Wnt5a signaling in Malignant Melanoma

Ekström, Elin

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Wnt5a signaling in Malignant Melanoma

Elin Ekström

Academic dissertation
By due permission of the Faculty of Medicine, Lund University, Sweden to be defended at the main lecture hall, Pathology building, entrance 78, Skåne University Hospital, Malmö, on Friday 16th of December, 2011, at 13.00 for the degree of Doctor of Philosophy, Faculty of Medicine.

Faculty opponent:
Gunnar Schulte, Ph. D. Associate Professor
Department of Physiology and Pharmacology
Karolinska Institute, Stockholm, Sweden
Wnt5a signaling in malignant melanoma

Abstract
The Wnt signaling pathway is highly conserved and crucial during embryonic development. Aberrant Wnt signaling is important in several different disease processes. One such disease process is the development and progression of cancer. Malignant melanoma is a highly aggressive tumor type, in which Wnt signaling is essential. Once a melanoma tumor has spread and formed metastasis, there are few treatment options and the mean 5-year survival rate is less than 5%. Activation of the non-canonical Wnt signaling pathway via Wnt5a has been correlated with increased migration and invasion as well as a poor prognosis in malignant melanoma patients.

In this thesis, we investigate different processes downstream of Wnt5a that can lead to increased aggressiveness of malignant melanoma. Our studies show that the secreted Wnt modulator, SFRP3 is down-regulated in malignant melanoma patients. Using melanocyte and malignant melanoma cell lines, we demonstrate that the down-regulation is due to methylation. Functional studies show that SFRP3 can inhibit Wnt5a signaling and induce a decrease in migration and invasion. Furthermore, by analyzing the effects of Wnt5a on the malignant melanoma proteome, we provide evidence for a cell context dependent reprogramming of cellular metabolism induced by Wnt5a. Activation of Wnt5a signaling in melanoma cell lines resulted in a pro-tumorigenic effect by favoring aerobic glycolysis.

Finally, we investigate the relationship between Wnt5a and secreted pro-tumorigenic factors. We found that Wnt5a could activate regulated secretion of IL-6 and VEGF, dependent on cytoskeletal re-arrangements mediated via calcium signaling and Cdc42 activation.

Taken together these data suggests that, in melanoma, an altered Wnt5a signal can induce different pro-tumorigenic effects. This thesis highlights the complexity of Wnt signaling in general and Wnt5a signaling in melanoma in particular and extends our knowledge of the mechanisms behind progression of malignant melanoma.

Key words: Wnt5a, Malignant melanoma, migration, invasion, methylation, SFRP3, Glycolysis, IL-6, VEGF

Signature: Elin Ekström
Date: 2011-11-07
Wnt5a signaling in Malignant Melanoma

by

Elin Ekström

LUND UNIVERSITY
Faculty of Medicine
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List of papers

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

I. Methylation and loss of Secreted Frizzled-Related Protein 3 enhances melanoma cell migration and invasion

_Elin J. Ekström, Victoria Sherwood and Tommy Andersson_
PLoS ONE 6(4) e18674. doi:10.1371/journal.pone.0018674

II. Non-canonical Wnt5a Signaling is a Novel Regulator of Melanoma Cell Metabolism

_Victoria Sherwood, Shivendra Chaurasiya, Elin J Ekström, Sofie Mattson, Qing Lui, Tomas Köck, Karin Hansson, Margrét Agnarsdóttir, Michael Bergqvist, Karin Jirström, Fredrik Ponten, Peter James, Tommy Andersson_
Manuscript

III. Wnt5a induces Ca\(^{2+}\)-dependent exocytosis of immunomodulatory and pro-angiogenic factors in malignant melanoma.

_Elin J Ekström, Verena von Bulow, Caroline Bergenfelz, Tommy Andersson and Karin Leandersson_
Manuscript
### List of abbreviations

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<tr>
<td>5′AZA</td>
<td>5-aza-2’deoxycytidine</td>
</tr>
<tr>
<td>Akt</td>
<td>v-akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical protein kinase C</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>β-TRCP</td>
<td>b-transducin-repeat-containing-protein</td>
</tr>
<tr>
<td>Bcl</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-raf murine sarcoma viral oncogene homolog B</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CamKII</td>
<td>Ca²⁺/calmodulin-dependent protein kinases II</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division control protein 42homolog</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine phosphate Guanine</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine rich domain</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyriboucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyl transferase</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FrzB</td>
<td>Frizzled motif associated with bone development</td>
</tr>
<tr>
<td>FZD</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GC</td>
<td>Guanine cytosine</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEO</td>
<td>Gene expression omnibus</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>GSK3b</td>
<td>Glycogen synthase kinase 3b</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulphate proteoglycan</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun terminal kinase</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LDHV</td>
<td>Lactate dehydrogenase 5</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphocyte enhancing factor</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>LRP</td>
<td>Low-density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia associated transcription factor</td>
</tr>
<tr>
<td>MLH1</td>
<td>MutL homolog 1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>Myc</td>
<td>v-myc myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NLK</td>
<td>Nemo-like kinase</td>
</tr>
<tr>
<td>NTR</td>
<td>Netrin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptors</td>
</tr>
<tr>
<td>Rac</td>
<td>ras-related c3 botulinum toxin substrate</td>
</tr>
<tr>
<td>RGP</td>
<td>Radial growth phase</td>
</tr>
<tr>
<td>Rho</td>
<td>ras homolog gene family member</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>ROR</td>
<td>RAR-related orphan receptors</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RYK</td>
<td>Related to receptor tyrosine kinase</td>
</tr>
<tr>
<td>SARP</td>
<td>Secreted apoptosis related protein</td>
</tr>
<tr>
<td>SFRP</td>
<td>Secreted frizzled related protein</td>
</tr>
<tr>
<td>SNAP</td>
<td>Synaptosomal-associated protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>STAT-3</td>
<td>Signal transducers and activators of transcription protein 3</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell factor</td>
</tr>
<tr>
<td>TMA</td>
<td>Tumor microarray</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>UVR</td>
<td>Ultra violet radiation</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle associated membrane protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VGP</td>
<td>Vertical growth phase</td>
</tr>
<tr>
<td>WIF</td>
<td>Wnt inhibitory factor</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless-related MMTV integration site</td>
</tr>
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</table>
Background

Basic concepts in tumor biology

The development of a tumor from normal cells is a process in multiple steps. Certain crucial cell characteristics are acquired and others are lost and these have been reviewed extensively elsewhere but will be briefly covered here. However the focus of this introduction will be the processes of tumor development and spread that are relevant to the topics covered in papers I, II, and III. These are migration and metastasis, methylation, tumor metabolism and angiogenesis.

Several processes that take place during tumor development and progression have been suggested as hallmarks of cancer (Hanahan and Weinberg 2011). Crucial for tumor development is the loss of growth control, which is generated in different ways. These are insensitivity to apoptosis inducing signals, suppression of growth inhibitors and an intrinsic capability to induce proliferation without the need for extrinsic growth factors. Genomic instability, the capability to replicate continually and a change in the cellular metabolism also support tumor development. The cells of the tumor stroma can also aid the development of the tumor. Often the tumor cells secrete factors that can affect these cells but the tumor cell can also utilize the functions of the stromal cells to promote tumor development. The ability to evade destruction of the immune system is another characteristic that is acquired during tumor development but inflammatory components can also act in a tumor-promoting manner. Induction of angiogenesis and invasion and metastasis are also crucial steps in the progression of tumors. A lot of these characteristics are generated by activating mutations in genes that have a stimulatory effect on the cells such as constitutive activation of proliferation. These genes with the ability to promote cancer are termed oncogenes. Contrary to this are inactivating mutations in tumor suppressors. These are genes whose activation would have had restrictive effects on the cancer cell such as activation of apoptosis or inhibition of the cell cycle. Mutations in oncogenes and tumor suppressors are also involved in the control of the cell cycle and when this is lost the cells can undergo uninhibited division.

Migration and metastasis

In cancer, particularly in solid tumors, it is usually not the primary tumor that causes lethality but the spread of the tumor to distant sites and the formation of metastases. In order for a tumor to spread to distant sites, the tumor cells must acquire a set of characteristics that increase their ability to metastasize. They must be motile and move away from their primary site, this process require cytoskeletal rearrangements and a change in adhesion. The cells need to be able to detach and break away from its surrounding cells and extracellular matrix. For tumor cells to move from their primary tissue, the adherence to the surrounding is important. Cells need to be able to grab hold of the surrounding tissue but too much adhesion makes the cells unable to move over the surface of the surrounding cells (Gassmann et al 2004). The inherent adhesion of the cells to their surrounding tissue will determine whether or not an increase in adhesion will lead to more or less migration. The detachment of tumor cells
from their surrounding cells can occur via a change in the expression of cell-cell adhesion molecules such as E-cadherin which is crucial in the attachment of one cell to another. (Christofori and Semb 1999). Another important factor in the detachment is integrins, these are cell surface receptors that attach the cell to the extracellular matrix but they can also communicate with the intracellular environment about the outside environment. A change in the expression of integrins is necessary for the cells capability to migrate away from the primary tissue and invade a foreign secondary tissue (Guo and Giancotti 2004). Yet another important factor for the invasion of the tissue surrounding the tumor cells and in the secondary site is the ability to break down and remodel the surrounding extracellular matrix. This is in part performed via the action of proteases such as matrix-metalloproteinases that can degrade proteins in the extracellular matrix (for example collagen) but also most other components of the extracellular matrix (Kessenbrock et al 2010).

Once the tumor cells have broken away from their primary tissue they need to intravasate into blood vessels. Now they reach surroundings that might be hostile to them since they are adapted to survive only in their primary tissue. The cells that have acquired characteristics to survive in this environment will be able to exit the blood vessels through extravasation and invade a new tissue. Again the cells need to be able to survive in this new tissue and through changes in adhesion, motility and tissue invasive capabilities, the tumor cells can colonize, proliferate and form metastases in the secondary tissue. Additionally, for a tumor cell to be able to metastasize, the contribution of stromal cells is now known to be important for most of the processes described above. The stromal cells such as fibroblasts and macrophages can secrete factors that render the tumor cells more aggressive. These can be growth factors to sustain uninhibited cell growth, factors that can aid in the degradation of the extracellular matrix (ECM) and the surrounding tissue and factors that can promote angiogenesis. Stromal cells have been shown to facilitate intra and extravasation in blood vessels, which is a crucial process for the spread of the tumor. Stromal cells also seems to mediate the formation of metastases in the secondary tissue by making it more hospitable for the tumor cells (Joyce and Pollard 2009).

The cytoskeleton in tumor cell adhesion, migration and vesicle transport

Cells that migrate usually do so in a polarized manner with a leading edge and a trailing edge. The leading edge forms a protruding lamellipodium, which requires adhesion and is the driving force for migration. Upon moving forward the trailing edge will contract with the help of stress fiber formation, the cell will detach and move in the direction of the lamellipodia. The cytoskeleton mediates all these processes by polymerizing and re-arranging actin filaments, this leads to the formation of protrusions such as lamellipodia and filopodia that can attach to the ECM (Yilmaz and Christofori 2010). Extracellular signaling, such as the signaling activated by chemotactic molecules in order to generate directional migration, can regulate this. However, cells in culture can also exhibit random migration (Pankov et al 2005). The microtubule cytoskeleton is also important during migration especially during polarized migration where it mediates organization of the organelles. This is required for polarized migration that occurs in wound healing and in a scratch induced in cells grown in a 2D monolayer (Hall 2009). The re-arrangement of the actin cytoskeleton is not only important in cell movements but also in the release of secreted proteins. This
requires a change in the arrangement of the cytoskeleton to allow for the trafficking of proteins from the endoplasmatic reticulum (ER) and Golgi to the cell membrane (Ridley 2001, Vitale et al 1995).

There are several proteins and signaling pathways that are important for the regulation of the cytoskeleton. Of these proteins, the Rho GTPase proteins are key regulators of migration and secretion by activating polymerization and rearrangements of the cytoskeleton via the activation of signals from the extracellular environment via membrane receptors. The cycling between the active GTP bound state and the inactive GDP bound state controls the activity of Rho GTPases. The cycling is regulated by the activating Guanine nucleotide Exchange Factors and the inactivating GTP activating proteins (Jaffe and Hall 2005). The Rho GTPases Rac and Cdc42 can regulate the polarization of actin and the formation of lamellipodia, and filopodia whereas Rho can regulate the rearrangements that lead to the retraction of the trailing edge (Nobes and Hall 1999).

Cytoskeletal changes are important for secretion, re-arrangements in the actin cytoskeleton are needed for secretory granules to reach the plasma membrane where they attach and the content of the granule can be secreted out of the cell. Most cytokines are suggested to be released in a regulated manner (Stanley and Lacy 2010). They are packaged in secretory vesicles in the Golgi and upon the proper stimuli these vesicles are transported to the plasma membrane, where the docking of the vesicle is dependent on several different SNARE proteins. One type of SNARE protein, the VAMP proteins are located in the vesicle and upon docking they can bind to other types of SNAREs, the SNAP and Syntaxin proteins, which are located in the plasma membrane. The contents of the vesicle are now secreted out of the cell. This process is dependent on actin remodeling that, in certain types of exocytosis is fueled by Cdc42 and/or Rac1 and that might be induced by an increase in intracellular calcium. Cdc42 has been suggested to regulate exocytosis in different ways, such as controlling membrane transport and interacting with VAMP proteins but the exact mechanism of regulation is still unclear, however it is probably important in regulating the re-arrangements of F-actin necessary for vesicle fusion with the plasma membrane that is required for secretion (Alberts et al 2006, Brown et al 1998, Gasman et al 2003, Kroschewski et al 1999).

Methylation

Oncogenes and tumor suppressors are important in the development and progression of tumors and these can be regulated by mutations but also by silencing and re-expression of these genes. The expression of these genes in many tumors can be regulated by epigenetic modulation. Specific promoter hypermethylation, global hypermethylation, histone acetylation and other epigenetic processes are frequently seen in cancer. This can activate the expression of oncogenes but the most common effect in cancers is the silencing of tumor suppressors. The methylation of DNA occurs in regions of the gene promoter that contains CpG dinucleotides, which can become methylated by DNA Methyl Transferases (DNMTs). In mammals these DNMTs that are thought to be active in mediating de novo methylation that occurs during tumorigenesis are DNMT3A and B (Taby and Issa 2010).
When the CpG region in a gene becomes methylated, Methyl-CpG binding proteins are recruited, these in turn can recruit histone deacetylases (HDACs) that removes acetyl groups from histones and Histone Methyl Transferases (HMTs), which methylates histones. The removal of the acetyl groups form the histones and the addition of methyl groups makes them more positively charged and the negatively charged DNA then wraps more tightly around the histone. This renders the DNA inaccessible for the transcriptional machinery and inhibits transcription from the gene in question (Baylin and Jones 2011). The silencing of tumor suppressor genes in this fashion has been shown in several tumors such as colon cancer, lung cancer, breast cancer, malignant melanoma and many more. In colon cancer, the silencing of, among others, mismatch repair genes such as MLH1 is frequently seen. In colon cancer several of the players in Wnt signaling are also methylated (van Engeland et al 2011). The tumor suppressor p16/INK4 is silenced by methylation in several different types of cancer (Myohanen et al 1998). The loss of expression of the estrogen receptor (ER) and the progesterone receptor (PR) due to methylation of the promoter region has been seen in ER and PR negative breast tumors (Gaudet et al 2009). Several of the SFRP genes are silenced due to methylation in cancer as described below (Marsit et al 2005, Marsit et al 2010, Suzuki et al 2002, Suzuki et al 2004).

Tumor metabolism

The ability to grow and proliferate in an uncontrolled fashion is one the earliest acquired characteristics during tumor development. In order for a cell to grow and divide in this fashion, a lot of energy and biomass is required which can be generated from the processing of glucose. Normal cells in an oxygen saturated environment process glucose via glycolysis to pyruvate, which is then shuttled into the mitochondria where it is converted into carbon dioxide via oxidative phosphorylation. This process provides the cells with sufficient amounts of ATP that it needs at the given moment and is tightly regulated and important for the normal function of the cell. In a situation with low oxygen pressure, glycolysis is increased but the pyruvate formed is not transformed into carbon dioxide but instead to lactate via LDH, which is a much less oxygen consuming process. The lactate is transported out of the cell via transporters. In tumor cells this process is often reprogrammed to favor glycolysis even in an oxygen rich environment termed aerobic glycolysis and this is what is known as the Warburg effect (Vander Heiden et al 2009). To support the high-energy demand of cancer cells despite the fact that glycolysis generates less ATP compared to oxidative phosphorylation, the players that mediate glycolysis are thought to be up-regulated which generates an increased glycolysis and more production of ATP this way. It has also been suggested that the re-programming of tumor cell metabolism to favor glycolysis is carried out to support the need for nucleotides, amino acids and lipids required in highly proliferating cells (Vander Heiden et al 2009). Many processes that are known to occur in tumor development can affect the rearrangement of cell metabolism to favor glycolysis, other than hypoxia, different oncogenic signals can confer this change such as activated Akt signaling, which has been shown to activate aerobic glycolysis (Elstrom et al 2004).
Angiogenesis

Formation of new vessels to provide cells with oxygen and nutrients and remove metabolites and carbon dioxide is necessary for tumor growth. It also facilitates the spread of the tumor by providing a route by which the tumor cells can escape the primary tumor and invade distant tissues in the body and form metastases. Angiogenesis is induced by several secreted and diffusible factors, the expression of which is activated by hypoxia and tumorigenic signaling such as growth factors and inflammatory molecules. The secretion of pro-angiogenic factors such as VEGF activates sprouting of new vessels from existing ones via the binding of VEGF to VEGF receptors in endothelial cells (Carmeliet 2005). This causes endothelial cells in existing vessels to proliferate and migrate towards the source of the angiogenic stimuli and form a new vessel. The new vessels formed in tumors in this manner are often simple, leaky and highly permeable (Nagy et al 2009). These vessels can now provide the tumor with nutrients and oxygen to sustain growth and survival of the tumor cells and even a means of escape in order to facilitate the metastatic process. VEGF also induces vascular permeability of already existing vessels, which facilitates tumor spread (Nagy et al 2007).

VEGF is one factor that can be secreted from the tumor cells and affect angiogenesis and also metastasis of the tumor. Another such factor is IL-6; the normal function of this interleukin is as an immunomodulatory factor, which has both pro-inflammatory and anti-inflammatory effects. IL-6 has been shown to play a role in many different tumor forms such as colorectal cancer, hepatocellular carcinoma, breast cancer, multiple myeloma and lung cancer (Grivennikov et al 2010, Hodge et al 2005). In cancer, IL-6 is suggested to promote both tumor-associated inflammation and aid the escape of tumor cells from destruction by
immune cells and can function both in an autocrine and paracrine manner. Upon activation of the receptor complex consisting of the IL-6 receptor and gp130, IL-6 activates STAT-3 signaling via Janus kinases but has also been shown to activate PI3K and MAPK signaling (Grivennikov and Karin 2008). Activation of IL-6 downstream signaling via STAT-3 has different outcomes, which are context dependent, but in tumors has been shown to activate transcription of CyclinD1, Myc, VEGF and Bcl-xL (Aggarwal et al 2006). This leads to increased proliferation, angiogenesis and inhibition of apoptosis.
Wnt proteins are a family of proteins that are highly conserved during evolution. The importance of Wnts in mammals were discovered when Varmus and Nusse discovered that DNA from the tumorigenic mouse mammary tumor virus (MMTV) was inserted at same site in the genome in several different tumors and that this insert was important for tumor development in these mice (Nusse and Varmus 1982). This site was the gene for Int1; drosophila int1 was later discovered to be identical to the drosophila gene wingless, wg, mutations in this gene leads to, as the name implies, loss of one of both wings and Int1 and wingless became Wnt (Rijsewijk et al 1987). In the fly wg was speculated to be a secreted protein from the fact that wg mutant cell placed in a wild type environment behaved as the wild type cells (Morata and Lawrence 1977). The wingless protein also contained a hydrophobic signal peptide and cysteine rich sequence which was another indication that the protein might be a secreted signaling protein (Rijsewijk et al 1987). This cysteine rich domain (CRD) is conserved in every Wnt protein from drosophila and the nematode C. elegans to humans. Later, the Moon laboratory discovered that overexpression of Wnt1 in xenopus embryos generated axis duplication and frogs with two heads (McMahon and Moon 1989). More proteins belonging to the Wnt family were discovered and some of these did not induce axis duplication but instead could inhibit the axis duplication induced by Wnt1. It was now known that Wnt proteins could induce at least 2 different signaling pathways that were termed canonical Wnt signaling and non-canonical Wnt signaling (Veeman et al 2003). Taken together the early Wnt discoveries show the importance for Wnt signaling in development and tumorigenesis. Since then Wnt signaling has been shown to control cell fate, cell movements and other processes that are important during both development and tumorigenesis. Wnt signaling functions transiently in development and is also important in self-renewal of adult tissue such as the gut, the hematopoietic system, hair and bone (Clevers 2006).

To date, there are 19 mammalian Wnt proteins, 10 members of the Frizzled family of 7 pass trans membrane receptors (FZD) and other receptors such as LRP5/6, Ror1/2 and RYK that might function as co-receptors (Rao and Kuhl 2010, Schulte 2010). Wnt ligands and receptors can interact using different mechanisms and these interaction mechanisms are partly how Wnt signaling is controlled. Wnt ligands are highly glycosylated and can associate with heparan sulphate proteoglycans (HSPG), such as glypicans and syndecans at the cell surface these modifications are important for the functions of Wnt ligands (Kurayoshi et al 2007). HSPGs can serve as modulators of Wnt signaling by keeping them at the cell surface however they might also act as inhibitors of signaling by keeping Wnt ligands away from their receptors, it is unclear whether these interactions are specific (Lin 2004). Wnt ligands are also lipid modified by covalently attached palmitoyl groups at conserved cysteine residues. This makes the Wnt ligand hydrophobic and is thought to be needed for Wnt function (Kurayoshi et al 2007, Nishita et al 2006, Nusse et al 2003). This makes Wnt ligands insoluble and difficult to purify, something that has halted the research on the specific effects of Wnt ligands. However, when ligands could be purified, the complexity of Wnt signals were further understood such as in the case for Wnt5a, previously thought to signal via the non-canonical Wnt signaling pathway, that was discovered to be
able to activate canonical Wnt signaling depending on the receptor context (Mikels and Nusse 2006).

**Wnt signaling**

The interactions between Wnt ligand and receptors activate several different downstream signaling pathways that are highly context dependent. Wnt signaling is usually divided into the β-catenin independent, canonical pathway and the independent non-canonical pathway. Although this is an oversimplification of the actual situation as there is plenty of crosstalk and other signaling pathways, it still remains the most common nomenclature for describing Wnt signaling (Kestler and Kuhl 2008).

**Canonical signaling**

The canonical Wnt signaling is by far the most studied Wnt signaling pathway, it was also the first Wnt pathway to be discovered. Without the presence of an activating canonical Wnt protein β-catenin is coupled to a degradation complex (Behrens et al 1998, Zeng et al 1997). Via the scaffolding effects of the destruction complex, GSK3β and Casein Kinase1 phosphorylate β-catenin which leads to the E3 ubiquitin ligase subunit β-TRCP1 mediated ubiquitination and degradation of β-catenin in the proteasome (Kimelman and Xu 2006). Upon binding of a canonical Wnt ligand to one of the Frizzled receptors and its co-receptors LRP5/6, Disheveled is phosphorylated and this leads to the disruption of the destruction complex (Bhanot et al 1996). Now the degradation of β-catenin is inhibited, the protein is stabilized and transported into the nucleus where it acts as a co-activator to transcription factors of the TCF/Lef family. This leads to the transcription of Wnt responsive genes like CyclinD1 and Myc. β-catenin also functions as a link between cadherins and the actin cytoskeleton at the cell membrane in order to establish cell adhesion (Hulsken et al 1994).

![Figure 2, Overview of canonical β-catenin dependent Wnt signaling](image-url)
Non-canonical signaling

The non-canonical pathways, that are often β-catenin independent, are less well described than the canonical pathway. Just as canonical Wnt signaling it is mediated via Frizzled receptors, co-receptors and Disheveled although possibly via interactions between different FZDs and co-receptors and different domains of Disheveled (Wallingford and Habas 2005). The most well known members of the non-canonical pathway are Wnt5a and Wnt11 but Wnt5b and Wnt4 are also activating non-canonical pathways. None of these ligands induce a secondary axis in Xenopus or zebrafish but upon Wnt5a or Wnt11 overexpression in zebrafish embryos a calcium signal was induced (Slusarski et al 1997, Westfall et al 2003). The non-canonical ligands could also inhibit the axis duplication induced by canonical ligands (Westfall et al 2003). The non-canonical proteins are crucial for cell polarity, correct convergent extension and axis formation; a lot of these processes are governed by changes in polarized cell movements. Non-canonical signaling is also important during gastrulation in the movements of neuronal cells and closure of the neural tube (Tada and Kai 2009, Wada and Okamoto 2009).

The binding of a non-canonical Wnt ligand to its putative receptors, leads to activation of non-canonical signaling via phosphorylation of Disheveled. Heterotrimeric G-proteins are thought to mediate parts of the non-canonical Wnt signaling (Kilander et al 2011, Slusarski et al 1997). Just as all Wnt signaling, the downstream activation is highly context dependent but certain different non-canonical pathways has been identified. One of these pathways is the Wnt calcium pathway where the binding of a Wnt ligand leads to the increase in intracellular calcium. The activation of calcium signaling can lead to activation of PKC and the calcium sensitive CaMKII and calcineurin (Kuhl et al 2000, Sheldahl et al 1999). CamkII is upstream of the activation of NLK and its activation via Wnt5a can inhibit β-catenin mediated transcription activated by a canonical ligand (Ishitani et al 2003). The RTK Ror2 was found to be required for Wnt5a mediated inhibition of β-catenin stabilization (Mikels et al 2009). The transcription factor NFAT is also a target downstream of the calcium signaling, (Dejmek et al 2006, Murphy and Hughes 2002, Saneyoshi et al 2002). Cyclic GMP has been suggested to be part of the non-canonical calcium signaling (Ma and Wang 2006).

The drosophila Planar Cell Polarity Pathway is activated by non-canonical ligands and activates Rho and Rac, JNK and Rho Kinase which is similar to the vertebrate JNK pathway in which non-canonical ligands also activates the JNK pathway and this is thought to be via the activation of Rho, Rac and Cdc42 and downstream of these is also Rho kinase (Veeman et al 2003). Furthermore non-canonical signaling can also lead to activation of the transcription factor CREB via cAMP and PKA, mediated via activation of heterotrimeric G-proteins (Hansen et al 2009). Taken together the activation of non-canonical signaling leads to changes in the cytoskeleton with effects on polarized cell movements and cell division orientation, it can also inhibit canonical signaling leading to changes in cell proliferation and cell fate. This is shown by the need for non-canonical signaling in mediating convergence and extension along the anterior-posterior axis (a process that depends upon polarized cell movements) of the developing mouse, Xenopus and zebrafish embryo and that β-catenin signaling is increased in Wnt5a knockout mice (Topol et al 2003). Non-canonical signaling can also induce other signaling pathways, thus is great complexity and depending on the cell
and tissue type, receptor and modulator context the effects of the signaling varies.

**Wnt5a**

Wnt5a is by far the most widely studied Wnt protein that induces non-canonical or β-catenin independent signaling. The gene for mouse Wnt5a was found in a search for Wnt related genes and 3 years later a human Wnt5a was cloned (Clark et al 1993, Gavin et al 1990, Yamaguchi et al 1999). The importance for Wnt5a during development is demonstrated by the homozygous knockout of Wnt5a in mice that is perinatally lethal; these mice probably die from asphyxiatation. The mouse embryos have defects in all tissues that exhibit outgrowth such as the snout, tongue and in the fore and hind limbs that lack digits. These mice display shortened anterior-posterior axis, the length of the body is truncated and they have lost the tail (Yamaguchi et al 1999). Thus Wnt5a knockout mice show that Wnt5a in essential in extension of the anterior-posterior axis formation and in the development of proximal-distal extension of the limb. In a study of the transformational effect of Wnt proteins on mouse mammary epithelial cells Wnt5a was found not to transform these cells as opposed to the canonical Wnt proteins Wnt3a and Wnt1 (Wong et al 1994). Just as the other Wnt proteins Wnt5a is a very sticky protein that is glycosylated and palmitoylated, these modifications are required for correct signaling, Wnt5a can also associate with HSPGs (Kurayoshi et al 2007). Wnt5a is important in cell migration and adhesion of several different cell types; it is thought to exert these effects by affecting the cytoskeleton. In fibroblasts grown in a monolayer, scratching of the monolayer leads to Wnt5a activated formation of complex of Disheveled and aPKC, that gives rise to a polarized movement, where the polarization is dependent on localization of Cdc42 (Schlessinger et al 2007). This was mediated by Wnt5a via Ror2, as was the induction of polarized migration in fibroblasts shown by Nishita et al (Nishita et al 2006). Polarized migration induced by Wnt5a was also seen in NIH3T3 cells (Nomachi et al 2008). Several other studies has shown an effect on polarized migration in cells, the mechanism of which seems to be cell context dependent judging by the different receptors and downstream players that are involved in the induction of migration (Matsumoto et al 2010, Nishita et al 2006, Nomachi et al 2008, Schlessinger et al 2007).

The Wnt5a-induced inhibition of β-catenin dependent signaling is mediated via several mechanisms. Knockdown of Ror2 has shown the need for this receptor in the inhibition (Mikels et al 2009). The activation of NLK downstream of Wnt5a phosphorylates transcription factors activated by β-catenin and this inhibits their action and thus the effects of canonical signaling (Ishitani et al 2003). The inhibition of β-catenin mediated transcription has also been seen to be mediated via RORα activation of PKC downstream of Wnt5a (Lee et al 2010). Also competitive binding of FZDs has been shown to inhibit canonical signaling via internalization of receptors upon Wnt5a binding to FZD2 (Sato et al 2010).

Processes in the adult tissue that Wnt5a are to be involved in are the maturation of the terminal end buds in the mammary gland (Roarty and Serra 2007). This process is similar to that of the maturation of the lungs were Wnt5a is important (Li et al 2002). Wnt5a also plays a role in adult tissue wound healing where it can induce invasion and regeneration of the wounded tissue (Fathike et al 2006). In mammary epithelial cells Wnt5a has been shown to activate Cdc42, which counteracted the calcium dependent activation of NFAT (Dejmek et
al 2006). Wnt5a has also been shown to increase adhesion leading to decreased migration of mammary epithelial cells and breast cancer cells by increasing their adhesion to a collagen coated surface via the Discoidin domain receptor1 (Jonsson and Andersson 2001).

Depending on the receptor expression in a specific cell, Wnt5a has been shown to activate different pathways. Upon overexpression of FZD5, Wnt5a can induce axis duplication (He et al 1997). LRP and FZD4 overexpression could also induce canonical signaling upon Wnt5a stimulation (Mikels and Nusse 2006). FZD2 has been studied for its role in Wnt5a induced calcium signaling and Wnt5a also induces internalization of FZD2 (Sato et al 2010, Sheldahl et al 2003, Slusarski et al 1997). Wnt5a has been shown to signal via different receptors but Ror2 has lately been shown by many researchers to be crucial for some of the effects of Wnt5a. The Ror2 knockout mouse shows similar phenotype to the wnt5a knockout mouse (Takeuchi et al 2000). Ror2 is also involved in several other Wnt5a mediated effects such as polarized cell migration and convergent extension (Nishita et al 2006, Schambony and Wedlich 2007).

Figure 3, Overview of non-canonical β-catenin independent Wnt signaling
Wnt modulation

The crucial importance of Wnt signaling during development and disease progression requires strict mechanisms for control. At the extracellular level there are several Wnt modulators that can function as Wnt inhibitors, they can also aid in the formation of Wnt gradients and they have also been shown to act as Wnt activators (Leyns et al. 1997, Mii and Taira 2009, Wang et al. 1997a). The Wnt inhibitory factor (WIF), is an extracellular Wnt inhibitor discovered by its ability to inhibit the axis duplication induced by canonical Wnt overexpression in xenopus embryos. WIF proteins inhibit Wnt signaling extracellularly via a WIF domain that shares homology with the WIF domain of the Wnt co-receptor RYK (He 2004, Hsieh et al. 1999). Another group of Wnt inhibitors that was discovered by their ability to inhibit axis duplication is the Dickkopf proteins (Glinka et al. 1998). They act as Wnt inhibitors by inhibiting the interaction between the Frizzled receptor and the co-receptor LPR5/6 upon Wnt binding leading to inhibition of signal activation (Bafico et al. 2001). In 1996, a search for genes with FRZ homology resulted in the finding of a group of secreted proteins with a cysteine rich domain, the Secreted Frizzled Related Receptors (SFRP) (Hoang et al. 1996).

Secreted Frizzled Related Receptors

SFRPs have been shown to inhibit Wnt signaling in different ways. They can to bind Frizzled receptors directly via the CRD and also by binding to Wnt ligands (Dann et al. 2001). The binding of SFRPs to Wnt ligand can inhibit the ligand receptor interaction but also make the fairly insoluble Wnt ligands more soluble and more capable of diffusion to distant sites (Mii and Taira 2009). The first SFRP to be discovered was SFRP3 that was found in a study of cartilage where Hoang et al were searching for secreted molecules that played a role in skeletal formation. They found a protein that contained a domain that shared homology with Frizzled receptors and they named it Frizzled motif associated with bone development (FrzB) (Hoang et al. 1996). Later that year a xenopus FrzB and found to be able to bind Wnt and inhibit the axis duplication induced by Wnt (Leyns et al. 1997, Wang et al. 1997a). Since FrzB had no transmembrane domain it was speculated to be an extracellular inhibitor. Four other similar genes were soon found in mouse and human tissue and now there was a family of SFRP proteins, SFRP1-5 where FrzB is SFRP3 (Chang et al. 1999, Finch et al. 1997, Rattner et al. 1997). Previously a group of proteins had been cloned that were linked to apoptosis in breast cancer called SARPs (Melkonyan et al. 1997). These genes turned out to be SFRP 1, 2 and 5. There are SFRPs found in all vertebrates analyzed (Bovolenta et al. 2008).

All 5 SFRPs are located on different chromosomes, SFRP 1, 2 and 5 belong to one subgroup and are encoded by three exons and SFRP 3 and 4 that make up another subgroup are encoded by 6 exons, they all have a size between 33 and 40kDa. These two groups differ the most in their C-terminal domains in which all SFRPs a netrin domain (NTR) that shares homology with Netrin-1, which is an axon guidance molecule and with tissue inhibitors of matrix metalloproteinases (Banyai and Patthy 1999). The spacing of certain cysteine residues in the NTR differs between SFRPs 1, 2 and 5 and SFRPs 3 and 4 giving them structural differences (Chong et al. 2002). In the N-terminal domain, all 5 SFRPs also have
SFRP3/FrzB

SFRP3 was the first gene in the SFRP family to be found, in 1996 in a study were Hoang et al were searching for genes for secreted proteins that had effects on the skeleton. They found a protein they named FrzB that was later re-named SFRP3. It is located on chromosome 2 (2q31-33) and acted as a chondrogenic factor in the development of the cartilage (Hoang et al 1996). The SFRP3 knockout mouse shows no developmental abnormalities, which is surprising considering its expression during development but suggests that there is redundancy between the SFRPs (Hoang et al 1998, Ladher et al 2000, Tylzanowski et al 2004). However upon skeletal stress the FrzB mice displayed a differential phenotype with increased cartilage damage and lesser inclination to voluntary exercise (Lories et al 2007, Lories et al 2009). Two single nucleotide polymorphisms (SNPs) in FrzB have been associated with osteoarthritis (Loughlin et al 2004, Min et al 2005, Valdes et al 2007). One of these SNPs was associated with a diminished ability to inhibit Wnt signaling (Loughlin et al 2004). SFRP3 is expressed during development and in xenopus was found to inhibit axis duplication induced by canonical Wnt signaling (Wang et al 1997a). SFRP3 was also found to be able to inhibit and oppose the effects of Wnt5a during development in mouse (Liu et al 2008, Qian et al 2007).
Wnt and cancer

Aberrant Wnt signaling plays a role in cancer, the fact that they were discovered in a search for genes that were affected by a tumor-inducing virus in the mouse mammary gland shows this very clearly (Nusse and Varmus 1982). After this finding it was shown that the Wnt signaling pathway was often constitutively active in colon cancer. This was found to be due to an early event in the development of the inherited form of colon cancer Familial Adenomatous Coli; this was the inactivating mutation of the tumor suppressor APC that leads to a truncation of the protein (Groden et al 1991, Nishisho et al 1991). The truncated protein can no longer function as a scaffolding protein in the destruction complex that phosphorylates β-catenin, which is not degraded but is free to act as transcription factor. Since then, constitutive activation of β-catenin has been detected in 85% of all colon cancers and in many other human tumor forms such as breast cancer, leukemia, gastric cancer, ovarian cancer, endometrial cancer and esophageal cancer (Giles et al 2003).

Activated β-catenin in tumors is in many cases correlated with a decreased survival of patients. The canonical pathway appears to be important during the development of tumors by controlling self-renewal and proliferation and mutations affecting its players are often occurring early in tumor progression. But recently, studies have shown that activation of canonical Wnt signaling is not universally correlated to poor prognosis and a negative outcome in patients. In malignant melanoma and medulloblastoma, patients with activated canonical Wnt signaling had a more positive prognosis outcome compared to patients without active canonical Wnt signaling (Chien et al 2009, Ellison et al 2005, Fattet et al 2009). Instead of generally considering activated β-catenin in tumors as pro-tumorigenic, the situation is probably more complex, depending on the developmental origin of the cells and the effects of β-catenin on the cell type in the normal setting. If β-catenin induces cell fate and differentiation for instance, activated signaling in the tumor might keep the cells more differentiated and result in a less aggressive tumor.

Aberrant signaling of the non-canonical pathway seems to play a role later during tumor progression by controlling migration, invasion and metastasis (Jessen 2009). Mutations of the non-canonical signaling proteins are not common in cancer instead it seems that in tumors there up or down regulation of these proteins to create a situation with aberrant non-canonical signaling. In fact, no mutations in Wnt ligand or receptors are found in tumors, instead it is proteins up- or downstream of the ligand-receptor interactions that are affected (Giles et al 2003).

Wnt5a and cancer

The context dependent effects of Wnt5a are clearly demonstrated by its different roles in different in cancer. Aberrant Wnt5a expression and signaling has been shown in several different tumor types such as breast, colon, gastric, pancreatic cancer and melanoma (Da Forno et al 2008, Dejmek et al 2005, Jonsson et al 2002, Kurayoshi et al 2006, Roman-Gomez et al 2007, Weeraratna et al 2002).
Wnt5a has been suggested as a tumor suppressor in different cancer types. Wnt5a heterozygous mice develop myeloid leukemia and B-cell lymphomas and loss of Wnt5a expression was shown in lymphoid malignancies and leukemia (Liang et al 2003, Roman-Gomez et al 2007). In dukes B colon cancer, loss of Wnt5a protein expression was associated with shortened survival (Dejmek et al 2005). In breast cancer cells, Wnt5a has been shown to increase cell adhesion and migration (Jonsson and Andersson 2001). Loss of Wnt5a protein expression was correlated with an early relapse in ductal carcinoma and a Wnt5a agonist was shown to decrease the formation of metastasis in a mouse model of breast cancer (Jonsson et al 2002, Safholm et al 2008).

Wnt5a has also been correlated with increased aggressiveness of tumors as in malignant melanoma, gastric cancer, pancreatic cancer and nasopharyngeal cancer by, for example increasing cell migration and invasion (Kurayoshi et al 2006, Ripka et al 2007, Zeng et al 2007).

The effects of Wnt5a in tumors are highly context dependent as all Wnt signaling. As Wnt5a affects migration in many cell types, Wnt5a induced changes in motility and migration that are dependent on adhesion will be different in cells that has a different degrees of adhesion to its surroundings. For instance in a cell that is rather tightly adhered an increase in adhesion will lead to a loss of motility whereas in a cell that is loosely attached an increased adhesion will increase its ability to move, thus the same signal in different cells can have a widely different outcome. Also, in cells with similar adhesion capabilities, differences in Wnt ligands, Wnt receptor or Wnt modulators will affect the outcome of Wnt5a signaling whether it inhibits canonical signaling, activates calcium signaling or activates other parts of the non-canonical signaling. All these different scenarios will lead to a different outcome for the development and progression of cancer.

Wnt modulators in cancer

Since Wnt signaling plays a role in cancer the modulators of Wnt signaling are also important. In 2002 Suzuki et al discovered that several of the SFRPs were down regulated in tumor tissue compared to normal tissue in colon cancer (Suzuki et al 2002). The down regulation was due to promoter methylation of the genes for SFRP 1, 2, 4 and 5 and this down regulation due to methylation was later found in breast cancer and other tumor types such as non-small-cell-lung cancer, bladder cancer, gastric cancer and mesotheliaoma (Fukui et al 2005, Lee et al 2004, Marsit et al 2005, Nojima et al 2007, Suzuki et al 2008). An additional mechanism for the down regulation of these proteins in cancer was speculated to be loss of heterozygozity (LOH), which is frequently seen in the chromosomal regions that harbor SFRP genes (Leach et al 1996, Wolf et al 2004, Wright et al 1998). This was shown to lead to an increase in canonical Wnt signaling which can be important during the development of these tumor types (Nojima et al 2007, Suzuki et al 2004, Suzuki et al 2008). The re-expression in tumors that displayed methylated SFRP promoter regions was shown to lead to decreased anchorage independent growth and decreased tumor growth in mouse models (Chung et al 2009) (Suzuki et al 2008). SFRP 1 and 4 has also been seen to be up regulated in tumor tissue when compared to normal tissue in invasive breast cancer and in endometrial cancer and later in uterine leiomyomas and prostate cancer (Abu-Jawdeh et al...
1999, Fukuhara et al 2002, Wissmann et al 2003). This further shows the complexity of the involvement of Wnt pathway components in cancer and the fact the characteristics and the origin of the tumor is crucial in determining the role of Wnt signaling in each specific tumor.

**SFRP3/Fzβ in cancer**

SFRP3 plays a role in different tumor types such as bladder cancer, mesothelioma, prostate cancer, renal cell cancer, osteogenic sarcoma, and fibrosarcoma and medulloblastoma (Guo et al 2008, Kongkham et al 2010, Mandal et al 2007, Zi et al 2005). In prostate cancer, SFRP3 was found to inhibit tumor growth in a mouse xenograft model presumably via reversal of epithelial-to-mesenchymal transition and decrease in MMP activity (Zi et al 2005). In fibrosarcoma SFRP3 suppresses tumor formation in mice and inhibits canonical Wnt signaling (Guo et al 2008). SFRP3 has also been suggested to be a tumor suppressor in other tumors for example was the expression of a non-functional SFRP3 associated with increased risk for colon cancer (Shanmugam et al 2007). In the original study were SFRPs were found to be down regulated in tumors due to methylation of the promoter region SFRP3 was not found to be methylated. Following this, several studies reported methylation of SFRPs in different tumors but not of SFRP3. Some of these studies did however report down-regulation of SFRP3 in tumors compared to normal tissue, it was speculated to due to LOH but the authors did not investigate whether or not it the down regulation was due to methylation (Lee et al 2004, Mandal et al 2007). Since then, other studies have shown methylation of SFRP3 in different tumor types and SFRP3 has been speculated to be regulated in cancers as the other members of the SFRP family (Kongkham et al 2010, Marsit et al 2010, McDonald et al 2009).
Cutaneous malignant melanoma is a highly aggressive disease of the melanocytes of the skin. The spread of melanoma to metastatic melanoma indicates a very poor prognosis in patients and there are few treatment options, however if the tumor is dissected early before spread, the prognosis is positive. The 5-year survival of metastatic malignant melanoma is less than 5% (Cummins et al 2006). Cutaneous malignant melanoma originates from melanocytes in the skin; these melanocytes are located in the lower part of the epidermis, the upper layer of the skin. They are dispersed among the keratinocytes and produce the pigment melanin, which is transported to the keratinocytes to provide hair and skin color and to protect the skin from damage from UV irradiation damage (Garibyan and Fisher 2010). Developmentally melanocytes are derived from the highly migratory cells of the neural crest that undergo epithelial-to-mesenchymal transition and migrate away from the neural tube; these cells also give rise to other cells such as cartilage, peripheral neurons and glial cells. Wnt signaling is important in this process and mice that lack expression of the canonical Wnt ligands Wnt1 and Wnt3a have neural crest cells with an altered fate that display a decrease in melanocytes (Ikeya et al 1997). β-catenin signaling is important for the development of melanocytes from their neural crest derivatives and the stimulation of human embryonic stem cells with Wnt3a, Endothelin1 and stem cell factor was enough to induce their differentiation into melanocytes (Fang et al 2006) (Dorsky et al 1998, Dunn et al 2000). Activation of β-catenin mediated transcription activates the transcription of proteins that are crucial for the differentiation of melanocytes such as MITF, which is a crucial differentiation factor for melanocytes (Sommer 2011).

Risk factors for developing malignant melanoma are light skin and blond hair, a family history of melanoma and also damaging UVR exposure from frequent sunburns or tanning beds. The melanin produced in the melanocytes of blond, light skinned people is not effective in its ability to absorb UV irradiation, which can cause DNA damage such as inactivating mutations in tumor suppressors, activating mutations in oncogenes and production of reactive oxygen species (ROS) (Garibyan and Fisher 2010). Another risk factor is preexisting benign nevi, the actual number of nevi in the skin is correlated with an increased risk of developing melanoma (Cummins et al 2006).

The progression of melanoma is divided into different phases. Increased proliferation in normal melanocytes that leads to the development of nevi. The majority of these are harmless and do not progress, however a few develop into atypical, dysplastic nevi which upon the accumulation of further tumorigenic damage can develop into early, pre-malignant stages of melanoma, termed Radial Growth Phase (RGP) melanoma. Invasion of RGP is restricted to the epidermis. However, some melanomas are thought to arise directly from melanocytes without the formation of dysplastic nevi. RGP melanomas can progress into the more malignant state termed Vertical Growth Phase (VGP) melanoma; tumors in this stage have a high risk of developing into metastatic tumors (Gray-Schopfer et al 2007). The transition from RGP to VGP is associated with a significantly worse prognosis compared to the earlier stages and requires a significant amount of further pro-tumorigenic events to take
place within and between the tumor cells and the tumor cell environment. In VGP melanoma, the tumor cells breaks through the basement membrane and invade the underlying tissue. These tumors are often vascularized through angiogenesis, which provides the tumor cells with a route by which they can spread and form metastases in distant tissues such as bone or the brain.

The earliest mutations that take place in melanoma are activating or inactivating mutations in proteins that can affect the cell cycle such as loss of PTEN and loss of expression of the CDKN2A gene, which leads to a loss of control of cell cycle induction and apoptosis. PTEN expression is lost by deletion or inactivating mutation in 5-20% of melanomas and this allows for an active PI3Kinase signaling (Wu et al 2003). Loss of expression of the CDKN2A gene is detected in 30-70% of melanomas (Sharpless and Chin 2003). Another early event in melanoma development is a mutation that causes constitutive activation in the MAPK pathway. The V600E mutation of the BRAF gene that causes constitutive activation of the kinase activity of BRAF is detected in about 50-70% of melanomas. Proliferation and survival induced via MAPK-ERK signaling in melanocytes is mainly governed via active BRAF. This is why an activating mutation in this gene is thought to have such a big impact on melanoma development and progression. V600E BRAF mutations lead to a dramatic and pronounced increase in cell proliferation but can also induce angiogenesis via induction of VEGF expression and the expression of several other pro-tumorigenic factors in melanoma (Davies et al 2002, Gray-Schopfer et al 2005, Sharma et al 2005). Activating mutations in NRAS are also detected in melanoma, observed in approximately in 15-30% of patients. NRAS and BRAF mutations are mutually exclusive presumably because they induce similar downstream effects and there is limited survival advantage to harbor mutations in both genes (Tsao et al 2004). The targeted inhibition of BRAF V600E with specific inhibitors has had positive results in patients showing shrinking of tumors, however the acquirement of resistance has been seen in patients treated with these targeted BRAF inhibitors (Chapman et al 2011, Flaherty et al 2010, Solit and Rosen 2011).

Concomitantly with the VGP transition the melanoma cells loose adhesion and break free from their primary tissue, but they also proliferate in the primary tissue to produce a larger primary tumor. Upon VGP transition there is a decrease in expression of E-cadherin, which is important in the interaction and adhesion between melanocytes and keratinocytes (Silye et al 1998). The cells also start to express markers important for the interactions between melanocytes and fibroblasts and melanocytes and endothelial cells, facilitating migration and invasion of melanoma cells such as N-cadherin (Hsu et al 2000). The expression of N-cadherin in melanoma cells is correlated with a more motile and aggressive state and this is thought to be part of the VGP transition as well as a change in the expression of integrins towards a more motile expression pattern (Kuphal et al 2005, Qi et al 2005). Coupled to VGP and growth of the tumor is also the formation of new vessels. In order to supply the growing tumor with nutrients and oxygen the melanoma cells can secrete several factors that promote angiogenesis the most prominent of which is VEGF (Ria et al 2010). VEGF and increased angiogenesis is correlated with a poor prognosis in melanoma (Erhard et al 1997).

In melanoma patients, IL-6 was correlated with a higher tumor burden and a poor prognosis and in melanoma cells treatment of IL-6 has a growth inhibiting effect (Lu et al 1992,
Mouawad et al 1996). However in melanoma cells from metastatic and aggressive melanoma the cancer cells themselves were shown to start expressing IL-6 and it was shown to be stimulatory similar to the behavior of Wnt5a in melanoma (Lu and Kerbel 1993). IL-6 has just as VEGF been shown to affect melanoma angiogenesis, an effect that correlated to the transition of melanoma from RGP to VGP and to a poor prognosis (Erhard et al 1997).

Wnt5a and melanoma

Wnt5a was first connected to melanoma when mRNA expression of Wnt5a was found to be increased in melanoma compared to normal skin (Iozzo et al 1995). Later high Wnt5a mRNA expression was correlated to aggressive melanoma cell lines and tumors (Bittner et al 2000). Over-expression of Wnt5a in melanoma cell lines lead to an increase in their aggressiveness as measured by changes in cell shape and increased migration and this was suggested to be mediated via increased activation of PKC signaling. This study also showed that Wnt5a expression was correlated with higher tumor grade. The authors speculate that since activation of PKC affects cell motility in melanoma and inhibitors of PKC can inhibit melanoma invasion, then this is the likely mechanism by which Wnt5a promotes melanoma aggressiveness (Weeraratna et al 2002). Wnt5a was also correlated with a poor prognosis in melanoma patients and suggested to be a prognostic marker (Da Forno et al 2008). In cell culture experiments, the addition of Wnt5a to cells with low endogenous expression can increase their migratory and invasive capacity (Dissanayake et al 2007, Jenei et al 2009). Knockdown of Wnt5a using siRNA can reduce the migration of melanoma cells (Dissanayake et al 2007). Witze et al showed that Wnt5a could mediate the directional migration of melanoma cells in the presence of a chemokine gradient and that Wnt5a did so by inducing the recycling of membrane components to specific surface sites via RhoB and Rab4, which was dependent on active PKC (Witze et al 2008). The knockdown of Ror2 in mouse xenograft experiments shows a decrease in the metastasis formed in the lungs, suggested to be caused by the fact that Wnt5a can not mediate its effects without its receptor (O’Connell et al 2009).

Chien et al showed in 2009 that active canonical Wnt signaling (as measured by nuclear β-catenin) was correlated with a positive prognosis. Wnt3a over-expression in a mouse
model of melanoma induced smaller tumors and a higher degree of differentiation of the melanoma cells; Wnt5a could antagonize this (Chien et al 2009).

Microarray analyses have shown that Wnt5a is important in a model that has been suggested for the progression of melanoma termed phenotype switching (Hoek et al 2006, Hoek et al 2008). This model describes the progression of melanoma as switching back and forth between a proliferative and an invasive state, which can explain the heterogeneity of melanoma and the difficulty to treat the disease. In the proliferative state, genes connected to melanocyte differentiation such as MITF and genes that are downstream of canonical Wnt signaling such as Bcl2 are expressed. While in the invasive state genes that are capable of inhibiting canonical signaling such as Dkk and Wnt5a are highly expressed (Hoek et al 2006, Hoek et al 2008). In the invasive state, Wnt5a plays a role and Hoek et al speculate that this is possibly downstream of TGF-β, which is thought to be a regulator of phenotype switching (Hoek et al 2008). Wnt5a has previously been shown to be a downstream mediator of TGF-β signaling (Jenei et al 2009, Roarty and Serra 2007). Differential expression of the transcription factors TCF4 and LEF1 has been suggested play a role in the switching between the proliferative and invasive phenotype by regulating the expression of different genes downstream of activated β-catenin (Eichhoff et al 2011).

These studies suggest that high Wnt5a expression in melanoma is correlated with increased aggressiveness and poor prognosis of melanoma possibly by inducing increased cell motility and invasiveness. However in contrast with this, other studies have shown that Wnt5a expression is high in nevi but is decreased in melanomas and is not correlated with poor prognosis (Bachmann et al 2005, Pham et al 2003). The phenotype-switching model might be an explanation to the discrepancy between these and other studies since according to Hoek et al Wnt5a does not appear to play a role in melanomas in the proliferative state (Hoek et al 2008). This model might also explain why several studies has not correlated activated β-catenin to a positive outcome in melanoma patients but instead seen an increase in canonical signaling in benign nevi and melanoma compared to normal skin and melanocytes.
The general objective of this thesis was to further the knowledge of the downstream effects of Wnt5a signaling and the modulation of Wnt5a signaling in malignant melanoma.

Specific aims

*The specific aims were*

I. To investigate the effects of SFRP3 in malignant melanoma and how it interacts with Wnt5a in malignant melanoma cells.

II. To investigate the effects of Wnt5a on the malignant melanoma proteome and what signaling pathways that are affected by the addition of rWnt5a.

III. To investigate how Wnt5a affects the transcription and secretion of IL-6 in malignant melanoma
Results and Discussion

Paper I

Methylation and Loss of Secreted Frizzled-Related Protein 3 Enhances Melanoma Cell Migration and Invasion

Results Paper I

In Paper I we started by investigating the expression of SFRP3 in publicly available microarray datasets from normal skin and benign nevi and malignant melanoma patients. The normalized values of the expression levels were collected from the GEO profiles website and were compared using the students t test. We found that the expression of SFRP3 was significantly lower in malignant melanoma compared to normal skin and benign nevi. We next investigated the expression levels of SFRP3 in 3 melanoma cell lines A375, A2058 and HTB63 and an immortalized non-transformed melanocyte cell line, Hermes 3A. The melanoma cell line A2058 is derived from the lymph node of a patient with metastatic melanoma and expresses low basal levels of Wnt5a. However with increasing passage number and when grown to confluency the expression levels of Wnt5a are increased and the cells stop responding to treatment with rWnt5a. In this study A2058 cells were only used when the Wnt5a levels were low and when the cells were shown to respond to Wnt5a by inhibition of Wnt3a signaling in the Topflash assay. HTB63 cells were also derived from a metastatic site in a malignant melanoma patient and express high levels of Wnt5a. The A375 cells are derived from the skin of a patient with malignant melanoma and have a moderate level of basal Wnt5a expression. All these three melanoma cell lines harbor the activating V600E mutation. The Hermes 3A cells are normal melanocytes immortalized by overexpression of hTERT and inactivation of p16. The mRNA levels of SFRP3 were found to be low in the melanoma cells and higher in the melanocytes. Using Elisa, the secreted protein levels of SFRP3 were found to be significantly lower in the melanoma cells than in the melanocytes. This pattern was also seen using immunohistochemistry. Because other SFRPs are down regulated in tumors compared to normal tissue due to methylation, we used the USCS genome browser to analyze the SFRP3 promoter region for CpG rich areas. We analyzed the detected areas for methylated cytosines using methylation Specific PCR and found more methylated DNA in the melanoma cells compared to the melanocytes. We detected a decrease in methylated DNA after treating the melanoma cells with the demethylating agent 5’aza-deoxy-cytidine (5’AZA), which also greatly increased the mRNA expression of SFRP3 in the melanoma cells. The protein expression and secretion of SFRP3 was also significantly increased after treatment with 5’AZA. Since SFRP3 was down regulated in the melanoma cells compared to normal cells we speculated that cells not expressing SFRP3 might have growth or progression advantage compared to cells that express SFRP3. If the effects of SFRP3 in melanoma were due to Wnt inhibition and Wnt has been shown to affect proliferation and progression of melanoma we investigated the effects of SFRP3 on melanoma proliferation, invasion and migration. There was no effect of
SFRP3 on the proliferation of A2058 melanoma cells but migration and invasion could be decreased. Since Wnt5a can increase migration and invasion we investigated the effects of SFRP3 on Wnt5a signaling using the TopFlash assay. SFRP3 had no effect on the activation of canonical signaling by Wnt3a. However SFRP3 could inhibit the inhibitory effects Wnt5a has on canonical signaling. We also found that SFRP3 could inhibit Wnt5a induced increase in intracellular calcium signaling as measured with FURA-2. We determined that when Wnt5a was depleted from A2058 cells there was no decrease in migration upon SFRP3 addition. Also SFRP3 could inhibit the increase in migration induced by Wnt5a. In HTB63 cells that express a high level of Wnt5a the addition of 1ug/ml of SFRP3 was not enough to inhibit migration in these cells, instead the conditioned media from cells over expressing SFRP3 could decrease migration in HTB63 cells.

**Discussion Paper I**

SFRP3 has been found to be down regulated in tumors compared to normal tissue in previous studies and suggested to act as a tumor suppressor in several tumor forms (Guo et al 2008, Kongkham et al 2010, Lee et al 2004, Mandal et al 2007, Marsit et al 2010, McRonald et al 2009, Zi et al 2005). Our finding that SFRP3 is less expressed in melanoma compared to benign nevi and normal skin and in a higher degree in melanoma cells further supports this. Since this study was accepted for publication, another study showing down regulation of SFRP3 in melanoma has been published providing further support to the notion that SFRP3 is down-regulated in melanoma and might act as a tumor suppressor (Mauerer et al 2011). In some of these studies the objective was to investigate the methylation of SFRPs in cancer and a decrease in expression was found in all SFRPs compared to normal tissue. Suzuki et al reported down-regulation of SFRP 1, 2, 4 and 5 due to methylation (Suzuki et al 2002). They did not investigate the methylation status of SFRP3 in this study since they found basal expression level of the gene in colorectal cancer cell lines and they discriminated between genes that could be methylated and genes that couldn’t be by searching the promoter region for CpG islands, which they couldn’t find in the SFRP3 gene. They defined CpG islands by a GC content >60% and the ratio of CpG to GpC >0.6 and a minimum length of 200bp (Suzuki et al 2002). Other studies following this did, based upon this finding, not investigate the methylation status of SFRP3 in spite of finding a down regulation of the gene in tumor samples and tumor cell lines. However using the UCSC genome browser we found a CpG island present in the promoter by the definition GC content > 50% the ratio of CpG to GpC >0.6 and a minimum length of 200bp and the one found in the SFRP3 gene had 66.7% suggesting that it fulfills all the requirements set up by Suzuki et al (Kent et al 2002). However the earliest available version of the genome browser today is from 2003 and it is possible that at the time that Suzuki et al performed their analysis this information was not available. However recently the importance of CpG island according to these definitions has been questioned (Baylin and Jones 2011). Several studies including ours have shown that SFRP3 indeed can be down-regulated due to methylation (Kongkham et al 2010, Marsit et al 2010, McRonald et al 2009). This might also have been the cause for the down-regulation in the other studies that did not investigate if methylation indeed was the cause for down-regulation of SFRP3 in tumors. We suggest that this is a plausible reason for the down-regulation of SFRP3 in melanoma.
Wnt5a signaling in Malignant Melanoma

We primarily used the A2058 cells to investigate the effects of SFRP3 on melanoma. This cell line is highly metastatic and expresses moderate levels of Wnt5a. SFRP3 did not induce a change in cell numbers but using assays designed to measure migration and invasion a decrease in migration and invasion was detected. This decrease was relatively small but similar to the increase in migration and invasion induced by Wnt5a in A2058 cells (Jenei et al 2009). Also the A2058 express relatively small levels of Wnt5a compared to HTB63 cells. In this cell line the same concentration of rSFRP3 that decreased migration in A2058 did not induce a change a migration. We used conditioned media from HTB63 cells that overexpressed SFRP3 with a presumably higher concentration of SFRP3 than 1μg/ml and this could inhibit random migration in HTB63 cells. This suggests that the small change in migration and invasion induced by SFRP3 is due to the small levels of Wnt5a expressed in the A2058 cells.

The inhibition of Wnt5a by SFRP3 has been shown during development in some studies and not in others and a study of the binding capabilities of SFRPs to Wnt ligands did not show the binding of SFRP3 to Wnt5a (Liu et al 2008, Qian et al 2007, Wang et al 1997b, Wawrzak et al 2007). This shows the differences in the effects of SFRP3 in different contexts but that SFRP3 might not inhibit Wnt5a signaling via direct binding to SFRP3 as shown by Wawrzak et al but the inhibition might be via binding of receptors instead. However, this study was performed in a cell free assay system and might provide different results from an environment containing cells (Wawrzak et al 2007).

We show that in malignant melanoma, SFRP3 is down regulated compared to normal tissue due to methylation. In melanoma cell lines, SFRP3 can inhibit Wnt5a signaling and suppress the effects on migration and invasion induced by Wnt5a. This is probably context dependent and might be highly specific to cells expressing Wnt5a and the correct receptors to mediate this effect. This suggests that SFRP3 expression in melanoma might have growth suppressive effects by inhibiting Wnt5a signaling. Its down regulation by methylation might provide a growth advantage in melanoma cells by increasing the ability of Wnt5a to signal without the presence of this inhibitor.
Paper II,

Non-canonical Wnt5a Signaling is a Novel Regulator of Melanoma Cell Metabolism

Results Paper II

In order to investigate the effects of Wnt5a on the proteome of malignant melanoma cells, we first determined a time point at which Wnt5a had an effect on the proteome. Wnt5a could induce an increase in migration in A2058 cells at 24 h. At 6 h and 12 h, Wnt5a induced a biphasic increase in adhesion, which is most likely needed for the increase in migration. When pre-treating the cells with protein synthesis inhibitors, only the 12 h increase in adhesion and not the 6 h, was inhibited, suggesting that at 12 h there is a need for protein synthesis to support the increase in adhesion that is prior to migration induced by Wnt5a treatment in melanoma cells. We therefore performed the proteomics analysis at 12 h after Wnt5a treatment of A2058 cells that express low endogenous levels of Wnt5a. Before performing the analysis we determined that the A2058 cells could respond to Wnt5a by measuring their ability to activate intracellular calcium signaling. In order to determine the specificity of the effects of the Wnt5a treatment we also analyzed the effects of Wnt5a in combination with Box5, a peptide shown to be able to antagonize the effects of Wnt5a. Box5 in combination with Wnt5a could inhibit the migration and adhesion induced by Wnt5a alone. The protein samples to be analyzed were tagged with Tandem Mass Tags and changes in peptide expression were analyzed from quantitative changes in peptides levels using reverse phase liquid chromatography tandem mass spectrometry, LC-MS/MS. 174 proteins were found to be differentially expressed following Wnt5a treatment using this method and Box5 inhibited most of these changes. By utilizing gene ontology tools the function of these proteins was analyzed and cellular metabolism and particularly glycolysis was found to be the cellular process mostly affected by Wnt5a treatment. Analyses of the cellular metabolism of A2058 cells showed that they mainly rely on aerobic glycolysis for their energy production and that citrate synthase activity was lowered following Wnt5a treatment, which is indicative of increased glycolysis.

Certain isoforms of LDH processes pyruvate from glycolysis to lactic acid and its levels were increased in the Wnt5a treated proteomics samples; this was also seen using western blotting. There was an accompanying increase in LDH activity and in the levels of lactate secreted into the culture medium, however there was no difference in the mRNA levels of LDH. Knockdown of Wnt5a in HTB63 cells and in A2058 cells could decrease the levels of lactate secreted into the culture medium. In order to find the underlying mechanism behind the increase in glycolysis, the expression levels of p-Akt were analyzed and they were increased following Wnt5a treatment. By using an inhibitor of the Akt downstream target mTOR, rapamycin, the increase in lactate levels were inhibited.
To test the clinical relevance of the relationship between Wnt5a signaling and cellular metabolism we utilized a tissue microarray containing melanocytic neoplasms. We analyzed the expression of Wnt5a and the expression of LDHV, which catalyzes the reaction where pyruvate is converted to lactate. We made the novel finding that the expression of Wnt5a and LDHV significantly correlated in this clinical material. Furthermore, we found in our TMA a correlation between expression of Wnt5a and reduced disease-free survival, thus confirming previous studies (Weeraratna et al 2002). LDHV alone correlated with T-stage, which is a marker of poor prognosis. Wnt5a and LDHV together correlated strongly with ulceration, which is another marker of poor prognosis.

To investigate the effects of Wnt5a on cellular metabolism in other tumor types we used MDA-MB-468 breast cancer cells treated with Wnt5a but found no differences in secreted lactate but there was an increase in oxidative phosphorylation.

**Discussion Paper II**

Wnt5a mediated increases in migration are preceded by biphasic increases in adhesion at 6 h and 12 h were the 12 h adhesion increase is dependent on protein synthesis. Therefore, it is likely that Wnt5a induces a change in protein synthesis at 12 hours and indeed proteomics analysis showed increased levels of several proteins most of which could be inhibited by Box5. Wnt5a treatment for 12 h increased protein levels of proteins important for different cellular processes; one of the most prominent of these was energy metabolism. The increases and decreases in LDH protein and activity seen upon addition of Wnt5a and knockdown of Wnt5a in melanoma cells are indicative of an increased aerobic glycolysis. In breast cancer cells Wnt5a appears to increase oxidative phosphorylation and there was no increase in lactate secretion suggesting that there is no increase in glycolysis upon Wnt5a treatment in breast cancer cells, but an alteration in energy metabolism nonetheless. The differences in the effects of Wnt5a on breast cancer and melanoma cells is not surprising as the protein expression of Wnt5a previously has been shown to be a marker of positive outcome in breast cancer patients while Wnt5a expression is a marker of poor prognosis in melanoma (Jonsson et al 2002, Weeraratna et al 2002).

These data suggest that Wnt5a can affect tumor cell metabolism in cancer in different manners depending on cell type. In melanoma, Wnt5a can either reprogram cellular metabolism to favor aerobic glycolysis or up regulate the players important in aerobic glycolysis to provide a higher capacity to produce ATP via glycolysis in cells already favoring this process for their energy production. Tumor cells often display re-programming of cellular metabolism to favor glycolysis in aerobic conditions even though this process usually only takes place in normal cells under anaerobic conditions. This is far less energy efficient than oxidative phosphorylation which is the usual source for energy in cells in aerobic conditions but might be more efficient in providing the biomass required in highly proliferative tumor cells (Vander Heiden et al 2009). A2058 cells have previously been shown to rely on glycolysis as a source for energy to promote cell motility (Beckner et al 1990) and since Wnt5a increases the capacity for glycolysis in these cells this might be one of the mechanisms by which Wnt5a increases migration in melanoma cells. One downstream target of Wnt5a is activation of the Akt signaling and the PI3K-Akt-mTOR signaling pathway can re-program
The Present Investigation

cell metabolism to favor glycolysis in cancer cells (Elstrom et al 2004, Kawasaki et al 2007). We found that Akt was activated by measuring its phosphorylation status following addition rWnt5a at 12 h. mTOR as part of the mTORC1 complex is often activated downstream of Akt and mTORC1 can be inhibited by rapamycin. Rapamycin could inhibit the increased lactated secretion induced by Wnt5a.

Elevation of LDH levels in the serum of melanoma patients is a marker of decreased survival (Balch et al 2001). Analysis of clinical data showed that the expression of the LDH isoform that catalyzes the conversion of pyruvate to lactate a process, LDHV, which is important for aerobic glycolysis was correlated to expression of Wnt5a and also to poor prognosis. LDHV has previously been associated with disease progression in malignant melanoma (Zhuang et al 2010). This further strengthens our notion that in malignant melanoma, Wnt5a is associated with increased aggressiveness of the disease as well as a reprogramming of cellular metabolism towards aerobic glycolysis.

This study suggest Wnt5a can increase the glycolytic capacity of melanoma cells via activation of Akt and mTORC1 and that this might be part of the mechanism of how Wnt5a induces increased aggressiveness of malignant melanoma. The difference in the effect of Wnt5a on tumor metabolism between breast cancer cells and melanoma cells is in line with its effect on tumor aggressiveness in these tumor forms. The inherent differences in cellular metabolism in these cells might be one cause of the difference but different downstream effects of Wnt5a in the cells can also explain this. It might be a specific effect of activation of Akt and mTORC1 via direct Wnt5a signaling. However, canonical Wnt signaling has been shown to play a role in cellular metabolism (Sethi and Vidal-Puig 2010) and the effects displayed by Wnt5a in this study might be mediated via inhibition of canonical signaling.
Paper III

Wnt5a induces Ca\(^{2+}\)-dependent exocytosis of immunomodulatory and pro-angiogenic factors in malignant melanoma.

Results Paper III

Here we investigate the relationship between Wnt5a and secretion of pro-tumorigenic factors such as IL-6. Previously, the expression of Wnt5a has been associated with increased levels of IL-6 in cell culture media from malignant melanoma cells, but the biological mechanism behind this was never investigated. (Dissanayake et al 2008). To further examine this relationship, we analyzed the Wnt5a expression levels of 5 melanoma cell lines. Out of these, we utilized two cell lines with low to moderate Wnt5a expression, MeWo and A375 and one cell line with high Wnt5a expression, HTB63. MeWo cells are derived from the lymph node of a patient with metastatic melanoma and do not harbor the V600E BRAF mutation but the A375 and HTB63 cells do. In MeWo cells, treatment with recombinant Wnt5a increased the levels of IL-6 in the culture media while treatment with rWnt3a did not increase IL-6 levels. In time-course experiments, IL-6 was robustly increased at 3 h, but also over a longer time period up to 48 h. In contrast, IL-6 transcription was not up regulated since IL-6 mRNA levels remained unaffected. The levels of the secreted protein VEGF were also increased in the culture media while there was no up regulation of VEGF at the mRNA level. The same pattern was seen in A375 melanoma cells, however these cells showed increased expression levels of Wnt5a with higher passage numbers and we decided to focus on the MeWo cells and HTB63 cells in this study. To further assess the effects of Wnt5a on the levels of IL-6 and VEGF we used siRNA against Wnt5a in HTB63 cells. Cells that had been transfected with Wnt5a siRNA secreted significantly lower levels of IL-6 and VEGF while the mRNA levels of IL-6 or VEGF remained unchanged.

To investigate the functional effects of IL-6 and VEGF secretion, we utilized an endothelial cell branching assay. MS1 endothelial cells were co-cultured with HTB63 cells transfected with Wnt5a siRNA or control siRNA. Knockdown of Wnt5a decreased the length and amount of tubes formed compared to cell transfected with control siRNA. Also cells that had been pre-treated with Bapta-AM displayed a decrease in the length and amount of tubes formed. Treatment with rWnt5a only did not affect the endothelial cell branching.

MeWo cells that were treated with Wnt5a for 3 h and paraffin imbedded were used to investigate the mechanism behind the increased secretion of VEGF and IL-6. The Wnt5a treated cells displayed a more diffuse cell cortex and a distinctly less prominent cell border compared to cells treated with carrier alone. Phalloidin staining also showed a re-organization of the F-actin cytoskeleton in cells treated with Wnt5a towards a more cytoplasmic and less cortical organization. In protein lysates the intracellular levels of IL-6 were decreased following Wnt5a treatment for 3 h. To confirm that the Wnt5a induced increase of IL-6 in cell culture media was not due to an increased transcription, we utilized the transcription inhibitor Actinomycin D in combination with Wnt5a. At 12 h there was no significant decrease in secreted levels of IL-6 in cells treated with Wnt5a and Actinomycin.
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D, as compared to cells treated with Wnt5a alone. By chelating intracellular calcium using Bapta or inhibiting PKA using H89, the Wnt5a induced IL-6 secretion could be inhibited.

Wnt5a signaling has been shown to activate small Rho GTPases that control rearrangements of the cytoskeleton and are involved in secretion. We transfected MeWo cells with dominant negative versions of the Rho GTPases Cdc42 and Rac1 to investigate the effects of these proteins on secretion downstream of Wnt5a. The Wnt5a induced secretion of IL-6 was significantly reduced in cells transfected with the dominant negative versions of Cdc42 and Rac1. Tetanus toxin inhibits secretion by inhibiting certain SNARE proteins, however tetanus toxin did not inhibit the secretion of IL-6 or VEGF suggesting that this was regulated by a tetanus toxin insensitive VAMP. TI-VAMP is one of these and has previously been shown to be involved in regulated exocytosis of cytokines. By extracting TI-VAMP protein and mRNA expression data from publicly available databases, we found that TI-VAMP expression was increased in samples from melanoma patients both at the protein and mRNA level. Upon Wnt5a treatment for 3 h, increased levels of TI-VAMP were found in the cytoskeletal fractions of MeWo cell lysates. These data indicates that the secretion of IL-6 in melanoma cells induced by Wnt5a can be mediated via TI-VAMP.

Discussion Paper III

The production of IL-6 has previously been suggested to be high in melanoma cells that express high levels of Wnt5a (Dissanayake et al 2008). We show that in melanoma cells expressing low levels of Wnt5a, increased secretion of IL-6 and VEGF was induced upon addition of rWnt5a. In melanoma cells with high Wnt5a expression the depletion of Wnt5a decreased the secretion of IL-6 and VEGF. Neither the decrease nor the increase in IL-6 and VEGF secretion was accompanied with a change in mRNA expression levels. The general transcription inhibitor Actinomycin D did not inhibit the secretion. These data show that Wnt5a induces secretion of pre-formed IL-6 and VEGF in the cells without inducing gene transcription. In support of this idea and indicating that Wnt5a did not increase IL-6 translation, we detected less intracellular IL-6 in cell lysates from cells treated with Wnt5a for 3 h than in untreated cells.

Wnt5a can activate intracellular calcium signaling in melanoma cells (Jenei et al 2009), which has been shown to be important for secretion. Upon chelation of intracellular calcium, the increase in secreted IL-6 was inhibited; indicating that activation of intracellular calcium signaling is needed in this process. A change in the intracellular calcium levels triggers the release of the contents of a secretory vesicle that is already bound to the plasma membrane. The increase in intracellular calcium can also regulate cytoskeletal re-arrangements by regulating proteins that control the polymerization of F-actin (Malacombe et al 2006, Stanley and Lacy 2010). Activation of PKA downstream of cAMP can activate regulated exocytosis and the secretion of IL-6 can be induced by PKA (Kiriyama et al 2001, Seino and Shibasaki 2005). Wnt5a signaling via heterotrimeric G-proteins can activate cAMP (Hansen et al 2009), which in turn activates PKA, and indeed, the PKA inhibitor H89 could inhibit the secretion of IL-6 induced by Wnt5a. However, since H89 is not highly specific, the effect of the PKA inhibitor on Wnt5a induced secretion might also be mediated via some of the other kinases that are inhibited by H89 such as ROCKII (Lochner and Moolman 2006). H89 has
also been suggested to inhibit transport from the ER to Golgi and also from the Golgi to the cell surface (Muniz et al 1997). To fully conclude that PKA mediates the secretion of IL-6 induced by Wnt5a, the effects of other PKA inhibitors needs to be analyzed.

Cytokine secretion is often preceded by a change in the cytoskeleton (Li et al 2003, Vitale et al 1995) and indeed we did detect a re-arrangement of the cytoskeleton towards a more cytoplasmic organization, a loss of the cortical organization and the prominent cell border seen in untreated cells. The rearrangement of the cytoskeleton is thought to be performed in order to allow secretory vessels to reach the cell membrane and has been shown to be regulated by RhoGTPases, especially Cdc42 and Rac1 (Malacombe et al 2006). Wnt5a signaling can activate RhoGTPases and affect the cytoskeleton, both in normal cells and cancer cells such as melanoma (Dejmek et al 2006, Schlessinger et al 2007, Witze et al 2008). Our data suggest that Wnt5a signaling via Cdc42 activates secretion possibly by inducing the cytoskeletal re-arrangements needed for this process. Tetanus toxin did not inhibit the effects on IL-6 and VEGF secretion induced by Wnt5a. This limits the mechanisms by which Wnt5a can induce this effect to the exocytosis mechanisms that are mediated by tetanus insensitive SNAREs such as the vesicle SNARE, TI-VAMP (Stanley and Lacy 2010). TI-VAMP expression was increased in malignant melanoma tumor samples suggesting that it plays a role in malignant melanoma. After Wnt5a treatment, TI-VAMP could be found to a higher extent in the cytoskeletal fraction, indicating that TI-VAMP is indeed involved in the secretion induced by Wnt5a, further supporting our notion that Wnt5a can induce regulated secretion via tetanus insensitive SNAREs.

The secretion of immunomodulatory and pro-angiogenic proteins by melanoma cells can have several different outcomes in tumors. IL-6 is associated with a higher tumor burden and poor prognosis in melanoma and can promote pro-tumorigenic inflammation and tumor cell evasion from destruction by immune cells (Lazar-Molnar et al 2000, Mouawad et al 1996). VEGF secretion in melanoma is associated with VGP and induction of angiogenesis, both of which are correlated with a poor prognosis (Erhard et al 1997). In order to test if the increased levels of secreted IL-6 and VEGF could have a functional effect in melanoma we utilized an endothelial cell branching assay. In co-culture experiments, using endothelial cells grown together with melanoma cells depleted of Wnt5a, there was a decrease in endothelial cell branching. This might be an indication of that secreted factors induced by Wnt5a expression is enough to affect endothelial cell branching. Wnt5a itself has been suggested to affect the survival and proliferation of endothelial cells (Masckauchan et al 2006). However, in this study, treatment with rWnt5a in endothelial cells grown without melanoma cells, did not affect the length or amount of endothelial cells formed.

This study indicates that Wnt5a can affect melanoma aggressiveness by inducing secretion of the pro-tumorigenic factors IL-6 and VEGF. We suggest that this effect is mediated by activation of PKA and intracellular calcium signaling and facilitated by a cytoskeletal re-arrangement induced by Cdc42 and Rac1. It might be specific for these cytokines or a general effect on secretion in melanoma cells upon activation of Wnt5a signaling. If this is the case, then Wnt5a might cause secretion of a wide array of pro-tumorigenic factors, which might provide further explanation as to why Wnt5a expression is correlated with poor prognosis in malignant melanoma patients.
Conclusions

I. In malignant melanoma SFRP3 is down regulated due to methylation. SFRP3 decreases migration and invasion in malignant melanoma cells and does so by inhibiting Wnt5a signaling.

II. Wnt5a signaling affects the malignant melanoma proteome by re-programming cellular metabolism to favor aerobic glycolysis. However, Wnt5a has a different outcome on tumor metabolism depending on cellular context.

III. Wnt5a signaling in malignant melanoma cells can induce a calcium dependent regulated exocytosis of the immunomodulatory and pro-angiogenic factors IL-6 and VEGF.
Wnt5a signaling in Malignant Melanoma

I detta avhandlingsarbete har vi fokuserat på effekten av cellsignalering via ett protein, Wnt5a, i utveckling och metastasering av malignt melanom. Wnt proteiner är väldigt viktiga under fosterutvecklingen och även för normala funktioner i olika celltyper. Avvikelser från normal Wnt signalering kan påverka många olika cancertyper. Denna signaleringsväg har även visat sig kunna påverka utvecklingen och spridningen av melanom på olika sätt. Högt uttryck av Wnt5a i malignt melanom är kopplat till en mer aggressiv tumör.

I den första studien har vi undersökt uttrycket av en extracellulär Wnt inhibitor, SFRP3, i melanomtumörer. SFRP3 var mindre uttryckt i malignt melanom jämfört med normal hud, detta kunde vi också bekräfta i melanocyt och melanomcelllinjer. I melanomcelllinjer kunde vi se att SFRP3 minskar rörligheten av melanomceller och att SFRP3 utför detta via inhibering av signalering aktiverad av Wnt5a. Detta tyder på att SFRP3 uttryck i malignt melanom är nedreglerat, vilket kan ge tumören ökad möjlighet att spridas eftersom Wnt5a får mer möjlighet att aktivera sin signalering okontrollerat.

Vidare undersökte vi hur Wnt5a påverkar proteinuttrycket i melanom celler. Här fann vi att aktivering av den här signaleringsvägen kan förändra cellens energimetabolism. Wnt5a visade sig kunna förändra hur cellernas metabolism mot en typ av energimetabolism som är fördelaktig för tumörceller. Vi detekterade även att i tumörprover från patienter med malignt melanom var uttrycket av Wnt5a starkt korrelerat med uttrycket av ett protein som driver tumörcellerna mot en, för tumören, mer fördelaktig typ av energimetabolism.
I det tredje arbetet undersöks hur uttrycket av två signaleringsproteiner, IL-6 och VEGF var kopplade till signalering aktiverad av Wnt5a. Wnt5a kunde aktivera signalvägar i melanomceller som leder till att IL-6 och VEGF som finns färdigproducerat i cellerna kunde utsändas ut ur cellen. Förekomst av dessa proteiner är kopplade till dålig patientprognos i malignt melanom. En, av Wnt5a inducerad, utsändring av dessa proteiner kan sätta igång processer inte bara i tumören utan även i tumörens omgivning som kan påverka metastasering av malignt melanom.

En ökad förståelse av dessa processer krävs för utvecklingen av nya behandlingar med möjlighet att förhindra spridning av cancer. I våra studier har vi identifierat nya mekanismer för hur Wnt5a kan påverka aggressiviteten och spridningen av malignt melanom. Resultaten understryker generellt hur komplex funktionen av Wnt signalering i cancer kan vara men framförallt hur komplex Wnt5a signalering kan vara i malignt melanom.
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