Vascular tumors of bone and related lesions: from gene fusions to tumor models

Ong, S.L.M.

CHAPTER 1

General introduction

1.1 Genetic classification of sarcoma

Sarcomas are rare forms of cancer, which arise predominantly in soft tissue and bone. They comprise approximately 2% of all malignancies. Benign mesenchymal tumors (such as lipoma or hemangioma) are approximately 10 times more common than malignant sarcomas (such as liposarcoma or angiosarcoma). Sarcomas constitute a heterogeneous group of tumors with over 50 subtypes categorized by the WHO. The classification is based on the presumed line of differentiation in the tumors [1]. As such, the group of vascular tumors displays endothelial cell differentiation at variable levels. The exact pathogenesis of most sarcomas is incompletely understood. Surgery is the mainstay of treatment, complemented with chemo- and/or radiotherapy in selected subtypes, depending on histological grade and stage. Elucidating the genetic drivers of sarcomas has improved their classification and diagnostic accuracy. This effort has aided in identifying novel therapeutic targets in a few of the subtypes, ultimately improving treatment outcomes [2]. Based on cytogenetic analysis, sarcomas can be roughly categorized into two groups: sarcomas with simple genetic alterations or sarcomas with a complex genome.

1.1.1 Mesenchymal tumors with simple genome

Simple genetic alterations driving bone and soft tissue tumors can be subclassified into tumor-specific translocations, specific amplifications, and activating mutations. Tumor-specific translocations result in the formation of fusion genes, encoding fusion proteins with gained oncogenic activities. These tumor-specific genetic alterations only occur in tumor cells, making them distinguishable from non-tumor cells. For example, in synovial sarcoma (SS) with *SS18::SSX1/2/4* translocation, expression of *SS18::SSX* is essential in driving tumorigenesis [24]. Likewise, the *EWSR1::FLI1* translocation in Ewing sarcoma is required for oncogenic transformation and tumor cell survival [25]. Specific amplification is, for example, seen in atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDLPS) with amplification of *MDM2* and/or *CDK4* [1]. Lastly, an activating mutation driving tumorigenesis is, for example, observed in gastrointestinal stromal tumors (GIST) with a mutation in *KIT* or *PDGFRA,* causing tyrosine kinase activation leading to oncogenesis [3, 4]. Also, simple genomes are frequently seen among the group of vascular tumors. For instance, *MAP2K1* activating mutations are associated with arteriovenous malformation/hemangioma (AVM), resulting in the activation of the *MAP2K1* (*MEK1*) [5] and mutations in the *GNA* family of genes are seen in a diverse group of benign vascular tumors [6, 7]. Gene fusions are frequently seen in vascular tumors, particularly within the category of hemangioendotheliomas, as extensively reviewed in **Chapter 2**.

1.1.2 Sarcomas with Complex Genome

In sarcomas exhibiting a complex genome, tumorigenesis is driven by the collective effect of multiple genomic changes or inactivation of a few critical genes, such as *TP53* or *RB1*. Complex karyotypes in sarcoma can be caused by genomic instability, resulting in multiple chromosomal rearrangements and copy number alterations. Genomic instability can be caused by sporadic replication or repair errors. Genomic instability in cells increases the chances of genome alterations during cell division, yielding complex karyotypes. As a result, copy number variations are frequently seen, including gain (partial or whole chromosome) or loss (deletion) of genetic material. The prototype of a complex genome sarcoma is high-grade conventional osteosarcoma. In central osteosarcoma, chromothripsis and kataegis are observed in more than 90% and 50% of cases, respectively [8, 9], and among a plethora of genetic changes, *TP53* inactivation is the most frequently observed [1, 10]. Chromothripsis is characterized by massive, chaotic, and complex chromosomal alterations [11], and kataegis is caused by localized hypermutations [12]. The presence of extensive molecular alterations in osteosarcoma makes developing alternative therapeutic strategies for osteosarcoma challenging. Another example of a tumor type with high genomic instability is leiomyosarcoma [13]. The most commonly observed genetic alterations in leiomyosarcoma resulting from genomic instability are the loss of tumor suppressor genes *PTEN*, *RB1,* and *TP53* [1]. Among the group of vascular tumors, only angiosarcoma can have complex karyotypes with somatic mutations (*KDR*, *PTPRB*, and *PLCG1*), rare mutations (*RAS* genes), gene amplifications (*MYC* and *FLT4*), or some recurrent chromosomal changes [14-16].

1.2 Vascular tumors, endothelium and angiogenesis

Vascular tumors are rare neoplasms that can occur in bone or soft tissues. They consist of many subtypes characterized by simple genetic alterations or complex and unbalanced karyotypes (Table 1). The prognosis of vascular tumors is variable depending on subtype, tumor size, and local extent, with limited therapeutic options available for aggressive tumors that can not be treated surgically. Vascular tumors are classified as such based on their resemblance to normal endothelium and the expression of endothelial markers suggesting endothelial differentiation [1]. These endothelial markers include CD31, CD34, or ERG. The cell of origin for vascular tumors is therefore hypothesized to be the endothelial cell or its precursor [17, 18]. Knowledge of normal angiogenesis is needed to understand vascular neoplasia better.

Angiogenesis is defined as the formation of new blood vessels from existing vessels. Tumor cells send angiogenic signals, such as VEGF, FGF, and ANG-2, to quiescent

vessels to initiate angiogenesis [19]. The quiescent endothelial cells degrade the basement membrane with the help of matrix metalloproteases (MMP), and pericytes detach from the vessel wall in the presence of ANG-2, increasing endothelial cells permeability and sprouting [20]. There are crucial signaling pathways (ANG, TIE, NOTCH, and WNT), growth factors (VEGF, PDGF, and FGF) and their receptors (VEGFR1/2 and PDGFRβ), involved in angiogenesis. In vascular neoplasia, these pathways involved in normal angiogenesis are often affected. For instance, gene mutations can be found that involve the angiogenic signaling pathway, growth factors, and growth factor receptors, such as VEGF/VEGFR, to promote tumor growth and metastasis in angiosarcoma [21]. Furthermore, in sarcoma with complex karyotypes, such as angiosarcoma, has shown genetic alterations involving *TP53* and the PIK3CA/AKT/mTOR pathway [15]. The PIK3CA/AKT/mTOR pathways regulate cell growth, survival, angiogenesis and others [22].

Angiogenesis is not only involved in normal development and affected in vascular neoplasia, it is also important for solid tumor growth in general, irrespective of the tumor type. Cancer cells need nutrients, and therefore, angiogenesis resulting in the formation of a neovasculature is needed to provide these [23, 24]. Tumor angiogenesis begins with the initiation of angiogenic response, causing the degradation of the extracellular matrix, which enables endothelial cell migration, proliferation, and tube formation, and finally, the maturation of neovasculature [25]. The neovasculature has been extensively investigated as a potential target to treat solid tumors: targeting angiogenesis and depriving the tumor of nutrient supply will affect its growth capacities [26, 27]. An example is pazopanib, which is approved for treatment of soft tissue sarcoma [28] and has been shown to selectively inhibit vascular endothelial growth factor receptor (VEGFR)-mediated angiogenesis [29].

Table 1. WHO classification of vascular tumors of soft tissue and bone

Classification	Vascular tumor	Genetic alteration
Benign	Hemangioma/vascular malformation	*EWSR1::NFATC2*
	Epithelioid Hemangioma	*FOS* or *FOSB* rearrangement, *FOXO1* fusion
	Tufted angioma	*GNA14* mutation
Locally aggressive	Kaposiform Hemangioendothelioma	*GNA14* mutation
Rarely metastasising	Retiform Hemangioendothelioma	*YAP1::MAML2* and *YAP1* rearrangement
	Papillary Intralymphatic Angioendothelioma	-
	Composite Hemangioendothelioma	*PTBP1/YAP1::MAML2* and *EPC1::PCH2*
	Kaposi Sarcoma	*HHV8* related
	Pseudomyogenic Hemangioendothelioma	*FOSB* rearrangement

Table 1. Continued.

Classification	Vascular tumor	Genetic alteration
Malignant	Epithelioid Hemangioendothelioma	*WWTR1::CAMTA1*
	Hemangioendothelioma With *YAP1::TFE3* Fusion	*YAP1::TFE3*
	Angiosarcoma	*MYC* or *FLT4* amplification, *PTPBR* or *PLCG1* mutation

1.3 Gene translocations and promiscuity in vascular tumors

Chromosomal translocation can result in overexpression or loss of function of the resultant fused genes. Gene fusions are quite common in vascular tumors, especially epithelioid hemangioma (EH), pseudomyogenic hemangioendothelioma (PHE), *TFE3*-rearranged hemangioendothelioma and epithelioid hemangioendothelioma (EHE) (extensively reviewed in chapter 2). While it was previously thought that gene fusions are specific for a tumor type, such as *SS18/SSX1/2/4* in synovial sarcoma, for most gene fusions this is not the case. Identical gene fusions occur in distinct neoplasms with different morphologies, molecular features, and aggressiveness. An example is the *ETV6::NTRK3* fusion gene, which has been identified in a broad spectrum of mesenchymal neoplasms, from benign lipofibromatosis-like neural tumors to malignant infantile fibrosarcoma [1]. *EWSR1::NFATC2* rearrangement is another example of identical gene fusions occurring in distinct neoplasms with different characteristics.

EWSR1 encodes EWS RNA binding protein 1, which regulates transcription and RNA splicing, as well as various other cellular processes. Together with *FUS/TLS* and *TAF15*, *EWSR1* belongs to the FET (*FUS*, *EWSR1*, and *TAF15*) family of RNA/DNA binding proteins. *EWSR1* is a highly promiscuous gene and can fuse to many different partners in various neoplasms including Ewing sarcoma, myoepithelioma, extraskeletal myxoid chondrosarcoma, and even leukemia [30]. Ewing sarcoma is a small round cell sarcoma with gene fusions involving a member of FET family genes and the ETS family of transcription factors [1].

Up till recently, *EWSR1/FUS::NFATC2* fusions were thought to be specific for the group of round cell sarcomas (RCS) with EWSR1-non-ETS fusions [1]. NFATC2 is a transcription factor that induces gene transcription during an immune response. EWSR1 is thought to serve as a robust N-terminal trans-activator for its C-terminal fusion partner. The exact mechanism of *EWSR1*-driven sarcomagenesis is incompletely understood. *EWSR1*::*NFATC2* fusion might be constitutively expressed and, in turn,

dysregulate *NFATC2* expression and transcriptional activity. NFATC1 also belongs to the NFAT family of transcription factors and has similar function as NFATC2 [31]. *EWSR1/FUS::NFATC1* rearrangement has been described in vascular neoplasm [32-34]. As for *EWSR1/FUS*::*NFATC2* gene rearrangement, it has been described in benign simple bone cysts (SBC) [35, 36] and now also in vascular tumors [37]. Table 2 illustrates a list of diseases involving *EWSR1/FUS::NFATC2* fusion.

Gene rearrangement involving *TFE3* are also promiscuous, and the various TFE3 fusion genes are not specific. TFE3 belongs to the microphthalmia/transcription factor E (MiT/TFE) family members of transcription factors. The MiT/TFE family consists of four distinct genes, MITF, TFEB, TFE3, and TFEC [38]. All four proteins share a common structural characteristic, the basic-helix-loop-helix leucine zipper (bHLH-LZ) domain, as shown in Figure 1. Rearrangements involving *TFE3* were identified in tumors such as renal cell carcinoma (RCC), alveolar soft part sarcoma (ASPS), EHE, or perivascular epithelioid cell tumor (PEComa). The list of neoplasms with *TFE3* fusion and their fusion partners is listed in Table 3. Chromosomal translocation in *TFE3* is mostly intergenic breakpoints, primarily in intron 2 or 3, retaining the C' terminal bHLH functional domain of TFE3 (Figure 1). The function of recurrent *TFE3* fusion partner varies from splicing (*SFPQ*, *RBM10*, *NONO*, *MATR3*, *LUC7L3*, and *KHSRP*), involved in hippo (*YAP1*), or Wnt (*DVL2*) signaling pathway. TFE3 protein is ubiquitously expressed at low levels in various tissues, therefore TFE3 nuclear expression may not correlate to presence of *TFE3* translocation. For example, a subset of approximately 9% of RCC lacking *TFE3* rearrangement showed TFE3 expression [39-41]. Furthermore, TFE3 nuclear staining is observed in 15% of PEComa cases [42]. As such, it is recommended to use other methods to identify *TFE3* rearrangement such as fluorescent *in situ* hybridization (FISH).

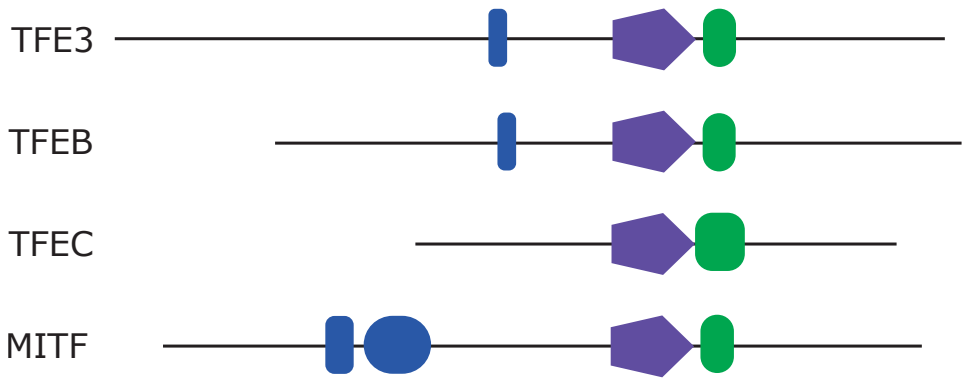

Figure 1. Common structural domains in MiT/TFE family. Conserved protein domains TFE3, TFEB, TFEC, and MITF. AD: Activation domain (blue); bHLH: basic helix–loop–helix (purple); LZ: leucine zipper domain (green).

Table 2. Neoplasms involving *EWSR1* or *FUS* in *NFATC2* rearrangement

Disease	Recurrent *NFATC2* fusion partners
Round cell sarcoma with *EWSR1*-non-*ETS* fusions	*EWSR1 [43-49], FUS [47, 50-54], FUS [55, 56]*
Simple bone cyst	*EWSR1 [35, 36, 57], FUS [36, 57] FUS [48]*
Epithelioid vascular neoplasm	*EWSR1* and *FUS [33]*

Table 3. Neoplasms with *TFE3* translocation

Disease	Recurrent *TFE3* fusion partners	A single case of *TFE3* fusion partner
Epithelioid hemangioendothelioma (EHE)	*YAP1 [58]*	
Alveolar soft part sarcoma (ASPS)	*ASPSCR1 [59]*	*DVL2 [60], HNRNPH3 [60], PRCC [60], UXT [56]*
Perivascular epithelioid cell tumor (PEComa)	*ASPSCR1 [61], RBM10 [62], SFPQ [63]*	*DVL2 [64], NONO [65], RBMX [66], VCP [67]*
TFE3-rearranged renal cell carcinomas (tRCC)	*NONO [68], SFPQ [68], PRCC [69], ASPSCR1 [70], DVL2 [65], EWSR1 [71], KHSRP [72], LUC7L3 [72], MATR3 [73], MED15 [74], PARP14 [75], RBM10 [76], VCP [77]*	*FUBP1 [73], ARID1B [78], CLTC [79], GRIPAP1 [74], KAT6A [80], SETD1B [41], NEAT1 [80], KHDRBS2 [72]*
Paediatric B-cell precursor acute lymphoblastic leukaemia (BCP ALL)		*NONO [81]*
Hormone Receptor–Positive Breast Cancer		*WDR45 [82]*
Myofibroblastic sarcoma		*RREB1 [83]*
Ossifying fibromyxoid tumor (OFMT)	*PHF1 [84]*	
Cutaneous fibromyxoid neoplasms (CFMN)		*YAP1 [85]*

1.4 Model systems for vascular tumors

Prolonged culture of normal endothelial cells is challenging, and only few primary endothelial cell models are available, including human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC). However, these cell lines are limited in their lifespan *in vitro* up to merely 16 passages. In addition, previous studies by others have demonstrated functional changes in HUVEC after passage 10 [38, 86]. This further restricts the use of these cells for genetic manipulation, as several passages are needed and good amount of cells are required for therapeutic drug testing. Human induced pluripotent stem cells (hiPSC) are also widely used for disease modeling. hiPSCs can differentiate into endothelial cells (EC) through a series of steps in the presence of growth factors [87]. As detailed in Orlova *et al.*, mesoderm is induced by mesoderm-inductive factors such as Activin A, BMP4, VEGF,

and CHIR. After three days, mesenchymal cells are formed, and vascular specification is initiated by supplementing cells with VEGF and TGF-β inhibitor. At the end of the vascular specification, endothelial cells and pericytes are formed. These endothelial cells maintain their endothelial characteristics by expressing the endothelial-specific marker CD31, VE-cadherin, and von Willebrand factor (vWF) [87]. As such, endothelial cells can be isolated from pericytes using Dynabeads conjugated with anti-CD31. A previous study showed that hiPSC is ideal for modeling vascular tumors [88]. Genetically manipulated hiPSC-derived EC harboring a specific translocation can be utilized to identify specific drug sensitivities. Over the years, a small number of *in vitro* and *in vivo* vascular tumors models were generated (Table 4).

Table 4. Gene-edited *in vitro* and *in vivo* models for vascular tumors

Vascular tumor type	Genetic alteration	Method	*In vitro* or *in vivo*	Publication year and reference
Epithelioid hemangioma (EH)	Truncated *FOS*	Overexpression	*In vitro*	2017 [89]
Pseudomyogenic hemangioendothelioma (PHE)	*SERPINE1::FOSB* translocation	Gene-edited	*In vitro* and *in vivo*	2020 [88]
Epithelioid hemangioendothelioma (EHE)	*WWTR1::CAMTA1* transcript	Overexpression	*In vitro*	2016 [90]
	WWTR1::CAMTA1 transcript	Overexpression	*In vitro* and *in vivo*	2021 [91]
***TFE3*-rearranged hemangioendothelioma**	*YAP::TFE3* transcript	Overexpression	*In vitro* and *in vivo*	2021 [91]

Note: Angiosarcoma cell lines are listed in Table 5.

1.4.1 Prolonged in vitro culture of patient-derived vascular tumor cell lines

Prolonged *in vitro* culture of primary vascular tumors is considered highly challenging. No patient-derived models for vascular tumor types are commercially available, except for a few angiosarcoma cell lines [92-97]. Reported angiosarcoma cell lines are listed in Table 5. Unger and colleagues carried out an expression profile of an established angiosarcoma cell line (HAEND) and showed that their immunohistochemical profile changed from what was initially published [95]. Even though the angiosarcoma cell line (HAEND) had undergone more than 100 population doublings, its molecular characteristics are not stable. Over the past decade, our laboratory has attempted to culture patient-derived vascular tumor lines in various media compositions. However, none of the cell culture were able to survive more than five passages *in vitro*. Limited *in vitro* passaging capabilities of patient-derived tumor cells might be caused by senescence. Cellular senescence is a state of irreversible growth arrest that may

result from the progressive shortening of telomeres [98]. Senescence can additionally be induced by a variety of cellular stresses, including oxidative and environmental stress. One of the first responses to such stress is mediated by the activation of P53 protein that leads to the arrest of cell growth or early cell death via apoptosis [99]. Previous *in vitro* studies in primary chick embryo fibroblasts have shown that the loss of P53 function is correlated with increasing cellular proliferative lifespan [100]. In line with this, proliferation of vascular tumor cells could potentially be increased by the inactivation of P53 protein through mutating *TP53*.

Table 5. Angiosarcoma cell lines published

Cell line name	Tumor location	Age/Sex	Immunohistochemical analysis and technique	Genetic characteristic	Publication year and reference
AS-M	Skin	80/M	Immunofluorescence and RT-PCR - Positive [CD31, vWF] expression		2003 [92]
BM 2.2.1	Liver	60/M	Immunofluorescence staining - Positive [Vimentin, αSMA] and negative [Desmin, SMA, CK19, vWF] expression	Homozygous missense mutation in codon 179 (CAT→CTT) was detected in the *p53* gene.	2000 [93]
HAEND	Liver	61/M	Flow cytometry analysis, RT-PCR and immunofluorescence - Positive [Flt-1, ICAM-1, VCAM-1] and negative [vWF, CD31, CD34, KDR, E-selectin] expression		1993 and 2002 [94, 95]
ISO-HAS	Skin	84/M	Flow cytometry analysis and immunofluorescence – Positive [CD31, vWF, UAE-1, CD34, Flt-1, KDR, ICAM-1, E-selectin] and negative [VCAM-1] expression	Point mutation in codon 250 was detected in the *p53* gene.	1999 and 2002 [95, 96]
KU-CAS3	Skin	73/M	Flow cytometry analysis and immunofluorescence - Positive [CD31, ADRβ2, vWF, Claudin-5] and negative [CD34, CD45] expression		2022 [97]

1.4.2 CRISPR/Cas9 genome editing to generate a chromosomal translocation

Over the past decade, genome editing technologies have developed from zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), to the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems. In recent years, the CRISPR/Cas9 system has become the most widely used genome editing technology due to its high editing efficiency. The CRISPR/Cas9 systems consists of two key molecules, Cas9 protein and guide-RNA, which act together in precise induction of a double-stranded DNA-break. In the nucleus, the Cas9 protein

recognizes guide-RNA and together they form a complex. The Cas9-guide-RNA complex attaches to matching genomic DNA sequence and cuts the double-stranded DNA. The repair of double-strand DNA breaks occurs in either of two ways, non-homologous end-joining (NHEJ) and homology-directed repair (HDR). The NHEJ repair mechanism does not utilize a repair template and DNA is repaired randomly. As a results, random insertions and/or deletions of base pairs are likely to occur during the repair process. Homologous directed repair mechanism employs the presence of a homologous sequence as a repair template for more accurate repair. The CRISPR/Cas9 technology has limitations such as off-targeting, low editing efficiency and toxicity. Hard-to-transfect cells pose a limitation to deliver CRISPR/Cas9 gene editing components into the nucleus thereby decreasing gene editing efficiency.

1.5 Thesis outline

Several vascular tumor entities present with recurrent chromosomal translocations. With limited therapeutic options available for aggressive tumor subtypes, it is all the more important to map out the uncharted territories of genotypes that underpin these conditions. Identifying genetic alterations in tumors and understanding underlying molecular mechanisms can improve therapeutic strategies and effectiveness. This thesis therefore aims to:

1. explore promiscuity of gene fusions (i.e. *EWSR1::NFATC2*) in vascular tumors and related lesions (**Chapters 3 and 4**)
2. develop methods to study the underlying molecular mechanisms of genetic alterations and potential therapeutic strategies in vascular tumors (**Chapters 5 and 6**)

Chapter 2 extensively reviews gene fusions in vascular tumors and their underlying molecular mechanism. Furthermore, key characteristics that can be helpful in the diagnosis of these tumors and the use of these translocations in molecular diagnostics to assist in the differential diagnosis are highlighted. Gene fusion partners can be promiscuous, and similar fusion types can occur in distinct neoplasms. An example is the *EWSR1/FUS::NFATC2* rearrangement that was encountered during routine diagnostic work-up in vascular malformations of the rib as well as the femur of a 63-year-old female. Thus far, *EWSR1/FUS::NFATC2* fusion was reported in malignant round cell sarcoma and benign solitary bone cyst. In **Chapter 3**, the frequency of *EWSR1/FUS::NFATC2* rearrangement in vascular tumors was examined.

Solitary bone cyst is a benign cystic lesion of bone lacking endothelial lining. Most *EWSR1/FUS*::*NFATC2* rearrangements outlined in SBC are located in the long bone, while only one case of jaw SBC was previously investigated [101]. Jaw SBC is previously referred to as a non-neoplastic traumatic bone cyst, suggesting the absence of a causative genetic aberration. In **Chapter 4**, jaw SBC cases were collected to determine whether jaw SBCs represent the same entity as SBCs of the long bones, with *EWSR1::NFATC2* rearrangement, or a separate – potentially reactive - entity or with different molecular signature.

Induced pluripotent stem cells can be differentiated towards endothelial cells and as such provide a nice basis for gene editing to introduce translocations and study the effect after endothelial differentiation and after drug treatment. To reliably evaluate changes in gene expression in this specific model, stably expressed housekeeping genes that remain stable in hiPSC and hiPSC-EC during drug treatment must first be identified. These are crucial to compare the relative expression of the gene of interest between drug-treated and non-treated samples. However, there is a lack of studies on housekeeping genes that remain stable in hiPSC and hiPSC-EC. **Chapter 5** aims to identify candidate housekeeping genes that remain stable during drug treatment in hiPSC and hiPSC-derived EC.

Chapter 6 aims to create a vascular model for EH, EHE, and angiosarcoma and describes the two strategies that were applied. The first strategy utilizes CRISPR/Cas9 technology to induce translocation in hiPSC. Using the CRISPR/Cas9 technology, various attempts with different methods were used to induce the *FOS::VIM*, *YAP1::TFE3*, or *WWTR1::CAMTA1* translocation in hiPSC to study translocation-drivers in vascular tumors. The second strategy aims to establish a patient-derived vascular tumor line by inducing *TP53* mutation in primary tumor cells. As optimizing media composition seems insufficient to prolong the culturing of patient-derived lines, the CRISPR/Cas9 technology is utilized to introduce a DNA double-stranded break in *TP53* to generate frameshift mutations causing permanent gene disruption to prolong the *in vitro* proliferative lifespan of the cells.

Lastly, the summary and concluding remarks of the thesis and future perspectives are discussed in **Chapter 7**.

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