UNIL | Université de Lausanne
Faculté de biologie et de médecine

Centre hospitalier universitaire vaudois

SCHOOL OF MEDICINE

MASTER THESIS N°5772

STUDY OF THE IMPACT OF WHSC1 AND CEP55 GENES SILENCING IN MYXOFIBROSARCOMA CELLS

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Ecublens, December 2018

Abstract

Myxofibrosarcoma is one of the most common soft tissue sarcomas, contributing to more than 5% of all adult sarcomas. This neoplasm most often develops in the dermis or subcutaneous tissue of the extremities. However, it may also be deep-seated and arise in other locations such as the head, neck or trunk (1,2). Although adult sarcomas have received more attention in recent years, myxofibrosarcoma remains vastly understudied (about 400 articles referenced on PubMed since the 1950s). Therefore, further knowledge about the tumour-initiating capacity of primary myxofibrosarcoma tumour cells is greatly needed to identify targets that could be susceptible for specific treatments.

By using gene expression microarrays, two genes (WHSC1 and CEP55) were found to be overexpressed in the more aggressive and metastatic primary myxofibrosarcoma cell population "SpA", as compared to the non-metastatic cell population "DMEM" derived from the same primary myxofibrosarcoma tumour. The present study was aimed at assessing the importance of these two genes for the survival of primary myxofibrosarcoma cells. These genes were silenced using the short hairpin RNA (shRNA) technique in two cell populations, cultured in different conditions (DMEM and SpA). Cell cultures with stable WHSC1 or CEP55 depletion were then injected into the renal capsule of mice to evaluate their tumorigenic capability. Although tumour formation *in vivo* was decreased to some extent in shRNA-treated cells, as compared to control cells, the tumour-forming ability was not abolished. As it could be partly due to residual protein expression, we designed a complete gene knockout by utilizing another silencing technique, the genome editing CRISPR CAS9 system. To date, these constructs targeting WHSC1 and CEP55, respectively, have not yet been evaluated in the target cells. This study may open the way to a better understanding of the tumorigenesis of myxofibrosarcoma, as well as the role of WHSC1 and CEP55 genes, by studying the impact of their silencing both *in vitro* and *in vivo*.

Keywords: myxofibrosarcoma, WHSC1, CEP55, shRNA, CRISPR Cas9

1. Introduction

1.1 Soft tissue sarcomas: Myxofibrosarcomas

According to data from the National Institute for Cancer Epidemiology and Registration (NICER), more than 16'000 people in Switzerland were diagnosed with cancer in 2014 (3). With a mortality rate of over 40%, cancers are a major cause of mortality and hence there is a need to improve prevention, early detection and therapeutic strategies tailored to the patients' needs. Research has led to a better understanding of the biological mechanisms underlying tumour development which is essential in order to develop effective treatments.

Soft tissue sarcomas are malignant neoplasms that arise primarily in connective tissues. Compared to malignant tumours of, for example, breast or lung, which are very common, soft tissue sarcomas are rare and constitute less than 1% of all tumours (3). Nevertheless, sarcomas are aggressive, have a high mortality rate and lack specific treatment options. Among the adult soft tissue sarcomas, myxofibrosarcoma (previously named malignant fibrous histiocytoma) is one of the more common entities. It represents more than 5% of all adult sarcomas and mainly affects patients between the ages of 60 and 80. This neoplasm most often develops in the dermis or subcutaneous tissue of the extremities, although it may also be deep-seated and arise in other locations such as the head, neck or trunk (1,2).

Histologically, myxofibrosarcoma has a heterogeneous appearance with gelatinous or firm nodules and myxoid areas rich in hyaluronic acid and collagen. It contains atypical pleomorphic cells, an infiltration of inflammatory cells and variable mitotic activity according to the grade of the tumour. One of the characteristics of myxofibrosarcoma is the presence of thin-walled blood vessels that are elongated and curvilinear. Because of a poor delimitation with an infiltrative margin, the local recurrence rate after surgical resection is around 50-60%. Neoadjuvant or adjuvant therapy such as radiotherapy or chemotherapy can reduce the local recurrence rate to about 25-30% (4,5). Low-grade tumours almost never metastasize, whereas high-grade tumours display high metastatic proclivity (1).

Whereas paediatric sarcomas typically carry only few genetic aberrations, myxofibrosarcomas display a complex pattern of mostly non-recurrent genomic aberrations (6). This makes it challenging to delineate the underlying tumorigenic mechanisms of most importance, and to develop specific treatments. Although adult high-grade sarcomas have received more attention in recent years, myxofibrosarcoma remains largely understudied (about 400 articles

referenced on PubMed since the 1950s). Therefore, further knowledge about the tumour-initiating capacity of primary myxofibrosarcoma tumour cells is needed to identify targets that could be susceptible to novel therapeutic strategies.

1.2 Project background

The tumour cells studied in the current project were isolated from a primary high-grade myxofibrosarcoma that was surgically removed from a patient. The tumour tissue was mechanically and enzymatically dissociated, and its extracellular matrix digested. The tumour cells were then cultured under two different conditions; as an adherent monolayer or as a sphere-forming suspension culture. *Both the adherent and sphere-forming cell cultures were observed to form myxofibrosarcoma-like tumours when injected beneath the renal capsule of immunocompromised mice.* However, a significant difference in the behaviour of these cells was noted. The sphere-forming cells frequently gave rise to metastasis in the mice, whereas the adherent cells were not observed to form metastasis (unpublished data). Sphere-forming and adherent xenograft-derived cultures could be re-established from the corresponding dissociated mouse tumours and when re-injected into new mice, were able to form myxofibrosarcoma-like tumours again (unpublished data).

Whereas the primary adherent cells (hereby referred to as "DMEM") proliferated well *in vitro*, the primary sphere-forming cells where difficult to maintain after the first passage. To alleviate this problem, the latter was instead maintained as an adherent culture (referred to as sphere-adherent cells, "SpA"). The SpA cells were also able to form myxofibrosarcoma-like tumours, as well as metastasis, when injected into mice (unpublished data).

The SpA and DMEM cell populations were analysed using gene expression microarray to compare the gene expression profiles. The gene expression microarray demonstrated differences in the expression levels of numerous genes between DMEM and SpA cells. Some of the genes that were more highly expressed in SpA cells may be implicated not only in myxofibrosarcoma tumorigenesis, but also in development of metastasis, as the SpA cells displayed metastatic capacity *in vivo*. Among the genes over-expressed in SpA cells, a subset was over-expressed also in primary myxofibrosarcoma tumours, as compared to histologically similar tumours (unpublished data). Two of these genes were WHSC1 and CEP55. Therefore,

the current project was aimed at assessing the importance of the corresponding proteins for the survival of the primary myxofibrosarcoma cell populations SpA and DMEM.

1.3 WHSC1 role in cancer

The WHSC1 gene (Wolf-Hirschhorn syndrome candidate 1), also known as NSD2 and MMSET, plays an essential role in early embryonic development. Indeed, this gene is particularly expressed in tissues with a high cellular growth rate, such as the epithelium of the gut (7,8). The deletion of the WHSC1 gene, which is located on chromosome 4p16.3, is associated with the Wolf-Hirschhorn syndrome (8). This gene is also the key molecular target of the translocation t(4;14)(p16.3;q32.3) in multiple myeloma (9).

WHSC1 encodes a methyl transferase, and its overexpression is associated with an increase in histone H3 lysine 36 dimethylation (H3K36me2), an activating histone mark, and a decrease in trimethylated H3K27 (H3K27me3), a repressive mark (10). WHSC1 has previously been shown to be over-expressed in many different types of tumours, including multiple myeloma, prostate, breast, colon, lung and ovarian cancers (11). This gene is involved in many cellular functions, including cellular proliferation via the p53 pathway, apoptosis, DNA repair and cell adhesion, and its overexpression has been associated with cancers bearing poor prognosis (10–13). Accordingly, WHSC1 silencing in cancer cells has been shown to decrease cell proliferation, increase apoptosis and sensitize the cells to treatment with chemotherapeutic agents. The latter is due to a decrease in the expression of genes involved in DNA repair mechanisms, such that a cell that has accumulated DNA damage due to chemotherapeutic agents will go into apoptosis (10,13).

The WHSC1 methyl transferase exists as several isoforms whereof the predominant ones are referred to in the literature as “MMSET-I” and “MMSET-II”. The full-length isoform MMSET-II has an approximate size of 150 kDa and contains the SET domain which affects histone modifications, whereas the MMSET I isoform has an approximate size of 80 kDa and lacks the SET domain. The histone methylation pattern altered by WHSC1 leads to a more open chromatin structure and active gene transcription. Whereas the global influence on histone modifications can account for some of WHSC1 oncogenic functions, the contribution of either WHSC1 isoform to tumorigenesis is not entirely clear. Both isoforms are expressed in cancer cells and seem to play an oncogenic role (11).

1.4 CEP55 role in cancer

The CEP55 gene (centrosomal Protein, 55-KD), also known as MARCH and CT111, plays an important role in the regulation of cell division (15). CEP55 is particularly important for the midbody structure during the terminal phase of cytokinesis (16,17). Given its pivotal role in cell cycle progression, the over-expression of CEP55 has been associated with increased cell division and invasion in various tumour types, such as breast, ovarian, lung, thyroid, colon, prostate, renal and liver cancers (18,19). Moreover, CEP55 is a poor prognostic marker for many cancers, as it promotes metastasis, resistance to treatment and risk of recurrence (19). CEP55 knockdown in cancer cells has been shown to decrease their cell proliferation, invasion and tumorigenicity (20–22).

1.5 Gene silencing by shRNA and CRISPR Cas9 systems

In order to silence the WHSC1 and CEP55 genes, we used two different techniques: gene silencing by short hairpin RNA (shRNA) and CRISPR Cas9 systems. The first technique does not allow a definitive silencing, as it acts only after the RNA transcription step. Thus, it does not permanently modify the DNA sequence. Nevertheless, this technique is interesting when it is not possible to do a complete knockout (in cases where a gene would be too important for the survival of the cell). As we were not sure of the importance of the studied genes, we utilized the shRNA-mediated knockdown technique as our initial approach.

For this technique, we used the Dharmacon RNAi Consortium (TRC) Lentiviral shRNA system (Figure 1).

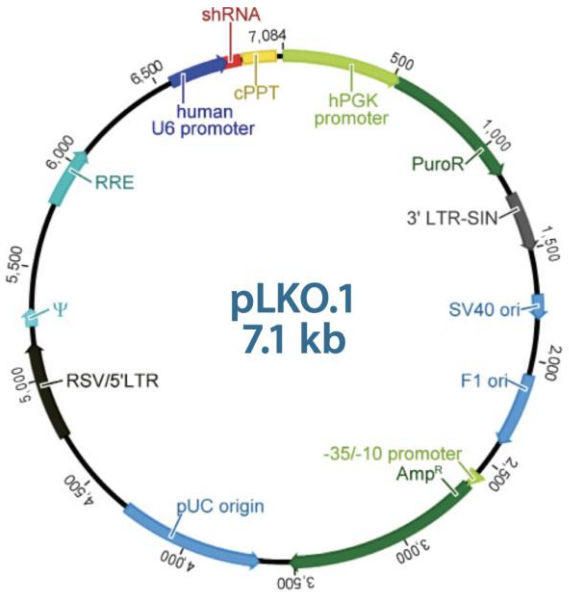


Fig. 1: The lentiviral vector backbone pLKO.1 used for the shRNA-mediated silencing (23)

When the shRNA construct is introduced into the genome of the host cell, it can be transcribed into pre-shRNA and then exported out of the nucleus. Once in the cytosol, this sequence adopts a hairpin-like structure. An enzymatic complex degrades the shRNA loop into a double-stranded sequence named small interfering RNA (siRNA). Then, the 5'-3' strand of this siRNA is degraded and the remaining 3'-5' sequence (named guide strand) is then recognized by the RNA-induced silencing complex (RISC). This enzymatic complex locates the guide strand to the complementary messenger RNA, leading to the degradation of the latter by endogenous nucleases. Thus, the target transcript is not translated, and its expression is silenced (24).

The second technique, the CRISPRCas9 system ensures complete gene silencing, as it acts directly at the genomic level. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeat and is constituted by two elements: a genomic DNA nuclease (Cas9) that cuts both DNA strands and a short RNA molecule that positions Cas9 over the genomic DNA. The end result of the CRISPRCas9 activity is a stable modification of the genome. Gene silencing by means of the CRISPRCas9 system can occur as a consequence of the non-homologous end joining (NHEJ) repair pathway activation, which leads to random insertions or deletions (INDEL) around the site of the DNA damage. If there is a frame shift before the tolerant region of the nonsense-mediated mRNA decay (NMD) pathway, the mRNA will harbour a nonsense mutation and undergo a decay process (NMD). Alternatively, if the frame shift does not occur or it happens in the NMD tolerant region, the silencing can still take place

if the INDELs targeted and modified an essential domain of the proteins. Therefore, a suitable guide RNA (gRNA) design should target both a functional domain and the NMD restrictive region.

2. Aim of the study

The study aimed to evaluate the importance of the WHSC1 and CEP55 genes for the survival of primary myxofibrosarcoma cells. To investigate this, the cell populations DMEM and SpA were lentivirally infected with specific shRNAs targeting WHSC1 or CEP55 and the knockdown efficiency was evaluated by Western blot. Stable cultures of cells bearing WHSC1 or CEP55 depletion were then injected beneath the renal capsule of immunocompromised mice to evaluate their tumorigenic capability. As the injected cells still were able to form tumours, at least partly due to residual protein expression, we decided to achieve complete gene knockout by utilizing the genome editing CRISPR CAS9 system.

3. Material and Methods

3.1 shRNA-mediated knockdown

For the shRNA-mediated gene silencing, constructs were purchased from the Dharmacon TRC Lentiviral shRNA library (GE healthcare) as glycerol stocks. Two or three shRNA constructs for each gene were chosen; for WHSC1: TRCN0000019816 (targeting exon 4) and TRCN0000019818 (targeting exon 6) and for CEP55: TRCN0000061973 (targeting exon 8), TRCN0000061976 (targeting exon 5) and TRCN0000061977 (targeting exon 9). The shRNAs against WHSC1 are both targeting the 5' end of the transcript, thus capturing the main "MMSET-I" (around 80 kDa) and "MMSET-II" (around 150 kDa) isoforms of WHSC1. Glycerol stocks were cultured for plasmid preparation and colonies screened by restriction enzyme digestion according to the manufacturer's instructions. Colonies were then sequence verified by Sanger sequencing. Positive colonies were obtained for WHSC1 shRNA constructs 19816 and 19818, however only from one of the CEP55 shRNA constructs (61977). To obtain larger quantities of DNA, Maxiprep was performed according to the manufacturer's instructions

(JetStar 2.0 Plasmid purification kit) and plasmid DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop technologies).

3.2 HEK 293T, DMEM and SpA cell culture

HEK293T packaging cells and primary adherent DMEM cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin (PS), and 1% non-essential amino acids (NEAA) in T-75 tissue culture flasks (Corning). The primary sphere-forming cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% KnockOut serum replacement (KO), 1% PS, 1:10 000 epidermal growth factor (EGF) and 1:10 000 fibroblast growth factor (FGF) in T-75 ultra-low attachment flasks (Corning). The SpA cells derived from the sphere-forming cells were cultured in IMDM medium supplemented with 10% FBS and 1% PS in 15cm Petri dishes. All cells were cultured at 37°C and 5% CO_2 in a humidified chamber, and frozen in 90% FBS and 10% Dimethyl sulfoxide (DMSO).

3.3 Virus production

HEK293T packaging cells were transfected with the envelope plasmid pMD2G (Addgene), the packaging plasmid pCMVDR8.74 (Addgene) and the respective shRNA encoding plasmid, using the FuGENE 6 Transfection reagent (Promega) according to the manufacturer's instructions. Seventy-two hours after transfection, the supernatant was filtered through a 0.45 μM filter to remove cellular debris, and the virus particles were concentrated by adding the Lenti-X Concentrator (Clonetech Takara Bio) according to the manufacturer's instructions. The mixture was incubated overnight. The virus pellet was then collected and resuspended in 250 μl of cell culture medium.

To evaluate knockdown efficiency, two different shRNA clones for WHSC1 (19816 and 19818), and one for CEP55 (61977), were first evaluated in HEK293T cells. The shRNAs showing the highest degree of knockdown, here shWHSC1 19818 and shCEP55 61977, were then used to infect the target cells; the primary myxofibrosarcoma DMEM and SpA cells. A non-targeting shRNA (shGFP) was used as a control. For the infection of HEK293T and primary cells, 1/1000 of Polybrene (Sigma Aldrich) was added to the culture medium 30 min before virus infection.

200 µl (for a T-75 flask) or 250 µl (for a 15cm Petri dish) virus suspension (in the LentiX concentrator) was added to each culture flask/dish. Twelve hours after virus infection, the cell medium was changed, and 48h-72h after infection, cells were selected with Puromycin. For the shRNA test infection, HEK293T cells were selected with 1 µg/ml Puromycin for 24h and cell pellets were collected after 72h and snap frozen for protein lysate preparation. For the primary DMEM and SpA test infections, cells were selected with 1 µg/ml Puromycin and collected at 48h, 72h and 96h after infection. The primary cells had previously been evaluated for their respective sensitivity to selection antibiotics. Samples were snap frozen for preparation of protein lysates. For the generation of primary DMEM and SpA cultures with stable knockdown of WHSC1 and CEP55, cells were infected as described above, selected with 0.5 – 1 µg/ml Puromycin for three days and then maintained with 0.2 µg/ml Puromycin. Samples were snap frozen for preparation of protein lysates after around 2 weeks, and then the cells were prepared for mice injection after another 2 weeks (the cells had been in culture around 1 month at the time of mice injection). The cells were kept in culture for around 1 week after the mice injection and new samples were snap frozen for protein lysate preparation.

3.4 Western blot

The efficiency of shRNA-mediated knockdown of WHSC1 and CEP55 in HEK393T cells, as well as primary myxofibrosarcoma cells, was evaluated by western blot. For each condition, one million cells were lysed in nuclear lysis RIPA buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing Protease and Phosphatase inhibitors (Roche, Sigma). After 30 minutes of incubation on ice, the samples were sonicated 3 cycles of 10 seconds at 30% power. Total protein content was quantified using the Protein assay dye reagent (Bio-rad) at 595 nM absorbance.

20-40 µg of protein was separated by electrophoresis through a 12% polyacrylamide gel and transferred onto an Amersham Protran 0.2 µm nitrocellulose blotting membrane (GE healthcare) at 250 mA for 90 minutes, at 4°C. The membrane was blocked in 1x Tris-buffered saline (TBS) with 0.05% Tween-20 (TBS-T) and 5% milk for 1 hour, and then incubated with the respective primary antibody diluted in TBS-T with 5% milk at 4°C overnight. Membranes were then washed 3 times 10 min in TBS-T and hybridized with sheep anti-mouse horseradish

peroxidase-conjugated secondary antibodies (GE healthcare) diluted 1:25 000 in TBS-T with 5% milk for 1h at room temperature. Protein signals were visualized using the WesternBright Sirius (Advansta) or SuperSignal West Femto (Thermo scientific) detection kits. The primary antibodies used were: mouse anti-WHSC1 antibody ab75359 (Abcam) at 5 µg/ml concentration, mouse anti-CEP55 antibody B01P (Abnova) at 1:500 dilution and mouse anti-ACTB antibody A5316 (Sigma) at 1:5000 dilution as loading control. The anti-WHSC1 antibody is raised against amino acids 1-647 of WHSC1, thus potentially recognizing both "MMSET-I" and "MMSET-II" isoforms of around 80 and 150 kDa, respectively. The Pageruler Prestained Protein Ladder (Thermo Fischer) or the SeeBlue Plus2 Prestained Standard (Invitrogen) were used as protein size markers. The expected protein sizes are around 80 or 150 kDa for WHSC1, 55 kDa for CEP55 and 42 kDa for Actin Beta (ACTB).

3.5 Injection of shRNA-treated primary cells into mice

Experimental protocols involving mice were approved by the Veterinary Service of the Canton of Vaud under authorization number VD2488.1. The mice used for cell injections were 5-8-week-old NOD-SCID-common-γ-KO (NSG) female mice. The cells were injected using a Hamilton syringe in 20 µl volume of culture medium beneath the renal capsule (left kidney). Mice were sacrificed when tumours reached 1 cm^3 or when the animals showed signs of pain or stress. The mice were monitored for tumour growth for 6 months.

Primary DMEM and SpA cultures with stable knockdown of WHSC1 and CEP55 were used for mice injection. The cells were kept in culture for around one month before the mice injection and the level of knockdown was evaluated by western blot two weeks before the mouse injection. As the SpA cells were strongly affected by both knockdowns, 500 000 cells of the SpA shCEP55/shWHSC1 cultures were injected. Of the other conditions (SpA shGFP and DMEM shGFP/shCEP55/shWHSC1), both 100 000 and 500 000 cells were injected. Four or five mice were used/condition.

3.6 CRISPR Cas9-mediated knockout

For the design of guide RNAs (gRNAs) we carefully assessed the intron/exon structures, and the location of the putative functional domains, of WHSC1 and CEP55. We also designed the gRNA as to target the exons common to all known isoforms of the proteins (Table 1 and 2). As

a result of this analysis, we decided to design gRNAs targeting exons 5 and 19 of WHSC1 and exons 7 and 8 of CEP55 (Fig. 2 and 3).

Table 1: Known isoforms of the WHSC1 gene, according to the NCBI gene database (7)

Histone-lysine N-methyltransferase (NSD2) isoforms	Accession number	Description
Isoform 1 (MMSET-II)	**NM_001042424.2**	*Has a novel 5' non-coding exon, which results in a different 5' UTR*
Isoform 4 (MMSET-I)	**NM_007331.1**	*Lacks exons 12 to 25 and has an additional fragment beyond the 3' end of exon 11. This results in a shift of the reading frame*
Isoform 1	**NM_133330.2**	*Contains only 24 exons, as it lacks exon 12*
Isoform 1	**NM_133331.2**	*Lacks exon 2 in the 5'UTR (but encodes the same isoform as variant 1 and 3)*
Isoform 3	**NM_133334.2**	*Encodes a short isoform with a different C-terminus, as it lacks most of the exons from 3'. Has also a different 3' UTR*
Isoform 1	**NM_133335.3**	*Misses exon 2 and 3 in the 5' UTR and encodes the same isoform as variants 1 and 2*

Table 2: Known isoforms of the CEP55 gene, according to the NCBI gene database (15) Note that the isoforms affect only the exon 1.

Centrosomal protein of 55 kDa (CEP55) isoforms	Accession number	Description
Isoform 1	**NM_001127182.1**	*Is the result of the use of an alternate splice donor site for exon 1*
Isoform 2	**NM_018131.4**	*Is the result of the use of an alternate splice donor site for exon 1*

For WHSC1, the gRNAs targeted the important functional domains of the protein, i.e. the HMG box and the SET domain. The chosen CRISPRCas9 sequences were located on exon 5 and exon 19. These locations provided a good efficiency of silencing, because of the low rate of off-targets sequences and the low rate of self-complementarity sequences (Fig. 2).

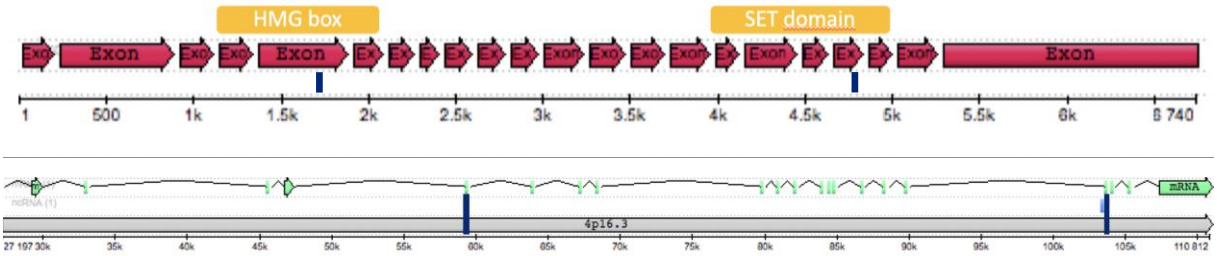


Fig. 2: WHSC1 gene. The known important functional domains (represented by orange boxes on this figure) are the HMG box (a DNA binding domain) encoded by exons 4 to 6 and the SET domain (a methyl transferase) encoded by exons 16 to 20. The chosen CRISPR sequences on exon 5 and exon 19 are shown by dark blue dashes.

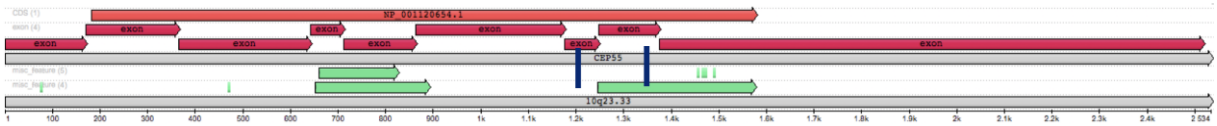


Fig. 3: CEP55 gene. Domains that are important for the formation of the midbody ring are encoded by exons 8 and 9 (mapped by green boxes). Without these domains, the resulting protein wouldn't be functional. As isoforms affect only the exon 1, we have chosen CRISPR sequences on exon 7 and exon 8 for their good efficiency (showed by dark blue dashes).

For CEP55, one of the chosen CRISPRCas9 sequence, which is on the exon 7, is located outside of the functional exons (Fig. 3). Indeed, if a premature termination codon is located on the last exon, or in the 50-55 nucleotides before the last exon-exon junction, the mechanism based on the nonsense-mediated mRNA decay (NMD) can't take place *(25)*. In order to take advantage of the NMD pathway, a CRISPR sequence located on exon 7, which is just before the "tolerant region" has been chosen (Fig. 4).

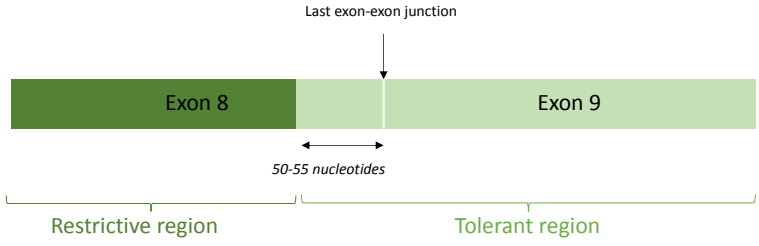


Fig. 4: CEP55 tolerant region (includes last exon and the 50-55 nucleotides before the last exon-exon junction), where the nonsense-mediated mRNA can't take place.

For the cloning of gRNAs into the lentiviral vector LentiCRISPRv2 backbone, two oligonucleotides (reverse and forward) for each exon were designed with the CHOPCHOP web service (26) using the standard parameters, with exception of GN or NG as 5' requirements for sgRNA, (Table 3).

Table 3: Oligos Design.

	1 20 PAM
WHSC1 **Exon 5**	**5'-**GAAGAGTACTCCTCAAAAGA**CGG-3'**
Forward primer	CACCG**GAAGAGTACTCCTCAAAAGA**
Reverse primer	C**CTTCTCATGAGGAGTTTTCT**CAAA
WHSC1 **Exon 19**	**5'-**GAATGTCACAGACGGCAAAC**AGG-3'**
Forward primer	CACCG**GAATGTCACAGACGGCAAAC**
Reverse primer	C**CTTACAGTGTCTGCCGTTTG**CAAA
CEP55 **Exon 7**	**5'-**GCAGCAAGAAGAACAAACAA**GGG-3'**
Forward primer	CACCG**GCAGCAAGAAGAACAAACAA**
Reverse primer	C**CGTCGTTCTTCTTGTTTGTT**CAAA
CEP55 **Exon 8**	**5'-**GTTGGAATCCTTGGTGAGTC**TGG-3'**
Forward primer	CACCG**GTTGGAATCCTTGGTGAGTC**
Reverse primer	C**CAACCTTAGGAACCACTCAG**CAAA

The oligos were cloned according to the protocol of the Lentiviral CRISPR Toolbox GeCKO by Zhang Lab (27). Oligos with 5' phosphorylation were ordered from Microsynth and annealed to a *Bsm*BI (Thermo Scientific) digested LentiCRISPRv2 vector (Fig. 5) and sequence verified by Sanger sequencing.

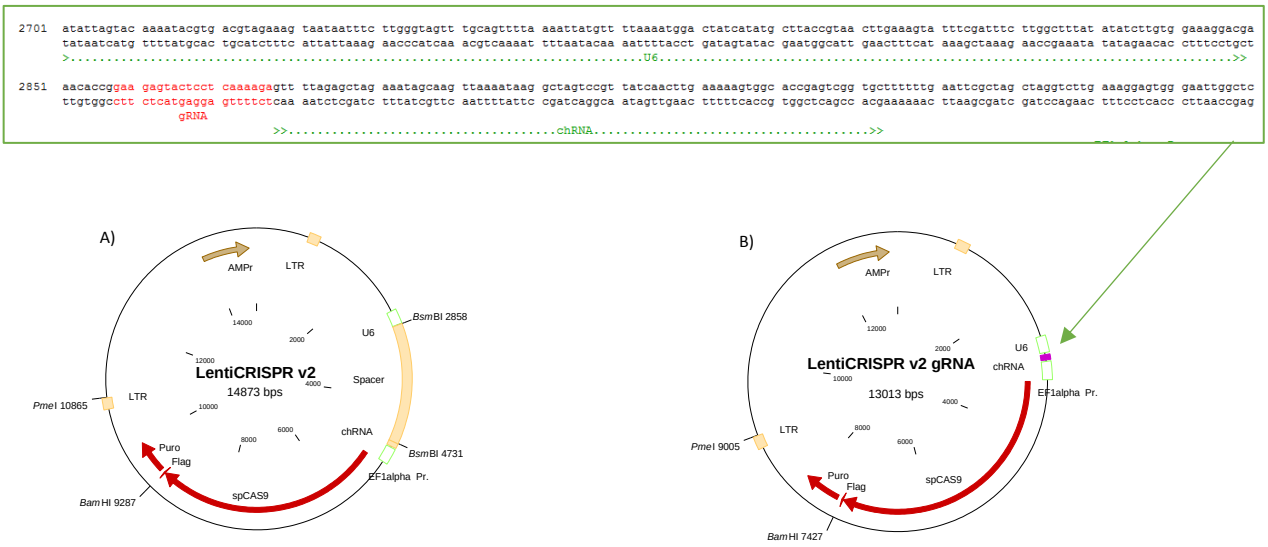


Fig. 5: (A) The LentiCRISPRv2 backbone. (B) The site (violet) of the vector backbone where the annealed oligos are inserted. The sequences in red shows the oligo that corresponds to the gRNA.

4. Results

4.1 shRNA-mediated knockdown of WHSC1 and CEP55

The knockdown efficiency of the shRNA constructs was evaluated in HEK293T cells. As shown by Western blot, the shWHSC1 construct 19818 was more efficient than the construct 19816 in providing a good knockdown. The shCEP55 construct 1977 also provided a good knockdown (Fig. 6A). The constructs 19818 (shWHSC1) and 1977 (shCEP55) were subsequently used to infect the primary myxofibrosarcoma cells DMEM and SpA. For the primary cell test infection, cells were collected at different time points (48h, 72h, 96h after infection) and it was evident that a high degree of depletion was achieved also in these cells especially at the later time points 72h and 96h (Fig. 6B). WHSC1 is expressed as several isoforms, of which MMSET-I (around 80 kDa) seems to be the predominant isoform in these cells. Whereas the HEK293T cells tolerated the knockdown of these proteins well, the primary myxofibrosarcoma cells were visibly affected. Many cells died, and the remaining living cells were proliferating much more slowly than the shGFP infected control cells (data not shown).

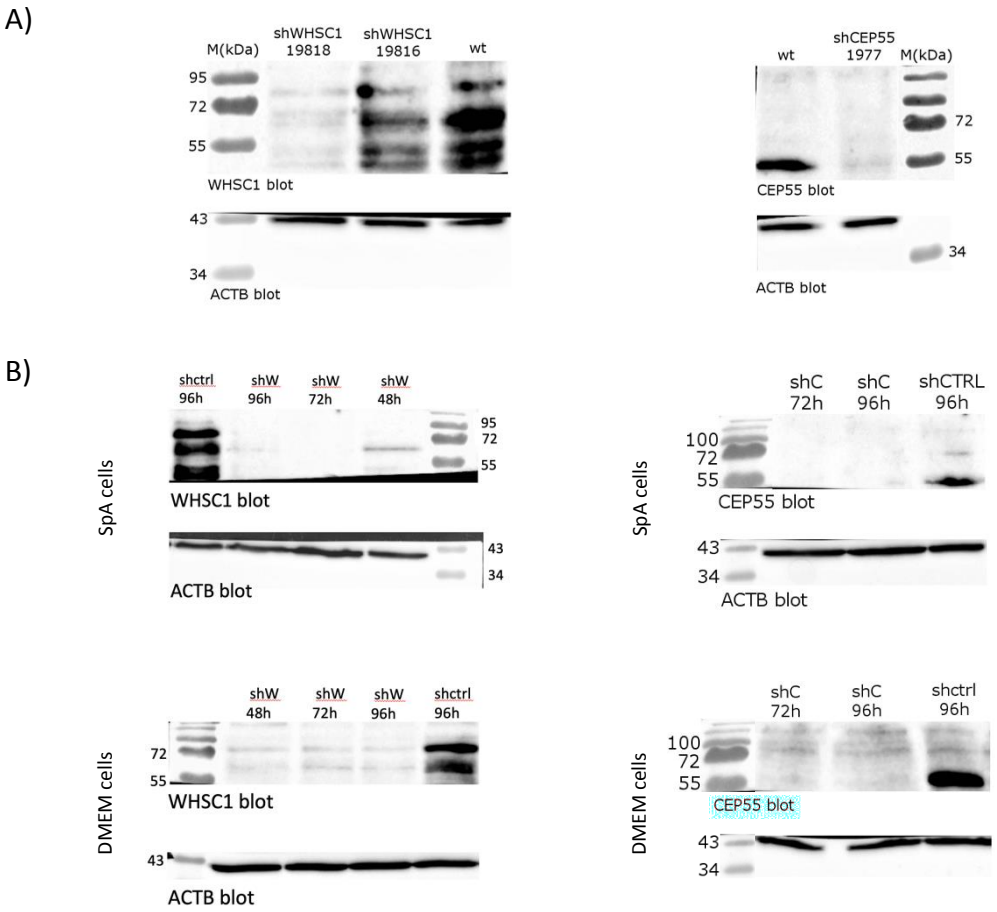


Fig. 6: Western blot analysis of cells infected with shRNAs targeting either WHSC1 or CEP55. A) HEK293T cells infected with two different shRNA constructs (19818 and 19816) against WHSC1 (left), and one construct (1977) against CEP55 (right). Cells were collected 72h after virus infection and protein expression was almost completely absent with constructs 19818 (shWHSC1) and 1977 (shCEP55). Protein lysate from wild-type (wt) cells was loaded for comparison. B) Primary myxofibrosarcoma cells SpA (top) and DMEM (bottom) infected with shWHSC1 19818 (left) and shCEP55 1977 (right), versus control non-targeting shRNA (shGFP), collected at different time points (48h, 72h, 96h) after virus infection. The protein expression was absent in these cells from 72h after infection. WHSC1 is expressed as several isoforms, whereof the ~80 kDa signal corresponding to the MMSET-I isoform seems to be the predominant isoform in these cells. ACTB was used as a loading control.

Primary cell cultures with stable knockdown of WHSC1 or CEP55 were then generated. The protein expression was evaluated by Western blot before the stable cultures were injected into mice. For both SpA and DMEM cells, the maintained knockdown of both genes was evident (Fig. 7). As mentioned above, the proliferation of the primary cells was slowed down in the shCEP55/shWHSC1 cells as compared to shGFP cells. Moreover, the SpA cells seemed to be more affected than the DMEM cells, as it was difficult to propagate the SpA

shCEP55/shWHSC1 stable cultures. We also had the impression that the morphologies of the SpA shCEP55/shWHSC1 cells were more elongated and spindle shaped as compared to shGFP control cells, whereas DMEM shCEP55/shWHSC1 cell morphologies were not noticeably different from the DMEM shGFP control cells (Fig. 8).

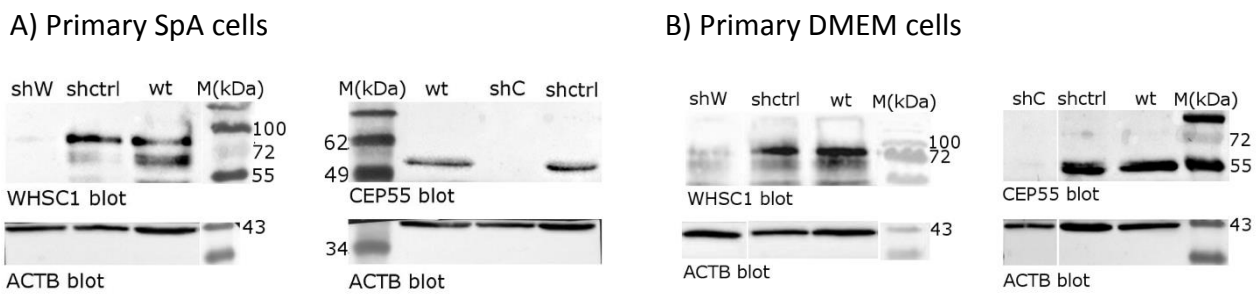


Fig. 7: Western blot analysis of primary myxofibrosarcoma cells SpA (A) and DMEM (B) with stable knockdown of WHSC1 and CEP55, respectively. The cell cultures showed a maintained knockdown of both genes. ACTB was used as a loading control.

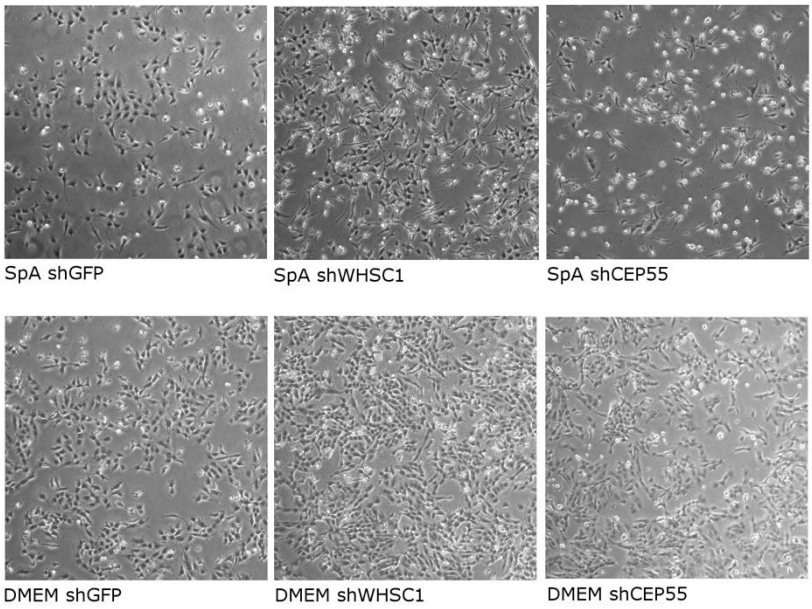


Fig. 8: Cell culture images (40x) of primary myxofibrosarcoma cells SpA (top) and DMEM (bottom) with stable knockdown of CEP55 and WHSC1, respectively, versus shGFP control cells. The shWHSC1/shCEP55 SpA cells have a more spindle shaped morphology as compare to shGFP cells, whereas the shWHSC1/shCEP55 DMEM cells did not display a clear difference in morphology as compared to shGFP cells.

4.2 Mouse injection of shRNA-treated primary myxofibrosarcoma cells

DMEM and SpA cell cultures with stable knockdown of WHSC1 and CEP55, respectively, were injected into the renal capsule of mice. Although these genes were initially efficiently knocked down in these cells (Fig. 7), the tumour-forming capacity was retained. The tumorigenic potential of the SpA cells was not affected by shWHSC1 and shCEP55 treatment although fewer mice developed metastasis, as compared to control wt and shGFP SpA cells that developed metastasis in all mice injected. For the DMEM cells the tumour formation was overall decreased in terms of number of mice developing tumours, and the DMEM shWHSC1 cells showed the least tumour forming capacity (Table 4).

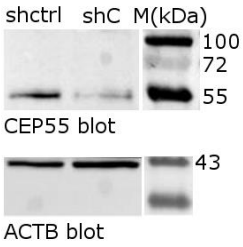
Table 4: Summary of the results obtained from injecting shRNA-treated primary myxofibrosarcoma SpA and DMEM cells into the renal capsule of mice.

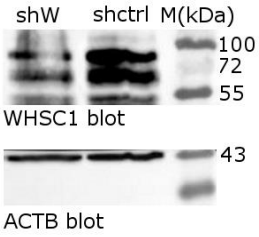
Condition (no. of mice)	No. of cells	Tumors	Time (months)	Metastasis	Lost mice[1]
SpA wt (3)	100 000	3/3	2,5	3/3	
SpA shGFP (3)	100 000	2/3	3	2/3	1/3
SpA shGFP (2)	500 000	2/2	2-4	2/2	
SpA shWHSC1 (5)	500 000	5/5	2,5-4	3/5	
SpA shCEP55 (5)	500 000	4/5	3	3/5	
DMEM wt (3)	100 000	3/3	2-2,5	0/3	
DMEM shGFP (3)	100 000	2/3	2,5-4	0/3	1/3
DMEM shGFP (2)	500 000	1/2	4	0/2	1/2
DMEM shWHSC1 (2)	100 000	0/2	NA	NA	
DMEM shWHSC1 (2)	500 000	1/2	3,5	0/2	1/2
DMEM shCEP55 (2)	100 000	1/2	4	0/2	
DMEM shCEP55 (2)	500 000	2/2	3	0/2	

[1]*Mice lost due to sickness of unrelated reasons*

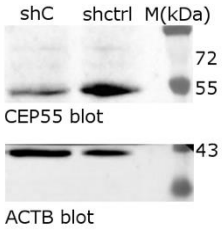
Additional cell pellets were collected from the cell cultures around 1 week after the mice were injected and when these were re-evaluated for protein expression by western blot, it was observed that all the shCEP55/shWHSC1 cell cultures indeed contained some residual protein expression (Fig. 9).

A) Primary SpA cells





B) Primary DMEM cells



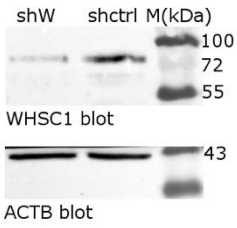


Fig. 9: Western blot analysis of primary myxofibrosarcoma cells SpA (A) and DMEM (B) with knockdown of WHSC1 and CEP55, respectively, around one week after mice injection. The cell cultures showed some residual protein expression of both genes. ACTB was used as a loading control.

4.3 CRISPR Cas9-mediated knockout

As the residual protein expression in the stable shRNA-mediated cell cultures is a plausible explanation for retained tumorigenicity of the target cells, we sought to achieve complete knockout by utilizing the CRISPRCas9 system. This system should reduce the possibility of revertants, as the genomic modification once established becomes permanent. Only the cells that escape the DNA damage or that manage a successful DNA repair can potentially take over the cell population. In order to minimize the risk of these latter events, we cloned two separate gRNAs targeting known functional domains of the proteins. Up to this date, these LentiCRISPRv2 constructs targeting WHSC1 and CEP55, respectively, have not yet been evaluated in the target cells.

5. Discussion

In the current study, we addressed the importance of the WHSC1 and CEP55 proteins for myxofibrosarcoma primary tumour cells via an shRNA-mediated knockdown approach. The knockdown was efficient in both HEK293T cells and in the primary DMEM and SpA tumour cells, 96h after viral infection protein expression was not detectable by Western blot.

It was observed that the primary myxofibrosarcoma cells were affected by shCEP55 and shWHSC1 treatment. If the WHSC1 and CEP55 proteins are essential for myxofibrosarcoma tumorigenesis, silencing either of these two genes should decrease the tumour-forming capacity of the tumour cells. To evaluate this, we injected primary DMEM and SpA cell cultures with stable shWHSC1 or shCEP55 knockdown beneath the renal capsule of mice. Although

tumour formation *in vivo* was decreased to some extent in shRNA-treated cells, as compared to wt and shGFP control cells, the tumour-forming ability was not abolished.

However, we did observe that shCEP55/shWHSC1 SpA cells gave rise to metastasis in fewer mice than the shGFP control cells. Moreover, the shWHSC1 DMEM cells had a reduced tumour-forming ability, as compared to shGFP control cells. But as a small number of mice were used/condition and since the behaviour of the shRNA-treated tumour cells was variable, it is difficult to draw any conclusions about the effect CEP55 or WHSC1 knockdown on the tumorigenic potential of the tumour cells. It could be of value to inject at least the DMEM shWHSC1 cells in a larger number of mice to adequately evaluate differences in number of tumour developed and/or time required for tumour formation, as compared to shGFP control cells.

There are several explanations for the retained tumour-forming ability of the shRNA-treated cells:

1) It is possible that the shRNA-mediated knockdown was incomplete. This technique, unlike the CRISPRCas9 system, does not permanently affect the genome. It produces shRNAs that will lead to mRNA degradation, *i.e.* once they have been transcribed from the genome. Theoretically, it is therefore possible that some mRNAs escape recognition by the RISC complex and are translated into proteins. Indeed, when re-evaluating the CEP55 and WHSC1 protein expression in the stable shCEP55 and shWHSC1 cell cultures, it was observed that the cells had retained some protein expression. Thus, our stable shCEP55/shWHSC1 cell cultures were most likely composed of heterogeneous cell populations containing cells with different degrees of gene knockdown. If so, the cells with some amount of protein expression would proliferate faster and be selected for over time. One way of circumventing this problem could be to establish cultures from single-cell derived colonies after shRNA infection and to assay them individually for their degree of gene knockdown. It would also be interesting to evaluate xenograft-derived cultures for their expression of CEP55 and WHSC1.
2) Another possibility is that even if the gene silencing may have worked well *in vitro*, the tumour cells may have activated other oncogenic pathways enabling the cells to bypass the effect of the silencing and retain tumorigenic capacity *in vivo*. Even if those

cells may be very few in culture, injecting them into mice may select them if they display a better capacity to survive and proliferate, allowing them to become the predominant cell population *in vivo*. To investigate this hypothesis, it would be interesting to further study the tumour cells derived from the xenografts. For example, it would be necessary to confirm that the global genomic aberrations of the xenograft-derived tumour cells are the same as the original cell populations. As tumour cells are often genomically unstable, additional aberrations may have been acquired during cell culture and/or tumour development *in vivo*.

3) The primary myxofibrosarcoma cells were clearly affected by the WHSC1 and CEP55 knockdown, suggesting that these proteins indeed are important for these tumour cells. However, as myxofibrosarcoma tumours display numerous genomic aberrations and many genes were differentially expressed between the DMEM and SpA cells, there may be many other genes that are potentially important for tumorigenicity and metastasis.

As future avenues of research, it would be interesting to silence CEP55 and WHSC1 in the myxofibrosarcoma cells by means of CRISPRCas9 and evaluate the effects on cell survival and proliferation *in vitro* and tumour-forming ability *in vivo*. If this hinders tumour growth, we would have evidence that these genes are particularly important for the tumorigenesis of myxofibrosarcoma. However, it is possible that this technique will not work. If these proteins are truly important for the survival of these tumour cells, they might not survive a complete knockout.

5.1 Implications of this work

Studies aimed at understanding the importance of specific genes for tumorigenesis are very important to understand which oncogenic pathways should be targeted therapeutically. In the future, it may be possible to identify important genes for each tumour entity in order to develop targeted treatments. These treatments could involve finding small, chemical molecules that would target particular proteins of importance. Another approach could be to direct the patient's immune cells to target specific antigens expressed on the surface of the tumour cells to slow or even stop the tumour growth. Today, these techniques are already

used in several types of cancers, with advantages and disadvantages. Tumours carrying many mutations or displaying a high level of genomic instability are particularly difficult to target, because they can utilize other oncogenic pathways to circumvent the targeted pathway or acquire additional mutations and thus become resistant to therapy. Immunotherapies are interesting because they are dynamic and could theoretically be adapted to target any antigen on the cell surface. However, the targeted antigens would need to be very specifically expressed by the tumour cells, in order not to eliminate any normal cells. Unfortunately, these therapies have many side effects nowadays. Indeed, they are not specific to tumour cells and sometimes affect normal cells, leading to auto-immune diseases. We should therefore encourage research for new, ever more effective and safe treatments.

6. Acknowledgements

I would like to thank the Professor Ivan Stamenkovic for giving me the opportunity to do this master thesis in his laboratory; Emely Möller for all her huge help, her attentive relecture and her patience; Carlo Fusco, Luisa Cironi, Patricia Martin and all the other members of the lab for their help and their kindness; my family and my friends for their support.

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