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Identification and Validation of Therapeutic Targets in Sarcoma Models

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“Wisdom was created before everything;
prudent understanding subsists from remotest ages.”
(Ecclesiasticus 1:4)

"*There is nothing I cannot do in the One who strengthens me.*"
(Philippians 4:13)

Year of Saint Paul

ABSTRACT

In spite of the low incidence of sarcomas, they are highly aggressive tumors, with high metastatic potential, and resistance to both chemotherapy and radiotherapy, leaving surgery as the only treatment option. There is evidently an urgent need in identifying molecular targets and oncogenic pathways in sarcomas that can be used for the development and evaluation of drugs directed against these tumors. The present projects have been developed to approach this clinical problem.

We have evaluated the effect of sorafenib, a receptor tyrosine kinase inhibitor, on soft tissue sarcoma cell lines of various histological subtypes, and we have shown that the growth of rhabdomyosarcoma, in particular, can be suppressed by treatment with sorafenib *in vitro* and in tumor xenografts, which was mediated through the IGF-1R-axis. These findings open up new possibilities for rhabdomyosarcoma patients, as treatment with sorafenib or anti-IGF-1R agents could be of clinical value.

Synovial sarcomas seldom carry p53 mutations. Knowing that HDM2 might have a role in the tumorigenesis of these tumors, we investigated the role of SS18-SSX1 on the function of p53. We found that SS18-SSX1 stabilizes HDM2, thus disrupting the stability and tumor suppressive function of p53 by proteosomal degradation. Inhibition of HDM2 expression could counteract this protective effect of SS18-SSX1 and restore p53. Therefore, targeting the p53-HDM2 autoregulatory loop in could be of benefit in treatment of synovial sarcomas.

We made yet another attempt in trying to restore the p53 function in synovial sarcoma. Using tenovin-6, a newly discovered p53 reactivator, we found that p53 levels were restored and we observed an induced cell death upon treatment with tenovin-6 in synovial sarcoma cells. Although this was not directly due to the presence of SS18-SSX1, the expression of SirT2 was inhibited, indicating that agents like tenovin-6 and HDAC inhibitors could be successful in the treatment of synovial sarcoma.

Lastly, we have shown that SSX (a cancer-testis antigen) regulates cell cycle progression and is involved in tumor formation through its ability to activateβ-catenin signalling. Depletion of SSX inhibited tumor growth *in vitro* and in tumor xenografts. Based on its restricted expression, and together with our findings, we have identified and validated SSX is an ideal molecular target for drug development in anti-cancer treatment.

SAMMANFATTNING

För närvarande finns det inget behandlingsalternativ för många sarkom. Den huvudsakliga behandlingen består fortfarande av kirurgi som ges tillsammans med radioterapi och kemoterapi, och som misslyckas i att hämma tumörbildning. Resistens och metastasbildning är ett stort kliniskt problem, därför är det av störst vikt att förbättra vår förståelse av de molekylära mekanismerna som ger upphov till sarkom (liksom många andra former av cancer) för att bekämpa denna sjukdom.

Eftersom val av behandling beror på en tumörs molekylära signatur, kommer förståelsen av fusiongenernas roll i tumörbildning, cellulära signalleringsvägar och molekylära markörer, samt att evaluera behandlingar som visat sig vara effektiva att ge oss insikt i nya behandlingsmetoder och underlätta identifiering av vilka grupper av patienter som svarar särskilt bra.

Idag är det av stor vikt att identifiera vilka patienter det är som svarar på en viss behandling och att utveckla en andra generation behandlingar till att kombineras med de redan etablerade. För utveckling av nya behandlingsmetoder är vi nu i behov av bättre förståelse av tumörbiologi och droger med väldefinierade mål, selektivitet, konsekvens och effekt. Cancer kommer antagligen att bekämpas först när vi har medel som har olika mål i en tumör, det vill säga; kärlbildning, storma, hypoxia, tumörbildande celler och så vidare.

Trots den låga incidensen av sarkom är de klassade som höggradiga tumörer på grund av deras aggressiva beteende, resistens till kemoterapi och radioterapi, vilka leder till misslyckade behandlingar, hög metastatisk potential, och begränsar därmed förbättring av patientöverlevnad.

Ett antal nya målriktade mediciner utvecklas kontinuerligt för cancer med hög incidens, men för sarkompatienter är kirurgi ännu det enda behandlingsalternativet. Det finns därför ett brådskande behov av identifiering av molekylära mål och onkogena signalleringsvägar i sarkom som kan vara användbara i utvecklingen av nya mediciner riktade mot dessa tumörer. Det är även av stor vikt att evaluera redan befintliga mediciner på sarkom som har de mål medicinerna är utvecklade för. Denna avhandling och de projekt som ingår i denna har varit ämnade till att tillmötesgå detta kliniska problem.

Vi har evaluerat effekten av sorafenib, en receptor tyrosin kinashämmare, på tillväxt och celldöd i mjukledssarkomcellinjer. Vi har visat att tillväxten av rhabdomyosarkom kan hämmas med behandling av sorafenib både i cellmodeller och i musmodeller. Vi

har även identifierat IGF-R1 som mål för sorafenib. Våra resultat visar att rhabdomyosarkom, som överuttrycker IGF-1R, kan vara bra kandidater för behandling med sorafenib, samt öppnar upp för möjligheten att behandla dessa tumörer med mediciner som är riktade mot IGF-1R.

Synovialt sarkom karaktäriseras av kromosomtranslokationen SS18-SSX1 och vi har visat att denna fusion hämmar p53s stabilitet och tumörhämmande funktion genom att stabilisera HDM2, vilket tyder på nya behandlingsmöjligheter och utveckling av mediciner som hämmar samspelet mellan p53 och HDM2. Alltså, inhibering av HDM2 med små molekyler skulle kunna reaktivera p53s funktion och därmed leda till tumörcelldöd.

Baserat på dessa resultat, undersökte vi vidare om tenovin-6, en molekyl som reaktiverar p53, kunde återställa p53s funktion och inducera celldöd i synovialt sarkom. Faktiskt, vi fann att tenovin-6 hämmar tumörtillväxt och inducerar celldöd, samt aktiverar p53s funktion mycket effektivt. Denna effekt var däremot inte direkt beroende av SS18-SSX1, men på grund av att tenovin-6 hämmar SirT2, en sirtuin-familjemedlem med histon-deacetylas aktivitet. Våra fynd pekar på en möjlig klinisk fördel i användandet av mediciner som reaktiverar p53s funktion i synovialt sarkom.

Till slut, har vi identifierat och utvärderat cancer-testis antigenen SSX som ett molekylärt mål för utveckling av nya mediciner. I denna studie visar vi att SSX reglerar cellcykelprogression och att inhibering av SSX hämmar tumörtillväxt i musmodeller. Vi har även studerat samspelet mellan SSX och Wnt signalering och visat att SSX behövs för att fosforylera beta catenin, vilket leder till att målgener som är viktiga för tumörtillväxt aktiveras. Dessa resultat visar därför att inhibering av SSX, baserat på dess strikta uttryck, skulle vara ett steg mot flera cancertyper.

LIST OF PUBLICATIONS

I. Sorafenib inhibits tumor growth and vascularization of rhabdomyosarcoma cells by blocking IGF-1R-mediated signaling.
Wessen Maruwge, Pádraig D'Arcy, Annika Folin, Slavica Brnjic, Johen Wejde, Anthony Davis, Fredrik Erlandsson, Jonas Bergh, Bertha Brodin.
Journal of OncoTargets and Therapy 2008:1 67-78.

II. The oncoprotein SS18-SSX1 promotes p53 ubiquitination and degradation by enhancing HDM2 stability.
Pádraig D'Arcy, **Wessen Maruwge**, Brid Ann Ryan, Bertha Brodin.
Molecular Cancer Research 2008:6(1) 127-38.

III. Reconstitution of p53 function by small molecule p53 activator in synovial sarcoma cells.
Wessen Maruwge, Pádraig D'Arcy, Sonia Lain, Bertha Brodin.
Manuscript

IV. SSX activates β-catenin transcriptional function and sustains tumor cell proliferation and survival *in vitro* and *in vivo*.
Wessen Maruwge[*], Pádraig D'Arcy[*], Bertha Brodin.
Manuscript

[*]Equal contribution

Related publications

Effect of sorafenib in synovial sarcoma.
Wessen Maruwge, Pádraig D'Arcy, Annika Folin, Slavica Brnjic, Johen Wejde, Anthony Davis, Fredrik Erlandsson, Jonas Bergh, Bertha Brodin.
Manuscript

CONTENTS

1 Introduction 1
1.1 The history of cancer 1

2 Behavior and features of cancer cells 2
2.1 Aberrant cell growth 2
2.2 Oncogenes 3
2.3 Tumor suppressor genes 3
2.4 Growth factors and receptor tyrosine kinases 3
2.5 Circumventing cell death programs 4
2.6 Metastasis 5
2.7 Vascularization 5

3 Cell signalling 7
3.1 MAPK/Erk and PI3K/Akt signalling pathway 7
3.2 WNT signalling pathway, beta catenin, and EMT 8

4 The p53 tumor suppressor 10
4.1 Discovery 10
4.2 Function 10
4.3 Regulation 11
4.4 P53 and sarcomas 11

5 Cancer classification 12

6 Sarcomas 14
6.1 Molecular alterations 14

7 Rhabdomyosarcoma 16
7.1 Cytogenetics 16
7.2 PAX3 and PAX7 17
7.3 FOXO1 17
7.4 PAX3/7-FOXO1 17
7.5 Mouse models 18
7.6 Clinical outcome 18

8 Synovial sarcoma 19
8.1 Cytogenetics 19
8.2 SS18 20
8.2.1 Function 20
8.2.2 Protein-protein interactions 20
8.3 SSX 21
8.3.1 Cancer-Testis Antigen 22
8.3.2 Function 22
8.3.3 Protein-protein interactions 22

8.4 Pathogenesis 23
8.5 Clinical outcome 23

9 Aims of the thesis 24

10 Results and discussion 25
10.1 Paper I 25
10.2 Paper II 26
10.3 Paper III 27
10.4 Paper IV 28

11 Significance 29

12 Acknowledgements 31

13 References 34

LIST OF ABBREVIATIONS

AF10 (MLLT10)	Myleeoid/lymphoid/mixed.lineage leukemia transl. chr 10
Akt (PKB)	Protein kinase B
ARF	Alternative reading frame
ARMS	Alveolar rhabdomyosarcoma
ATP	Adenosine triphosphate
BAX	B cell associated X protein
Bcl-2	B cell lymphoma 2
BRG1	BRM-related gene 1
BRM	Human Brama homologue
CK1	Casein kinase 1
CTA	Cancer-testis antigen
CXCR4	CXC chemokine receptor
ECM	Extra cellular matrix
EMT	Epithelial-mesenchymal transition
Erk	MAP kinase
ERMS	Embryonal rhabdomyosarcoma
FKHR/ FOXO	Forkhead box
GSK3	Glycogen synthase kinase 3
HAT	Histone acelyl transferase
HDAC	Histone deacetylace
HDM2 (MDM2)	Human double minute 2 (murine double minute 2)
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
KRAB	Krüppel associated box
LEF	Lymphoid enhancer factor
LHX4	Lim homeo box protein 4
LIF-R	Leukemia inhibitor factor receptor
LRP	Low-density lipoprotein receptor-related proteins
MAPK	Mitogen activated protein kinase
MEL2	Melanoma associated antigen
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
Myc	Myelocytomatosis oncogene
PAX	Paired box
PCD	Programmed cell death
PcG	Polycomb group
PDGFR	Platelet-derived growth factor
PI3K	Phosphatidylinisitol 3 kinase
PUMA	p53 up regulated modulator of apoptosis
RAB3IP	RAB3 interacting protein
RB	Retino blastoma oncogene
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SDF-1	Stromal derived factor 1

SirT1	Sirtuin 1
SirT2	Sirtuin 2
SNH	SS18 N-terminal homology domain
SS18	Synovial sarcoma translocation chromosome 18
SSX	Synovial sarcoma X chromosome breakpoint
SSX2IP	SSX2 interacting protein
SSXRD	SSX repressor domain
SV40	Simian virus 40
SWI/SNF	Switch/sucrose non framing
TCF	T-cell factor
TGF	Tumor growth factor
TrkB	Tyrosine kinase B
VEGFR	Vascular endothelial growth factor receptor
Wnt	Wingless and Int

1 INTRODUCTION

1.1 THE HISTORY OF CANCER

Hippocrates (460-370 B.C.), the father of medicine, used the terms *carcinos* and *carcinoma* to describe tumors. Celcus (28 B.C – 50 A.D.) translated later these into the word *cancer*. The oldest description of cancer dates back to 3000-1500 B.C., it was found in Egypt on a writing that describes tumors or ulcers of the breast, and evidence of cancer have been found on bones of mummies, with the oldest specimen dating back to 1900-1600 B.C.

The causes of cancer have puzzled the human mind since the time of ancient Egyptians, ranging from Gods to body fluids, trauma, parasites, and later on from cells, and viruses. After the discovery of the DNA helix in early 1950s by James Watson and Francis Crick, and the subsequent understanding of the genetic code, together with the development of chemical and biological techniques, scientists were enabled to answer many questions regarding cancer biology. Although by this time it was already known that there were certain cancer-causing agents and familial cancers, scientists suddenly could point out the site of damage on the gene that caused the cancer, including inheritance of defective genes. Another breakthrough came in the 1970s and early 80s, when tumor suppressor genes (genes that slow down cell division, repair DNA damage and targets the cell to apoptosis) and oncogenes (mutated proto-oncogenes that normally control cell division and differentiation) were discovered.

The discovery of oncogenes and proto-oncogenes brought with it an explanation to how the proliferation of cells in cancer is driven, that is, through deregulated and constant flow of growth-stimulatory signals. Likewise, tumor suppressor genes (that usually are inactivated or lost in cancers) also control cell growth, but in order to suppress proliferation in response to growth-inhibitory and differentiation-inducing signals, as well as in response to metabolic imbalance and DNA damage.

The behavior of one cancer type differs largely from another. For example, different cancers are caused by different agents and thus grow at different rates and respond to therapies differently. This is the reason to why each type of cancer needs a certain treatment. Several biological strategies have been developed in an attempt to find a cure for cancer, by depriving them from the anti-apoptotic signals that maintain cancer cell growth. However, in spite of many successful examples of anti-cancer treatments, drug resistance is a major clinical problem.

2 BEHAVIOR AND FEATURES OF CANCER CELLS

Tumorigenesis involves several deregulated cellular processes, all of which cancer cells need to acquire for tumor development. These are; independence of proliferation signals, insensitivity to anti growth signals, evasion of programmed cell death (PCD), unlimited replicative potential, ability to evade, invade, and metastasize, and ability to sustain angiogenesis [1]. In addition to all these, cancer cells also show acquisition of drug resistance.

2.1 ABERRANT CELL GROWTH

Normally, cells grow and divide to produce more cells and differentiate to specialized cells only when the body needs them, that is, during embryogenesis, in childhood, in response to an injury, cell damage, or environmental changes. Growth and division of a normal cell are highly controlled processes that are carried out only in response to the right signals and only when the process of replication has been completed correctly. Moreover, the cell cycle itself includes phases that are controlled by several checkpoints, which ensure that there will not be a progress to the next phase of the cell cycle unless the previous one has been completed accurately. A cancer cell, however, is characterized by uncontrolled cell growth and division, escaping control signals, thus continuously forming abnormal cells that may evade, enter the blood stream or lymph vessels, invade and home in normal tissue, and metastasize.

Normal cells have the ability to sense their surroundings and thus respond to changes. For example, cells stop dividing when they sense being surrounded on all sides by other cells, a phenomenon called contact inhibition. Cancer cells, however, don’t behave in this way, they continue to grow and divide in spite of surrounding cells and genetic aberration, leading to tumor formation. Abnormal cell division can also be caused by aberrant growth factor signaling, an oncogene, a virus, or a non-functioning tumor suppressor. With other words, while normal cells that carry a damage (i.e., gene loss, translocation, mutation or epigenetic regulation) die, cancer cells instead have the ability to escape the repair machinery and become more malignant and even contribute to drug resistance over time.

2.2 ONCOGENES

An oncogene is a proto-oncogene that has acquired gain-of-function mutations (also a gene with abnormally increased expression caused by gene amplification – duplication of DNA region, or chromosomal translocation) that turn a cell into a cancer cell, by causing it to escape programmed cell death and instead survive and proliferate – a key event in malignant transformation. Proto-oncogenes normally function in regulating cell growth and differentiation [2]. Examples of oncogenes are Ras and Myc.

2.3 TUMOR SUPPRESSOR GENES

A tumor suppressor gene is a gene that regulates cellular functions, such as cell cycle and cell death, signal transduction, and DNA repair to protect a cell from becoming malignant [3]. Basically, what tumor suppressors do is, as the name implies, to suppress genes involved in cell cycle progression when cell damage is encountered, thus giving time for the repair machinery to repair the damage. If the damage cannot be repaired, then the cell is subjected to self destruction or programmed cell death. They may even block loss of contact inhibition and prevent cells from metastasizing. When inactivated, (e.g. by point mutations, deletions, and epigenetic silencing), tumor suppressors can no longer control cellular events that cause tumor formation [4, 5]. Examples of tumor suppressor genes are Rb and p53.

2.4 GROWTH FACTORS AND RECEPTOR TYROSINE KINASES

Cells need to receive growth-stimulatory signals from their surroundings to grow and divide. These signals are conveyed by growth factors (ligands) to cell surface receptors. By binding to the ectodomains of their monomer (single sub-unit) receptors, growth factors oligomreize the receptors, followed by cross phosphorylation of the kinase domain on the cytoplasmic receptor domains to induce cellular signaling and stimulate growth, proliferation, and differentiation. Growth factors function in all types of signalling, that is, in endocrine, paracrine, autocrine, and juxtacrine signalling.

Aberrations in growth factor signalling (mutations, truncated receptors, over expression of receptors and ligand-independent receptor activation, increased sensitivity to ligands, autocrine signalling, etc.) lead to abnormal cell behaviour. Cancer cells, in contrast to normal cells, have less dependency on the presence of growth factors for their growth and survival because they can create and use their own auto-stimulatory cell signalling; autocrine signalling, where they produce the growth factors they need for their growth and proliferation. In general one can say that normal cells do not produce and release the growth factor whose receptor they display. This is therefore a phenotype that cancer cells acquire to create an autocrine-loop for their own benefit. In fact, several cancer

types create more than one auto-stimulatory loop at the same time. An example is lung cancer that produces tumor growth factor alpha (TGF-α), stem cell factor (SCF), and insulin-like growth factor (IGF) [6-8].

Several studied have showed aberrant expression of Receptor tyrosine kinases (RTK) in sarcomas, where their concerted expression has been shown to promote tumor growth and survival [9-14].

2.5 CIRCUMVENTING CELL DEATH PROGRAMS

Programmed cell death is mediated and regulated by intracellular programs, and it plays an important role in tissue development. There are several different kinds of programmed cell death; apoptosis, autophagy, anoikis, and senescence (permanent cell cycle arrest caused by cellular stress). Besides these, another pathway has been described called caspase-independent programmed cell death or non-apoptotic programmed cell death [15].

Apoptosis

A very extensively studied programmed cell death is apoptosis, a crucial physiological phenomenon that plays a key role in both growth and development of an organism. Apoptosis involves blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and DNA fragmentation to eliminate defective cells whose damage cannot be repaired, hindering them from entering cell cycle and replication, and consequently hindering also tumor formation. Therefore, if apoptosis is insufficient or if it is inhibited, the result will be uncontrolled cell proliferation and acquisition of a more aggressive phenotype of cells carrying a damage that eventually will become a cancer. Many experimental models have shown the implication of p53 in apoptosis, for example by activation of pro-apoptotic genes and targets [16, 17].

Anoikis

When normal cells lose contact with the extracellular matrix (ECM) they die by anoikis, in contrast to cancer cells which can survive to invade and metastasize another tissue [18]. Anoikis is induced when anchorage dependent cells detach from the ECM because of loss of cell-matrix interaction [19, 20]. Unfortunately, resistance to anoikis is observed in cancer, for example in squamous cell carcinoma it has been found to be induced by hepatocyte growth factor (HGF) through the activation of ERK and PI3K [21]. Tyrosine kinase B (TrkB) can make tumor cells resistant to anoikis through activation of the PI3K/AKT signalling pathway, resulting in a blockade of caspases, promoting survival and migration of cells [22].

Senescence

Senescence is a form of cell cycle arrest that is permanent. As a “normal” cell cycle arrest, it is caused by cellular stress, for example by DNA damage, loss of replicative capacity, and oxidative stress. Characteristic for senescence are morphological changes (enlargement of nuclei, granulation, etc.) [23, 24]. Like apoptosis, p53 is involved in activation of signalling pathways that lead to senescence [25].

2.6 METASTASIS

Metastasis is the spread of tumor cells from one organ to another, creating a secondary or a metastatic tumor. Cancers are “leaky”, meaning that they have the ability to leave the primary tumor mass by degrading extracellular matrix proteins, enter the lymphatic system and/or blood vessels, migrate, and eventually extravasate the circulation system and settle down in healthy tissue elsewhere to create a secondary tumor. Normal cells, as well as tumor cells, are in constant need of nutrients, glucose, and oxygen, which are delivered through the blood to acquire survival growth. All these processes are dependent on a network of blood vessels, namely angiogenesis. Tumors therefore induce the formation of blood vessels to adequately sustain their dependency and need of nutrients and oxygen. Common sites of metastasis are liver, lungs, and bone; however, it can occur anywhere in the body. Far more people die of metastasis rather than primary tumors.

2.7 VASCULARIZATION

Vascularization is the formation of the vascular system. There are three kinds of blood vessel formation; i) vasculogenesis (spontaneous blood vessel formation), ii) angiogenesis (formation of blood vessels from already existing vasculature in tumors), and iii) intussusception (in itself growth, formation of new blood vessels by splitting the existing ones).

Sprouting angiogenesis is associated with intracellular junctions and it connects neighbouring vessels, enabling growth of new vessels. The receptors on the endothelial cells in already existing vessels are activated by angiogenic growth factors (e.g. vascular endothelial growth factor, VEGF – the main player in vascularization and angiogenesis, stimulates cellular response through tyrosine kinase receptors). Once the receptors have been activated, proteases are released to degrade the basement membrane and the endothelial cells can escape from vessel walls to form sprouts that connect neighbouring vessels.

Intussusception is when new blood vessels are formed by splitting of existing ones, through the extension of the capillary wall into the lumen and formation of an area of contact, followed by reorganization of existing cells, increasing the number of capillaries, microvascular growth pillars, leading to increased capillary surface [26].

A switch from sprouting to intussusception has been observed in tumor recovery; where an increased number of pillars lead to tumor recovery and is associated with pericyte recovery, a mechanism termed pruning. At the end of tumor recovery, sprouting is repeated anew again, etc. [27].

3 CELL SIGNALLING

Cellular signal transduction is the communication network between and within cells for coordination of all cellular activity. Cellular signalling pathways are interconnected, forming complex networks. There are four types of signals based on the distance they mediate between interactors; i) endocrine signalling (distant, blood borne), ii) paracrine signalling (near vicinity), iii) autocrine signalling, and iv) juxtacrine signalling (contact dependent). A correctly functioning cell signalling system is the basis for the development, repair, and immunity of cells and organs. There are various types of cell signalling cascades which are involved in the development and progression of cancer.

Once a signal has reached the cell surface, it is then integrated within the cell to regulate cellular processes like cell growth, motility, differentiation, protein synthesis, etc. Note that the same signalling molecule may result in different command or outcome, depending on the cellular context. This in turn requires an interaction between the signalling protein (ligand) and their corresponding cell surface receptor.

Thus, understanding basic cellular mechanisms and pathways behind signal transduction and how they are altered in cancer cells will provide us with knowledge to combat this and also other diseases. There are many targeted therapies that are directed against single molecules in the signal transduction pathways, a major problem is though, that even if a certain pathway is inhibited, cancer cells may still evade the inhibitor by activating other pathways to sustain their survival and growth. This is the reason to why combinational therapies are required for successful cancer treatments. There are various types of cell signalling cascades which are involved in the development and progression of cancer.

3.1 MAPK/ERK AND PI3K/AKT SIGNALLING PATHWAY

Many cancers utilize numerous RTK, such as VEGFR, plateled-derived growth factor receptor beta (PDGF-Rβ), and insulin-like growth factor receptor (IGF-R) to transmit extracellular signals to mediate biological responses. Overexpression of RTKs and aberrant growth factor signalling is a common feature, particularly in cancers of soft tissue origin, which promote their growth, proliferation, and motility. Two most characterised RTK mediated signalling cascades are the MAPK/Erk and PI3K/Akt pathways involved in promoting cell proliferation and cell survival [28-30]. Moreover, crosstalk between these pathways provides further levels of cellular regulation [31-33].

Mitogen activated protein kinase (MAPK) pathways couple signals from the cell surface receptors to regulate many physiological cellular processes, including cell proliferation (through the induction of cyclin D1 transcription), differentiation, cell cycle arrest and subsequent senescence, apoptosis (by inactivation of Bad protein), migration, and tumor progression and invasion [28, 34-38].

The phosphoinositide 3-kinase (PI3K) pathway is associated with metastasis, enabling the cytoskeleton to reorganize and thereby allow cells to move and invade. It can also stimulate angiogenesis within tumors during hypoxic conditions. The PI3K pathway has been implicated in many types of epithelial cancers and can be caused by a constitutive activation or the functional loss of upstream tumor suppressor proteins [39-41]. The PI3K is a major signalling component downstream of RTKs. It catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP3), that in turn activates the protein kinase B (Akt) (among other downstream targets) [42].

The PI3K/Akt signalling pathway is involved in the regulation of many cellular processes like cell proliferation, survival, growth, and motility, all of which are important for tumor development and can trigger tumor progression. Phosphorylated Akt promotes stabilization of HDM2 and consequently also degradation of p53, promotes sequesterization of the Bcl-2 family member pro apoptotic Bad protein, and promotes enhanced protein translation via the mTOR pathway [43-46]. Akt can also regulate cell cycle progression by inhibition of GSK3, and thus stabilize cyclin D1 and Myc [47, 48].

In fact, many cancers (including synovial sarcomas) show an aberrant activation of the MAPK/Erk and PI3K/Akt pathways, including the amplification of components within these signalling cascades. Both pathways serve as molecular targets for the development of targeted anti-cancer therapies, as both are implicated in oncogenic transformation and tumor cell invasion. Therefore, targeted therapies designed against the specific components could be an approach to inhibit cell proliferation and survival of cancer cells, and to induce apoptotic mechanism in a wide range of cancer.

3.2 WNT SIGNALLING PATHWAY, BETA CATENIN, AND EMT

The Wnt pathway is implicated in embryogenesis and tissue homeostasis [49]. One central and essential component of the canonical Wnt pathway is the β-catenin protein, which in its stable form (i.e. in the presence of Wnt ligands), functions as a transcriptional activator through its interaction with lymphoid enhancer factor/T-cell factor (LEF/TCF) in the nucleus and induces gene expression of genes involved in proliferation and differentiation, altered cell migration and cell polarity [50-52].

In the absence of Wnt signaling, β-catenin is targeted to ubiquitin-mediated proteosomal degradation through its phosphorylation at serine and threonine residues by a death complex composed of glycogen synthase kinase-3β (GSK-3β), adenomatuos polyposis coli (APC), axin and casein kinase 1 (CK1) [50, 51, 53]. Nuclear translocation of β-catenin is a result of intracellular β-catenin accumulation caused by disruption of the death complex through co-activation of the Frizzled and low-density lipoprotein receptor-related proteins (LRP) receptors, i.e. in the presence of Wnt signals [50, 54].

β-catenin is also involved in cell adhesion at the plasma membrane, where it forms a complex with the transmembrane protein E-cadherin and the adaptor protein α-catenin, and thus promotes cell adhesion [54, 55]. Tyrosine phosphorylation (by for example *KRAS* or MET kinase) dissociates this complex from the plasma membrane, leading to increased Wnt signaling and β-catenin-dependent transcription [50, 51, 54]. Tyrosine phosphorylation (by for example *KRAS* or MET kinase) dissociates this complex from the plasma membrane, leading to increased Wnt signaling and β-catenin-dependent transcription [51, 54, 56].

Dissociation of β-catenin from the E-cadherin/β-catenin/α-catenin complex at the plasma membrane leads to loss of E-cadherin function and, in turn, also to initiation of epithelial to mesenchymal transition (EMT) in epithelial cells, which is characterized by loss of cell-cell adhesion and increased cell motility [51, 54, 56]. EMT is the process in which cells acquire increased motility and it has been implicated in metastasis, where cells lose their adhesion along with E-cadherin repression and become mesenchymal-like in order to invade.

4 THE P53 TUMOR SUPPRESSOR

4.1 DISCOVERY

The p53 protein was discovered in the late 1970s in SV40 transformed cells, as a consequence of its co-immunoprecipitation with Large T-antigen [57, 58]. Very soon it was also discovered that p53 could immortalize and transform cells together with the Ras oncogene in cell culture, which led to the belief that p53 was an oncogene [59-61]. However, mutations of the p53 were later found in several tumor types, and the previous experiments could not be repeated with wild type p53 [62-64]. All this observation lead to the conclusion that p53 is a tumor suppressor. Exactly twenty years later, two more family members of p53 were discovered, namely p63 and p73.

4.2 FUNCTION

The p53 tumor suppressor protein is a transcription factor that regulates many important cellular functions, such as DNA-repair, cell cycle arrest, apoptosis, and senescence, in response to cellular stress like DNA damage, hypoxia, ribosomal stress, contact inhibition, and oncogene activation, to prevent cancer from developing [65-67]. All these properties prevent non-healthy cells from replication. p53 is therefore called "the guardian of the genome". The transcriptional activating function of p53 lies in its ability to bind numerable promoters in the genome and to regulate the expression of many target genes involved in many cellular signalling pathways and networks, resulting in either cellular arrest, to repair damaged DNA, or in cellular elimination [68].

The tumor suppressive function of p53 is owed to its ability to regulate the expression of p21 (which regulates cell-cycle arrest and senescence), and BAX, NOXA, and PUMA, (which regulate apoptosis) [66, 69]. It is therefore of interest for cancer cells to mutate p53 in order to overcome this and to gain growth advantage. Indeed, mutations in the p53 gene are very common in several types of cancers, leading to disruption of the stability and transactivating function and the loss of the tumor suppressive function of the p53 gene [70, 71]. Many efforts have been attributed to the reversal of mutant p53 action in cancers where the p53 is mutated.

4.3 REGULATION

In normal, non-stressed cells, wild type p53 is expressed at low levels and has a very short half-life because of a negative, reversible, feedback-loop where MDM2 (mouse double minute 2 [72], HDM2 in humans) plays a key role in its inactivation and degradation. When oncogenic and cellular stress is encountered, p53 is activated by post-translational modifications such as phosphorylation of p53 by PI3-kinases or phosphorylation of MDM2, [73-76]. Also other proteins (MDMX, ARF, Pirh2, and COP1) are in involved in the regulation of p53 by interfering with its stability and activity [77-79].

MDM2, which itself is a transcriptional target for p53, can regulate p53 stability through different mechanisms, that is, through binding to the N-terminal and interference with p53 trans activating function, ubiquitination and degradation of p53, and shuttling of p53 from the nucleus to the cytoplasm [80-82]. Transcriptional co-activators (for example p300) and acetylate the p53 C-terminal (exposing the DNA binding domain) and thus resulting in gene activation or repression. Deacetylation is carried out by sirtuins (silent information regulators).

MDM2 can bind to the transactivation domain of p53 and inhibit its growth inhibitory function by blocking the transactivation domain and transporting it from the nucleus out to the cytosol, thus acting as an E3 ubiquitin ligase, attaching ubiquitin to p53 C-terminal and marking it for proteosomal degradation [81-88]. This is the reason why in non-stressed cells p53 is maintained at low levels and cells are able to proliferate. A main player in the disruption of this loop is the ARF tumour suppressor that inhibits degradation of p53 mediated by MDM2 by binding to MDM2, preventing ubiquitination and degradation of p53, thus increasing its stability [89, 90].

4.4 P53 AND SARCOMAS

Soft tissue sarcomas seldom carry mutations in the p53 gene, however, it has been reported that HDM2 is commonly over expressed in some soft tissue sarcomas [91]. This has lead to the suggestion that in soft tissue sarcomas the function of p53 may be abrogated by the over expression of its negative regulator HDM2 [92]. Wild type p53 plays an important role in prevention of malignant transformation, but it is frequently inactivated in many cancers. Knowing the importance of tumor cell death, the constitution of wild type p53 activity in tumors with new drugs is of extensive interest as therapeutic strategy.

5 CANCER CLASSIFICATION

Classification of cancers is an attempt to predict the prognosis, and to develop and select therapeutic treatment protocols for a given type of malignancy. It also allows comparison of clinical trials conducted on defined groups of patients, and serves as a tool for basic molecular research. Many factors underlie classification of cancers, such as histological features, biological function of cell type of origin, site of origin, and degree of invasion.

Most tumors arise from epithelial tissue, which is found throughout the body, lining the walls and surfaces of cavities and organs, as well as the skin, and thus provides protection from injuries, facilitates secretion, absorption, subcellular transport, etc. Cancers of epithelial origin are the most common type of cancers and they are called carcinomas. They develop in organs and glands that are capable of secretion, such as in breasts, lungs, prostate, bladder, colon, etc. They originate in epithelial cells, and are divided into two major groups according to the biological function that is associated with epithelia; adenocarcinomas (epithelial cells that secrete protective substances, such as mucus in the stomach) and squamos cell carcinomas (epithelial cells that form protective cell layers).

Non-epithelial-tissue tumors are divided into three major classes; sarcomas, hematopoietic, and neuroectodermal tumors, and they are, unlike carcinomas, classified upon their histological appearance. Sarcomas are divided into two subgroups; bone sarcomas and soft tissue sarcomas. The hematopoietic (blood-forming) tissues give rise to hematopoietic malignancies; leukemias and lymphomas. While leukemias are cancers of the bone marrow, lymphomas develop in the glands or nodes of the lymphatic system. Neuroectodermal tumors arise from cells that form the central and peripheral nervous system, and among these tumors are found gliomas, glioblastomas, neuroblastomas, schwannomas and medulloblastomas. Table 1 shows cell of origin for different cancer types.

Table 1: Cancer classification based on cell of origin

Cancer	Cell of origin
Carcinomas	Epithelial
Adenocarcinomas	
Squamous cell carcinomas	
Sarcomas	Mesenchymal
Bone sarcomas	
Soft tissue sarcomas	
Hematopoietic	Hematopoietic
Leukemias	
Lymphomas	
Neuroectodermal	CNS and PNS
Gliomas	
Glioblastomas	
Medulloblastomas	
Neuroblastomas	
Schwannomas	
Others	

6 SARCOMAS

The term sarcoma comes from the Greek word *sarkoma* meaning “fleshy growth” [93]. Sarcomas (bone and soft tissue) are a diverse group of tumors with at least more than 50 histological subtypes, each of which displays different biological characteristics, and thus also clinical behavior and therapeutic response. Sarcomas originate from mesenchymal precursor cells, that is, fibroblasts, adipocytes, osteoblasts and myocytes. Sarcomas constitute about 1% of all cancers and they develop in supportive and connective tissues, such as bone, muscle, tendon (bands of fiber that connect muscles to bone), cartilage, synovial tissues (tissue around joints), blood vessels, nerves, and fat. Hence, sarcomas are grouped into soft tissue sarcomas and bone sarcomas, and they are classified according to the differentiation status they show (Table 2).

Table 2: Sarcomas and their tissue of origin

Tumor	Tissue of origin
Osteosarcoma	bone
Chondrosarcoma	cartilage
Leiomyosarcoma	smooth muscle
Rhabdomyosarcoma	skeletal muscle
Fibrosarcoma	fibrous tissue
Angiosarcoma	blood vessels
Liposarcoma	adipose (fat) tissue

6.1 MOLECULAR ALTERATIONS

Cytogenetic changes are very common in sarcomas. They may therefore be divided into two classes based on their cytogenetics. Characteristic for many sarcomas are specific genetic alterations, like translocations (Table 3), point mutations, deletions, and amplifications [94]. Many of these translocations generate fusion genes that produce functional transcripts and fusion proteins. Given that the fusion genes are specific for the type of cancer they appear in, it is thought that they are part of the cancer genesis. However, some sarcomas (for example osteosarcomas) do show very complex and non-specific gene alterations. The identification of fusion genes has facilitated the diagnosis of these tumors, as well as the basic research in understanding their biology and mechanisms of actions in development and progression, as well as the setting for developing targeted therapies. Independently of genetic alterations, both subclasses show poor prognosis and response to therapeutic options.

Table 3: Chromosomal translocations and fusion genes in sarcomas

Tumor	Translocation	Fusion product
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	PAX3-FOXO1
	t(1;13)(p36;q14)	PAX7-FOXO1
	t(2 ;2)(q35;p23)	PAX3-NCOA1
Ewing's sarcoma	t(11;22)(q24;q12)	EWS-FLI1
	t(21;22)(q22;q12)	EWS-ERG
	t(7;22)(p22;q12)	EWS-ETV1
Synovial sarcoma	t(X;18)(p11.23;q11)	SS18-SSX1
	t(X;18)(p11.21;q11)	SS18-SSX2
	t(X;18)(p11;q11)	SS18-SSX4
	t(X;20)(p11;q13.3)	SS18LI-SSX1

7 RHABDOMYOSARCOMA

Rhabdomyosarcomas are the most common soft tissue sarcomas in children and adolescents. There are two variants of rhabdomyosarcomas based on their histological appearance; embryonal (ERMS) and alveolar (ARMS). ERMS is the more common type of rhabdomyosarcoma, and occurs often in the bladder, vagina, prostate, testis, and the head and neck among young children, while ARMS occurs mainly among adolescents and young adults, often in the trunk, arms, and legs [95]. Of the two, ARMS is more aggressive, have a worse outcome with higher mortality, and is associated with early dissemination of tumor cells, bone marrow involvement, and well as metastasis [95-99].

The origin of rhabdomyosarcoma are primitive muscle cells, the rhabdomyoblasts, that fail to differentiate into striated muscle cells [100]. The specific immunohistological staining for muscle proteins and myofilaments of these tumors points at their myogenic differentiation [95].

7.1 CYTOGENETICS

ARMS carry the chromosomal translocation PAX3-FOXO1 (also known as PAX3-FKHR) in 70% of all cases, a translocation between chromosomes 2 and 13 t(2;13)(q35;q14) that generates a fusion protein [101-104]. Some ARMS express the chromosomal translocation PAX7-FOXO1 (also known as PAX7-FKHR) between chromosome 1 and 13 instead t(1;13)(p36;q14) [105-108]. Both PAX3 and PAX7 are transcription factors, and when fused with FOXO1 (also a transcription factor) give rise to a novel transcription factor with altered function, transcriptional targets, and posttranslational regulation.

Microarray studies have been done as an attempt to identify genes that are upregulated in rhabdomyosarcoma by PAX3-FOXO1 and the potential targets have been found to cluster as neural-related genes and transcription factors involved in mesodermal development, and as genes that take part in myogenic differentiation, muscle contraction, [96, 109-111].

7.2 PAX3 AND PAX7

PAX3 and PAX7 belong to the paired box (PAX) genes family of highly conserved transcription factors with DNA binding domains [95, 112]. These genes, which consist of nine exons each, code for very similar proteins that are important in tissue and organ development, and contain an N-terminal DNA binding domain with a paired box, a homeodomain, and a C-terminal domain that is rich in proline, serine, and threonine. In total there are nine genes in the PAX family, which are divided into four groups based on their similarities [95, 113].

7.3 FOXO1

The forkhead box (FOX) genes function in both embryonic development and the adult tissue and encode for proteins that are transcription factors that regulate the expression of genes involved in cell growth, proliferation and differentiation [95, 114, 115]. The FOXO1 consists of three exons, contains a forkhead domain, and a transcriptional activation domain [116]. The C-terminal of FOXO1 transactivation domain fuses to the N-terminal of the PAX3/7 DNA binding domain In rhabdomyosarcoma.

7.4 PAX3/7-FOXO1

The PAX3-FOXO1 nuclear fusion protein of 836 amino acids, is a more potent transcription factor than PAX3 and FOXO1 alone, meaning that the fusion protein can enhance the activation of PAX3 target genes and enhance growth, differentiation, and survival [95, 117-119]. The transforming properties of PAX3-FOXO1 have been demonstrated in chicken embryo fibroblasts and in NIH 3T3 cells, leading to anchorage-independent growth and the activation of myogenic transcription [111, 120]. It has been demonstrated that the fusion protein plays a role in cell survival and deregulation of the cell cycle [99].

Overexpression of both PAX3-FOXO1 and PAX7-FOXO1 in comparison to wild type PAX3/7 and FOXO1 has been demonstrated both at gene and protein levels in ARMS tumors, and it has been suggested that this is required for oncogenesis, as it contributes to oncogenesis through abnormal control of growth, apoptosis, differentiation, or motility [76, 121].

7.5 MOUSE MODELS

It has been shown in both *in vitro* and *in vivo* experiments that PAX3-FOXO1 alone cannot induce tumor formation, but rather contribute to transformation and oncogenesis, requiring additional genetic lesions [96, 122]. When PAX3-FOXO1 was ectopically expressed in rhabdomyosarcoma cell lines it resulted in an increased proliferation rate, decreased dependence on exogenous growth factors, and more invasive growth of the cells in mice xenografts [123].

A PAX3-FOXO1 transgenic mouse study resulted in developmental abnormalities but it was not sufficient to cause tumors [124]. This was also seen in a conditional mouse model for ARMS with a PAX3-FOXO1 knock-in allele (activated in late embryogenesis and postnatal in terminally differentiating skeletal muscle) that resulted in low tumor occurrence [125]. However, disrupting the Ink4a/ARF or Trp53 pathway, by conditional Trp53 or Ink4a/ARF loss of function, increased the frequency of ARMS tumor formation, which suggests that PAX3-FOXO1 requires additional alterations for ARMS tumor development.

7.6 CLINICAL OUTCOME

Why rhabdomyosarcomas metastasize to the bone marrow remains an important question to resolve. It is known that stromal-derived factor-1 (SDF-1) and hepatocyte growth factor (HGF), together with their receptors CXCR4 and c-MET respectively are expressed in rhabdomyosarcoma cells and are thought to play a role in metastasis by attracting the cells to the bone marrow and enabling their survival and expansion [98, 126, 127] . However, a blockade of SDF-1–CXCR4 and the HGF–c-MET did not result in complete inhibition of the cells' chemotaxis, pointing at the possible involvement of other factors involved in the metastasis of rhabdomyosarcoma [98, 126].

Current treatment for rhabdomyosarcoma is surgery, chemotherapy and radiotherapy. However, resistance to chemotherapy and radiation therapy is a limiting factor in the treatment of rhabdomyosarcoma, leading to therapeutic failure and limiting improved survival in these patients

8 SYNOVIAL SARCOMA

Synovial sarcoma was first used as a denomination for tumors that arise near joints, lacks however connection with synovial tissue. However, subsequent studies revealed that synovial sarcomas show features of epithelial differentiation [128]. A knock out mouse model based on the conditional expression of SS18-SSX2 in immature myoblasts resulted in development of synovial sarcoma tumors, and thus lead to the identification of myoblasts as potential cells of origin in synovial sarcoma [129]. Although the tissue of origin remains unknown, these tumors are thought to be of mesenchymal origin.

Synovial sarcomas are composed of two distinct cell types: spindle cells and epithelioid cells. This feature classifies these tumors into three subclasses based on their histological appearance; that is, i) biphasic, which are composed of both epithelial and spindle cells, ii) monophasic, which are composed of only spindle cells, and iii) poorly differentiated, which are an intermediate of epithelial and spindle cells [130]. Characteristic for synovial sarcomas is the immunohistological staining for the expression of the epithelial markers cytokeratins 7 and 19, the mesenchymal marker vimentin, and the anti-apoptotic protein Bcl-2 [131-133].

Synovial sarcomas (one of the most common soft tissue tumors in young patients) are a rare type of cancer that occurs at any age but affects mainly young adults and accounts for about 5-10% of all soft tissue tumors [134, 135]. These tumors can appear anywhere in the body, although they occur more frequent in the extremities, in close association to and around the large joints in the legs and arms. They metastasize mainly to the lungs, but also to the lymph nodes and bone marrow [134]. Synovial sarcomas are highly aggressive soft tissue tumors, killing at least 25% of the patients within the first 5 years after diagnosis, and five years overall survival has been reported to vary from about 40% to 70%, and the 10 years survival 20% [136-139].

8.1 CYTOGENETICS

In nearly all cases (more than 95%), synovial sarcomas are specifically characterised by the (X;18)(p11;q11) chromosomal translocation, where the SS18 gene (also known as SYT) on chromosome 18 fuses with one of the SSX genes on chromosome X (SSX1, SSX2 or SSX4), where the last eight amino acids of SS18 are replaced by the 78 amino acids on the C-terminal of SSX [137, 140-148]. In spite of the large portion of the SS18 protein that is retained in synovial sarcoma, an over expression wild type SS18 does not result in transformation of cells, which clearly shows that the fusion partner SSX is

needed for transformation [149].In general, biphasic synovial sarcomas show an expression of SS18-SSX1, while monophasic synovial sarcomas show the expression of SS18-SSX2 fusion gene [94].

8.2 SS18

SS18 is ubiquitously expressed in a wide variety of cell types during early stages of embryogenesis, in fetal human tissues and in the adult [150, 151]. The SS18 gene has 11 exons and encodes for a 418-amino acid nuclear protein, with no homology to any other known protein [134, 152].

8.2.1 Function

The SS18 protein contains a SYT N-terminal homology domain (SNH domain) and a C-terminal QPGY domain (which is rich in glutamine, proline, glycine and tyrosine), believed to have a transcriptional activating function [135, 141, 153, 154]. Deletion of the SNH domain enhances transcription, which suggests that this domain acts as an inhibitor of the C-terminal activation domain [154]. A study in which the SS18 gene was knocked out in mice resulted in an early embryonic lethality due to placental failure and blood vessel formation, with growth defects and gestation [155].

8.2.2 Protein-protein interactions

The SS18 contains numerous protein-protein interaction sites, including three SH2-binding motifs and one SH3-binding motif, and several interactions of SS18 with other proteins have been identified [156, 157].

SWI/SNF

The histone modifying protein complex SWI/SNF complex is highly conserved and it is involved in the ATP dependent chromatin remodeling and transcriptional activation [158]. The SWI/SNF complexes are implicated in cell cycle regulation, malignant transformation, and signal transduction [159]. The SS18 SNH domain interacts with the SWI/SNF complex components ATPases BRM (Brahma, regulates gene transcription by chromation remodelling) and its homologue BRG1 (BRM-related gene 1), Which suggests a role in the epigenetic regulation of gene expression [149, 154, 160-162]. Interestingly, BRM and BRG1 interact with RB, p53, and β-catenin [163-165].

AF10

The SS18 SNH domain interacts with the putative transcription factor AF10 (also known as MLLT10), which is deregulated in acute leukemia and functions in heterochromatin-dependent gene silencing [166]. The interaction between SS18 and AF10 could have a consequence on the normal regulation of SS18-mediated transcription.

mSin3A

The mSin3A is a co-repressor essential for embryonic development and regulation of proliferation and survival of cells, partly by controlling the expression of p21 and deacetylation of p53 [167]. The mSin3A is a histone deacetylase (HDAC) complex component that is involved in transcriptional repression and in rendering the chromatin inaccessible. The SNH domain in SS18 interacts with mSin3A, which results in regulation of the transcriptional activity of SS18 by repression [168].

p300

The p300 is a nuclear tumor suppressor protein with versatile transcriptional co-activating function with histone acetyl transferase (HAT) activity to regulate chromatin structure. It interacts with transcriptional activators and repressors, and coordinates these pathways through chromatin remodelling. The p300 has an essential role in cell cycle control, cellular differentiation, and development. SS18 interacts with p300 histone acetyl transferase and promotes cell adhesion to a fibronectin matrix, and results in the activation of β1 integrin (a major adhesion receptor) [169]. In this study, the formation of the SS18/p300 complex appeared to be transcription activation-independent, though the transcriptional repressor properties of the SS18/p300 complex in mediation of cell adhesion were not excluded as possible mechanisms.

8.3 SSX

SSX was initially identified as a melanoma associated antigen (MEL2), and later as fusion gene in synovial sarcoma [141, 170]. Currently there are nine identified highly homologous gene family members, and ten pseudo genes [171]. The SSX members show high similarity in nucleic acid and protein sequence [172]. All SSX genes (three of which are found as fusion partners with SS18 in synovial sarcoma) have 7 exons and encode for proteins of 188 amino acid that are rich in charged amino acids arginine and lysine [141].

8.3.1 Cancer-Testis Antigen

In normal tissue SSX is expressed in male germ cells, trophoblasts of the placenta, in fetal and adult mesenchymal stem cells, and in the thyroid gland [173-176]. In malignant cells, SSX is expressed in several tumor types; in melanomas and in tumors of mesenchymal, epithelial and neuroectodermal origin [171, 172, 177]. Due to their restricted expression in testis and tumors, and their ability to illicit an immune response, SSX are classified as cancer testis (CT) antigens.

8.3.2 Function

The SSX protein has two repressor domains, a Kruppel associated box (KRAB, a family of zinc finger DNA binding proteins) at the N-terminal and an SSX repressor domain (SSXRD) at the C-terminal [146, 178, 179]. The KRAB domain is deleted and replaced with SS18 in synovial sarcoma fusion transcripts. Despite the lack of a DNA-binding domain and a nuclear localization signals, the SSX protein localizes in the nucleus, but may also occasionally be targeted to the cytoplasm [135, 152, 176].

Although the function of SSX has not been defined, the SSX proteins that have been found interact specifically with several different proteins (and may therefore perform different functions), such as with the polycomb group proteins (PcG) proteins HPC2, BMI-1 and RING 1, and with core histones. The expression of SSX is believed to be epigenetically regulated by methylation and histone deacetylation [152, 153, 159, 180-182].

8.3.3 Protein-protein interactions

The highly conserved C-terminal SSX repressor domain SSXRD, a transcriptional repression domain, is important for the nuclear localization and association of the SSX proteins with heterochromatin, and consequently, also for the localization of SS18-SSX to the PcG nuclear domains [153, 178, 183]. PcG proteins complexes function as transcriptional repressors and are involved in gene silencing through structural changes of chromatin [36, 51]. The SSX proteins have been shown to repress transcription, which could be mediated by PcG complexes that it associates with [178]. Moreover, the C-terminal region of SSX1 binds strongly to core histones and oligonucleosomes [159].

RAB3IP

SSX interacts with RAB3IP (human homologue of Ras-like GTPase Rab3A interactor which interacts with SSX2) and SSX2IP (SSX2 interacting protein, which interacts with SSX2 and SSX3) proteins, forming nuclear co-localization [184]. The interaction

of SSX2 with these two proteins was localized to the N-terminal regions that are not present in synovial sarcoma. SSX2IP is also expressed in acute myeloid leukemia as an antigen [185].

LHX4

The putative transcription factor Lim homeo box protein LHX4 is a DNA-binding protein that is involved in transcriptional regulation and development [186]. The interaction between the C-terminal proteins of SSX1 and LHX4 is thought to repress transcription of target gene promoters that contain LHX4 binding sites [187].

β-catenin

Nuclear β-catenin is a transcriptional activator of genes involved in proliferation, differentiation, altered cell migration and cell polarity, whereas plasma membrane-bound β-catenin is involved cell adhesion [50-52, 54, 55]. When fused with SS18 in synovial sarcoma, SSX2 interacts with β-catenin and promotes β-catenin nuclear translocation without altering β-catenin transactivating function without activating canonical Wnt targets [188]. This mechanism was reversible, as depletion of SS18-SSX2 resulted in the loss of nuclear β-catenin. These findings reveal the involvement of β-catenin in the development of synovial sarcomas.

8.4 PATHOGENESIS

The transcriptional activating domain of SS18 and the transcriptional repressor domain of SSX are clearly thought to be of importance in the yet unknown pathogenesis-mechanism of synovial sarcomas. It is likely that the development of synovial sarcoma is due to deregulated transcription through chromatin remodelling. Since both SS18 and SSX lack a DNA-binding domain and a nuclear localization signal, its binding partners through protein-protein interactions, may have important implications for the mechanisms underlying transformation and malignant cellular growth of synovial sarcoma.

8.5 CLINICAL OUTCOME

Some studies have shown that patients with SS18-SSX1 fusion type show a worse prognosis and higher risk of relapse [94, 144, 190, 191]. However, whether it is SS18-SSX1 or SS18-SSX2, there is currently no treatment for synovial sarcomas, and the only option is surgical resection for tumor eradication.

9 AIMS OF THE THESIS

Given the fact that currently there are no treatment options for many sarcomas, and that the main treatment is still surgical resection together with radiotherapy and chemotherapy which fail to induce tumor regression and instead show resistance, whereby metastasis is a major clinical problem, it is of great importance to improve our understanding of the molecular mechanisms that underlie the formation of sarcomas (as well as other cancers) in order to combat this disease.

As choice of therapeutics depends on the molecular signature of a tumor, understanding the fusion gene mediated tumorigenesis, assessment of tumor signature (e.g. signalling pathways, molecular markers), and evaluation of effective therapies will provide insight into potential new therapeutic options, and identification of responding groups of patients.

Today it is of importance to identify the patients that do respond to current therapies, and to develop a second generation therapies to be combined with the already established ones. For development of new treatments, we are in need of a better understanding of tumor biology and drugs with well defined action, selectivity, impact/consequence, and effect. Combating cancer will probably occur when we have agents that target different sites of a tumor, that is, neovasculature, tumor stroma, hypoxic areas, tumor initiating cells / stem cell like cancer cells, etc.

This has been the underlying basis for this thesis, where the specific aims were as follows:

PAPER I To evaluate of the activity of newly developed drugs against receptor tyrosine kinases in soft tissue sarcomas.

PAPER II To study of the role of SS18/SSX on p53 function in synovial sarcoma as an attempt to identify molecular targets.

PAPER III Based on the results from paper II, we aimed at investigating if the reconstitution of p53 function with a newly developed small molecule p53 reactivator could induce death in synovial sarcoma cells.

PAPER IV To investigate the oncogenic function of SSX, and to validate its potential as a therapeutic target in tumor growth and progression *in vitro* and *in vivo*.

10 RESULTS AND DISCUSSION

10.1 PAPER I

Sorafenib inhibits tumor growth and vascularization of rhabdomyosarcoma by blocking IGF-1R mediated signaling

Rhabdomyosarcoma is the most common soft tissue sarcoma in children. Current treatment is surgery, chemotherapy and radiotherapy. However, resistance to chemotherapy and radiation therapy is a limiting factor in the treatment, leading to therapeutic failure and limiting improved patient survival. In this study we aimed to evaluate the effect of sorafenib, a receptor tyrosine kinase inhibitor, on cell growth and apoptosis in soft tissue sarcoma cell lines of various histological subtypes. As the growth of many soft tissue sarcomas is dependent on aberrant growth factor signaling, which promotes their proliferation and motility, we also analyzed the expression of RTKs that are potential targets for sorafenib in the soft tissue sarcoma cell lines.

We analyzed the response of soft tissue sarcoma cell lines to sorafenib by cell viability assays, the inhibition of Erk and Akt activation in response to treatment with sorafenib. We also investigated the involvement of RTKs in response to ligand stimulation and treatment with sorafenib. Finally, we also evaluated the effect of sorafenib in rhabdomyosarcoma xenografts.

We found that sorafenib effectively inhibited cell proliferation in rhabdomyosarcoma, synovial sarcoma and Ewing's sarcoma with IC_{50} values < 5 μM, and induced growth arrest in rhabdomyosarcoma, which was concurrent with inhibition of Akt and Erk signaling. Analysis of receptor expression showed the presence of IGF-1R, and several other RTKs. Studies of ligand induced phosphorylation of Erk and Akt in rhabdomyosarcoma cells showed that IGF-1 is a potent activator, which can be blocked by treatment with sorafenib. Treatment of rhabdomyosarcoma xenografts with sorafenib had a significant inhibitory effect on tumor growth, which was associated with inhibited vascularization and enhanced necrosis advanced to the tumor stroma and reduced proliferative capacity.

Our results demonstrate that *in vitro* and *in vivo* growth of rhabdomyosarcoma can be suppressed by treatment with sorafenib due to inhibition of IGF-1R mediated signaling. In addition to the anti-proliferative effect of sorafenib on the tumor cells, its effect on the tumor stroma is determinant for the growth and angiogenesis of these tumors. Our findings suggest that rhabdomyosarcoma, which tend to over express IGF-1R, may be good candidates for treatment with sorafenib.

10.2 PAPER II

The oncoprotein SS18-SSX1 promotes p53 ubiquitination and degradation by enhancing HDM2 stability

The p53 protein is a transcription factor that regulates many important cellular functions, such as cell cycle arrest and apoptosis, in response to genotoxic stress. Mutations in the p53 gene are very common in several types of cancers, leading to the loss of the tumor suppression function of this gene. Soft tissue sarcomas seldom carry mutations in the p53 gene, however, it has been reported in several independent studies, that HDM2, a p53 regulator, is commonly over expressed in some soft tissue sarcomas. This has lead to the suggestion that in soft tissue sarcomas the function of p53 may be abrogated by the over expression of its negative regulator HDM2. In this study we have evaluated the role of SS18-SSX1 in synovial sarcoma on p53 function.

We have sequenced primary synovial sarcoma samples for determination of p53 status, and analyzed the expression of p53 protein, its transactivating function, cell survival and growth in the presence and absence of SS18-SSX1 expression by western blot, immunoprecipitation, immunofluorescence, FACS assays, colony formation assays and pulse chase experiments.

Sequencing analysis showed no p53 mutations in the synovial sarcoma tumors. We found that p53 levels decreased with the over expression of SS18-SSX1, which also conferred survival and growth advantage. We demonstrated that this was due to stabilization of HDM2, through the inhibition of HDM2 autoubiquitination by SS18-SSX1, which consequently results in p53 ubiquitination and degradation. The transactivation of p53 target genes HDM2, PUMA, and NOXA, but not that of p21, following genotoxic stress was altered in the presence of SS18-SSX1. Cells expressing SS18-SSX1 displayed a higher proportion of cytoplasmic p53 and were resistant to apoptosis in response to genotoxic agents. Finally, inhibition of HDM2 expression could counteract the protective effect of SS18-SSX1 on the induction of apoptosis.

In conclusion; we have showed that in synovial sarcoma with wild type p53, the tumor suppressinve function of p53 is damaged by virtue of the SS18/SSX1 fusion gene that regulates the stability of p53 in a negative manner by stabilizing HDM2, and consequently promoting p53 for proteosomal degradation. The clinical impact of this finding is that drugs that reactivate the function of p53 by inhibiting its degradation, i.e. targeting HDM2 interactions, can be therapeutically effective in the treatment of synovial sarcoma.

10.3 PAPER III

Reconstitution of p53 function by a small molecule p53 activator in synovial sarcoma cells

The recent discovery of tenovins, which activate wild type p53 by inhibition of factor(s) upstream of p53, increasing p53 levels and inducing growth inhibition, opens a possibility of their usage in the treatment of synovial sarcomas which retain wild type p53. The molecular targets of tenovin-6 are sirtuins (silent information regulators) SirT1 and SirT2. Inhibition of sirtuins in this regard is of high interest, as the binding site for MDM2 on p53 is the same as the acetylation site on K382. Thus, acetylation will inhibit the degradation of p53 and consequently increase transcriptional activation of target genes. Based on the p53 status in synovial sarcomas and that tenovins can reactivate the function of p53, we aimed to enquire whether tenovins do reactivate the p53 function in synovial sarcoma, testing their potential in restoring of p53 in synovial sarcomas.

We have analyzed the effect of tenovin-6 (a p53 reactivator) in synovial sarcoma cell lines by western blot, FACS assays, colony formation, and cytotoxicity assays. We have also looked at the effect of tenovin-6 on p53 stability and its gene transactivation function by real time PCR. We further evaluated if the observed response to tenovin-6 was due to the expression of SS18-SSX1, and at the expression of sirtuins.

We found that tenovin-6 inhibits synovial sarcoma cell proliferation, and rapidly restores p53 protein levels and transactivatin function at very low concentrations. However, this response was not directly associated with the expression of SS18-SSX1, but with the histone deacetylase sirtuin SirT2. Tumor growth inhibition mediated by inhibition of sirtuins (and HDAC inhibitors) deserves closer investigation, as inhibitors like tenovin-6 may be of clinical interest in the treatment of synovial sarcoma, which retain an inactive wild type p53.

10.4 PAPER IV

SSX activates β-catenin transcriptional function and sustains tumor cell proliferation and survival in vitro and in vivo

SSX is a cancer-testis antigen, with restricted expression to male germ cells, trophoblasts of the placenta, mesenchymal stem cells, the thyroid gland (occasionally), and in several tumor types of various histological subtypes, in particular in mesenchymal tumors. SSX is a transcriptional repressor, and although its functions has not been defined, it is has been shown that SSX has a role in epigenetic regulation of gene expression, and in controlling cell renewal and differentiation. Here we have investigated the function of SSX on the Wnt pathway and on tumor growth *in vitro* and in mice xenografts.

We have used a conditional RNA interference doxycycline regulated system to silence SSX in a highly metastatic melanoma cell line and evaluated its oncogenic function by survival and growth assay, cell cycle analysis, the expression of cell cycle proteins, and the β-catenin status and its transactivating function in the presence and absence of SSX. We also evaluated the function of SSX in melanoma xenografts.

We found that knock down of SSX halts cell growth, without affecting cell viability, decreasing the number of cells in S phase and accumulating the cells in G2 and G1, phases along with a reduced expression of cyclin E and A. Moreover, we also found that the knock down of SSX down regulates the expression levels of MMP2 and cyclin D1. Analysis of the association between SSX and β-catenin showed that the loss of SSX associates with a reduced phosphorylation of β-catenin, protection of β-catenin from degradation, nuclear export of β-catenin, and a reduced expression of β-catenin target genes cyclin D1, MMP7 and c-Myc. SCID mice xenografts showed that knock down of SSX indeed reduces the tumor growth and the rate of proliferation, and induces necrosis.

In conclusion; SSX regulates the cell cycle progressing by enhancing tumor cell growth through promoting S phase entry, and depletion of SSX inhibits tumor growth *in vivo*. SSX contributes to tumor formation by activating β-catenin signalling, promoting its transactivating function. Our results prove the oncogenic function of SSX and its potential as a therapeutic target.

11 SIGNIFICANCE

In spite of the low incidence of sarcomas, they are considered to be high grade tumors because of their aggressive behaviour; resistance to chemotherapy and radiotherapy, leading to therapeutic failure and high metastatic potential, and thus limiting improvement of patient survival.

A number of new targeted drugs are emerging for the treatment of cancers with high incidence (mostly for carcinomas), for most sarcoma patients, however, surgery remains the only treatment option. There is therefore an urgent need to identify molecular targets and oncogenic pathways in sarcomas that can be used for the development of new drugs directed against these tumors. It is also of importance to evaluate already available drugs in sarcomas that have the targets that the drugs have been developed for. The present projects have been developed to approach this clinical problem.

We have evaluated the effect of sorafenib, a receptor tyrosine kinase inhibitor, on cell growth and apoptosis in soft tissue sarcoma cell lines, and we show that the growth of rhabdomyosarcoma can be suppressed by treatment with sorafenib. We also identified the IGF-1R as a target for sorafenib. Our findings suggest that rhabdomyosarcomas, which tend to over express IGF-1R, may be good candidates for treatment with sorafenib, and open up possibilities for treatment with agents that target the IGF-1R.

Synovial sarcomas are characterized by the chromosomal translocation SS18-SSX1 and we have shown that this fusion disrupts the stability and tumor suppressive function of p53 through the stabilization of HDM2, opening a new possibility for therapeutic development for the inhibition of the interactions between p53 and HDM2. Thus, inhibition of HDM2 stabilization with small molecules could reactivate the function of p53.

Based on these results, we further investigated whether tenovin-6, a p53 reactivator, could reconstitute the function of p53 and induce cell death in synovial sarcoma cells. Indeed, we found that tenovin-6 efficiently inhibits tumor growth and induces cell death along with induced activation of p53 target genes. This effect was, however, not due to the presence of SS18-SSX1, but because of the inhibition of Sir2, a sirtuins family member with histone deacetylase activity. Our findings point at a possible clinical benefit with the use of p53 reactivating agents in synovial sarcomas.

Finally, we have identified and validated the cancer-testis antigen SSX as a molecular target for drug development. In this study we have shown that SSX has a function in regulating the cell cycle progression, and that long term depletion of SSX inhibits tumor growth in xenografts. We have also shown a connection between SSX and the Wnt pathway, as it is required for the phosphorylation of β–catenin and its release from the plasma membrane, nuclear translocation, and consequently activation of target genes. These results therefore show that targeting SSX would be an approach to treat several cancer types.

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“My heart is stirred by a noble theme,
I address my poem to the king,
my tongue the pen of an expert scribe.
I thank you with all my heart,
Lord my God,
I will glorify your name for ever,
for your faithful love for me is so great.”
(Psalm 45:1, 86:12-13)

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