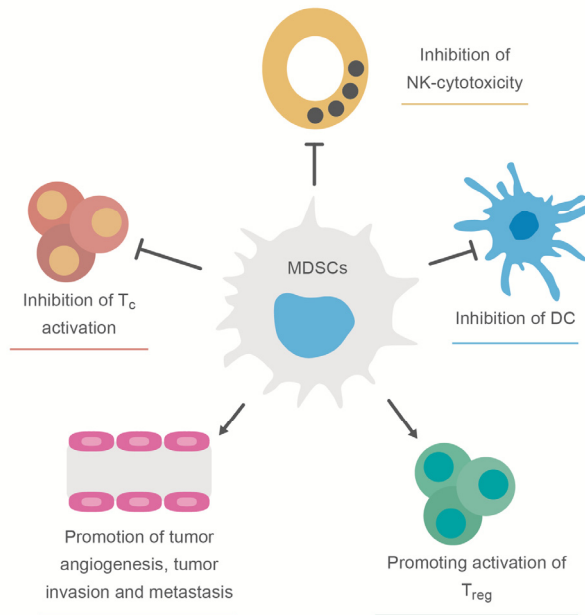
## *Leukocytes in the tumor microenvironment*

The inflammatory microenvironment in a malignant tumor, regarded to be the 7<sup>th</sup> hallmark of cancer, will influence the fate of the tumor and hence the clinical outcome of a cancer patient<sup>6</sup>. Tumor development can be triggered, promoted and prevented by a wide range of leukocytes belonging to either the innate or the adaptive immune system<sup>87,88</sup>. The innate and the adaptive immune system will be activated by damage associated molecular pathways (DAMPs) which include endogenous molecules, called alarmins, and pathogen associated molecular patterns (PAMPs) that refer to exogenous pathogens and bacterial endotoxin<sup>89</sup>. Lymphocytes belonging to the adaptive immune system can be divided into T lymphocytes and B lymphocytes<sup>90</sup>. The T lymphocytes are classified into CD4<sup>+</sup>T helper 1 cells (T<sub>H1</sub>) or T helper 2 cells (T<sub>H2</sub>)<sup>91</sup>. T<sub>H1</sub> cells are pro-inflammatory with an antitumorigenic function. They activate pro-inflammatory macrophages and produce interleukin 2 (IL-2), IL-12, TNF $\alpha$  and interferon  $\gamma$  (IFN $\gamma$ )<sup>92</sup>. Both T<sub>H1</sub> cells and IFN $\gamma$  have been correlated with good clinical outcome in many cancer types, including breast cancer<sup>91</sup>. IFN $\gamma$  and IL-2 further activate cytotoxic T (T<sub>c</sub>) cells that contain cytotoxic granules which kill tumor cells through programmed cell death<sup>91</sup>. T<sub>c</sub> and T<sub>H1</sub> cells can be activated by the major professional antigen presenting cell population, namely dendritic cells (DC) that are part of the immune surveillance and actively take up tumor antigens<sup>90,91,93,94</sup>. T<sub>H1</sub> can further promote immune surveillance by activating natural killer (NK) cells (lymphocytes belonging to the innate immune system) that normally are tumoricidal but eventually might acquire an anergic phenotype (unable to secrete IFN $\gamma$  and to kill tumor cells) due to the production of TGF $\beta$  by tumor cells<sup>87,91,92</sup>. T-cell- and NK markers in breast cancer were included in the 26-gene predictor developed by Finak *et al.*, and upregulation of these markers in the tumor stroma, indicating a T<sub>H1</sub> type immune response, correlated strongly with good clinical outcome<sup>95</sup>.

Contrary to T<sub>H1</sub> cells, T<sub>H2</sub> cells are anti-inflammatory and protumoricidal. A high infiltration ratio of T<sub>H2</sub> to T<sub>H1</sub> cells indicate poor clinical outcome among breast cancer patients<sup>87</sup>. T<sub>H2</sub> cells favor humoral immunity directed by B lymphocytes, inhibit T-cell mediated cytotoxicity and produce IL-4 and IL-13 that activate anti-inflammatory M2 macrophages<sup>90,92,96</sup>. Regulatory T (T<sub>reg</sub>) cells are yet another protumorigenic T cell population, correlated with higher tumor grade and with poor clinical outcome in breast cancer, which inhibit anti-tumor immunity through the production of TGF $\beta$  and IL-10<sup>92,97</sup>. The development and activation of T<sub>reg</sub> cells in the tumor can be induced by myeloid-derived suppressor cells (MDSCs)<sup>93,98</sup>.

MDSCs are a heterogeneous population of bone marrow derived immature myeloid cells that are found in the majority of cancer patients where they promote tumor progression<sup>98,99</sup>. In addition to inducing T<sub>reg</sub> cells they inhibit differentiation of DC and the function of NK cells (Figure 6)<sup>94</sup>. MDSCs further interfere with T cell anti-tumor activity and promote angiogenesis and tumor invasion<sup>93,98</sup>. They are able to mediate an anti-inflammatory and protumorigenic environment through the production of IL-10, TGFβ, arginase 1 and inducible nitric oxide synthase (iNOS)<sup>94,99,100</sup>. The MDSCs are recruited to the tumor and activated by IL-4, IL-6, IL-13, VEGF and TGFβ, produced by both the tumor cells and the tumor microenvironment<sup>100</sup>. In addition to having MDSCs, cancer patients have been reported to have an aberrant immunosuppressive CD14<sup>+</sup> monocyte population, producing IL-10 and TGFβ<sup>98,101,102</sup>.

Hence, there are a wide range of different leukocytes in the tumor which are either protumorigenic or antitumorigenic. However an additional major player in tumor development and progression are macrophages that are highly plastic in nature and abundantly found in a tumor where they promote tumor invasion, migration and angiogenesis<sup>103-105</sup>. High TAM infiltration has been found to correlate with poor outcome in many cancers including breast cancer<sup>106</sup>



**Figure 6.** MDSCs promote tumor progression by facilitating angiogenesis, tumor invasion and metastasis and by interacting with various leukocytes that are part of both the innate and adaptive immune system. (Adapted from Tadmor *et al*<sup>98</sup>.)

## *Tumor associated macrophages*

Macrophages can be divided into resident and recruited macrophages. Recruited macrophages are differentiated monocytes that are derived from myeloid progenitors in the bone marrow. Monocytes differentiate into either classical M1 macrophages or alternative M2 macrophages, depending on the environmental context<sup>105</sup>. Toll like receptor (TLR) ligands (i.e. alarmins) and IFN $\gamma$  differentiate monocytes (via STAT1) into pro-inflammatory M1 macrophages that produce IL-12, IL-23 and TNF $\alpha$ <sup>105,107</sup>. M1 macrophages are tumoricidal and have the capacity to recruit and drive polarization of T<sub>H</sub>1, hence amplifying a T<sub>H</sub>1 mediated reaction<sup>105</sup>. Monocytes that encounter IL-10, and the T<sub>H</sub>2-type cytokines IL-4 and IL-13, are on the contrary differentiated (via STAT3 and STAT6 respectively) into M2 macrophages which have a IL-12<sup>low</sup> IL-10<sup>high</sup> phenotype<sup>96</sup>. M2 macrophages are able to downregulate an inflammatory reaction, a capability they utilize in chronic inflammation and in wound healing. They can further promote angiogenesis, recruit fibroblasts and regulate connective tissue remodeling<sup>105,108,109</sup>. TAMs have many features in common with M2 macrophages; they have a IL-12<sup>low</sup> IL-10<sup>high</sup> phenotype, are anti-inflammatory, have a weak tumoricidal capacity and have many of the properties of M2 macrophages in wound healing (Figure 7)<sup>104</sup>.

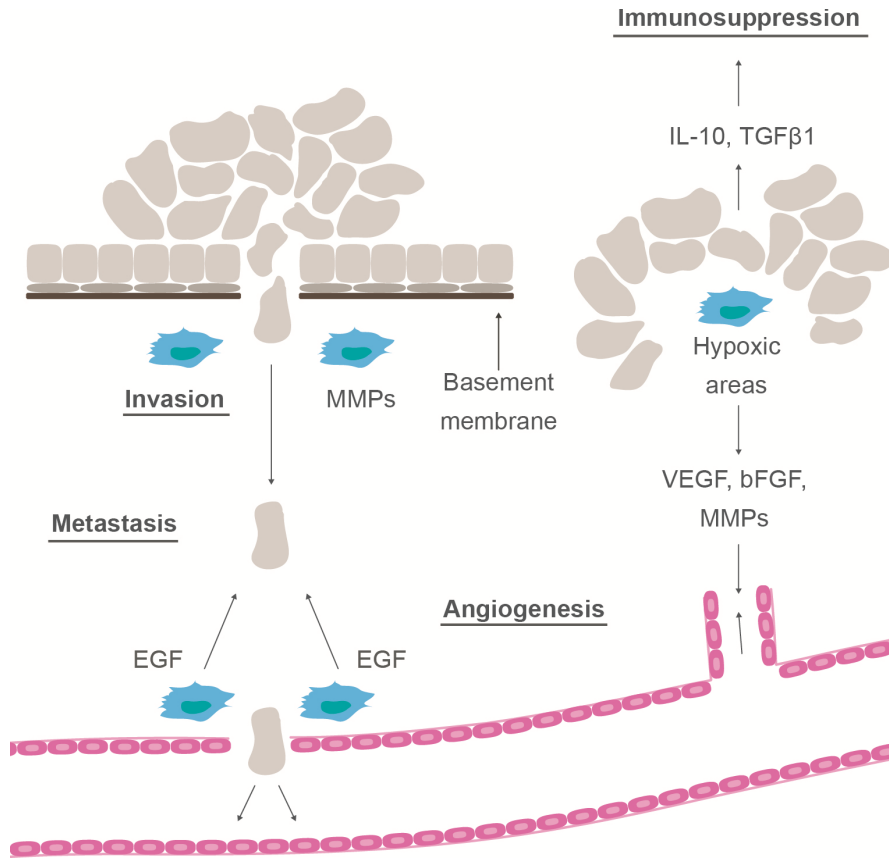
M2 macrophages are thus related to wound healing and tune an inflammatory response while M1 macrophages primarily promote inflammation<sup>88</sup>.

Chronic inflammation has been linked to various cancer types (e.g. colon and hepatocellular cancer)<sup>88,110</sup>. Chronic inflammation will recruit and initially differentiate monocytes into M1 macrophages that are able to eradicate potential tumor cells, together with other leukocytes belonging to the innate and the adaptive immune system<sup>111</sup>. If tumor elimination fails and the tumor prevails in the inflammatory environment, the M1 macrophage population will eventually contribute to genetic instability and a mutagenic environment through their production of the highly reactive compound nitric oxide and TNF $\alpha$ <sup>112,113</sup>. In addition, they produce IL-6 that together with TNF $\alpha$  promotes epithelial growth. The pro-inflammatory M1 macrophages in chronic inflammation can hence initiate tumor formation, but once the tumor is established it will primarily induce differentiation into an anti-inflammatory macrophage phenotype that will enhance malignancy<sup>112</sup>. The transcription factor NF- $\kappa$ B is a key player in the switch from pro- into anti-inflammatory macrophages<sup>110</sup>. The NF- $\kappa$ B family (NF- $\kappa$ B1 (p105/p50), NF- $\kappa$ B2 (p100/p52), RelA (p65), RelB and c-Rel) will be activated by TLR ligands, TNF $\alpha$  and IL-1 and upon activation induce production of additional inflammatory cytokines<sup>110,114</sup>. TAMs have been found to have defective NF- $\kappa$ B activity probably due to p50-homodimer formation (homodimer formation of p50 or p52 inhibit NF- $\kappa$ B activity) which will contribute to differentiation into a pro-

tumor M2 macrophage phenotype<sup>110,112</sup>. Importantly, TAMs infiltrating an established tumor are not pro-inflammatory but rather anti-inflammatory with the ability to enhance malignancy.

Although breast cancers are epidemiologically unrelated to chronic inflammation, they recruit and differentiate monocytes due to cellular damage, hypoxia and the activation of oncogenes<sup>6,112,115</sup>. The recruitment of monocytes begins in the early stage of tumor development and continues in tumors that are invasive and capable of forming secondary tumors<sup>104</sup>. An important chemoattractant for TAMs is the chemokine CC-motif ligand 2 (CCL2), that in addition to being produced by tumor cells can be produced by recruited TAMs, leading to an amplifying loop of TAM recruitment<sup>116, 117</sup>. CCL2 is upregulated and correlated with poor outcome in breast cancer<sup>116</sup>. Other important tumor-derived chemoattractants for TAMs are the colony-stimulating factor 1 (CSF1), upregulated and correlated with poor outcome in breast cancer, and VEGF, an important factor for angiogenesis<sup>104</sup>. Recruited TAMs infiltrate hypoxic areas in the tumor, where they produce VEGF, bFGF, the pro-angiogenic factor MMP-7 and induce ECM degradation, creating angiogenic fragments from hyaluronic acid<sup>94,116</sup>. The angiogenic growth factors provided by TAMs and the tumor microenvironment will stimulate nearby endothelial cells to migrate and proliferate, forming a new and more permeable vasculature in hypoxic areas of the tumor<sup>104</sup>. Leek *et al.* showed a significant correlation between angiogenesis and TAMs in breast cancer<sup>21</sup>. In addition to inducing angiogenesis, TAMs are able to attract tumor cells to the vasculature. Abluminal TAMs produce EGF, upon CSF1 stimulation, which will bind to the EGFR on the tumor cells, attract them and facilitate their entry into the circulation<sup>104,118,119</sup>.

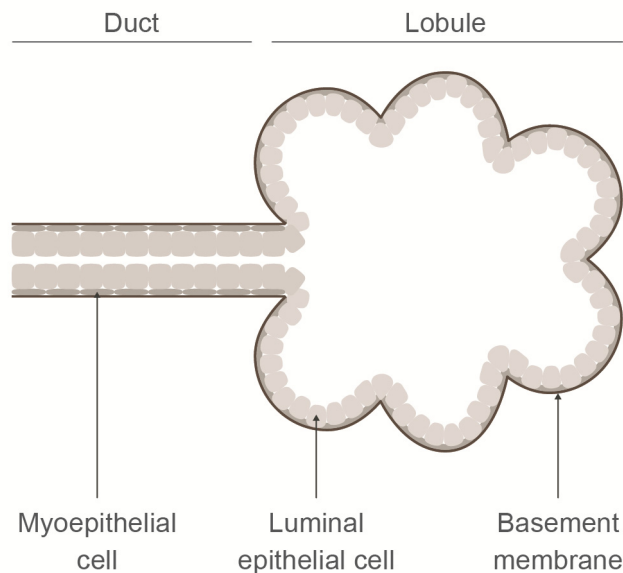
To conclude, macrophages do not only initiate tumor formation but they are also involved in tumor progression and facilitate the formation of secondary tumors.



**Figure 7.** The role of TAMs in tumor progression. They promote *invasion* through MMP- induced degradation of the basement membrane, allowing tumor cells to enter the stroma. TAMs are recruited to hypoxic areas where they promote *angiogenesis*. They inhibit anti-tumor immunity through the production of IL-10 and TGFβ, promoting *immunosuppression*. Finally TAMs secrete EGF that recruits tumor cells to the vasculature and thereby facilitates *metastasis*. (Adapted from Lewis *et al*<sup>104</sup>.)

# Breast anatomy and development

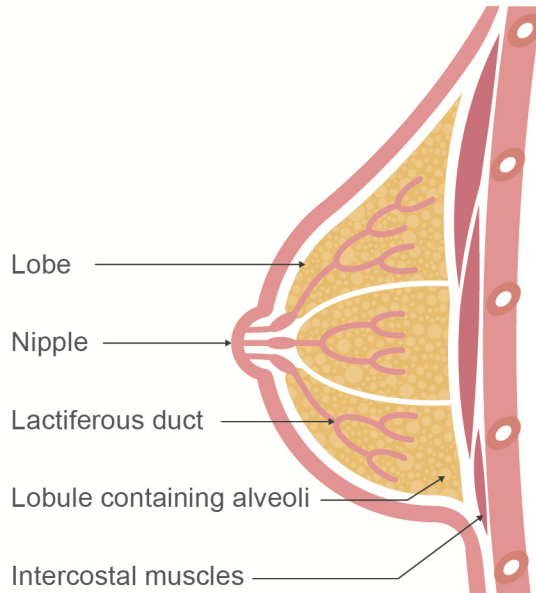
The development of the mammary gland begins during the second fetal month in the ectoderm layer<sup>120,121</sup>. At the end of the seventh intrauterine month two cell populations can be distinguished, an inner luminal cell population, from which breast cancers mainly arise, and an outer myoepithelial cell population<sup>122,123</sup>. During the prenatal period and from birth to puberty there are no differences between the development of the male and the female mammary glands<sup>123</sup>. Development of the female mammary gland will however begin again during puberty under the influence of elevated ovarian hormones<sup>124</sup>. There is an increase in the amount of stroma surrounding the ducts, made up of the ECM and stromal cells (fibroblasts, leukocytes and endothelial cells)<sup>121,125</sup>.



**Figure 8.** Normal mammary duct and lobule lined with inner luminal epithelial cells and outer myoepithelial cells on a basement membrane. (Adapted from Polyak *et al*<sup>125</sup>.)

The ducts, lined with a luminal and myoepithelial cell layer, elongate and lobular structures are formed through the fat pad (Figure 8)<sup>123,125</sup>. Branching of ducts continues and the breast grows even more during pregnancy due to the influence of hormones, in particular estrogen ( $E_2$ ), produced by the placenta<sup>124</sup>. Proper ductal development and branching depends on  $TGF\beta$ , a negative regulator in mammary development that upregulates  $Wnt5a$ .  $Wnt5a$  activates  $DDR1$  that in turn inhibits migration of mammary epithelial cells and thereby inhibit ductal outgrowth and contribute to controlled breast development<sup>126</sup>. There is additional growth of lobules under the influence of progesterone (Pg) once the development of the

ducts is completed. Pg will stimulate budding of alveoli and the cells in the alveoli gain the ability to produce milk and secret it into the lactiferous ducts (Figure 9)<sup>124</sup>.



**Figure 9.** Illustration of breast anatomy. (Adapted from Marieb<sup>7</sup>.)

The alveoli glands make up the lobules, which are part of 15 to 25 lobes that open at the breast nipple via the lactiferous ducts (Figure 9)<sup>7</sup>. While the lobules will decrease in size and number during involution, occurring after pregnancy and lactation, the ducts will not be affected<sup>123</sup>. Another period of involution takes place after menopause. The postmenopausal involution involves both the lobules and ducts, which are replaced by fat<sup>123</sup>. Hence, the mammary glands will go through intriguing developmental changes during the life of a woman, but are unfortunately also involved in the most common form of cancer affecting women worldwide, namely breast cancer<sup>4</sup>.

# Breast cancer

Breast cancer is the most common cancer among women worldwide with approximately 1,150,000 new cases each year and accounting for over 400,000 deaths per year<sup>4</sup>. Around 7000 women are annually diagnosed with breast cancer in Sweden<sup>127</sup>. Known risk factors for breast cancer are female gender, age, early menarche, older age at first full-term pregnancy (>30 years), late menopause, menopausal hormonal therapy and a family history, particularly women with a first degree relative (mother, daughter or sister)<sup>128,129</sup>. Between 5-10% of all breast cancer cases are due to inherited mutations or alterations in genes involved in the development of breast cancer<sup>127,128</sup>. BRCA1 or BRCA2 mutations account for 40% of all patients with hereditary breast cancer and give a 10-30 times higher risk of developing breast cancer compared to women in the general population<sup>130</sup>. Hereditary breast cancer will often develop at a younger age, being particularly true for women carrying the BRCA1 mutation, who often develop triple-negative breast cancer, a more aggressive molecular subtype of breast cancer<sup>131,132</sup>. The molecular subtype, histological type, grade and the TNM classification can predict the clinical outcome of breast cancer patients.

## *Breast cancer pathology and diagnostics*

### **Histological type**

The histological type of a tumor refers to a particular growth pattern, including morphological and cytological features. According to the World Health Organization (WHO) classification of breast cancer from 2003 there are 17 different histological types of breast cancer accounting for 25% of all breast cancers, including tubular carcinoma (<2%), mucinous carcinoma (2%), neuroendocrine carcinoma (2-5%), medullary carcinoma (1-7%) and infiltrating lobular carcinoma (5-15%)<sup>133</sup>. The major histological type, namely invasive ductal carcinoma accounts for the remaining 75% that do not fit into any other histological type<sup>134</sup>.

### **The Nottingham Histological Grade**

Tumors can be classified into being either well (grade I), moderately (grade II) or poorly (grade III) differentiated, according to the Nottingham histological grade (NHG) scoring system. Breast cancer patients with well differentiated tumors have a better prognosis compared to those with moderately or poorly differentiated breast cancer. The differentiation grade of a tumor will depend on the evaluation of tubule formation, degree of nuclear atypia and mitotic count<sup>135</sup>. NHG together with axillary lymph node status and tumor size (two important prognostic factors)

are included in the Nottingham prognostic index (NPI) which can predict which breast cancer patients will benefit from adjuvant therapy<sup>136</sup>

## **TNM**

Breast cancer is further classified according to the TNM staging method, which can predict the clinical outcome through the evaluation of the size of the primary tumor (T), number of infiltrated axillary lymph nodes (N) and the occurrence of metastases (M)<sup>137</sup>.

## **Molecular subclassification**

Breast cancer can be divided into five major molecular subtypes defined by gene expression array: luminal A, luminal B, basal-like, HER2<sup>+</sup>/ER<sup>-</sup> and normal breast-like breast cancer<sup>138-140</sup>. Luminal A and luminal B are estrogen receptor (ER) positive and express many genes that are related to breast luminal cells<sup>141</sup>. They have lower proliferation rate, are more differentiated and have a better clinical outcome compared to both HER2<sup>+</sup>/ER<sup>-</sup> and basal-like breast cancer<sup>142,143</sup>. Basal-like tumors express cytokeratins 5/6 and/or cytokeratin 17, which are typical features for myoepithelial cells (also called basal epithelial cells)<sup>141,144</sup>. The majority (77%) of the basal-like breast cancers have a triple-negative immunophenotype referring to that they are negative for ER, progesterone receptor (PgR) and HER2 when evaluated immunohistochemically<sup>144,145</sup>. Approximately 70% of the triple-negative breast tumors have a basal-like gene expression profile and the two subtypes have similar clinical behavior; they affect younger women (<50 years), are more aggressive and metastasizing more frequently to the lungs and brain compared to luminal tumors which mainly disseminate to the liver and skeleton<sup>132,144,145</sup>. Secondary tumors are the main cause of death for women with breast cancer<sup>142</sup>. Approximately 1500 woman die each year because of breast cancer, making it the number one cause of death among middle aged women in Sweden<sup>127</sup>. Fortunately, improvements have been made with both earlier detection and better treatment, giving an approximate overall 5-year survival at 90%<sup>127</sup>.

## *Breast cancer treatment*

### **Surgery and Radiotherapy**

Locoregional breast cancer can be cured with surgical removal of the primary tumor. Depending on the size of the primary tumor in relation to the breast, the surgical choice is either radical or breast conserving surgery. Breast conserving surgery, the more common choice, is followed by radiotherapy to reduce the risk of local and regional recurrence. Both surgical approaches include (in the case of a unifocal tumor) sentinel node biopsy to determine whether or not there are malignant cells in the sentinel lymph node. If none are found, further axillary dissection is not necessary. Surgical removal of the primary tumor will also

provide the opportunity to more closely evaluate the histological type and grade and hormone receptor status of the breast cancer and thereby facilitate the choice of further adjuvant therapy<sup>146</sup>.

### **Endocrine therapy**

Women with ER-positive breast cancer receive endocrine therapy that interferes with the E<sub>2</sub> signaling pathway. Tamoxifen is a selective estrogen receptor modulator (SERM) that binds to and acts as antagonist of ER signaling in breast cancer<sup>147</sup>. Treatment with tamoxifen is usually given for five years and can reduce the mortality from breast cancer by preventing local and regional recurrence. In women with a higher risk of recurrence, tamoxifen treatment will be combined with aromatase inhibitors (AI). AI's reduce the amount of circulating E<sub>2</sub> in the body by preventing the production of E<sub>2</sub> in adipose tissue, but do not have any effect on the ovarian production of E<sub>2</sub>, and are therefore only given to postmenopausal women<sup>146</sup>.

### **Chemotherapy**

Breast cancer patients with high risk of recurrence are also given adjuvant chemotherapy, according to a standard international protocol, called the FECregimen which includes fluorouracil, epirubicine and cyclophosphamide. Breast cancer patients with malignant cells in the axillary lymph nodes are further given docetaxel following the FECregimen<sup>148</sup>.

### **Monoclonal therapy**

Women with HER2 amplified breast cancer, accounting for approximately 10-30% of all breast cancers are given adjuvant monoclonal therapy with trastuzumab, a monoclonal antibody that will inhibit HER2 activity, for one year after completed adjuvant chemotherapy<sup>148</sup>.



# The Present Investigation

## Aims

The overall aim of the investigations was to study the impact of Wnt5a and tumor associated macrophages in breast cancer.

The specific aims were:

- I. To find out whether Wnt5a signaling affects the E-cadherin/ $\beta$ -catenin complex, which in part could explain how Wnt5a can inhibit recurrence in breast cancer.
- II. To analyze whether Wnt5a signaling induces pro-inflammatory or anti-inflammatory activity in monocytes-macrophages.
- III. To evaluate two macrophage markers in breast cancer and elucidate whether or not the tumoral localization of tumor associated macrophages have a clinical impact.

# Results and Discussion

## *Paper I*

### **Results**

In 2002 it was reported that reduced levels of the Wnt5a protein correlated with higher incidence of recurrence in invasive ductal carcinoma<sup>81</sup>. In paper I we wanted to elucidate whether Wnt5a signaling could promote the formation of the  $\beta$ -catenin/E-cadherin complex and in part explain how Wnt5a reduces the metastatic behavior of breast cancer.

To begin with we found that Wnt5a could increase the amount of  $\text{Ca}^{2+}$  dependent cell aggregates formed in the non-cancerous ductal breast epithelial cell line HB2 that had been stably transfected with Wnt5a (Wnt5a<sup>high</sup>HB2) compared to HB2 cells stably transfected with antisense Wnt5a (Wnt5a<sup>low</sup>HB2). We further saw that Wnt5a<sup>low</sup>HB2 cells stimulated with recombinant Wnt5a and Wnt5a<sup>high</sup>HB2 cells had elevated levels of both E-cadherin and  $\beta$ -catenin in the cell membrane. Wnt5a induced  $\beta$ -catenin/E-cadherin complex formation in both the non-cancerous ductal breast epithelial cell line HB2 and the ductal breast cancer cell line MCF-7.

To find out in what way Wnt5a signaling can promote  $\beta$ -catenin/E-cadherin complex formation we wanted to know if Wnt5a could affect tyrosine phosphorylation of  $\beta$ -catenin.  $\beta$ -catenin phosphorylated on Tyr 654 has a six fold reduced affinity for E-cadherin while phosphorylation on Tyr 142 interrupts the interaction between  $\beta$ -catenin and  $\alpha$ -catenin<sup>45</sup>. Wnt5a did not have any effect on the tyrosine phosphorylation of  $\beta$ -catenin, but could induce serine phosphorylation of  $\beta$ -catenin. In a previous paper it was shown that Wnt5a could activate the serine kinase CK1 $\alpha$ <sup>149</sup>. Prior to our paper, CK1 $\alpha$  phosphorylation of  $\beta$ -catenin on Ser 45 had been reported to induce  $\beta$ -catenin degradation by a multiprotein degradation complex including Axin, APC, CK1 $\alpha$  and GSK3 $\beta$ <sup>57</sup>. Although we saw a CK1 $\alpha$  dependent Ser 45 phosphorylation of  $\beta$ -catenin upon Wnt5a signaling we could not detect any additional degradation of  $\beta$ -catenin. More importantly, we could report that the Wnt5a induced  $\beta$ -catenin/E-cadherin complex formation was dependent on the phosphorylation of  $\beta$ -catenin at Ser 45 by CK1 $\alpha$ .

Wnt5a<sup>low</sup>HB2 cells that were transfected with Flag-tagged mutated Ser 45  $\beta$ -catenin and stimulated with recombinant Wnt5a did not have any reduced migration capacity although Wnt5a<sup>low</sup>HB2 cells transfected with Flag-tagged mutated Ser 33  $\beta$ -catenin had a significant reduction in migration capacity when stimulated with recombinant Wnt5a, hence further highlighting the importance of  $\beta$ -catenin Ser 45 phosphorylation for Wnt5a signaling.

Since we saw an elevation of membrane bound  $\beta$ -catenin upon Wnt5a stimulation in our cell lines we next analyzed the clinical relevance of our findings in a human breast cancer tissue microarray (TMA). In line with our experimental data we found that Wnt5a significantly correlated with the concentration of  $\beta$ -catenin in the cell membrane ( $P = 0.004$ ).

## Discussion

Downregulation of Wnt5a in primary ductal breast cancer correlated with poor outcome and higher tumor grade and was found to be an independent predictor of recurrence<sup>80,81</sup>. The ability of Wnt5a to inhibit tumor progression can partly be explained by the Wnt5a induced activation of DDR1 that promotes binding to collagen in the ECM and inhibits cell migration<sup>82</sup>. In paper 1 we show, in line with the data indicating a beneficial effect of Wnt5a in breast cancer, that Wnt5a can further inhibit tumor progression by inducing  $\beta$ -catenin/E-cadherin complex formation.

$\beta$ -catenin can exert an effect in both the nucleus where it binds to TCF/LEF and in the cell membrane where it forms a complex with E-cadherin promoting cell-cell adhesion<sup>36</sup>. The function of  $\beta$ -catenin depends on the phosphorylation status of certain residues.  $\beta$ -catenin phosphorylated on Tyr 142 will preferentially bind to TCF in the nucleus while  $\beta$ -catenin phosphorylated on Tyr 654 has a reduced affinity for E-cadherin<sup>45</sup>. Upon Wnt5a stimulation of the non-cancerous ductal breast epithelial cell line HB2 we did not detect any effect on tyrosine phosphorylation of  $\beta$ -catenin although we were able to show that Wnt5a can induce Ser 45 phosphorylation. Serine phosphorylation is important for the degradation of  $\beta$ -catenin. Ser 45, phosphorylated by CK1 $\alpha$ , and Ser 33 and Ser 37 phosphorylated by GSK3 $\beta$ , targets  $\beta$ -catenin for degradation<sup>57</sup>. We could not detect any elevation of  $\beta$ -catenin degradation but on the contrary we saw more  $\beta$ -catenin bound to E-cadherin in the cell membrane upon Wnt5a signaling.

We could further show that mutation of Ser 45 reduced the affinity of  $\beta$ -catenin for E-cadherin and abrogated the ability of Wnt5a to inhibit migration. Ser 45 is found on the N-terminal of  $\beta$ -catenin<sup>48</sup>. In line with our data indicating an important role for Ser 45 in  $\beta$ -catenin/E-cadherin, the N-terminal end has been shown to be required for the binding of  $\beta$ -catenin to E-cadherin<sup>48</sup>. In addition E-cadherin preferentially binds to  $\beta$ -catenin/ $\alpha$ -catenin dimers and we were able to show that Wnt5a signaling promoted the interaction between  $\beta$ -catenin and  $\alpha$ -catenin, hence further facilitating  $\beta$ -catenin/E-cadherin complex formation<sup>45</sup>.

Furthermore, we could show that lack of or low levels of Wnt5a protein in breast cancers strongly correlate with reduced levels of  $\beta$ -catenin in the cell membrane. The importance of  $\beta$ -catenin at the membrane in human breast cancer was evaluated in 2006 by Dollart-Filhart *et al*, who showed that low levels of  $\beta$ -catenin at the cell membrane correlated with poor patient outcome<sup>39</sup>.

Our data further strengthens the role of Wnt5a as a tumor suppressor in breast cancer and show that Wnt5a can not only induce cell-extracellular adhesion, but also promote  $\text{Ca}^{2+}$ -dependent intercellular adhesion through the  $\text{CK1}\alpha$ -induced Ser-45 phosphorylation of  $\beta$ -catenin promoting  $\beta$ -catenin/E-cadherin complex formation and thereby inhibit cell migration.

## *Paper II*

### **Results**

In paper II we analyzed what effect Wnt5a would have on primary human monocyte-macrophage (Mo-M) differentiation. Mo-M differentiated into M1 macrophages were treated with pro-inflammatory GM-CSF, LPS and  $\text{IFN}\gamma$  to mimic a microbial attack or with endogenous HMGB1, a typical alarmin that signal tissue damage. Anti-inflammatory M2 macrophages had been treated with GM-CSF and IL-4. While we did not find any effect on M2 differentiation upon Wnt5a treatment we noted that Wnt5a signaling inhibited M1 differentiation in favor of an anti-inflammatory Mo-M population that produced higher levels of IL-10, an anti-inflammatory cytokine. Wnt5a treatment also induced NF- $\kappa$ B p50 homodimer formation, causing defective pro-inflammatory NF- $\kappa$ B activity. Importantly, this was only seen in the presence of both a TLR4 ligand (LPS/HMGB1) and Wnt5a. Next we wanted to find out whether or not the Wnt5a mediated inhibition of NF- $\kappa$ B activity could be due to IL-10 causing an inhibitory feedback effect on the pro-inflammatory NF- $\kappa$ B activity<sup>150,151</sup>. Once we inhibited the IL-10 receptor we could not detect any Wnt5a induced reduction of NF- $\kappa$ B activity.

Although the anti-inflammatory Mo-M's found in the M1 macrophage population treated with Wnt5a had reduced cytotoxic T cell stimulatory capacity and higher pinocytosis capacity they did not have a M2 phenotype (levels of CD163, a M2 macrophage marker, remained unaffected) but rather a  $\text{CD14}^+\text{HLA-DR}^{-/\text{low}}$  and co-receptor<sup>-/low</sup> phenotype similar to the  $\text{CD14}^+$  Mo suppressor population found in cancer and sepsis patients<sup>101,102,152-154</sup>. Prior to our data it had been reported that Wnt5a could be involved in activation of pro-inflammatory Mo in sepsis<sup>155</sup>. The  $\text{CD14}^+$  Mo population found in sepsis patients had low levels of HLA-DR/co-receptors and was  $\text{CD163}^+$ . Although the Wnt5a treated M1 macrophages from healthy donors lacked CD163 we could detect a prominent elevation of the  $\text{CD163}^+$  population when we treated  $\text{CD14}^+$  Mo from sepsis patients with Wnt5a and LPS, hence supporting our prior data that Wnt5a has an anti-inflammatory effect in a pro-inflammatory environment. We also found a strong correlation between Wnt5a expression in tumor cells and the infiltration density of  $\text{CD163}^+$  TAMs in a human breast cancer TMA.

## Discussion

Exogenous pathogens and endotoxins (e.g. LPS) are included in pathogen-associated molecular patterns (PAMPs) that will activate the innate and adaptive immune system through their interaction with TLR<sup>89,109</sup>. TLR signaling will induce pro-inflammatory activity through NF- $\kappa$ B<sup>112,150</sup>. The immune system can also be triggered by endogenous molecules called alarmins (e.g. HMGB1) from damaged tissue<sup>89</sup>. Both microbial and endogenous molecules evoke pro-inflammatory activity, partly through the activation of macrophages that efficiently kill pathogens and tumor cells, and remove damaged tissue<sup>105,109</sup>. Macrophages are part of the innate immune system and they can differentiate into pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages<sup>105</sup>. The anti-inflammatory M2 macrophages will limit pro-inflammatory activity that in abundance would cause additional tissue damage<sup>109,156</sup>. Other anti-inflammatory Mo-Ms are TAMs and CD14<sup>+</sup> Mo suppressor cells found in cancer patients<sup>98,112</sup>. Although late stages of cancer are abundantly populated with anti-inflammatory Mo-Ms, early stages have mainly infiltration of pro-inflammatory Mo-Ms with tumoricidal capacity<sup>110</sup>. How Mo-Ms switch from pro-inflammatory to anti-inflammatory state in cancer or inflammation remains somewhat unclear although defective NF- $\kappa$ B activation (p50 homodimer formation) has been suggested and reported in both TAMs and M2 macrophages<sup>150</sup>. Another example of a Mo-M switch from a pro-inflammatory state to an anti-inflammatory state occurs during sepsis. Sepsis initiates an acute pro-inflammatory reaction that is immediately counteracted by anti-inflammatory activity. Prior to our paper Wnt5a was reported to be a pro-inflammatory factor upregulated in sepsis patients and important for the macrophage induced invasiveness in human breast cancer<sup>155,157,158</sup>. Data in paper II indicate, on the contrary, that Wnt5a promotes an anti-inflammatory reaction in a pro-inflammatory environment suggesting that Wnt5a is part of the reprogramming mechanism.

In sepsis patients this mechanism is caused by a phenomenon called endotoxin tolerance that occurs when low amounts of repeated endotoxin exposure reprogram Mo-Ms which are pro-inflammatory (from their initial endotoxin encounter), into anti-inflammatory tolerant Mo-Ms making them refractory to eventual future endotoxin challenge<sup>150</sup>. The tolerant Mo-Ms have low levels of the HLA-DR/co-receptors and like M2 macrophages they produce TGF- $\beta$  and IL-10<sup>150</sup>. The IL-10 gene can be activated by non-classical NF- $\kappa$ B activity (p50- or p52-homodimer formation) and IL-10 signaling has an inhibitory feedback effect on the pro-inflammatory activity of NF- $\kappa$ B activity<sup>159</sup>. IL-10 further accounts for the induction of endotoxin tolerance and M2 macrophage activation<sup>150,151</sup>. According to our data, Wnt5a induced IL-10 activity in M1 macrophages might be due to NF- $\kappa$ B p50 homodimer formation, which further inhibits the pro-inflammatory activity of NF- $\kappa$ B.

We also found a strong correlation between Wnt5a expression in tumor cells and infiltration of CD163<sup>+</sup> TAMs in a breast cancer TMA. We could further show that Wnt5a, together with the pro-inflammatory factor LPS reprogram primary CD14<sup>+</sup> Mo from sepsis patients into CD163<sup>+</sup> Mo-M. The CD14<sup>+</sup> Mo found in sepsis patients also have low levels of HLA-DR/co-receptors in line with our data concerning Wnt5a signaling in primary human Mo-M<sup>150</sup>.

In summary the work in paper II reveals how the non-canonical Wnt5a induces a tolerogenic macrophage phenotype in a pro-inflammatory environment and we could validate our *in vitro* data by showing the clinical relevance in both breast cancer and sepsis patients.

### *Paper III*

#### **Results**

In paper III we evaluated the accuracy of using CD163 as a M2/TAM marker and the importance of analyzing the localization of TAMs in human breast cancer.

We began to analyze the localization and infiltration density of CD163<sup>+</sup> and CD68<sup>+</sup> TAMs in tumor stroma (TS) and tumor nest (TN) respectively in a human breast cancer TMA, with tumors from 144 patients, using immunohistochemistry (IHC). We did not detect any correlation between the infiltration of TAMs in TS and TN. Within each tumoral compartment we found a significant correlation between CD163<sup>+</sup> and CD68<sup>+</sup> TAMs.

Dense CD163<sup>+</sup> TAM infiltration in TS correlated with higher grade, larger tumor size, higher proliferation index (indicated by Ki67 positivity), ER negativity, PgR negativity and granulin (GRN). We could further report that CD163<sup>+</sup> TAMs infiltration in TS positively correlates with triple-negative/basal-like breast cancer and inversely with luminal A breast cancer. The majority of the breast cancer patients in the TMA had luminal A breast cancer (79%). Only 11% of the patients in the TMA had triple-negative/basal-like breast cancer. Eight percent of the luminal A breast cancer patients, while eighty percent of the triple-negative/basal-like breast cancer patients had dense infiltration of CD163<sup>+</sup> TAMs in TS. Twenty percent of the triple-negative/basal-like breast cancer patients had dense infiltration of CD68<sup>+</sup> TAMs in TS. In a publically available gene expression array dataset we found that the gene expression levels of CD163 were significantly higher in basal-like breast cancer compared to luminal breast cancer and hence in line with our data. CD163 in TS conferred a prognostic value for overall survival and breast cancer specific survival for luminal A, but not for triple-negative/basal-like breast cancer patients.

We did not find any significant correlation between CD163<sup>+</sup> TAMs or CD68<sup>+</sup> TAMs in TN and clinicopathological features, overall survival, breast cancer specific survival or recurrence free survival.

Dense infiltration of CD68<sup>+</sup> TAMs in TS positively correlates with large tumor size, high grade and inversely with luminal A breast cancer. It further correlated with poor overall survival, breast cancer specific survival and recurrence free survival. Data from a publically available gene expression array dataset revealed that breast cancer patients who had recurrence had higher gene expression levels of CD68, hence in line with our recurrence free survival finding. Dense infiltration of CD68<sup>+</sup> TAMs was an independent prognostic factor for reduced breast cancer specific survival.

## Discussion

TAMs generally have a weak tumoricidal capacity and an anti-inflammatory function similar to alternatively activated M2 macrophages. CD163 has been reported to recognize M2 macrophages, while CD68, on the other hand, is a frequently used pan-macrophage marker that recognizes both pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages<sup>106,160</sup>. In paper III we evaluated CD163 as a TAM marker in human breast cancer and compared it to CD68. We further revealed and could highlight the clinical importance of analyzing the localization of TAMs in human breast cancer. While TAMs in the TN did not have any correlation with clinicopathological feature or patient outcome, we found TAMs in TS to be highly relevant.

CD163<sup>+</sup> TAMs turned out to be abundant among triple-negative/basal-like breast cancer patients and correlated with both ER negativity and PgR negativity. A recent paper reported that TAMs were able to downregulate ER through the activation of kinase cascades<sup>161</sup>. Yet another paper, Elkabets *et al.* reported that human breast cancer attracts GRN expressing hematopoietic cells from the bone marrow<sup>162</sup>. They further evaluated GRN in a breast cancer TMA (notably, the same that we used in our paper) and intriguingly GRN<sup>+</sup> hematopoietic cells and CD163<sup>+</sup> TAMs in TS correlated with the exact same clinicopathological features (e.g. triple-negative/basal-like breast cancer). This might imply that CD163<sup>+</sup> cells found in TS are in fact GRN<sup>+</sup> hematopoietic cells recruited from the bone marrow to the tumor where they activate fibroblasts that will promote malignancy<sup>162</sup>.

The infiltration pattern for CD163<sup>+</sup> TAMs and CD68<sup>+</sup> TAMs in the TS differed within the patient group having triple-negative/basal-like breast cancer. Although CD68 is regarded a pan-macrophage marker that recognize both pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages, TAMs might express less CD68 compared to CD163, hence explaining the difference in recruitment pattern. Yet another explanation might be that CD163 is a marker for another subset of cells, for example myeloid derived suppressor cells which have been

shown to enhance tumor progression or as mentioned above, related to GRN+ hematopoietic cells<sup>94,163</sup>. Hence the CD163 marker might recognize different subpopulations of cells but importantly they all promote tumor progression, indicating that CD163 could be used as a marker to recognize pro-tumorigenic cells.

Although we found a significant correlation between CD163<sup>+</sup> TAMs in TS and triple-negative/basal-like breast cancer prognosis, CD163<sup>+</sup> TAMs in TS was not prognostic factor for these patients. This could be due to the limited number of patients with triple-negative/basal-like breast cancer and their poor clinical outcome making it difficult to detect any difference in survival. It would hence be of value to analyze whether or not CD163 has a prognostic value in a larger triple-negative/basal-like breast cancer cohort. CD163<sup>+</sup> TAMs in TS did however have prognostic value for luminal A breast cancer patients.

Taken together our data reveal the clinical importance of analyzing the localization of TAMs in human breast cancer and while dense infiltration of CD163<sup>+</sup> TAMs correlated with unfavorable clinicopathological features, dense infiltration of CD68<sup>+</sup> TAMs in TS was an independent risk factor for reduced breast cancer specific survival.

## *Conclusions*

- I. Our data further strengthens the role of Wnt5a as a tumor suppressor in breast cancer and show that Wnt5a can mediate Ca<sup>2+</sup>-dependent intercellular adhesion via CK1 $\alpha$ -induced Ser-45 phosphorylation of  $\beta$ -catenin, promoting  $\beta$ -catenin/E-cadherin complex formation and thus inhibiting cell migration.
- II. Wnt5a induces a tolerogenic macrophage phenotype in a pro-inflammatory environment in both breast cancer and sepsis patients.
- III. Dense infiltration of CD163<sup>+</sup> TAMs in tumor stroma correlate with unfavorable clinicopathological features and dense infiltration of CD68<sup>+</sup> TAMs in tumor stroma is an independent risk factor for reduced breast cancer specific survival.

# Populärvetenskaplig sammanfattning

Bröstcancer är den vanligaste cancerformen bland kvinnor i Sverige. Varje år får cirka 7000 kvinnor diagnosen bröstcancer och drygt 1500 kvinnor dör årligen av sjukdomen.

Tumören i bröstet går i princip alltid att operera bort, men blir dödlig om cancer sprider sig till andra organ. För att detta skall kunna ske måste tumörceller lossna från sina grannceller och det underlag de sitter i. De måste också få egenskaper som gör att de kan förflytta sig genom omgivande bindvävnad och på detta sätt nå blodbanan och/eller lymfsystemet.

Bröstcancer börjar ofta i de celler som bildar bröstets mjölkgångar, s.k duktala celler. Dessa sitter normalt hårt bundna till varandra. Cellerna binder till varandra genom ett protein som sitter i cellmembranet, E-cadherin. En del av E-cadherinet sticker ut utanför cellmembranet och binder samman med granncellens E-cadherin. För att denna bindning ska bli så stark som möjlig och förhindra att cellen rör på sig måste E-cadherin delen på cellens insida binda till ett annat protein,  $\beta$ -catenin.

För drygt tio år sedan visade en forskargrupp att kvinnor med bröstcancer som saknade proteinet, Wnt5a, i tumören oftare hade återfall och spridning av sin cancer.

Vi har studerat hur Wnt5a kan hindra spridning av bröstcancer. Vi frågade oss om Wnt5a bidrar till att öka E-cadherin/ $\beta$ -catenin bindningen mellan celler och på detta vis får tumörcellerna i bröstcancer att binda hårdare till varandra och minskar deras möjlighet till spridning. I vår studie kunde vi visa att Wnt5a bidrog till att bilda fler E-cadherin/ $\beta$ -catenin bindningar. Vi kunde också visa att kvinnor vars tumörceller producerade lite eller inget Wnt5a hade mindre av  $\beta$ -catenin i tumörcellernas cellmembran och dessa kvinnor hade oftare återfall och spridning av sin cancer.

Spridningen och utvecklingen av cancerceller påverkas även av vita blodkroppar. Det finns olika typer av vita blodkroppar. Vissa har förmågan eliminera tumörceller medan andra gynnar cancerutveckling och förenklar spridningen av cancer. En typ av vita blodkroppar, monocytter, cirkulerar i blodbanan och kan mogna till olika typer av makrofager beroende på vilka signaler de får av sin omgivning. Förenklat kan man säga att de kan mogna till markofager som klarar av att döda tumörceller,; M1 makrofager, eller M2 makrofager som gynnar

cancerutvecklingen. M2 makrofager underlättar spridningen av cancer genom att bl a guida tumörcellerna ut i cirkulationen och blockera de vita blodkroppar som har förmågan att döda tumörceller.

I ett tidigt skede då tumören fortfarande är liten och inte har förmågan att sprida sig kan M1 makrofager eliminera tumörcellerna. Om M1 makrofagerna inte klarar detta kommer tumörcellerna i den inflammatoriska miljön, som M1 makrofager skapar, så småningom blir mer aggressiva och producera ämnen som gör att monocyter och M1 makrofager börjar likna M2 makrofager. Det är fortfarande något oklart vilka ämnen som bidrar till detta makrofagskifte. I vår studie har vi kunnat visa att Wnt5a kan göra att monocyter och M1 makrofager som befinner sig i en inflammatorisk miljö blir mer lika M2-makrofager. Vi kunde också visa att de tumörceller som producerade mycket Wnt5a även hade mer av M2-liknande makrofager runt sig.

Tumören är uppbyggd av tumörceller, vita blodkroppar samt bindvävnad som omger tumörcellerna. Bindvävnaden består bl a av kollagenproducerande celler, fibroblaster, endotelceller som bygger upp blodkärl, och makrofager. Makrofager kan alltså finnas bland tumörceller eller i bindvävnaden som omger dem. Vi har undersökt om makrofagernas lokalisering och koncentration har någon inverkan på överlevnadsprognosen för kvinnor med bröstcancer. Det visade sig att det endast var makrofagerna lokaliserade i bindvävnaden som hade betydelse. De kvinnor med mycket makrofager i bindvävnaden hade sämre överlevnadsprognos.

Vi har visat att Wnt5a kan hindra spridning av bröstcancer genom att öka bindningen mellan tumörceller. Wnt5a bidrar till att monocyter och M1 makrofager i en inflammatorisk miljö blir mer lika M2 makrofager, som förenklar spridning av cancer. Makrofager lokaliserade i tumörens bindvävnad ger en sämre överlevnadsprognos i bröstcancer.

# Acknowledgements

This work was carried out at Division of Experimental Pathology and the Center for Molecular Pathology, Department of Laboratory Medicine, Malmö, Lund University, Skåne University Hospital, Malmö, Sweden.

This work was funded by Swedish Cancer Society, the Medical Research Council, the UMAS Research Foundations, Gunnar Nilssons Cancer Foundation, Ollie and Elof Ericssons Foundation.

I would like to extend my gratitude to the following people...

*Karin Leandersson*, my supervisor, for always believing in me, for support and encouragement and for excellent guiding. For introducing me to tumor immunology and for giving me the opportunity to think big. I admire your enthusiasm and your ability to see opportunities in everything you do and I am grateful for our discussions about science and life. Thank you for being a good friend with a great way of looking at life.

*Tommy Andersson*, my first supervisor and later co-supervisor, for introducing me to the world of science when I was still a medical student. Thank you for all the opportunities you have given me and for sharing your deep knowledge of research.

To my co-authors *Göran Landberg*, *Helena Janols*, *Marlene Wullt*, *Anders Bredberg*, *Fredrik Pontén* and *Karin Jirström*, I'm greatly thankful for your contribution to my work. *Karin Jirström*, for taking the time to guide me through my work.

To my old colleagues at CRC. *Annette*, *Jeannette* for taking care of me when I first came to the group and for a great time in the lab together with *Caroline*, *Christian*, *Veronica* and *Simone*. *Jill* for understanding how wonderful pugs are and for taking the time to go through my thesis. *Lena* for your contribution in my first project. *Ann-Kristin* for help with administration and nice talks in our room.

All the colleagues at CMP for a nice working atmosphere. *Siv* and *Kristin* for all the help and for a nice time when working in the same room. *Elise*, for letting me occupy your computer and for taking the time to show me how to use the computer program. *Caroline* for always being cheerful and helpful and for showing me how to work with monocytes and macrophages. *Elin* for being so enthusiastic about research and for being a good friend and colleague.

*To my friends* for making life enjoyable. *Mikki* for being a good friend with an open mind and heart and for giving invaluable advices. *AK* for long talks about nothing and everything and for all the laughter throughout the years. *Anneli* for being a loyal and inspiring friend and for sharing your knowledge of nature.

*To my family, Mamma, Pappa, Martin, Nelly, Hugo and Ralph* for your big heart and support throughout the years and for the tremendous amount of laughter and joy you have brought and bring into my life.

*Calle* for invaluable help with figures and tables and encouragement. I love you and thank you for believing in my and for bringing laughter and creativity into my life and for making life to a wonderful adventure. Soon our biggest adventure will begin...

# References

1. World Health Organization. Cancer mortality and morbidity. [cited 2012 July 21]  
Available:[http://www.who.int/gho/ncd/mortality\\_morbidity/cancer/en/index.html](http://www.who.int/gho/ncd/mortality_morbidity/cancer/en/index.html).
2. Jemal, A., Center, M.M., DeSantis, C. & Ward, E.M. Global patterns of cancer incidence and mortality rates and trends. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 19, 1893-1907 (2010).
3. World Health Organization. Breast cancer: prevention and control. [cited 2012 July 21] Available:  
<http://www.who.int/cancer/detection/breastcancer/en/>
4. Kamangar, F., Dores, G.M. & Anderson, W.F. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 24, 2137-2150 (2006).
5. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* 144, 646-674 (2011).
6. Mantovani, A. Cancer: Inflaming metastasis. *Nature* 457, 36-37 (2009).
7. Marieb, E.N. *Human anatomy & physiology, Sixth edition*, (Pearson Education, Inc, , 2004).
8. Weinberg, R.A. *The biology of cancer*, (2007).
9. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* 100, 57-70 (2000).
10. Slamon, D.J., *et al.* Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235, 177-182 (1987).
11. Hanahan, D. & Coussens, L.M. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer cell* 21, 309-322 (2012).
12. Gorsch, S.M., Memoli, V.A., Stukel, T.A., Gold, L.I. & Arrick, B.A. Immunohistochemical staining for transforming growth factor beta 1

associates with disease progression in human breast cancer. *Cancer research* 52, 6949-6952 (1992).

13. Bieche, I., *et al.* Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay. *Cancer research* 59, 2759-2765 (1999).
14. Herbert, B.S., Wright, W.E. & Shay, J.W. Telomerase and breast cancer. *Breast cancer research : BCR* 3, 146-149 (2001).
15. Shay, J.W. & Bacchetti, S. A survey of telomerase activity in human cancer. *Eur J Cancer* 33, 787-791 (1997).
16. Greenberg, R.A., *et al.* Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. *Oncogene* 18, 1219-1226 (1999).
17. Harris, C.C. p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. *Carcinogenesis* 17, 1187-1198 (1996).
18. Lacroix, M., Toillon, R.A. & Leclercq, G. p53 and breast cancer, an update. *Endocrine-related cancer* 13, 293-325 (2006).
19. Pietras, K. & Ostman, A. Hallmarks of cancer: interactions with the tumor stroma. *Experimental cell research* 316, 1324-1331 (2010).
20. Butt, A.J., Firth, S.M. & Baxter, R.C. The IGF axis and programmed cell death. *Immunology and cell biology* 77, 256-262 (1999).
21. Leek, R.D., *et al.* Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer research* 56, 4625-4629 (1996).
22. Bergers, G. & Benjamin, L.E. Tumorigenesis and the angiogenic switch. *Nature reviews. Cancer* 3, 401-410 (2003).
23. Konecny, G.E., *et al.* Association between HER-2/neu and vascular endothelial growth factor expression predicts clinical outcome in primary breast cancer patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10, 1706-1716 (2004).
24. Thiery, J.P., Acloque, H., Huang, R.Y. & Nieto, M.A. Epithelial-mesenchymal transitions in development and disease. *Cell* 139, 871-890 (2009).
25. Christofori, G. & Semb, H. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends in biochemical sciences* 24, 73-76 (1999).

26. Berx, G., *et al.* E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. *Oncogene* 13, 1919-1925 (1996).
27. Polyak, K. & Weinberg, R.A. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nature reviews. Cancer* 9, 265-273 (2009).
28. Fidler, I.J. The biology of cancer metastasis. *Seminars in cancer biology* 21, 71 (2011).
29. Nass, S.J., *et al.* Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. *Cancer research* 60, 4346-4348 (2000).
30. Graff, J.R., Gabrielson, E., Fujii, H., Baylin, S.B. & Herman, J.G. Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *The Journal of biological chemistry* 275, 2727-2732 (2000).
31. Kaplan, R.N., *et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 438, 820-827 (2005).
32. Psaila, B. & Lyden, D. The metastatic niche: adapting the foreign soil. *Nature reviews. Cancer* 9, 285-293 (2009).
33. Wodarz, A. & Nathke, I. Cell polarity in development and cancer. *Nature cell biology* 9, 1016-1024 (2007).
34. Baum, B. & Georgiou, M. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *The Journal of cell biology* 192, 907-917 (2011).
35. Cowin, P., Rowlands, T.M. & Hatsell, S.J. Cadherins and catenins in breast cancer. *Current opinion in cell biology* 17, 499-508 (2005).
36. Heuberger, J. & Birchmeier, W. Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. *Cold Spring Harbor perspectives in biology* 2, a002915 (2010).
37. Yamada, S., Pokutta, S., Drees, F., Weis, W.I. & Nelson, W.J. Deconstructing the cadherin-catenin-actin complex. *Cell* 123, 889-901 (2005).
38. Drees, F., Pokutta, S., Yamada, S., Nelson, W.J. & Weis, W.I. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* 123, 903-915 (2005).
39. Dolled-Filhart, M., *et al.* Quantitative in situ analysis of beta-catenin expression in breast cancer shows decreased expression is associated with poor outcome. *Cancer research* 66, 5487-5494 (2006).

40. Incassati, A., Chandramouli, A., Eelkema, R. & Cowin, P. Key signaling nodes in mammary gland development and cancer: beta-catenin. *Breast cancer research : BCR* 12, 213 (2010).
41. Dupre-Crochet, S., *et al.* Casein kinase 1 is a novel negative regulator of E-cadherin-based cell-cell contacts. *Molecular and cellular biology* 27, 3804-3816 (2007).
42. Nelson, W.J. & Nusse, R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 303, 1483-1487 (2004).
43. Piedra, J., *et al.* p120 Catenin-associated Fer and Fyn tyrosine kinases regulate beta-catenin Tyr-142 phosphorylation and beta-catenin-alpha-catenin Interaction. *Molecular and cellular biology* 23, 2287-2297 (2003).
44. Brembeck, F.H., *et al.* Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions. *Genes Dev* 18, 2225-2230 (2004).
45. Brembeck, F.H., Rosario, M. & Birchmeier, W. Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. *Curr Opin Genet Dev* 16, 51-59 (2006).
46. Roura, S., Miravet, S., Piedra, J., Garcia de Herreros, A. & Dunach, M. Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. *The Journal of biological chemistry* 274, 36734-36740 (1999).
47. Du, C., Jaggi, M., Zhang, C. & Balaji, K.C. Protein kinase D1-mediated phosphorylation and subcellular localization of beta-catenin. *Cancer research* 69, 1117-1124 (2009).
48. Gottardi, C.J. & Gumbiner, B.M. Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. *J Cell Biol* 167, 339-349 (2004).
49. Nusse, R. Wnt signaling. *Cold Spring Harbor perspectives in biology* 4(2012).
50. Bienz, M. beta-Catenin: a pivot between cell adhesion and Wnt signalling. *Curr Biol* 15, R64-67 (2005).
51. Shimizu, H., *et al.* Transformation by Wnt family proteins correlates with regulation of beta-catenin. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* 8, 1349-1358 (1997).
52. Kikuchi, A., Yamamoto, H. & Kishida, S. Multiplicity of the interactions of Wnt proteins and their receptors. *Cell Signal* 19, 659-671 (2007).
53. Angers, S. & Moon, R.T. Proximal events in Wnt signal transduction. *Nature reviews. Molecular cell biology* 10, 468-477 (2009).

54. Rao, T.P. & Kuhl, M. An updated overview on Wnt signaling pathways: a prelude for more. *Circulation research* 106, 1798-1806 (2010).
55. Clevers, H. & Nusse, R. Wnt/beta-Catenin Signaling and Disease. *Cell* 149, 1192-1205 (2012).
56. Klaus, A. & Birchmeier, W. Wnt signalling and its impact on development and cancer. *Nature reviews. Cancer* 8, 387-398 (2008).
57. Amit, S., *et al.* Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes & development* 16, 1066-1076 (2002).
58. Li, V.S., *et al.* Wnt Signaling through Inhibition of beta-Catenin Degradation in an Intact Axin1 Complex. *Cell* 149, 1245-1256 (2012).
59. Zeng, X., *et al.* A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* 438, 873-877 (2005).
60. Davidson, G., *et al.* Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* 438, 867-872 (2005).
61. Brennan, K.R. & Brown, A.M. Wnt proteins in mammary development and cancer. *Journal of mammary gland biology and neoplasia* 9, 119-131 (2004).
62. Lin, S.Y., *et al.* Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proceedings of the National Academy of Sciences of the United States of America* 97, 4262-4266 (2000).
63. Wulf, G., Ryo, A., Liou, Y.C. & Lu, K.P. The prolyl isomerase Pin1 in breast development and cancer. *Breast cancer research : BCR* 5, 76-82 (2003).
64. Ryo, A., Nakamura, M., Wulf, G., Liou, Y.C. & Lu, K.P. Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. *Nature cell biology* 3, 793-801 (2001).
65. Sugimura, R. & Li, L. Noncanonical Wnt signaling in vertebrate development, stem cells, and diseases. *Birth defects research. Part C, Embryo today : reviews* 90, 243-256 (2010).
66. Kestler, H.A. & Kuhl, M. From individual Wnt pathways towards a Wnt signalling network. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 363, 1333-1347 (2008).
67. Semenov, M.V., Habas, R., Macdonald, B.T. & He, X. SnapShot: Noncanonical Wnt Signaling Pathways. *Cell* 131, 1378 (2007).
68. Komiya, Y. & Habas, R. Wnt signal transduction pathways. *Organogenesis* 4, 68-75 (2008).

69. Veeman, M.T., Axelrod, J.D. & Moon, R.T. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Developmental cell* 5, 367-377 (2003).
70. De, A. Wnt/Ca<sup>2+</sup> signaling pathway: a brief overview. *Acta biochimica et biophysica Sinica* 43, 745-756 (2011).
71. Minami, Y., Oishi, I., Endo, M. & Nishita, M. Ror-family receptor tyrosine kinases in noncanonical Wnt signaling: their implications in developmental morphogenesis and human diseases. *Developmental dynamics : an official publication of the American Association of Anatomists* 239, 1-15 (2010).
72. Katso, R.M., *et al.* Overexpression of H-Ryk in mouse fibroblasts confers transforming ability in vitro and in vivo: correlation with up-regulation in epithelial ovarian cancer. *Cancer research* 59, 2265-2270 (1999).
73. Kikuchi, A., Yamamoto, H., Sato, A. & Matsumoto, S. Wnt5a: its signalling, functions and implication in diseases. *Acta Physiol (Oxf)* 204, 17-33 (2012).
74. Sato, A., Yamamoto, H., Sakane, H., Koyama, H. & Kikuchi, A. Wnt5a regulates distinct signalling pathways by binding to Frizzled2. *The EMBO journal* 29, 41-54 (2010).
75. Mikels, A.J. & Nusse, R. Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS biology* 4, e115 (2006).
76. Topol, L., *et al.* Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation. *The Journal of cell biology* 162, 899-908 (2003).
77. Dejmek, J., Dejmek, A., Safholm, A., Sjolander, A. & Andersson, T. Wnt-5a protein expression in primary dukes B colon cancers identifies a subgroup of patients with good prognosis. *Cancer research* 65, 9142-9146 (2005).
78. Kremenevskaja, N., *et al.* Wnt-5a has tumor suppressor activity in thyroid carcinoma. *Oncogene* 24, 2144-2154 (2005).
79. Liu, X.H., *et al.* Expression of Wnt-5a and its clinicopathological significance in hepatocellular carcinoma. *Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver* 40, 560-567 (2008).
80. Dejmek, J., *et al.* Expression and signaling activity of Wnt-5a/discoidin domain receptor-1 and Syk plays distinct but decisive roles in breast cancer patient survival. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11, 520-528 (2005).

81. Jonsson, M., Dejmek, J., Bendahl, P.O. & Andersson, T. Loss of Wnt-5a protein is associated with early relapse in invasive ductal breast carcinomas. *Cancer research* 62, 409-416 (2002).
82. Jonsson, M. & Andersson, T. Repression of Wnt-5a impairs DDR1 phosphorylation and modifies adhesion and migration of mammary cells. *Journal of cell science* 114, 2043-2053 (2001).
83. McDonald, S.L. & Silver, A. The opposing roles of Wnt-5a in cancer. *British journal of cancer* 101, 209-214 (2009).
84. Weeraratna, A.T., *et al.* Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer cell* 1, 279-288 (2002).
85. Kurayoshi, M., *et al.* Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion. *Cancer research* 66, 10439-10448 (2006).
86. Huang, C.L., *et al.* Wnt5a expression is associated with the tumor proliferation and the stromal vascular endothelial growth factor--an expression in non-small-cell lung cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 23, 8765-8773 (2005).
87. Grivennikov, S.I., Greten, F.R. & Karin, M. Immunity, inflammation, and cancer. *Cell* 140, 883-899 (2010).
88. Balkwill, F., Charles, K.A. & Mantovani, A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer cell* 7, 211-217 (2005).
89. Bianchi, M.E. DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of leukocyte biology* 81, 1-5 (2007).
90. DeNardo, D.G. & Coussens, L.M. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast cancer research : BCR* 9, 212 (2007).
91. Fridman, W.H., Pages, F., Sautes-Fridman, C. & Galon, J. The immune contexture in human tumours: impact on clinical outcome. *Nature reviews. Cancer* 12, 298-306 (2012).
92. DeNardo, D.G., Andreu, P. & Coussens, L.M. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer metastasis reviews* 29, 309-316 (2010).
93. Gabrilovich, D.I., Ostrand-Rosenberg, S. & Bronte, V. Coordinated regulation of myeloid cells by tumours. *Nature reviews. Immunology* 12, 253-268 (2012).

94. Murdoch, C., Muthana, M., Coffelt, S.B. & Lewis, C.E. The role of myeloid cells in the promotion of tumour angiogenesis. *Nature reviews. Cancer* 8, 618-631 (2008).
95. Finak, G., *et al.* Stromal gene expression predicts clinical outcome in breast cancer. *Nature medicine* 14, 518-527 (2008).
96. Biswas, S.K. & Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nature immunology* 11, 889-896 (2010).
97. Bates, G.J., *et al.* Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 24, 5373-5380 (2006).
98. Tadmor, T., Attias, D. & Polliack, A. Myeloid-derived suppressor cells--their role in haemato-oncological malignancies and other cancers and possible implications for therapy. *British journal of haematology* 153, 557-567 (2011).
99. Ostrand-Rosenberg, S. & Sinha, P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 182, 4499-4506 (2009).
100. Gabrilovich, D.I. & Nagaraj, S. Myeloid-derived suppressor cells as regulators of the immune system. *Nature reviews. Immunology* 9, 162-174 (2009).
101. Vuk-Pavlovic, S., *et al.* Immunosuppressive CD14+HLA-DR<sup>low</sup>/monocytes in prostate cancer. *The Prostate* 70, 443-455 (2010).
102. Poschke, I., Mougiakakos, D., Hansson, J., Masucci, G.V. & Kiessling, R. Immature immunosuppressive CD14+HLA-DR<sup>low</sup> cells in melanoma patients are Stat3<sup>hi</sup> and overexpress CD80, CD83, and DC-sign. *Cancer research* 70, 4335-4345 (2010).
103. Condeelis, J. & Pollard, J.W. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124, 263-266 (2006).
104. Lewis, C.E. & Pollard, J.W. Distinct role of macrophages in different tumor microenvironments. *Cancer research* 66, 605-612 (2006).
105. Sica, A. & Mantovani, A. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation* 122, 787-795 (2012).
106. Bingle, L., Brown, N.J. & Lewis, C.E. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *The Journal of pathology* 196, 254-265 (2002).
107. Pollard, J.W. Trophic macrophages in development and disease. *Nature reviews. Immunology* 9, 259-270 (2009).

108. Mantovani, A. Molecular pathways linking inflammation and cancer. *Current molecular medicine* 10, 369-373 (2010).
109. Gordon, S. Alternative activation of macrophages. *Nat Rev Immunol* 3, 23-35 (2003).
110. Mantovani, A., Allavena, P., Sica, A. & Balkwill, F. Cancer-related inflammation. *Nature* 454, 436-444 (2008).
111. Dunn, G.P., Old, L.J. & Schreiber, R.D. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 21, 137-148 (2004).
112. Qian, B.Z. & Pollard, J.W. Macrophage diversity enhances tumor progression and metastasis. *Cell* 141, 39-51 (2010).
113. Lin, W.W. & Karin, M. A cytokine-mediated link between innate immunity, inflammation, and cancer. *The Journal of clinical investigation* 117, 1175-1183 (2007).
114. Sen, R. & Smale, S.T. Selectivity of the NF- $\kappa$ B response. *Cold Spring Harbor perspectives in biology* 2, a000257 (2010).
115. Leek, R.D. & Harris, A.L. Tumor-associated macrophages in breast cancer. *Journal of mammary gland biology and neoplasia* 7, 177-189 (2002).
116. Mueller, M.M. & Fusenig, N.E. Friends or foes - bipolar effects of the tumour stroma in cancer. *Nature reviews. Cancer* 4, 839-849 (2004).
117. Sica, A., Schioppa, T., Mantovani, A. & Allavena, P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer* 42, 717-727 (2006).
118. Wyckoff, J.B., *et al.* Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer research* 67, 2649-2656 (2007).
119. Condeelis, J. & Segall, J.E. Intravital imaging of cell movement in tumours. *Nature reviews. Cancer* 3, 921-930 (2003).
120. Curt Franksson, B.H. Frankssons Kirurig. Chapter 11, 864 (1992).
121. Arendt, L.M., Rudnick, J.A., Keller, P.J. & Kuperwasser, C. Stroma in breast development and disease. *Seminars in cell & developmental biology* 21, 11-18 (2010).
122. Bissell, M.J. & Radisky, D. Putting tumours in context. *Nature reviews. Cancer* 1, 46-54 (2001).
123. Howard, B.A. & Gusterson, B.A. Human breast development. *J Mammary Gland Biol Neoplasia* 5, 119-137 (2000).
124. Arthur C. Guyton, J.E.H. *Textbook of Medical Physiology, Tenth Edition*, (2000).

125. Polyak, K. & Kalluri, R. The role of the microenvironment in mammary gland development and cancer. *Cold Spring Harb Perspect Biol* 2, a003244 (2010).
126. Roarty, K. & Serra, R. Wnt5a is required for proper mammary gland development and TGF-beta-mediated inhibition of ductal growth. *Development* 134, 3929-3939 (2007).
127. Socialstyrelsen. Cancer i siffror 2009. Artikelnummer: 2009-126-127(2009).
128. Society, A.C. Breast Cancer Facts & Figures 2009-2010. (2009).
129. Hulka, B.S. & Moorman, P.G. Breast cancer: hormones and other risk factors. *Maturitas* 38, 103-113; discussion 113-106 (2001).
130. Shuen, A.Y. & Foulkes, W.D. Inherited mutations in breast cancer genes--risk and response. *J Mammary Gland Biol Neoplasia* 16, 3-15 (2011).
131. Lux, M.P., Fasching, P.A. & Beckmann, M.W. Hereditary breast and ovarian cancer: review and future perspectives. *J Mol Med (Berl)* 84, 16-28 (2006).
132. Foulkes, W.D., Smith, I.E. & Reis-Filho, J.S. Triple-negative breast cancer. *N Engl J Med* 363, 1938-1948 (2010).
133. Weigelt, B., Geyer, F.C. & Reis-Filho, J.S. Histological types of breast cancer: how special are they? *Molecular oncology* 4, 192-208 (2010).
134. Gruver, A.M., Portier, B.P. & Tubbs, R.R. Molecular pathology of breast cancer: the journey from traditional practice toward embracing the complexity of a molecular classification. *Archives of pathology & laboratory medicine* 135, 544-557 (2011).
135. Elston, C.W. & Ellis, I.O. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 19, 403-410 (1991).
136. Galea, M.H., Blamey, R.W., Elston, C.E. & Ellis, I.O. The Nottingham Prognostic Index in primary breast cancer. *Breast cancer research and treatment* 22, 207-219 (1992).
137. Singletary, S.E. & Connolly, J.L. Breast cancer staging: working with the sixth edition of the AJCC Cancer Staging Manual. *CA: a cancer journal for clinicians* 56, 37-47; quiz 50-31 (2006).
138. Hu, Z., et al. The molecular portraits of breast tumors are conserved across microarray platforms. *BMC genomics* 7, 96 (2006).
139. Polyak, K. Breast cancer: origins and evolution. *The Journal of clinical investigation* 117, 3155-3163 (2007).

140. Sorlie, T., *et al.* Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms. *BMC genomics* 7, 127 (2006).
141. Perou, C.M., *et al.* Molecular portraits of human breast tumours. *Nature* 406, 747-752 (2000).
142. Weigelt, B., Peterse, J.L. & van 't Veer, L.J. Breast cancer metastasis: markers and models. *Nature reviews. Cancer* 5, 591-602 (2005).
143. Sorlie, T., *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98, 10869-10874 (2001).
144. Badve, S., *et al.* Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 24, 157-167 (2011).
145. Bertucci, F., *et al.* How basal are triple-negative breast cancers? *International journal of cancer. Journal international du cancer* 123, 236-240 (2008).
146. SweBCG. National Guidelines for Breast Cancer Treatment. (2011).
147. Platet, N., Cathiard, A.M., Gleizes, M. & Garcia, M. Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion. *Critical reviews in oncology/hematology* 51, 55-67 (2004).
148. *Läkemedelsboken 2011-2012*, (Apoteket AB, 2011).
149. Dejmek, J., Sapholm, A., Kamp Nielsen, C., Andersson, T. & Leandersson, K. Wnt-5a/Ca<sup>2+</sup>-induced NFAT activity is counteracted by Wnt-5a/Yes-Cdc42-casein kinase 1alpha signaling in human mammary epithelial cells. *Molecular and cellular biology* 26, 6024-6036 (2006).
150. Biswas, S.K. & Lopez-Collazo, E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in immunology* 30, 475-487 (2009).
151. Hu, X., Chen, J., Wang, L. & Ivashkiv, L.B. Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. *Journal of leukocyte biology* 82, 237-243 (2007).
152. Filipazzi, P., *et al.* Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 25, 2546-2553 (2007).

153. Hintz, K.A., *et al.* Endotoxin induces rapid metalloproteinase-mediated shedding followed by up-regulation of the monocyte hemoglobin scavenger receptor CD163. *Journal of leukocyte biology* 72, 711-717 (2002).
154. Groselj-Grenc, M., Ihan, A. & Derganc, M. Neutrophil and monocyte CD64 and CD163 expression in critically ill neonates and children with sepsis: comparison of fluorescence intensities and calculated indexes. *Mediators of inflammation* 2008, 202646 (2008).
155. Pereira, C., Schaer, D.J., Bachli, E.B., Kurrer, M.O. & Schoedon, G. Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for the antiinflammatory action of activated protein C and interleukin-10. *Arteriosclerosis, thrombosis, and vascular biology* 28, 504-510 (2008).
156. Qualls, J.E. & Murray, P.J. A double agent in cancer: stopping macrophages wounds tumors. *Nature medicine* 16, 863-864 (2010).
157. Blumenthal, A., *et al.* The Wntless homolog WNT5A and its receptor Frizzled-5 regulate inflammatory responses of human mononuclear cells induced by microbial stimulation. *Blood* 108, 965-973 (2006).
158. Pukrop, T., *et al.* Wnt 5a signaling is critical for macrophage-induced invasion of breast cancer cell lines. *Proceedings of the National Academy of Sciences of the United States of America* 103, 5454-5459 (2006).
159. Goldsmith, M., *et al.* Synergistic IL-10 induction by LPS and the ceramide-1-phosphate analog PCERA-1 is mediated by the cAMP and p38 MAP kinase pathways. *Molecular immunology* 46, 1979-1987 (2009).
160. Holness, C.L. & Simmons, D.L. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood* 81, 1607-1613 (1993).
161. Stossi, F., Madak-Erdogan, Z. & Katzenellenbogen, B.S. Macrophage-elicited loss of estrogen receptor- $\alpha$  in breast cancer cells via involvement of MAPK and c-Jun at the ESR1 genomic locus. *Oncogene* 31, 1825-1834 (2012).
162. Elkabets, M., *et al.* Human tumors instigate granulysin-expressing hematopoietic cells that promote malignancy by activating stromal fibroblasts in mice. *The Journal of clinical investigation* 121, 784-799 (2011).
163. Maniecki, M.B., Moller, H.J., Moestrup, S.K. & Moller, B.K. CD163 positive subsets of blood dendritic cells: the scavenging macrophage receptors CD163 and CD91 are coexpressed on human dendritic cells and monocytes. *Immunobiology* 211, 407-417 (2006).