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Rocio K. RIVERA-Valentin

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HER4 PROMOTES A STEM-LIKE PHENOTYPE IN OSTEOSARCOMA

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HER4 PROMOTES A STEM-LIKE PHENOTYPE IN OSTEOSARCOMA

A DISSERTATION

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Health Science Center at Houston
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of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

December 2016

Dedication

This dissertation is dedicated to my dear daughter Norah, to my amazing husband Pablo, my loving parents Herminia and Angel and my beloved sisters Anmelys and Angelica. I love you with all my heart!

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First and foremost, I want to thank God for blessing me with so many opportunities and challenges throughout these four years. I can do hard things because He is by my side holding me through the tough times.

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HER4 PROMOTES A STEM-LIKE PHENOTYPE IN OSTEOSARCOMA

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Metastatic disease to the lungs is the primary cause of death for patients with pediatric osteosarcoma (OS). OS has a high degree of heterogeneity and genomic instability, making understanding the pathogenesis and drivers of metastasis of this disease challenging. In an effort to explain tumoral heterogeneity, the tumor initiating cell model (TIC) states that tumors are composed of cells that form the majority of the tumor and are terminally differentiated. This model however, attributes tumorigenesis, metastasis and chemoresistance to a distinct cell population with a stem-like phenotype that can be identified using selective markers. OS appears to follow this model where OS cells with tumor initiating potential can be identified by expression of Stro1, CD117 and embryonic stem cell transcription factors such as Sox2, Nanog and Oct3/4. Additionally, OS stem-like cells display high aldehyde dehydrogenase activity and sarcosphere formation under limited nutrient media and anchorage independence. These markers are not feasible targets for therapy due to their expression on normal tissue stem cells; however, upstream regulators of this phenotype may be targetable. Therefore, we investigated other modulators of the stem-like phenotype.

Her4, a transmembrane receptor of the EGFR family, has been recently studied for its role in cancer. Previously, we demonstrated that Her4 is highly expressed in neuroblastoma, and OS, while others have shown its importance in

Ewing sarcoma. This receptor is induced and required to survive stressors, like anchorage independence, serum starvation and chemotherapy treatment, which are similar *in vitro* conditions used to enrich for cells with tumor initiating potential. Therefore, we hypothesized that Her4 expression is an important regulator of a stem-like phenotype in OS.

In sarcosphere culture, Her4 expression is induced and precedes the induction of CD117 and Stro1. OS cells with Her4 deleted by CRISPR/Cas9 have decreased aldehyde dehydrogenase activity and cannot upregulate the pluripotency transcription factors Sox2, Oct3/4 and Nanog even when in sarcospheres. Overexpression of exogenous Her4 was able to cause upregulation of these transcription factors and increase expression of CD117 in monolayer culture. We examined Her4 expression in OS diagnostic biopsies and determined the correlation with metastasis free survival. Tumors with high Her4 expression have higher probability of developing metastatic disease.

In this dissertation, we demonstrate that Her4 expression is induced by conditions that enrich stem-like cells and its expression correlates with the ability to upregulate various OS TIC markers. Therefore, Her4 may contribute to pathogenesis of OS by conferring a stem-like phenotype.

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Chapter 1: Introduction

Osteosarcoma

Osteosarcoma (OS) is the most prevalent bone tumor among pediatric patients comprising around 60% of all bone tumors in children [1-3]. It affects mostly adolescents and males more often and earlier than females, but there is an additional peak incidence amongst patients older than 50, pointing at a possible role of bone remodeling in tumor initiation common at both ages [1]. Around 4.4 cases per million per year are diagnosed in the U.S. making this malignancy fairly uncommon [4]. However, this tumor is highly deadly due to its high metastatic capacity making OS the second most common cause of cancer related deaths among pediatric patients [5, 6]. Metastatic disease localizes primarily to the lungs and less frequently to bone and it is the primary cause of death of OS patients [7]. Patients who initially present with localized disease have a 5-year survival of around 70% compared to patients who present with metastatic disease upon diagnosis who have less than 20% survival at 5 years [1].

Management of the Osteosarcoma Patient

A common case presentation for OS is a young adolescent who presents with articular pain and swelling, commonly on the distal femur or proximal tibia, without any significant history of trauma [8]. Patients who present with OS suspecting lesions undergo biopsy and imaging studies as part of their initial workup. These typically include plain radiographs, which can show pathognomonic OS features like a sunburst pattern indicating bone destruction and deposition and presence of Codman's triangle or periosteum elevation around the tumor [1] However, MRI of the affected area is necessary to determine the extent of disease and neurovascular and

soft tissue involvement. CT of the lungs is typically used to determine the presence of metastatic lesions. Histologic evaluation of the tumor is the standard for OS diagnosis. Lace patterning is indicative and a specific feature of osteosarcoma indicating osteoid (unmineralized bone matrix) deposition [2].

After the diagnosis of OS has been made, patients are placed on induction chemotherapy. Besides achieving local tumor control for better surgical excision, response to induction chemotherapy is the only validated prognostic factor for OS patients [9]. Patients who present with higher than 90% necrosis after induction are classified as good responders and have a 5-year disease free survival (DFS) of around 68%. Poor responders show less than 90% tumor necrosis and 5-year DFS of 51% [1]. Standard chemotherapy for OS consists of high-dose methotrexate, doxorubicin (Adriamycin) and cisplatin, also known as MAP therapy. Recent trials have evaluated whether there is an added benefit of combining ifosfamide and etoposide with MAP therapy to improve outcome of poor responders but unfortunately no significant success was obtained, with survival unchanged for over 20 years since the discovery of neoadjuvant chemotherapy and surgical excision [9].

Osteosarcoma Pathogenesis

Unlike other malignancies, no specific recurrent mutations have been identified as drivers of spontaneous OS, but some genetic alterations predispose to OS. Patients with mutations in the Retinoblastoma (RB1) gene have an incidence of osteosarcoma 500 times higher than the normal population and it's the most common secondary malignancy these patients present [10-13]. Li-Fraumeni, a familial syndrome involving germline mutations in p53, confers patients an increased

risk in many cancers like breast, brain and osteosarcoma among others [14].

Overall, around 70-80% of OS patients present with some mutation in either of these genes [1]. Mutations in the RECQL4 (RecQ protein-like 4) gene, which encodes for a RecQ helicase are associated with Rothmund-Thomson syndrome and have an increased predisposition to OS [15, 16].

Secondary OS primarily occurs among patients that were treated for another malignancy during their childhood years. Ionizing radiation and anthracycline or alkylator containing chemotherapies for the treatment of childhood solid tumors have been associated with the development of OS in the following two decades after treatment [5, 17]. Secondary OS also occurs in around 1% of patients with osteitis deformans or Paget disease of the bone. Although there is a low probability of malignant transformation among this population, patients who do present secondary OS have a particular poor outcome [18].

Tumor Heterogeneity in OS

One of the major features of OS is genomic instability [19]. Many studies have elucidated the complexity of the genomic alterations in this disease by whole genome sequencing and genome wide association studies (GWAS) on patient samples compared to paired normal tissue. We have learned from these investigations that OS is one of the cancers with the highest degree of structural variation. OS presents with two important phenomena that greatly contribute to genomic alterations and heterogeneity. Kataegis refers to hypermutated chromosome regions that have undergone somatic rearrangement [20]. These regions have been found throughout OS lesions and are not exclusive of specific

chromosomes. The second event is chromothripsis where entire chromosomes are shattered or broken into small fragments of DNA, which are then reassembled through non-homologous end joining generating a “stitched up” chromosome with many translocations and aberrations[20]. These two events make studying and understanding the biology of OS extremely challenging since there are no specific recurring chromosomal rearrangements that can be considered driving mutations among OS patients.

Cancer Stem Cell Model: Understanding tumor heterogeneity

Two models that have been extensively studied in order to understand more about tumor progression and heterogeneity are the stochastic model and the cancer stem cell model (CSC). In the early days of cancer biology research, the stochastic model was widely accepted and many if not all malignancies were thought to follow its principle [21]. This model posits that all cells within a tumor are biologically equivalent and have the same tumorigenic potential, therefore tumors are essentially homogeneous. Heterogeneity then is explained in this model by unpredictable random alterations in individual cells or particular and unique responses to intrinsic and extrinsic factors [22]. The stochastic model assumes that it is impossible to isolate pure populations of cancer cells that do not have tumorigenic potential, since the microenvironment and other uncontrollable factors will come into play and generate cells capable of tumor formation [23]. The CSC model is now more widely accepted over the stochastic model. The CSC model was initially described by Dick and colleagues in leukemia [22, 24, 25] but since then many malignancies, both solid and hematologic, have been shown to follow this principle. In the CSC model,

tumor heterogeneity is intrinsic. There are broadly two populations within tumors: those that form the bulk of the tumor and those with tumor initiating potential (see Figure 1). Cells that form the majority of the tumor have undergone clonal expansion, are rapidly dividing and can only generate progeny through symmetrical division [23]. In contrast, there is a small subpopulation of cells that are at the top of the cellular hierarchy since they can generate, through asymmetrical cell division, cells at different stages of differentiation. These CSCs or stem-like cells are thought to resemble normal tissue stem cells in many aspects including self-renewal, multipotency and differentiation into multiple lineages. This stem-like cell population is thought to be responsible for chemotherapy resistance and relapse due to their ability to remain in a quiescent state and their capacity to effectively dispose of drugs. Markers that identify normal tissue stem cells and also unique markers for CSCs have been studied in an effort to isolate this population.

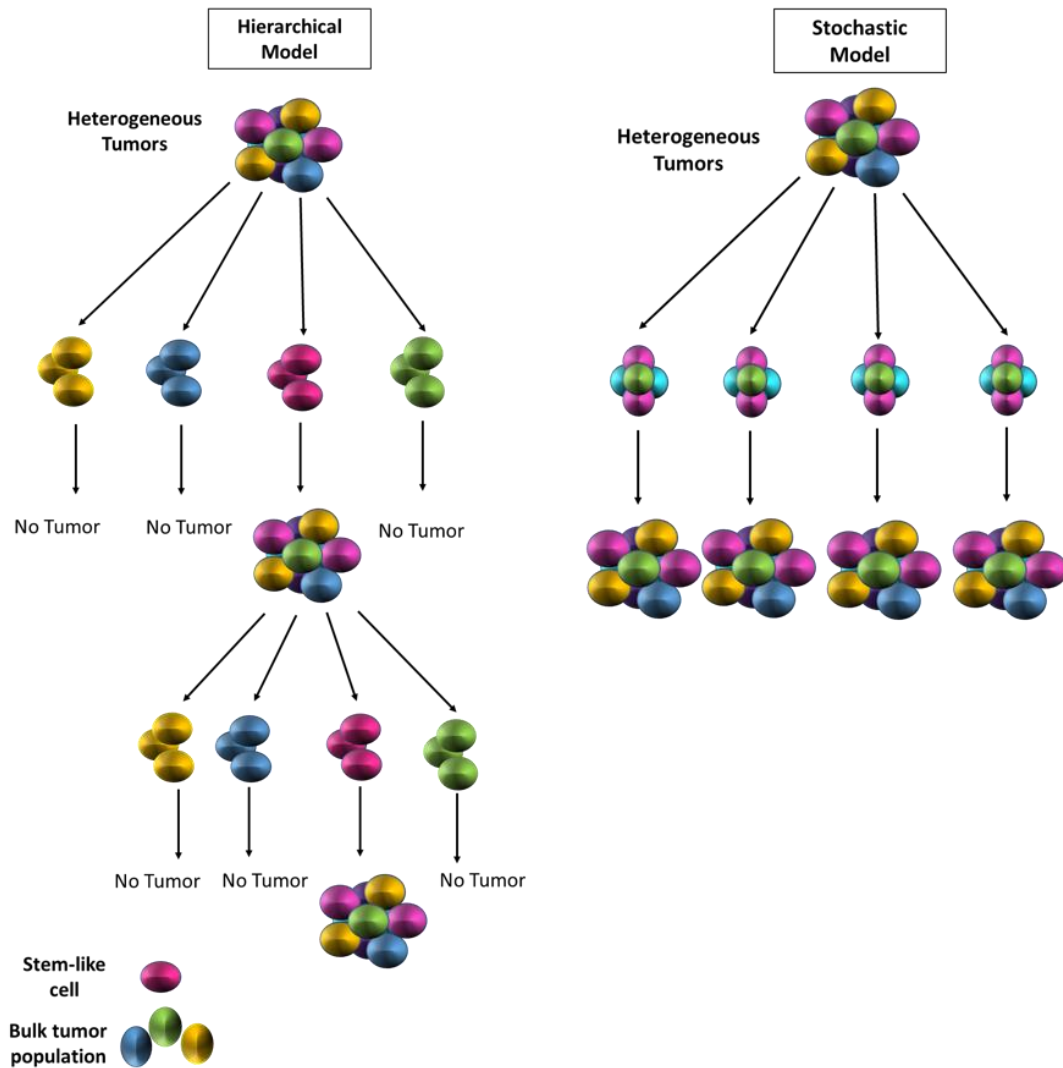


Figure 1: Hierarchical versus stochastic model to explain tumor heterogeneity.

The hierarchical model (left panel) states that tumor heterogeneity is due to the coexistence of various cell populations within a tumor. The bulk tumor is composed of cells that can be effectively targeted by chemotherapy and upon dissociation and serial transplantation in mice, these cells cannot effectively initiate tumors. A minority of cells within a tumor has tumor initiating potential and can be identified by markers used to identify stem cells. These termed stem-like cells can recreate tumors when serially transplanted and even when very low numbers of cells are injected.

Recurrence and metastatic disease therefore occurs by the inadequate targeting of this population. On the contrary, the stochastic model (right panel) assumes that every tumor cell has the same ability to initiate tumors and therefore intrinsic factors will determine which cell will be clonally expanded in order to achieve tumor survival.

Stem-like cells in OS

OS has been shown to follow a CSC model [26-31]. This discovery has paved the way for further identification of markers capable of selecting populations with high tumorigenic potential. Various CSC markers and capabilities, both unique to OS and shared between various malignancies, identify subpopulations of OS cells with stem-like features and tumor initiating potential. Table 1 summarizes key characteristics associated with a stem-like phenotype in OS.

Table 1: Validated markers that identify stem-like cells in OS

Markers	Important Findings	References
CD117 (Mast/stem cell growth factor receptor) and Stro1 (mesenchymal stem cell marker)	<ol style="list-style-type: none">1. Human and murine OS cells that expressed these markers were highly tumorigenic <i>in vivo</i>, highly metastatic and expressed self-renewal potential.2. They also expressed additional markers of stem-like cells like: CXCR4, ABCG2 (ABC transporter for drug efflux), etc.3. Displayed sphere forming ability.	[26, 29]
Aldehyde dehydrogenase enzyme	Cells with high Aldefluor expression	[32]

(ALDH)	in OS were chemoresistant, and displayed high tumor initiating frequency.	
Oct3/4, Sox2 and Nanog	1. Highly tumorigenic, sphere forming and related to metastasis ability	[26, 33, 34]
CD133 (Prominin-1)	<ol style="list-style-type: none"> 1. Overexpress Embryonic stem cell transcription factors 2. Invasive and metastatic 3. Express sphere forming ability 4. Displayed multipotency 	[27, 31, 35-37]
Membrane dye retention PKH26 or PKH67	<ol style="list-style-type: none"> 1. Able to form spheres under limited media 2. Form tumors <i>in vivo</i> under limiting dilution 	[38]
Hoechst-33342 dye excluding side population	<ol style="list-style-type: none"> 3. Display ability to form spheres under limiting dilution 4. Chemoresistant 	[39, 40]

Although OS has been consistently thought of as a malignancy that follows a tumor initiating cell model, recent studies have suggested that this phenotype can be inducible demonstrating that tumor cells can have some degree of plasticity allowing them to adapt to their microenvironment [41]. Similar observations have been made in melanoma [21], and neural tumors [42-44].

ErbB4 and EGFR Family

ErbB4 also known as Her4, is a type I transmembrane protein and receptor tyrosine kinase part of the Epidermal Growth Factor Receptor (EGFR) family. This family is composed of four members: EGFR, ErbB2, ErbB3 and ErbB4 which share a similar structural composition consisting of an extracellular domain that contains a ligand binding site, a transmembrane region and a cytoplasmic domain [45]. Signaling is initiated by ligand binding to the extracellular portion of the receptor, which triggers receptor activation and homo- or heterodimerization with other family members. There are ligands that activate multiple family members (betacellulin, heparin-like binding factor and epiregulin which can bind and activate both EGFR and Her4) and specific members: EGFR (Epidermal growth factor (EGF), amphiregulin, TGF- α), ErbB3 (Neuregulin 1 and 2) and ErbB4 (Neuregulin 3 and 4)[46, 47]. ErbB2 is an exception in the family because although it does contain an extracellular domain in its structure, no ligands or specific ligand binding sites have been identified. Upon activation, the EGFR family undergoes autophosphorylation of specific tyrosine residues that initiate a myriad of signaling cascades. ErbB3 however possesses very little, if any, kinase activity.

Her4 has an added layer of complexity due to exon splicing events that can generate various isoforms with specific cellular functions and differentially expressed within tissues. 4 different juxtamembrane isoforms of this receptor have been described termed Jma, Jmb, Jmc and Jmd (Figure 2). These isoforms differ by splicing of exon 16 that codes for a TACE (Tumor necrosis factor- α converting enzyme, also known as ADAM17) binding site rendering the receptor susceptible to

cleavage by this enzyme [48-50]. Only isoforms Jma and Jmd contain this exon and are therefore the cleavable forms of ErbB4 that allow this receptor to undergo a two-step proteolytic processing. After the extracellular fragment of Her4 is released upon TACE cleavage, the membrane bound receptor of around 100 KD is then the substrate of a second cleavage event by gamma-secretase. A soluble 80 KD fragment of Her4 (s80) is released into the cellular cytoplasm where it can affect many signaling pathways or go into the nucleus to regulate transcription. It is important to note that s80 Her4 can go into the nucleus due to its nuclear localization sequence, however this receptor lacks DNA binding domains. Therefore its effects in regulating transcription are mediated through binding positive and negative regulators of transcription [48, 50, 51].

Additional cytoplasmic isoforms of ErbB4 exist due to further exon splicing events. As represented in Figure 2, the CYT1 isoform contains exon 26, which encodes for a PI3K binding site and can activate both the MAPK and PI3K signaling pathway; the CYT2 isoform on the contrary does not contain this exon and can only activate the MAPK pathway upon receptor activation [52, 53].

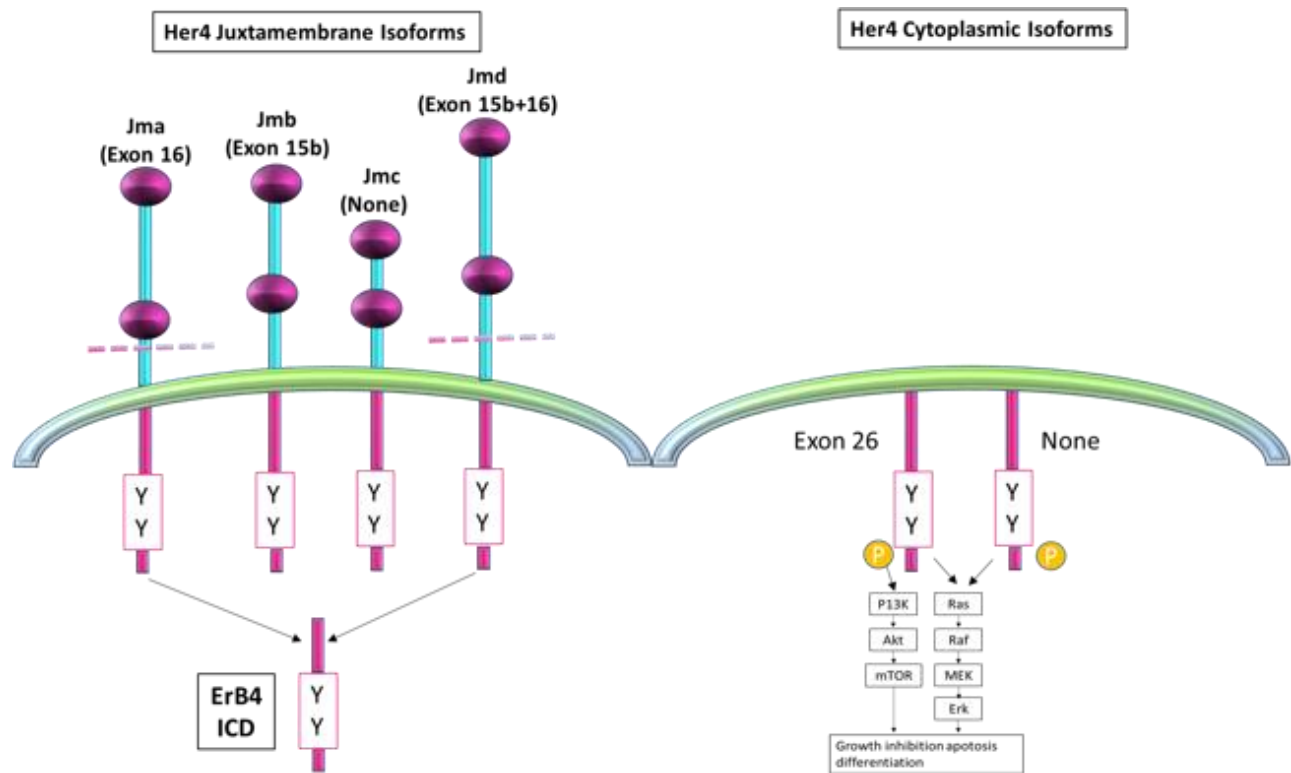


Figure 2: Her4 receptor juxtamembrane and cytoplasmic isoforms. The Her4 receptor can be expressed by a combination of various juxtamembrane and cytoplasmic isoforms that are generated by various splicing events. Expression of exon 16 allows for expression of a TACE cleavage site, allowing the receptor to release its extracellular domain and generating a membrane bound fragment that can be further cleaved by gamma-secretase. This leads to the release of a soluble 80 KDa cytoplasmic fragment that can go to the nucleus and act as a chaperone affecting transcription. This exon is only expressed in Jma and Jmd isoforms. Cytoplasmic isoforms differ by the expression of exon 26 of Her4 which codes for a PI3K binding domain. This exon is contained in the Cyt1 isoform, allowing signaling through both PI3K and MAPK signaling pathways. The Cyt2 isoform lacks this domain and can only mediate MAPK signaling.

Her4 in cancer and OS

Early reports on Her4 have been contradictory showing both tumor suppressor and oncogenic features on the same malignancy. One example of these incongruent observations has been Her4 expression in breast cancer. Some reports show that survival is markedly improved in patients that have Her4 positive tumors, while others point at Her4 as an oncogene with overexpression conferring poor

survival [46, 51, 54]. These seemingly disparate results have been explained by understanding the specific roles of the different Her4 isoforms during carcinogenesis. Studies comparing cleavable JmaCyt2 versus non-cleavable JmbCyt2 demonstrated a role in survival for Jma versus a role in cell death for Jmb. In addition, the cytoplasmic isoforms of the receptor have opposing effects with CYT1 promoting growth inhibition and CYT2 involved in proliferation. Also, the CYT2 isoform has a greater kinase activity than CYT1 and it is preferentially translocated to the nucleus [55].

Although Her4 has been mostly studied on breast cancer, other malignancies have demonstrated the important role this receptor plays in tumorigenesis. Some examples are in neuroblastoma [56], melanoma [57], Ewing sarcoma [58, 59] and more recently in lung cancer [60]. Particularly in Ewing sarcoma, reports from Poul Sorensen's group showed that Her4 expression mediates chemoresistance and survival under anchorage independence. In this model, Her4 is upregulated in response to cell-cell interactions modeled by anchorage independence that activate E-cadherin and Her4 expression in turn promotes PI3K signaling and survival. In this malignancy, Her4 expression has also been associated with reduced disease-free survival. Similar observations were published by our department in neuroblastoma [56].

Therefore, Her4 expression in various malignancies seems to be important to promote resistance to chemotherapy, anoikis and promotes development of metastasis.

Hypothesis

Because Her4 expression is thought to be necessary for the survival of tumorigenic cells when they are under various stressors including chemotherapy, anchorage independence and serum starvation [56, 58] and these conditions are used to enrich cancer cells with stem-like features, we sought to determine whether Her4 expression in OS conferred a stem-like phenotype.

We hypothesized that Her4 plays an important role in OS, causing aggressive and metastatic disease in part by regulating the expression of stem-like characteristics in OS.

Chapter 2: Materials and Methods

OS cell culture

Human OS cell lines CCHD, CCHK, CCHO, CCHM, Maos, HOS, MG63, SJSA, U2OS, SaOS2 were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad , CA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan ,UT) and 1% penicillin/streptomycin (Gemini Bio-Products, Woodland, CA). Cells were incubated at 37°C with 5%CO₂ and were kept in a humidified atmosphere. MG63, SaOS2, Hos, U2OS, SJSA were purchased from the American Type Culture Collection (ATCC, Manassas, VA). CCHD, CCHO, CCHM and CCHK are OS cell lines obtained from patients at the Children's Cancer Hospital at the University of Texas MD Anderson Cancer Center. Maos was derived from a patient at the University of Michigan. CCHD was obtained from a pretreatment biopsy of a proximal femoral lesion in an 18-year-old male with pulmonary metastases upon diagnosis. CCHO was obtained from a hip lesion of a 22-year-old male who presented T5 spinal metastasis. Unfortunately, additional information regarding the origin of remaining patient derived cell lines (CCHM, CCHK and Maos) is not available. Cells were routinely passaged upon reaching confluency of around 80% using trypsin (TrypLE Express, Invitrogen, Carlsbad, CA).

Sarcosphere Assay

Sarcosphere culture using OS cell lines was performed as previously described [30] [32] with some modifications . Briefly, 60,000 cells per well of 6-well poly-Hema (poly 2-hydroxyethyl methacrylate, Sigma, St. Louis, MO) coated plates were seeded and supplemented with DMEM/F12 (Gibco), B27 supplement (Life Technologies, Waltham, MA), 50 ng/uL Epidermal Growth factor (EGF, Life

Technologies) and 50 ng/uL basic Fibroblastic Growth factor (bFGF, Life Technologies). Sarcospheres were supplemented EGF and bFGF every other day.

Flow cytometry

To measure the expression of surface markers Stro1, CD117 and Her4, OS cell lines in monolayer culture were washed with Phosphate Buffered saline (PBS, Gibco) and detached from culture plates using enzyme-free cell dissociation buffer (Gibco). Cells were counted using Trypan blue exclusion in a Vi-Cell automated cell counter (Beckman Coulter). For single cell dissociation, sarcospheres were collected, washed once in PBS and enzyme-free cell dissociation buffer was added. Cells were pipetted gently up and down until a uniform cell suspension without aggregates was observed. Cells were counted manually using a hemacytometer and trypan-blue (Thermo) exclusion. Cells were suspended in blocking buffer consisting of PBS, 10% mouse serum (Thermo) and 5mM EDTA (Life Technologies) and the following antibodies were added: anti-human Stro1 PE or anti-human Stro1 PerCP/Fitc (Santa Cruz Biotechnology, Santa Cruz, CA), anti-human CD117 PeCy7 (eBioscience, San Diego, CA) and anti-human Her4 APC (Novus Biologicals, Littleton, CO). Samples were stained for 30 minutes in the dark and on ice and were later washed twice with blocking buffer and either analyzed immediately using the BD LSRFortessa cell analyzer (BD Biosciences) or fixed in 1% paraformaldehyde (Electron Microscopy Services) and fixed for later analysis. Analysis was performed using FlowJo software (Tree Star Inc.).

Immunofluorescence staining

OS sarcospheres were grown as described for 4 days. At this time, sarcospheres were collected, washed twice with PBS and incubated in a solution containing 0.1% Triton (Thermo Scientific) in PBS for 20 minutes at room temperature to achieve permeabilization. Spheres were washed twice with PBS and incubated for one hour at room temperature in a protein block solution containing PBS, 10% goat serum (HyClone) and 0.1% Triton. Following blocking of unspecific binding, primary antibodies were added at the following dilutions: anti-human Stro1 (Santa Cruz Biotechnology, 1/100); anti-human Her4 (abcam HFR-1, Cambridge, MA, 1/200); anti-human CD117 (Stem Cell Technologies, Cambridge, MA, 1/100) and were left incubating overnight at 4 degrees Celsius. Sarcospheres were washed three times in PBS and secondary antibodies conjugated to Alexa-Fluor 488 (CD117 or Stro1) or Texas Red (Her4) were added and incubated at room temperature for 1.5 hours in the dark. Cells were washed three times in PBS; one drop of DAPI was added (Thermo Fischer) and sarcospheres were transferred to slides and visualized using a Leica Fluorescent microscope.

Western Blot

Whole cell lysates from human OS cell lines were obtained as follows: cells were detached from culture plates using cell scrapers (Falcon) and washed twice with cold PBS. Cell pellets were obtained by centrifugation at 13,000 rpm for 5 minutes. These pellets were resuspended in lysis buffer containing: 25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, 5% Glycerol, phosphatase inhibitor cocktail (Sigma) and protease inhibitor tablets (Roche Diagnostics). Cell pellets were

incubated with lysis buffer at 4 degrees Celsius with constant rotation for 30 minutes. Then, were centrifuged at 13,000 rpm for 10 minutes and supernatant was collected. Protein concentration was quantified by BCA assay (Thermo Scientific) using albumin standards to generate a standard curve. Equal lysate concentration between samples was loaded on 8% SDS-PAGE gels to allow protein separation. Proteins were transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen). Full-length Her4 expression was detected by incubating nitrocellulose membranes with anti-human Her4 antibody (Abcam) at a dilution of 1/1000 in 5% Bovine serum albumin (BSA, Thermo Scientific) in TBST. Anti-rabbit secondary antibody conjugated to horseradish peroxidase was used. Pierce ECL (Thermo Scientific) western blotting substrate was used to detect chemoluminescent signal.

Aldefluor Assay

Aldefluor kit (Stem Cell Technologies) was used to measure the activity of aldehyde dehydrogenase enzyme and the assay protocol provided was followed. Briefly, after detaching cells from plastic culture dishes, cells were counted and one million cells were aliquoted in a separate tube. Cells were centrifuged, media was removed and the remaining cell pellet was resuspended in 1mL of Aldefluor Assay buffer and placed in a micro centrifuge tube. In a separate micro centrifuge tube 5uL of the DEAB control were aliquoted. The cell suspension was added 5uL of the Aldefluor substrate, immediately mixed by pipetting and 500uL of the cell suspension were added to the DEAB control. Both microcentrifuge tubes were incubated at 37 degrees for 30 minutes, then centrifuged at 1.4 rpm for 5 minutes. Supernatant was

discarded and the cell pellet was resuspended in 300uL fresh Aldefluor Assay buffer and analyzed using the BD LSRFortessa.

Real-time Polymerase Chain Reaction (RT-PCR)

To determine the expression of embryonic stem cell transcription factors in OS cell lines with either Her4 knockout or overexpressing constructs, polymerase chain reaction (PCR) was used. Total RNA was extracted from OS cell lines using the RNeasy Mini Kit (Qiagen) and cDNA was prepared through reverse transcription using the Omniscript Reverse Transcriptase Kit (Qiagen) supplemented with oligo(dT)s (Invitrogen) and RNase inhibitor (New England Biolabs) and according to the instructions provided. Real-time PCR was performed using the Lightcycler 480 (Roche) using relative quantification analysis and the following Taqman probes (Applied Biosystems): Nanog: Hs04260366_g1; Sox2 : Hs01053049_s1 and Oct4: Hs04260367_gH. PCR reactions were prepared using Taqman gene expression master mix (Applied Biosystems) and following the provided protocol.

Statistical Analyses

Triplicate samples were analyzed in each experiment and every experiment was performed at least three times unless otherwise specified. Statistical analysis was performed using Student's t test (GraphPad Software Inc.). Assessment of survival curves was done with log-rank test. P-values of <0.05 were considered significant.

Chapter 3: Her4 is highly expressed and a possible prognostic factor in OS

Rationale

Previous studies have demonstrated the importance of the Her4 receptor in mediating an aggressive phenotype in many malignancies [46, 58, 59, 61-64]. Moreover, pediatric cancers like Ewing sarcoma and neuroblastoma have increased Her4 expression and this correlates with resistance to chemotherapy and metastatic disease [58, 59]. Expression of the EGFR family has been reported in OS and among the family, Her4 is frequently highly expressed in both primary and metastatic OS lesions [65, 66]. However, no data is available specifically correlating survival among OS patients that express high Her4 expression in their primary tumors. In addition, no specific data is available regarding prevalence of the various Her4 isoforms in OS. Therefore, we pursued to understand how Her4 affects survival of OS patients and specifically which isoforms are differentially expressed in patient-derived and commercially available OS cell lines.

Results

Her4 expression correlates with decreased metastasis free survival

In order to determine correlation between Her4 expression and OS patient survival we analyzed the Mixed Osteosarcoma Kuijjer database set [67, 68] using the R2 Genomics Analysis and Visualization platform. This publicly available online tissue microarray contains gene profiles of 84 primary diagnostic osteosarcoma biopsies. We generated Kaplan-Meier survival curves with Her4 high (n=40) and low (n=28) expressing patient biopsies that did not present with metastasis at diagnosis and that were followed for development of metastasis over a period of 240 months (20 years). Cutoff for high and low Her4 expression was determined by comparison with osteoblasts and mesenchymal stem cells used as controls. From the generated curves we observed that patients with high Her4 expression had a significantly decreased metastasis free survival that remained steady at 50% after 48 months (Figure 3). In comparison, patients with low Her4 expression had a significantly higher metastasis free survival probability of around 90%.

Metastasis free survival

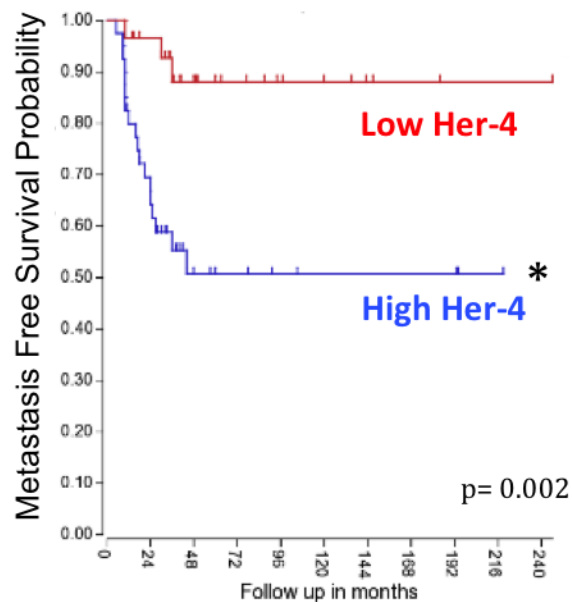


Figure 3: Metastasis free survival is decreased in OS patients with high Her4 expression. The R2 Genomics Analysis and Visualization platform (Academic Medical Center; <http://r2.amc.nl>) was used to create Kaplan-Meier metastasis-free survival curves using the 'Mixed Osteosarcoma - Kuijjer - 127 - vst - ilmnhwg6v2' dataset which comprises genome-wide analysis of 84 high-grade osteosarcoma diagnostic biopsies. These primary tumors were analyzed for Her4 high and low expression using osteoblasts (n=3) and mesenchymal stem cells (n=12) as controls. The R2 generated "scan" cut-off modus was used to determine the threshold point that most significantly separates high relative gene expression and low relative gene expression. * $p \leq 0.05$.

Her4 juxtamembrane isoforms are variably expressed in OS cell lines

Next, we wanted to determine the expression of the different juxtamembrane isoforms of the Her4 receptor in OS. As previously discussed, the complex processing of this receptor can generate four different juxtamembrane isoforms of which only two (Jma and Jmd) can undergo a two-step proteolytic cleavage event releasing a soluble Her4 cytoplasmic fragment. This 80KDa peptide can then localize to the nucleus to regulate transcription by binding several transcription factors like STAT5, YAP1 among others [48-50]. We previously showed that Her4 is

highly expressed and primarily localized to the cytoplasm in patient-derived tumor biopsies [65, 66]. However, we were lacking information on specific isoform expression. To have more information regarding the abundance of the juxtamembrane isoforms of the Her4 receptor, we performed absolute quantification PCR using primers specifically designed to distinguish between the different cleavable and non-cleavable isoforms. We noted from this experiment that compared to established commercially available OS cell lines, 4 out of 5 patient-derived OS cell lines have a higher copy number of Her4 overall (Figure 4). In addition, these cell lines with high Her4 express preferentially the cleavable juxtamembrane isoform Jma.

Copy Number of Her4 juxtamembrane isoforms in OS cell lines per 1 million copies of GAPDH

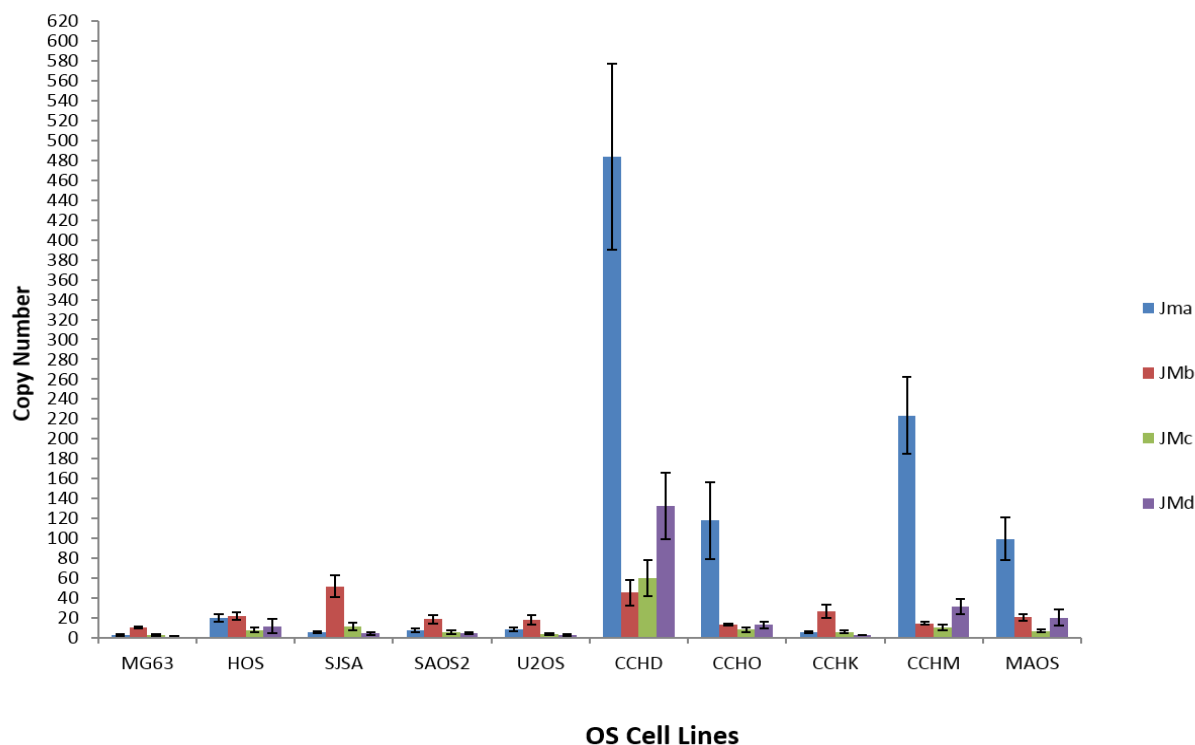


Figure 4: OS cell lines have a variable expression of Her4 with the cleavable isoform Jma preferentially expressed. Quantitative real-time PCR was performed using standard curve analysis to determine copy number of the various juxtamembrane isoforms of the Her4 receptor. Patient derived cell lines CCHD, CCHO, CCHM and Maos have a higher copy number overall of the Her4 receptor compared to established OS cell lines MG63, HOS, SJSA, Saos2 and U2OS. Furthermore, in cell lines that express high Her4 copy number the most prevalent isoform is Jma, which contains exon 16 and encodes for a TACE cleavage site. This data suggests that Her4 expression in patient derived cell lines goes in accordance with Her4 expression in OS patient diagnostic biopsies.

Summary

Although reports of Her4 expression in OS showed increased expression of this receptor in patient biopsies, specific correlation on patient outcome and which specific isoform is preferentially expressed was lacking. For this purpose, we performed Kaplan-Meier survival curves using gene array data from primary OS biopsies and found that Her4 expression correlates with patient outcome indicating that Her4 may be a prognostic factor. Patients with high Her4 expression had a higher probability of developing metastasis than those with low expression of this receptor as shown in Figure 3. This data has translational applicability since current assessment on prognosis for OS patients is done after 12 weeks of induction chemotherapy where evaluation of tumor necrosis takes place [1, 9]. However, with Her4 expression being an important determinant for development of metastatic disease, patients that present Her4 upon diagnosis can be treated more aggressively under the expectation that their tumors are likely to metastasize.

Also, we identified the cleavable juxtamembrane isoform Jma to be preferentially expressed in cell lines derived from patient biopsies compared to established OS cell lines. This supports a potential role of intracellular Her4 signaling in OS and is consistent with previously published data showing that Her4 expression in OS tumors is primarily localized to the cytoplasm.

Chapter 4: Her4 is induced by sarcosphere culture and leads to up-regulation of markers that identify OS cells with a stem-like phenotype

Rationale

Tumor cells able to form distant metastatic lesions need to withstand adverse environments on the road to metastasis [69, 70]. One important aspect of metastasis is anoikis, which is described as the ability of cancer cells to grow effectively under anchorage independence. *In vitro*, this capacity is studied by growing cancer cells without allowing them to adhere to plastic surfaces [71]. Under these conditions, Her4 is upregulated in two pediatric malignancies, Ewing sarcoma and neuroblastoma. These studies showed that cells that have Her4 expression besides forming spheroid structures under anchorage independence, are highly chemoresistant and metastatic compared to those that do not express this receptor [58, 59].

Furthermore, survival under anchorage independence has become a staple of cells with tumor initiating potential or stem-like cells [26, 72]. Subpopulations of cells thought to be stem-like display many distinct attributes including, but not limited to, growth as spheres under anchorage independence with limited nutrient media, expression of markers of pluripotency, expression of markers associated with normal tissue stem cells, high aldehyde dehydrogenase activity and high expression of ABC transporters leading to high drug clearance and increased chemotherapy resistance [33, 44, 73-75].

Since Her4 expression in select pediatric malignancies leads to various attributes that characterize tumor initiating cells *in vitro*, we wanted to determine whether Her4 expression in OS correlates with expression of validated makers used to characterize and isolate OS stem-like cells.

Results

Sarcosphere culture induces Her4 expression

In Ewing sarcoma cell lines, growth in suspension causes an increase in E-cadherin signaling which ultimately leads to activation of Her4 as a downstream effector [58]. Similar to these findings, we were able to observe Her4 upregulation in patient derived OS cell lines after just 4 days in sarcosphere culture, as shown in Figure 5. For CCHO, Her4 upregulation was observed after 7 days in spheres. However, unlike previous studies focusing on anchorage independence only, our sarcosphere culture conditions were more strict and restrictive and included defined media without fetal bovine serum and supplemented with essential vitamins, minerals, EGF and FGF (see materials and methods section). These sarcosphere conditions have been described as stressors that allow selection and enrichment of cells with tumor initiating capabilities [26, 30]. In addition, *in vitro* sarcosphere formation is accepted as a more representative model of actual disease in cancer patients than monolayer culture [71]. Therefore, this data validates a possible significance of Her4 in stem-like populations in OS. Since OS stem-like cells are more metastatic and chemoresistant than cells that do not express these traits [26, 29, 30], correlation of Her4 with a stem phenotype in OS provides a feasible mechanism for our observations of Her4 as a prognostic factor in OS.

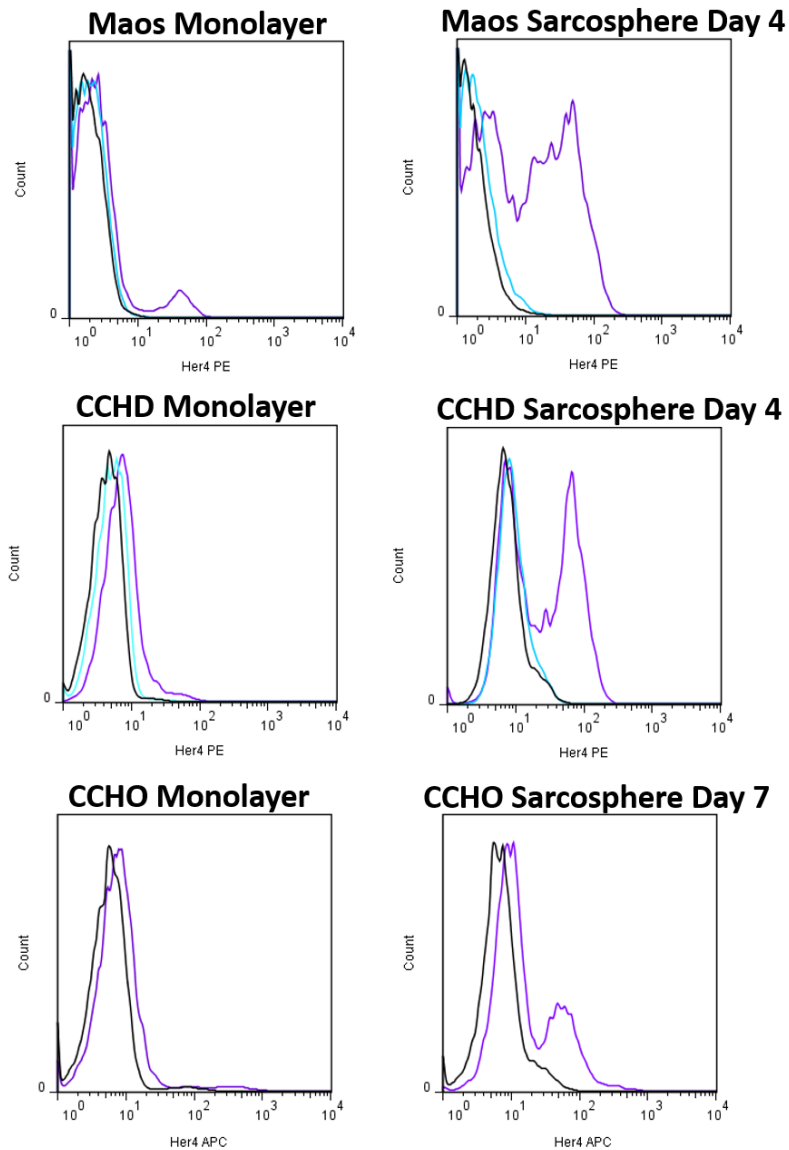


Figure 5: Her4 is inducible by sphere culture. Patient derived cell lines Maos, CCHD and CCHO were stained with Her4 antibody for flow cytometry analysis both in monolayer and sphere culture. Maos and CCHD cells show upregulation of Her4 after 4 days under anchorage independence and limited media. CCHO upregulates Her4 after seven days in these conditions.

Her4 upregulation in spheres precedes upregulation of stem-like markers Stro1 and CD117

Subpopulations of cells with Stro1 and CD117 expression in OS have a higher metastatic potential and can form serially transplanted tumors in very low numbers recapitulating the initial tumor heterogeneity [29]. Therefore, since these markers have been specifically studied in OS cells with stem-like phenotype, we sought to determine whether there was a correlation between Her4 expression and expression of these markers. For this, we performed flow cytometry staining for Her4 and stemness markers Stro1 and CD117 on various OS cell lines in monolayer culture and at different time points in sarcosphere. This experiments allowed us to observe specifically when Her4 upregulation occurs relative to upregulation of these markers. As Figure 6 shows, Her4 expression is consistently induced early after cells are placed under sarcosphere conditions but Stro1 expression occurs at later time points and mostly in cells that are Her4 positive. We also performed immunofluorescence staining on CCHD sarcospheres and observed that Stro1 expression was limited to cells that co-expressed Her4 (Figure 7).

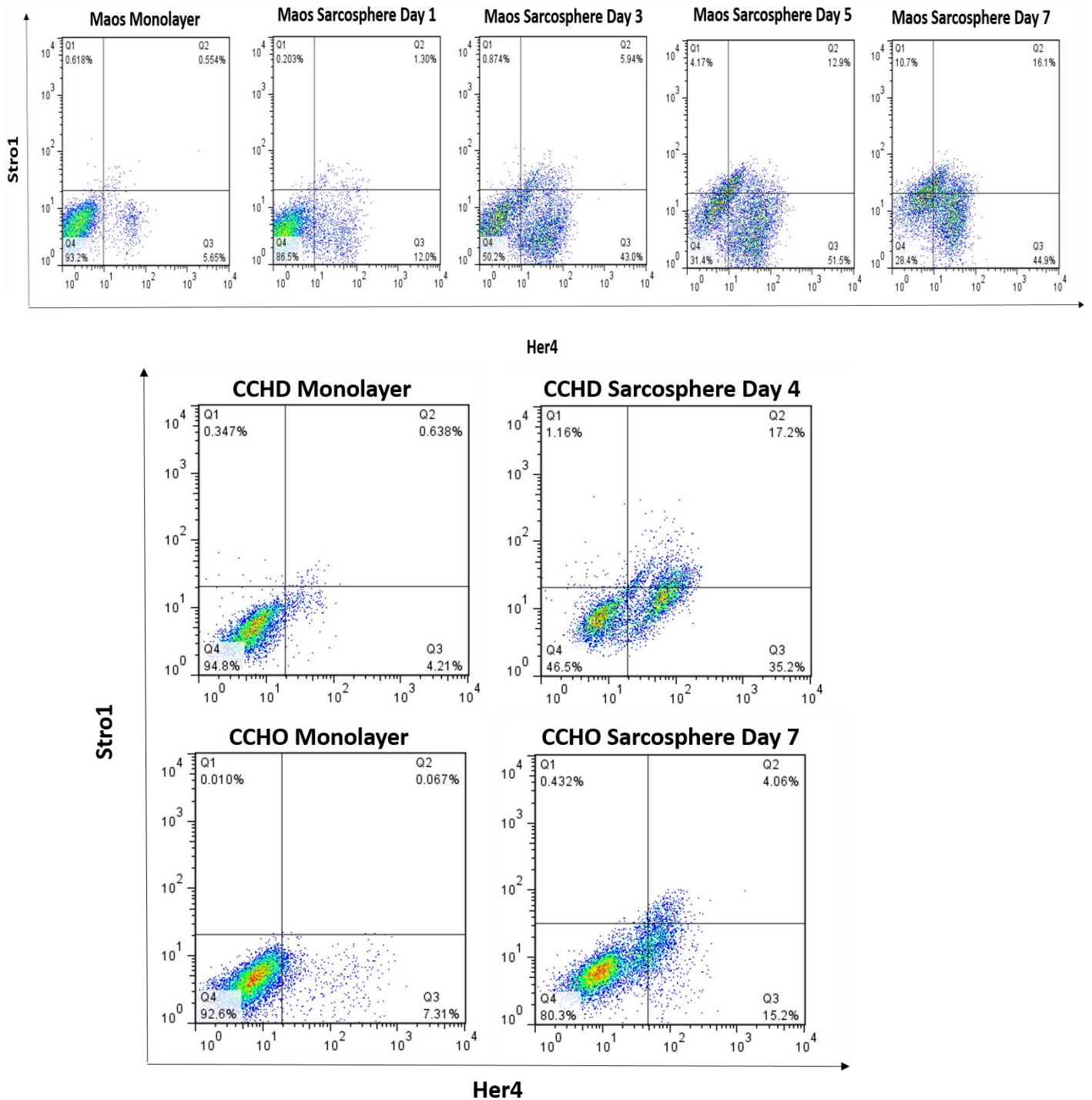


Figure 6: Her4 expression in OS sarcospheres is induced prior to expression of OS stemness marker Stro1. Maos OS cells were placed under sarcosphere culture and at day 1, 3, 5 and 7 cells were collected, dissociated into single cells, stained for Her4 and Stro1 and analyzed by flow analysis. In spheres, Her4 is induced at day 3 of sphere culture but Stro1 expression is not increased until day 7 (top panel). CCHD and CCHO show similar findings at day 4 and day 7, respectively (bottom panel).

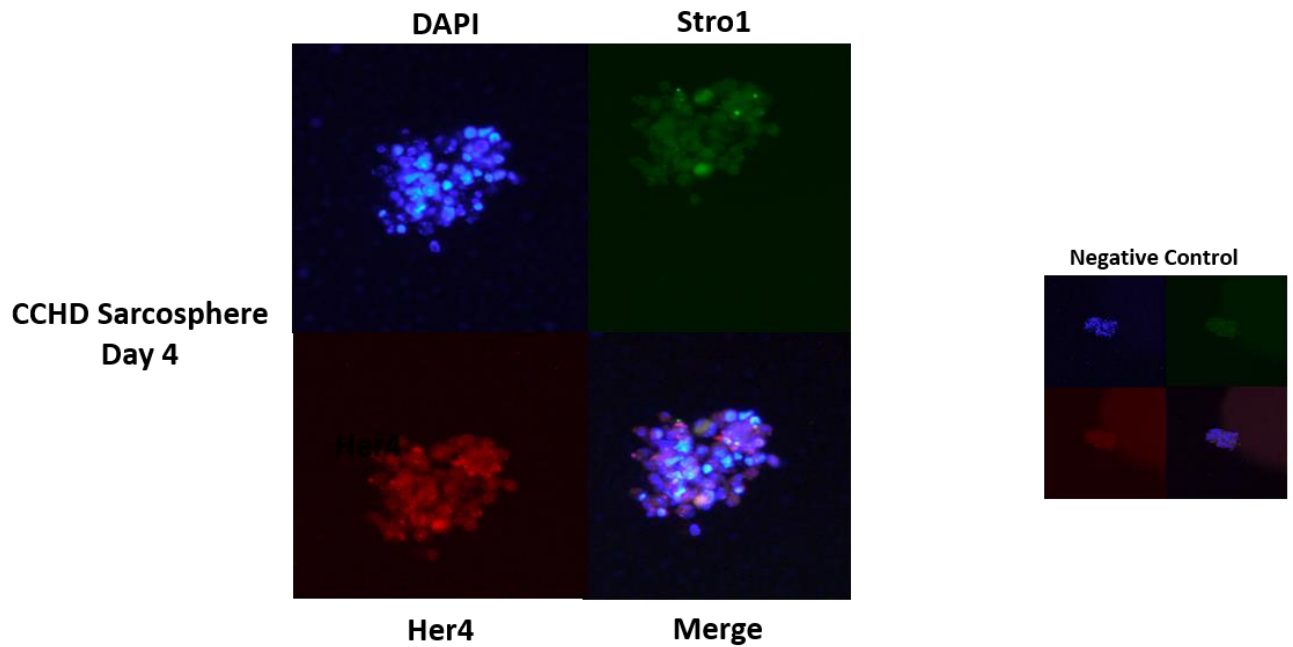


Figure 7: Stro1 expression co-localizes to Her4 positive cells in sarcospheres. Immunofluorescence staining was performed for Stro1 and Her4 expression on CCHD OS cells after 4 days in sarcosphere culture. Her4 expression was upregulated compared to Stro1. However, Stro1 expression was only found on Her4 positive cells.

After these observations, we decided to analyze the expression of Stro1 specifically gating on Her4 positive and negative cells. Figure 8 shows representative data for cell line CCHO. As expected, cells with higher expression of Her4 were also Stro1 positive. Similar results were obtained for CCHD (not shown).

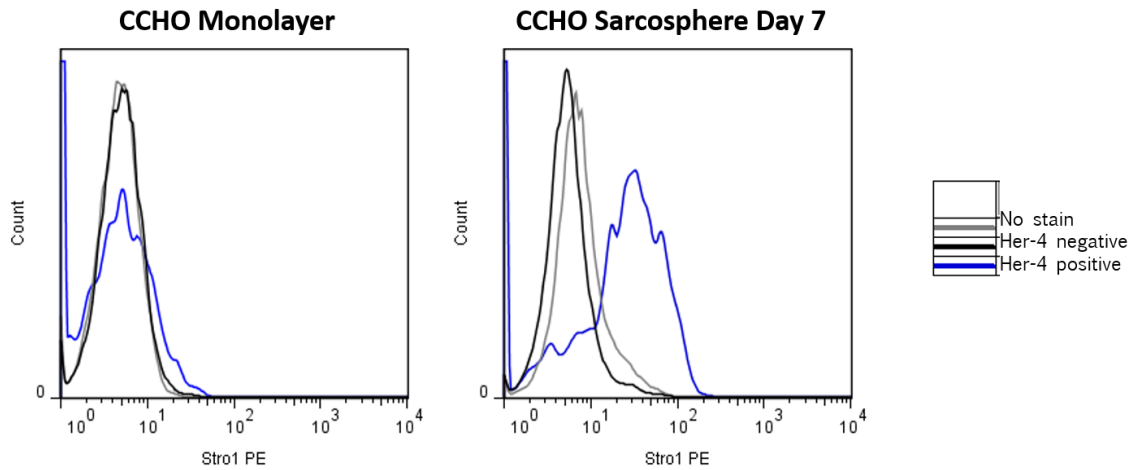


Figure 8: Her4 positive cells are also Stro1 positive. Representative data for cell line CCHO is shown. Positive cells for Her4 expression were gated using the isotype control as a negative Her4 population. Then, histograms were created for both Her4 positive and negative populations showing increased Stro1 expression among Her4 positive cells. Similar findings were observed with CCHD (data not shown).

We also performed similar experiments comparing Her4 to CD117, an additional marker of stem-like cells in OS, using the CCHO OS cells and observed similar findings (Figure 9).

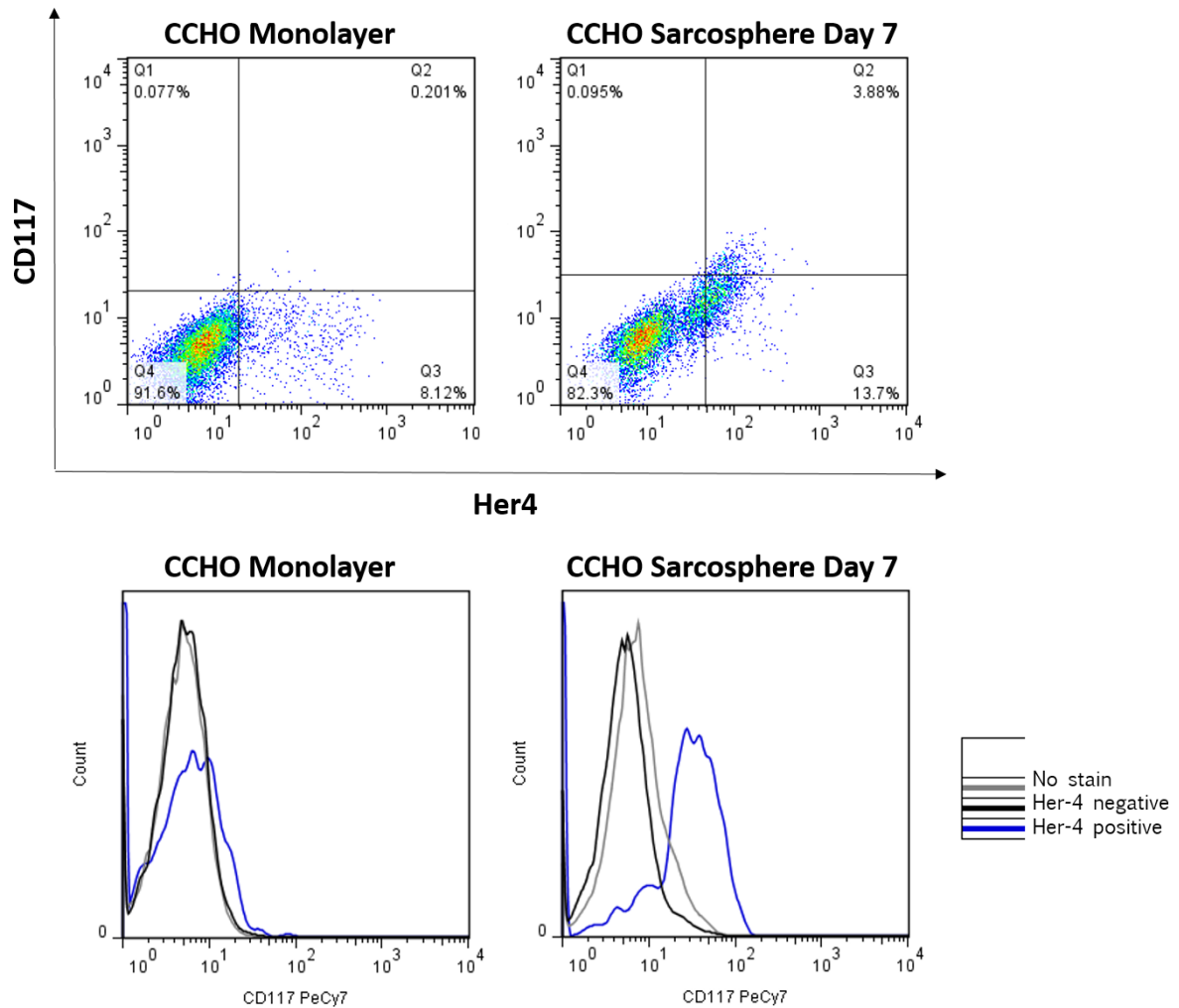


Figure 9: Her4 expression in sarcospheres correlates with CD117 expression. OS cell line CCHO was analyzed by flow cytometry after staining with Her4 and CD117 antibodies seven days after seeding under sarcospheres. There is a distinct population of cells that upregulate both Her4 and CD117 (top panel). Gating on Her4 positive and negative populations in CCHO demonstrates that CD117 positive cells are also Her4 positive (bottom panel).

Summary

A variety of surface markers have been identified to effectively characterize cells with tumor initiating potential in OS (see Table 1). Stro1 expression has been observed on mesenchymal stem cells and particularly in OS, this marker has been shown to be preferentially expressed on cells with tumor initiating potential, generating aggressive tumors *in vivo* with metastases to the lung [29]. CD117 is also expressed on stem-like cells in OS and double expression of these markers has been published to effectively characterize murine OS cells with a stem-like phenotype, although Stro1 expression is more relevant in human OS cell lines [29].

In this chapter, we have demonstrated that Her4 is upregulated under sarcosphere culture conditions used to enrich stem-like cells in OS. Different OS cell lines upregulate Her4 at various time points ranging from 4 to 7 days. One common finding was that when cells were double stained for Her4 and Stro1 or CD117, cells that express any of these markers became Her4 positive before expressing them. To our knowledge, this is the first study to link expression of the Her4 receptor to markers identifying stem-like populations.

Chapter 5: Genomic deletion of Her4 using CRISPR/Cas9 negatively impacts expression of stemness markers in OS

Rationale

To understand the specific contributions of the Her4 receptor in OS we designed CRISPR (Clustered Regularly Interspaced Palindromic Repeats) /Cas9 constructs targeting this receptor. The CRISPR/Cas9 system was discovered in bacteria and has been successfully implemented in molecular biology due to its convenience and stability [76]. Briefly, the CRISPR/Cas9 system works by inserting a vector in target cells containing a guide RNA sequence and Cas9 DNA. When the Cas9 protein is expressed it recognizes the guide RNA and the flanking palindromic repeats of the target DNA to bind and make double strand breaks around the gene of interest [76]. When the cell tries to repair these breaks it generates premature stop codons, not allowing the process of translation to take place or translating an aberrant non-functional form of the protein of interest. In addition, since this system works at the genomic level, the changes are considered stable and permanent [76].

Since Her4 has been shown to play an important role in survival under adverse culture conditions and stressors including anoikis and confers metastatic capability in pediatric malignancies and we observed an increased expression of Her4 in conditions used to enrich a stem-like population in OS (see Chapter 4), we wanted to decipher the specific contributions of this receptor in a stem-like phenotype in OS. In this chapter, we focused on exploring how different validated OS markers used to identify cells with tumorigenic potential are affected after Her4 knockout.

Generation of Her4 CRISPR/Cas9 clones

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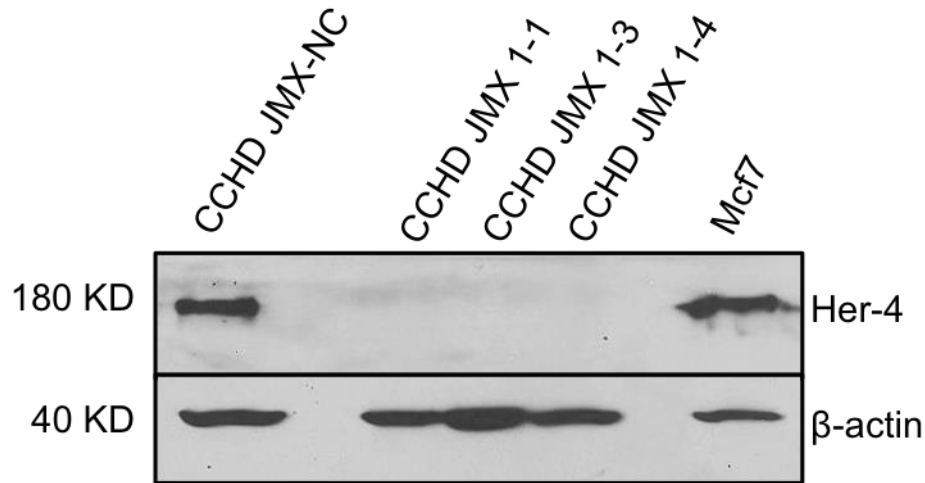


Figure 11: CRISPR constructs targeting Her4 show high knockout efficiency. CRISPR/Cas9 vectors were transfected using lentivirus into CCHD. Parental cell line with guide RNA not targeting any specific gene is labeled Nonsense control (NC). Three different clones are depicted above (JMX-1-1, 1-3, and 1-4). Mcf7 is a breast carcinoma cell line and Her4 positive control. 60ng of protein lysate were loaded.

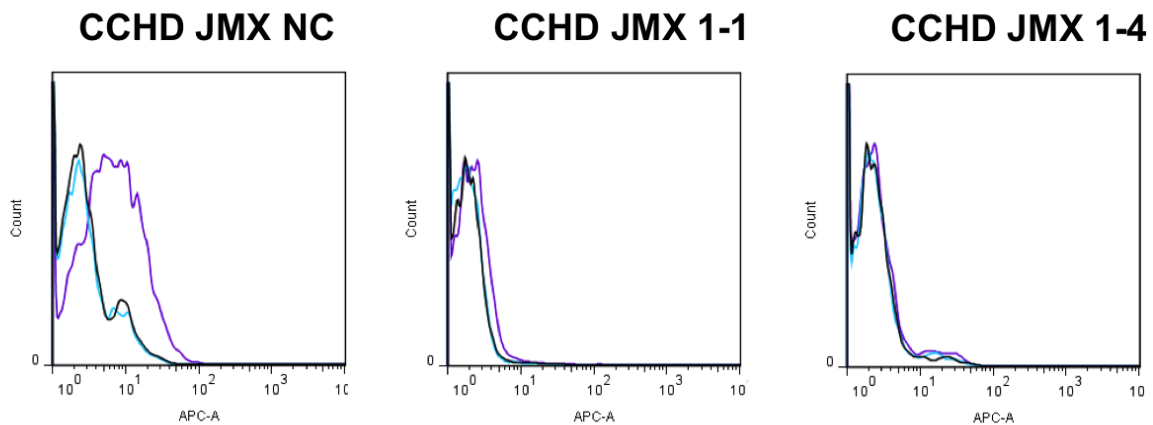


Figure 12: Her4 knockout verification by flow cytometry corroborates effective deletion. Using an antibody that specifically recognizes Her4 surface expression we observed that this receptor was efficiently deleted at the genomic level. Black lines show unstained negative control, blue is isotype and purple is Her4 APC expression level.

Surface expression of tumor initiating cell markers is not affected by Her4 knockout

Next, we wanted to determine whether Her4 knockout affected the expression of validated surface markers for OS stem-like cells. For this, we used flow cytometry and measured the level of stem-like markers CD117 and Stro1 together with Her4 expression in CRISPR clones 1-1 and 1-4 compared to nonsense control. Figure 13 shows that in monolayer culture the expression of these markers is so low that there is not an obvious effect of genomic deletion of Her4. Therefore, we proceeded to generate sarcospheres for both nonsense control and CRISPR clones in order to determine whether changes in expression of these markers occur after sarcosphere culture induces its expression, as has been previously demonstrated [29]. While sarcospheres were formed by the CRISPR clones, upon dissociation of these CRISPR spheres into single cells most of these cells are non-viable. We interpret this to mean that Her4 expression is necessary for cells to be able to withstand dissociation from spheres.

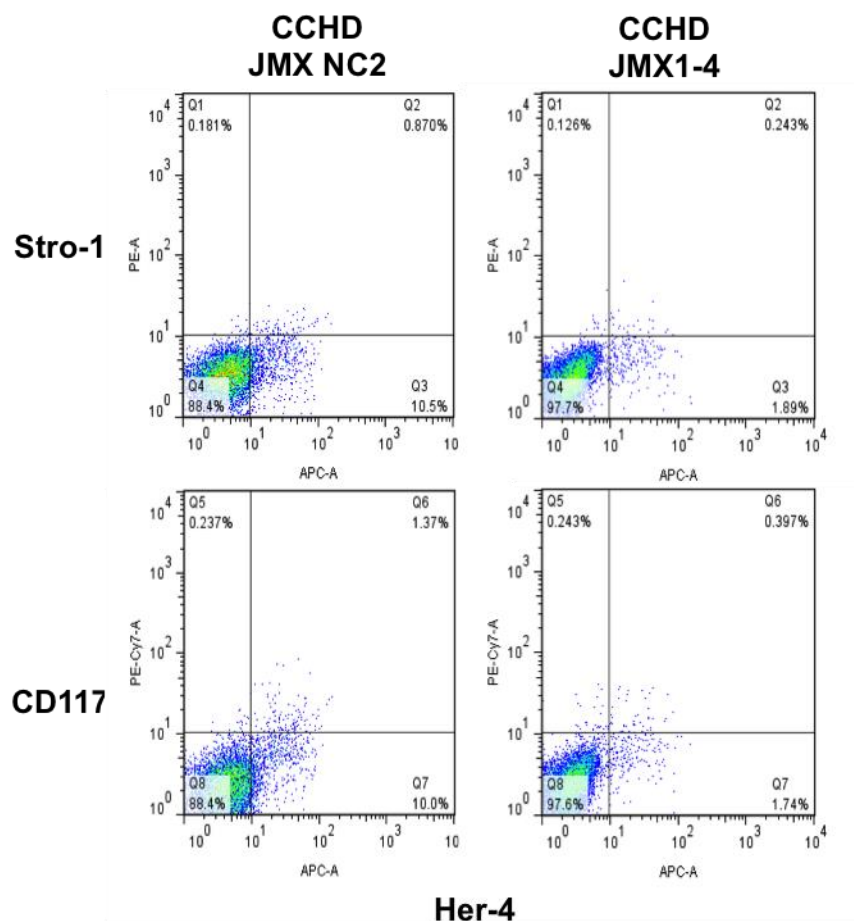


Figure 13: Expression of OS stemness markers CD117 and Stro1 is not affected in monolayer culture after Her4 knockout. Flow cytometry staining of CCHD nonsense control and CCHD CRISPR clone 1-4 does not show significant effect of Her4 knockout in stem cell marker expression since basal levels of these markers are extremely low. Attempts to obtain single cell suspensions after sarcosphere culture in Her4 knockout clones were unsuccessful due to low cell viability.

Her4 knockout mitigates Aldehyde dehydrogenase activity

Aldehyde dehydrogenase (ALDH) activity has been studied extensively as a marker for cancer cells with tumor initiating potential in many tumor types, including OS [32, 77, 78]. It has also been validated as a marker for OS stem-like cells and it confers tumor cells with an increased detoxifying capability making them more resistant to chemotherapy and more tumorigenic [32]. We therefore analyzed the

activity of this enzyme in our control and CRISPR clones by measuring the percentage of Aldefluor bright cells after addition of the Aldefluor substrate. Figure 14 and Figure 15 demonstrate that the percentage of Aldefluor bright cells in the CRISPR Her4 clones CCHD 1-1, 1-3 and 1-4 is significantly decreased compared with the CCHD nonsense control cells. In addition, we also looked at the activity of this enzyme after growth under sarcosphere conditions and observed that CCHD nonsense control cells are able to slightly increase their Aldefluor bright population whereas the Her4 CRISPR clone JMX 1-1 cannot (see Figure 16).

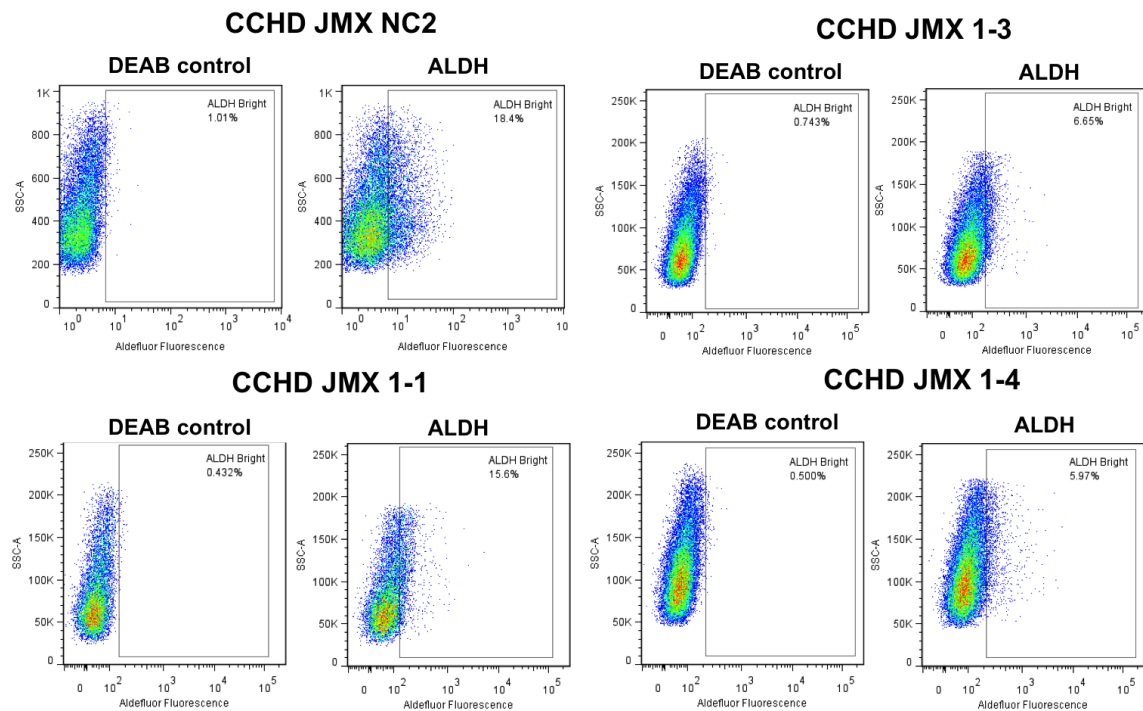


Figure 14: Aldehyde dehydrogenase activity is decreased after deletion of Her4. Using Aldefluor fluorescence as a surrogate for aldehyde dehydrogenase activity, we were able to observe that Her4 CRISPR knockouts had a diminished percentage of cells with high activity of this enzyme after addition of a fluorescent substrate, which can only be processed by isoforms of ALDH.

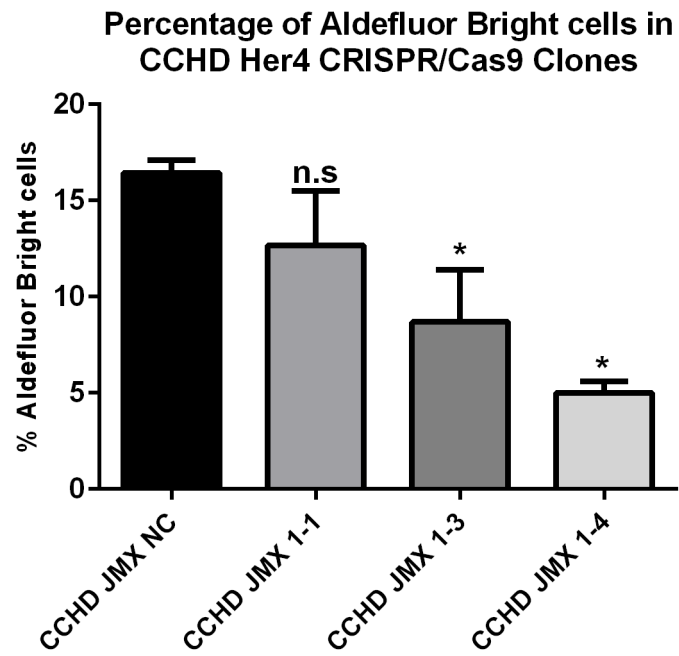


Figure 15: Quantification of ALDH Bright cells in CCHD nonsense and Her4 CRISPR Clones presented in Figure 14.

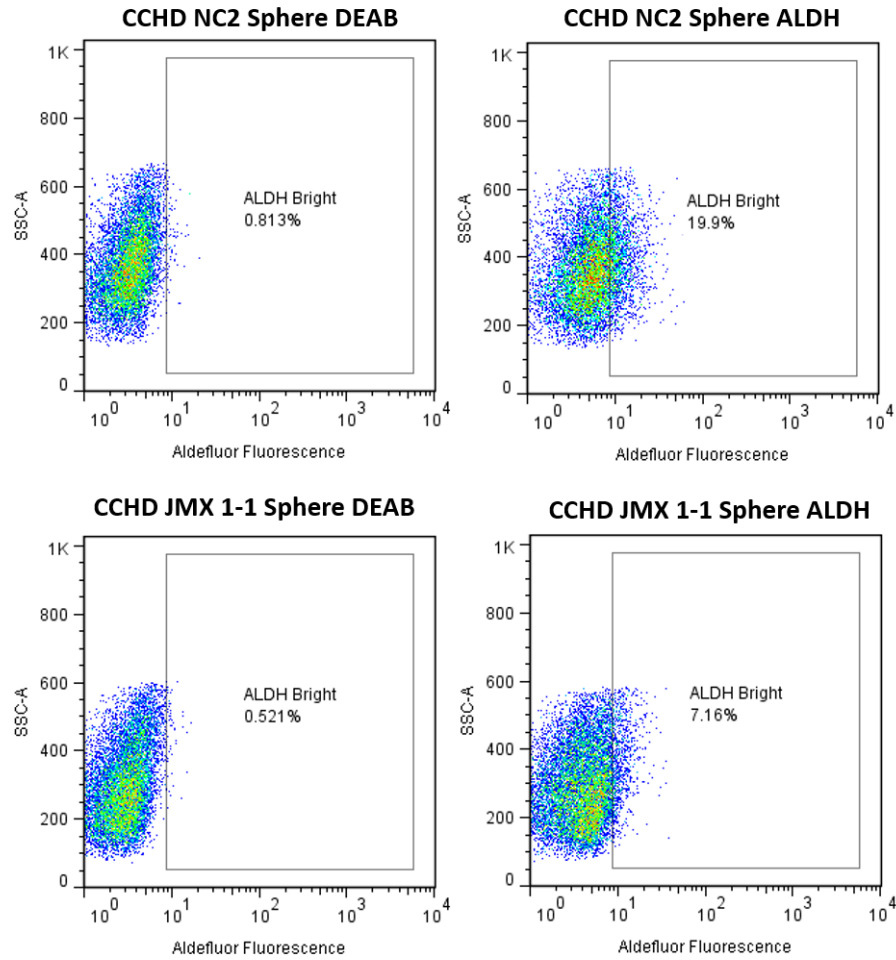


Figure 16: Aldehyde dehydrogenase in Her4 knockout remains diminished after sarcosphere culture. CRISPR clone CCHD JMX 1-1 and nonsense control were grown under sarcospheres, dissociated into single cells and added aldefluor substrate to measure activity of this enzyme. Enzymatic activity in the Her4 knockout remain decreased compared to the nonsense control.

Expression of embryonic stem cell transcription factors is decreased in Her4 CRISPR clones even after sarcosphere culture

Sox2, Oct3/4 and Nanog are embryonic stem cell transcription factors important in maintaining pluripotency and self-renewal in both normal tissue stem cells and tumorigenic stem-like cells [31, 33]. These transcription factors were initially observed in OS stem-like cells by Gibbs et al. who demonstrated that OS sarcospheres upregulated expression of Oct4 and Nanog. To determine if the expression of these transcription factors was affected after Her4 deletion, we performed quantitative real-time PCR using validated hydrolysis probes. After growth under sarcospheres the CRISPR clones were not able to upregulate the expression of these factors when compared to the nonsense control (Figure 17).

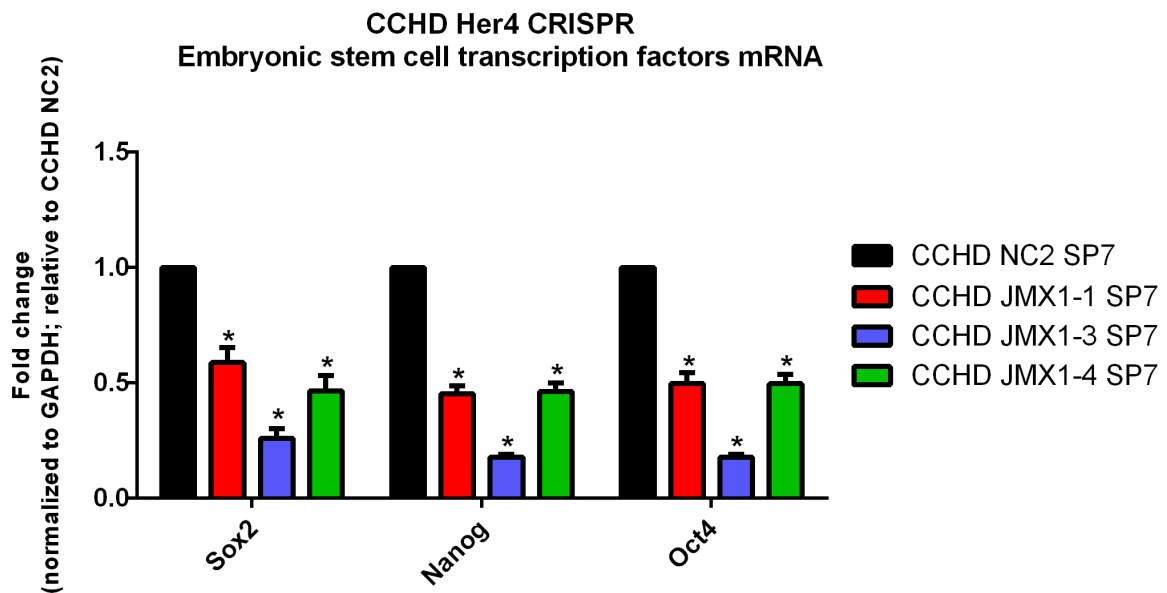


Figure 17: Expression of pluripotency markers Sox2, Nanog and Oct4 decreases with Her4 genomic deletion. mRNA expression of Sox2, Nanog and Oct4 was measured after seven days in sarcosphere culture for CCHD nonsense control and CRISPR clones JMX 1-1, 1-3 and 1-4. Data was normalized to GAPDH as internal control and compared relative to CCHD nonsense control. (*) denotes p-value of less than 0.05.

Summary

Validated markers for OS stem-like cells identify different subsets of cells within a tumor with the ability to confer aggressive disease and to initiate tumors even when implanted in very low numbers and these tumor cells can be serially transplanted in mice each time recapitulating the heterogeneity of the initial tumor [26]. Although no specific marker can accurately identify the entire pool of cells with tumor initiating potential, it is thought that a combination of several markers can provide a better understanding of this stem-like population [75, 79]. Through the analysis of several markers for stem-like cells in OS, our data indicates that aldehyde dehydrogenase activity and expression of embryonic stem cell transcription factors are compromised following Her4 knockout. Therefore, this data suggests that Her4 can modulate expression of markers associated with high tumorigenic potential, thus validating our prognostic data (see Chapter 3) that correlates Her4 with poor metastasis-free survival.

Chapter 6: Overexpression of Her4 Jma isoforms in OS cell lines promotes expression of stem-like markers

Rationale

In the previous chapter, we observed how genomic deletion of Her4 in the CCHD OS cell line mitigated the expression of markers that characterize cells with stem-like activity. In this chapter, we wanted to explore how cells would be affected after Her4 overexpression. Taking into consideration that the most common Her4 isoform found in OS cell lines is the juxtamembrane isoform Jma (see Chapter 3), we wanted to observe whether there were specific effects mediated by the two Her4 cytoplasmic isoforms: Cyt1 and Cyt2. These two cytoplasmic isoforms differ by mRNA splicing of exon 26 which encodes for a PI3K binding site and is expressed in Cyt1 but not in Cyt2 [49, 50]. There have been various studies that have demonstrated that these isoforms have complementary but distinct roles in the same tissue, with Cyt2 mainly mediating proliferation and Cyt1 mediating survival and chemotaxis [51, 55, 80, 81]. Therefore, to uncover how Her4 is mediating its effects in OS and to have an idea of the possible signaling pathways involved, we decided to generate Her4 JmaCyt1 and JmaCyt2 overexpressing OS cell lines and measure how the expression of embryonic stem cell transcription factors and OS stem-like cell surface markers were affected.

Results

Generation of Her4 JmaCyt1 and JmaCyt2 overexpressing cell lines.

To generate cell lines overexpressing the two different Her4 cytoplasmic isoforms with the juxtamembrane isoform Jma, we used the vector shown in Figure 18 to transfect OS cell lines Maos and CCHM. These cell lines were chosen due to their efficacy for transfection and their moderate level of Her4 expression among our panel of patient derived OS cell lines. Selection of Her4 expressing cells was performed by either GFP or puromycin selection.

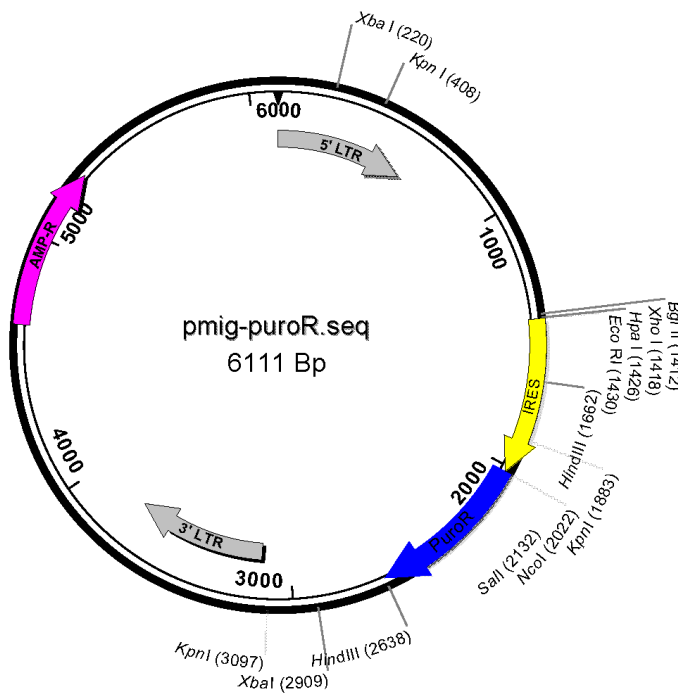


Figure 18: Her4 JmaCyt1 and JmaCyt2 vector used for transfection. OS cell lines Maos and CCHM were transfected using lentivirus using the vector depicted. Selection of transfected cells was performed by either GFP expression or puromycin selection.

Her4 JmaCyt1 isoform overexpression causes increased expression of pluripotency markers Sox2, Nanog and Oct4.

Maos and CCHM OS cell lines containing either MigR1, Her4 JmaCyt1 or Her4 JmaCyt2 were analyzed by quantitative real-time PCR for expression of pluripotency markers Sox2, Nanog and Oct4 since we wanted to determine if by overexpressing this receptor we were able to cause a shift towards a stem-like state in these OS cells. Indeed, we observed that with the Her4 JmaCyt1 isoform there was increased expression of these markers as shown in Figure 19. As a reminder, published data with these transcription factors in OS demonstrated that growth under sarcosphere conditions induced their expression and this correlated with expression levels *in vivo* and with tumor initiation potential [26, 30].

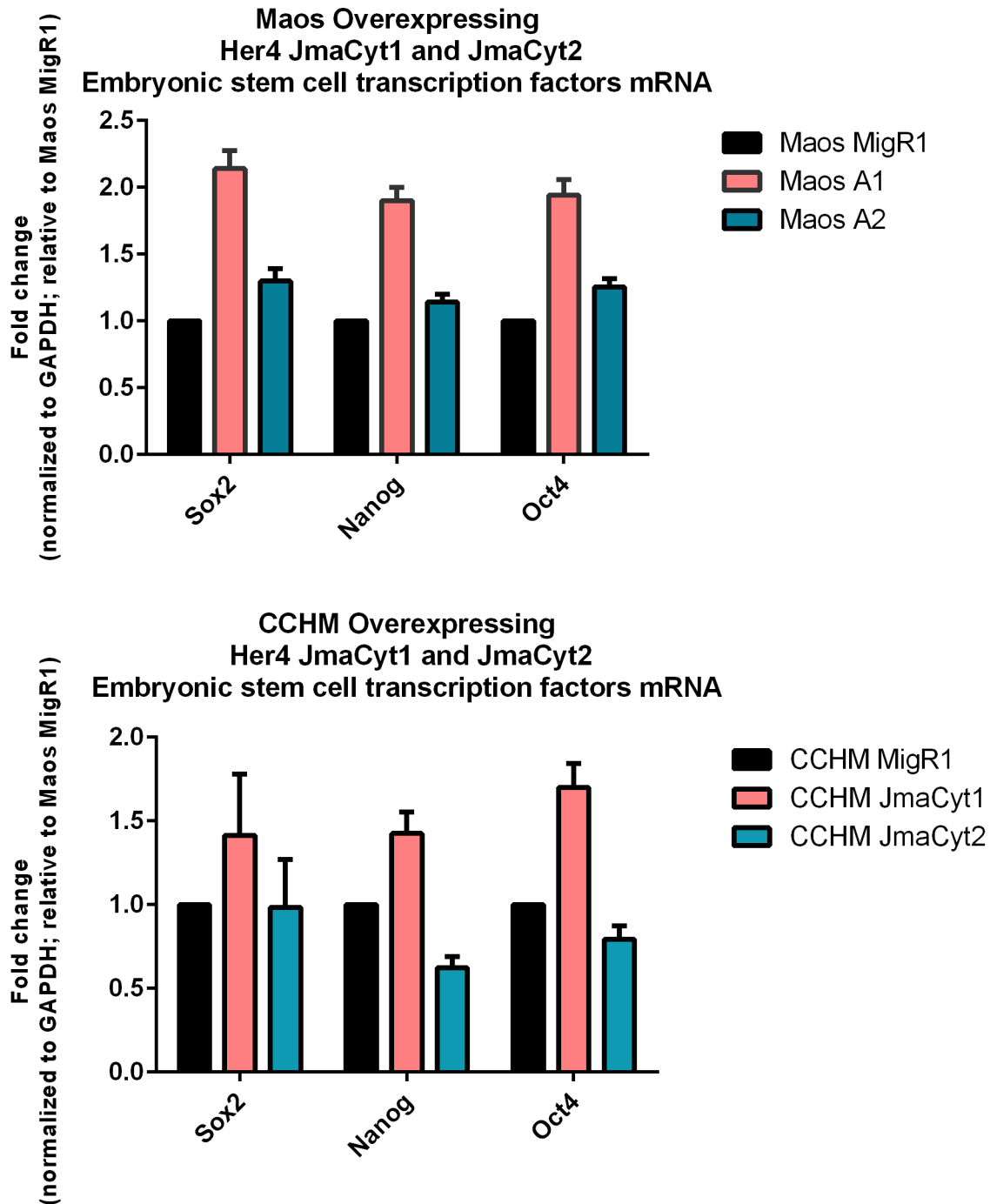


Figure 19: Overexpression of the Her4 JmaCyt1 isoform upregulates embryonic stem cell transcription factors. Real-time PCR was performed using RNA extracted from OS cell lines Maos and CCHM with either MigR1 vector or overexpressing Her4 JmaCyt1 or Her4 JmaCyt2. Her4 JmaCyt1 overexpressing cells were able to upregulate expression of these markers in monolayer culture without sarcosphere culture necessary for induction.

Both Her4 JmaCyt1 and JmaCyt2 isoform overexpression in OS cell lines lead to increased CD117 expression

Next, we wanted to investigate whether increasing Her4 expression in OS affected expression of the surface markers Stro1 and CD117. Figure 20 shows that there is low to moderate basal expression of CD117 in both OS cell lines Maos and CCHM. In Maos MigR1 around 15% of the population expresses CD117 and 40% in CCHM MigR1. When Her4 JmaCyt1 is overexpressed in Maos cells there is not an evident increase in the total percentage of CD117 positive cells, however, 7% of the population is now double positive for both Her4 and CD117 in the JmaCyt1 construct compared with 0.1% in the control MigR1 cells. With Maos JmaCyt2, the total percentage of CD117 positive cells increased from 15% to 23% with now around 20% of the population staining for Her4 and CD117 simultaneously. In CCHM, the majority of the CD117 positive cells also stained for Her4 in the JmaCyt1 construct and with the JmaCyt2 construct the total percentage of CD117 positive cells increased from 40% to 64% with the majority staining for Her4 as well. Therefore, these results indicate that overexpression of Her4 JmaCyt1 and JmaCyt2 in OS cells results in increased numbers of Her4/CD117 double positive cells and an increase in the total percentage of CD117 positive cells.

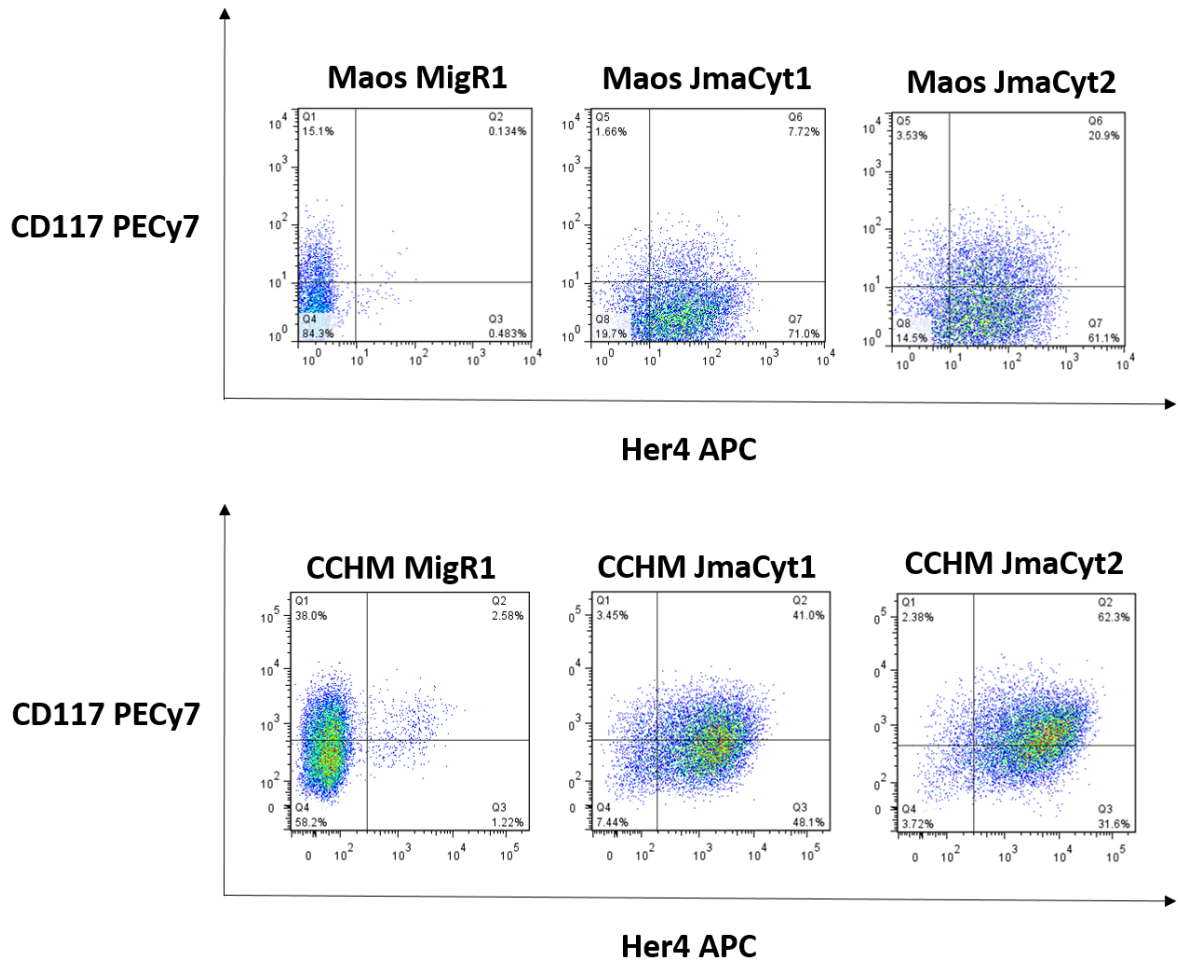


Figure 20: Her4 positive cells co-localize with CD117 positive cells. Maos and CCHM OS cell lines overexpressing Her4 JmaCyt1 or JmaCyt2 grown in monolayer culture were stained for flow cytometry analysis with Her4 and CD117. Both cell lines have basal expression of CD117 as seen in the MigR1 vector. However, when Her4 is overexpressed the majority of the CD117 positive cells also express Her4.

Summary

In this chapter, we have observed the direct effects of Her4 overexpression in OS cell lines. As others have reported in other tumor types, we observed that the two cytoplasmic isoforms of Her4 lead to distinct effects *in vitro*. In terms of expression of embryonic stem cell transcription factors, increasing the levels of the Her4 JmaCyt1 isoform, but not JmaCyt2 lead to increased levels of Sox2, Nanog and Oct4. However, in terms of surface expression of the stemness marker CD117, although Her4 JmaCyt1 seems to co-stain with CD117 positive cells, overexpression of the Her4 JmaCyt2 isoform caused an increase in the total number of CD117 positive cells. Therefore, this data suggests that these two different isoforms might mediate a stem-like phenotype in OS by different but converging signaling pathways.

Chapter 7: Discussion and Future Directions

Development of metastatic disease and distant tumor initiation is a complex process that requires resilience and survival of cancer cells under adverse environments on the route to metastasis [69, 70, 82-84]. This process requires that cancer cells locally invade, intravasate and survive in circulation and arrest and extravasate in the metastatic niche [85]. Research studies in hematologic and solid malignancies have demonstrated that the majority of cancer cells within a tumor do not possess this ability; therefore tumors are considered heterogeneous conglomerates of malignant cells [26, 72]. Tumor cells with the innate ability to initiate tumors and with higher metastatic potential have been identified *in vitro* and *in vivo* by their ability to grow and travel as small clusters of cells and by using markers and enzymatic abilities that are attributed to normal stem cells, hence they have been termed cancer stem cells or stem-like cancer cells. The plasticity of this phenotype has been demonstrated in melanoma [21, 77] and other tumors including osteosarcoma [41], and regulators of this phenotype would allow for better understanding of metastasis progression and to develop effective targetable therapies that would benefit these patients.

Although relatively little is known about Her4 and its contribution in cancer compared to other EGFR family members like Her2, recent reports have shown that this receptor is highly expressed in malignancies like breast cancer, melanoma, colon and lung cancer and its expression correlates with aggressive disease and metastasis [57, 60, 61, 86-89]. In pediatric malignancies like Ewing sarcoma and neuroblastoma, Her4 is necessary for anoikis resistance and survival under noxious stimuli [56, 58, 59]. Her4 is overexpressed in OS patients in both primary tumors and

metastatic lesions [65, 66], however the relevance of this receptor in OS has not been previously described. Since Her4 expression seems to be an important factor for survival in many malignancies mediating its effects by conferring resilience under stressors and similar conditions are used to enrich cancer cells with a stem-like phenotype, we sought to determine the importance of Her4 in OS and whether its effects could be mediated by the promotion of a stem-like phenotype in OS.

In Chapter 3 we demonstrated that Her4 expression has prognostic significance in OS patients, where high Her4 expression in their diagnostic biopsies correlated with a diminished metastasis free survival. This finding has potential for clinical translation in patients initially diagnosed with OS. Upon diagnosis, OS patients undergo 2-3 cycles of induction with high-dose methotrexate, doxorubicin and cisplatin (MAP) chemotherapy comprising a period of 10-12 weeks [9, 90]. Then, tumor response is evaluated by imaging and histologic analysis and patients are stratified in good and poor responders based on the level of necrosis in their tumors. This entails that patients have to wait at least three months to know if their chemotherapy regime is adequate in order to treat their tumor. If Her4 indeed has prognostic potential, upon OS diagnosis Her4 expression can be assessed and patients that express Her4 in their tumors can be offered aggressive chemotherapy initially since it is expected that these patients will have a higher probability of developing metastatic disease. Further experiments to validate Her4 as a potential prognostic marker need to show how Her4 expression is comparable to percent necrosis after induction chemotherapy.

Although these observations correlating Her4 expression to decreased metastasis free survival may suggest a link between Her4 and formation of metastasis, it is necessary to directly address whether Her4 expression is sufficient to promote development of metastatic disease to the lungs in OS. For this, we can design an *in vivo* experiment with CCHD OS cell line with and without Her4 and Fas expression. Fas expression in OS has been demonstrated to be vital for development of metastasis [91, 92], with primary OS tumors having variable Fas expression but Fas negative OS cells are selected for metastatic growth. Taking advantage of this, we can observe whether it will be possible to rescue metastatic potential in Fas positive cells after Her4 overexpression. This experiment will then establish Her4 as a sufficient mechanism to drive OS metastatic disease.

In Chapter 3 we also investigated the Her4 juxtamembrane expression level in a panel of commercially available OS cell lines and patient-derived cell lines. In this experiment we demonstrated that patient derived cell lines have comparable Her4 expression to that of OS patient biopsies. Furthermore, published data on Her4 expression in OS suggests that this receptor is mainly localized in the cytoplasm of tumor cells [65, 66], where it could be possibly mediating its effects. Our data from patient-derived OS cell lines is consistent with these observations since we showed that Jma is the highest expressing Her4 isoform in these cell lines. As discussed in Chapter 1, only the juxtamembrane isoforms Jma and Jmd can undergo proteolytic cleavage by ADAMS17 and gamma-secretase to release a soluble intracellular fragment of the Her4 receptor [49]. Experiments to understand the impact of expression of cleavable Her4 isoforms in OS should further analyze the level of

expression of these cleavable isoforms in more patient derived cell lines and/or patient tissue samples and if Jma or Jmd are validated as the most prevalent isoforms, specific mutations on exon 16 (the ADAMS17 binding site) could be generated and determine if there is any impact on *in vivo* tumor formation. Alternatively, non-cleavable isoforms of Her4 (Jmb and Jmc) could be overexpressed and similar experiments could be performed. Furthermore, to truly understand the pathobiology of Her4 in OS, *in vivo* models using patient xenografts should be performed, as they will more accurately represent actual disease and Her4 expression in OS patients.

To understand whether Her4 expression was affected by conditions used to enrich cells with a stem-like phenotype, in Chapter 4 we studied Her4 expression with validated markers used to identify cells with tumor initiating potential in OS. From this set of experiments we observed that Her4 expression is inducible after sarcosphere culture and that expression of CD117 and Stro1, published markers for OS stem-like cells [29], is mainly exhibited by Her4 expressing cells. Although previous experiments have demonstrated that Her4 becomes upregulated under anchorage independence [56, 58], our results are the first to directly study Her4 under conditions that are used to enrich cancer stem-like cells. Sarcosphere conditions have important translational implications. Recent studies have uncovered that the majority of tumor cells able to form metastatic disease travel to their niche as small cellular clusters [70, 83, 84], and thus this culture system serves as a useful *in vitro* model of tumor cell interaction. In addition, these findings support a possible

targetable mechanism to identify cancer stem-like cells in OS, which are thought to display increased metastatic potential [26, 30].

In Chapter 5 we assessed the specific contributions of Her4 to OS stem-like populations by generating CRISPR/Cas9 constructs targeting this receptor and causing genomic deletion. Our studies with Her4 knockout clones from the patient derived OS cell line CCHD demonstrated that when Her4 expression is abrogated there is decreased aldehyde dehydrogenase activity and decreased expression of embryonic stem cell transcription factors Sox2, Nanog and Oct4. These markers have been shown to identify highly tumorigenic subpopulations in OS [30] [28]. Therefore, this data further supports a possible role of Her4 as an upstream mediator of a stem-like phenotype. Further experiments to corroborate these observations are needed and should mainly focus on *in vivo* models. *In vitro* models, although useful to test hypothesis in a cost-effective manner, have certain limitations that become more evident when studying cells with stem-like attributes. As discussed in Chapter 1, characterization of cells that have the ability to initiate tumors should not focus only on a specific surface marker or enzymatic ability. Rather, studying a collective array of abilities and molecules expressed in these cells allow for better understanding and more accurate representation of this population. Additionally, the gold standard to assess tumor-initiating capacity is self-renewal [22, 70, 75]. This refers to the ability that cancer stem-like cells have, like tissue stem cells, to undergo asymmetric cellular division to generate both identical and more differentiated progeny, thereby allowing single cancer cells to recapitulate tumor heterogeneity. Using *in vivo* models can only accurately represent this ability. An

ideal experiment to assess whether Her4 affects tumor-initiating capacity should be performed by injecting Her4 high and low OS cells in low numbers (around 100 cells) intratibially in mice. Since it is thought that even one cancer cell with stem-like features can recapitulate its parental tumor with similar tumor heterogeneity [75], this first experiment will allow us to see if by having Her4 expression the ability of OS cells to initiate tumors is enhanced, even when OS cells are injected in low concentration. Furthermore, to demonstrate self-renewal in mouse models, serial transplantation should be performed by isolating OS cells from primary bone lesions and sorting for Her4 high and low expressing OS cells. These cells should then be injected intratibially in a second set of mice. If Her4 expressing cells have self-renewal capacity and represent a subset of OS cells with tumor initiating potential, they should be able to efficiently recapitulate tumor heterogeneity in secondary tumors whereas Her4 low OS cells should not. Currently, initial *in vivo* experiments are undergoing in our lab where we performed intratibial injection of CCHD nonsense control and CCHD CRISPR clone JMX 1-1 OS cells in NOD/SCID mice in order to understand the effects of Her4 knockout in terms of primary tumor size, metastatic disease burden and expression of OS stem-like markers. Taking into account our observations from *in vitro* experiments, we expect to see that the CRISPR mice will develop decreased metastatic lesions compared to our nonsense control group. Additionally, we expect to observe decreased expression of OS stem-like markers in these tumors.

To further demonstrate the importance of Her4 for a stem-like phenotype, we overexpressed the isoforms JmaCyt1 and JmaCyt2 as depicted in Chapter 6. The

Jma isoform was selected for overexpression due to its abundance across patient derived OS cell lines as shown in Chapter 3. Upon overexpression of these two Her4 isoforms we observed that expression of embryonic stem cell transcription factors Sox2, Nanog and Oct4 increased with Her4 JmaCyt1 overexpression. Previous experiments with OS cell lines showed upregulation of these transcriptions factors after sarcosphere culture [30, 36] and our observations indicate that overexpressing Her4 is enough to cause these factors to be upregulated. Also, upon Her4 JmaCyt2 overexpression we observed that CD117 expression increased. These findings suggest that when Her4 is highly expressed, OS cells acquire a more aggressive phenotype, as these markers have been linked to metastasis in OS and other tumor models [26, 28, 29]. Also, these observations validate that different Her4 isoforms may mediate different cellular effects as observed in breast cancer [53]. Clear understanding of the roles of the various Her4 isoforms in OS and signaling pathways activated is necessary. Although the Her4 Cyt1 isoform containing a PI3K binding site has been demonstrated to mediate survival effects compared to more proliferative effects of Her4 Cyt2, the latter preferentially binds transcription regulators and translocates to the nucleus to regulate transcription [49, 50, 93]. The Yes-associated protein 1(YAP1) is one such transcription coactivator that selectively binds Her4 Cyt2 in the nucleus, leading to transcription of a genetic signature to promote migration [94, 95]. YAP1 in turn, has been associated with a cancer stem cell phenotype on breast cancer [96-98]. Therefore, we predict that effects of the cytoplasmic isoforms of Her4 in OS are specific but complementary to promote a stem-like phenotype.

In conclusion, the observations presented herein suggest that Her4 can be an important mediator of aggressive disease in OS through the induction of a stem-like phenotype. These findings are of great translational potential and can lead to the development of new and targeted therapies in order to improve survival among OS patients, which has remained unchanged for the past 20 years [1]. More so, individualized chemotherapy regimens can be designed for OS patients who are at risk for developing metastasis. Conversely, in those patients with low Her4 and a better prognostic outcome the chemotherapy exposure may be able to be reduced in terms of number of post-op cycles of chemotherapy agents that can diminish quality of life and may lead to development of secondary malignancies during adulthood years.

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Vita

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