Chemokines in Ewing sarcoma

Sand, L.G.L.

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Author: Sand, Laurens
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Chapter 8

Summary and future perspectives
Nederlandse samenvatting
Curriculum Vitae
List of publications

SUMMARY AND FUTURE PROSPECTIVE

Ewing sarcoma (EWS) is an aggressive primary malignant bone tumor with high degree of tumor vascularization, to the exert that it was originally supposed to be of vascular origin [1]. High vascularization is often a result of an intensive interaction between the tumor and its microenvironment. For the understanding of its interactions within the microenvironment detailed characterization of EWS at the molecular level is needed. This thesis focusses on chemokine signaling. In addition, characterization of EWS at the genome, epigenome and transcriptome levels may potentially help to identify new druggable targets and prognostic markers. In **Chapter 2** results of so far published large sequencing studies were combined to provide a comprehensive overview that can facilitate the use of large datasets generated from EWS. At genome level, besides the characteristic *EWSR1-ETS* translocation, no frequently recurring structural rearrangements were identified to be present in EWS. The number of gene mutations detected in EWS was very low and only two gene mutations, *TP53* and *STAG2*, were frequently observed in multiple studies with frequencies of 5.2-7% and 9-21.5%, respectively [2-4]. The distribution of these mutations across the gene sequence showed an interesting pattern. The most frequent *TP53* mutation in EWS -c.527G>T (p. C176F)- was not listed as hot spot mutation in the *TP53* mutation database of the IARC, which collects mutations from many types of cancer [5]. It may be speculated that these *TP53* mutations are appearing due to or are related to the EWS-ETS fusion protein [6]. One clear hot spot mutation c.646C>T (p.R216X) was identified in *STAG2*, which was present in 25% of the analyzed cases. These mutations together with structural alterations including 1q gain, are clinically relevant as they can be used as prognostic markers for survival [2,7-9]. In addition, *CDKN2A/CDKN2B* locus deletion, observed in 12% of the analyzed cases was not associated with survival which is conflicting with the results of earlier studies [2,3,7]. The overall low number of mutations in EWS suggests that the cell death pathways, in contrast to most other tumors, are not genetically but rather functionally defective. Intracellular or extracellular reactivation of these pathways by the DNA damage recognition system or immune cells, respectively, might be feasible, creating additional opportunities for chemo- and immunotherapy (**Chapter 2**) [10].

The impact of the EWSR1-ETS fusion protein on cellular processes was observed both at epigenome and transcriptome level. EWS specific epigenetic patterns were observed at distant enhancer and super-enhancers sites [11]. In addition, EWSR1-ETS binds at specific GGAA satellites and requires interactions with various epigenetic modifiers to be able to bind and act as oncogenic driver [12]. These epigenetic modifiers would be therefore be ideal therapeutic targets for EWSR1-ETS specific treatment [13]. The blocking of the interaction sites of EWSR1-ETS or of the epigenetic modifiers which are involved epigenetic regulation are both plausible therapies that have been investigated; YK-4-279 as blocking agent for EWSR1-ETS, and HCI2509 as reversible blocking agent for lysine-specific histone demethylase 1 (LSD1) [13,14].

At transcriptome level -besides a specific Ewing sarcoma gene expression pattern- a specific non-coding RNA expression pattern and alternative splicing events were identified, all influenced by the EWSR1-ETS fusion protein. The fusion protein up- and downregulates several cellular processes. For the studies presented in this thesis, downregulation of extracellular signaling, including chemokines and chemokine receptors signaling was highly relevant and investigated in more detail.

The downregulation of specific chemokines and their receptors might be used as a treatment option for EWS. In **Chapter 3** the lack of chemokine CCL21 and CCR7 expression in

EWS cells makes this tumor a prime candidate for CCL21 activation-based immunotherapy. The CCL21-CCR7 axis is often activated by the immune system that improves the anti-tumor immune response. However, it has been reported that when tumor cells express CCL21 and/or its receptor CCR7, a pro-tumorigenic response can occur upon CCL21 immunotherapy with an adverse effect. *CCL21*, in addition, might be used as a prognostic marker in EWS since increased RNA expression of *CCL21* was associated with decreased event free survival and overall survival in therapy-naïve primary EWS samples and inversely correlated with $CD4^+/CD8^+$ T-cell ratio (**Chapter 3**).

Although most chemokines and chemokine receptors are downregulated in EWS, upregulation of CXCR4 has been described in EWS [15]. CXCR4 is involved in all the major processes of the tumor microenvironment: angiogenesis, tumor growth, metastasis and immune surveillance [16]. In EWS its expression has been examined at RNA and protein level with conflicting results [17,18]. At the RNA level high CXCR4 expression was associated with metastasis, but at the protein level no expression in metastasis was observed. By studying the *CXCR4-CXCR7* axis in two patient cohorts we investigated the role of CXCR4 in EWS in broader perspective and demonstrated a role for the chemokine factors that inhibit CXCR4 activation, namely chemokine receptor CXCR7 and chemokine CXCL14 (**Chapter 4**). Increased expression of these factors showed a negative correlation/association with the development of metastases and with improved overall survival in one cohort (**Chapter 4**). These data confirmed the importance of CXCR4 activity in EWS and in its microenvironment.

CXCR4 expression and activity are regulated at various levels, including alternative splicing, post-translational modifications, dimerization and cellular localization. Previously, two *CXCR4* splice variants had been reported (*CXCR4-1* and *CXCR4-2*) but their role in the tumor microenvironment was undetermined. The balance between splice variants is known to be important in protein function [19,20]. In **Chapter 4** we demonstrated that the *CXCR4-1*/*CXCR4-2* ratio is increased in tumor samples compared to cell lines and this increased ratio was associated with an improved patient survival. CXCR4 can form dimers or even oligomers. We propose that a heterodimer formed by CXCR4-2 and CXCR4-1 isoforms might influence activation of CXCR4-2 [21]. Besides these two known splice variants, we have identified two novel splice variants (annotated as *CXCR4-3*, *CXCR4-4*) in EWS cell lines by using whole transcriptome sequencing (**Chapter 5**). Interestingly, all identified CXCR4 splice variants showed variations only at the N-terminal end of CXCR4 implying an important role for this N-terminal region in signaling. For the CXCR4-2 variant, this role has been confirmed by modeling experiments [22].The expression of these, novel splice variants were detected in both other tumor and normal tissue samples, indicating that their expression is not EWS specific but they are regular splice variants. Further validation at protein and functional level revealed that ectopic expression resulted in a dominantly unstable intracellular protein. However, the CXCR4-3 isoform in the T7 transformed human embryonic kidney cell line HEK293T demonstrated some cell membrane localization and activity upon CXCL12a stimulation.

CXCR4 receptor activation by CXCL12 ligand binding is dependent on the subcellular localization of the receptor and its highly regulated cellular trafficking. Only cell membrane located CXCR4 receptor is accessible for ligand binding and the cell membrane expression levels have been associated with a degree of metastasis [23]. As in EWS conflicting CXCR4 protein and RNA expression levels are reported, a peptide-based staining method was applied and compared with antibody-based staining method (**Chapter 6**). The peptide-based method could qualitatively and quantitatively detect the CXCR4 cell membrane expression and was linearly correlated to the *CXCR4* RNA expression levels where the antibody-based method de-

tected the same cell membrane expression levels in EWS cell lines with varying RNA expression levels. In addition, the CXCR4 receptor internalized upon binding of the used peptide Ac-TZ14011-Cy5. Upon CXCL12 binding the intracellular domains of the transmembrane receptor are modified by ubiquitination and phosphorylation leading to CXCR4 internalization [24]. Receptor internalization and intracellular trafficking is a complex process achieved via multiple pathways and has been shown to be a cell/tissue specific process [25,26]. Since binding of the previous used peptide Ac-TZ14011-Cy5 to CXCR4 resulted internalization of the receptor-peptide complex, this peptide was used as chemical backbone for the synthesis of an activatable CXCR4 endocytosis tracer to study the intracellular trafficking. The tracer contained, besides the Ac-TZ14011 peptide, a double fluorescence labeling that is appropriate for Förster resonance energy transfer (FRET) analysis. The disulfide bond between the two fluorophores enabled to measure the endocytosis process as function of the reducing environment (**Chapter 7**). *In vivo* validation of the tracer revealed that the peptide internalized with the receptor and could be used to measure the internalized CXCR4 qualitatively and quantitatively. The development of such novel tracers will allow to further study the role and regulation of CXCR4 in different tumor types.

In conclusion, this thesis illustrates the importance of chemokines in EWS tumor cells and its tumor microenvironment. *CCL21 CXCL14*, *CXCR7* and the ratio between *CXCR4-1* and *CXCR4-2* have been identified as candidate prognostic markers, CCL21 immunotherapy as potential therapy and CXCR4 as potential therapeutic target in EWS. In addition, the presented peptide-based life cell imaging methods improve the ability to study CXCR4 cell membrane expression and dynamics qualitatively and quantitatively. This approach might be helpful for the measurement of anti-CXCR4 therapy efficacy. This work identified specific the chemokine signaling pathways that can be used to target Ewing sarcoma and its tumor microenvironment.

The present EWS treatment regimen with surgery and intense chemotherapy has led to a survival of around 70% when EWS is localized but worse for patients with a metastatic disease at diagnosis or relapse. In addition, the regimen results in severe long-term effects on the health of these patients [27]. To stratify patients in the clinic only classic prognostic markers are used. The inclusion of targeted - and immunotherapy in the EWS treatment regime, such as YK-4-279 and CCL21 dendritic cell therapy, and inclusion of genetics and tumor-microenvironment related markers, such as TP53 and CXCL14 could lead to an increase the overall survival. . Especially the combined analysis of these different kinds of markers could further help to improve the patient stratification and survival prediction. To measure the expression and spatial-temporal activity of these markers new methods are needed. Examples of these methods are described in this thesis. Further development for successful application of these techniques will require a multi-disciplinary approach involving multiple fields of expertise such as radiology, (molecular) pathology and molecular biology.

EWS is a EWSR1-ETS driven tumor with low number of additional mutations and in which almost all pro-inflammatory chemokines are downregulated. Due to these characteristics, activation-immunotherapy may have great potential as additional treatment for EWS and could have a long lasting effect. Earlier lymphocyte recovery, high number of infiltrating CD8+ T-cells and high expression of CCL21 are all associated favorable overall survival in EWS patients (Chapter 3)[28,29]. The ideal target would be the EWSR1-ETS protein. However, its native peptides are poor immunogenic, MHC class I cell membrane expression is lacking in advanced-stage EWS and reactive cytotoxic T-cells which are present in EWS

tumors are exhausted [30-32]. For the optimal response a active immune microenvironment in combination with a high MHC class I expression and immunogenic EWSR1-ETS peptides to prime the T-cells would be required.

The tumor microenvironment is of key importance for understanding the behavior of the tumor, predicting its reaction on therapeutics and for predicting patients' survival. This thesis has shown that not only the targeted receptor but the complete interaction network should be considered. Although, next generation sequencing and proteomics have identified several new DNA, RNA and protein variants, like the novel CXCR4 RNA splice variants presented in this thesis, tools to study their biological relevance in the complex tumor microenvironment are lacking. Ex vivo model systems that allow recapitulation of the tumor microenvironmental conditions in patients might improve our knowledge of the interaction network between the tumor and its microenvironment. Next generation sequencing and proteomics could be used to monitor changes in these ex vivo models if certain conditions were changed, for example altering pH, metabolic products or bringing in additional cell types or (novel) therapeutics. At last, these ex vivo systems could provide a platform to validate molecular markers which were obtained by retrospective research, like the novel markers presented in this thesis, at a cell biological level to understand their predictive value.

Research on the tumor microenvironment of EWS has been performed in a multicenter approach (NWO TOP GO 854.10.012) and presented in this thesis hopefully leading to a stronger scientific basis for development of immunotherapy based strategies for the high-risk and relapsed patient groups.

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NEDERLANDSE SAMENVATTING

Het Ewing-sarcoom is een agressieve primair maligne bottumor met een hoge graad van tumor vascularisatie. Zelfs in zo'n hoge mate dat het oorspronkelijk was verondersteld dat het een vasculaire oorsprong had. Voor dit soort uitgebreide vascularisatie is een intensieve interactie en samenspel tussen de tumor en zijn micro-omgeving nodig. Om die interacties binnen de tumormicro-omgeving te begrijpen is gedetailleerde karakterisering op moleculair niveau van het Ewing-sarcoom geboden. In dit proefschrift is binnen de tumormicro-omgeving gefocusseerd op de chemokine signalering. Een gedetailleerde karakterisering van het Ewing-sarcoom op genomisch, epigenomisch en transcriptomisch niveau kan daarnaast mogelijk helpen om nieuwe behandelbare doelwitten en voorspellende factor te identificeren. In **Hoofdstuk 2** zijn de resultaten van de tot nu toe gepubliceerde grote sequentie analyse studies samengevoegd tot een overkoepelend overzicht die het gebruik van deze grote, Ewing-sarcoom specifieke, datasets kan vergemakkelijken. Op genomisch niveau zijn in het Ewing-sarcoom, naast de karakteristieke *EWSR1-ETS* translocatie, geen andere meermaals terugkerende structurele herschikkingen geïdentificeerd. Het totaal aantal genmutaties gedetecteerd in het Ewing-sarcoom was erg laag en slechts twee genmutaties, *TP53* en *STAG2*, zijn meerdere keren waargenomen in verschillende studies met frequenties van respectievelijk 5.2-7% en 9-21,5%. De verdeling van deze mutaties over de twee genen resulteerde een interessant patroon in beide gevallen. De meest voorkomende *TP53* mutatie in Ewing-sarcoom -c.527G > T (p. C176F)- is niet vermeld als hotspotmutatie in de *TP53* mutatie databank van het internationaal agentschap voor onderzoek naar kanker, het IARC, waarin de *TP53* mutaties van vele soorten kanker zijn opgenomen. Deze resultaten zouden te maken kunnen hebben met het EWSR1-ETS fusie-eiwit en zijn effect op de functie van het TP53 eiwit. In *STAG2,* was een duidelijke hotspotmutatie - c.646C > T (p.R216X)- geïdentificeerd en was goed voor 25% van de *STAG2* mutaties. De *STAG2* en *TP53* mutaties zijn beiden klinisch relevant en kunnen dienen als voorstellende factoren voor de prognose van de patiënt. Het algeheel lage aantal mutaties in het Ewing-sarcoom suggereert dat de celdood signaleringscascades, in tegenstelling tot in de meeste andere tumoren, niet genetisch defect zijn. Intracellulaire of extracellulaire activatie van deze signaleringscascades door respectievelijk het DNA beschadiging herkenningssysteem en immune cellen zou hierdoor mogelijk zijn. Dit biedt grote kansen voor effectiviteit van chemo- en immunotherapie (**Hoofdstuk 2**).

Het effect van het EWSR1-ETS fusie-eiwit op de cellulaire processen is zowel op epigenoom als transcriptoom gebied duidelijk zichtbaar. Het Ewing-sarcoom heeft zijn eigen epigenetische patronen bij sommige ver gelegen versterker en superversterker gebieden. Daarnaast bindt EWSR1-ETS op specifieke GGAA satelliet sequenties wat belangrijke factor is in het functioneren als oncogen [8]. Om te kunnen binden is het afhankelijk van interacties met verschillende epigenetische modificerende eiwitten en deze eiwitten en het interactiegebied zijn daarom ideale therapeutische doelen voor een specifieke EWSR1-ETS behandeling.

Op transcriptoom gebied -naast een Ewing-sarcoom specifiek gen expressie patroon- zijn er Ewing-sarcoom specifieke niet-coderende RNA expressie patronen en alternatieve splicing gebeurtenis geïdentificeerd. Dit alles komt grotendeels door het EWSR1-ETS fusie-eiwit. Het verhoogt en verlaagt de activiteit verscheidene cellulaire processen. Voor de studies gepresenteerd in dit proefschrift was de verlaging van de extracellulaire signalering -met name de chemokines en chemokinereceptoren signalering- relevant en in meer detail onderzocht.

De gereguleerde expressieverlaging van chemokines en hun receptoren kan ook juist in het voordeel zijn voor de behandeling van de EWS. Het in **Hoofdstuk 3** besproken ontbreken van

chemokine CCL21 en CCR7 expressie in Ewing-sarcoom cellen maakt deze tumor een uitstekende kandidaat voor actieve immunotherapie met CCL21. De CCL21-CCR7 signaleringsas wordt namelijk vaak geactiveerd door het immuunsysteem en dat verbetert de anti-tumor immuunrespons. Echter, wanneer tumorcellen CCL21 en/of CCR7 tot expressie brengen kan er een pro-tumor respons optreden bij een CCL21 immunotherapie met een ongunstig effect van dien. *CCL21* RNA expressie kan tevens mogelijk gebruikt worden als een prognostische marker in het Ewing-sarcoom omdat verhoogde RNA expressie van *CCL21* in therapie-naïeve primaire tumormonsters retrospectief geassocieerd was aan een langere levensduur en omgekeerd gecorreleerd met verhouding tussen $CD4^+$ en $CD8^+$ T-cellen (**Hoofdstuk 3**).

In tegenstelling tot de meeste chemokines en chemokinereceptoren receptoren, is verhoging van expressie van CXCR4 in het Ewing-sarcoom beschreven. CXCR4 is betrokken bij alle belangrijke processen van de tumormicro-omgeving: angiogenese, tumorgroei, uitzaaiing op afstand en immuunsurveillance. Op het gebied van associatie van CXCR4 expressie met deze processen zijn er in het Ewing-sarcoom tegenstrijdige resultaten gevonden op RNA en eiwit niveau. Hoge RNA expressie was geassocieerd met uitzaaiingen, hoge eiwit was geassocieerd met groei en geen aanwezigheid van metastase was waargenomen. Door het bestuderen van de *CXCR4-CXCR7* -as op RNA niveau in twee patiënt cohorten hebben we de rol van CXCR4 in het Ewing-sarcoom in een breder perspectief onderzocht. In deze studie hebben we een rol aangetoond voor de chemokine receptor CXCR7 en chemokine CXL14 die beiden een remmende werking hebben op CXCR4 activering (**Hoofdstuk 4**). Verhoogde genexpressie van deze factoren bleek een negatieve correlatie/associatie met de ontwikkeling van uitzaaiingen in beide cohorten en met verbeterde overleving in één cohort en bekrachtigt het belang van CXCR4 activiteit in de tumormicro-omgeving van het Ewing-sarcoom (**Hoofdstuk 4**).

CXCR4 wordt op vele niveaus gereguleerd, inclusief alternatieve splicing, post-translationele modificaties, dimerisatie en cellulaire lokalisatie. In het verleden zijn er twee *CXCR4* splice varianten gerapporteerd (*CXCR4-1* en *CXCR4-2*), maar hun rol in de tumormicro-omgeving was onbekend. Het is bekend dat het evenwicht tussen splice varianten van belang zijn voor functie van een eiwit. In **Hoofdstuk 4** is gedemonstreerd dat de *CXCR4-1/CXCR4-2* verhouding verhoogd was in weefselmonsters van de tumor ten opzichte van cellijnen en deze verhoogde ratio was geassocieerd met een betere overleving. Wanneer CXCR4 kan dimeriseren of zelfs oligomeriseren stellen wij voor dat er een CXCR4-2 en CXCR4-1 heterodimer gevormd kan worden deze de activering van CXCR4-2 kan beïnvloeden. Naast deze twee bekende splice varianten, we hebben met behulp van transcriptoom sequentieanalyse twee nieuwe splice varianten (geannoteerd als *CXCR4-3*, *CXCR4-4*) in Ewing-sarcoomcellijnen (**Hoofdstuk 5**) gevonden. Deze varianten kwamen ook tot expressie in andere tumor en normale weefselmonsters en hieruit blijkt dat hun expressie niet Ewing-sarcoom specifiek is. Validatie op eiwit en functioneel niveau openbaarde dat ectopische expressie in een overwegend onstabiel intracellulaire eiwit resulteerde. De CXCR4-3 isovorm in HEK293T-cellijn bleek echter gedeeltelijk gelokaliseerd te zijn op het celmembraan en actief wanneer gestimuleerd werd met CXCL12a. Interessant is dat alle geïdentificeerde CXCR4 splice varianten alleen variëren in het N-terminale eind van CXCR4 en dit impliceert een belangrijke rol voor dit N-terminale gebied in de CXCR4 signaaltransductie. Voor de CXCR4-2 variant is deze rol al bevestigd door model-experimenten.

CXCR4 activatie door zijn ligand CXCL12 is afhankelijk van de subcellulaire lokalisatie van de receptor en zijn sterk gereguleerde cellulaire transportatie. Alleen celmembraan gelokaliseerd CXCR4 is toegankelijk voor ligand binding en de celmembraanexpressie niveaus zijn geassocieerd met de vorming van metastases. Sinds in het Ewing-sarcoom conflicterende

CXCR4 RNA en eiwit expressie is gerapporteerd is een op peptide-gebaseerde kleuring gebruikt en vergeleken met een antilichaam-gebaseerde kleuring (**Hoofdstuk 6**). De peptide-gebaseerde kleuring kon zowel kwalitatief als kwantitatief CXCR4 celmembraanexpressie detecteren en deze was in Ewing-sarcoomcellijnen lineair gecorreleerd met de *CXCR4* RNA expressie niveaus. Bij de antilichaam-gebaseerde kleuring werd daarentegen geen verschil in celmembraanexpressie gedetecteerd in dezelfde Ewing-sarcoomcellijnen. Daarbij werd CXCR4 als gevolg van binding het gebruikte peptide Ac-TZ14011-Cy5 geïnternaliseerd. Na CXCL12 binding worden de intracellulaire domeinen van CXCR4 gemodificeerd door ubiquitinatie en fosforylering wat leidt tot internalisering van CXCR4. Receptor internalisering en intracellulair transport is een complex proces waarbij meerdere signaaltransductiepaden betrokken zijn en het is aangetoond cel/weefsel specifiek te zijn. Binding van het peptide Ac-TZ14011-Cy5 leidde tot internalisatie van het CXCR4-peptide complex en was daarom gebruikt as chemische basis voor de synthese van een CXCR4 endocytose tracer dat als techniek kan helpen bij de bestudering van CXCR4 intracellulair transportatie. De tracer bestond naast het Ac-TZ14011 peptide uit twee fluorophores verbonden door middel van een zwavelbrug en deze dimeer was geschikt voor Förster resonance energie transfer (FRET) (**Hoofdstuk 7**). Deze zwavelbrug maakte het mogelijk om de endocytose te meten op gebied van fluorescentie omdat deze zwavelbrug verbroken wordt in een reducerend milieu. Internalisatie van de tracer was *in vivo* geobserveerd in Ewing-sarcoomcellijnen. Dit kon zowel kwalitatief als kwantitatief bepaald worden. Deze methode zou kunnen helpen bij de verdere studies naar de rol en de regulering van CXCR4 in andere tumortypes.

Concluderend, illustreert dit proefschrift het belang van chemokines in het Ewing-sarcoom en haar tumormicro-omgeving. *CCL21 CXCL14*, *CXCR7* en de verhouding tussen *CXCR4-1* en *CXCR4-2* zijn retrospectief geïdentificeerd als prognostische markers, CCL21 immunotherapie als potentiële therapie en CXCR4 als potentieel therapeutisch doel in het Ewing-sarcoom. De twee gepresenteerde methodes, peptide-gebaseerde CXCR4 celmembraankleuring en CXCR4 endocytose tracer, kunnen helpen bij de experimentele validatie van de anti-CXCR4 therapie in het Ewing-sarcoom en andere tumoren. Dit proefschrift biedt een perspectief voor aanpakken van de tumormicro-omgeving van het Ewing-sarcoom en handvaten voor verbeterde stratificatie van Ewing-sarcoom patiënten wat misschien kan resulteren in betere prognose voor de patiënten op korte en lange termijn.

CURRICULUM VITAE

Laurens Gerard Leo Sand was born on February 13, 1988 in Breda, the Netherlands. After graduating at the secondary school Mencia de Mendoza in Breda with a broad subject cluster, he started his Bachelor program Life Science & Technology at Technical University of Delft in 2006. During his Bachelor program he did his internship at the Medical Pharmacology Department of the Leiden Amsterdam Center for Drug Research (LACDR) and received his Bachelor degree in 2009. Directly thereafter he started the Research master program Life Science & Technology in Leiden University and followed subjects at multiple institutes, including the Kluyver Center, LACDR and LUMC. His first research internship was at the Department of Medicinal Chemistry of the LACDR on G protein-couple receptor NIACR1 affinity studies. The second internship was in Basel at the Biologics department of the Novartis Institutes of BioMedical Research (NIBR) on antibody display in *Saccharomyces cerevisiae*. He accomplished his Master program with honor in 2011 and started in 2011 with his PhD at the Department of Pathology under supervision of Dr. Karoly Szuhai and Prof. Pancras C.W. Hogendoorn. Results obtained during this PhD are described in this thesis. Currently, he is pursuing a career in research & development at Janssen Infection Disease and Vaccines.

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ABBREVIATIONS:

EWS	Ewing sarcoma
PNET	peripheral primitive neuroectodermal tumor
RMS	Rhabdomyosarcoma
MSC	Mesenchymal stem cell
EWRS1-ETS	Fusion gene/protein consisting of Ewing sarcoma breakpoint region-1 and a member of the erythroblast transformation-specific family of transcription factors
miRNA	micro-RNA
lncRNA	Long non-coding RNA
NGS	Next generation sequencing
SNP	Single nucleotide polymorphism
SNV	Single Nucleotide Variant
LOH	Loss of heterozygosity
DSB	double-strand break
GPCR	G-protein coupled receptor
NK	Natural killer
OS	Overall survival
EFS	Event free survival
MHC	major histocompatibility complex
WGBS	whole-genome bisulfite sequencing
ChIP-seq	chromatin immunoprecipitation sequencing
MeDIP-seq	methylated DNA immunoprecipitation sequencing
ChIP-exo	chromatin immunoprecipitation-exonuclease
UTR	Untranslated region
RT-Q-PCR	real-time quantitative-reverse transcriptase PCR
FISH	Fluorescence in situ hybridization
FRET	Förster resonance energy transfer
FFPE	Formalin fixed paraffin embedded
IHC	Immunohistochemistry
CFU	Central flow cytometry fluorescence units
PBA	PBS 5% BSA
SPECT	Single-photon emission computed tomography