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Identification of Cancer Stem Cells in Sarcoma

BY:

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RTRG Group picture 2013: left to right first row ***(Dr Alya Ul-hassan, Dr David Hammond, Dr Abdulazeez Salawu, Dr Karen Sisley*** (head of RTRG group)***, Dr Nawal AlShammari and Dr Afnan Al Kathiri)***. Second row left to right: (***Dr Safar kheder and Dr Meshal Alhajimohammed).***

ABSTRACT

Sarcomas are a very diverse group of cancers, with over 50 subtypes. However, they fall into three main types: soft tissue sarcomas (STS), primary bone sarcomas and gastrointestinal stromal tumours (GIST). They are also known to be resistant to chemotherapy and radiotherapy. Because of their diversity comparatively little is known about them. Furthermore, the role of cancer stem cells in sarcomas is unknown and has not been investigated in depth. It has been however been claimed that some sarcomas develop from transformed tissues, and these cells have some stem cells like properties.

One of the aims of this study was to investigate these sarcoma subtypes and look for the presence of these stem cells. Immunophenotyping by FACS was used to identify CD markers that potentially stain stem cells in enriched populations. The second aim was to develop a novel technique that possibly identifies these stem cells in culture, called the stress assay. Comparisons between post-starved cells and pre-starvation was performed, using a different variety of methods to study differences that may occur, such as aCGH, MTT, FACS with different monoclonal antibodies and sorting. Furthermore, modified gating strategies and statistical methods (linear regression) were applied to the FACS data. A total of twenty-one cultures of sarcomas were tested and were derived from different sarcoma subtypes.

The primary data suggest differences between pre-and-post cells starvation (stress assay) among the sarcoma subtypes who survived this assay. FACS confirmed these sub-populations, using the modified gating strategy to only focus on large cells, were enriched for CSC markers. Linear regression and co-expressions of these CD markers were analysed and contributed to a potential hierarchal association of sarcoma subtypes. Finally the effect of post starvation on cells demonstrated genetic changes they may suggest the post-recovered populations were more like the parental tumour. Although it is not conclusive the findings of these studies seem to indicate that the stress assay may be a useful method to select for CSC in sarcomas. Further studies are required to more fully investigate and explore the true nature of the cells recovered.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ABC Transporter	ATP-binding cassette transporter
ABCG2	ATP Binding Cassette Subfamily G Member 2
ABS	Albumin Bovine Serum
ALDH	Aldehyde Dehydrogenase
AML	Acute myeloid leukeamia
APC	Allophycocyanin
BAA	BODIPY aminoacetate
BAAA	BODIPY aminoacetaldehyde
BAAA-DA	BODIPY- aminoacetaldehyde-diethyl acetate
B cell	B lymphocytes are a type of white blood cells
Bmi-1	Polycomb complex protein BMI-1 or RING finger protein 51 (RNF51)
β-catenin	Beta-catenin is a dual function protein, regulating the coordination of cell–cell adhesion and gene transcription
CD marker	Cluster of differentiation (cluster of designation)
CDH1	Protein Coding gene (Cadherin 1)
CIS	Cancer initiating cells
CML	Chronic myelogenous leukaemia
CSC	Cancer stem cells
CSCM	Cancer Stem cells Medium
CV	Crystal violet
CXCR4	Chemokine receptor
c-Myc	Multifunctional transcription factor
DEAB	Diethylaminobenzaldehyde
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacitate acid
Em	Emission
EMT	Epithelial-mesenchymal transition
ESCs	Embryonic stem cells
Ex	Excitation
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
FL1	Fluorescence channel 1

FL2	Fluorescence channel 2
FL3	Fluorescence channel 3
FL4	Fluorescence channel 4
GIST	Gastro-intestinal stromal tumours
G6PD	Glucose-6-phosphate dehydrogenase
hADSCs	Adipose tissue derived stem cells
h-ESC's (hESC)	Human embryonic stem ells
HIF-1	Hypoxia inducible factor-1
***Hox* family**	Homeobox family proteins
HSCs	Hematopoietic stem cells
LDH-5	Lactate dehydrogenase-5
MDR	Multi-drug resistant
MET	Mesenchymal-epithelial-transition
MSC	Mesenchymal stem cells
NANOG	Transcription factor protein involve in self renewal of undifferentiated embryonic stem cells in human
2N HCL	Hydrochloric acid
Nm	Nanometer
Notch	Is a highly conserved cell signalling system present in most multicellular organisms
NOX	NADPH/NADH-oxidase
OCT3/4	Octamer-binding transcription factor 4, also known as POU5F1 (POU domain, class 5, transcription factor 1)
PBS	Phosphate Buffer Solution
PE	R-phycoerythrin PE
PerCP	Peridinin chlorphyll protein
POSTN	Periostin
Post-SA	Post stress assay
Pre-SA	Pre stress assay
PTEN	Phosphatidylinositol-3, 4, 5-trisphosphate 3-phosphatase
PSA	Post stress assay
rpm	Round per minute
mRNA	Messenger ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RTRG	Rare Tumour Research Group
SA	Stress assay

SC	Stem cell
Shh	Sonic hedgehog
SOX2	SRY (sex determining region Y)-box 2 a transcription factor essential for pluripotency and a member of the SOX family
SP	Side population a subpopulation distinct from the main population (in flow cytometry)
SSC	Side Scatter
STS	Soft tissue sarcomas
T cell	T lymphocytes are a type of white blood cells
TERT	Telomerase reverse transcriptase
UL-MSC	Umbilical cord mesenchymal stem cells
Wnt	A group of signal transduction pathways made of proteins

PUPLICATIONS

- Salawu A, Fernando M, Hughes D, Reed MW, Woll P, Greaves C, Day C, Alhajimohammed M, Sisley K (2016). Establishment and characterization of seven novel soft-tissue sarcoma cell lines. *Br J Cancer* doi:10.1038/bjc.2016.259.

Consideration for publications:

In progress, working on the manuscripts but have not submitted anything yet.

- New markers to detect cancer stem cells in sarcoma?
- Novel procedure to isolate cancer stem cells?

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- The 9th Saudi Student Conference- Birmingham. (14th and 15th February 2016).
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CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 CANCER: AN OVERVIEW

A Neoplasm is a new growth; cancer is known as a malignant neoplasm, which is the term used to describe a broad group of diseases (Tarin, 2011). Cancer is a term that originates from a Latin word meaning 'crab'; the swollen veins found to be surrounding a tumour have similarities to the appearance of crabs' legs (Hecht, 1987). Worldwide, cancer is considered a major health problem and a leading cause of death. There are estimated to be about 19.3 million new cases annually; in 2020, 10.0 million deaths were caused by cancer (Jemal et al. 2011, Siegel et al., 2013, Cronin et al., 2018, DeSantis et al., 2019, Sung et al., 2021). Cancer affects both developed and developing countries. The most commonly diagnosed type of cancer for women is breast cancer, which accounts for (11.7%) new cases of the total number of cancer cases (estimated 2.3 million new cases) and 6.9% of cancer deaths among females. In males, lung cancer is the leading cause of death, accounting for around (11.4%) of new cancer cases and 18% of cancer deaths (estimated by 1.8 million deaths). Furthermore, new diagnosed cancer cases such as colorectal cancer was (10.0% new cases), followed by prostate cancer (7.3%), and stomach (5.6%). However, the leading cause of death still lung cancer and followed by colorectal cancer (9.4%) deaths, liver (8.3%), stomach (7.7%). Both cervical cancer and breast cancer death rates were found to be higher in developing countries compared to developed countries. The cancer cases expected to increased in 2040 to 28.4 million cases, a 47% higher than 2020 due to many factors. (Jemal et al., 2011, Siegel et al., 2013, Cronin et al., 2018, DeSantis et al., 2019, Sung et al., 2021).

The causes of cancer are diverse, whereby when the cells lose their growth control mechanisms and gene alteration occurs, a malignant tumour is formed (Fink, 1979, Siegel et al., 2013). This uncontrolled growth of cells consumes the entire nutrient leading to the death of the surrounding cells and tissues and eventually causing patient death (Siegel et al., 2013). If we consider that cellular and genetic changes, as well as unregulated growth control are a feature of tumours. Studying these changes has a huge impact in terms of understanding the fundamental biological background of these tumours and the resulting rates of patient survival (Yang et al., 2006). These changes are important in determining whether a tumour is benign or malignant, with invasive properties and the ability to spread to other sites within the body; a process known as Metastasis. Benign tumours do not have the capacity to invade the surrounding tissues. However, they can develop and turn

malignant in later stages, and spread through the lymphatic system or bloodstream to more distant tissues in the body, resulting in a poor prognosis for the patient (Yang et al., 2006).

1.2 CANCER DEVELOPMENT AND HALLMARKS

Cancer development is governed by different and multi-complex elements that control its transformation from normal to malignant. Interpreting the role of these elements, their effect, as well as interactions, will help in-depth understanding of cancer biology (Hanahan and Weinberg, 2011), in their paper, they point out hallmarks that are involved in cancer development, such as sustaining proliferating signaling, resisting apoptosis, inducing angiogenesis, escaping growth suppression, activating invasion and metastasis, self-renewal, and microenvironmental interactions. As normal tissue has controlled cell proliferation it prevents cells from excess growth, but cancer on the other hand does not, which leads to uncontrolled cell number and size through cell deregulation (such as through growth factors). In addition, cancer cells have the ability to maintain self-renewal by secreting growth factors ligands (autocrine stimulation) or they can recruit normal cells for this purpose, and by deregulation for receptor signaling (Bhowmick et al., 2004, Cheng et al., 2008, Hanahan and Weinberg, 2011). Figure 1.1.

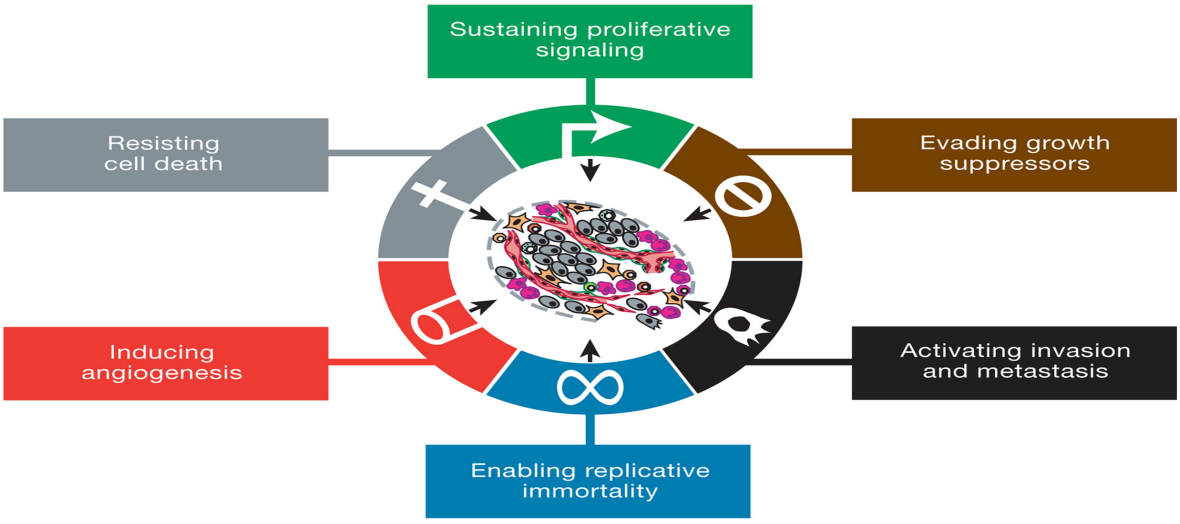


Figure 1. 1: The Hallmarks of Cancer. (Hanahan and Weinberg, 2011).

Hanahan and Weinberg (2011) also discussed emerging hallmarks that could act as enabling characteristics and contribute to the progression and continued development of cancers. These include the inflammatory response, increasing genetic instability and promotion of angiogenesis. Figure 1.2.

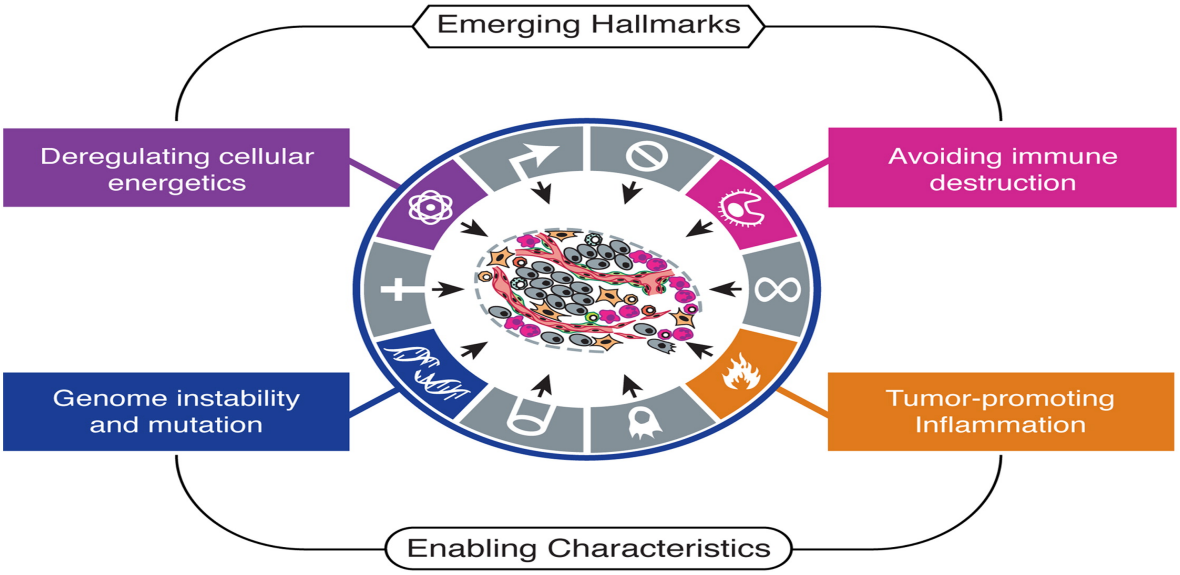


Figure 1. 2: Emerging Hallmarks and Enabling Characteristics. (Hanahan and Weinberg, 2011).

1.3 THE ROLE OF ONCOGENES

The regulation of cancer development is under genetic control and cancer has long been known to be a genetic disease and the process outlined, as hallmarks can be both promoted or suppressed through the actions of genes (Vicente-Duenas et al., 2013). Oncogenes are a group of genes that have the potential ability to cause cancer. The overexpression or mutation to gain function of oncogenes and their proteins usually leads to uncontrolled cell proliferation in cancer. For example, RAS, RAF and MYC will inhibit cell senescence and apoptosis (Zheng et al., 2013, Liu et al., 2018).

Furthermore, tumour suppressor genes (TSG) are considered as the genes that can negatively control cancer development (i.e. suppress) and are often involved in the production of the proteins that regulate cell division, such as responsible for correcting DNA damage or directing cells to programmed cell death (apoptosis). Famous examples of tumour suppressor genes are the RB gene (retinoblastoma-associated) and P53, both playing key roles in cellular regulation (Ong et al., 2004, Chau et al., 2008, Benson et al., 2014). RB intervenes directly in cell proliferation and can if required induce apoptosis (Wang et al., 2021). TP53 on the other hand, acts as part of the regulation of genomic integrity, detecting any genomic damage and pausing the cell cycle until the defect is corrected, otherwise if the damage is excessive TP53 can initiate apoptosis (Burkhart and Sage, 2008, Shukla et al., 2017).

Another example of TSG regulation of cancer development is the transforming Growth Factor Beta gene (TBF-ß) which can play a role in cell proliferation inhibition, and switching the TBF-ß signalling pathway from proliferation suppression is part of cellular re-programming and transformation, known as Epithelial-mesenchymal transition (EMT) (Ikushima and Miyazono, 2010).

1.4 Epithelial-Mesenchymal Transition (EMT) AND Mesenchymal Epithelial Transition (MET)

Epithelial-mesenchymal transition (EMT) is the complex reprogramming of epithelial cells that includes molecular and cellular changes, and enables epithelial cells to transit into a mesenchymal state. As a consequence epithelial cells gain characteristics such as the ability to migrate and becoming more invasive, resistance to apoptosis, increased production of enzymes responsible for the degradation of extracellular matrix and evading the immune system. EMT has a crucial role in normal cell biology in the development of an embryo, with transition to roaming cells to facilitate morphogenesis (Nieto, 2013, Jayachandran et al., 2016).

Evidence suggests that tumour cells capable of causing metastasis express both EMT and stem cell markers (Sannino et al., 2017). Therefore any activation of EMT transcription factors leads to the development and production of countless numbers of cancer stem cells (CSC) and the formation at distant sites of secondary tumours, which retain their ability to switch back to the epithelial state by so called mesenchymal-epithelial-transition (MET) (Kahlert et al., 2017). EMT induction is triggered by exogenous transcription factors such as epithelial growth factors (EGF), transforming growth factor-ß (TBF-ß) and fibroblast growth factor (FGF) with signaling pathways such as NF-kB, Wnt/ ß-catenin and hedgehog invoked (Gurzu et al., 2015). In addition, cancer cell with no stem cell like abilities, exposed to TBF-ß results in the transformation of these cells to mesenchymal cells or CSC like-cells, and by inhibition of this factor, cells switch back to the epithelial phenotype and loose the gained characteristics (Doherty et al., 2016). Some cytokines, such as Interleukin-6 for instance are also capable of inducing EMT (Sun et al., 2014, Bharti et al., 2016). In lung carcinoma exposure to IL-6 and mediation by a STAT3 signalling pathway leads to low expression of E-cadherin and up-regulation of N-cadherin and the induction of EMT by IL-6 influences cell plasticity (Bharti et al., 2016). Similarly in prostate cancer following EMT migration and invasion is enhanced or impaired by up/down-regulation of N-cadherin (Doherty et al., 2016, Yadav and Desai, 2019). Finally increased expression of EMT transcription factors

can promote tumour formation. For example, a high level of SNAI1 (SNAIL), in cancer cells increases both tumour initiation and metastatic potential (Ye et al., 2015).

1.5 SARCOMA: AN OVERVIEW

In general, sarcomas are rare tumours. They originate from mesenchymal cells, which consist of mesodermal and ectodermal germ layers (Mackall et al., 2002). They account for around 21% of all pediatric solid tumours (11% of childhood and 14% of teenager tumours) and around 55% of sarcomas affect the limbs, and 15% affects the head and neck. Less than 1% are adult solid tumours. This means that there are a higher proportion of pediatric cases compared to adult cases. Due to the fact that sarcomas arise from connective tissue, they can affect diverse tissues such as muscles and bone and are found in different parts of the body (Grunewald et al., 2020). Furthermore, certain types of sarcoma can be easily misdiagnosed as sports injuries (Mackall et al., 2002, Burningham et al., 2012). As a result of their origin, sarcomas are histologically diverse tumours; there are more than 100 subtypes of sarcoma. Some sarcomas have an unknown histogenesis, with some types have similar normal connective tissue features depending on their origin (Burningham et al., 2012, Fiedorowicz et al., 2018, Thompson and Franchi, 2018).

1.6 ETIOLOGY

Sarcoma aetiology is ambiguous, but genetic mutations play a major role in forming some sarcomas, and are associated with known cancer syndromes such as mutations in the retinoblastoma gene correlating with osteosarcoma (Abramson et al., 1984, Grunewald et al., 2020). Other factors that may cause sarcoma are exposure to specific chemicals or radiation, like vinyl chloride and ionizing irradiation. It is worth mentioning that race plays a role in sarcoma. For instance, the white race has more incidences of Ewing's sarcoma, while the Asian and African races have fewer incidences. This is related to genetic differences (Worch et al., 2010). Other causes of sarcomas are hormonal changes, infections, job types, and environmental risk factors (Balarajan and Acheson, 1984, Kedes et al., 1996, Fioretti et al., 2000, Comba et al., 2003).

1.7 SARCOMA TYPES

Although there are many sarcoma subtypes, they fall under three main types: soft tissue sarcomas (STS), gastrointestinal stromal tumours (GIST) and primary bone sarcomas. Despite STS being relatively rare, most of the common subtypes of sarcomas, which patients can develop, are under this type. STS may occur at any age. However, ageing increases the incidence of STS, it reaches a peak after people have reached their sixties. Approximately 50% of STS patients are over 65 years old (F. Farshadpour and R. Otter

2005). In addition, almost half of STS cases with an intermediate- or high-grade tumour metastasize. On average, the survival rate after five years is 50% (Jansen-Landheer et al., 2009, Grimer et al., 2010). In the table below we summarized some sarcoma subtypes and discussed some major sarcoma subtypes also, which fall under two main categories, as it is difficult to list and discuss all the subtypes. Table 1.1.

Soft-tissue tumours are classified into:	Bone tumours are classified into:
▪ Adipocytic tumours ▪ Fibroblastic/myofibroblastic tumours ▪ Fibrohistiocytic tumours ▪ Smooth muscle tumours ▪ Pericytic (perivascular) tumours ▪ Skeletal muscle tumours ▪ Vascular tumour ▪ Chondro-osseous tumours ▪ Gastrointestinal stromal tumours ▪ Nerve sheath tumours ▪ Tumours of uncertain differentiation ▪ Undifferentiated/unclassified sarcomas.	▪ Chondrogenic tumours ▪ Osteogenic tumours ▪ Fibrogenic tumours ▪ Fibrohistiocytic tumours ▪ Ewing sarcoma ▪ Haematopoietic neoplasms ▪ Osteoclastic giant cell-rich tumours ▪ Notochordal tumours ▪ Vascular tumours ▪ Myogenic, lipogenic and epithelial tumours ▪ Tumours of undefined neoplastic nature.

Table 1. 1: A list of some sarcoma subtypes that falls under two categories (soft-tissue and bone sarcomas). Adapted from (Beckingsale and Shaw, 2017).

1.7.1 Soft Tissue Sarcoma

Soft tissue sarcomas consist of a large group of heterogeneous mesenchymal tumours. Most of them are very aggressive. Mutation in genes such as ATM, ATR and TP53 are linked to the sensitivity increment to ionizing radiation which leads to the development of sarcoma (Ballinger et al., 2016). For example, 10% of the patients investigated with neurofibromatosis type one, GIST and malignant peripheral nerve sheath tumours develop an autosomal dominant mutation in the TP53 gene encoding p53, and lead to the development of tumours and on one third were sarcomas in the total cases (Farid and

Ngeow, 2016). Moreover, some studies claimed that there are also some genetic risk factors or gene damage associated with sarcomas such as BRCA2, ATM, ATR, and ERCC2 (Ballinger et al., 2016) Tables 1.1 and 1.2.

Diagnosis	Chromosomal abnormality (Translocations)	Involved genes
Alveolar rhabdomyosarcoma	t(2;13) (q35;q14)	PAX3-FKHR
	t(1;13) (p36;q14)	PAX7-FKHR
Alveolar soft part sarcoma	t(X;17) (p11.2;q25)	TFE3-ASPL
Angiomatoid fibrous histiocytoma	t(12;16) (q13;p11)	FUS-ATF1
	t(12;22)(q13;q12)	ATF1, EWSR1
	t(2;22)(q33;q12)	CREB1, EWSR
Clear cell sarcoma	t(12;22) (q13;q12)	EWS-ATF1
Congenital fibrosarcoma/ Congenital mesoblasticnephroma	t(12;15) (p13;q25)	ETV6-NTRK3
Dermatofibrosarcoma protuberans	t(17;22) (q22;q13)	PDFGB- COL1A1
Desmoplastic small round cell tumour	t(11;22) (p13;q12)	EWS-WT1
	t(21;22)(q22;q12)	ERG, EWSR1
Endometrial stromal sarcoma	t(7;17) (p15;q21)	JAZF1-JJAZ1
	t(6p;7p)	JAZF1, PHF1
Ewing's sarcoma/	t(11;22) (q24;q12)	EWS-FLI1
Peripheral primitive	t(21;22) (q22;q12)	EWS-ERG
Neuroectodermal tumour	t(7;22) (p22;q12)	EWS-ETV1
	t(17;22) (q12;q12)	EWS-FEV
	t(2;22) (q33;q12)	EWS-E1AF
	t(16;21) (p11;q22)	FUS-ERG
Inflammatory myofibroblastic tumour	t(1;2) (q22;p23)	TPM3-ALK
	t(2;19) (p23;p13)	TPM4-ALK
	t(2;17) (p23;q23)	CLTC-ALK

Infantile fibrosarcoma	t(12;15)(p13;q26)	ETV6, NTRK3
Low-grade fibromyxoid sarcoma	t(7;16) (q33;p11)	FUS-CREB3l2
	t(11;16) (p11;p11)	FUS, CREB3L1
Myxoid chondrosarcoma	t(9;22) (q22;q12)	EWS-CHN
	t(9;15) (q22;q21)	TFC12-CHN
	t(9;17)(q22;q11)	TAF2N-CHN
Myxoid liposarcoma	t(12;16) (q13;p11)	TLS-CHOP
	t(12;22) (q13;q12)	EWS-CHOP
Synovial sarcoma	t(X;18) (p11;q11)	SSX1-SYT
		SSX2-SYT
		SSX4-SYT

Table 1. 2: Show Common Chromosomal translocations known in sarcoma. Adapted from (Thway, 2009) and (Beckingsale and Shaw, 2017).

1.7.1.1 Fibroblastic Sarcoma

Under this type, there are several subgroups of sarcoma such as Malignant fibrous histiocytoma, Myxofibrosarcoma, Fibrosarcoma, Dermatofibrosarcoma, developed in the fibrous tissue, diagnosed mostly in people over 65 years old, with equal sex distribution (Willems et al., 2006). However, around 6% of young people less than 5 years old are diagnosed with fibrosarcoma. The most common type in the STS subtype is myxofibrosarcoma (Willems et al., 2006). This type-specific area is in the deep soft tissue of the extremities, trunk and head and neck. The histopathology picture shows monomorphic fibroblastic cancer cells in the collagenous matrix (Grunewald et al., 2020). A rare mutation was seen in alveolar soft tissue myxofibrosarcoma in NF1 and TP53 genes (Potter et al., 2018). Myxofibrosarcoma fusion areKIAA2026-NUDT11, CCBL1-ARL1, and AFF3- -PHF1 (t[9;X][p24;p11]; t[9;12][q34;q23]; t[2;6] [q12;p21], respectively) (Sbaraglia and Dei Tos, 2019) Tables 1.1 and 1.2.

1.7.1.2 Gastrointestinal Stromal Tumours

GISTs are the most common mesenchymal tumours of the gastrointestinal tract; they occur from the oesophagus to the rectum, the main site is the stomach (60%) and small intestine (25%), mostly occur in elderly men over 70 years old (Soreide et al., 2016). Histopathology contains broad-spectrum, however, mainly spindle shape cells and epithelioid cells in about 20% of the cases, in some cases it shows mixed histology picture which characterized by the differentiation toward interstitial cells of Cajal (Corless, 2014). The markers that stain this type are CD117 (KIT) and DOG1. The main frequent mutations are KIT in 70% of GISTs and PDGFRA (10%) (Duensing et al., 2004, Boikos et al., 2016) Tables 1.1 and 1.2.

1.7.1.3 Leiomyosarcoma

LMS, account for around 10-20% of STS in adults, specific areas for this type are seen in the peritoneum and uterus and rarely found in bone that affects middle-aged and older people (Burns et al., 2020). Histopathology cells picture mesenchymal, blunt-ended spindle shape cells and smooth muscle differentiation with SMA, desmin and h-Caldesmon positive (Grunewald et al., 2020). This type has a highly complex karyotype leading to a genomic instability picture. However, losses in the tumour suppressor genes RB1 at the 10q and phosphatase and tensin homolog (PTEN) at 13q were detected (El-Rifai et al., 1998). Also, whole-exome sequencing analysis of LMS and uLMS show a heterogeneous picture with common alterations in the tumour suppressor genes RB1, TP53, PTEN and cadherin-1 (CDH1) (Agaram et al., 2016) Tables 1.1 and 1.2.

1.7.1.4. Liposarcoma

LPS is common in the adult STS subgroup account around 20%(Toro et al., 2006). This type of location is variable, the most common site is in the retroperitoneal space and morphologically the cancer cells are heterogeneous show adipocytic differentiation within the vascularization stroma (Grunewald et al., 2020). LPS most common subgroups are well-differentiated (WDLPS) and dedifferentiated liposarcoma (DDLPS), both share genetic aberrations displaying a 12q21-15 amplicon, which contain the MDM2 proto-oncogene (MDM2) and cyclin dependant kinase 4 (CDK4) cell cycle oncogenes (Kanojia et al., 2015). Also in WDLPS COAS and PRUNE oncogenes are both often amplified in the 1q21-22 region (Arrigoni and Doglioni, 2004). In addition, the rare subgroups of LPS are myxoid liposarcoma (MLPS) and pleomorphic liposarcoma (PLPS) (Burns et al., 2020). MLP translocations found in t(12,16)(q13;p11), and less commonly t(12,22) (q13;q12), that fuse FUS RNA binding protein (FUS) or EWS binding protein-1 (EWSR1) and DNA damage-inducible transcript-3 (DDIT3) on chromosome 12 ,as a result, adipocytic differentiation is inhibited (Powers et al., 2010, Fiedorowicz et al., 2018) Tables 1.1 and 1.2.

1.7.1.5 Rhabdomyosarcoma

RMS is a skeletal muscle malignant tumour and accounts for around 40% of STS in children and 3-4% of all malignancies in this age group (Hoang et al., 2018). However, the specific site of this type is variable; histopathology, shows mesenchymal phenotype and variable myogenic differentiation morphology. These cells were found to be positive for myogenin and MYOD(Grunewald et al., 2020). Translocations in RMS are in PAX3 or PAX7 with FOXO around 77% of these tumours. Some mutations were observed such as CDKN2A/B deletions, mutations in FGFR4 and PIK3C A, TP53 and MDM2 amplification, some of them have high GLII expression (Hoang et al., 2018) Tables 1.1 and 1.2.

1.7.1.6 Undifferentiated Pleomorphic Sarcoma

UPS is the most common sarcoma in older people between 50-70 years old and rare in children; the most common site is the extremities (de Paiva et al., 2018). Although the origin of the malignant cells is not clear, histopathologically, the cancer cells are undifferentiated and contain a high degree of cellular atypia and pleomorphism (Grunewald et al., 2020). UPS mutations are detected in KRAS and PIK3CA, also, activated protein kinase B (AKT) was screened in some cases (Lahat et al., 2011, Li et al., 2015) Tables 1.1 and 1.2.

1.7.1.7 Synovial Sarcoma

Commonly seen in the deep soft tissue of the extremities in young adults (El Beaino et al., 2017). Cells morphology is spindle shape with variable mesenchymal and/or epithelial differentiation (Grunewald et al., 2020). The specific chromosomal aberration for this subtype is in t(X;18) translocation, and this translocation causes the fusion of oncogene SS18-SSX1/2/4 fusion, which is specific for synovial sarcoma, also known as SYT, chromosome 18 (El Beaino et al., 2017) Tables 1.1 and 1.2.

1.7.2 Bone sarcoma

1.7.2.1 Chondrosarcoma

CHC is found in cartilage, bone surface and centre and morphologically, show lobules contain malignant chondrocyte inside the chondroid matrix with calcified foci (Grunewald et al., 2020). The involved mutations for this type are IDH1/2 and EXT1/2. However, the role of p53 is unclear, but the presence of p53 protein overexpression, 17p1 chromosomal aberration, and TP53 mutations is present in almost all poorly differentiated CHS. Moreover, TP53 mutations occur in later stages. This was investigated and confirmed 12q13 (MDM2) amplification and loss of 9p21 (CDKN21/p16/INK4A and INK4A-p14ARF)

(Zajac et al., 2021). Also, half of the CHC cases show a mutation in the IDH1 (isocitrate dehydrogenase 1) or IDH2 genes (Lugowska et al., 2018) Tables 1.1 and 1.2.

1.7.2.2 Ewing Sarcoma

This type is found in the long and the flat bones around 85% of the cases and on extraskeletal around 15% of the cases, it accounts for 6-8% of bone tumours and affects young people of European origin (Gargallo et al., 2020). Histopathology show undifferentiated small round cells CD99 and PAS +ve cytoplasm (Grunewald et al., 2020). Mutations are somatic FET-ETS translocations EWSR1-FLI1 (85%), EWSR1-ERG (10%) and 5% rare subtypes. The most frequent translocation is t(11;22)(q24;q12) and the EWSR 1 gene is located at 22q12 and FLI1 at 11q24 (Kim and Park, 2016, Cidre-Aranaz et al., 2017, Gargallo et al., 2020) Tables 1.1 and 1.2.

1.7.2.3 Osteosarcoma

OS localized on the surface and centre of the bone. Histopathology picture, show neoplastic cells with a mesenchymal appearance and constant polymorphism (i.e. epithelioid, fusiform, round, spindle shape), these are combined with an extracellular osteoid matrix (Grunewald et al., 2020). Karyotype appearance is unstable with multi-chromosomal aberrations (Rickel et al., 2017, Grunewald et al., 2020). The most constant mutations are TP53, RB amongst others known as BRCAness, RECQL4, RAS signal pathways genes (EGFR, GNAQ, GNAS, ALK, PDGFRA, PDGFRB, PIK3CA, AKT2, PIK3R1, PTEN, TSC2, VHL, CBL); notch signalling pathways genes (NOTCH1-4, MAML2, FBXW7, PDPK1, AKT1, E1F4B) and others. The primary inducer genes for OS are TP53, NOTCH1, MYC, FOS, NF2, WIF1, BRCA2, APC, PTCH1, and PRKAR1A (Rickel et al., 2017, Fiedorowicz et al., 2018) Tables 1.1 and 1.2.

1.8 SARCOMA AND EMT/MET

As sarcomas are mesenchymal in origin and as mentioned earlier they can develop in bone, cartilage, and connective tissue (fat, fibrous, muscle and other tissues); they show phenotypic plasticity, and as such a plasticity with the transition between two different cellular phenotypes; epithelial-mesenchymal transitions (EMT) and mesenchymal-epithelial transitions (MET). In carcinomas EMT and MET are known to be regulated by many transformation factors, such as SNAIL, SLUG, TWIST1, and ZEB1/2 (Gurzu et al., 2015). In sarcoma however comparatively little is known about MET/EMT and the factors and the stages that the cells should undergo.

A study of gene expression of STS (around 250 samples) looking at epithelial-like or mesenchymal-like markers, found that epithelial-like positive markers had a better prognosis compared to mesenchymal-like markers (Somarelli et al., 2016). Recently Periostin was suggested as a prognostic biomarker for EMT in some of the STS, such as leiomyosarcoma, myxofibrosarcoma and undifferentiated pleomorphic sarcoma (Piano et al., 2020). Moreover, SNAI1 (SNAIL) expression has been found to be correlated with poor prognosis in sarcoma, and its expression by fibroblasts was related to development of sarcoma (Alba-Castellon et al., 2014). In Chondrosarcomas SNAIL and N-cadherin up-regulation correlated with MERK/ERK and PI3K/AKT signaling and also 80% had expression of both CXCR4 and survivin, with potential as therapeutic targets (Yang et al., 2015b). More is known about EMT /MET in Osteosarcomas than most sarcomas. Osteosarcomas show a high level of E-cadherin and low vimentin expression, due to HIF-1α-mediated hypoxia, and this was found to be associated with higher proliferation and more invasive cells (Sun et al., 2015). Also in osteosarcoma, ZEB1 was found at a high level and even higher in metastatic osteosarcoma (Shen et al., 2012) and TGF-ß treatment triggered MET in osteosarcoma associated with SNAI1 activated through estrogen-related receptor alpha-dependent (Chen et al., 2017). Furthermore, genes such as TIM3, ST6GAL1, TRIM66 and UHRF1 in osteosarcoma play an important role in cell-cell interaction, cell-matrix adhesion and EMT in general (Chen et al., 2015, Meng et al., 2015, Feng and Guo, 2016, Liu et al., 2016).

1.9 TREATMENT

The diversity of sarcomas, together with patient history and response to treatment, affects their management. For example, some elderly patients receive no treatment. These elements improve the treatment outcome, the survival rate, and help to avoid a recurrence of the tumour. Surgery, chemotherapy and radiotherapy are the available standard treatments for sarcoma. The treatment decision is made according to many factors such as the grade, location of the tumour, and age (Kasper et al., 2007, Jansen-Landheer et al., 2009, Grimer et al., 2010 Grunewald et al., 2020). Furthermore, improvements in finding new therapies, rather than using the traditional treatment, have been beneficial and have shown a shift toward molecular targeted therapy, which increases the prediction of tumour biological behaviour in many sarcoma subtypes (Kasper et al., 2007, Grunewald et al., 2020).

1.10 CANCER STEM CELLS (CSC)

A stem cell (SC) is a cell that has the ability to proliferate and differentiate into different cell populations (Tang, 2012). In order to gain an understanding of cancer stem cells (CSC), it is a necessity to first understand the characteristics of normal adult stem cells. In any normal tissue or developed organ, there is a hierarchy of cells; including importantly the presence of a specific type of cell, called an adult stem cell, which can share all, or some, of the properties of stem cells. Somatic adult stem cells can trigger and play a major role in their environment, and cellular growth and homeostasis of normal tissues is managed by normal SCs. This means that the SCs are crucial for preventing tissues from degeneration, and supporting tissue development and cell division occurs through specific signaling pathways. (Figure 1.3), These progenitor cells fluctuate between the self-renewal and non-self-renewal states: see Figure 1.3 and the pathways in stem cell biology have been studied extensively in the last few years using a combination of new CD markers (cluster of differentiation) (Majeti et al., 2007, Notta F, 2011, Tang, 2012, Najafi et al., 2019a, Thankamony et al., 2020). It was thought to be possible to purify a population of SCs from a mixture of SCs and mature progenitors. For example, markers such as Aldehyde dehydrogenase (ALDH) can be used in the Aldefluor assay in combination with a side population (SP) that enriches for SC's, such as CD34, CD49f and CD90. Other markers like CD38, CD45RA, also could isolate a small population of cells that contain SC's properties (Schatton et al., 2009, Najafi et al., 2019a, Thankamony et al., 2020).

Cancer stem cells (CSC) are thought to be a subpopulation in the tumour, characterized as having an undifferentiated phenotype that possesses the ability of self-renewal, or of being able to differentiate into committed daughter cells that are resistant to chemotherapy and radiotherapy (Schatton et al., 2009, Najafi et al., 2019a, Thankamony et al., 2020). They, therefore, resemble normal SCs in their ability to renew and differentiate, but importantly are considered to initiate tumour relapse or distant metastasis. Also, they are not controlled by homeostasis (as a result of the tumour); the population of CSC is heterogenic with genomic and epigenetic instability, which leads to genetic plasticity (Clarke and Fuller, 2006, Dalerba et al., 2007, Bomken et al., 2010, Najafi et al., 2019a, Yadav and Desai, 2019, Thankamony et al., 2020). As a result, we can suggest that CSC resemble SC in their various capabilities (Moghbeli et al., 2014, Tinhofer et al., 2014, Najafi et al., 2019a, Thankamony et al., 2020).

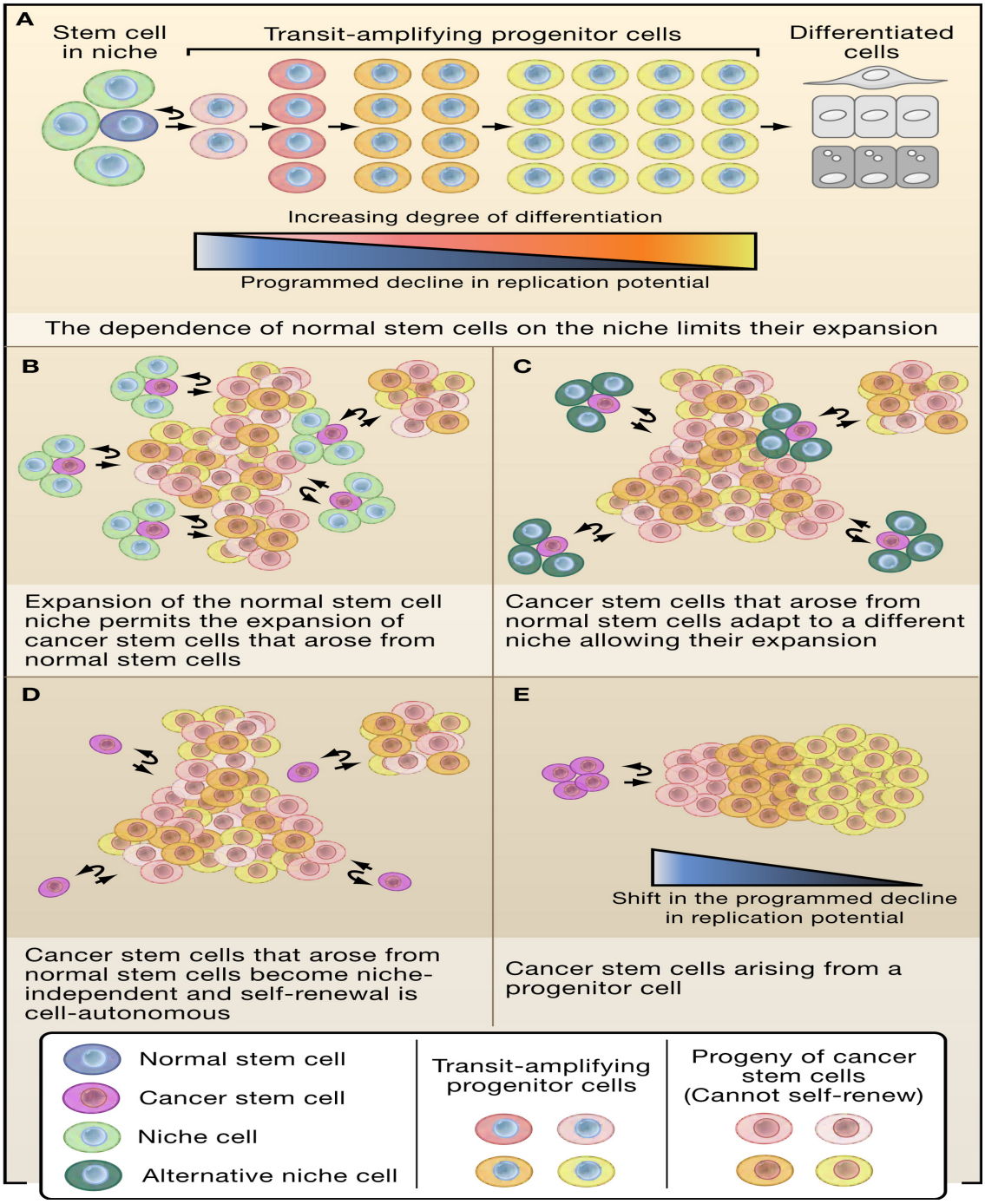


Figure 1. 3: Shows the possible origins of cancer stem cells. (Clarke and Fuller, 2006).

(a) Interactions between Normal SC and niche. (*b*) Expansion of the SC niche. (*c, d*) Alterations in CSC and genetics alterations promote them to niche-independent (*e*) CSC may arise from progenitor cells due to mutations.

There are two theories for the development of CSC. It was originally hypothesized that CSC could potentially arise from normal stem cells. As a result, CSC are thought to be somatic adult stem cells transformed through acquired mutations under certain conditions (Lobo et al., 2007). This assumption was based on observations of leukaemia (Fialkow, 1990,

Lapidot et al., 1994). Also, some adult cells in the lymphoid system can be described as having a stem cell-like hierarchy, according to their functions. T and B cells in this system are divided into memory cells and effector cells based on the surface marker expression. The status of the cells, and in what stage of development they are in, can easily be determined by this expression's profile. The memory T and B cells (long-term) encounter self-renewal, sharing this property with the hematopoietic stem cell at the molecular level. This means that the common transcription factors play a significant role in not only maintaining the cells but in determining which cells can undergo self-renewal (Luckey et al., 2006).

The second theory argues that CSC evolve from progenitors and early progenitors, which have the ability to renew themselves, and then de-differentiated over the course of the tumour initiation and progression, resulting in their transformation into CSC and the tumour source (Reya et al., 2001, Clarke and Fuller, 2006).

As mentioned before, any malignant tumour is found to be a heterogenic population composed of different cells with diverse properties. Self-renewal is a significant property that allows CSC to be regulated by the microenvironment signals just as normal cells are. These signals maintain the cell division to give rise to more cells, whether these are normal cells, adult SCs or some CSC. This process, however, is inhibited in normal tissue to restrain ungoverned cellular growth (Morrison et al., 2002, Morrison and Kimble, 2006), whilst no such constraints exist for cancers. Based on what has been already mentioned about CSC theory, CSC could be defined as a restricted subpopulation of cancer cells that have the ability to govern the growth and spread of the tumour. These cells can also develop into more differentiated cells, and at some point, they may stop proliferating or die due to their limited ability. This leads us to assume that the cellular hierarchy observed in normal tissue, could also be seen in the tumour as will be shown in the next sections.

1.11 CSC IN CANCER

1.11.1 CSC and Hematopoietic Cancer

According to a study on chronic myelogenous leukaemia (CML) (Fialkow, 1990), CML disease starts with a high number of mature abnormal white blood cells. By the time the disease progresses and becomes aggressive, the white cells have accumulated in the bone marrow and are called blasts. Fialkow investigated their lineage by using glucose-6-phosphate dehydrogenase G6PD isozymes, which indicated that pluripotent stem cells become malignant progeny. Red blood cells and plasma B cells also have the same G6PD

isozyme, which means they share the same origin. These outcomes were probably the first conclusive evidence of the presence of CSC (Fialkow, 1972, Fialkow et al., 1981, Fialkow, 1990). In addition, many attempts have been made to isolate CSC, for example, the pioneering studies by John Dick's group in 1997. They isolated what were thought to be hematopoietic stem cells (HSCs) in acute myeloid leukaemia (AML). Based on what is known today as monoclonal antibodies, they were able to isolate a subset of human AML cells that were CD34+CD38- in immunocompromised mice. These cells continued to grow to mature cells (Lapidot et al., 1994, Bonnet and Dick, 1997), suggesting that a malignant population can give rise to a hierarchical system.

1.11.2 CSC and Solid Tumours

Most of the body's organs are assumed to have an adult stem cell population that governs their development and contributes to the cellular hierarchical system of each organ by organized pathways. In addition, most of the organs or tissues are known to maintain a balance that stabilizes after development preventing neoplastic development; for instance, adult SC subpopulations have been proven to exist in the brain and muscle and can be triggered in certain circumstances (Collins et al., 2005b, Ming and Song, 2005, Sugihara and Saya, 2013, Atashzar et al., 2020, Thankamony et al., 2020).

Based on these findings, many studies have tried to investigate subpopulation of CSC in different tumours. Some of the most investigated and potentially valuable markers are CD44 and CD133, which have been found in breast, brain, prostate, head and neck cancers (Table 1.3). CD44 is a glycoprotein receptor that is widely known as a surface marker associated with CSC migration, adhesion and metastasis of cancer cells (Shipitsin et al., 2007). On the other hand, CD133 is a surface antigen known as prominin-1, which is a glycoprotein with five transmembrane domains. It is widely used as a prospective CSC marker (Grosse-Gehling et al., 2013). Expression of these markers in subpopulations was found to be correlated with high clonogenicity and stem cell characteristics (Najafi et al., 2019a, Yadav and Desai, 2019, Thankamony et al., 2020). In breast cancer populations of cancer cells with different phenotypes can be found, and CD44 expression has been investigated using immunocompromised mice as a model to test these diverse populations (Trapasso and Allegra, 2012). The results from these tests showed that there is a tumorigenic subpopulation called tumour-initiating cells, with a distinct surface marker expression that can be distinguished from the non-tumorigenic cancer cells. The isolated cells ($CD44^{+}CD24^{-/low}$) were noted to have occurred in eight patients out of nine and accounted for 11%-35% of the total number of cells (Al-Hajj et al., 2003). In addition, these

cells had the ability to form tumours in mice while the other cell lineages failed to initiate tumours. Most significantly, the mice's tumours from ($CD44^{+}CD24^{-/low}$) cancer cells were found to be mixed populations representing the same phenotypic picture as the original tumour (Al-Hajj et al., 2003).

Cancers	Cancer stem cells phenotype	References
Breast	CD44, CD24, EPCAM, CD133, ALDH	Al-Hajj et al., 2003; Qiu et al., 2019
Glioblastoma	CD133, CD15, CD44, A2B5	Singh et al., 2004; Dirkse et al., 2019
Head and Neck	CD44, CD133, CD98, ALDH, Side population	Prince et al., 2007; Peitzsch et al., 2019
Lung	CD44, CD133, ALDH, CD90	Leung et al., 2010; Maiuthed et al., 2018
Colorectal	CD44, CD24, CD133, CD166, ALDH, EPCAM	Ricci-Vitiani et al., 2007; Zhou et al., 2018
Gastric	CD44, CD24, CD133, LGR5, CD90, CD71	Yang et al., 2007; Bekaii-Saab and El-Rayes, 2017
Pancreatic	CD44, CD24, CD133, ESA, DCLK1, ABCB1	Li et al., 2007; Di Carlo et al., 2018
Hepatocellular	CD44, CD133, CD13, CD45, CD90, EPCAM	Terris et al., 2010; Wang and Sun, 2018
Renal	CD105, CD133, ALDH1	Bussolati et al., 2008; Peired et al., 2016
Ovarian	CD44, CD24, CD117, EPCAM, ABCB1, ABCB2	Zhang et al., 2008; Roy and Cowden Dahl, 2018
Endometrial	CD44, CD117, CD55, CD133	Giannone et al., 2019
Prostate	CD133, CD44, α2β1, ABCG2, ALDH	Collins et al., 2005; Skvortsov et al., 2018
Melanoma	CD133, ALDH, CD271, ABCG2, JARID1B, CD20	Fang et al., 2005; Kumar et al., 2017
Leukemia	CD34, CD38, CD123, CD47, CD96	Lapidot et al., 1994; Wang et al., 2017

Table 1. 3: Cancer stem cells identified according to stem cell markers in different tumours (Thankamony et al., 2020).

Similarly, with regard to head and neck tumours, findings suggest that CD44 could be relied upon as a potential marker to diagnose cancer and the tumour could possibly be eradicated by targeting these cells (Yuce et al., 2011, Cruz et al., 2012, Joshua et al., 2012). There are

however obvious differences in how CD44 expression is interpreted as a prognostic marker in different regions of the head and neck cancer. The poor prognosis of laryngeal tumours is correlated with CD44 high expression and has a significant correlation with the ability of these cells to metastasize to the lymph nodes, tumour recurrence and resistance to therapy (Sun et al., 2010). For other locations in the head and neck region, the situation is not so well established. For example, the low expression of CD44 by squamous cell carcinoma in the oral cavity has been associated with a high potential for metastasis and recurrence of cancer, which may indicate a bad prognosis (Wang *et al*., 2009, Kokko *et al*., 2011). In contradiction, other articles suggest that high expression of CD44 and poor prognosis are correlated (Fonseca et al., 2001, Kokko et al., 2011), whilst other studies find no relationship (Carinci et al., 2002, Rajarajan et al., 2012). Therefore, CD44 has the potential as a marker for diagnosis and prognosis, but more studies are required to determine its value.

The other marker frequently studied is CD133. Further understanding of CSC has come from studying Glioblastoma multiforme (GBM) that can express $CD133^{+}$ in around 5% - 30% of the tumour. By purifying and xenografting $CD133^{+}$ cells into immunocompromised mice, they showed an increased ability for self-renewal and differentiation (Galli et al., 2004, Dirks, 2005). Much of what we have gained as an insight into CSC however comes from the study of Prostate cancer. In prostate cancer, CD133 has also proved to be useful as a potential marker of CSC, and CD44 and CD133 are used to isolate the prostate CSC along with a few other markers, for example, beta-1 integrins and the ABCG2-associated drug-resistance proteins. Prostate cells that express CD44 and CD133 are able to form prostasphers (Tu and Lin, 2012). Most importantly, Collins *et al* (2005) in their study, isolated CSC from primary human prostate cancer that had the hallmark of SC properties by using (CD44+ /alpha2beta1^{high}/CD133+) markers (Collins et al., 2005a). Nevertheless, this does not mean that the CD133 and CD44 are specific as CSC markers, and are correlated with SCs (stemness).

1.12 CSC AND SARCOMA

Although there are numerous studies of potential stem cell markers in solid tumours, there have been relatively few investigations of sarcoma. As the term sarcoma is used to describe a diverse and large group of cancers under the same type of tumour (sarcomas), it is not necessarily the case that the findings of one type can be applied to another due to this diversity (Figure 1.4).

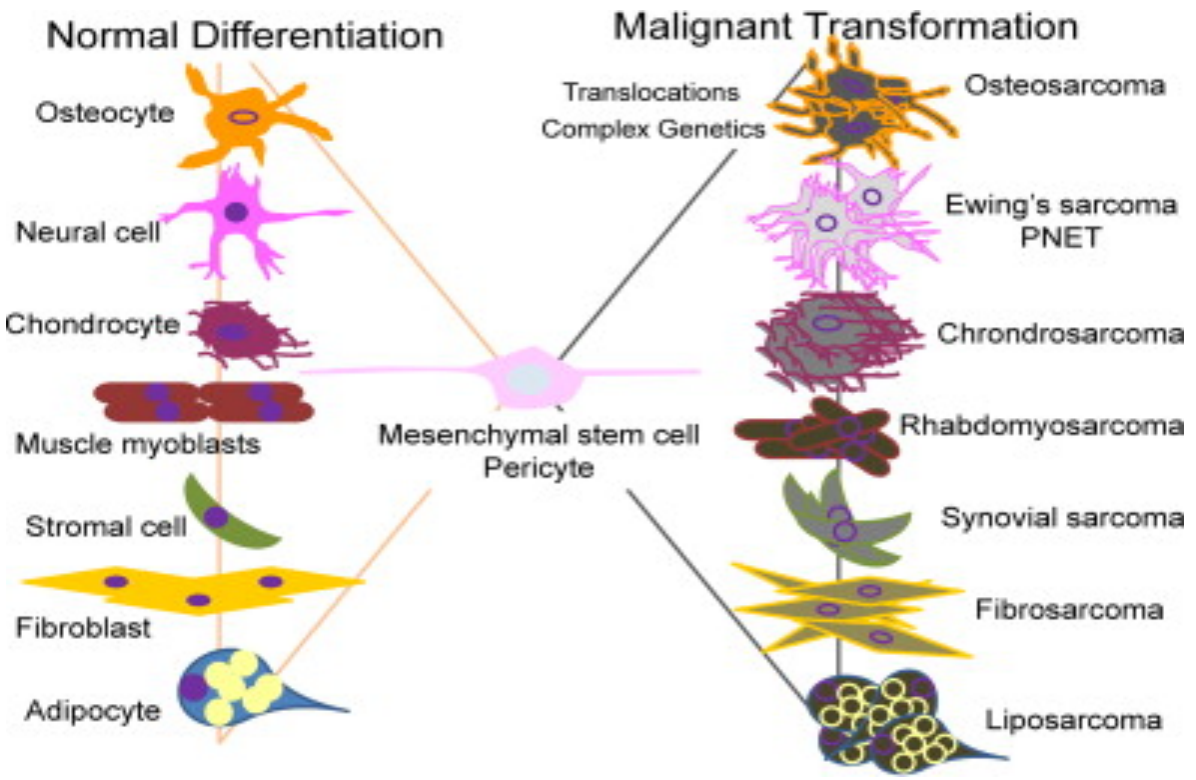


Figure 1. 4: Showing a schematic depicting the pluripotency of mesenchymal stem cells or pericytes. These cells reside in all tissue and give rise to many normal differentiated cell types; however, when chromosomal translocations or complex genetic alterations occur, mesenchymal stem cells or pericytes may give rise to the diverse types of sarcoma (Teicher, 2012).

Further complications may arise for some types of sarcoma, such as neuroectodermal sarcoma, as the actual origin is controversial (Fabian et al., 2009). The major issue with identifying a CSC population in sarcomas is; how to selectively isolate them, considering that no pure CSC population has been identified that hold stem cell properties. For example using a combination of CD133 and ALDH to purify CSC (Hess et al., 2006) and (Silva et al., 2011), found that the lack of surface antibodies made it difficult to establish whether potential CSC were the same cells causing relapse, metastasis and resistance to therapy in sarcoma. This has led to the use of a range of different markers in order to identify and isolate the CSC small subpopulation (Trucco and Loeb, 2012).

1.12.1 Markers Detecting CSC in Sarcoma

One of the powerful tools to identify and isolate CSC is by using surface markers (CD markers), which is the most common technique to identify both normal and tumour cells. Cell lines derived from sarcoma can be analyzed, according to the expression of these CD markers, and the level of individual and co-expression staining can also be performed; enabling determination of the phenotype and cell sorting. As a result, other assays can be

accomplished based on sorted cells such as clonogenic assay, sphere formation assay, invasion assay, xenografting in immunocompromised mice and molecular approaches. The expression can also be linked to transcription factors and controlling pathways that play a role in tumour maintenance and formation. This profiling enables us to understand how to potential select CD markers for CSC and how to interpret the findings. Subsequently, the need to find specific markers, particularly for sarcoma is crucial. The most commonly used markers for sarcoma are CD133, CD44 and ALDH. As mentioned previously, only a few studies have looked at CSC in sarcoma and these are as follows:

1.12.1.1 CD44

The presence of this marker in numerous tumour types and its use as a putative CSC identifier has been discussed above. However, the expression should be associated with other markers for better resolution. In sarcoma, CD44 is used as the preliminary marker to isolate what has been thought of a CSC (Trucco and Loeb, 2012, Henderson et al., 2018).

1.12.1.2 CD133

CD133 is a suggested putative marker of different human malignant tumours, as discussed above. In addition, different sarcoma types exhibit CD133 positivity, such as osteosarcomas, chondrosarcomas, osteochondrosarcomas, fibrosarcomas, synovial sarcomas, liposarcomas, leiomyosarcomas and chordomas (Tirino et al., 2011, Veselska et al., 2012).

1.12.1.3 ALDH

Aldehyde dehydrogenase (ALDH) is a catalyzing enzyme that works on the oxidation of intracellular aldehydes in numerous cell types (Veselska et al., 2012). ALDH expression has been found in many tumours (Atashzar et al., 2020, Thankamony et al., 2020). Sometimes the activity of ALDH is correlated with CD44 expression (Clay *et al*., 2010). However, the cells with high and low ALDH expression differ in terms of their stemness abilities. In addition, CSC show a high level of ALDH expression and chemotherapy resistance. This subpopulation was also identified in Ewing's sarcoma and tested as a tumour-initiating tumour using different methods such as clonogenic assay, sphere formation assay, and through tests on immunocompromised mice (Clay et al., 2010, Veselska et al., 2012).

1.12.1.4 Nestin

Nestin is a neuronal protein for stem cell class VI of the intermediate filaments. Usually, during embryonic stem cell development, the expression is observed in nervous tissue. In

spite of this, the expression has been found to be positive in some solid tumours, which could be suggested as a potential CSC marker (Krupkova et al., 2010).

1.12.1.5 ABC Transporter

The ATP-binding cassette ABC transporter is a part of the plasma membrane found in many cell types and plays a role in protecting the cell by excluding antineoplastic agents, and this means cells can become multi-drug resistant (MDR). However, in sarcoma, the expression is questionable, because in some studies ABCG2 was found to be positive (Tirino et al., 2008), whereas in other studies it was negative (Olempska et al., 2007). The same variability was found in osteosarcoma between two in-house cell lines, the MG-63 osteosarcoma and the 3AB-OS osteosarcoma cell line (Di Fiore et al., 2009).

1.12.1.6 Other Markers Detecting CSC

CD24 is a mucin-like adhesion molecule labelling neutrophils, pre-B lymphocytes and solid tumours. It is known to increase the potential of cancer cell spread. High expressions of CD24 have been found to be associated with adenocarcinoma of the colon, stomach, gallbladder and ovaries (Lim and Oh, 2005). Moreover, CSC subpopulations are not only detected with the CD24 and CD90 co-expression but have also been found to have the ability to metastasize (Malanchi et al., 2012). Also of potential value are CD117 and Stro-1 used to detect bone marrow osteogenic progenitor cells, whereby co-expression is a possible indicator for osteosarcoma (Adhikari et al., 2010). Finally, CXCR4, which is a chemokine receptor, can be considered as a CSC marker for osteosarcoma (Adhikari et al., 2010, He et al., 2012). There are also other less well-studied markers, but to conclude, all these putative CSC markers are expressed differently in different tumours and suggest that each tumour may have a specific phenotype for the CSC: see Table 1.4 (Mannelli and Gallo, 2012).

Tumour Type	Technique	Summary of findings	Reference
Osteosarcoma	CD133	CD133+ cells demonstrated increased sphere formation, growth in soft agar, expression of OCT3/4, and the presence of a side population	Tirino et al, 2008; Tirino et al, 2011; Wang et al., 2020
	CD117, Stro-1	Double positive (CD117+ and Stro-1+) cells were seen with a higher incidence in spheres, and they showed higher tumorigenicity as well as chemoresistance	Adhikari et al, 2010;Cai et al., 2017.
	ALDH	ALDH-high cells isolated from xenografts established from cell line OS99-1 had increased tumorigenicity, self-renewal, and an ability to produce differentiated progeny, and expressed increased levels of OCT3/4A, NANOG, and SOX-2	Wang et al, 2011; Belayneh and Weiss, 2020.

	SP	SP was seen in 1 of 7 osteosarcoma cell lines tested. The SP population had increased sphere- and colony-forming activity and increased tumorigenicity	Murase et al, 2009; Yi et al., 2015.
Ewing's sarcoma	CD133	133+ cells showed increased tumorigenicity, an ability to establish a heterogeneous population and differentiation, and increased expression of OCT4, SOX2, and NANOG. There was a correlation in primary tumours between the higher expression of CD133 and chemoresistance	Suva et al, 2009; Jiang et al, 2010; Skoda et al., 2016; Skoda and Veselska, 2018
	ALDH	ALDH-high cells showed increased sphere- and colony-forming ability, chemoresistance, SP, and tumorigenicity	Awad et al, 2010; Skoda and Veselska, 2018
Rhabdo-myosarcoma	CD133	Serial passages of rhabdomyosarcoma spheres enriched for cells with increased expression of OCT4, NANOG, c-Myc, SOX2, and PAX3 and increased expression of CD133 and CD133+cells showed increased tumorigenicity, an ability to differentiate, and to display chemoresistance. The CD133 expression also correlated with poorer survival in patient samples	Walter et al, 2011; Skoda et al., 2016
Synovial - sarcoma	CD133	5 of 5 primary tumour samples and 3 of 3 cell lines demonstrate a CD133+ subpopulation, but no TIC properties were tested	Terry and Nielsen, 2010; Martinez-Delgado et al., 2020
Multiple-sarcomas	SP	The size of SP in primary tumour samples correlated with the tumour grade. The SP cells from 1 osteosarcoma, 1 synovial sarcoma, and 2 malignant fibrous histiocytosis samples showed increased tumorigenicity and an ability to produce a heterogeneous cell population (SP and non-SP)	Wu et al, 2007; Skoda and Veselska, 2018; Martinez-Delgado et al., 2020

Table 1. 4: Studies, showing the isolation of sarcoma-initiating cells (CSC). Adopted Table. (Trucco and Loeb, 2012).

The accumulative evidence suggests the presence of subpopulations with stem cell abilities, and much of this support comes from studies using specifically CD133, CD44, and ALDH. For instance, in osteosarcoma, a CD133+ve subpopulation was isolated in several osteosarcoma cell lines that also retained the transcription factor OCT3/4 (associated with stem cell properties) in contrast to the CD133-ve subpopulation (Tirino et al., 2008). Moreover, in Ewing's sarcoma, a subpopulation of CSC was analyzed again using the same surface marker, and CD133+ve cells exhibited stem cell characteristics and hierarchy even when they underwent xenografting and re-culturing. Furthermore, CD133+ve cells were found to be able to differentiate into osteogenic, adipogenic, and chondrogenic cell types. Also, the CD133 +ve cells expressed intracellular markers, which are found in normal stem cells, such as OCT3/4, NANOG and SOX2 (Suva et al., 2009, Riggi et al., 2010). Finally, ALDH is found in some of Ewing's sarcoma cell lines (around 2% of the total). The subpopulation showed resistance to chemotherapy (doxorubicin), more so than the ALDH-low did, and a capacity to form colonies (Awad et al., 2010). Also, (Lohberger et al., 2012) obtained the same results when they evaluated the ALDH expression in several

sarcoma cell lines. (SW-684, SW-872, SW-982, SW-1353, and TE-671), as well as the chemotherapy drugs doxorubicin, epirubicin, and cis-diammineplati-num (II) chloride (cisplatin) (Lohberger et al., 2012, Nakahata et al., 2015).

1.13 PATHWAY SIGNALLING PLAYS A ROLE IN THE MICRO ENVIRONMENT OF CSC

Both CSC and normal stem cells require self-renewal; therefore, they have to share certain mechanisms to regulate this stem cell ability. A variety of pathways play a key role in this process. In sarcoma, however, due to the lack of studies investigating CSC, it is difficult to investigate the pathways of these subpopulations. Pathways relevant to stem cell biology, whether it is cancer or normal cells are presented in Table 1.5; (Lobo et al., 2007). For instance, Wnt signalling is required to prevent differentiation of embryonic stem cells (ESCs) to epiblast stem cells, which means overexpression of Wnt preserve the cells in the stem cell status; Wnt is also essential in self-renewal (Lohberger et al., 2012). Indeed, the Wnt pathway is known to regulate stem cell maintenance in different tissues such as mammary glands and tumours (Malanchi et al., 2008, Zeng and Nusse, 2010). Moreover, Malanchi *et al* (2012), showed not only the necessity of Wnt to self-renewal, but also the interaction between different ligands of Wnt and periostin (POSTN) in boosting metastasis. When a small population was isolated, the cells managed to expand and form a secondary tumour; first, the CSC induced POSTN and then Wnt signalling increased through POSTN to maintain the expansion (Malanchi et al., 2012). This growing evidence supports the theory that the interaction of CSC and their microenvironment niche govern CSC survival.

Signalling pathways associated with normal stem cells and cancer

Pathway	Normal stem cells	Cancer
Bmi-1	Bmi-1 is required for the self-renewal of hematopoietic and neural stem cells	Bmi-1 is upregulated in AML and overexpressed in medulloblastoma
	Bmi-1 downregulates the Ink4a/Arf locus	Overexpressed Bmi-1 and cell proliferation induces self-renewal of leukemic stem cells
Shh	Involved in the maintenance of hematopoietic stem cells and the expansion of progenitors	Activation of SHH is implicated in skin and brain carcinogenesis, including basal cell carcinoma of skin and medulloblastoma
	Crucial in embryonic development of skin, hair follicles, and sebaceous glands	Mutation of SHH causes Gorlin's syndrome
	Involved in postnatal and adult brain development	
Wnt/β-catenin	Involved in the maintenance and self-renewal of hematopoietic stem cells and progenitor cells	Overexpression of WNT is seen in many human cancers
	Regulates the maintenance of normal intestinal epithelial cells	Accumulation of β-catenin is associated with breast cancer, melanoma, sarcoma, myeloid leukaemia, multiple myeloma, and brain tumours
	Implicated in regenerative responses	Mutations in β-catenin are found in

	during tissue repair	endometrial carcinomas, prostate carcinoma, and hepatocellular carcinomas
		Mutations of both β-catenin and APC genes are common in colorectal cancer
Notch	Mediates the self-renewal of hematopoietic and neural stem cells	Mutations or aberrant activation of Notch1 are known to cause T-ALL in humans and mice
	Activates Notch target genes involved in T cell differentiation and self-renewal	
***Hox* family**	Involved in the self-renewal of hematopoietic stem cells and the proliferation and differentiation of precursor cells	Overexpression of HOXA9 is found in AML patients with poor prognosis
		Overexpression of HOX11 is described in T-ALL with chromosome translocations
		Hoxb3, Hoxb8, and Hoxa10 are associated with leukemogenesis in a mouse model
Pten	Implicated in the maintenance of hematopoietic stem cells and neural stem cells	Loss of PTEN leads to the formation of a variety of tumours, including myeloproliferative disease, and the emergence of transplantable leukaemia
		Mutation and/or LOH causes glioblastoma multiforme, prostate carcinoma, and endometrial carcinoma
Efflux transporters	Marker proteins are found in self-renewing stem cells, such as ABCG family proteins, responsible for the side-population phenotype	Upregulated ABCG2, ABCB1, and CEACAM6 are found in cancer cells from the gastrointestinal system
		Upregulated ABCG is implicated in broad-spectrum chemoresistance of cancer cells, such as AML cells
Telomerase	Expressed at a high level in normal self-renewing populations in the blood	Expressed at a high level in tumour cell populations with upregulated mRNA expression
		hTERT is involved in tumorigenic transformation
		Upregulated telomerase activity is found in glioblastoma

Table 1. 5: Show signalling pathways associated with normal stem cells and cancer*. (Lobo et al, 2007).

* Abbreviations used: AML: acute myeloid leukaemia; APC: adenomatous polyposis coli; hTERT: human telomerase reverse transcriptase; LOH: loss of heterogeneity; PTEN: phosphatase and tensin homolog deleted from chromosome 10; Shh/SHH: sonic hedgehog; T-ALL: T cell acute lymphoblastic leukaemia; WNT: Wingless-Int. (Lobo *et al*, 2007).

1.14 SOME OF THE THERAPEUTIC IMPLICATIONS OF CSC In RESEARCH

Cancer is well known for its heterogeneity in terms of phenotype and function (Thankamony et al., 2020, Atashzar et al., 2020) and CSC are the cells that have the defining characteristics (self-renewal and giving rise to different tumour cells) that govern tumour development. The resistance of CSC to traditional cancer therapies, such as chemotherapy and radiotherapy is therefore considered to be one of the biggest challenges, as remaining CSC are potentially responsible for the post-treatment relapse (Yang et al., 2014b, Yang et al., 2015). Any advance in targeting methods for those cells could potentially lead to full cancer eradication (Yadav and Desai, 2019, Atashzar et al., 2020, Cole et al., 2020).

1.14.1 CSC Surface Markers Targeting

One of these methods is to directly target CSC, or by directing them to a non-CSC fate or apoptosis. For example AML was eliminated in a mouse model, by targeting these CSC, using the TIM-3 surface marker as a target, found on AML but not normal haemopoietic stem cells (HSCs) (Kikushige et al., 2010). CD47 known to protect cells from phagocytosis was also used as a CSC directed therapy by inducing phagocytosis in AML (Jaiswal et al., 2009, Aikawa et al., 2010, Kikushige et al., 2010, Yadav and Desai, 2019).

1.14.2 CSC Niche Targeting

A second method is by attacking the CSC niche, which is a rich microenvironment that supports CSC development directly and indirectly. Vascular endothelial growth factor (VEGF) is supposedly released from CSC to support the network of tumour blood vessels (Bao et al., 2006, Phi et al., 2018), and neutralization of VEGF, using Bevacizumab prevented endothelial cells migrating and forming a tube in a Glioblastoma mouse model (Burkhardt et al., 2012). Moreover, CSC stemness is possibly activated through gene activation under hypoxic conditions. Hypoxia-inducible transcription factors (HIFs) are activated, and HIF-1α known as angiogenesis regulator plays a major role in the maintenance of the CSC by protecting CSC against DNA damage, and increasing NOTCH signaling pathway to promote cell stemness (Koshiji et al., 2004, Quail et al., 2012, Yadav and Desai, 2019).

Furthermore, other studies have shown that by controlling cell proliferation inside the niche and reducing the impact of the immune system and stromal cells in protecting the CSC microenvironment therapeutic action is more effective (Scadden, 2006, Oskarsson et al., 2014, Shukla et al., 2017) Other studies have targeted CXCR4 and chemokine CXCL12 (referred to as metastatic agents in some cancers) and have also been shown to have value (Burger and Peled, 2009, Hwang-Verslues et al., 2009, Sugihara and Saya, 2013).

1.14.3 Targeting of CSC Signaling pathways

In section 1.13 and Table1.5 above, pathways that regulate cell stemness were mentioned. Normal stem cells undergo a very restricted self-renewal process, CSC do not. Understanding the factors that regulate this process are still under investigation, and the dysregulation could lead eventually to tumourigenesis by the transition of normal cells to tumour cells. For example, high expression of the NOTCH signaling pathway is thought as carcinogenic, and found in many cancers such as solid tumours and leukaemias (Lobry et al., 2011, Lobry et al., 2014, Yadav and Desai, 2019). Inhibition of NOTCH-3 that is highly expressed in lung cancer results in decreased proliferation and tumour mass

(Osanyingbemi-Obidi et al., 2011, Hassan et al., 2013). Targeting other pathways such as Wnt and Hedgehog (see Table 1.5) may also help in eradicating CSC and preventing chemo-radiotherapy resistance and improving cancer survival as the endpoint.

1.14.4 CSC Targeting by Inhibition of ATP-Binding Cassette Transporters

The role of ATP-BC was mentioned above in section 1.12.1.5. First and second generation inhibitors have bee tested, however, it is necessary to develop less toxicity and fewer side effects, selecting properties essential to CSC growth. The third generation of ATP-BC inhibitors (ABCC1, ABCG2 and ABCB1) are still under investigation (Thomas and Coley, 2003, Yadav and Desai, 2019).

1.14.5 CSC Oncogenes Targeting

The usual approach is by RNA interference (RNAi); to suppress the gene, also known as gene silencing, and the siRNA binds with complementary mRNA to degrade it. For example, CSC were inhibited in cervical cancer using lentiviral short hairpin RNA by silencing gene E6 in the human-papilloma virus (Gu et al., 2011). Moreover, CD133 +ve CSC in colon cancer were targeted with knockdown of HMGA1 oncogene, which restored normal stem cells characteristics, increasing p53 expression and reducing self-renewal (Puca et al., 2014). Repeated cytotoxic treatments were reported to make CSC quiescent (Francescangeli et al., 2012). The radio-chemo therapy usually target cells in the proliferating status (active cells) for apoptosis and the quiescent CSC used this pathways to protect them selves and resist conventional therapeutics. Using some therapies to target cell cycle such as vinorelbine, vincristine, docetaxel and vindesine were found to be less effective because of the quiescent CSC (Viale et al., 2009). However, CD133+ Glioblastoma stem cells were shown to have quiescent gene expression profile, which may lead to more insight to knockdown these genes (Liu et al., 2009).

1.15 HYPOTHESIS AND AIMS OF THE STUDY

1.15.1 Hypotheses

It has been hypothesized that CSC are stem cell-like cells that are responsible for the initiation and maintenance of the tumour. These cells are thought to have the properties of self-renewal, being able to differentiate into daughter cells, and being resistant to chemo-radiotherapy. Understanding these specific subpopulations and confirming their existence can improve our knowledge of how to diagnose, treat and target them and push tumour treatment to the next level, which is the ultimate goal. Therefore, the hypothesis for this

project is that sarcomas contain CSC capable of driving their development and contributing to their resistance to treatment.

1.15.2. Aims

The initial aim is to study different sarcoma subtypes, and see is CSC can be identified in all sarcomas or certain subtypes, if so to isolate and characterize them. A secondary aim, and leading to other studies is to understand their role in resistance to therapy and the ability to promote sarcoma development and metastasis. Therefore, the specific aims of this project are to:

a) Investigate potential markers in sarcoma to see if they can identify a CSC sub population, using single and co –expression studies, and to determine if they are of relevance to all sarcomas or maybe certain subtypes.

b) To explore and develop possible new methods to potentially isolate CSC using the so-called “stress assay”.

c) To isolate and analyse potentially enriched CSC populations and characterize their behaviours, using a combination of flow cytometry, clonogenic and MTT assays, and to explore potential genetic differences using array CGH.

d) To investigate whether CSC are diluted by continued culture; using a combination of short-term and established cell lines of sarcomas.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Disposable Materials and Laboratory Equipment

Standard reagents and disposable plastic ware are detailed in table 2.1

Items	Manufacturer
60 mm tissue culture dishes	Becton Dickinson- Nunc
25cm and 75cm tissue culture plastic flasks	NUNC
5ml, 10ml and 25ml pipets	Fisher Scientific
15ml and 50ml polypropylene tubes	Fisher Scientific
10µl, 200 µl and 1000 µl pipette tips	Fisher Scientific
0.6 x 30mm sterile needles	Becton Dickinson
Nitrile gloves	Microflex- Supreno
5ml and 10ml Plastic syringes	Becton Dickinson
Disposable needles	Becton Dickinson
Flow cytometry tubes	Becton Dickinson
1.5ml micro-centrifuge tube	Eppindorf
22x50 mm cover slips	VWR International
22x22 mm	VWR International
Sterile disposable scalps	Swann morton
Cryo vials 1.8ml	Sarstedt-Nunc
Beckman coulter tubes-sample cup	Beckman Coulter
Ethanol	Analar Volumetric Solution
Methanol	Analar Volumetric Solution
Acetone	Fisher Scientific
0.22 µm sterile filter	Millipore
Nunc flat bottom test tube	NUNC

Table 2. 1: A list of Disposable Materials and Laboratory Equipment were used in this study.

2.1.2 Buffers and Solutions for Tissue Culture and Staining

Phosphate Buffer Solution

Was purchased as a 500ml sterile solution of Dulbecco's PBS from LONZA Biowhittaker to be stored in +4°C.

Dimethyl Sulfoxide DMSO

A 100ml DMSO BTL from Sigma Aldrich stored at room temperature.

Trypsin/Ethylenediaminetetraacitate acid (EDTA)

Purchased as 0.05% trypsin/EDTA from Invitrogen, aliquoted into 15ml polypropylene tubes (Fisher Scientific) and stored at -20C°.

Trypan Blue

Purchased as a 0.4% trypan blue solution from sigma and diluted in PBS. The staining solution made by 5ml of trypan blue added in 10ml of distilled water and filtered using a

syringe with a 0.22µm filter. The solution then dispensed into 15ml tubes and stored at room temperature.

Crystal Violet

Crystal violet (CV) powder from sigma and stored at room temperature. The stain was prepared by adding 50mg (0.05g) of CV powder to 5ml ethanol and 45ml of a distilled water (water filtered using Triple Red Laboratory Technology equipment- Barnstead). The solution was kept at room temperature in a 100ml bottle.

Cells Fixative

A 50ml of methanol mixed with 50ml of acetone and stored at room temperature. The fixative solution placed at 4°C for 10-15 minutes before use.

2.1.3 Tissue Culture Media

2.1.3.1 Established Cell Line Culture

Established cell lines were cultured in sterile Dulbecco's modified eagle medium (DMEM) from (LONZA Biowhittaker with 4.5g/L and glucose with L-glutamine CAT No. BE12-604F), supplemented with 10% fetal calf serum from Invitrogen, 10kU/ml penicillin/streptomycin (10kU/ml, LONZA) 1% amphotericin B (Lonza). The number of cells seeded in each flask (T75) was 0.4 - $1x10^6$. The sarcoma established cell lines U2OS, SKUT-1, SKLMS-1 and SW1353 with the Prostate cell line PC3 were purchased from the American Tissue Type Collection (ATTC). In addition, Human epithelial retinal cells hTERT-RPE-1 was cultured and treated as an established cell line and purchased from S. Collis Institute for cancer.

2.1.3.2 Primary Cell Lines (Sarcoma cell Lines)

Sarcoma cell lines were cultured in Roswell Park Memorial Institute (RPMI) medium from LONZA Biowhittaker without L-glutamine CAT. No. BE12-167F supplemented with 20% fetal calf serum from Invitrogen, 10kU/ml penicillin/ streptomycin (10kU/ml, LONZA) 1% amphotericin B (Lonza), 200mMol glutamine in0.85% NaCl (LONZA) and 2.2ml 0.4% glucose (45% solution) from Sigma CAT. No. RNBC2785. The number of cells seeded in each flask (T75) was 0.4 - $1x10^6$.

2.1.3.3 Cancer Stem cells Medium, Serum-free

Cancer Stem cells Medium, serum-free (CSCM) from Provitro (CAT. No. 2131001) and supplemented with L-glutamine, BIT-100 supplement and antibiotics according to the

manufacturer instructions. Primary sarcoma cell lines were seeded in in T75 flasks at 0.5 - 1x $x10^6$.

2.1.4 FACS Analysis

2.1.4.1 Albumin Bovine Serum (ABS) Blocking /Washing Buffer

A blocking buffer or washing buffer was made by adding 0.25g ABS powder from sigma to 50 ml PBS solution, and stored at 4°C and was used to wash cells after staining with antibodies.

2.1.4.2 Antibodies

Antibodies and their respective negative controls for the flow cytometry studies were purchased from different manufacturers (Table 2.2). For different experiments the fluorochrome dye labelling any given antibody may be altered to allow more combinations to be studied. The dilution of these antibodies and other details will be discussed in sections 2.2.5 and 2.2.7. Table 2.2.

Antibodies	Description	Source
Mouse IgG1 Isotype (Negative control)	* Against CD44 conjugated to APC fluorescent dye.	Miltenyi. Clone (130-095-177)
Mouse IgG1 Isotype (Negative control)	* Against CD133 recognized epitope 1, APC fluorescent dye.	Miltenyi. Clone (130-090-826)
Mouse IgG1 Isotype (Negative control)	* Against CD133 conjugated to PE fluorescent dye.	Miltenyi. Clone (130-095-400
Mouse IgG1 Isotype (Negative control)	* Against CD24 conjugated to PerCP fluorescent dye.	Miltenyi. Clone (130-099-271)
Mouse IgG1 Isotype (Negative control)	* Against CD90 conjugated to FITC fluorescent dye.	Miltenyi. Clone (130-095-403)
Mouse IgG1 Isotype (Negative control)	* Against CD71 conjugated to FITC fluorescent dye.	Miltenyi. Clone (130-095-403)
Mouse IgG1 Isotype (Negative control)	* Against CD34 conjugated to APC fluorescent dye.	Miltenyi. Clone (130-095-402)
CD44	Anti human monoclonal antibody conjugated to APC florescent. The concentration was used at 1:50	Miltenyi. Clone (130-095-177)
CD133	Anti human monoclonal antibody conjugated to epitope 1 with APC florescent tag. The concentration was used at 1:10	Miltenyi. Clone AC133

ALDEFLUOR (Aldehyde Dehydrogenase Based cell Detection) Kit	A reagent kit used to identify human cells to express the level of ALDH enzyme in hematopoietic and non-hematopoietic cells.	Stemcell. Cat No.01700
CD133	Anti human monoclonal antibody conjugated to epitope 1 with APC florescent tag. The concentration was used at 1:10	Miltenyi. Clone (130-111-756)
CD24	Anti human monoclonal antibody conjugated to PerCP florescent. The concentration was used at 1:11	Miltenyi. Clone (130-097-914)
CD90	Anti human monoclonal antibody conjugated to FITC florescent. The concentration was used at 1:11	Miltenyi. Clone (130-095-403)
CD34	Anti human monoclonal antibody conjugated to APC florescent. Ready to use	Beckman Coulter Clone (IM2472U)
CD71	Anti human monoclonal antibody conjugated to FITC florescent. Ready to use	Beckman Coulter Clone (IM0483U)
CD117	Anti human monoclonal antibody conjugated to APC florescent. Ready to use	Beckman Coulter Clone (IM3638)
CD154 (CD40L)	Anti human monoclonal antibody conjugated to FITC florescent. Ready to use	Beckman Coulter Clone (IM2216U)

Table 2. 2: List of the antibodies and their isotypes used throughout the course of the FACS study, showing descriptions, manufacturing sources and clones of these antibodies.

*All Mouse IgG1 Isotype (Negative controls) were monoclonal mouse IgG1 primary against (selected antibody). The Isotype antibody is already diluted in 22 µg/ml by the manufacturer.

2.2 METHODS

All the cell lines used in this study are detailed in Table 2.3. In addition, angiosarcomas were established and studied with small panels of antibodies. These samples were the focus of another research team at the time, but were also included as part of this study.

2.2.1 Patient Samples (Angiosarcomas) Short Term Sarcoma Cultures Established as Part of The Study

Samples of angiosarcoma were obtained with ethical approval from patients treated at the Royal Hallamshire Hospital. The National Research Ethics Committee approval to use these tissues in research was under number 09/H1313/52.

These fresh samples of tumours were taken from patients who received surgical treatment at the Royal Hallamshire Hospital. Tumours were taken to histopathology department and sampled by an experienced sarcoma's histopathologist Dr Malee Fernando, who confirmed the sarcoma diagnosis. A small piece from the tumour then removed for culturing and placed in PBS.

Tumours were dissociated manually, using a scalpel in a Petri dish in a small volume of fresh media, into small pieces approximately 10mm x10mm x5mm. The tissues pieces were transferred into two 15ml tubes (the first tube treated with RPMI media while the other tube treated with CSCM media). Then washed with each respective media by spinning for 10 minutes at 1000rpm. The supernatant was removed and resuspended with fresh media and seeded into two T25 flasks and one Nunc flat bottom test tube (slopes). The amount of media is 5mls in the T25 and 1.5 in the slopes. Finally, the cultures were incubated at 37°C 5% CO_2. Cells were monitored regularly by an inverted microscope and were left to become confluent, changing media if required. Before, the cells in T25 flasks become almost confluent, there will be some cells not attached to the flasks or the slopes (alive floating cells). During the media being changed, instead of discarding these floating cells the media was placed in a centrifuge tube, spun for 10 minutes at 1000rpm and the supernatant was removed. The pellet was resuspended in new fresh media and cultured in T25 and incubated in 5% CO_2 at 37°C. This sub-culture was called a wash (W). The cell count of seeded cells was not performed in this procedure.

2.2.1.1 Routine Tissue Culture of Tumours Cell Lines of Sarcoma and Passaging

All media mentioned was stored at 4°C and before use warmed to room temperature.

Cell lines were cultured in tissue culture flasks T25 and T75 in 5% CO_2 at 37°C and were

passaged when approximately 90% confluent. Cells were washed with RPMI media once and twice with sterile PBS. After that cells were trypsinized by adding 2-3ml of trypsin for 2-5 minutes until they dissociated and placed in a 15ml universal tube. Cells were centrifuged for 5 minutes at 1000rpm in room temperature conditions. The supernatant then removed and the pellet resuspended with fresh RPMI media and cultured in T75 flasks. The number of cells seeded in each flask (T75) was between 0.3 - 1 $x10^6$. The amounts of media in these flasks are double (10mls). During cells seeding, the number of the flasks used varies; and depends on how the cells are growing. If cells are growing fast, then they are split into T75 flasks, and if not, T25 flasks will continue to be used.

Established cell lines are usually passaged once-per-week, but primary cell lines take more time to grow as they are in the early passages and not adapted to the media. As a general guide, established cell lines proliferate faster and were split at a higher ratio i.e. between 1:10 and 1:30. Primary cell lines (angiosarcomas) were spilt at 1:2 and were used in real time for the limited studies performed. All established and Sheffield derived cell lines were bulked up and frozen stocks were prepared; so that all experiments could be performed within a limited passage range to ensure continuity. Cell lines were regularly tested for mycoplasma by the technical team. The majority of the cell lines were established by Dr Abdulazeez Salawu, and primary cultures were established by either Dr Karen Sisley or Meshal Alhajimohammed. The characterization of the lines was previously undertaken and the data published (Salawu et al, 2016).

Cell Line	Sarcoma Type	Supplier/Established in Culture	Passage No. Used
Established Cell Lines			
U2OS	Osteosarcoma	ATTC/1953	63-91 102-110
SW1353	Chondrosarcoma	ATTC/1977	40-63 69-71
SKUT-1	Leiomyosarcoma	ATTC/1972	66-71 89-96
SKLMS-1	Leiomyosarcoma	ATTC/1971	134-152 168-174
PC3	Prostate cancer	ATTC	25-83 30-36
hTERT-RPE-1	Human epithelial retinal cells	S. Collis Institute for cancer	14-16 20-30
Sheffield derived Sarcoma Cell Lines			
02/11 W1	Leiomyosarcoma	Sheffield patient, RTRG (Dr. A Salawi)/2011	60-70
02/11 WS	Leiomyosarcoma	Sheffield patient, RTRG (Dr. A Salawi)/2011	61-78
21/11 W2	Myxofibrosarcoma	Sheffield patient, RTRG (Dr. A Salawi)/2011	45-47 50-57
09/10	Dedifferentiated	Sheffield patient, RTRG (Dr.	75-85

	Liposarcoma	A Salawu)/2010	
13/12W2	Pleomorphic NOS sarcoma	Sheffield patient, RTRG (Dr. A Salawu)/2012	13v-25v
13/12 W1	Pleomorphic NOS sarcoma	Sheffield patient, RTRG (Dr. A Salawu)/2012	16-31
09/11	Pleomorphic Sarcoma NOS	Sheffield patient, RTRG (Dr. A Salawu)/2011	38-41
14/10	Pleomorphic Sarcoma NOS	Sheffield patient, RTRG (Dr. A Salawu)/2010	75-83 104-114
06/11 WS	Pleomorphic Sarcoma NOS	Sheffield patient, RTRG (Dr. A Salawu)/2011	92-106
06/11 W2	Pleomorphic Sarcoma NOS	Sheffield patient, RTRG (Dr. A Salawu)/2011	78-91
06/11	Pleomorphic Sarcoma NOS	Sheffield patient, RTRG (Dr. A Salawu)/2011	81-99
20/11	Dedifferentiated Liposarcoma	Sheffield patient, RTRG (Dr. A Salawu)/2011	23-35
04/13 S	Angiosarcoma of the breast	Sheffield patient, RTRG (Meshal Alhajimohammed)/2013	2-6
04/13	Angiosarcoma of the breast	Sheffield patient, RTRG (Meshal Alhajimohammed)/2013	2-6
08/13 W1	Sarcoma	Sheffield patient, RTRG (Dr. K Sisley)/2013	6-8
07/13	Angiosarcoma of the breast	Sheffield patient, RTRG (Dr. K Sisley)/2013	4-6
07/13SW1	Angiosarcoma of the breast	Sheffield patient, RTRG (Dr. K Sisley)/2013	4-6
07/13S	Angiosarcoma of the breast	Sheffield patient, RTRG (Dr. K Sisley)/2013	4-6
05/13	Angiosarcoma of the breast	Sheffield patient, RTRG (Meshal Alhajimohammed)/2013	2-6

Table 2. 3: A list of Sarcoma Cell lines used in this study, showing established cell lines (commercial), Sheffield derived sarcoma cell lines and primary cell lines. Supplier and passage ranges for each cell line is detailed.

*h-TERT-RPE-1 is a retinal epithelial cell line donated from the Academic Unit of Molecular Oncology and could be maintained in both culture media described above.

Figure 2.1: An example of one of the Sheffield primary sarcoma cell lines (13/12W2) growing in culture showing a confluent monolayer of adherent cells. Photograph at x4 magnification taken by light microscope.

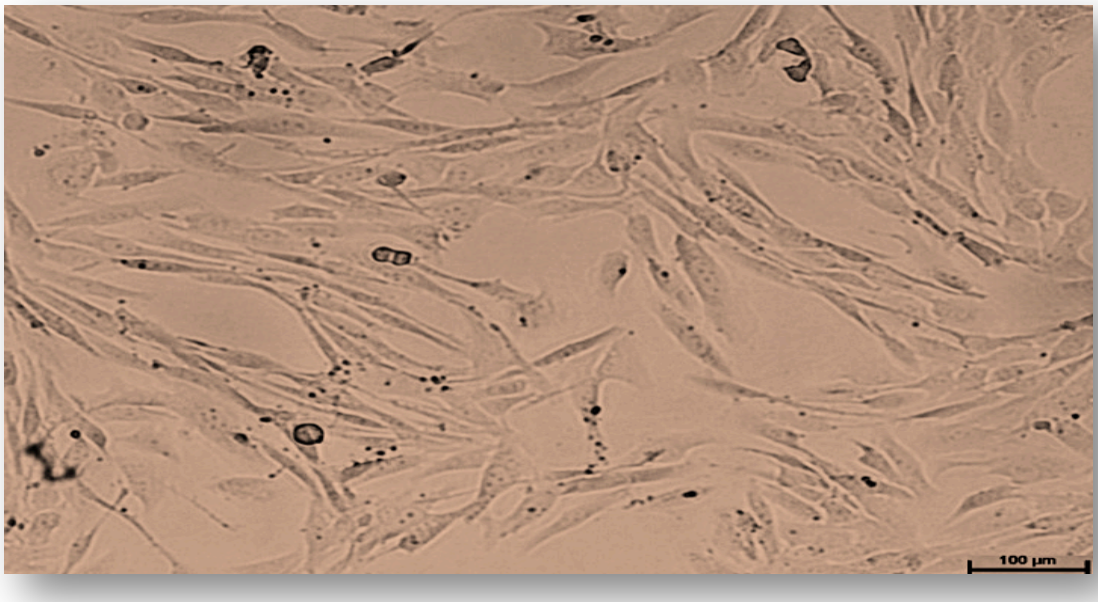


Figure 2.2: An example of another Sheffield primary sarcoma cell line (02/11W1), grown in culture, showing adherent cells, confluency and spindle shape cells, photograph at x10 magnifications taken by light microscope.

2.2.2 Storage of Cell Lines

Cell lines were frozen at -80°C to build up a cell lines stock. Cultured cells are trypsinized, washed and centrifuged. Following centrifuging, cells were resuspended in 1.8ml fetal calf serum. Finally, DMSO was added as 200ul (20% in the total volume). Also, they can be stored in liquid nitrogen if necessary for longer storage.

2.2.3 Cells Counting

2.2.3.1 Manual

Cells were trypsinized, washed (as mentioned in section 2.2.1.1), resuspended in 10ml of media and counted manually using a hemocytometer chamber from Scientific Laboratory Supplies SLS by counting viable (blue) cells. First, the trypan blue was added to the hemocytometer chamber carefully. After 1-2 minutes at room temperature, using a light microscope, cells were counted in four 1x1 mm squares of one chamber and the average number of cells was determined per square. (The chamber is divided into nine 1 mm^2 squares). The viability should be around 95% and calculated according to this equation.

% of viable cells = (Number of blue cells ÷ Number of total cells) × 100.

The number of viable cells per ml and the number of cells to be seeded in each dish, prior to the clonogenic assay, was calculated by using the two formulas below:

1- $\frac{Average\ No.of\ viable\ cells\ x10^5/ml}{5x10^2 cells/ml(dilution)=(*500\ cells)} = X1000 = DF(dilution\ factor)$

2- $\frac{**20ml(volume\ of\ media\ that\ the\ cells\ will\ be\ added\ to)}{DF} = ml\ x1000 =$

$\rightarrow$*** µl (volume of cell suspention per µl added to 20ml media)

* DF calculation was determined based on this number of cells as a baseline.

**This volume varies depending on cell proliferation and cell count. Preferably 10ml or 20ml used for dilution.

*** Volume of a cell taken from the original cell suspension tube to be resuspended in 20ml new media.

2.2.3.2 Automated

This was assessed by using an automated cell counter (Vi-cell XR Cell viability Analyzer) from Beckman Coulter will give an absolute Number of live cells x10^6).

2.2.4 Clonogenic Assay

Colony formation is governed by seeding cells at a low concentration. Cell lines were trypsinized and resuspended in media and centrifuged. Cells were counted either manually or by the automated cell counter with a concentration around 0.5 to 1x10^6 cell/ml prior to seeding. Cells were seeded into 60mm Petri dishes at different cell concentrations (250, 500, 1000, 2000 and 3000 cells). This will allow the cells to form colonies (7 - 14 days) following incubation in 5% CO_2 at 37°C and the different cell concentrations were used to determine the optimal plating number for each cell line.

Following incubation, (colonies were stained by using CV), first, by removing media, washing twice with PBS, then 2mls of cell fixative was added and left for 10 minutes. After incubation cells were washed 1x with PBS and 2mls of CV stain added and incubated for 30 minutes in the hood. Following incubation colonies were washed with distilled water and counted. Figure. 2.3.

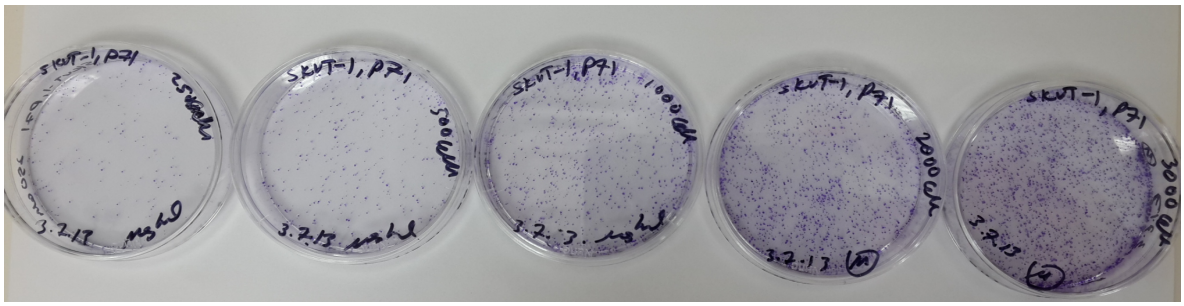


Figure 2. 3: Example of a clonogenic assay performed on one of the commercial cell lines (SKUT-1) with different concentration of cells seeded (250- 500-1000-2000-3000) cells per petri dish. Each Petri dish was left between 7 to 14 days in 5% CO_2 at 37°C to grow and to form colonies, each small blue dot represents a colony, colony numbers were counted for each petri dish. The optimal seeding density was then established for the clonogenic assays. In this example a seeding density of 1,000 cells was selected as optimal.

2.2.5 ALDH Assay

2.2.5.1 ALDH Assay (Aldefluor Kit), Optimization, Expression, and Gating Strategy

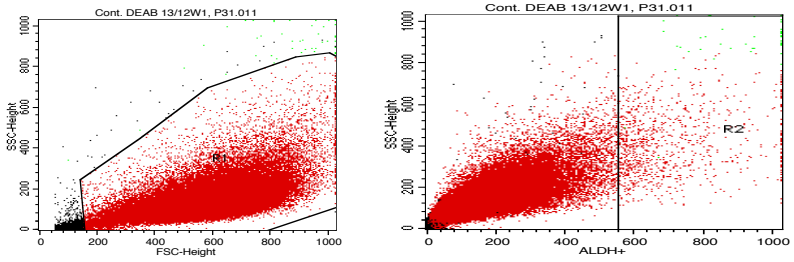
The Aldefluor assay has a substrate called BODIPY aminoacetaldehyde (BAAA) metabolized by ALDH, which can enter the cells through passive diffusion and become aminoacetate (BAA), a fluorescent substance. This substance can be recognizable by flow cytometry. The kit also consists of an inhibitor to prevent any BBA loss. According to the

manufacturer instructions, the dry aldefluor from BODIPY-aminoacetaldehyde-diethyl acetate, (BAAA-DA), is provided as inactive, and must therefore be activated first. At room temperature, 25 µl of dimethylsulphoxide (DMSO) is added to the dry aldefluor vial mixed and left for 1 minute. Then a 25 µl of Hydrochloric acid 2N HCL was added, mixed and incubated for 15minutes. Finally, a 360 µl of aldefluor assay buffer was added to the same vial and mixed. During the use, the activated aldefluor should be stored at 2-8°C otherwise it should be stored at -20°C. The working concentration was 300 µM of the activated Aldefluor. Cell lines were prepared and used at a concentration of $1x10^6$ (see section 2.2.3) and then transferred into a new tube, washed and centrifuged twice for 5 minutes. The supernatant was removed and 1ml of aldefluor buffer was added. The suspended cells were split into two tubes, each containing $5x10^5$ cell concentration (control and test). Only the cells in the control tube were inhibited by adding 10 µl Diethylaminobenzaldehyde (DEAB) and incubated for 10 minutes at room temperature. This step is required to inhibit the ALDH activity of the cells in this tube to be considered as negative control. Following 10 minutes incubation, a 2.5 µl of aldefluor substrate was added to both control and test tubes and incubate for 20-35 minutes at 37°C. Cells were washed by adding 1ml of aldefluor buffer and centrifuged for 5minutes at 1000rpm. Cells were resuspended in 300µl -500µl aldefluor buffer and read by flow cytometry analyzer FACScalibur from Becton Dickinson. Prior to the acquisition of the cells, a dot plot of a Forward (FSC) and Side (SSC) Scatter were created and the voltage of the FSC and SSC were adjusted to create an R1 region. Following that fluorescence channel, 1 FL1 was created VS SSC based on the R1 FSC and SSC dot plot. Based on the DEAB control tube any shift is considered ALDH+. The laser wavelength is 488nm and the bane pass filter was 530/40.
In addition, some cell lines were sorted based on ALDH+/- by FACS Aria (Becton Dickinson) and cultured for further investigations. Figure 2.4. Tables: 2.2, 2.5 and 2.6.

2.2.6 Determining Clonogenicity Post- Sorting of ALDH+ve Cells

Based on ALDH positivity, twelve cell lines were selected for sorting (five established – U2OS, SKLMS-1, SW1353, SKUT-1 and h-TERT-RPE-1) and (seven Sheffield derived primary cell lines – 13/12W1, 06/11, 09/10, 02/11W1, 21/11W2, 09/11 and 14/10). Due to the technical issues associated with using a large number of cell lines the number of cell lines was reduced. Cell lines where no distinct ALDH+ population was detected were therefore not included. Cell lines used were U2OS, 06/11, 09/10, 09/11, 14/10, 02/11W1 and 13/12W1. After the cell sorting by FACS Aria based on ALDH+. Cells were seeded in T25 flasks and then a clonogenic assay was performed in 60mm petri dishes.

A)



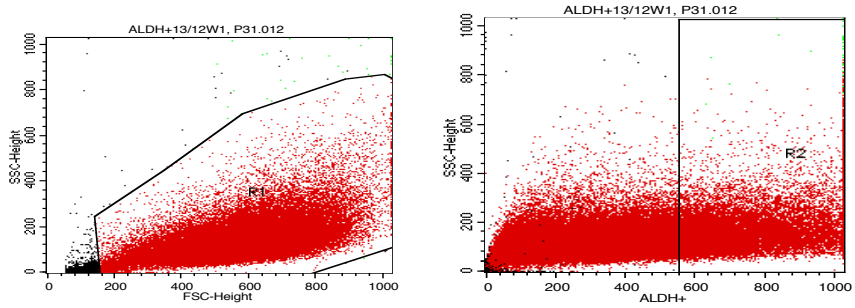
Region Statistics

File: Cont. DEAB 13/12W1, P31.011
Gate: G1
Total Events: 104768

Sample ID: Cont. DEAB 13/12W1, P31
Gated Events: 102421

Region	Events	% Gated	% Total
R1	102421	100.00	97.76
R2	931	0.91	0.89

B)



Region Statistics

File: ALDH+13/12W1, P31.012
Gate: G1
Total Events: 104906

Sample ID: ALDH+13/12W1, P31
Gated Events: 102583

Region	Events	% Gated	% Total
R1	102583	100.00	97.79
R2	27570	26.88	26.28

Figure 2.4 A dot plot diagram of Forward (FSC) and Side (SSC) Scatter showing an example of the activated ALDH+ staining, expression and the gating strategies that were applied on one of the Sheffield primary sarcoma cell line 13/12W1.

R1 (all cells except the dead cells) represents all alive cells been gated; while R2 (right side) represents ALDH+ cells. The negative control lines were applied on cells stained with ALDH to count the positivity percentages using FACScalibur from Becton Dickinson. The total event on the machine software usually sets on 10000 events. In this example each tube contains 5x105 cell concentration prior reading and resuspended in 300µl -500µl; the total gated event for control (A) was 102421 events and 102583 total events for the ALDH +ve (B) showing 27% positivity. The reading time was around from 2 to 3 minutes.

A) Illustrates DEAB (negative control), while **B)** shows ALDH+ expression which, count around 27% positivity.

2.2.7 Standard Staining for Expression of CD Markers and Gating Strategy

To measure the expression of CD marker cells were first stained with primary antibody (CD44, CD133, CD24, CD90) purchased undiluted from Miltenyi, while (CD71, CD34, CD117, CD154) were purchased diluted and ready to use from Beckman Coulter. Each antibody and its respective isotype antibody was conjugated to a fluorochrome dye. CD44, CD133, CD34, CD154 and CD117 were conjugated to an Allophycocyanin APC. CD133 was also conjugated to R-phycoerythrin PE in other panels. CD24 was conjugated to Peridinin chlorophyll protein PerCP fluorochrome. CD90 and CD71 were conjugated to Fluorescein isothiocyanate FITC fluorochrome.

Prior to staining the undiluted antibodies were diluted and aliquoted according to the manufacturer instructions. Then the CD marker antibody was added according to the manufacturers based on the dilution calculations.

CD Marker	Aliquots	Experimental Use
CD44	22 µg/ml	1:50
CD133	22 µg/ml	1:10
CD24	20 µg/ml	1:11
CD90	20 µg/ml	1:11
CD71	-	Purchased diluted and ready to use
CD34	-	Purchased diluted and ready to use
CD117	-	Purchased diluted and ready to use
CD154	-	Purchased diluted and ready to use

Table 2. 4: The list of monoclonal antibodies and dilutions require for use in this study.

In addition, a fresh washing buffer (blocking buffer) of 0.5% ABS was prepared. Two tubes were labelled, the first tube was: 1. Isotype tube (control tube). And the second tube was: 2. Antibody tube (test tube). Cells were prepared as in section 2.2.1.1. The media was discarded and the cell pellet washed for a second time to remove any residual trypsin. The supernatant was removed and 100 µl of the blocking buffer was added for 10-20 minutes. This step is to block any non-specific binding sites that might occur. Prior to incubation, the selected antibody was added to the test antibody tube and the isotype control added to the

control tube. Both tubes were directly incubated at room temperature for 15 minutes in the dark. Stained cells were washed by adding 1ml of the blocking buffer in each tube, and followed by centrifuging the cells for 5 minutes at 1000 rpm. The supernatant was removed and 1ml of blocking buffer was added as a second wash and centrifuged again. Lastly, the supernatant was removed and the cells were resuspended in 300 µl -500 µl blocking buffer with around 0.5 - $1x10^6$ cells in each tube and analyzed using flow cytometry (FACSCALIBUR) Becton Dickinson with 488nm wavelength laser.

2.2.8 Expression of CD44 and Gating Strategy

Cells were treated and stained as mentioned above in (section 2.2.7), and CD44 was used experimentally at 1:50 dilution.

Dot plot of FSC vs. SSC was created and a gating strategy was made to detect the APC florescent through side scatter against FL4, with a 650 nm excitation (Ex) and 660 nm emission (Em) bandpass filter. This is to provide optimumal detection for the fluorescent dye and minimize the excitation of other sources and blocking light in the fluorescence emission band. Figures 2.5 and 2.6. Tables 2.2, 2.4, 2.5 and 2.6.

2.2.9 Expression of CD133 and Gating Strategy

Cells were prepared as in section 2.2.7, and CD133 was diluted and used at 1:10 dilution.

Dot plot of FSC vs. SSC was created and the gating strategy was applied to detect the APC and PE fluorescent through side scatters against FL4 and FL3, respectively. As mentioned APC Ex was 650 nm, the Em was 660 nm, and for the PE Ex was 480, 565 nm and Em was 575 nm, bandpass filter excitation. Figure 2.5. Tables 2.2, 2.4, 2.5 and 2.6.

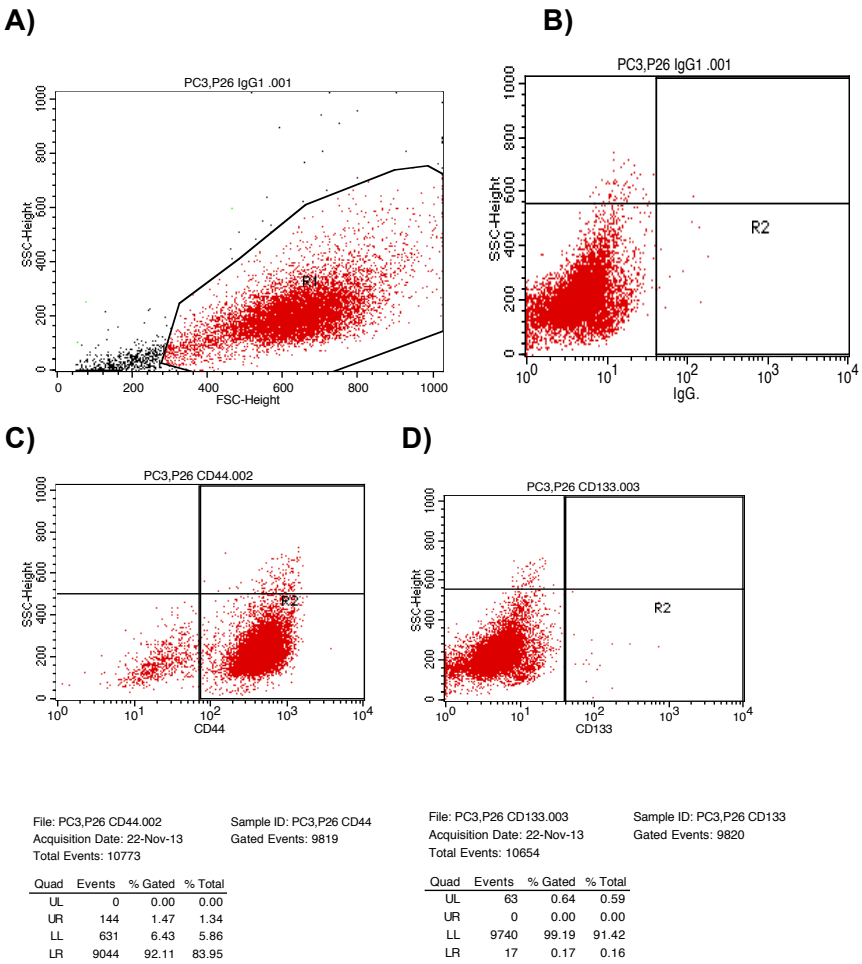
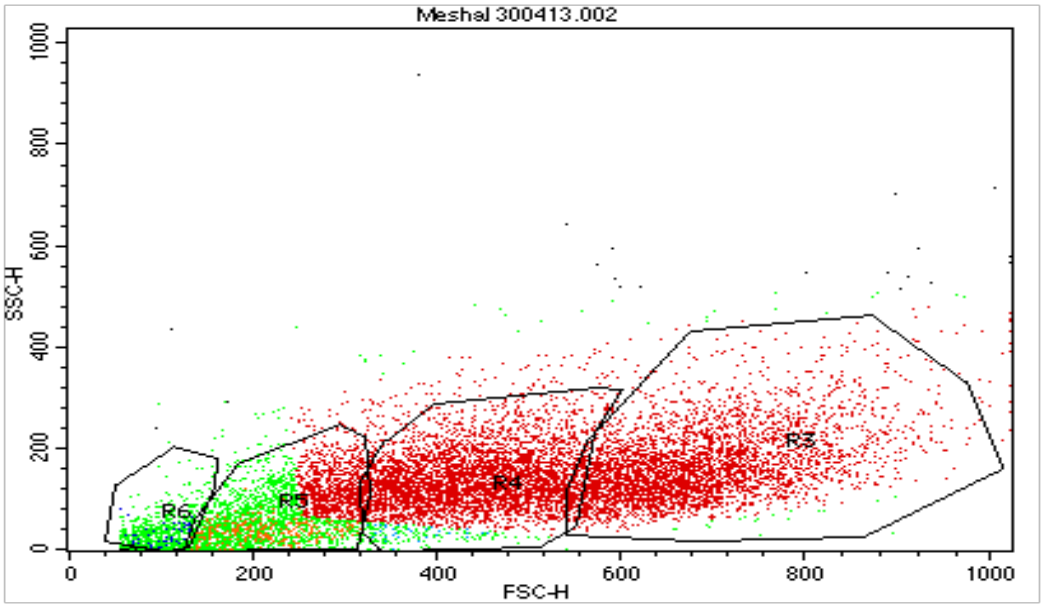


Figure 2.5: A dot plot diagram of Forward (FSC) and Side (SSC) Scatter showing an example of the CD44 and CD133 staining, expression and the gating strategies were applied on one of the commercial cell line (PC3). R1 represents all alive cells been gated while R2 represents positive cells. The negating control lines were applied on cells stained with antibodies to count the positivity percentages using FACScalibur from Becton Dickinson. The total event on the machine software usually sets on 10000 events. CD44 total events are 10773 (UR AND LR) on the dot plot with 93% positivity. CD133 total events are 10654 (UR AND LR) with 0.2% (negative). In this example each tube contains around 0.5 - 1x10^6 cell count prior reading and resuspended in 300µl -500µl. Reading time was around 2-3 minutes.

A) This graph is showing the R1 region (population) gating all the alive cells PC3 cells. **B)** The negative control (isotype) showing no false positivity. **C)** and **D)** are the CD44 (93%) and CD133 (0.2%) expression with the percentages positivity sup-population. In this dot plot set any positivity should be seen on UR AND LR parts.

A) FSC/SSC dot plot format



B)

Control applied on different gates based on the FCS/SSC dot plot

C) CD44 applied on different gates based on the FCS/SSC dot plot.

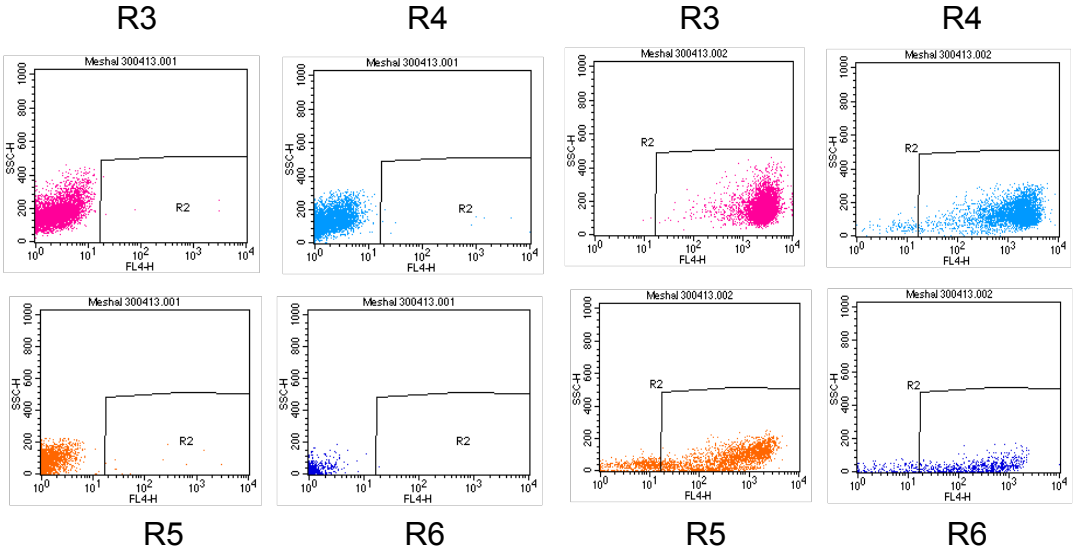


Figure 2.6: A multiple dot plot diagram analysis of Forward (FSC), Side (SSC) Scatter and antibody positivity gating against SSC; showing different gates (dot plot platforms) based on the cells size.

This example is of CD44 staining, expression and gating strategy on PC3 and observes the expression of CD44 on small and large cells on the same dot plot. A) R1 gate represents all the alive cells, R2 gate represent positive cells after the application of the negative isotype control reading for the respected gates in (B) (R3, R4, R5 and R6), using FACScalibur from Becton Dickinson. (C) The positive CD44 for each gates (R3, R4, R5 and R6). This analysis was performed to detect the intensity of CD44+ve cells compared to control on different dot-plot platforms and different gating based on cell sizes appearance. (C) The gates R5 and R6 show small cells with less brightness compared to large cells on R3 and R4 gates. The total event on the machine software usually sets on 10000 events. CD44 total events are 10773 (UR AND LR) on the dot plot with 93% positivity. In this example each tube contains around 0.5 - 1x10^6 cell count prior reading and resuspended in 300µl -500µl. Reading time was around 2-3 minutes.

Technique	Description
Surface markers (e.g. CD133 and CD44)	Cells incubated with fluorescent antibodies for specific surface markers. Flow cytometry used to isolate cells expressing the surface marker
Aldehyde Dehydrogenase (ALDH)	Cells incubated with a reagent that was activated to a fluorescent state by ALDH. Flow cytometry used to isolate the cells with the most ALDH activity
Side population analysis (dye exclusion)	Cells incubated with a fluorescent dye. Flow cytometry used to select a population that excludes the dye. Referred to as the "side population" because of its location on the left-most portion of the flow-plot

Table 2. 5: Techniques used to isolate sarcoma-initiating cells (CSC) reported in the literature. Adapted from (Trucco and Loeb, 2012).

2.2.9.1 Determining Clonogenicity Post- Sorting of CD133+ve cells

CD133 positivity for five established lines – U2OS, SKLMS-1, SW1353, SKUT-1 and h-TERT-RPE-1, and seven Sheffield derived primary cell lines – 13/12W1, 06/11, 09/10, 02/11W1, 21/11W2, 09/11 and 14/10 was established. Only Seven-sarcoma cell lines were selected for determining clonogenicity post cell sorting (U2OS, 06/11, 09/10, 09/11, 14/10, 02/11W1 and 13/12W1) due to the issues discussed previously. After the cell sorting by FACS Aria based on CD133+ve. Cells were seeded in T25 flasks and then a clonogenic assay was performed in 60mm Petri dishes.

2.2.10 Expression of CD24 and Gating Strategy

A dot plot of FSC vs. SSC was created and a gating strategy was made to detect the PerCP florescent through side scatter against fluorescence-3 FL3. The PerCP fluorochrome Ex was 490 nm and the Em was 675 nm bandpass filter. Tables: 2.2, 2.4 and 2.6.

2.2.11 Expression of CD90 and Gating Strategy

See (section 2.2.9) for more details. CD90 was diluted and aliquoted and prepared at 1:11 (10 µl added to 100 µl in the blocking buffer). A dot plot of FSC vs. SSC was created and a gating strategy was made to detect the FITC fluorescent through side scatter against fluorescence-1 FL1. The FITC fluorochrome Ex. was 494 nm and the Em was 518 nm bandpass filter. Tables: 2.2, 2.4 and 2.6.

2.2.12 Expression of CD71 and Gating Strategy

See (section 2.2.7) for more details for preparation and staining. CD71 was purchased diluted and ready to use. A dot plot of FSC vs. SSC was created and a gating strategy was made to detect the FITC fluorescent through side scatter against fluorescence-1 FL1. The FITC fluorochrome Ex. was 494 nm and the Em was 518 nm bandpass filter. Tables: 2.2, 2.4 and 2.6.

2.2.13 Expression of CD34 and Gating Strategy

See (section 2.2.7) for more details for preparation and staining. CD34 was purchased diluted and ready to use. A dot plot of FSC vs. SSC was then created and a gating strategy was made to detect the APC fluorescent through side scatter against fluorescence-4 FL4. The APC fluorochrome Ex. was 650 nm and the Em was 660 nm bandpass filter. This is to provide optimal detection for the fluorescent dye and minimize the excitation of other sources and blocking light in the fluorescence emission band. In addition, the compensation was high 61.4 (FL3) and detector/amplifier 690 (FL4). Tables: 2.2, 2.4 and 2.6.

No	Fluorochrome	Excitation Ex (nm)	Emission Em (nm)
1	Fluorescein isothiocyanate FITC	494	518
2	R-phycoerythrin PE	480, 565	575
3	Peridinin chlorphyll protein PerCP	490	675
4	Allophycocyanin APC	650	660

Table 2. 6: Summary list of all the fluorochromes, excitations and emissions used in this study.

2.2.14 Expression of CD117 and Gating Strategy

See (section 2.2.7) for more details for preparation and staining. CD117 was purchased diluted and ready to use.

A dot plot of FSC vs. SSC was created and a gating strategy was made to detect the APC fluorescent through side scatter against fluorescence-4 FL4. The APC fluorochrome Ex. was 650 nm and the Em was 660 nm bandpass filter. The compensation was high 61.4 (FL3) and detector/amplifier 690 (FL4). Tables: 2.2, 2.4 and 2.6.

2.2.15 Expression of CD154 and Gating Strategy

See (section 2.2.7) for more details in terms of preparation and staining. CD154 was purchased diluted and ready to use. A dot plot of FSC vs. SSC was created and a gating strategy was made to detect the APC fluorescent through side scatter against fluorescence-4 FL4. The PE fluorochrome Ex. was 480, 565nm and the Em was 575 nm bandpass filter. In addition, the compensation was high 61.4 (FL3) and detector/amplifier 690 (FL4). Tables: 2.2, 2.4 and 2.6.

2.2.16 Co-expression of The Antibodies

2.2.16.1 Optimization for Co-expression of The Antibodies

To measure the co-expression of antibodies in one tube, cells were prepared as previously detailed and the same dilutions of isotypes and primary antibodies was used as detailed in, sections 2.2.5 to 2.2.15. To increase the accuracy of the reading, the first two tubes were the control tubes (no staining only the cells in the first tube and in the second tube was negative control-isotype). The rest of the controls tubes were isotype only with one fluorescent dye. The antibodies panel used for each cell lines was as follows:

1- Control-1 (cells without staining).
2- Control -2 (cells stained with isotype contain the four fluorescence FITC/PE/PerCP/APC).
3- Isotype FITC only.
4- Isotype PE only.
5- Isotype PerCP only.
6- Isotype APC only.
7- Antibody tube FITC.
8- Antibody tube PE.
9- Antibody tube PerCP.
10- Antibody tube APC.
11- Antibody tube FITC.
12- CD 90/133/24/34.
13- CD 90/133/24/44.
14- CD 71/133/24/44.
15- ALDH/CD 133/24/44.
16- CD 71/154/19/34 (performed only once on 13/12W2 cell line).
17- 71/154/19/117 (performed only once on 13/12W2 cell line).

In all instances use was according to the manufacturer instructions and a fresh washing buffer (blocking buffer) of 0.5% ABS was prepared.

To ensure there was sufficient stocks (within the same passage range) of all cell lines for the FACs analysis sufficient stocks of each line were prepared in advance. As mentioned in sections 2.2.1.1 and 2.2.2. Cells were prepared as detailed in sections 2.2.7 and 2.2.5. However, the ALDH staining is different from the rest of the antibodies see section 2.2.5. In order to obtain the maximum excitation of the antibodies and ALDH require incubation at 37°C and this result may affect the other antibodies excitations. The co-expression was performed in two attempts. Tables: 2.2 and 2.5.

Dot plots of FSC vs. SSC were created and a gating strategy assessed all through side scatter against fluorescence-1, 2, 3 and 4 FL1, FL2, FL3 and FL4 respectively. The detection was made firstly for each control and antibody separately and then the co-expression was performed. This is to calibrate the FACS machine properly.

2.2.16.2 Co-expression Staining with ALDH

As ALDH staining follows a different protocol and may affect the other antibodies the effect of staining with antibodies first or second was explored. Cells were trypsinized, dissociated and stained with antibodies and ALDH as described in section 2.2.5 up to section 2.2.13. The ALDH used was Fluorescein isothiocyanate FITC so the rest of the antibodies used R-phycoerythrin PE (CD133) and Allophycocyanin APC (CD44). The flow cytometry set up voltage (detector/ amplifier) was 580 and 1.8 compensation (low). This co-expression experiment was performed on 13/12W2, P20v cell line. The only difference was whether the cells with stained first with ALDH or the other antibodies.

The results show that when cells were stained with antibodies and then ALDH, the antibodies lost some of their intensity and the expression was less when compared with the second experiment. In the second experiment (ALDH then antibodies) there was excellent excitation and the expression was no different when the antibodies were stained alone. There were however some cells that seemed to have shrunk (based on flow cytometry dot plot readings) as a result of this protocol, but the staining was still robust and significant.

2.2.17 Cell Proliferation Assay (MTT)

2.2.17.1 MTT Solution Preparation

MTT stock (thiazolyl blue tetrazolium bromide) 5mg/ml from sigma with Catalogue Number

M5655-1G stored at 2-8°C was diluted with sterile PBS, wrapped in foil to protect from direct light and stored at 4°C. The working solution was 1ml of the MTT stock added to 4mls of sterile PBS.

2.2.17.2 MTT Assay

The MTT ((4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a colourimetric assay used to assess cell metabolic activity or proliferation. It is based on the principle that only the viable cells can metabolize tetrazolium to form blue (or purple colour) crystals of insoluble formazan. In brief, cells were seeded in equal numbers into a 96 well plate with MTT added to the cultures. Any formazan crystals built up will be dissolved after DMSO has been added. Cultured cells were compared with the control and read by spectrophotometer to obtain the absorbance and as a result, the number of viable cells that remain in the culture. This equation was used to calculate the density of the cells been added to each well.

$$= \frac{Cell\ count * 1{,}000{,}000 * (amount\ of\ suspended\ cells\ added(\mu l)\ in\ a 10ml\ new\ media)}{50{,}000/\mu l}$$

Triplicates were set up of around 50,000 cells in 100µl of media in each well of a 96 well plate. Three 96 well plates were one for each of the time points (24hours, 48hrs and 72 hours). At each time point 100µl of MTT was added to each well and the plate incubated wrapped in foil in an incubator at 5% CO_2 / 37°C for two hours. Then, the mixture (media and MTT) was removed completely from each well by a multi-pipette aspirator and 100µl of 99.9% DMSO was added. Plates were incubated for 30 minutes wrapped in foil and placed on a shaker with a gentle agitation at room temperature. The measurement of the optical density absorbance was performed for both control (only MTT no cells were added) and test cells by a spectrophotometer at 570nm. The spectrophotometer was adjusted to suit the experiment. The parameters Emission filters (white) and excitation (red) were put in place. Then FLUO star galaxy software for reading was used, and excel sheets of the absorbance results were produced. The calculation of the absorbance ratio was performed and an average of three replicate wells was obtained. Statistics for significant results ($p<0.05$) were done by t-test, using GraphPad Prism® software v7.0.

2.2.18 Stress Assay (SA)

The Stress Assay used in this study was developed in three phases. Briefly, cells were seeded in flasks and left to become completely confluent. Then cells were left in a “stressed status”, without feeding or changing media. Cultures were observed regularly by an

inverted microscope and pictures were taken to monitor which cells can survive the stress and what changes could happen to the cells. Both established (adapted) and Sheffield derived cell lines were tested.

2.2.18.1 Stress Assay (SA)/ Phase One

This was the initial exploratory phase. For each cell line T75 flasks were used. Double the usual amount of media was used (10mls) and the number of cells seeded was low, around 0.3-0.5x $x10^6$ to allow observation of the cells ability to form colonies. Cultures were incubated in 5% CO_2 at 37°C for 8-9 months and monitored by an inverted light microscope once a week. At the end of the 8-9 months period, cells were pictured and then washed –off and the wash retained in a new flask. Both the original flask and the washes were re-feed and incubated in 5%CO2 at 37°C and any growth / recovery was regularly monitored.

2.2.18.2 Stress Assay (SA)/ Phase Two

In phase 2 the number of cells seeded in each flask was 1x $x10^6$ and the period of their stress was shortened to 2 to 3 months without changing the media. Cells were monitored and pictured on the 7^{th} day, 14^{th} day, 21^{st} day, at confluency and when they appeared dead. For each cell lines nine flasks were set up, three T75 flasks for the stress assay (triplicate) three flasks to be grown to monitor confluency and provide the maximum viable cell number at the point of the stress assay. Three T75 flasks were set up one for DNA extraction, continued culture and last flask for back up and freezing down. When the cells were confluent, RPMI media was changed and left for 24-72 hours to give the cells more room to grow. Cell counting (maximum viable cell number MVCN) was performed, to determine the average of live cells in each flask using an automated cell counter (Vi-cell XR Cell viability Analyzer) from Beckman Coulter. After the period of stress (2-3 months), the old media was removed and transferred into a universal tube and centrifuged for 5 minutes at 1000rpm in room temperature conditions. Cells then were resuspended in a new media and cultured in T25 flasks as a wash-off of the post-stress assay (WPOST-SA). Culture flasks were then monitored to observe any growing cells and pictures were taken. Cells that proliferated and become confluent were subsequently cultured in new flasks and maintained for other assays. The originally stressed flasks were rinsed with fresh RPMI media and re-fed with new media. Flasks labelled as post-stress assay (POST-SA). Cells observed for any growth and left to become confluent and then cultured and maintained for other assays. Figure 2.7.

2.2.18.3 Stress Assay (SA)- Phase Three

In the final phase three adjustments were made based on the findings of the 2 earlier phases, shown in Figures 2.8 and 2.9.

In phase three cells were stressed for 4-months. To minimize drift as a result of a different starting population one flask was used to bulk up the number of flasks required for all aspects of the study (to be performed in triplicate). The starting population flask was then passaged into four T75 flasks, three of these flasks were then split to a further three flasks in preparation for the assay and fourth was maintained in culture to assess for genetic drift as a result of extended culture. From each of these sets one was selected to establish the MVCN using the MTT assay, and Clonogenic assay, array CGH and flow cytometry were all performed to establish the characteristics prior to stress recovery and compare populations.

The flasks for stressing were monitored and pictured on the 7th day, 14th day, 21st day, when confluent and when they appeared dead. Stage E is the period where the cells had been stressed for 4 months and stage F was for post-stress assay (POST-SA). Each flask had the media changed (POST-SA) and the washes retained WPOST-SA). Pictures were taken before and after the media were changed. Cells then monitored for any growth and pictures were taken. Cells that proliferated and become confluent were cultured in new flasks and maintained for other assays. The same Assays were performed before the stress assay was repeated on the POST-SA cells.

2.2.19 Determining Proliferation for Pre and Post Stress Assay and Expression of ALDH+ and CD44+ Cells

Two sarcomas cell lines were selected (SKUT-1 and 02/11W1) for co-expression staining for ALDH and CD44 cell sorting of pre and post-stress assay populations. Other antibodies were not selected based on their low expression and because of time constraints the study was limited to just the two lines. Cells from pre and post-stress assay's populations were as described in sections (2.2.4, 2.2.5, 2.2.7 2.2.8, 2.2.10, 2.2.11 and 2.2.13). After the sorting by FACS Aria based on ALDH+ve and CD44+ve; Cells were seeded in T75 flasks and then a clonogenic assay was performed in 60mm Petri dishes.

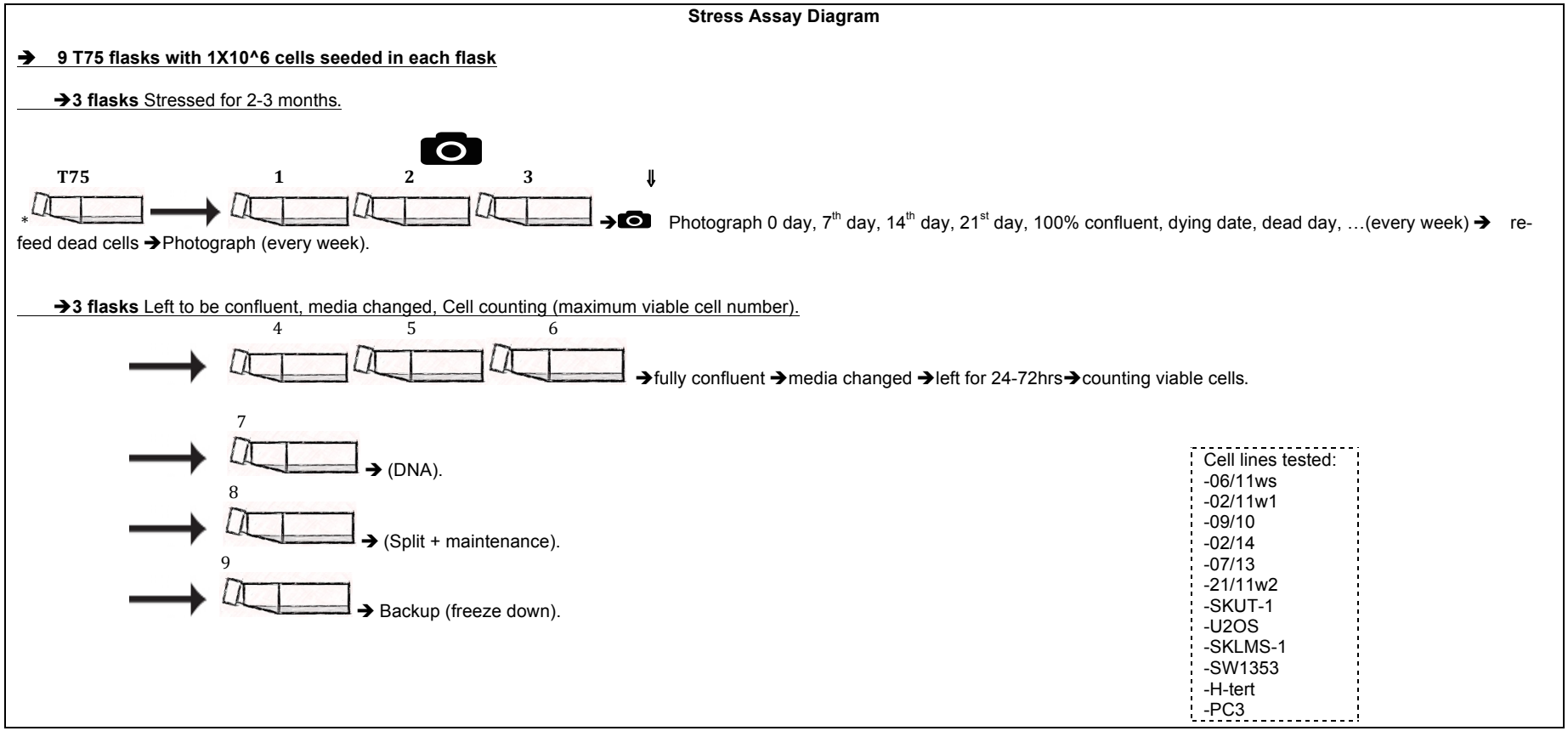


Figure 2.7: A diagram shows in details, the flow work was performed and developed on cell lines during the stress assay experiments in the second phase.

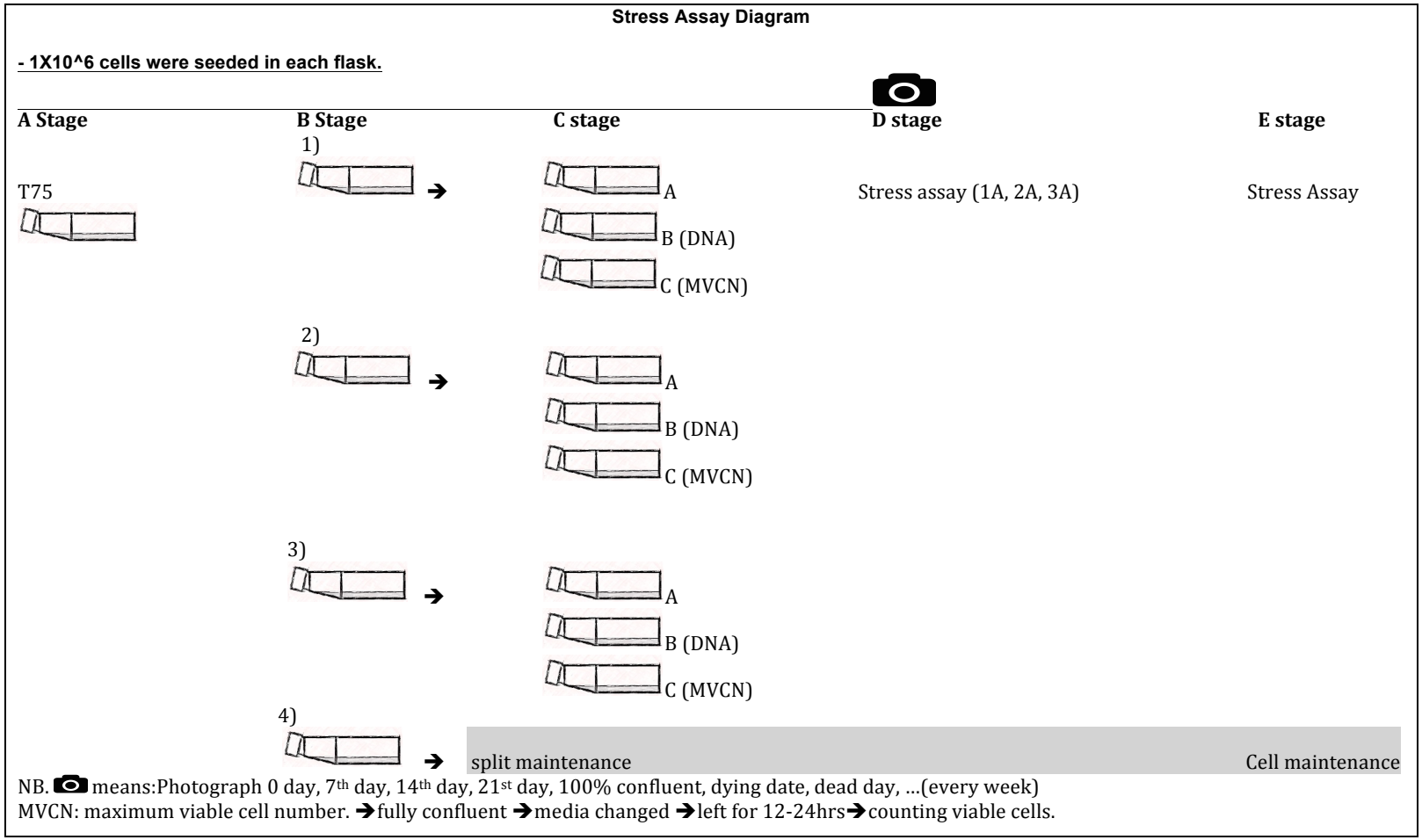


Figure 2.8: A diagram shows in details the amended version of the flow work were performed and developed on cell lines during the stress assay experiments in phase two (was amended later see next page).

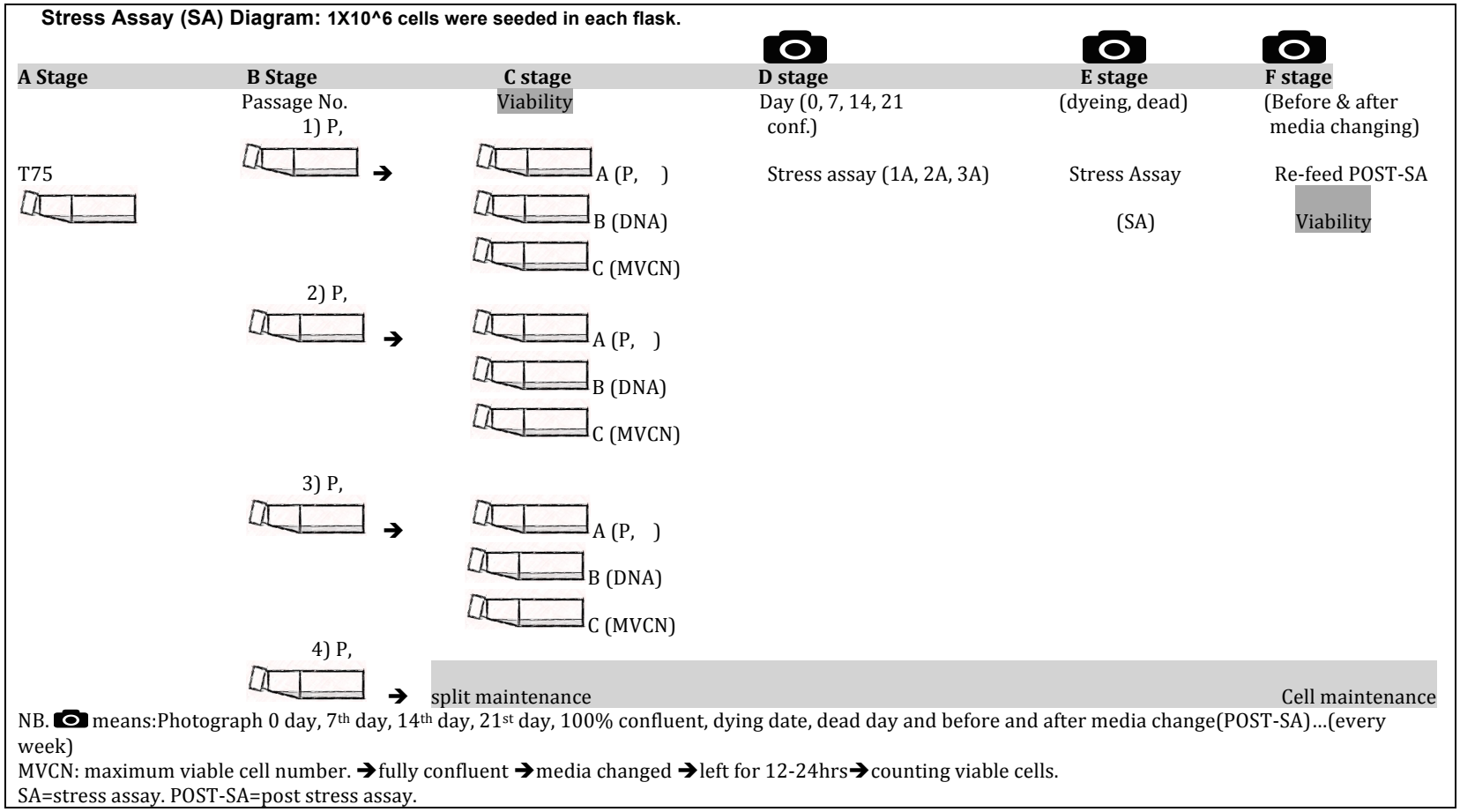


Figure 2.9: A diagram shows in details, the last amended flow work that was performed and developed on cell lines during the stress assay experiments in phase three.

CELL LINES: _____ Cells were stressed for_____

EXPERIMENTS LIST SUMMARY (Established cell lines)

	Cell lines	Stressed date	Cell count	Volume(mls)= No.of cells(50,000cells) will be seeded in this volume	Stress assay 1x10^6 in each flasks & Date of stress assay start	Viability (trypan blue) before SA	Cell count flasks for stress	DNA	MVCN	Pic Before changing the media	Viability after SA	Date of re-feed	Pic After changing the media day 1, 3, 7, 14.	Pics of the maintained cells 7,14,21,	Comments
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12															
13															
14															
15															
16															
17															
18															

Table 2. 7: This table shows in details, the flow work of monitoring established (commercial) cell lines during stress assay experiments in phase three.

CELL LINES: _____ Cells were stressed for_____

EXPERIMENTS LIST SUMMARY (Primary cell lines)

	Cell lines	Stressed date	Cell count	Volume(mls)= No.of cells(50,000cells) will be seeded in this volume	Stress assay 1x10^6 in each flasks & Date of stress assay start	Viability (trypan blue) before SA	Cell count flasks for stress	DNA	MVCN	Pic Before changing the media	Viability after SA	Date of re-feed	Pic After changing the media day 1, 3, 7, 14.	Pics of the maintained cells 7,14,21,	Comments
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12															
13															
14															
15															
16															
17															
18															

Table 2. 8: A table shows in details the flow work of monitoring Sheffield primary cell lines during stress assay experiments in phase three.

2.2.20 DNA Extraction (Isolation and Purification from Cultured Cells)

The DNA from cultured cells was extracted by using Qiagen DNeasy® Blood and Tissue Kit. In summary, the kit performs on the principle of lysing alkaline (protease), protein digestion, DNA releasing, washing, elution, and DNA purification by loading it on a silica-based membrane; which contain the ability of DNA selection in the presence of chaotropic salt in high concentrations. The content was then centrifuged followed by two washes to remove any contaminants, and then the pure DNA was eluted.

Cells were obtained from confluent flasks; trypsinized, dissociated, resuspended and centrifuged as shown in section 2.2.2. Cells were resuspended in 200µl of sterile PBS in an Eppendorf tube (1.5ml), then 20µl of proteinase K was added with 200µl of AL buffer (lysing buffer). The mixture was vortexed and incubated on a heat block (56°C) for 30 minutes. Usually, the mixture becomes clear after this time, if not either additional proteinase K was added or the time of incubation was increased to facilitate lysing. 200µl of absolute ethanol was added and vortexed and the mixture transferred into a single labelled DNeasy® mini spin column placed inside a collection tube and centrifuged for 1minute at 8,000 rpm. 500µl of washing buffer 1 (AW1) was added, and a new collection tube was placed underneath and centrifuged for 3 minutes at 14,000 rpm. The step was repeated but washing buffer 2 (AW2) was used instead. DNA from cultured cells was eluted in elution buffer (AE)

After the second wash, the mini spin column was placed in a new-labelled Eppendorf tube (1.5ml) and centrifuged for 1 minute at 8,000 rpm. Then, the mini spin column was discarded and the tube with DNA was stored in the fridge at 4°C.

2.2.20.1 Quantification and Purity Assessment of The DNA

The DNA concentration and purity were acquired by using UV/VIS spectrophotometry as NanoDrop® ND-1000 (Thermo Fisher Scientific, USA). It was set up and used to measure double-stranded DNA. The amount of DNA concentration was Auto-calculated by loading only 1µl and using Beer-Lambert modified equation:

$$c = (A * e)/b$$

Where c represents the concentration in ng/µl; A for absorption at 260nm, e for wavelength-dependent absorbance coefficient (50ng· cm/µl for double-stranded DNA) and b is path length obtained in cm. The Absorbance ratio was at 260nm/280nm to

acquire DNA purity that indicates the absence of any protein contamination. The other parameter 260nm/230nm indicate the absence of other organic complex contamination such as salt. The importance of obtaining ideal DNA concentration is because; it is a critical element in terms of having to array CGH excellent labelling and data in a later stage.

NanoDrop® optical surface was cleaned with special lint-free wipes, then 2 µl of initializing nuclease-free water. Then the instrument was blanked with 2 µl AE buffer and then DNA samples were loaded at the same amount, the optical surface was cleaned between samples to avoid any contaminations. The absorbance ratio was within the acceptable range (1.8-2). DNA Samples then stored at 4°C or -20°C or -80°C (for tissues).

2.2.21 Genome Analysis by Array-based Comparative Genomic Hybridisation (aCGH)

2.2.21.1 aCGH Array

Oligonucleotide Array-based Comparative Genomic Hybridisation considered being a recent powerful molecular technique is used to examine the whole genome to distinguish copy number aberrations in tumour DNA. In brief, both matched reference DNA (control) and cell line sample DNAs were labelled with two different fluorescent dyes Cyanine 5 (Cy5™) and Cyanine 3 (Cy3™). They were hybridized together on an off-shelf microarray slide on 180,000-oligonucleotide probes array that screen in high resolution the whole genome. The next step was washing the array and scanning. Then quantifications of both DNAs samples ratio were obtained by a digital analysis system. The main steps, in general, are exhibited in Figure 2.10.

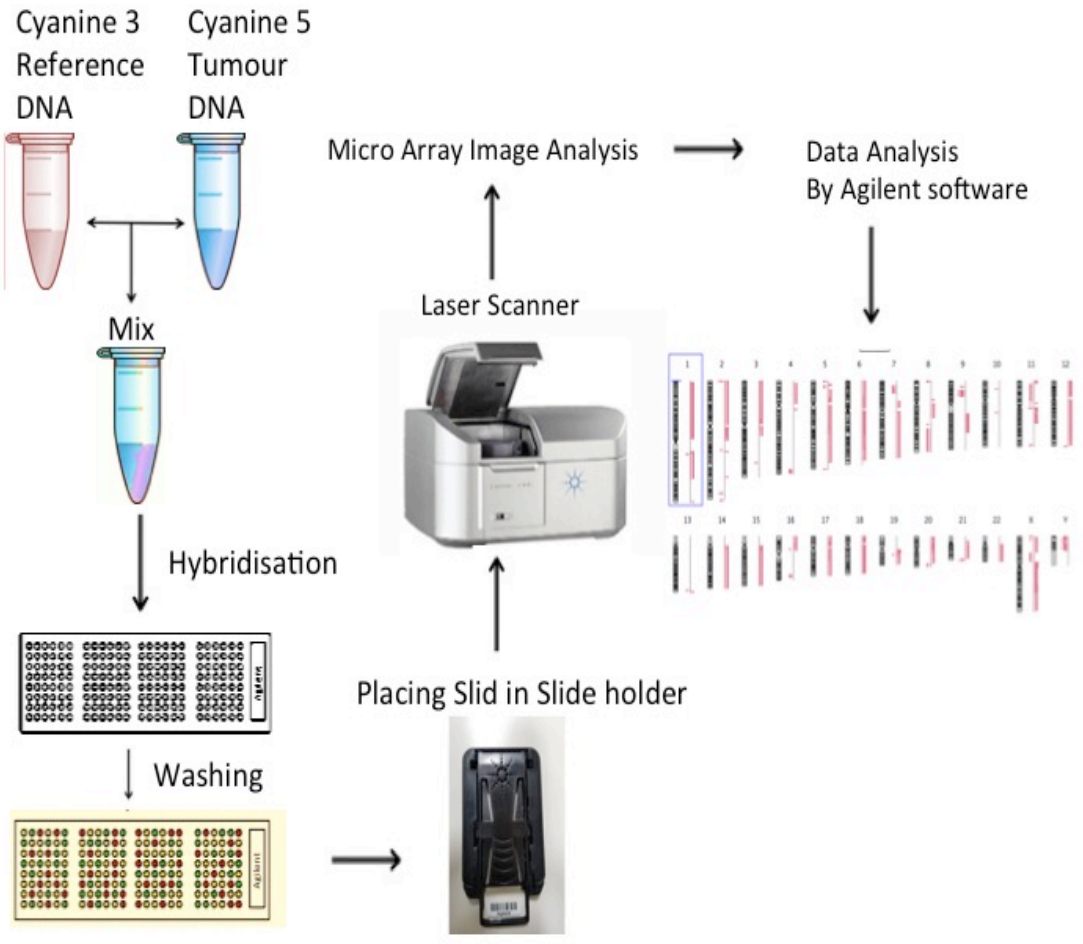


Figure 2.10: A diagram shows the steps of the Genome Analysis by Array-based Comparative Genomic Hybridisation (aCGH) performed in this study.

Start with labelling then hybridization, washing and then loading the slid inside the laser scanner. Finally, data were assessed and analyzed. (Laser scanner image was imported from http://www.agilent.com).

2.2.21.2 DNA Labelling by (Random Priming with Exo-Klenow)

Extracted DNA from the sarcoma cell lines was compared against commercial reference DNA. Both were labelled by using an enzymatic method, random priming with Exo-Klenow, which contain different fluorescence that labels nucleotides. Then Cot-1 was used for blocking any repetitive binding before the hybridisation of the DNAs to the genomic array was carried out. The ratio of the fluorescent signals for both DNAs samples detected. For an Ideal array experiment amount of 0.5 - 1µg should be labelled separately for both DNA samples (test and reference). As described in section 2.2.22 DNAs quality were obtained.

2.2.21.3 Restriction Digestion on The DNA

An amount of 1µg DNA for 4 x 180k microarray slide needed for eight samples (4 tumours and 4 reference DNA) were added in 0.2 cleaned PCR tubes, and made up to a final volume of 20.2µl with nuclease-free water.

The first step was DNA digestion into smaller strands for all samples. The master mix was prepared as detailed in Table 2.9. Each sample (5.8µl in total) contains nuclease-free water (2µl), restriction buffer 10x buffer RE (2.6µl), Acetylated BSA (10µg/µl) for stabilizing the reaction, Alu-1 (0.5µl) and RSA-1 (0.5µl), Alu-1 and RSA-1 must be placed on ice. Each tube received a 5.8µl of the master mix added to the previous amount of 20.2µl gDNA, which makes a total of 26µl. PCR tubes then placed on a PCR instrument (Eppendorf Mastercycler Thermal Cyclers, USA) figure 2.7, incubated for two hours at 37°C. then 20 minutes at 65°C lastly, hold at 4°C. Samples were kept in a -20°C freezer overnight for the next step (labelling).

	Per tube (µl)	*For 9 samples (µl)
Nuclease free water	2.0	18
10x duffer RE	2.6	23.4
Acetylate BSA (10µg/µl)	0.2	1.8
Alu-1 (10µg/µl)	0.5	4.5
RSA-1 (10µg/µl)	0.5	4.5
Total	5.8	52.2

Table 2. 9: Master mix components for DNA digestion in preparation for array CGH.

2.2.21.3.1 Enzymatic Labelling Reaction

5µl of the random primer was added to all 8 tubes, mixed by pipetting to reach a total volume of 31µl. Tubes were then incubated on PCR instrument figure 2.11, for 3 minutes at 95°C, and held at 4°C whilst preparing the master mix.

The labelling master mix was prepared by using Cy-3 dUTP and 5Cy5 dUTP in different tubes each placed on ice and covered from direct light, Table 2.10.

	Per tube (µl)	*For 5 samples (µl) Cy-3dNTP (Reference DNA)	*For 5 samples (µl) Cy-5dNTP (Tumour DNA)
5 x reaction buffer	10	50	50
10 x dNTB	5	25	25
Cyanine-3 dUTP	3	15	-
Cyanine-5 dUTP	3	-	15
Exo-Klenow fragment	1	5	5
Total	19	95	95

Table 2. 10: Master mix of Exo-Klenow Labelling for array CGH.

19µl of the master mix was added to each tube (makes a 50µl total volume), mixed by gently pipetting. Tubes were placed on the PCR instrument for 2 hours at 37°C to allow the labelling reaction to take place, then inactivating the enzyme reaction at 65°C for 10 minutes. Samples were held at 4°C.

2.2.21.3.2 Clean-up of Exo-Klenow Labelled DNA

The labelled genomic DNAs were cleaned using Amicon® 30kDa filters. These filters are designed to trap DNA fragments based on their size. Each labelled sample was transferred to the Amicon filters and washed by adding TE buffer 430µl (PH 8), mixed gently by pipetting and centrifuged for 10 minutes at 14,000 rcf. Then the collection tube was discarded and the filter placed in a new collection tube and 480µl of the TE buffer was added, mixed gently by pitting and centrifuged at 14,000 rcf for 10 minutes. Filters then inverted and placed in new tubes centrifuged for 1 minute at 1,000 rcf in room temperature conditions. The harvested volume was usually around 21µl of labelled DNA. Samples foiled and stored in -20°C freezer overnight.

2.2.21.4 Measurement of DNA Labelling Efficiency

Prior to determining the fluorescent activity of the labelled DNA, samples were thawed out and placed in Speed Vac for 30 minutes to evaporate any liquid and obtain a consistent volume. Then samples were resuspended with TE buffer in 21µl.

NanoDrop® ND-1000 UV-VIS Spectrophotometer was used to measure the fluorescent activity of each labelled DNA sample. Based on the absorbance the Spectrophotometer calculates DNA concentration, Cy-3 and Cy-5 dyes at specific wavelengths and corresponds with each element at 260, 550 and 650nm, respectively. Array software was set up on double-stranded DNA 50ng·cm/µl (DNA-50) to determine the absorbance coefficient. Prior to loading, the samples spectrophotometer was blanked with 1.5-2µl of nuclease-free water then with TE buffer, 1.5µl of each sample was loaded. The gDNA yield and dye amount per microgram collected to calculate dye specific activity by applying the equation stated below with the ideal values table 2.11:

$$DNA\ Yield\ (\mu l) = \frac{DNA\ concentration\ (ng/\mu l) * sample\ volume(\mu l)}{1000\ ng/\mu l}$$

$$Dye\ Specific\ Activity = \frac{dye\ concentration\ (pmol/\mu l) * 1000}{gDNA\ concentration\ (\mu g/\mu l)}$$

	Cyanine-3 (pmol/µg)	*Cyanine-5 (pmol/µg)
Dye Specific Activity (pmol/µg)	25-50	20-40
DNA Yield (µg)	5.0-10.0	5.0-10.0
Input gDNA (µg)	0.5-1.0	0.5-1.0

Table 2. 11: DNA labelling efficiency values for dye specific activity and yield.

If labelling was ideal, both DNA samples (tumour and reference DNA) were combined in a new 0.2 microfuge PCR tube with a 19.5µl each, in preparation for the hybridisation step.

2.2.21.5 Pre Hybridisation Blocking

An aliquot of 10 x blocking agent was prepared (nuclease-free water (1350µl) containing lyophilized 10x blocking agent), left for 60 minutes at room temperature

condition. Agilent Oligo aCGH Hybridisation Kit was used according to the manufacturer's instruction to block the repetitive DNA sequence by Cot-1DNA.

Tumour and reference DANs were combined as mentioned before were transferred to a new 0.2ml PCR tube. The master mix for the hybridisation was prepared as shown in table 2.12. A 71µl of the master mix was added to each PCR tube, mixed gently by pipetting to a final volume of 110µl. PCR tubes then pulsed by centrifugation for a few seconds and placed on PCR instrument Figure 2.7, for 3minutes at 95°C, then for 30 minutes at 37°C, and held at the same temperature whilst waiting for the hybridisation master mix to be prepared.

	Volume in (µl) per tube (sample)	Volume x 5 tubes
Human Cot-1 DNA	5	25
10 X aCGH Blocking Agent	11	55
2 X 111-RPM Hybridisation buffer	55	275
Total	71	355

Table 2. 12: Hybridisation Master mix preparation components used on labelled samples for array CGH.

2.2.21.6 Hybridisation for Array CGH

A clean 4 x microarray gasket slide was placed inside a SureHyb® chamber base and the gasket label facing up and aligned with the rectangular part of the chamber. Samples were very slowly loaded inside the gasket. With care a microarray slide was placed on with the Agilent labelled side facing down on the gasket slide. The SureHyb® chamber was fitted over and sandwiched together and the slide carefully clamped and tightened. Then the assembly was rotated to make sure that trapped air bubbles would move freely over the slide when rotated. The next step was placing the chamber assembly inside a hybridisation oven at 65°C, which contain a rotator rack that rotates 20 rpm, and the samples were rotated for exactly 24 hours. Figure 2.11.

Figure 2.11: Some of the instruments and tools were used in the aCGH Experiment.

A) Washing step. **B)** PCR instrument used (Eppendorf* Mastercycler* Thermal Cyclers, USA). **C)** SureHyb® chamber base. **D)** Plastic slide holder can be fitted into array scanner for reading. **E)** Hybridisation oven.

2.2.21.7 Post Hybridisation Washing and Scanning

Prior to washing all the glass dishes, racks, magnetic stir and bottles used were rinsed with high-quality Milli-Q ultrapure water. Washing was prepared before removing the hybridisation assembly from the oven. The first wash basically, contains a glass dish 1 and a second glass dish (2) filled with an aCGH Buffer 1 at room temperature. The glass dish 2 contains a rack and a magnetic stirrer. Glass dish 3 contains stirrer filled with Agilent Oligo aCGH/Chlp- on -Chip Wash Buffer 2 pre-warmed at 37°C a day

before in a sterile bottle and placed on a magnetic stirrer. After the 24 hours hybridisation the slide assembly was collected from the oven and observed for the presence of the air bubbles. The SureHyb® chamber was placed horizontally and the clamp unscrewed very gently. The slide was removed and placed in glass dish 1; to separate the array gasket sandwich plastic forceps were used. The array gasket was left in glass dish 1 and the array slide immediately removed into glass dish 2 on the rack and left to wash for 5 minutes at room temperature. The rack was then transferred into glass dish wash 3 and left only for 1 minute at 37°C. Next, the rack was removed very slowly to avoid any droplet formation on the slide. The slide was placed carefully on a plastic holder and scanned immediately. The slide was scanned using Agilent Surescan® high-resolution technology with control software (version 10.5.1), a configuration of Agilent was performed according to manufacturer recommendations. The data were saved in a TIFF format file to determine any damages or artifacts during the hybridisation. See Figure 2.11.

2.2.21.8 Microarray Data Processing

2.2.21.8.1 Quality Assessment and Feature Extraction for Array

Feature Extraction software version 11.0.1.1 was used to analyze images scanned at high resolution (3μm). Fluorescent intensity was normalized and the ratio for both dyes was also, calculated by the software, to produce a logarithmic scale (probe $\log_2$-ratio). The logarithmic scales then exported as a Text (.txt) form. Quality control was constructed by the FE software to examine, the reproducibility and reliability of the array experiment. Many parameters such as threshold values and statistical metric were accessed in order to accept the array data as valid. Derivative Log Ratio Spared DLRS is considered as the most effective parameter in order to obtain microarray reliability. The $\log_2$ Standard deviation between consecutive oligonucleotide probes was measured. A small SD value means low background noise. While other elements such as signal to noise S/N, and average background noise ratio, were examined throughout to calculate the mean signal intensities of all genomic probes for both dyes, by comparing them to non-hybridised control probs.

Signal Intensity to noise SNR (both dyes)	Determined by dividing signal intensity over background noise and detect the real signal	>30-100
Derivative Log_2 Ratio SD (both dyes) DLRSD	Determined by calculate SD and assess the noise array measurement	<0.2-0.3
Background noise	To calculate SD on negative control probes	<5-10

Table 2. 13: QC Metric thresholds for Array CGH Experiment.

2.2.21.8.2 Micro-array Data Analysis

The Data were analysed by Agilent Genomic Workbench software v7.0.4.0 license and the download was obtained from the Agilent web site. Specific format files were imported and used to perform the data. They were Agilent Feature Extraction (*.Txt) data files. Then, the algorithm was imposed to calculate the data QC metric table 2.12. Then the data were analysed by using the Aberration Detection Method (ADM-2) algorithms and threshold set up to 6. Finally, a genomic viewer with chromosome ideograms exhibited the genomic data.

2.2.22 Statistical Analysis, Linear Regression and Correlations

The linear regression is a descriptive statistic to calculate variable that are not linearly related, and transfer this relationship into a linear form, by an equation to predict some variables, through plotting line graphs with one X-axis (independent variable) and other one with Y-axis (dependent variable) against each other. The analysis was performed using Microsoft Excel version 2011 to find equations that fit the data. The prediction could be made based on that model. When the data shows the correlation coefficient of determination (R^2) that predicts the future outcome, a scatter plot will form a straight line. The equation has the form Y'=a + b X, usually calculated automatically by the excel program and the data were interpreted. This will explain if there are any associations between the two variables. The range for R^2 from -1 to 1. A value close to 1 shows a positive or strong association.

After inserting the data into the columns in Excel with no blank cell, two columns of choice (dependent and independent) were highlighted, click on chart layout then chose to scatter and then marked scatter a chart will appear. The chat format is adjusted by clicking on chart quick layout and then click on f x layout to give a more descriptive

layout on the chart to be filled with other variable information. This will create a linear regression line. Then the equation for the slop (the linear line) will appear with the calculated coefficient of determination (R^2). Then line estimation was used to calculate and to make sure that the slop line and the association given is correct, by choosing to calculate regression and regression statistic that appears under the scatter chart. To highlight the regression enter =, then highlight dependent and independent variables, a comma between each segment, then entered the number one for true (for statistics) and another number one lastly click on enter. The Excel will give the regression calculation showing that the slop equation calculation is correct and allowing correlation coefficient (R) to be calculated with error in m, coefficient of det (R sqt), F stat and regression sums of squares.

Therefore this study tried to find a relation or correlation between different variables of the suggested CSC markers, considering new markers single expression and co-expression with different gating strategies on pre-SA and POST-SA cell lines. The first column was the name of the cell lines used; the rest of the columns were the CD markers percentage's single expression (CD44, CD133, CD24, CD90, CD34, CD71 and ALDH) and co-expression columns of (CD44/CD71, CD44/ALHD, and CD44/CD34). Lastly, detecting the correlation between the suggested CSC markers and the new markers, was determined by choosing the CD marker percentage columns required to find their relationship to each other on all cells and large cells, as well as pre-SA and POST-SA. After the data was filled the designated column were selected, and the chart layout was selected as mentioned above.

CHAPTER THREE

INVESTIGATION OF PUTATIVE STEM CLL MARKERS IN SARCOMA SUBTYPES

3.1 INTRODUCTION

The identification of markers for cancer stem cells is a challenge for most cancers, and no more so than for sarcomas, where the large numbers of subtypes mean markers for these cells remains elusive. Generally, for sarcoma, because of the large number of sarcoma subtypes, the reported expression of any antibody in the literature is highly variable. Sometimes, cells with a high proportion of a specific marker do not necessarily have high tumorigenicity or are enriched with CSC (Skoda et al., 2016). Moreover, not all sarcoma subtypes have been studied, which makes it complicated to find /reach a definite conclusion.

The individual and co-expression of CD markers for CSC, found to be of use in other cancers, are still under investigation for their value to sarcoma, where their relevance is disputed (Abbaszadegan et al., 2017, Akbarzadeh et al., 2019). For sarcomas, one of the most studied of these markers, CD133, is expressed by many cancers and considered to identify CSC (Tirino *et al*., 2008, Di Fiore et al., 2009, Tirino *et al*., 2011, He *et al*., 2012). Naturally, CD133 (glycoprotein) is found in the plasma membrane, but reports claim in some sarcomas that it is, also found in the cytoplasm and the nucleus (Nunukova et al., 2015). The role of CD133 is still not fully understood, which makes it controversial and difficult to consider as a true CSC marker. It has been suggested that CD133 is an inadequate marker for all sarcoma subtypes, but that it may be of relevance to some (Tirino *et al*., 2008, Di Fiore et al., 2009, He *et al*., 2012).

Other markers have also been proposed as identifying CSC in different sarcoma subtypes, including CD90, CD24, CD117, CD34, SSEA4, CD49f, ALDH and CD57 (Harris et al., 2009, Abbaszadegan et al., 2017, Brown et al., 2017, Skoda and Veselska, 2018,). Noticeably, some of these markers are found in normal stem cells or progenitor cells (Akbarzadeh et al., 2019), but for sarcomas, their investigation was either focused on one sarcoma subtype or the results were contradictory. Placing reliance on the expression of a single marker could therefore make identification of CSC challenging. Combining two markers, or more could however enhance the identification of CSC. Nestin, for example, is known to be positive in some sarcoma tissue samples, and co-expression with CD133 was found to give promising results in sarcoma, not only for diagnosis but also in terms of the prognosis of the disease (Sana et al., 2011 He et al., 2012, Zambo et al., 2016,). ALDH, widely reported to be CSC marker for many types of cancer, as well as for stem cells and progenitor cells (Silva et al., 2011), is also expressed by some sarcomas subtypes. For instance, Ewing's and

synovial sarcomas both express a high level of ALDH activity (Nakahata et al., 2015, Lohberger et al., 2012). The expression however is variable even, for the same cell line, and this maybe as a result of heterogeneity of the CSC, or due to the different ALDH isoforms (Zhou et al., 2019). As mentioned previously (1.11.1.3), the use of ALDH on its own (like the single-use of CD133) is controversial and should be considered as being a poor CSC marker. However, as ALDH is strongly expressed in some sarcoma subtypes, its potential as a CSC marker may therefore be of relevance to some sarcomas. Alternatively, and potentially more reliable is ALDH co-expression with SOX2, found recently to be of values in Ewing's sarcoma and rhabdomyosarcoma, possibly associated with increased tumorigenicity (Nakahata et al., 2015, Zambo et al., 2016).

In this study, cell lines from different types of sarcoma (both commercial and those established in Sheffield as cell lines or short term cultures), were investigated to see if the presence of CSC could be reliably determined using either single markers or combinations. Initially, CSC markers previously thought of as of relevance to sarcoma were investigated on their own, leading to a second phase that included expansion of the markers' panel and their use in combination. In this chapter, the characterization of sarcomas by flow cytometry analysis with a single expression, co-expressions and the study of cell hierarchy was undertaken, and an overview of the workflow is shown in Figure 3.1. Furthermore, sarcoma subpopulations expressing different markers were specifically selected and studied for their ability to form colonies as a measure of the capacity for self-renewal.

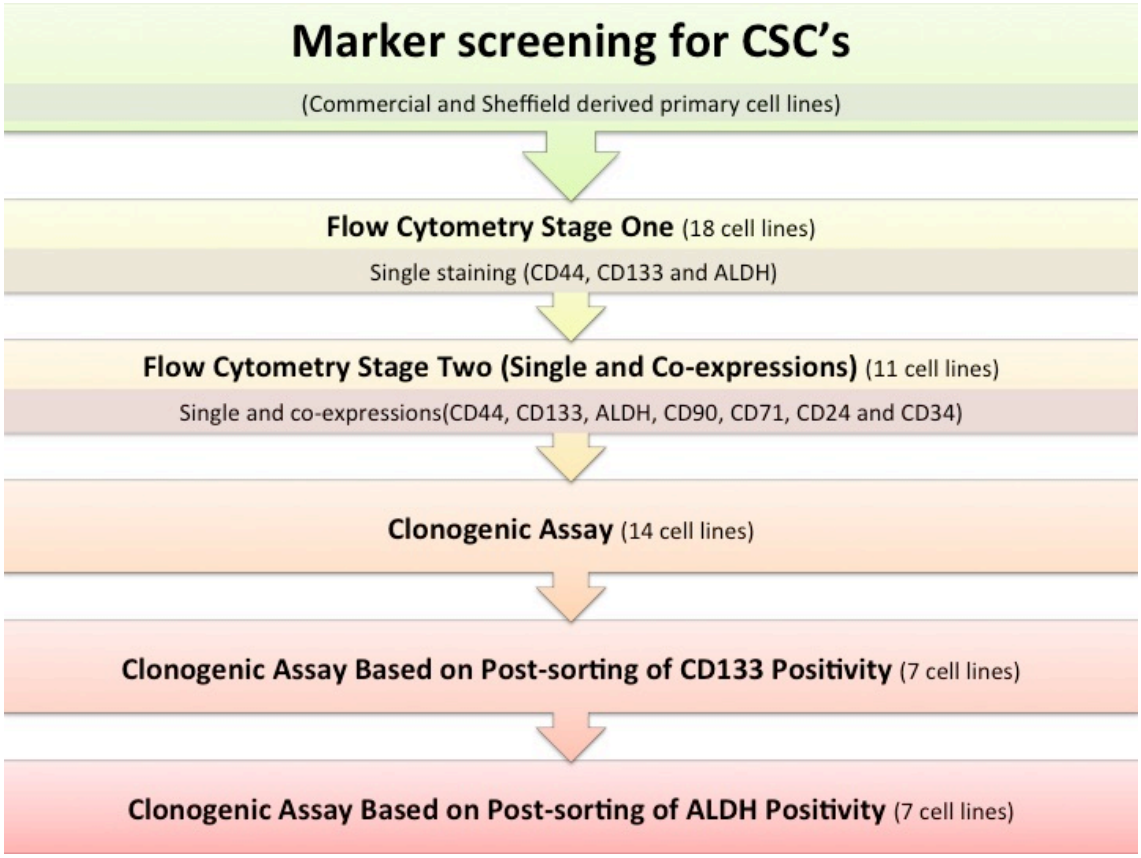


Figure 3. 1: Overview of Chapter 3 work-flow,

The initial phase of the studied looked at markers previously proposed as of relevance to sarcoma and studied a wide range of subtypes to see if the expression was subtype-specific. In the second phase, an expanded number of emerging markers were investigated, including their co-expression. Finally, the ability to form colonies was compared between populations highly expressing either CD133 or ALDH and those non-expressing cells.

3.2 RESULTS

3.2.1 Sarcoma Cell Lines and Selection of Markers

In this study, two groups of cell lines were used to investigate the expression of various CD markers using flow cytometry as an analytical tool (see section 2.2.). Established cell lines comprise both; commercial STS and other sarcomas and cell lines used as controls. The aim of using these commercial cell lines was to compare the findings with more recently established cell lines and primary STC that had been developed as cultures in Sheffield (Table 2.3). The majority of samples were set up and cultured by Dr Abdulazeez Salawu, but STC of angiosarcomas were established as part of this investigation. The CD markers used were CD44, CD133, ALDH, CD90, CD34, CD71 and CD24. CD117 and CD154 (CD40L) were also used, but the analysis was only performed on one cell line (13/12W2) due to the limited time. The gating method for flow cytometry dot plots was to include all cells except the debris (arising due to the mechanical processing of the cells) and dead cells (usually shrunk cells). These accumulate in the lower-left LL of the dot plot area, and can be easily differentiated by comparing to controls or positively stained cells. Staining was carried out in two stages as described below, and cells lines were cultured as in section 2.2.

3.2.2 Stage One: Single Expression of CD44, CD133 and ALDH

In this stage, only a certain number of monoclonal antibodies were investigated for a single expression. The antibodies used were (CD44, CD133 and ALDH); and had all been suggested as possible CSC markers for sarcoma. As a starting point, it was hoped to provide an overview of putative CSC in sarcomas of different subtypes, latterly additional CD markers were proposed as putative CSC markers became available and were included in phase 2. All experiments were carried out in triplicate on three different passages unless otherwise stated. The expression of certain markers by one distinct homogenous population was found for the sarcomas, meaning that the cells have the same size and complexity, and these cells are in the same proliferation stage Figure 3.2.

3.2.2.1 SKUT-1 (Human Uterus Leiomyosarcoma (GIII))

This cell line was supplied by ATTC/1971 as a grade 3 leiomyosarcoma from the uterus of a 75-year-old lady and was derived in 1972. Expression of CD44 was high, around 57% positive, and appears to identify one distinct homogenous population. CD133 was very low to negative at 0.4%. ALHD activity shows a high-level of

expression, approximately 68% positivity Figure 3.3.

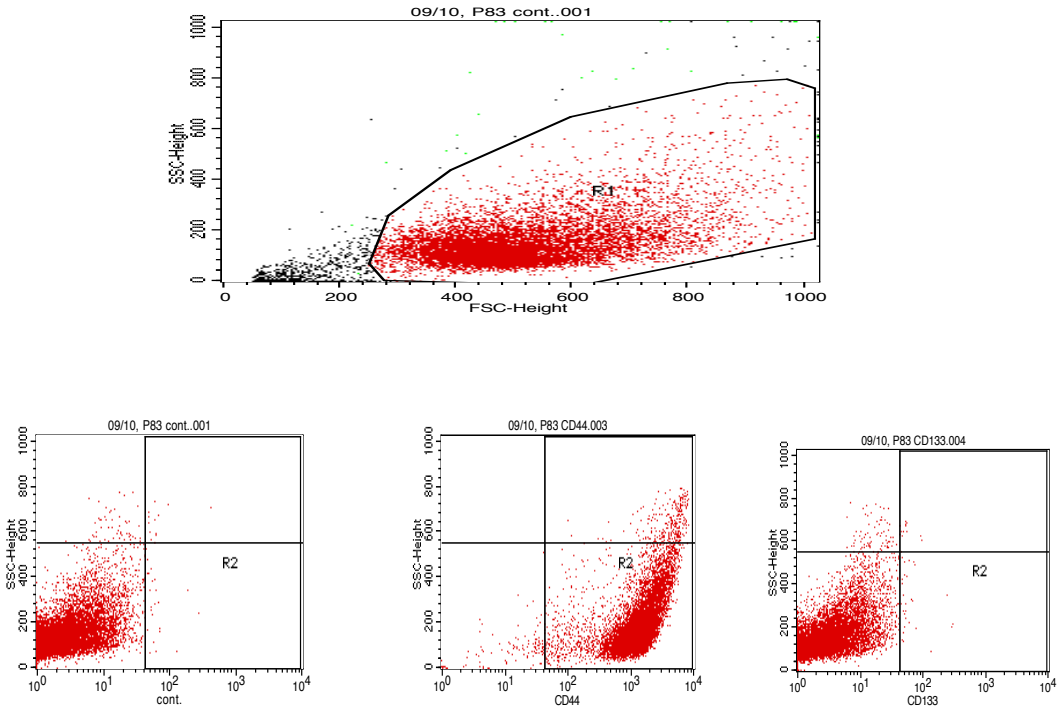


Figure 3. 2: These figures show examples of CD44 and CD133 expressions and analysis on one of the primary sarcoma cell lines (09/10) using flow cytometry staining.

These expressions were based on one distinct cell population. The CD44 expression was 99.3% ± 6% (n=3 SEM) and CD133 was 0.3% ±0.1 (n=3 SEM). The data correspond to mean ± SEM from three independent experiments unless otherwise stated.

3.2.2.2 SW1353 Chondrosarcoma

This cell line was supplied by ATTC/1977 and was chondrosarcoma taken from the right humerus of a 72 years old lady and derived in 1977. A very high level of CD44 expression (99.9% positive) was found and associated with one distinct homogenous population. CD 133 expression was very low to negative (0.4%), and ALDH activity expression was also low at 2% (Figure 3.3).

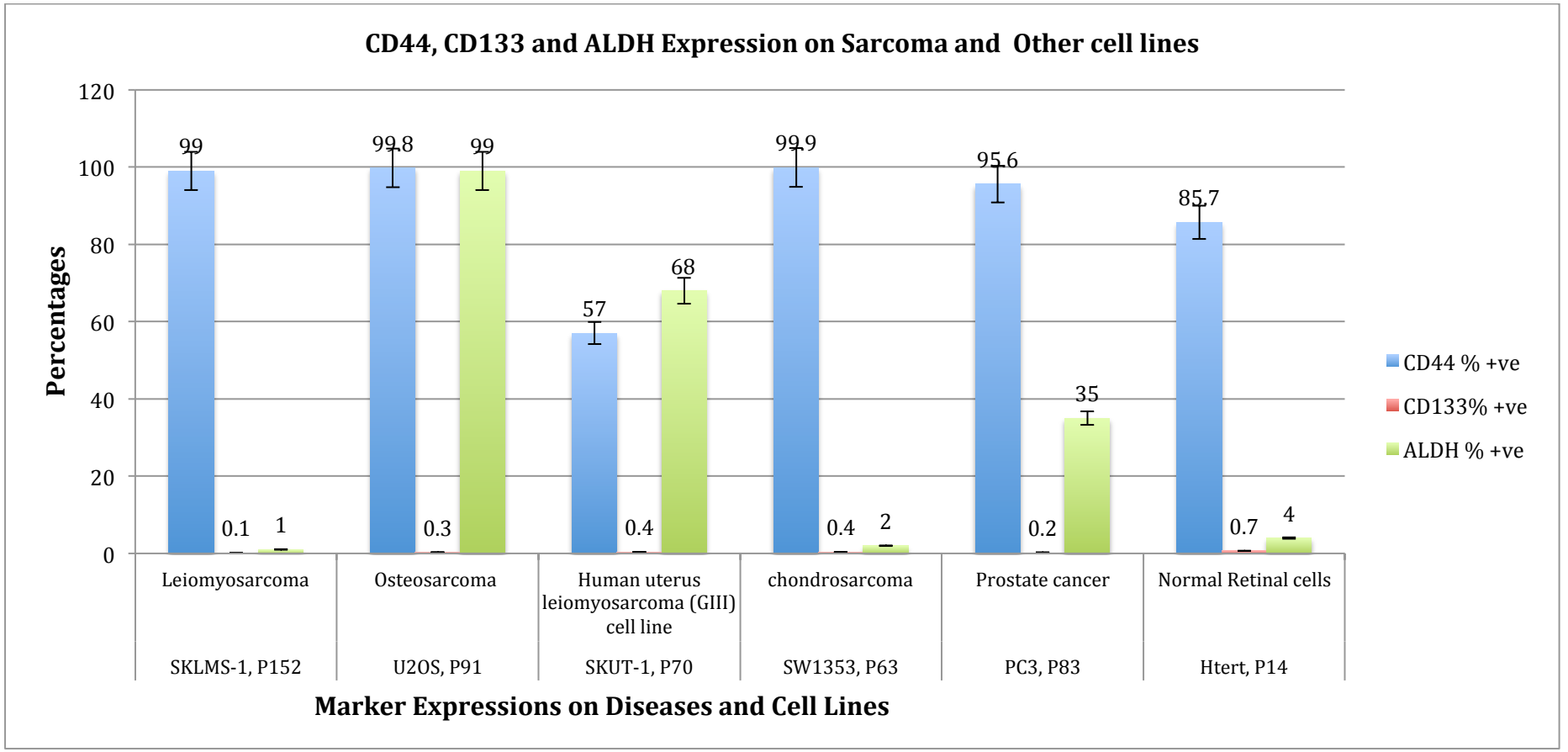


Figure 3. 3: Overall, summary of The Established Cell lines, PC3 and Normal Cell line h-TERT expression of CD44, CD133 and ALDH.

The expression of CD44 was highly positive (57%-99.9%) ±2% (n=3 SEM). The CD133 shows very week expression (0.1%-2.1%) ±0.1% (n=3 SEM). The ALDH expression is varied (1%-99%) ±3% (n=3 SEM). The data correspond to mean ± SEM from three independent experiments unless otherwise stated.

3.2.2.3 SKLMS-1 Leiomyosarcoma

This cell line was supplied by ATTC/1971 and was a Leiomyosarcoma from the vulva of a 43-year-old lady derived in 1971. In this cell line, the expression level of CD44 was again very high (99.9% positive) and appeared to be expressed in one distinct homogenous population. CD 133 expression was very low to negative at 0.1%, and ALDH activity was also very low at 1% (Figure 3.3).

3.2.2.4 U2OS Osteosarcoma

This cell line was supplied by ATTC/1953 as osteosarcoma from the bone of a 15 year old girl, and was originally cultured in 1953. Again, a very high level of CD44 expression (99.8 % positive) was expressed by one distinct homogenous population. CD133 was very low to negative (0.3 %), but in contrast to the STS cell lines, ALHD showed a very high level of activity, with expression at approximately 99% positivity (Figure 3.3).

3.2.2.5 Prostate Cancer Cell Line PC3

Supplied by ATTC, and derived from a prostate cancer this line has been used extensively as a model representative of CSC behaviour; and was therefore included in this study as a positive control for the expression of CSC markers. CD44 expression was very high (95.6 % positive) and as has been consistently observed, appears to be expressed by one distinct homogenous population. CD133 was very low to negative at 0.2 %, and again in contrast to the commercial STS lines, ALHD showed moderate activity, with expression approximately 35% positivity Figure 3.3.

3.2.2.6 Human Epithelial Retinal Cells hTERT-RPE-1

This cell line was supplied; by S. Collis (Department of Oncology and Metabolism) and was used as a normal human control cell line. hTERT-RPE-1 was immortalized by transfection with human telomerase making the cells capable of renewing themselves (Bodnar et al., 1998, Yang et al., 1999). Early passages were used for this study, as there was a drift in responses over the extended passages. As with the other commercial lines, a very high level of CD44 expression (85.7 % positive) in one distinct homogenous population was observed. CD133 was very low to negative at 0.7 %, and ALHD showed a low level of activity to negative expression at 4% Figure 3.3.

3.2.2.7 STS (13/12) Pleomorphic NOS Sarcoma

The cell line was derived in Sheffield, and the tumour tissue (13/12W); was kindly donated by an 80-year-old man with a grade 3 (GIII) pleomorphic NOS sarcoma, which is considered to be a sub-type of undifferentiated sarcoma (Fletcher, 2014, Jo and Fletcher, 2014). This sarcoma was very aggressive and metastasized to the lung. The cell line was established and cultured in 2013 in our laboratory with two variants 13/12W1 and 13/12W2 developed and both were tested. As found previously for all lines CD44 expression was very high on both cell lines, 13/12W1 and 13/12W2, at 99.8 % and 99 % respectively, and expressed in one distinct homogenous population. CD133 expression was very low to negative 0.3 % for 13/12W1 and 0.2 % for 13/12/W2, and ALDH activity was moderate around 32 % for 13/12W1 and 28 % for 13/12W2 positivity. An example of the gating and analysis for ALDH is shown in Figure 3.4, and the results are tabulated in Figure 3.5.

3.2.2.8 STS (09/10) Dedifferentiated Sarcoma

This tumour tissue; was kindly donated by a 68-year-old lady who sadly passed away in 2012. The diagnosis was grade 3 (GIII) retroperitoneal dedifferentiated liposarcoma. The cell line was developed in 2011. CD44 expression was very high (99.9%) positive with one distinct population. CD133 expression was very low to negative 0.5 %, and ALHD had a low level of activity at 9% positivity only Figure 3.2 and Figure 3.5.

3.2.2.9 STS (14/10) Pleomorphic NOS Sarcoma

This tumour tissue was kindly donated by a 54-year-old lady with pleomorphic sarcoma NOS; from the left thigh and the cell line was developed in 2010. CD44 expression was very high (99.9%) positive with one distinct population, and CD133 expression was very low to negative 0.2 %, whilst ALHD shows a moderate level of activity at 23% positive Figure 3.5.

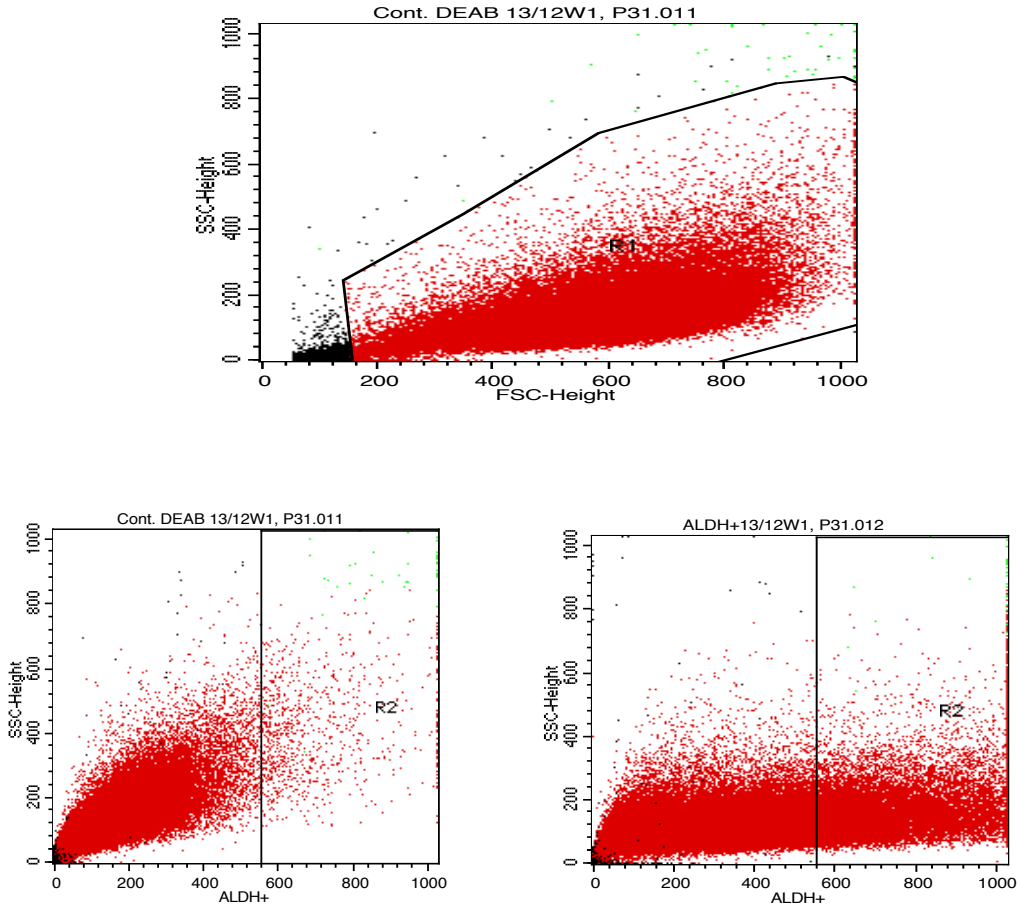


Figure 3. 4: These figures show examples of ALDH expression, gating and analysis on one of the primary sarcoma cell lines (13/12W1) using flow cytometry staining.

The expression was based on one distinct cell population. The ALDH expression was 31.99% ± 4% (n=3 SEM) based on DEAB negative control. The data correspond to mean ± SEM from three independent experiments unless otherwise stated.

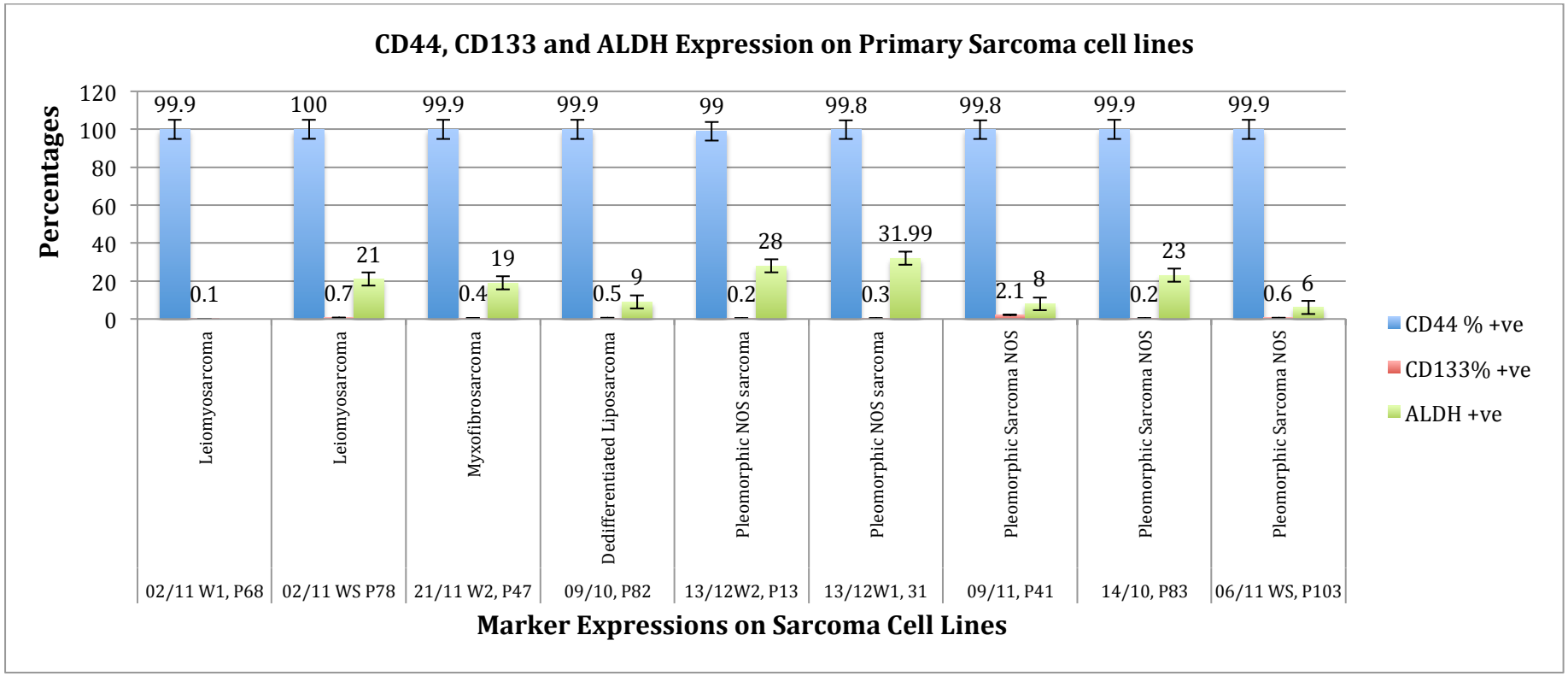


Figure 3. 5: CD44, CD133 and ALDH average expression on all Sheffield derived primary sarcoma cell lines.

The expression of CD44 was highly positive (99.8%-99.9%) ±1% (n=3 SEM). The CD133 shows very week expression (0.1%-2.1%) ±0.1 (n=3 SEM). The ALDH expression is varied (6%-31.99%) ± 5% (n=3 SEM). The data correspond to mean ± SEM from three independent experiments unless otherwise stated. The ALDH for 02/11W1 cell line was not performed.

3.2.2.10 STS (02/11) Leiomyosarcoma

This tumour tissue was kindly donated by a 62-year-old lady with a grade 3 (GIII) vaginal leiomyosarcoma. The cell line was established and cultured in 2011 in our laboratory with two variants 02/11W1 and 02/11WS developed. CD44 expression was very high on both cell lines 02/11W1 and 02/11WS 99.9 % and 100 % respectively, and appears to be expressed in one distinct homogenous population. CD133 expression was very low to negative 0.1 % for 02/11W1 and 0.7 % for 02/11/WS, and ALDH expression was moderate, around 21% for 02/11WS. Unfortunately, ALDH activity on 02/11W1 was not performed due to technical issues Figure 3.5.

3.2.2.11 STS (20/11) Dedifferentiated Liposarcoma

This tumour tissue was kindly donated by a 70-year-old lady who was diagnosed with dedifferentiated liposarcoma from the right thigh. The patient received radiotherapy treatment prior to the operation. The cell line was established and cultured in 2011 in our laboratory. Unfortunately, due to repeated fungal infection and slow growth, only the clonogenic assay was performed on this cell line.

3.2.2.12 STS (09/11) Pleomorphic NOS Sarcoma

This tumour tissue was kindly donated by a 66-year-old lady patient who was diagnosed with pleomorphic NOS sarcoma from the right thigh. The cell line was established and cultured in 2011. The patient sadly passed away after only 3 months with evidence of lung metastasis. CD44 expression was very high, 99.8% positive with one distinct population. CD133 expression was very low 2.1 %, and ALHD had low-level activity at 8% positive Figure 3.5.

3.2.2.13 STS (06/11) Pleomorphic NOS Sarcoma

This tumour tissue was kindly donated by a 76-year-old man who was diagnosed with pleomorphic NOS sarcoma from the right thigh. The cell line was established and cultured in 2011 with three different clones 06/11, 06/11WS and 06/11W2. The patient sadly passed away a year later.

CD44 expression was very high on all three-cell lines 06/11, 06/11WS and 06/11W2 were 99.8%, 99.9% and 99.9% positive respectively. One distinct homogenous population expressed the marker. CD133 expression was very low to negative 0.1% for 06/11, 0.1 % for 06/11W2 and 0.6% for 06/11WS. ALDH activity expression was also low at 5%, 6% and 4% for 06//11, 06/11WS and 06/11W2 respectively. Figure 3.5 and Figure 3.6.

3.2.2.14 STS (21/11) Myxofibrosarcoma

This tumour tissue was kindly donated by a 75-year-old man who was diagnosed with myxofibrosarcoma grade 3 (GIII) from the left arm. The cell line was established and cultured in 2011. CD44 expression was very high 99.9% positive, with one distinct population. CD133 expression was very low to negative at 0.4 %. ALDH shows a moderate level of expression and was 19% positive Figure 3.5.

3.2.2.15 STS (04/13) Angiosarcoma

This tumour tissue was kindly donated by a 42-year-old lady who was diagnosed with angiosarcoma of the breast. No other information was provided for the patient. The cell line was established and cultured in 2013, two clones were cultured 04/13 and 04/13S. CD44 expression was very high on both cell lines 04/13 and 04/13S were 95.8% and 99.6% positive, respectively and one distinct homogenous population expressed. CD133 expression was very low to negative 0.3% for 04/13 and 0.2% for 04/13S. Unfortunately, ALDH activity was not tested because the cell lines were growing very slowly. In later stages, these cell lines were excluded from this study Figure 3.6.

3.2.2.16 STS (07/13) Angiosarcoma

This tumour tissue was kindly donated by a lady who was diagnosed with angiosarcoma of the breast. No other information was provided for the patient. The cell line was established and cultured in 2013 and two clones were cultured 07/13 and 07/13SW1. Both cell lines express 99% positivity of CD44 with one distinct population. CD133 was negative on both 07/13 and 07/13SW1. ALDH shows a low level of activity at 7% positive on both cell lines. In later stages, these cell lines were excluded from this study due to the long time taken to reach confluency Figure 3.6.

3.2.2.17 STS (05/13) Angiosarcoma

This tumour tissue was kindly donated by a lady who was diagnosed with angiosarcoma of the breast. No other information was provided for the patient. The cell line was established and cultured in 2013. CD44 expression was very high 99% positivity. One distinct population was analyzed. CD133 was negative 0% and ALDH expression was at a low level of 4% positive. In later stages, the cell line was excluded from this study because the cell line failed to proliferate Figure 3.6.

3.2.2.18 STS (08/13W1) Angiosarcoma

This tumour tissue was kindly donated by a lady who was diagnosed with angiosarcoma of the breast. No other information was provided for the patient. The cell line was established and cultured in 2013. CD44 expression was very high 87% positivity, and one distinct population was analyzed. CD133 was negative 0% and ALDH expression was low at 7% positive. This experiment was performed once, and this cell line was excluded from this study because the cell line was unable to proliferate Figure 3.6.

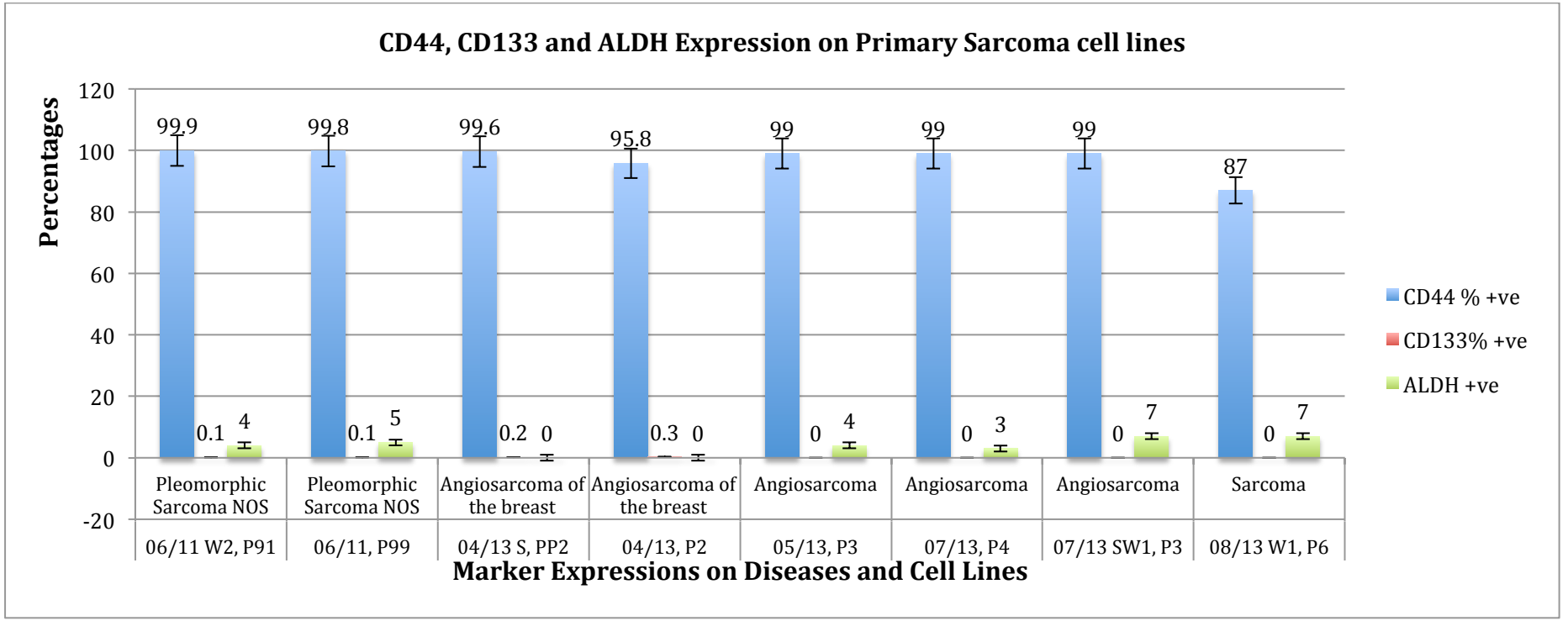


Figure 3. 6: CD44, CD133 and ALDH average expression on all Sheffield derived primary sarcoma cell lines.

The expression of CD44 was highly positive (87%-99.9%)±1% (n=3 SEM). The CD133 shows very week expression to negative (0%-0. 3 %) ±0.1% (n=3 SEM). The ALDH expression is varied (3%-7%)±1% (n=3 SEM). The data correspond to mean ± SEM from three independent experiments unless otherwise stated. Some Angiosarcoma cell lines were not tested for ALDH, because cells were unable to proliferate.

3.2.3 Summary of Findings from The Single Expression of CD44, CD133 and ALDH

The original marker selection was based on reports at the start of the project that suggested CD44, CD133 and ALDH may be the most promising markers for the identification of putative CSC in sarcoma (Al-Hajj *et al*., 2003, Tirino *et al*., 2008, Di Fiore et al., 2009, Silva et al., 2011, He *et al*., 2012). Universally all cell lines, including controls and non-sarcoma lines, had very high expression of CD44, and either weak expression or were negative for CD133. The expression of ALDH was the most variable with the osteosarcoma line having the highest.

3.2.4 Stage Two: Single Expanded Screening for CSC Markers

In this stage, some primary cell lines were eliminated due to time pressures and the inability of some cell lines to proliferate quickly enough, such as the Angiosarcoma cell lines. The same commercial cell lines were used as controls, but only 5 of the sarcoma lines developed in Sheffield were selected and included representation of different types of sarcoma (13/12W2, 09/10, 14/10, 02/11W1 and 21/11W2). New markers suggested to identify and label CSC were added to this study (CD90, CD71, CD24 and CD34 Section 2.2.16). In addition, to a single expression of an expanded panel of putative CSC markers, co-expression of markers was also performed but was restricted to the suitability of available dyes for the antibodies to be used in the co-expression studies. As mentioned in section (2.2.16) the panel combination was used to detect any co-expression between the newly added markers and the markers already tested in stage one. As CD44 was highly positive for all lines it was used a baseline or as a pan-cancer marker and the other combination of markers used to detect any sub-population or side population (SP), which could stain CSC and isolate them. It was also hoped that it may be possible to see if there was any hierarchy between these cells and the role they played in determining their fate. The co-expression aria is demonstrated in the dot plot upper right (UR) Figure 3.7). The panel of combinations were:

CD90/CD133/CD24/CD34,
CD90/CD133/CD24/CD44,
CD71/CD133/CD24/CD44,
ALDH/CD133/CD24/CD44

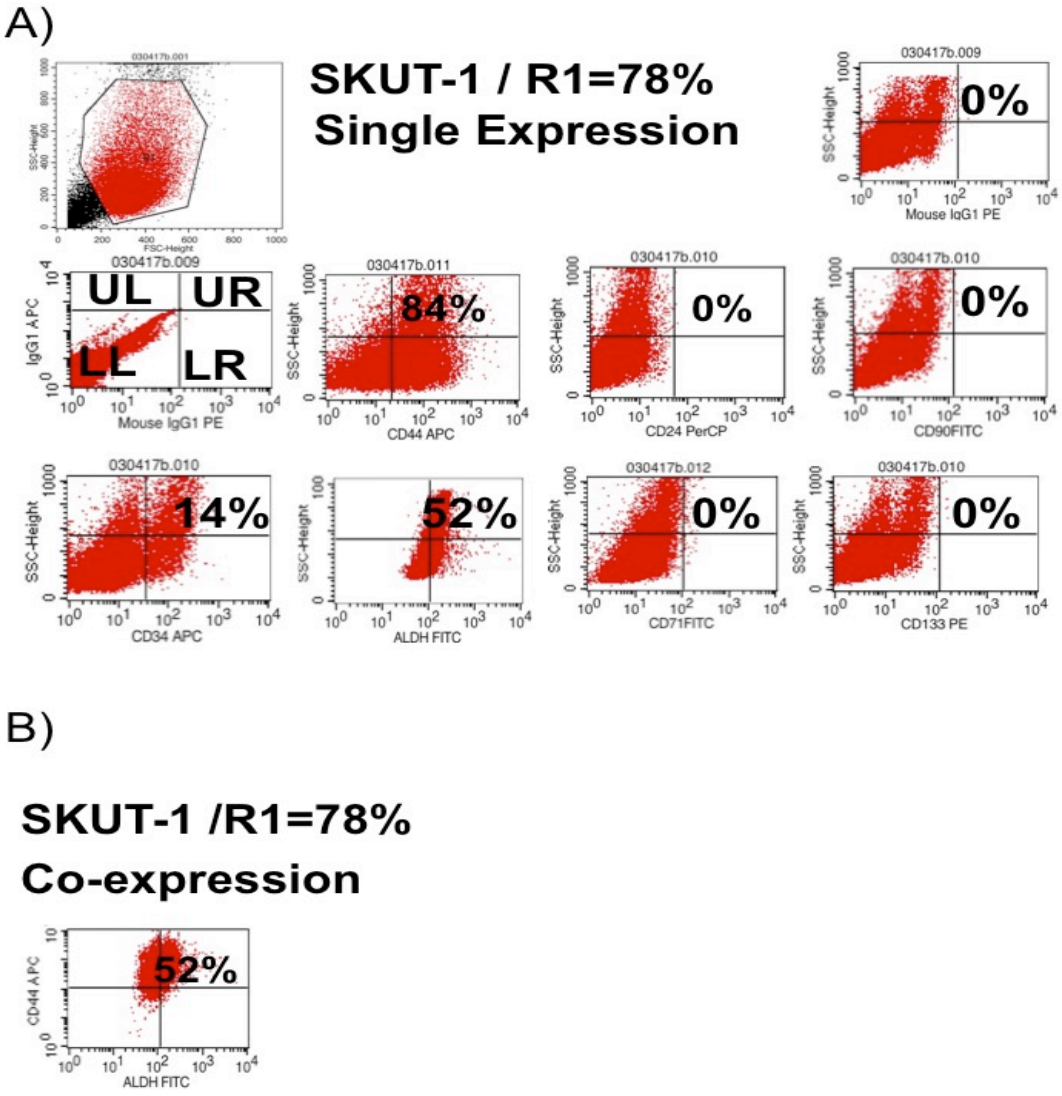


Figure 3. 7: These figures show the dot plot analysis of different CD markers expression and co-expression on a commercial cell line (SKUT-1) using flow cytometry staining.

The expression was based on one distinct cell population. The CD44 mean expression was 83.7% ± 0.3% (n=3 SEM); CD34 mean expression was 13.7% ±0.3 (n=3 SEM) and ALDH mean activity was 49.7% ± 0.3% (n=3 SEM). Co-expression was CD44/ALDH=49.7% ±0.3. The data correspond to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculated was done using Graph Pad® Prism software (version 7).

3.2.4.1 SKUT-1 (Human uterus Leiomyosarcoma (GIII))

The percentage of the gated and analyzed cells was R1= 78% cells (as shown in figure 3.7). The expression results of CD44, CD133 and ALDH activity were almost the same as previously presented in stage one, CD44 (84%), CD133 (0%) and ALDH (52%). The extra markers CD34, CD90, CD24 and CD71 were 14%, 0%, 0% and 0% respectively. Co-expression for CD90, CD133, CD71 and CD24 was not significant because they were negative. All 52% of positive cells of ALDH were co-expressed with CD44. With regards to CD34 (14%) it is presumed that the whole population was all co-expressed with CD44, as this expression was high anyway (84%). As CD44 and CD34 had the same fluorochrome dye colours (APC) they were stained in a different test tube (UR in dot plot) Figure 3.7 and Table 3.1.

3.2.4.2 Rest of Established and Sheffield Derived Primary Cell Lines Flow cytometry Screening

Gating percentages were above 50% for all the cell lines (established and Sheffield derived). The expression results of CD44 and CD133 were almost the same as previously tested in stage one for all cell lines. The exception was for ALDH activity which was had more variability and positivity than in stage one: observed to be in highest for 02/11W1 (99%) and negative for SW1353. This could possibly be due to passage numbers differences and adaptation to culture. The extra markers such as CD24 and CD90 showed no significant outcome because they were negative on all cell lines tested. Moreover, CD34 was also variable, the highest result was found on 21/11W2 (52%) and negative on four cell lines (SKLMS-1, U2OS, h-TERT-RPE-1 and 14/10). In addition, CD71 expression results were also variable and positivity was only observed for the Sheffield derived and the PC3 cell lines. The highest was for 13/12W2 (Figure 3.9). Co-expression for CD71 and ALDH with CD44 was also found. CD34 and CD44 markers had the same fluorochrome dye colours (APC), hence impossible to stain them in the same test tube). Hypothetically as all cell lines were highly positive for CD44 it can be assumed that there is a similar positive co-expression for these two markers. As was shown in section (2.2.16), the co-expression panel was limited due to fluorochrome dyes. Also, some negative co-expression results were not included in the table due to indeterminate outcome Figure 3.8, Figure 3.10 and Table 3.1.

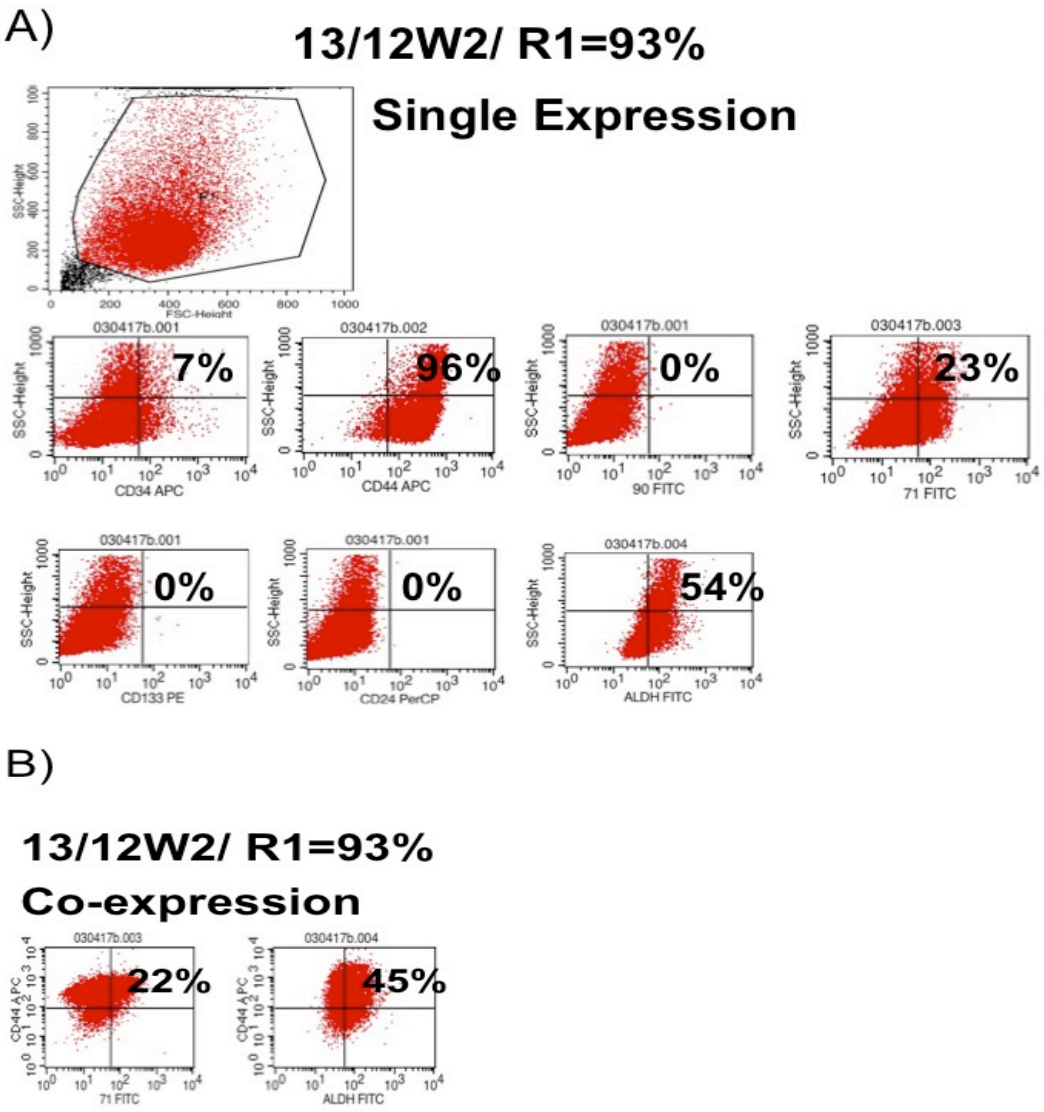


Figure 3. 8: These figures show the dot plot analysis of different CD markers expression and co-expression on a primary cell line (13/12W2) using flow cytometry staining.

The expression was based on one distinct cell population. The CD44 mean expression was 96.3% ± 0.3% (n=3 SEM); CD34 mean expression was 6.7% ±0.3 (n=3 SEM), CD71 mean expression was 22.7% ±0.3 (n=3 SEM) and ALDH mean activity was 53.7% ± 0.3% (n=3 SEM). Co-expression was CD44/ALDH=44.7% ±0.3 and CD44/71=21.7% ±0.3. The data correspond to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculated was done using Graph Pad® Prism software (version 7).

Cell line	(Gated Cells) Out of 100%	CD44	ALDH	CD71	CD34	CD133	CD90	CD24	CD44 / ALDH	CD71 / CD44	CD24 / CD34	CD90 / CD34	ALDH/ CD133	CD90 / CD44
SKUT-1 (Leiomyosarcoma)	78%	**84% ******	**52% ******	0% (N)	**14% ****	0% (N)	0% (N)	0% (N)	**52% ******	0% (N)	0% (N)	0% (N)	0% (N)	0% (N)
SW1353 (Chondrosarcoma)	55%	**91% *******	**1% ***	**1% ***	**8% ****	0% (N)	0% (N)	0% (N)	**1% ***	**1% ***	0% (N)	0% (N)	0% (N)	0% (N)
SKLMS-1 (Leiomyosarcoma)	41%	**96% *******	**64% ******	0% (N)	**1% ***	0% (N)	0% (N)	0% (N)	**64% ******	0% (N)	0% (N)	0% (N)	0% (N)	0% (N)
U2OS (Osteosarcoma)	81%	**90% *******	**19% *****	0% (N)	**1% ***	0% (N)	0% (N)	0% (N)	**19% *****	0% (N)	0% (N)	0% (N)	0% (N)	0% (N)
PC3 (Prostate cancer)	78%	94% *****	91% *****	**7% ****	**32% *****	0% (N)	0% (N)	0% (N)	91% *****	**7% ****	0% (N)	0% (N)	0% (N)	0% (N)
h-TERT-RPE-1 (Human epithelial retinal cells)	71%	**95% *******	**43% *****	0% (N)	**1% ***	0% (N)	0% (N)	0% (N)	**43% *****	0% (N)	0% (N)	0% (N)	0% (N)	0% (N)
13/12W2 (Pleomorphic NOS sarcoma)	93%	**96% *******	**54% ******	**23% *****	**7% ****	0% (N)	0% (N)	0% (N)	**45% *****	**22% *****	0% (N)	0% (N)	0% (N)	0% (N)
09/10 (Dedifferentiated Liposarcoma)	81%	**99% *******	**31% *****	**20% *****	**30% *****	0% (N)	0% (N)	0% (N)	**30% *****	**20% *****	0% (N)	0% (N)	0% (N)	0% (N)
14/10 (Pleomorphic NOS sarcoma)	79%	**95% *******	**95% *******	**23% *****	**1% ***	0% (N)	0% (N)	0% (N)	**92% *******	**22% *****	0% (N)	0% (N)	0% (N)	0% (N)
02/11 W1 (Leiomyosarcoma)	82%	**99% *******	**99% *******	**16% *****	**12% ****	0% (N)	0% (N)	0% (N)	**99% *******	**16% *****	0% (N)	0% (N)	0% (N)	0% (N)
21/11W2 (Myxofibrosarcoma)	57%	**99% *******	**56% ******	**4% ***	**52% ******	0% (N)	0% (N)	0% (N)	**56% ******	**4% ***	0% (N)	0% (N)	0% (N)	0% (N)
02/11 W1 (POST-SA2) (Leiomyosarcoma)	82%	**99% *******	**99% *******	**17% *****	**13% ****	0% (N)	0% (N)	0% (N)	**99% *******	**17% *****	0% (N)	0% (N)	0% (N)	0% (N)
06/11WS (Pleomorphic NOS Sarcoma)	56%	**100% *******	**6% ****	N/A	N/A	1%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 3. 1: A summary of CD markers expressions and gating percentages out of 100% for all cell lines, gating all cells on some selected commercial and primary (Sheffield derived) cell lines. (Gating strategy - phase one). N/A = not available, N= 0% Negative, *(1%-5% = very weak (negative), **(6%-15%) = weak, ***(16%-49% = moderate, ****(50%-89 = strong, *****(90%-100% = very strong. Markers expression percentage given is ± 0.3% (n=3 SEM). The data correspond to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculated was done using Graph Pad® Prism software (version 7).

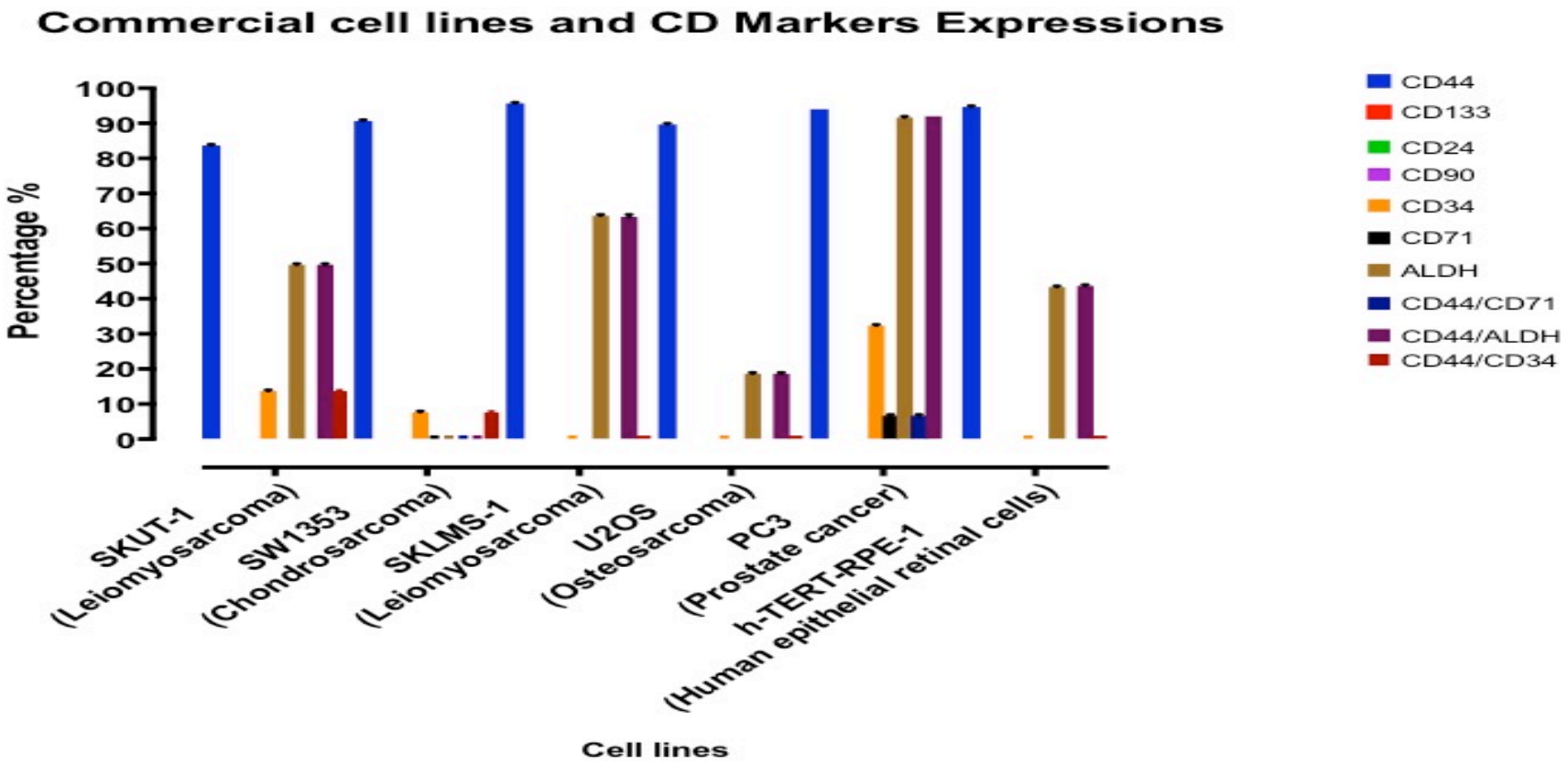


Figure 3. 9: These bar charts summaries different CD markers expression and co-expression results on commercial cell lines using flow cytometry staining. The expression was based on one distinct cell population.

The data correspond to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculated was done using Graph Pad® Prism software (version 7).

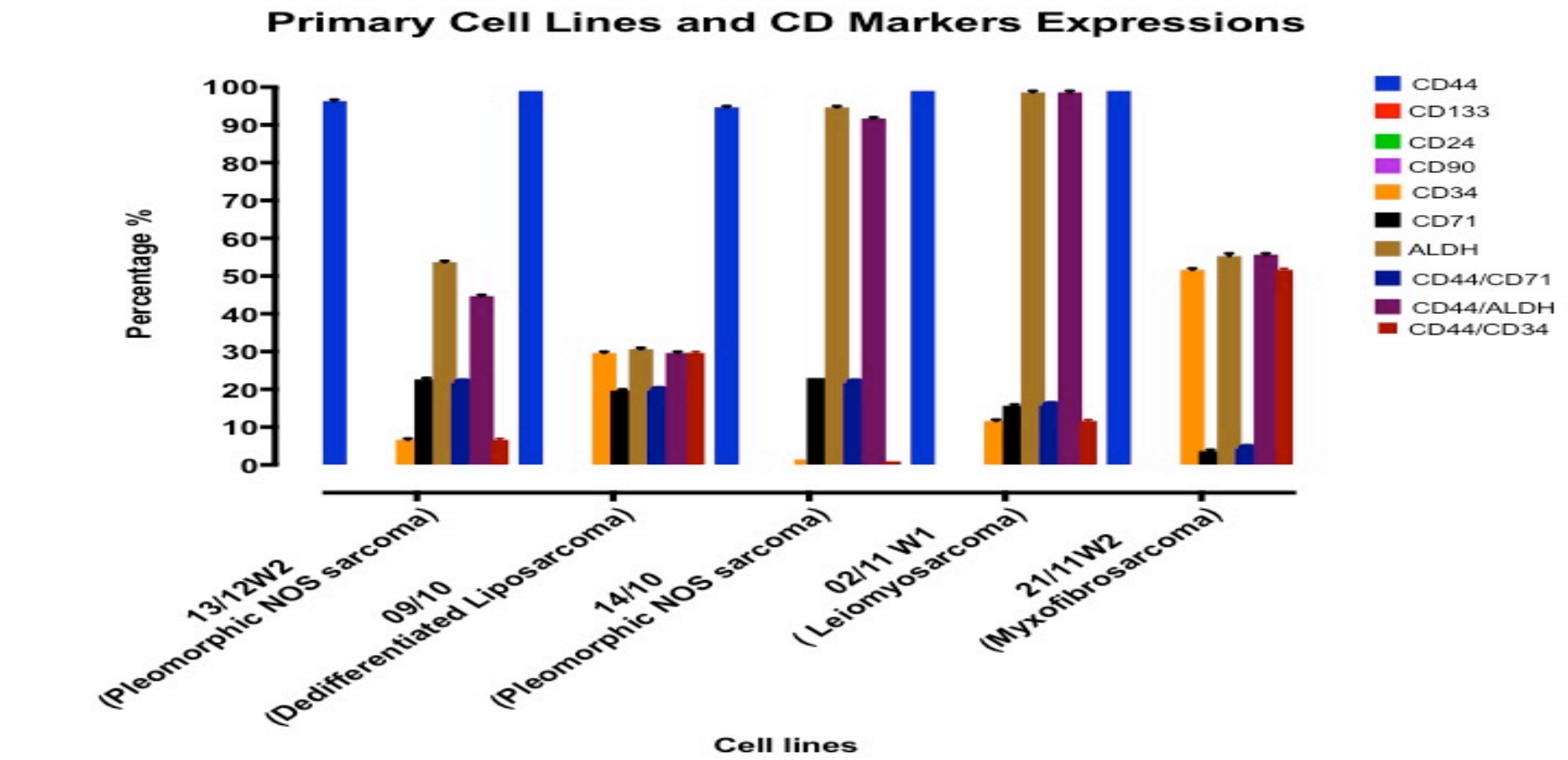


Figure 3. 10: These bar charts summaries different CD markers expression and co-expression results on primary cell lines using flow cytometry staining.

The expression was based on one distinct cell population. The data correspond to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculated was done using Graph Pad® Prism software (version 7).

3.2.5 Clonogenic Assay

This method was used to detect the ability of these cell lines to produce colonies from single cells. Initially, as detailed in the method section, several different concentrations were tested to find the optimum density of cells, so that a low-density seeding could be determined, and as such each colony was therefore most likely to arise from one single cell. (See section 2.2.4). Examples of these initial studies are presented in table 3.2.

Number of cells seeded	Number of colonies for U2OS, P63	Clonogenic efficiency No. for U2OS	Number of colonies for 06/11W2, P78	Clonogenic efficiency No. for 06//11W2
250	154	0.616	159	0.636
500	213	0.426	283	0.566
1000	467	0.467	513	0.513
2000	575	0.288	714	0.357
3000	616	0.205	1184	0.395
Average Clonogenic efficiency		0.400		0.493

Table 3. 2: shows an example of U20S (established) and 06/11W2 (primary) sarcoma cell lines with different numbers of cells seeded, the colonies counted and the clonogenic efficiency at each density and the average density in numbers.

Images of colonies formed as a result of low seeding are shown in figure 3.11

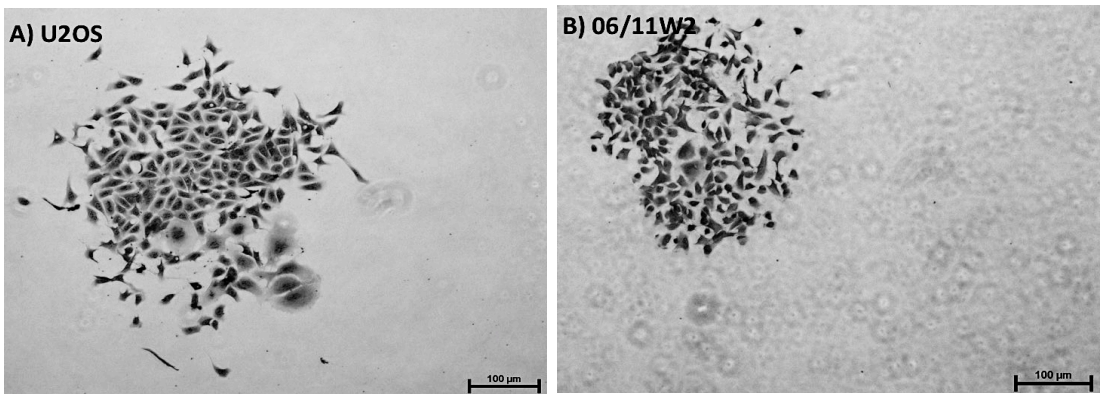


Figure 3. 11: An example of colony formation from an established and a primary sarcoma cell.

A) Photograph x4 magnification taken by light microscope of a U2OS cell line showing a distinct colony.
B) Photograph x4 magnification taken by light microscope of a 06/11W2 cell line showing a distinct colony.

The clonal efficiency, which is the calculated percentages of proliferation or survival of colony numbers divided by the number of cells seeded grown in culture, is presented in Table 3.3.

Cell lines	Type of Cancer	% of average Clonal Efficiency of all densities
SKLMS-1	Leiomyosarcoma	32.3
U2OS	Osteosarcoma	40
SKUT-1	Human uterus leiomyosarcoma (GIII) cell line	84.4
SW1353	Chondrosarcoma	34.8
02/11 W1	Leiomyosarcoma	24.5
02/11 WS	Leiomyosarcoma	47.2
09/10	Dedifferentiated Liposarcoma	40.4
13/12W2	Pleomorphic NOS sarcoma	9.8
09/11	Pleomorphic Sarcoma NOS	28.4
14/10	Pleomorphic Sarcoma NOS	54.3
06/11 WS	Pleomorphic Sarcoma NOS	23
06/11 W2	Pleomorphic Sarcoma NOS	49
06/11	Pleomorphic Sarcoma NOS	33.6
04/13	Angiosarcoma of the breast	2

Table 3. 3: This table shows a list of sarcoma cell lines with an average % of clonal efficiency of the cells that were seeded in all densities (500, 1000 and 2000).

The established cell lines, and higher primary passage number, show a higher percentage clonal efficiency compared to the low passages. The data correspond from three independent experiments unless otherwise stated.

For all cell lines, the preferable cell seeding density was determined to be between 500 and 1000 cells (Figures 3.11 and 3.12). At 3000 cells, a monolayer formed and colonies were uncountable. All cell lines formed colonies, but early passages of the Sheffield primary cell lines took on average seven days longer than later passages.

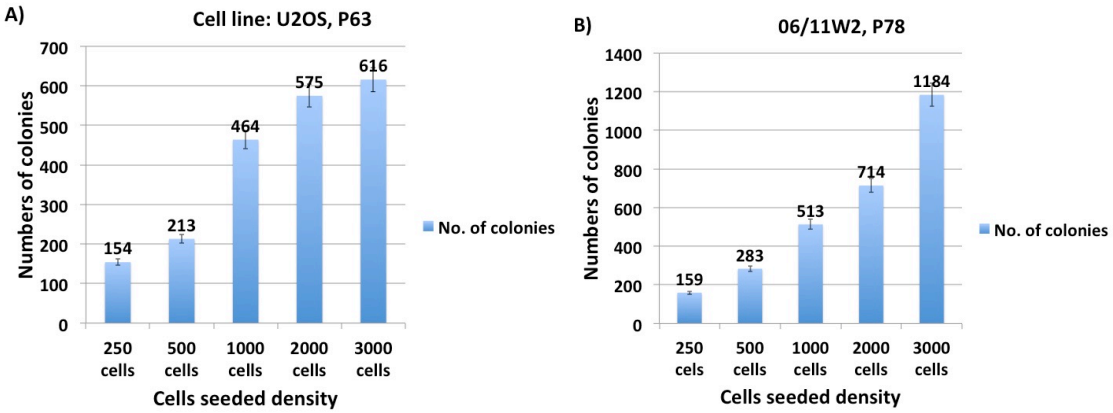


Figure 3. 12: An example of an established and primary sarcoma cell lines clonogenic assay colony counting shows the number of colonies counted in several different densities.

A) U2OS established cell line. B) 06/11W2 primary cell line.

3.2.5.1 CD133+ve Cells Have Variable Clonogenicity, but ALDH-Hi Cells Have High Clonogenicity

In order to determine whether sorted CD133+ve and ALDH +ve primary and established cell lines had higher clonogenicity, cell lines were cultured from 7 to14 days immediately post sorting using FACS. The CD133 and ALDH +ve sorted cells and matched unsorted cells were then grown in Petri dishes for 7-14 days depending on the cell line. Some of the CD133 +ve post sorted cell lines had a significantly higher clonogenicity rate, in comparison to unsorted cell lines. For example Pleomorphic NOS sarcoma cell line (06/11) and one of the established cell lines U2OS (Osteosarcoma). The ALDH +ve post sorted cells however had for most cell lines (5/7) significantly more colonies than the unsorted controls (figures 3.13 and 3.14). As stated in sections 2.2.6 and 2.2.9.1 only seven cell lines were selected out of twelve cell lines due to technical issues in handling so many lines and the time constraints. Cell line (09/11) was excluded from the ALDH staining due its slow-growing. Also, for the cell line (02/11W1) CD133 was not detected in post sorting, due to background similarity between the control and test tube Table 3.4. Sorted cells were obtained from U2OS and 06/11 had noisy background and some difficulty, but manageable was faced during sorting and drawing the line between the negative control and the actual positive cells. This was one of the reasons that some cell lines were excluded.

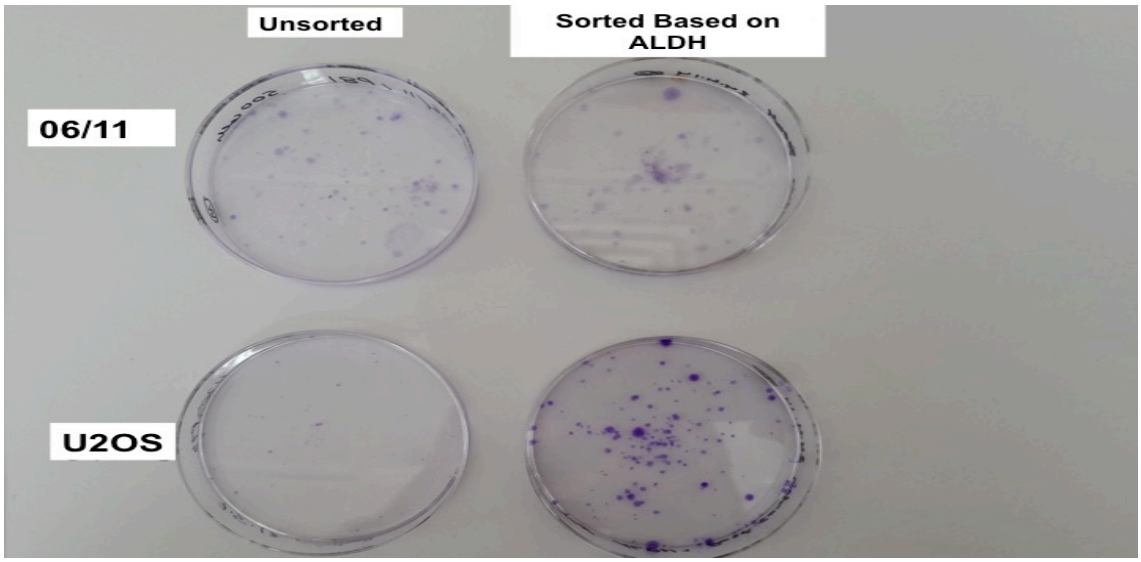


Figure 3. 13: A photograph showing clonogenic assay of a post sorting sarcoma cell lines (06/11 primary and U2OS established) compared with unsorted for the same cell lines based on ALDH expression. The number of cells being sorted for 06/11 cell line based on ALDH positivity was 33,000 cells and U2OS cell line was 24,000 cells.

Cell lines	No. of cells after Sorting based on CD133 +ve	No. of cells after Sorting based on ALDH +ve
U2OS	24,000	90,000
06/11	33,000	100,000
09/10	100,000	60,000
14/10	4,800	30,000
09/11	20,000	30,000
02/11W1	-	50,000
13/12W1	20.000	20,000

Table 3. 4: This table shows a list of sarcoma cell lines being sorted based on CD133 and ALDH antibodies positivity with the number of cells been sorted. Cell count prior cell sorting was between 0.4 and 2 $x10^6$, sorting time for all cell lines was varies around 15 to 25 minutes, depending on the cell count in the suspended solutions and markers positivity. Test tubes cell suspensions were around 0.5 ml on all of them, and cell concentration was adjusted around 1 $x10^6$.

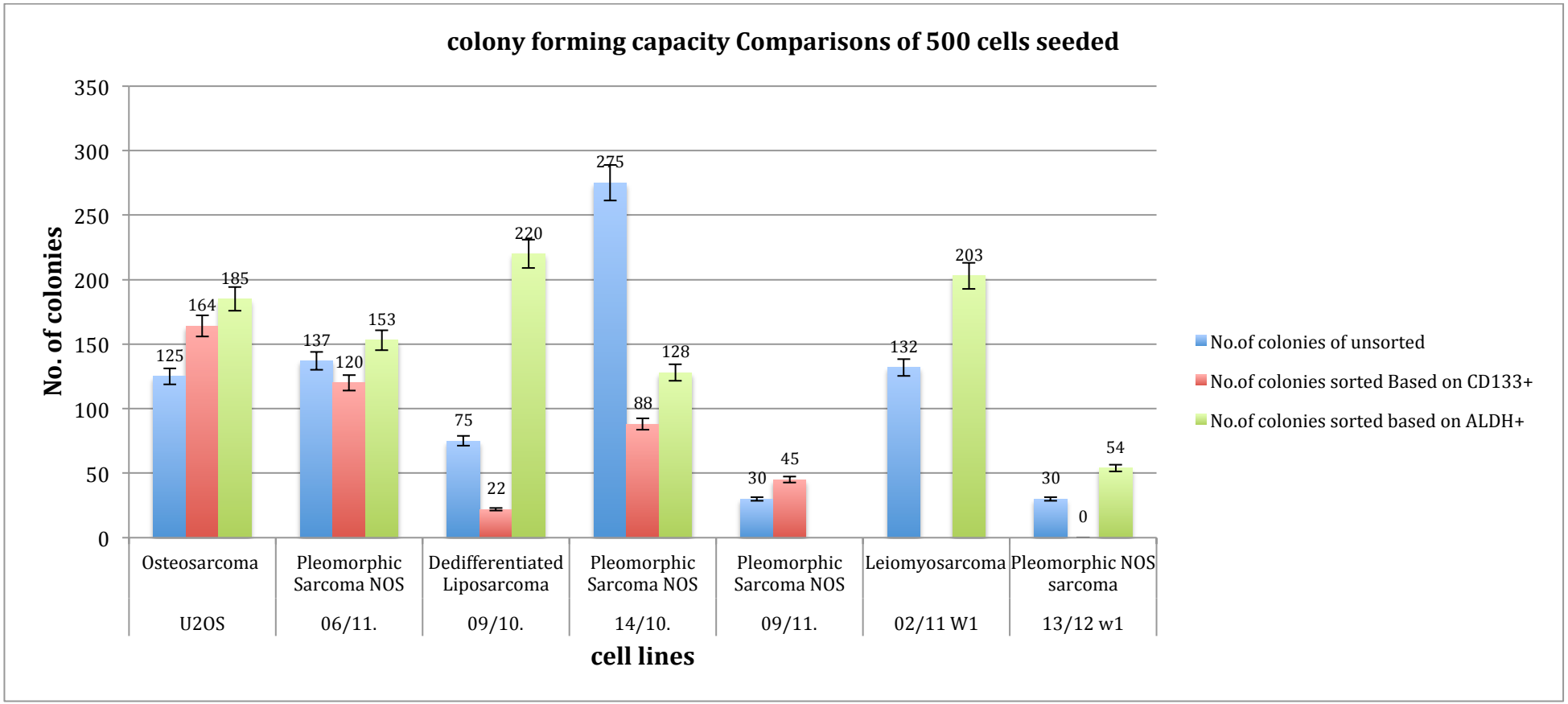


Figure 3. 14: Clonogenic assay comparisons of sarcoma cell lines post sorting based on ALDH and CD133 expression using flow cytometry.

The cell lines post-sorted based on ALDH expression shows a significantly higher ability to form colonies. While the cell lines post sorted based on CD133 expression were variable. Sorting based on ALDH for 09/11 cell line and sorting based on CD133 on 02/11W1 cell line were not performed due to technical issues. See Table 3.4. showing in details the number of cell being sorted for each cell line in this experiment.

3.3 DISCUSSION

3.3.1 CD44 Expression as a Possible CSC Marker for Sarcoma

CD44 was considered as the main CD marker in many solid tumours such as breast, head and neck, pancreas, prostate and other (Al-Hajj *et al*., 2003, Yuce *et al*., 2011, Cruz *et al*., 2012, Joshua *et al*., 2012, Mannelli and Gallo, 2012, Trapasso and Allegra, 2012,) however, few studies have investigated sarcoma. In this study, PC3 was used as a positive control because it has been reported to express high levels of CD44 *(Tu and Lin, 2012)*, and h-TERT (Normal Retinal cells) was used to observe the expression on normal cells. No previous studies have investigated CD44 expression on h-TERT. The high levels of CD44 expression in one distinct homogenous population, was found for all cell lines between 87% and 99%. The CD44 gating strategy was based against isotype control and by using the forward scatter and side scatter dot plot. By comparing the findings, CD44 flow data suggest that CD44 is also associated not only with cancer cells but found to be highly expressed on normal cells as well (Figure 3.3 and Figure 3.9). These findings suggest that CD44 expression is not significant for CSC in sarcoma and cannot be used singly, and as such was not deemed of value for the assessment of colony-forming ability post sorting. As CD44 has been clearly identified as a marker for mesenchymal stem cells its high expression in sarcomas is not altogether surprising (Ullah et al., 2015). It is however surprising that the normal retinal cells also expressed CD44. These normal retinal cells have become immortalized by HTERT and recent evidence however suggests that TERT interacts with stem cell markers and hence immortalization using TERT could switch on expression. The correlation between CD44 and TERT, although not reported may still exist. Markers such as CD24, CD133, ALDH1A2 and ALDH1A1 were found to correlate with TERT (Wazir et al., 2019).

For CD44 a number of studies have linked its high expression to poor prognosis; using Immunohistochemistry of tumour samples and correlating patient survival with CD44 expression pre and post-treatment (Fonseca *et al*., 2001; Wang *et al*., 2009, Kokko *et al*., 2011). Other studies have found no relationship (Carinci *et al*., 2002, Rajarajan *et al*., 2012), and in contrast,(Humphrey *et al*., 1999) indicated that low CD44 expression in rhabdomyosarcoma associated with a poor outcome and according to (Peiper *et al*., 2004) patients with low or negative CD44 expression correlated with the risk of metastasis and relapse. All sarcomas in this study had high expression of CD44 (Table 3.1) but some of the patients had already developed metastatic disease, seemingly

indicating that low expression of CD44 for the tumours in this series cannot be considered as a prognostic indicator. This raises the question of the real role of CD44 as CSC marker. There is no clear evidence in the literature linking CD44 with self-renewal or pluripotency. On the other hand, it is linked to regulated gene activation in stem cells, adhesion, dormancy and metastatic properties (Zoller, 2011) and this may explain the correlation between CD44 and metastasis: or it could be related to the CD44 standard and different isoform used. More studies are required on a bigger scale to verify these outcomes and compare with flow cytometry results. In this investigation CD44 was used as a possible pan CSC marker to check this expression on a variety of sarcomas and to observe if CD44 was switched on in co-expressed populations. CD44 is however not of value for sarcoma by itself. It is possible that by only selecting some cell lines for further investigation that there is bias in the findings. The selection of the cell lines was based on their proliferative ability and to provide representations of different types of sarcomas. Therefore, slower growing cell lines such as the angiosarcomas were either too slow, or as short term cultures had started to die around passage 5. It is therefore possible that by selecting faster growing cell lines the results were biased, or that different versions of the same tumour / and subtypes may have different expressions. For CD44 however this seems unlikely as it was ubiquitously highly expressed even for the short term and much slower growing angiosarcomas.

3.3.2 CD133, CD90 and CD24 as Possible CSC Markers for Sarcoma

Previously some positivity for CD133 in sarcoma has been reported (Tirino *et al*., 2011). In this study, the results showed universally very low positivity to negative (0.1% to 2.1%) for the majority of the cell lines, and the positive percentages did not represent a distinct population due to the low expression levels (Figure 3.3, Figure 3.5, Figure 3.6, Figure 3.9 and Figure 3.10). The finding suggests that the CD133 is also expressed at a similar level by the normal cell line (h-TERT). Reports of CD133 expression in sarcoma are variable, with some positive expression in osteosarcoma (immunohistochemistry), while by flow cytometry negative expression was found (He *et al*., 2012). Other studies were more consistent with the findings reported here, and one of them used osteosarcoma MG-63 cell line (Tirino *et al*., 2008, Di Fiore et al., 2009, Tirino *et al*., 2011). It is therefore difficult to rely on CD133 by itself because of this very-low expression seen in most lines, although this could in theory represent a very low number of cells with CSC characteristics present in sarcoma.

CD90 has been reported as a positive human embryonic stem cells surface marker (hESC's) (Xu et al., 2001, Draper et al., 2002, Skottman et al., 2005), and for hematopoietic stem cells, involved with neuron differentiation and T-activation cells and playing a role in their classification (Yin et al., 1997, Adewumi et al., 2007). Cells that express CD90 in hepatocellular carcinoma tend to display more tumorigenic capabilities, and co-expression with CD45 associated with the production of tumour nodules (Yang et al., 2008). Furthermore the few cells that were isolated and that co expressed CD90 and CD24 were metastatic (Malanchi et al., 2012), but the preliminary data isolating the CSC dot plot of CD24+ve / CD29 +ve ^{high} or CD24+ve /CD49f +ve, could not be verified as it was not included in the article.

CD24 is also associated with hESC's (Bhattacharya et al., 2004, Skottman et al., 2005, Adewumi et al., 2007, Assou et al., 2007, Lian et al., 2007), and as a CSC surface marker with metastasizing ability (Malanchi et al., 2012,) potentially expressed by the CSC of many carcinomas with poor prognoses, such as breast, lung, liver, bladder and ovarian, colorectal and pancreas (Aigner et al., 1997, Karahan et al., 2006, Gao et al., 2010, Yeung et al., 2010, Stratford et al., 2011, Yang et al., 2014, Rao and Mohammed, 2015). CSC subpopulations in breast cancer isolated based on a combination between CD44 and CD24 markers, that express CD44+ve / CD24 $^{low/-ve}$ were found to be chemotherapy and radiotherapy resistance (Phillips et al., 2006, Lu et al., 2011). Although in this study CD133, CD90 and CD24 were considered to be negative to very low in all sarcoma cell lines been tested, the subpopulation percentages were however comparable to those previously reported in other cancers (Phillips et al., 2006, Lu et al., 2011, Tirino *et al*., 2011, Malanchi et al., 2012). Further investigations are needed to validate this outcome.

3.3.3 ALDH Activity as a Possible CSC Marker for Sarcoma

ALDH activity could be a useful marker to investigate CSC due to the association with normal stem cell function. The expression level of ALDH was found to be variable and can be split into three groups accordingly. Group one: the established sarcoma cell lines like U2OS (Osteosarcoma) and SKUT-1 (Human uterus leiomyosarcoma (GIII) cell line) illustrate high ALDH +ve expression 99% and 68%, respectively. Also, PC3 was 35% for ALDH. Group two: Sheffield primary cell lines Myxofibrosarcoma, some Leiomyosarcoma and some pleomorphic NOS sarcoma cell lines appear to show moderate ALDH positivity between 19% to 31.99%. Group three: the rest of the

sarcoma cell lines including the normal cell line, demonstrate week expression for ALDH from 1%to 9% (Figure 3.2, Figure 3.5, Figure 3.6, Figure 3.8 and Figure 3.10).

ALDH activity or expression has been widely used in CSC research and measured in solid tumours and hematopoietic and neuronal cells (Adhikari *et al*., 2010). ALDH was found at a high level of about (45%) in some osteosarcoma (OS99-1, Saos-2, HuO9, and MG-63) cell lines as a distinct subpopulation (Wang *et al*., 2011), and similar levels were reported for liposarcomas (Stratford *et al*., 2011). In this study, expression was classified into three groups according to the expression intensity. ALDH ^{high} found in osteosarcoma (U2OS) 99% and (Human uterus leiomyosarcoma (GIII) (SKUT-1) 68%. ALDH^{moderate} expression was in Myxofibrosarcoma (21/11) 19%, some Leiomyosarcoma (02/11WS) 21% and some Pleomorphic NOS sarcoma (13/12W1) 31.99%, (13/12W2) 28%, (14/10) 23%. ALDH ^{low} was in angiosarcoma (05/13, 07/13 and 07/13SW1) 4%, 3%, 7%, respectively. The other Pleomorphic NOS sarcoma (06/11, 06/11W2 and 08/13), Leiomyosarcoma (SKLMS-1), Chondrosarcoma (SW1353) Dedifferentiated Liposarcoma (09/10) cell lines also exhibit ALDH^{low} expression from1%to 9% (Figure 3.4). This outcome means that even with the same tumour (with different variants) 13/12W1(31.99%) and 13/12W2(28%) cell lines there were some differences. These differences could be due to more variation in some subtypes of sarcoma or may reflect changes over time in culture. Finally, All ALDH +ve were co-expressed with CD44 except the Chondrosarcoma SW1353. Table3.1. Interestingly, in this study it was noticed that when cell lines become more adapted to the culture (higher passages); the ALDH expression was found to be higher. Therefore, is ALDH expression linked more to cell proliferation and adaptation? Or linked more to CSC in later stages? Or is it because CSC populations were diluted in culture? Or it is not linked to the CSC as a marker? This study, could not answer all these questions and more future work needs to be performed Table 3.5. Having said that as some of the slowly growing angiosarcomas expressed ALDH at the same levels as the much faster Sheffield derive lines (Table 3.5) the balance of probability it is about cultural selection when increase were found in higher passages.

Cell line	ALDH expression	Proliferation to be confluent as estimated
Sheffield cell line		
02/11WS	21%	4-7 days
21/11W2	19%	4-7 days
09/10	9%	4-7 days
13/12W2	28%	4-7 days
13/12W1	32%	4-7 days
09/11	8%	4-7 days
14/10	23%	4-7 days
06/11WS	6%	4-7 days
06/11W2	4%	4-7 days
06/11	5%	4-7 days
Angiosarcoma		Around 3-4 weeks
04/13S	0%	Around 3-4 weeks
04/13	0%	Around 3-4 weeks
05/13	4%	Around 3-4 weeks
07/13	3%	Around 3-4 weeks
07/13SW1	7%	Around 3-4 weeks
08/13W1	7%	Around 3-4 weeks

Table 3. 5: This table shows a list of sarcoma cell lines (established and primary Sheffield sarcoma cell lines) with ALDH expression and time for proliferation to be confluent as estimated. Data were extracted from table 3.1 and section 2.2. Markers expression percentage given is ± 0.3% (n=3 SEM). The data correspond to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculated was done using Graph Pad® Prism software (version 7).

3.3.4 CD34 and CD71 as Possible CSC Markers for Sarcoma

CD34 is widely known as a marker for hemopoietic progenitors cells, leukemic progenitor cells, as well as CSC surface marker and may play a role in cell fate and hierarchy (Dao and Nolta, 2000, Yang et al., 2015). CD34 and CD71 have never been investigated intensively before in sarcoma. CD34 expression was generally lower in the established cell lines, between (1%-14%), while the Sheffield derived cell lines demonstrated higher expression (1% - 52%), as well as the prostate and h-TERT-RPE-1 cell lines.

CD71 (transferrin receptor-TFR) is known in the literature as an activate marker of progenitor cells and proliferating cells, and is also found to be positive on erythroid or erythroblast cells in the early stages (An and Chen, 2018, Acharya and Kala, 2019).

CD71 is often used to detect leukemic and lymphatic blast cells using flow cytometry (Della Porta et al., 2006, An and Chen, 2018, Acharya and Kala, 2019). The majority of hemopoietic progenitor cells detected as CD34+ve were co-expressed with CD71. However, the mean percentages isolated were around 4.6%±2.5 of CD34 +ve cells (Darena et al., 1996). Furthermore, normal hematopoietic stem cells and leukemic stem cells were CD34+ve but CD71 –ve (Blair et al., 1998, Blair and Sutherland, 2000, Bonnet, 2005). In addition, CD71 was investigated in cervical carcinoma cell lines in combination with other markers and showed weak expression in the isolated side population (Villanueva-Toledo et al., 2014). Interestingly, in gastric cancer cells with CD71-ve were tumorigenic, chemoresistant and more invasive than CD71+ve cells (Ohkuma et al., 2012). However, in colorectal adenocarcinoma putative CSC displayed positivity around 7% of co expressed CD71+ve/CD90+ve (Gisina et al., 2011).

CD71 expression in this study appeared to be negative to weak on established cell lines (0%-7%) compared to the moderate primary cell lines expression (4%-23%). These findings again suggest there appear to be differences between more recently established cell lines and those many years previously, particularly when you consider that many of the lines were established in the 1970's over 40 years ago. If this is the case both CD 34 and CD71 may have potential as markers of CSC for sarcomas, and repeated analysis overtime would indicate if there was a possible cultural dilution effect, or selection of sub populations and genetic drift. It would also be worthwhile repeating some of the published studies using the Sheffield derived lines in comparison with the established lines, especially those with co-expression on different osteosarcoma cell line mentioned before (Wang *et al*., 2011). There are many possible variables, such as the gating strategy, whether it is FACs compared to IHC, as study suggest differences for CD133 depending on the method (He *et al*., 2012). Off course in the end it could be done to the cell line used and the conditions it is cultured under. What is clear from this study is that sarcoma cell lines established over 40 years ago share some characteristics with the Sheffield derived lines, but there are distinct differences that implies that prolonged culture has effected the expression of some possible CSC markers.

3.3.5 ALDH ^{high} and CD133 +ve in Some Sarcoma Cell Lines are Significantly More Clonogenic and Possibly Enriched with CSC

The sarcoma cell lines show varying abilities to form colonies. The proliferative rate for the sarcoma cell lines however varies, which means that the time to form colonies was quicker for some cell lines than others, and potentially more highly proliferative lines would have comparably higher clonogenicity. Lohberger *et al*., 2012 found that ALDH^{high} chondrosarcoma (SW1353) cells demonstrated higher proliferation than ALDH^{low} and according to (Stratford *et al*., 2011), CD133^{high} and ALDH^{high} sorted cells were found to create more tumours than other subpopulations in mice. Based on CD133 +ve and ALDH^{high} in this study, seven-sarcoma cell lines were sorted and cultured for clonogenicity (Figure 3.2, Figure 3.5, Figure 3.6, Figure 3.8, Figure 3.10, Figure 3.14 and Table 3.4), and in general, post sorted ALDH had higher clonogenicity and formed colonies quicker, suggesting increased proliferation. Findings were more variable for CD133 post-sorted sarcomas, but taken together the findings are broadly supportive of the limited previous reports for these markers in sarcoma. All work carried out in this study was restricted to comparable passage numbers for each individual cell line. Post sorting increases in clonogenicity and proliferation, may however by association correlate with these cell lines having slightly higher passage number such as (02/11W1, 13/12W1 and 09/10), even though the range of passage numbers were restricted as much as feasibly possible. It would be interesting to see what effects extended culture of the Sheffield derived lines had on expression of ALDH and CD133 and repeat proliferation assays to more directly correlate increased proliferation with enhanced clonogenicity.

3.3.6 SUMMARY

The different findings of this study were broadly supportive of the limited information previously published on the use of markers to identify CSC in sarcomas. Certainly, CD44 has no clear value, but CD133 and ALDH may have some impact. Equally, other markers were negative and so again of no value, but the expression of CD71 restricted to the Sheffield derived lines was of interest. Co-expression analysis was restricted by the different dyes available for the combinations, but could be repeated based on the initial observations of this current study. The use of a large panel of sarcoma cell lines does however demonstrate how variable the expression for some such as ALDH and

CD71 can be. The expressions of these markers in the normal cell line also suggest that there may be issues with the reliability of these markers. These findings need to be validated to confirm the importance of using these markers and methods in identifying CSC populations in sarcomas, and additional non-transformed normal cells should also be included. The evidence from post sorting and the expression of CD71, not found in the established lines, all helps to suggest that cultural adaption as expected over time may complicate issues. Given the findings of this study, the question arises as to whether the use of established cell lines is likely to identify markers as CSC, since they may be deselected over time in culture, and may explain why there are so many differences between reported data based on whether cell lines or tumour sections have been studied. As the use of markers had not been successful, a different approach was taken to see if CSC populations could be identified in sarcomas.

CHAPTER FOUR

DEVELOPMENT OF A FUNCTIONAL ASSAY TO ISOLATE AND ENRICH FOR CANCER STEM CELLS "THE STRESS ASSAY"

4.1 INTRODUCTION

Stem cells are known as primitive cells with the ability of self-renewal to maintain status, and the capacity to differentiate into more cells in a hierarchy order. CSC have been suggested to be a small population of tumour initiating stem cells with SC properties, giving rise to CSC and differentiating into other cancer cells types (non-CSC). Based on studies that show expression of putative markers the number of isolated cells claimed to be CSC is very low, not more than (7%) (Malanchi et al., 2008). Other studies have suggested that the incidence of CSC in cancers is positive without stating any percentages (Brown et al., 2017, Dinavahi et al., 2019, Tarhriz et al., 2019). CSC are considered responsible for maintaining, relapsing and the metastasis of the tumours (Gupta *et al*., 2009, Ratajczak et al., 2018), which make them very important for investigation in cancer research. In addition, CSC properties may differ depending on the malignancy stage and progression of the disease (Gupta *et al*., 2009, Harris *et al*., 2009, Ratajczak et al., 2018).

Detecting CSC in tissues, cell suspensions or even circulating in the blood are emerging areas, but the identification and isolation of these cells is challenging due to the low number and lack of specific detection methods. In the previous chapter (three), markers were used to identify CSC in sarcoma without much reliability. Besides the use of markers, isolation of CSC by other methods have been proposed including targeting signalling pathways, such as Nanog, Stat3, and Wnt/β-catenin, as well as functional assays (Bekaii-Saab and El-Rayes, 2017, Akbarzadeh et al., 2019). The multitude of sarcoma subtypes and variable behaviour makes the identification of CSC even harder in sarcoma. (Skoda and Veselska, 2018); as markers were not of value it is possible therefore that functional assays may be more beneficial. The ability of tumour cells to resist chemotherapy and radiotherapy is the main reason for relapse and metastasis and CSC are thought to be central to this ability (Pinedo and Verweij, 1986, Mackall et al., 2002, Ray-Coquard and Le Cesne, 2012, Ratajczak et al., 2018). Sarcomas however do not universally respond to either radiotherapy or chemotherapy so using either to potentially enrich for CSC in sarcomas is likely to be problematic although found to enrich in other cancers for CSC (Crawford et al., 2019).

Hypoxia has also been shown to enrich CSC (Crawford et al., 2019). A hypoxic microenvironment usually arises when the tumour becomes large in size and heterogeneous promoting cancer progression, and through hypoxia-inducible factor-1 HIF1, maintaining differentiation and potentially enhancing CSC behaviour (Petrova et

al., 2018). In addition, HIF1 levels could be used as a prognostic marker and high-levels associate with reduced disease period and affects the survival of the patients, such as has been shown in rectal tumours (Ullmann et al., 2019). In sarcoma, HIF1 activation and increased expression correlates with invasion and metastasis (El-Naggar et al., 2015, Ouyang et al., 2016) but may not be consistent for all subtypes due to their diversity. Alternatively, it has been known almost that deprivation of glucose and oxygen (the Warburg effect) in tumour cells results in cell death (Schwartz et al., 2017).

Therefore, as this project is studying different types of sarcoma, starvation of the cells is a sure way to promote cell death in all sarcoma subtypes and to potentially mimic cancer relapse. In this chapter, an assay based on prolonged starvation, the so-called "stress assay" was developed. Initially, cell lines from different types of sarcoma (both commercial and those established in Sheffield), were set up in cultures and starved for prolonged periods to promote cell death. The methodology was refined in the second and third phases, see section 2.2, and comparisons were undertaken of the tumour population before the stress assay and the population of surviving cells post-stress assay. An overview of the workflow is shown in Figure 4.1.

Figure 4. 1: Overview of Chapter 4 workflow

The initial phase of the studied was about drawing the baseline of this starvation method (stress assay), in the second phase the stress assay was amended based on the initial findings. The last phase was more refined and based on the earlier findings and focused on fewer cell lines. Also, investigations were carried out prior to and after the stress assay, such as MTT, DNA extraction and picturing the cells during the process. Finally, the assays were documented to record timings and images of the re-culturing of the post-stress assay surviving cells.

4.2 RESULTS

4.2.1 Stress Assay Phase One

In this study 15 Sheffield derived cell lines and 6 commercial cell lines were initially studied. In total 21 cell lines in triplicate (around 61 flasks) were included. This first phase was to observe the effect of prolonged culture without feeding, or supplementing the media with any supporting elements, and to see whether cells could survive following refeeding. Cells were treated as mentioned in section 2.2.18.1. The period of this study was 8-9 months and the cells were observed once a week. Unfortunately, contaminations of some flasks occurred during this study and affected replicates. To ease the procedure cells lines used were separated into two groups. For each line three flasks with three different passages number (minimum) were set up for each cell line, except 04/13S, 04/13 and 04/13WS, which due to the very low proliferation rate of the cells made it difficult to passage the cells in adequate numbers. Table 4.1.

After 8-9 months, all the flasks were examined under the light microscope and pictured. The observations of all cell lines at 8-9 months were consistent, with none surviving the so-called stress assay. The media was consumed (very bright yellow coloured), and floating single dead cells and sheets of dead cells and cell fragments were present. Furthermore, “Ghosts” or the remnants of cell shapes and colonies were also seen. At this point, floating cells were transferred to separate flasks and media was added to both the original and washed off cells. Refed cells were then monitored for three months but exhibited no sign of cell growth. For example, Figures 4.2.

	Commercial cell lines (Phase One SA)	Group one (Phase One SA)	Group two (Phase One SA)
Cell lines were set up	SKUT-1 U2OS SW1353 SKLMS-1 PC3 hTERT-RPE-1	06/11W2 P83, 06/11W2 P84, 06/11W2 P88 06/11 P91, 06/11 P92, 06/11 P94 13/12W1 P17, 13/12W1 P19, 13/12/W1 P20 20/11 P24, 20/11 P25, 20/11 P26 02/11WS P72, 02/11WS P73, 02/11WS P74, 02/11WS P76, 04/13 WS P2 09/11 P38, 09/11 P39, 09/11 P38 04/13S P2 04/13 P2 14/10 P77, 14/10 P78. 14/10 P79	02/11W1 P64, 02/11W1 P66, 02/11W1 P67 06/11WS P95, 06/11WS P96, 06/11WS P97, 06/11WS P100 09/10 P76, 09/10 P77, 09/10 P78, 09/10 P88 13/12W2 P14v, 13/12W2 P15v, 13/12W2 P19v, 13/12W2 P40v 21/11W2 P42, 21/11W2 P43, 21/11W2 P44, 21/11W2 P49

Table 4. 1: Representing the entire commercial and primary cell lines used in the phase one stress assay experiment.

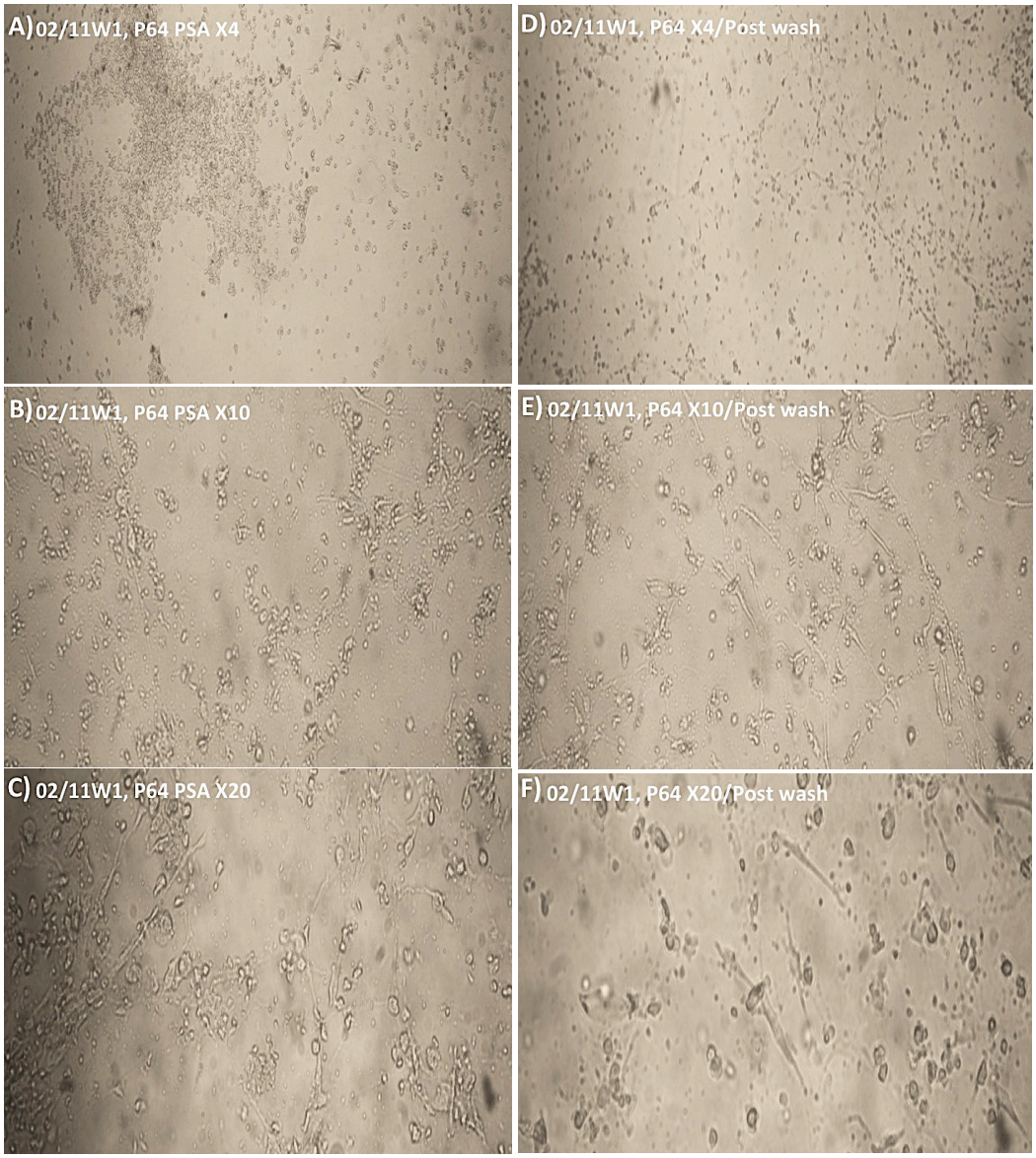


Figure 4. 2: Microscopic images (Phase-contrast) showing **02/11W1** Primary cell line cells in culture Post-stress assay (phase one) for 8-9 months pre-washed and post washed with new media.

Cells shown are at passage 64 and were derived from the first wash (W1) from 02/11 tissue. **A** and **D** Images were captured at x4 magnification, showing dead colonies on the flasks' surface. **B** and **E** Images were captured at x10 magnification, showing loose floating dead cells and dead cells on the flasks' bottom. **C** and **F** Images were captured at x20 magnification, showing starved, floating dead cells and cell fragments with undefined cell shapes. Experiments were done in triplicate on three different passages.

4.2.2 Stress Assay Phase Two

The stress assay was amended in the next attempt (phase two) and is described in sections 2.2.18 and 4.2.2 Figures 2.7, 2.8 and 2.9, Tables 2.7 and 2.8. There were two attempts in this phase. First variants of cell lines were excluded, and other lines were added. The newly derived angiosarcomas were also included as well as SKUT-1, U2OS, SKLMS-1, SW1353, h-TERT-RPE-1 and PC3, and Sheffield cell lines 06/11WS, 02/11W1, 09/10, 02/14, 07/13 and 21/11W2. It was thought that the direct comparison between commercial cell lines that had been established for many years and the newly developed lines could help in better understanding how best to isolate CSC. In phase two, a specific number of cells were seeded into each flask for each cell lines (1x 10^6) prior to stress assay. The second amendment carried out was to shorten the period for the assay to 2-3 months. Increased monitoring and imaging of the cells was also undertaken, to gain a better insight on the time to induce starvation of the entire cell population for each individual line. Monitoring took place on the 7th, 14th, and 21st day, after lines were confluent and when they died. Three different passages (flasks) were seeded. In addition, three flasks were set up to be assessed later for the viable cell number when confluent, after changing the media and leaving for 24-72 hours, in order to determine the maximum viable cell number (MVCN) for each cell line at the point of stress. It was planned that this could act as an additional comparison for the number of cells with the capacity to recover post stress. Finally, one flask was maintained and routinely passaged as a control to those that survived the stress assay. Additional flasks were grown also, for DNA extraction and as a back up (frozen down) for future tests and a reference point of zero time. See section 2.2.18.2 and Figure 2.7.

Some cell lines were problematic to work with, 02/14 stopped proliferating at early passages, and 07/13 was proliferating so slowly there were not enough cells to perform the assay. Both cell lines were excluded from this study, on the other hand, cell lines such as 02/11W1 had shown promising results after been stressed for around two months. Later this cell line was included in the stress assay phase three as 02/11W1 POST-SA2 to compare it with the original cell line and to observe whether the cells will show the same results after repeated stress. Other issues are raised in section 2.2.18.2 and Figure 2.8, and due to infection issues, this phase entirely failed and was unsuccessful.

4.2.3 Stress Assay Phase Three

As phase 2 failed, the assay was repeated as phase 3, but with refinements based on observations made in the previous 2 phases Figure 2.9. The maintained flasks were set up for a comparison at the end of this study with the stressed flasks from each line tested Figure 2.9. This was to provide a comparison with genetic drift and heterogeneity of the cell lines. Unfortunately, due to health issues and time limits these were not analyzed. To ensure this final phase worked a dedicated incubator was purchased to avoid any contaminations and cross-contamination, and cells were monitored on a regular basis by phase-contrast microscope.

4.2.3.1 Commercial Established Cell Lines and Controls (Other than SKUT1).

For all these lines (SW1353, h-TERT-RPE-1, PC3, SKLMS-1, SW1353, and U2OS), the MTT Proliferation assay, clonogenic assays, DNA extraction and the MVCN was determined in conjunction with the stress assay, but there were no surviving colonies post-stress (Figure 4.3).

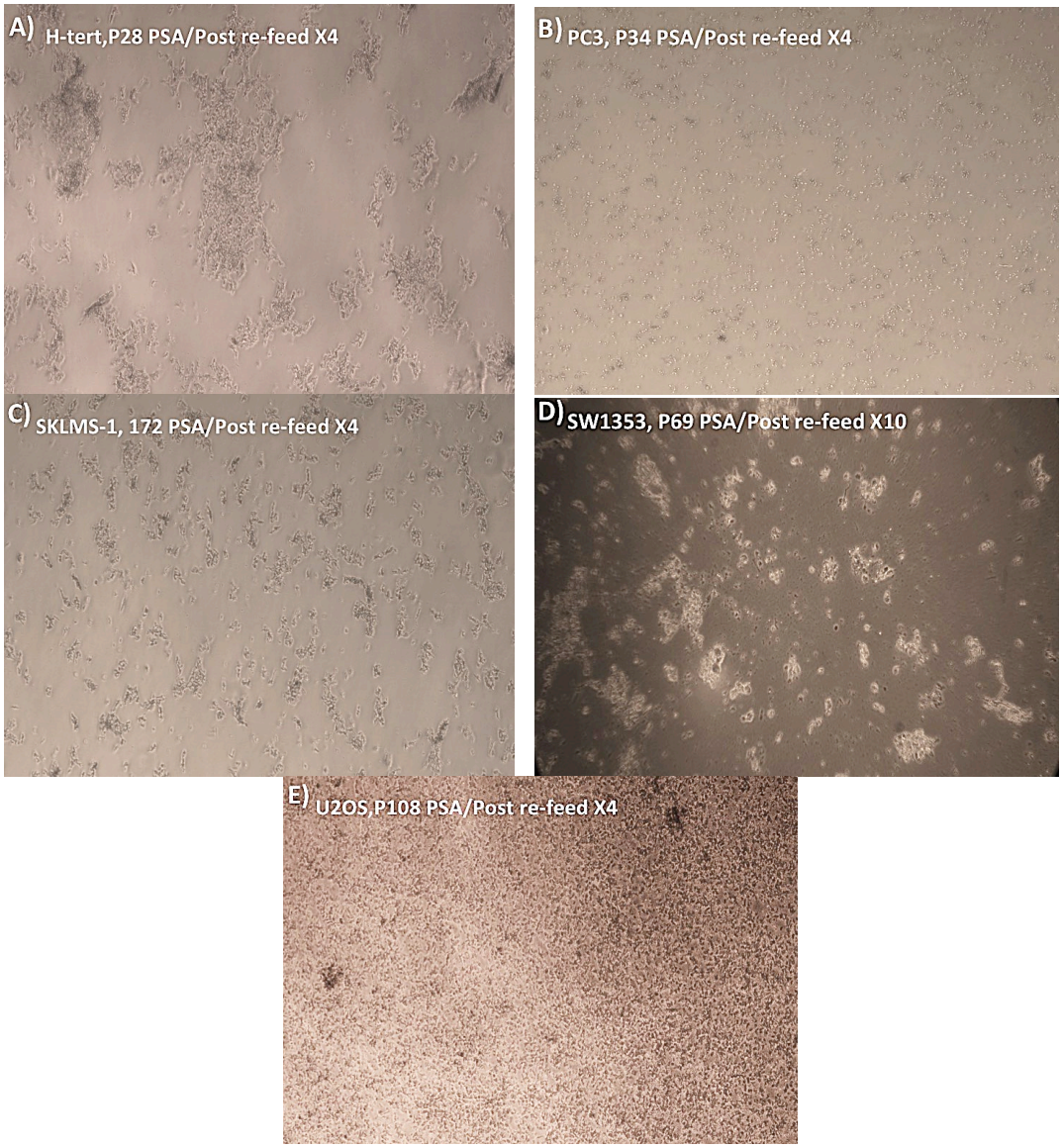


Figure 4. 3: Microscopic images (Phase-contrast) showing monitoring of **(H-tert, P28, PC3, P34, SKLMS-1, P172, SW1353, P69, U2OS, P108)** POST-SA commercial cell lines cells in culture post 4 months of stress assay (phase three) POST-SA.

A- H-tert, P28, B-PC3, P34, C- SKLMS-1, P172, D- SW1353, P69 and E- U2OS, P108, Images were captured at x4 magnification showing no growth post-re-feed (after 2-3 months). Experiments were done in triplicate on three different passages.

4.2.3.2 SKUT-1 (Human Uterus Leiomyosarcoma (GIII))

Of the commercial long time established cell lines, only the SKUT-1 line worked in the stress assay. SKUT-1 was the same as all the commercial established cell lines, and they all had fast proliferation in culture and need passaging after 4-5 days. Based on the MTT proliferation assay, the doubling time was assessed at 38.73 hours. Figure 4.4D. Cells grow as a mixture of fibroblast-like spindle-shaped and epithelial morphology and tend to grow in small compacted packed colonies with clear edges Figure 4.4 C.

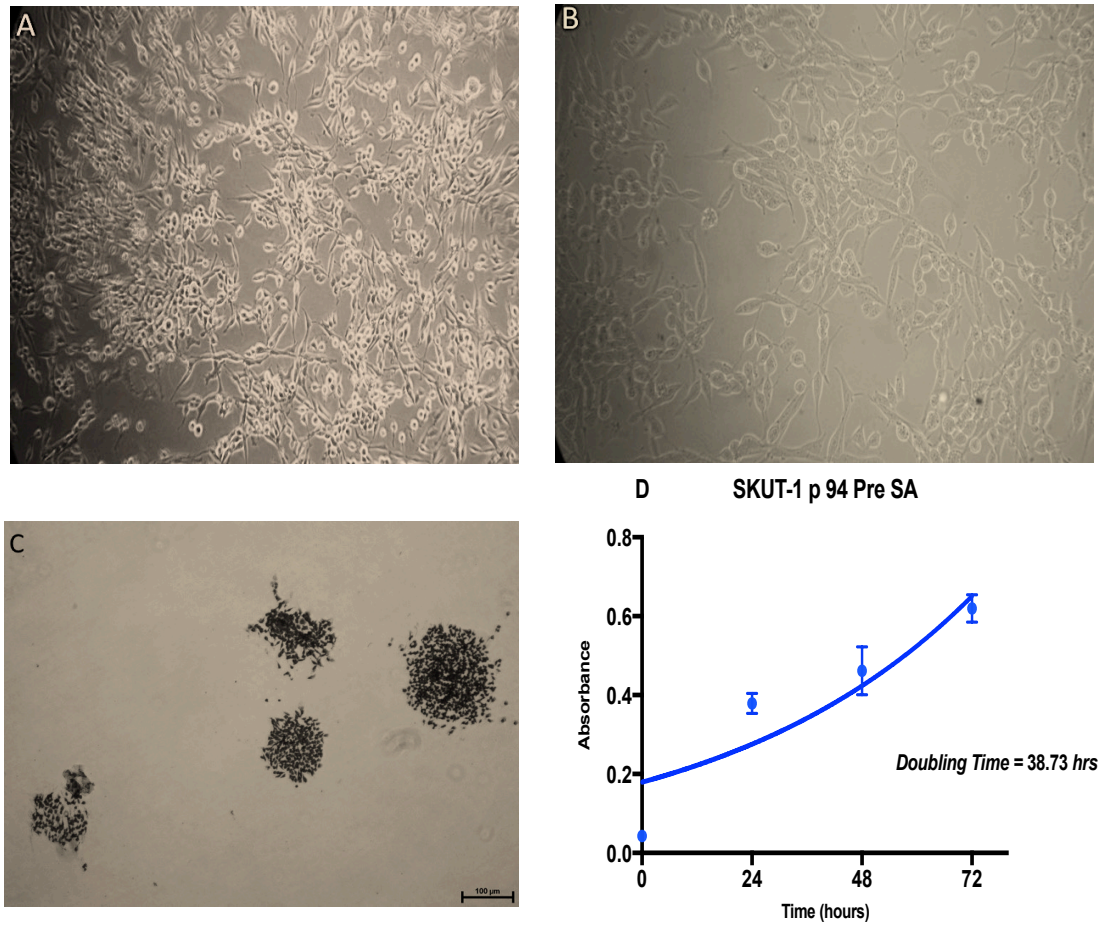


Figure 4. 4: Microscopic images (Phase-contrast) showing **SKUT-1** Established cell line cells in culture and Pre-Stress Assay cell growth evaluated by MTT Proliferation Assay.

Cells shown were at passage 89 and were supplied by ATTC/1971. **A** – Image was captured at x10 magnification showing the pattern of growth in loose colonies. **B**- Image was captured at x20 magnification showing the mixture of fibroblast-like spindle-shaped morphology of most cells with more distinct nuclei. **C**- Image was captured at x4 magnification showing the pattern of growth and colonies shapes. **D**- Cell growth evaluated by MTT proliferation assay. Cells were at passage 94. Data is representative of experiments done in triplicate on three different passages. Curve fitting was done using Graph Pad® Prism software (version 7) and the doubling time calculated by using the same software and using Exponential Growth Equation as well. N.B For some points, the error bars would be shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars. To see the error bars, make the symbols smaller.

Clonogenic assays were performed for all commercial cell lines at the point of the pre-stress assay (Pre-SA) stage as a comparison with different concentrations. For the Clonogenic, colonies out of 250, 500, 1000 and 2000 cells seeded were 101(± 1.53), 170 (±5.6), 220.7(±1.7) and 232.7(±2.3) respectively. Clonal efficacy CE based on the clonogenic assay colonies results was 27.15% (± 0.32) Figure 4.5 and Figure 4.6.

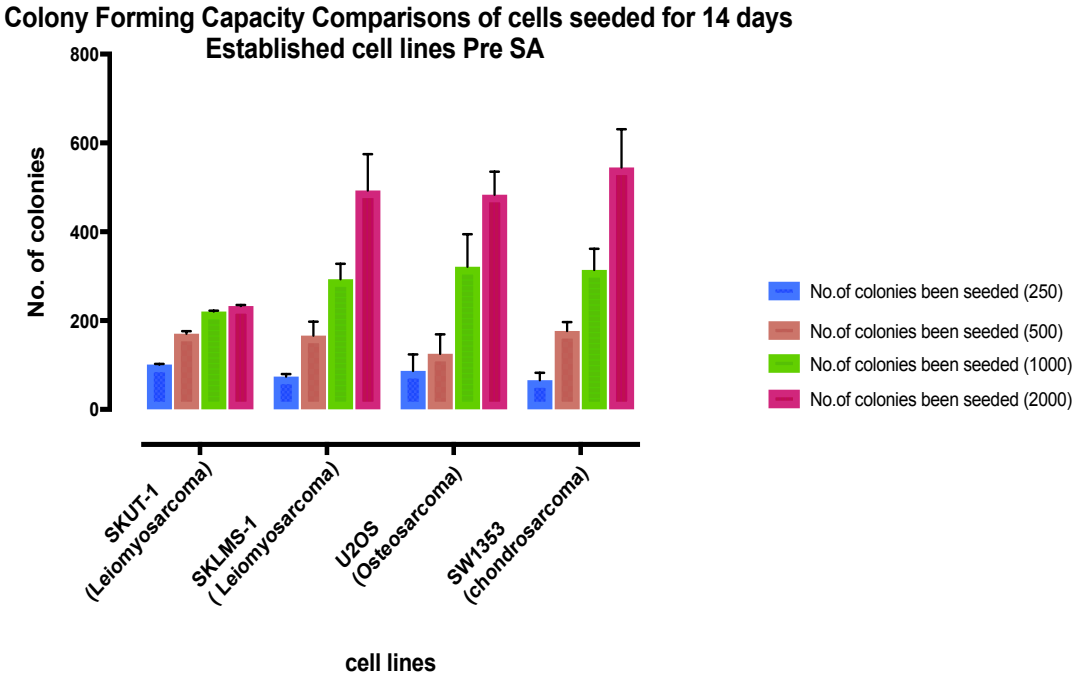


Figure 4. 5: A bar chart representing a comparison of the colony-forming capacity of commercial cell lines seeded for 14 days prior to the pre-stress assay at different densities.

Cell lines were seeded at different densities (250, 500, 1000, and 2000) cells per Petri dishes. **SKUT-1** Number of colonies out of 250, 500, 1000 and 2000 cells seeded were 101(± 1.53), 170 (±5.6), 220.7(±1.7) and 232.7(±2.3) respectively. **SKLMS-1** was 74(±5.9), 166(±31.2), 293(±35) and 493(±81.6) colonies. **U2OS** was 87(±37.3), 125(±43.8), 321(±73) and 483(±51) colonies. **SW1353** was 66(±16.7), 177(±19.9), 314(±47) and 545(±85.8) colonies for each cell concentration mentioned above (n=3 SEM). Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

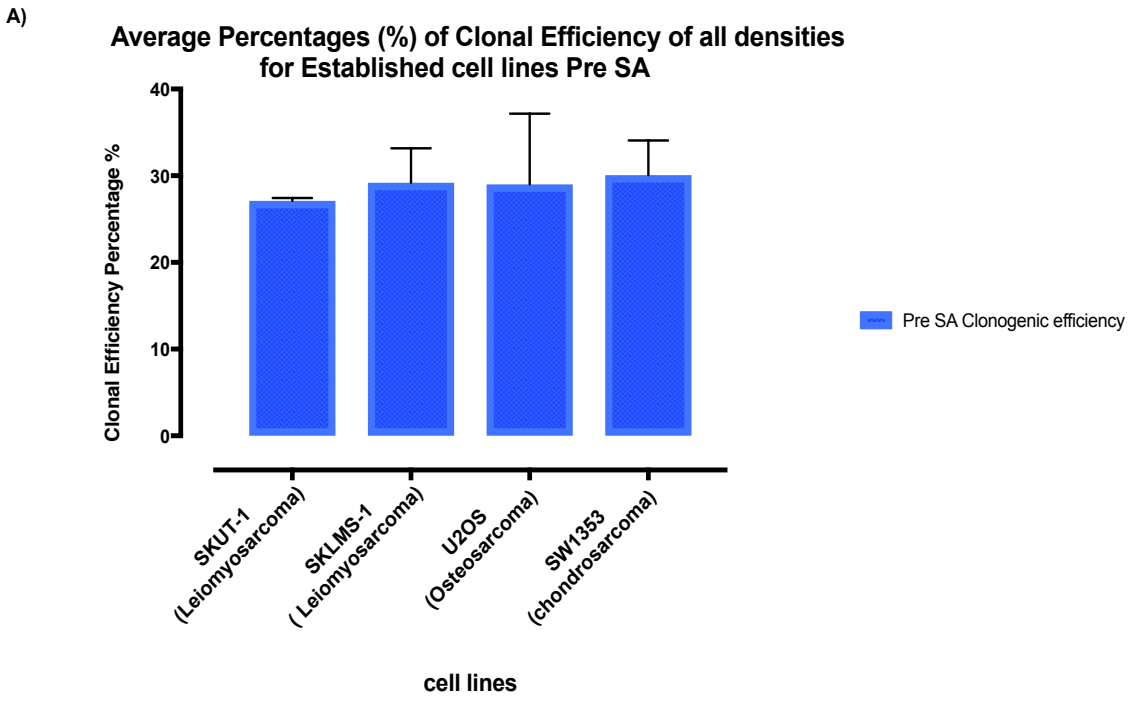


Figure 4. 6: A bar chart represents a comparison of average percentages for clonal efficiency (CE) at all densities for different commercial cell lines prior to the stress assay.

Cell lines were seeded in different densities (250, 500, 1000, and 2000) cells per Petri dishes. **SKUT-1** CE was 27.15% (± 0.32). **SKLMS-1** CE was 29.2%(±4). **U2OS** CE was 29%(±8.2). **SW1353** CE was 30%(±4), (n=3 SEM).. Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

DNA Extraction

DNA extraction was performed as described in Section 2.2.20 using three different passages and was kept in the fridge at 4°C for future investigations. DNA purity and the absorbance ratio was within the acceptable range, DNA concentrations for SKUT-1 were 87.42, 84.47 and 58.43 ng/µl, and absorbance ratios were 2.03, 1.92 and 2.1 respectively.

Stress Assay

Cell lines were then set up for stress assay as in Figure 2.9. One million cells in each flask for each passage were seeded. Maximum viable cell number (MVCN) was determined after the flasks become fully confluent, media changed and left for 12-24 hours, it was approximately 99% average. Cells then were pictured and monitored on a weekly basis. Cells start to show senescence after the 20th day (Figure 4.7 C and G). Cell lines were then observed until the cells were completely dead after approximately two months (Figure 4.7 D and H). The pictures show packed and rounded dead cells, cell fragments, undefined cell shapes and dead colonies, detached dead cells and

dead floating sheets of cells in a very distinct bright yellow media (consumed) Figure 4.7.

Post-Stress Assay (POST-SA)

After 4 months, cells were washed and re-feed with new media. The wash-off was also cultured in a new flask. Both flasks were kept in culture and observed, some of the floating cells in the wash settled dawn. The viability test showed less than 2% viability from the wash-off. Cells start to grow after only 5 days post-re-feed and produced an average of 121 colonies out of 1 million cells had been seeded. The surviving cells could be seen under dead cells as larger in size compared to the original cells, and eventually grew out from the ghosts of the dead cells as colonies. Based on the formation of colonies that were assumed from the observations as arising from single cells, the percentage of cell survival was around 0.0121%, for all flasks (three original flasks and three wash-off flasks) that had produced colonies Figure 4.8.

Figure 4. 7: Microscopic images (Phase-contrast), showing monitoring of **SKUT-1** a commercial cell line during four months of stress assay (phase three).

Cells shown are at passage 94. **A and E**- Images were captured at x4 and x20 magnification on the 7th-day post-seeding day, showing around 90% confluent, healthy cells and a mixture of fibroblast-like spindle-shaped morphology of most cells. **B and F**- Images were captured at x4 and x20 magnification on the 14th day post seeding day, showing 100 % confluency. **C and G**- Images were captured at x4 and x20 magnification on the 21st day post-seeding day, showing over 100 % confluency and a formation of the second layer of cells. **D and H**- Images were captured at x4 and x20 magnification after few weeks, showing rounded dead cells, cell fragments and undefined cell shapes and colonies. Experiments were done in triplicate on three different passages.

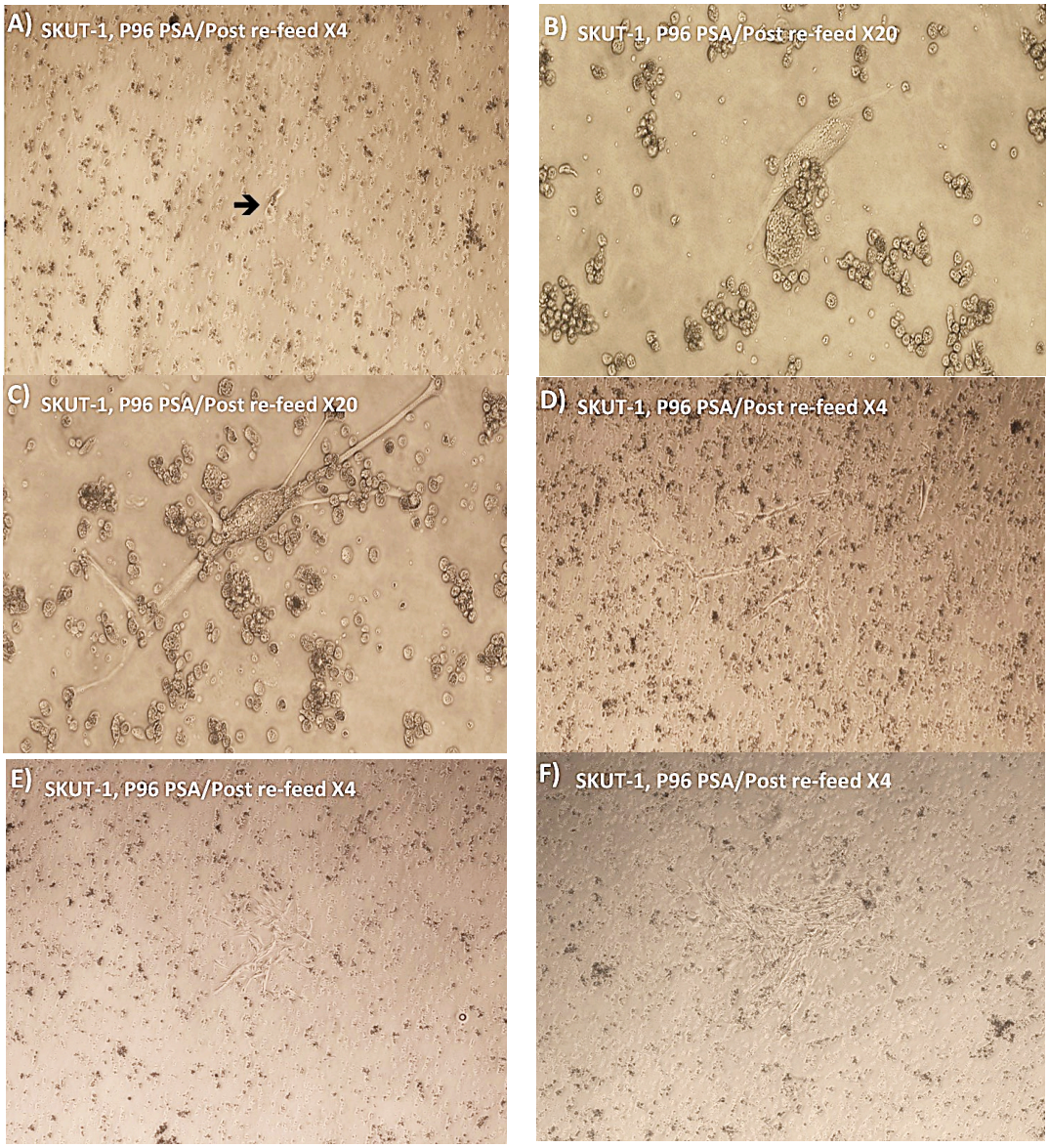


Figure 4. 8: Microscopic images (Phase-contrast), showing monitoring of **SKUT-1 POST-SA** a commercial cell line post four months of stress assay (phase three) POST-SA.

Cells shown are at passage 96. **A and B**- Images were captured at x4 and x20 magnification showing a single fibroblast-like spindle-shaped morphology cell under a layer of dead cells post-re-feed with new media. **C and D**- Images were captured at x20 and x4 magnification after few days of re-feed showing a few cells growing with stretched out protrusions. **E and F**- Images were captured at x4 magnification representing the formation of colonies. Experiments were done in triplicate on three different passages.

4.2.3.3 Other Cell Lines Surviving The Stress Assay: The Sheffield Derived Lines

As mentioned previously the other commercial long term established cell lines did not show colony recovery after the stress assay. The Sheffield more recently derived cell lines however mainly produced recoverable colonies post-stress. Cells in these cell lines grow as a mixture of fibroblast-like spindle-shaped and epithelial morphology Figure 3.11. AS an example, the findings for STS (13/12W2) are presented below.

The STS 13/12W cell line needs to be passaged around every 4-5 days, and cells grow as a mixture of fibroblast-like spindle-shaped and tend to grow in single cells and some small packed colonies with no clear-edges Figure 4.9 B and C. Based on MTT proliferation assay, the doubling time was assessed at 41.87 hours. Figure 4.9.

For the clonogenic assay the number of colonies out of 250, 500, 1000 and 2000 cells seeded were 16(±2.9), 30(±6.4), 54(±15.9) and 75(±13.9), respectively. Clonal efficacy CE based on the clonogenic assay results was 5.3%(±1.2) Figure 4.11 and Figure 4.12. DNA extraction of three different passages was performed and stored for future investigations. DNA purity and the absorbance ratio was within the acceptable range, DNA concentrations were (56.86, 106.21 and 63.91) ng/µl and absorbance ratios were (2.04, 2.13 and 1.96) respectively.

Stress Assay

Cell lines were set up for stress assay as in Figure 2.9. One million cells in each flask for each passage were seeded. The MVCN was determined after the flasks become fully confluent, media changed and left for 12-24 hours; it was approximately 99% confluent on average. Cells were pictured and monitored on a weekly basis and started to show senescence after the 14th day (Figure 4.20 B and F). Cells became senescent and were observed until the entire flask appeared completely dead, that was after approximately two months (Figure 4.10 D and H and Figure 4.10).

Post-Stress Assay (POST-SA)

After 4 months (post-stress assay) cells were washed and re-feed with new media. The wash-off was also cultured in a new flask. Both flasks were kept in culture and observed, some of the floating cells in the wash-off settled down. The viability test was less than 2% viability determined from the wash-off. Cells started to grow after only 15

days post-re-feed and produced an average of 149 colonies out of 1 million cells that had been seeded. The surviving cells were seen under dead cells and they were larger in size compared to the original cells. The percentage of cell survival was around 0.0149%, all the three original flasks had produced colonies, but none from the wash-off flasks Figure 4.13 and Table 4.2.

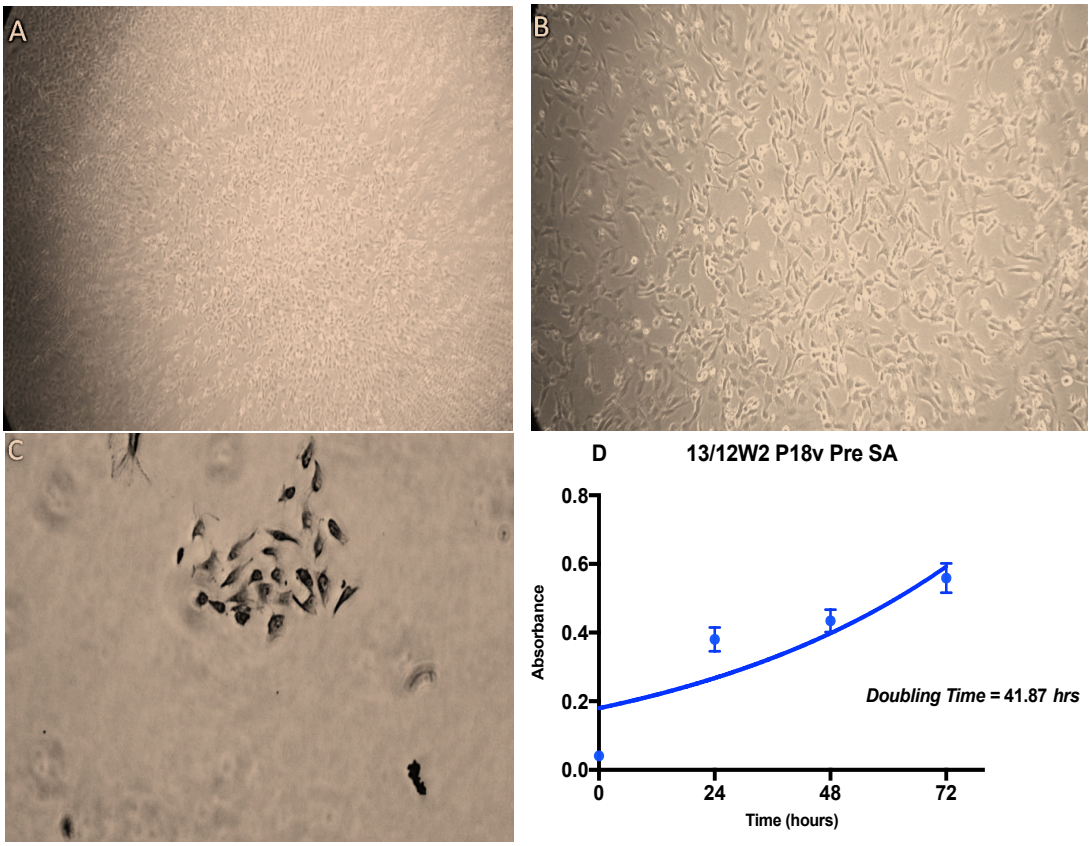


Figure 4. 9: Microscopic images (Phase-contrast) showing **13/12W2** Primary cell line in culture and pre-stress assay cell growth evaluated by MTT proliferation assay.

Cells shown were at passage 14 and the variant (v) was derived from the second wash (W2) of the cell culture grown in the lab from the 13/12 tissue. **A**- Image was captured at x4 magnification showing the pattern of growth in loose colonies. **B**- Image was captured at x10 magnification showing the mixture of fibroblast-like spindle-shaped morphology of most cells. **C**- Image was captured at x10 magnification showing the pattern of growth and colonies shapes. **D**- Cell growth evaluated by MTT proliferation assay at passage 18. Data is representative of experiments done in triplicate on three different passages. Curve fitting was done using Graph Pad® Prism software (version 7) and the doubling time calculated by using the same software and using Exponential Growth Equation as well. N.B For some points, the error bars would be shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars. To see the error bars, make the symbols smaller.

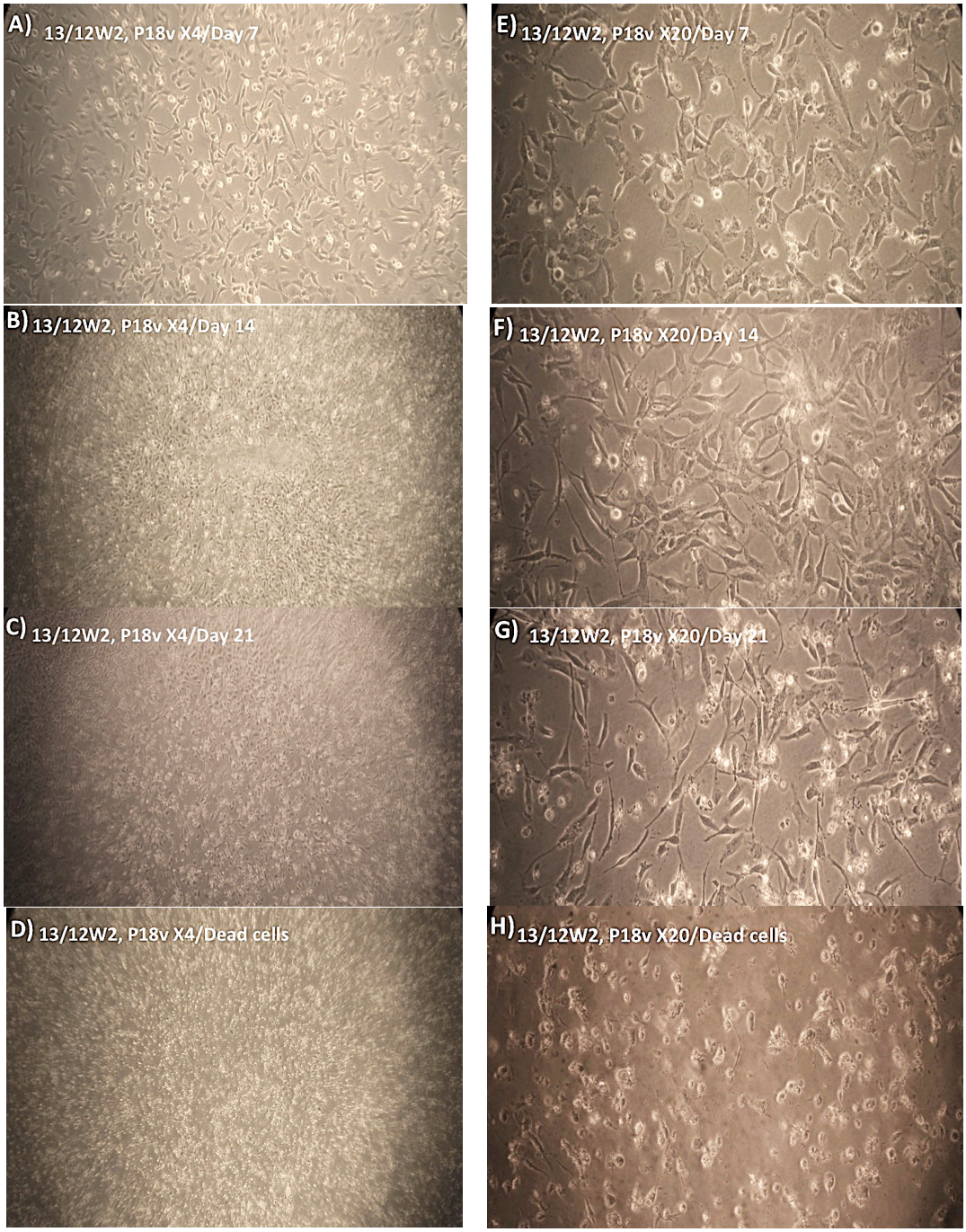


Figure 4. 10: Microscopic images (Phase-contrast), showing monitoring of **13/12W2** primary cell line in culture during the 4 months of the stress assay (phase three).

Cells shown are at passage 18. **A and E**- Images were captured at x4 and x20 magnification on the 7th-day post-seeding day, showing around 50%-60% confluent cells and a mixture of fibroblast-like spindle-shaped morphology of most cells. **B and F**- Images were captured at x4 and x20 magnification on the 14th day post-seeding day, showing 90%-100 % confluency. **C and G**- Images were captured at x4 and x20 magnification on the 21st-day post-seeding day, showing the formation of a second layer of cell and some floating cells. **D and H**- Images were captured at x4 and x20 magnification after a few weeks, showing rounded dead cells, cell fragments and undefined cell shapes and colonies. Experiments were done in triplicate on three different passages.

The ability of all cell lines to form colonies post stress, including the established SKUT-1 line, are presented in Table 4.2. below.

Cell line POST-SA	Forming Colonies Duration after re-feed	Average of Colonies No. Formation after re-feed	No. of cells were seeded Pre-Stress Assay	Percentage of Survived cells %	No. of Flasks Survive the SA	No. of Wash off Flasks growing cells (Floating cells)
SKUT-1 (Leiomyosarcoma)	5 days	121 colonies	1,000,000 cells	0.0121	3 Flasks	3 Flasks
02/11 W1 (Leiomyosarcoma)	15 Days	140 colonies	1,000,000 cells	0.014	3 Flasks	3 Flasks
13/12W2 (Pleomorphic NOS sarcoma)	15 Days	149 colonies	1,000,000 cells	0.0149	3 Flasks	0
14/10 (Pleomorphic NOS sarcoma)	15 Days	13 colonies	1,000,000 cells	0.0013	1 Flask	0
09/10 (Dedifferentiated Liposarcoma)	30 Days	72 colonies	1,000,000 cells	0.0072	1 Flask	0
21/11W2 (Myxofibrosarcoma)	37 Days	3 colonies	1,000,000 cells	0.0003	3 Flasks	0
02/11 W1 (POST-SA2) (Leiomyosarcoma)	15 Days	103 colonies	1,000,000 cells	0.0103	3 Flasks	3 Flasks
06/11WS (Pleomorphic NOS Sarcoma)	No Survival	No Survival	1,000,000 cells	-	No Survival	No Survival

Table 4. 2: The percentage of colonies formed post stress assay from each line that recovered.

The period taken for each cell line to form colonies after being re-feed with new media is shown, along with the average colonies calculated, as a percentage of the original number of cells initially seeded. Experiments were done in triplicate on three different passages.

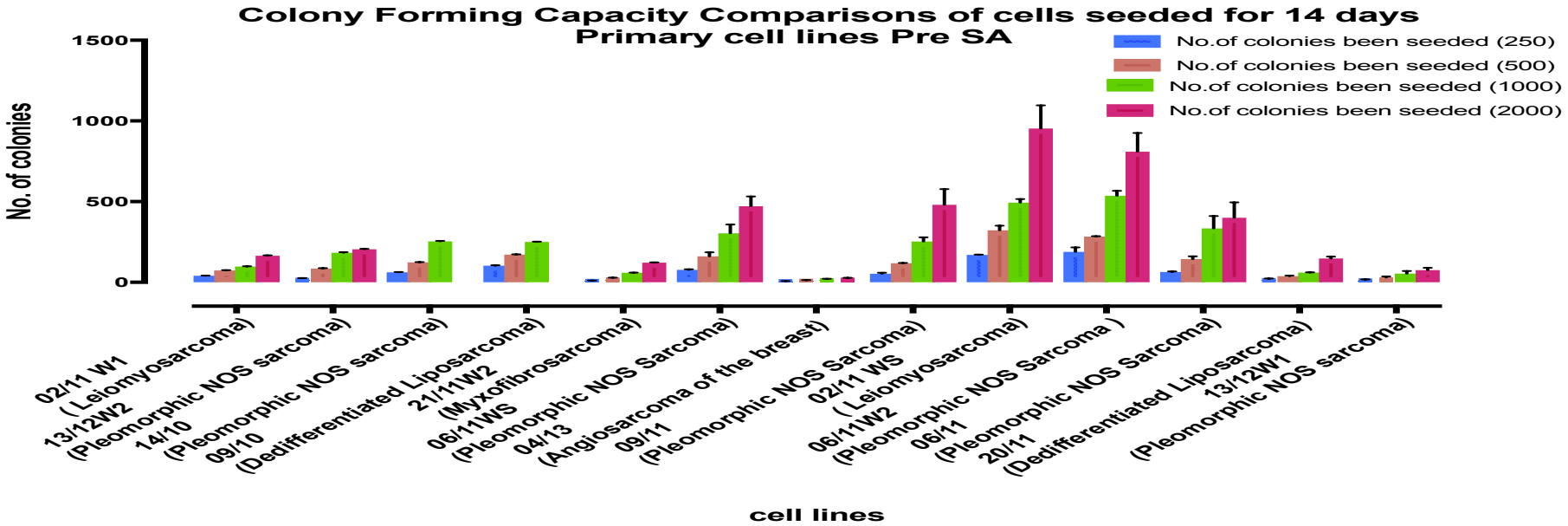


Figure 4. 11: A bar chart representing a comparison of the colony-forming capacity of Sheffield derived cell lines seeded for 14 days pre-stress assay at different densities.

Cell lines were seeded at different densities (250, 500, 1000, and 2000)/cells per Petri dishes. 02/11W1 Number of colonies out of 250, 500, 1000, 2000 cells seeded were 40.7(± 1.2), 73 (±2.4), 98(±1.2) and 164(±2.6) respectively. 13/12W2 was 24(±2.3), 85(±2.7), 184(±2.7) and 204(±2.6) colonies. 14/10 was 62(±1.5), 123(±2.4) and 253(±2.3) colonies. 09/10 was 103(±2), 171(±1.9) and 250(±1.7) colonies. 21/11W2 was 10(±0.8), 28(±1.2), 59(±1.2) and 122(±1.2) colonies. 06/11WS was 77(±3.6), 161(±24.9), 304(±54) and 472(±60) colonies. 04/13 was 7(±0.8), 12(±1.5), 20(±2.4) and 27(±1.9) colonies. 09/11 was 53(±6.6), 118(±1.5), 253(±26.3) and 480(±97.5) colonies. 02/11WS was 170(±1.5), 323(±28.4), 494(±22) and 953(±144) colonies. 06/11W2 was 189(±27), 283(±2.6), 536(±29.9) and 809(±116) colonies. 06/11 was 64(±3.8), 144(±17), 334(±76) and 400(±95) colonies. 20/11 was 21(±3.2), 38(±3.5), 60(±2) and 147(±11.9) colonies.13/12W1 was 16(±2.9), 30(±6.4), 54(±15.9) and 75(±13.9) colonies (n=3 SEM). Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software. (14/10 and 09/10 cell lines) 2000 cells seeded dish could not be counted due to the overgrowth.

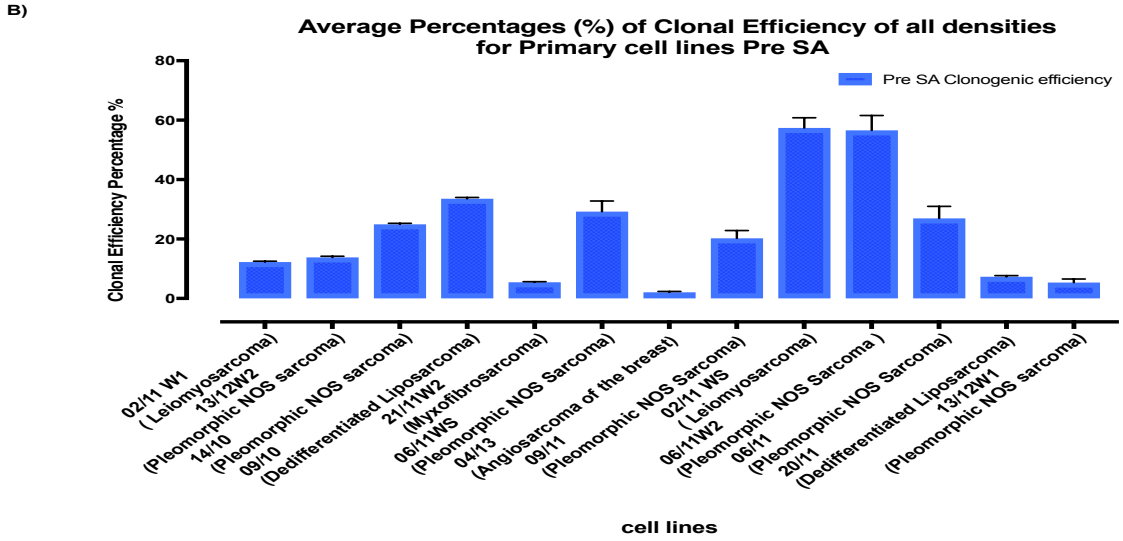


Figure 4. 12: A bar chart representing the comparison of average percentages for clonal efficiency **(CE)** of all densities for different primary cell lines prior to stress assay.

Cell lines were seeded in different densities (250, 500, 1000, and 2000) cells per Petri dishes. **02/11W1** CE was 12.2%(± 0.3). **13/12W2** CE was 13.8%(±0.4). **14/10** CE was 25%(±0.3). **09/10** CE was 33.5%(±0.4). **21/11W2** CE was 5%(±0.1). **06/11WS** CE was 29%(±3.6). **04/13** CE was 2%(±0.3). **09/11** CE was 20.3%(±2.6). **02/11WS** CE was 57.4%(±3.4). **06/11W2** CE was 56.6%(±5). **06/11** was 26.9%(±4). **20/11** CE was 7%(±0.3). **13/12W1** CE was 5.3%(±1.2) (n=3 SEM). Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software. (14/10 and 09/10 cell lines) 2000 cells seeded dish could not be counted due to the overgrowth).

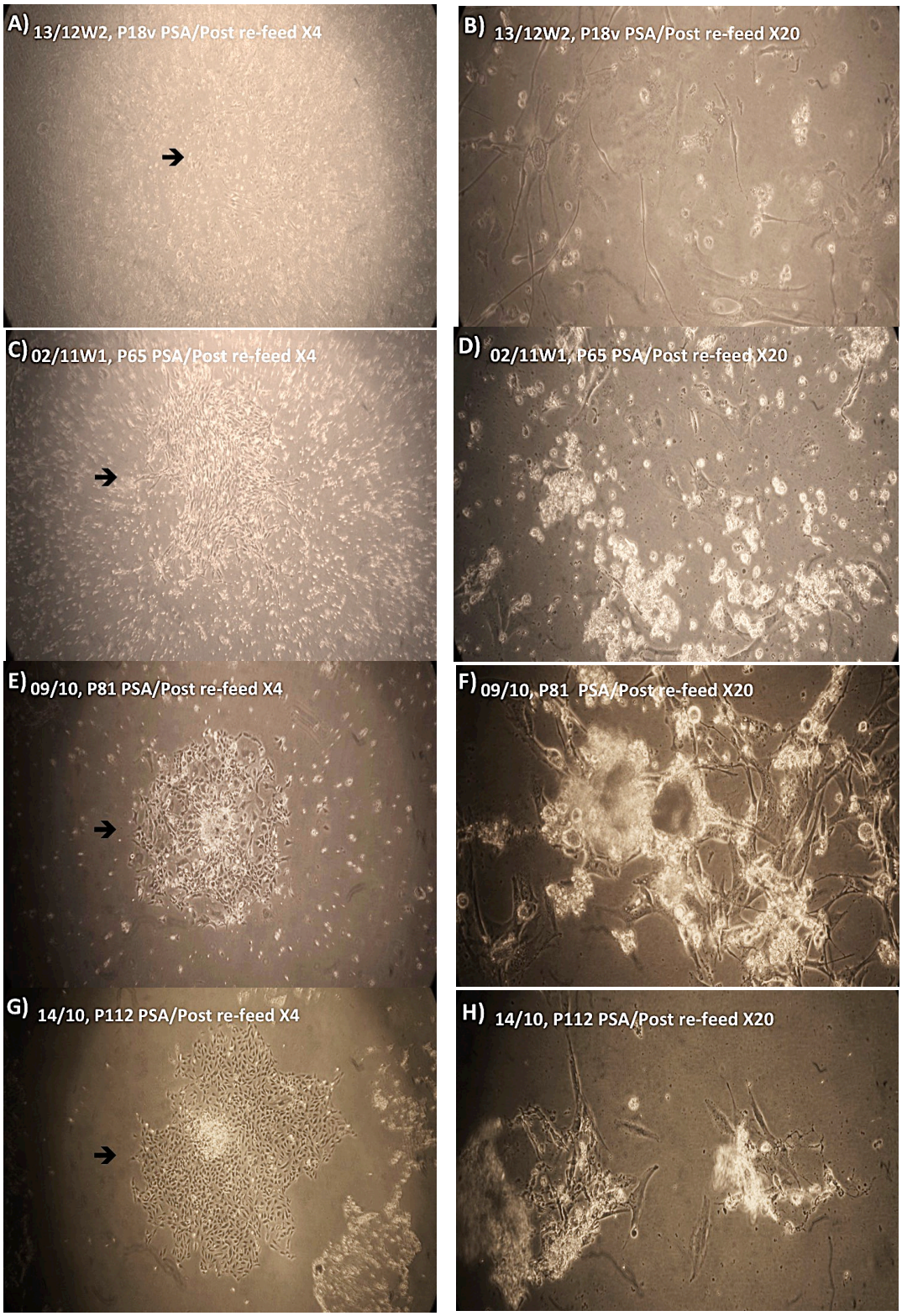


Figure 4. 13: Microscopic images (Phase-contrast), showing monitoring of **Sheffield derived Primary cell lines POST-SA** in culture post four months stress assay (phase three) POST-SA.

A and B - 13/12 W2, P18 POST-SA, C and D- 02/11W1, P65 POST-SA, E and F- 09/10, P81 POST-SA and E and F- 14/10, P112 POST-SA, Images were captured at x4 and x20 magnification for each cell line, and shows fibroblast-like spindle-shaped morphology cells emerging and growing under a layer of dead cells in the post-re-fed flasks. Experiments were done in triplicate on three different passages.

4.3 DISCUSSION

In this study sarcoma cell lines were set up in a stress assay, a method developed to challenge cell survival and enriched for possible CSC, and based on the requirement for tumours cells to have nutrients (Ratajczak et al., 2018, Skoda and Veselska, 2018). Various amendments were made to the procedure; and there were also problems arising due to contamination with the need to keep cultures for the extended period of the assay. Despite the harsh conditions of starvation for up to four months, six cell lines had cells that retained the capacity to survive and were able to give rise to colonies. The number of surviving cells that produced colonies were between (3-149), and the duration after being re-fed was between (15-37) days for the Sheffield primary cell lines and 5 days for the only established cell line (SKUT-1).

The established cell lines, having been derived mainly in the 1970s had comparable clonal efficiencies (27% - 30%) CE (Figure 4.6), whereas the Sheffield primary cell lines were more variable (2%-57%) CE (Table 4.2, Figure 4.12) There was an association between higher passage number and an increased colony-forming abilities of these cell lines, possibly reflecting becoming more adapted to the culture. The ability to form colonies pre-stress was not however related to the ability to produce colonies after starvation since only one of the established commercial cell lines managed to retain cells post-stress. As the assay was only developed to it full in the final phase, it is possible that cell survival may not have been accurately determined in all cases, and potentially explains why SKUT-1 alone amongst the commercial lines was capable of producing colonies post-stress. The SKUT-1 cell line had around the same doubling time as the same commercial established lines used in this study, so it is unlikely that there were any differences due to the depletion of media. Indeed, the control lines (PC3 and HTERT) also had comparable doubling times (data not shown). In addition, the ability to proliferate, and the potential to use up nutrients does not appear to relate to the potential to have cell populations capable of surviving post-stress.

It is certain that the Sheffield derived cell lines, in terms of length of time since establishment, were newly derived. It is possible that the commercial cell line SKUT -1 that was capable of forming colonies post-stress was actually from stocks that were more newly derived, i.e. lower passages. On the whole, the passages for the established lines were all around 100 or above, but it cannot be assumed that the passage numbers are as relatable as those from the Sheffield derived lines. Certainly,

there is clear evidence of cell drift following extended and prolonged culture, and also adaption to different cultural environments that may exist between laboratories (Ben-David et al., 2018).

It is therefore possible that any CSC population within cell lines will become effectively diluted to levels far lower than capable of detection in this study. These findings presented here, however, suggest that within some sarcoma cell lines a small subpopulation retain the ability to recover from starvation, and could as an approximation be responsible for relapse post-treatment. Certainly, the levels found in this study capable of producing colonies (ranging from 3 – 149 per million (Table 4.2), are comparable to the incidence reported in some studies for CSC, but lower than others, and may represent dilution following culture (Tirino et al., 2011, Malanchi et al., 2012a, , Veselska et al., 2012). The ability of sarcomas to recover post stress was not subtype specific. It was of interest that all the UPS (NOS) parental cell lines were all capable of recovery, but not all variants. These sarcomas are inherently the most dedifferentiated and potentially stem cell-like, and it is possible that the variants became differentiated in such a manner as to lose this stem cell like characteristic (F. Farshadpour and R. Otter 2005).

Although the findings presented here need to be validated to confirm the importance of using a stress assay as a method to identify CSC populations in sarcomas, the findings do provide evidence for functional characteristics that are associated with CSC, namely the ability to self renew (Ratajczak et al., 2018, Skoda and Veselska, 2018). Certainly the findings were more encouraging than the use of markers alone. In order to understand a little more about these populations, in the next chapter, an in-depth comparison between pre and post-stress assay for the cell lines that survive was undertaken.

4.4 SUMMARY

In this chapter sarcoma cell lines were set up for the stress assay, a method developed to challenge cell survival and enrich for possible CSC. The methodology was amended during the course of the study, and refined to more accurately establish the timings required to effectively stress the cell lines. Increased monitoring and photography was undertaken to image the key points, and additional tests for changes in behavior such as colony forming ability were included. Prior to the stress assay, cell lines were documented by pictures, MTT, DNA extraction and clonogenic assay. Only six cell

lines were able to survive the extended period of nutrient depletion (four months) and give rise to colonies. The number of cells to give rise to colonies was between 3-149, and the duration after refeeding to give rise to colonies was between 15-37days for the Sheffield primary cell lines and only five days for the established cell line. Colony forming ability for the established cell lines were almost the same, between 27% - 30% CE, while the Sheffield derived cell lines were more variable 2%-57% CE. As the passage number increased, a correlation with an increased CE was observed. As cell lines become more adapted to culture there will be drift which could complicate the ability to detect such cells in long established cell lines as was found here.

These findings suggest that a subpopulation within tumour cell lines could be responsible for the evolution of the lines in culture as they were capable of recovery in a manner akin to tumour relapse, and that these cells may possess the characteristics of CSC. These findings need to be validated to confirm the importance of using the stress assay as a method for identifying CSC in sarcomas. In order to understand this in more depth, in the next chapter comparisons were made between the pre and post-stress populations of the cell lines to investigate the changes that might happen. Given the findings of this study the question arises as to whether these cells hold CSC characteristics or not and how they escape apoptosis? Also, based on the observations in this chapter of the larger cell morphology of recovering cells, the gating strategy for FACS was refined to potentially target any CSC subpopulation, since the use of markers by gating all cells had not been successful as discussed in chapter 3.

CHAPTER FIVE

COMPARISON OF PRE AND POST STRESS SURVIVAL CELL POPULATIONS FOR ENRICHMENT OF CANCER STEM CELLS

5.1 INTRODUCTION

Although there have been many advances made in the development of cancer therapies and diagnosis, more advances are required and the improvements have not been equal, with some cancers, such as sarcomas still lagging behind and therapeutic options limited (Van Hoeck et al., 2019). Methods to identify and then eradicate CSC offer new hope for many cancers, but for sarcomas with so many subtypes this hope may be elusive (Ray-Coquard and Le Cesne, 2012, Skoda and Veselska, 2018). A common method that may help in the identification of CSC relies on the use of potential markers for stemness specifically and looking for distinct subpopulations that possibly contain CSC. Hematopoietic adults stem cell and non-hematopoietic cancer both shared expression of CD133 (Miraglia et al., 1997, Yin et al., 1997, Sana et al., 2011). Also, cells with CD71 and CD44 positive expression are more likely to possess adhesive and metastatic characteristics (Zoller, 2011, Ohkuma et al., 2012). Despite co-expression of these potential CSC markers isolating this rare population is still problematic due to cell heterogeneity and sample quality (Yang et al., 2014b, Gokhale et al., 2015, Lee, 2018), and when studying cell lines it has been reported that prolonged culturing of cells has a direct effect and changes the characteristics of the cells. (Engelholm et al., 1985, Vonhoff, 1988, Fridborg et al., 1999, Kim et al., 2019).

In this chapter, cell lines from different types of sarcoma that survived the stress assay (see chapter four section 4.2 assay development and chapter two section 2.2.18), were investigated to see if the surviving colonies capable of regrowth had differences to the parental population and had been enriched for markers of CSC; either of single markers or combinations (co-expression). Comparison of the pre and post-stress assay populations was undertaken, see sections 2.2.5-2.2.19 for more details. Initially, proliferation by MTT assay was performed, followed by clonogenicity assays and aCGH. In this chapter, the detection methods for marker characterization were changed to include different FACS gating strategies and was based on the results of chapter 3 (Figure 2.6 see section 2.2.8). Linear regression was performed to detect possible correlations and finally a cell hierarchy assessment was undertaken based on the results expressions. Furthermore, two sarcoma subpopulations expressing different markers were specifically selected and studied for their ability to form colonies, as a measure of the capacity for self-renewal. Also, a limited side flow cytometry experiment (pilot study) was carried out to study possible new markers for sarcoma. An overview, of the workflow is shown in Figure 5.1.

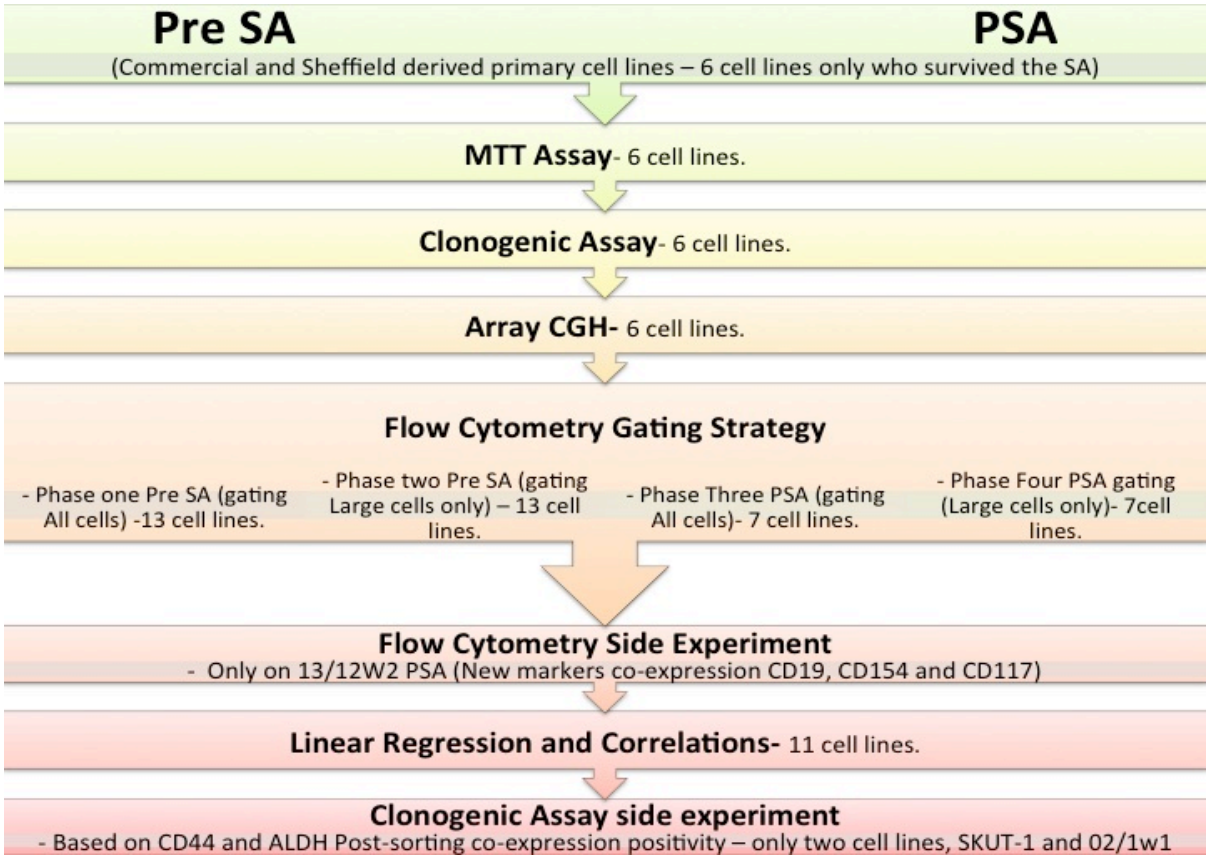


Figure 5. 1: An overview of Chapter 5 workflow, summarizing the methodology used to assess the differences between Pre-SA and POST-SA in this study.

Initially, commercial and Sheffield derived cell lines that survived the stress assay were set up for comparison testing between pre-SA and Post-SA at the same passages number. These populations were studied by MTT, clonogenicity, array CGH, four flow cytometry gating strategies, followed by a side experiment on only one cell line (13/12W2, due to the time limit). Linear regression was undertaken to investigate if there was any correlation between CD's marker expressions and the different gating for pre and post-stress assay populations. Finally, limited clonogenicity assay was carried out on two cell lines to assess pre and Post-SA differences sorting of two monoclonal antibodies (CD44 and ALDH).

5.2 RESULTS

5.2.1 MTT (Pre-SA vs Post Stress Assay POST-SA)

In this section, MTT proliferation (cell growth and doubling time) was assessed only on cell lines that survived the stress assay, a comparison between pre-SA and post-stress assay (POST-SA) results were then compared. All cell lines were cultured, maintained, harvested and re-cultured in the same conditions as mentioned in section 2.2.3.1 and 2.2.17. Stress assay (phase three) was performed on the selected cell lines for four months without changing the media after seeding 1 $x10^6$ as described in section 2.2.18.3. The number of cells seeded in each flask (T75) for cell maintenances and culturing was between 0.3 - 1 $x10^6$ prior to MTT assay, for both pre and post-stress assay (section 2.2.18). In the MTT assay 50,000 cells in each well were added, and 100µl of DMSO was added as a fixative in the final step before reading see section 2.2.17. The MTT results for the pre stress assay populations were obtained prior to the stress assay phase three setup, as mentioned in the protocol in chapter two and in chapter four. Each cell line was treated in triplicate for three different MTT assays; the results presented were an average of three individual experiments out of three runs (n=3). The same passage number, or as close to, was used for the pre and post stress assay populations, with pre stress populations analysed whilst the stress assay was underway. The post stress assay populations were then passaged to the same number for comparison, rather than freezing and thawing the pre stress populations, which may recover different populations. Due to the enormous amount of graphs for all cell lines, only SKUT-1 detailed graphs were presented below as an example.

Cell line	Pre-SA Doubling time In hours	POST-SA Doubling time In hours	Absorbtion Incubation period (Significance)
SKUT-1	38.73	42.95	(Higher POST-SA DT) 24, 48 and 72
13/12W2	41.87	56.17	(Higher POST-SA DT) 24, 48 and 72
09/10	40.15	42.62	(Higher POST-SA DT) 24, 48 and 72
14/10	47.02	51.82	(Higher POST-SA) 24, 48 and 72
02/11W1	37.86	46.55	(Higher POST-SA DT) 24, 48 and 72
21/11W2	62.97	52.54	24, 48 and 72

Table 5. 1: Comparison between Pre and Post-Stress Assay (POST-SA), cell growth (doubling time) by MTT Proliferation Assay for Established and Sheffield derived cell lines.

Data is representative of experiments done in triplicate on three different passages (n=3). The analysis was done using Graph Pad® Prism software (version 7) and the doubling time calculated by using the same software and using Exponential Growth Equation as well.

5.2.1.1 SKUT-1 (Human Uterus Leiomyosarcoma (GIII))

Based on the MTT proliferation assay, the doubling time was assessed at 38.73 hours for pre-SA, section 4.2.3.2 and 42.95 hours for POST-SA. Analysis of the t-test showed the P-value was < 0.05 in 24hours and 72 hours, indicating slower proliferation and longer doubling time in the POST-SA Figure 5.2, Figure 5.3 and Table 5.1.

5.2.1.2 STS (13/12W2) Pleomorphic NOS Sarcoma

The doubling time was at 41.87 hours for pre-SA, as showed in section 4.2.3.3 and 56.17 hours for POST-SA. The data demonstrated a clear difference in the doubling time measured between pre-SA and POST-SA. Analysis of the t-test showed P-value was < 0.05 in 24hours and 72 hours, indicating slower proliferation and longer doubling time in the POST-SA Figure 5.4 and Table 5.1.

5.2.1.3 STS (09/10) Dedifferentiated Sarcoma

The doubling was 40.15 hours for pre-SA and 42.62 hours for POST-SA (MTT data analysis not shown). According to the t-test, analysis data demonstrated no significant difference in the doubling time measured between pre-SA and POST-SA. P-value was > 0.05 in all Figure 5.4 and Table 5.1.

5.2.1.4 STS (14/10) Pleomorphic NOS Sarcoma

The doubling time was 47.02 hours for pre-SA and 51.82 hours for POST-SA. The data demonstrated a clear difference in the doubling time measured between pre-SA and POST-SA (MTT data analysis not shown). Analysis of the t-test showed P-value was < 0.05 in 24hours and 48 hours, showing slower proliferation and longer doubling time in the POST-SA Figure 5.4 and Table 5.1.

5.2.1.5 STS (02/11W1) Leiomyosarcoma

The doubling time was 37.86 hours for pre-SA and 46.55 hours for POST-SA. The data demonstrated a clear difference in the doubling time measured between pre-SA and POST-SA (MTT data analysis not shown). Analysis of the t-test showed P-value was < 0.05 in 48 hours and 72 hours, showing slower proliferation and increased doubling time for the POST-SA. Also, the 02/11W1 POST-SA2 cell line was included in this study and the P-value was < 0.05 in all readings (24, 48 and 72) Figure 5.4 and Table 5.1.

5.2.1.6 STS (21/11W2) Myxofibrosarcoma

The doubling time was 62.97 hours for pre-SA and 52.54 hours for POST-SA (MTT data analysis not shown). According to the t-test analysis, data showed no significant difference in the doubling time measured between pre-SA and POST-SA. P-value was > 0.05 in all Figure 5.4 and Table 5.1.

In conclusion, all the pre-stress cell lines being tested for MTT had a higher proliferation compared to the post-stress except for on cell line (21/11W2) that showed no significant proliferation results between pre and post-stress Table 5.1 and Figure 5.2. However, this slight elevation in the proliferation results for pre-stress has to be treated with care as the MTT assays were not performed at the same time so experimental differences may have caused the variations seen in pre and post stress results. The rationale however was that recovering cells from frozen to more directly compare at the same time point would potentially introduce another variable. If time permitted a fuller comparison would be to compare analysis pre stress assay, and then recover stocks and perform analysis of the post stress assay population as well as the pre stress population at the same time using the same passages. The need to repeat these outcomes is necessity to validate these changes on the same cell lines and on different cell lines.

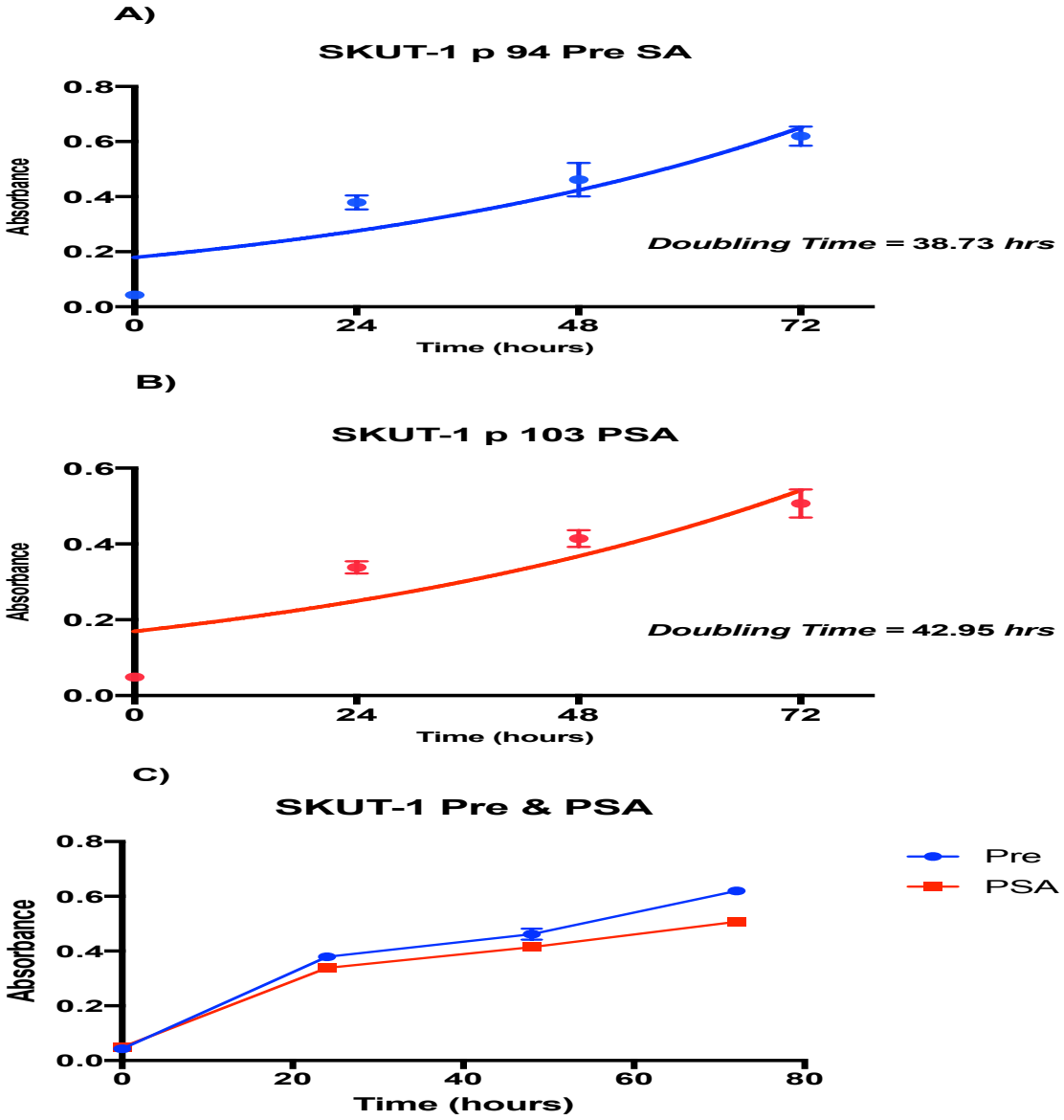


Figure 5. 2: SKUT-1 Comparison of the growth of SKUT-1 established cell line Pre and Post-Stress Assay, evaluated by the MTT Proliferation Assay.

A) Pre-Stress Assay. **B)** Post-Stress Assay. **C)** Pre and Post-Stress Assay. Cells were at passage 94 and 103 in pre and post, respectively. Data is representative of experiments done in triplicate on three different passages (N=3) (passage range 94-103). Curve fitting was done using Graph Pad® Prism software (version 7) and the doubling time calculated by using the same software and using Exponential Growth Equation as well.

N.B For some points, the error bars would be shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars. To see the error bars, make the symbols smaller.

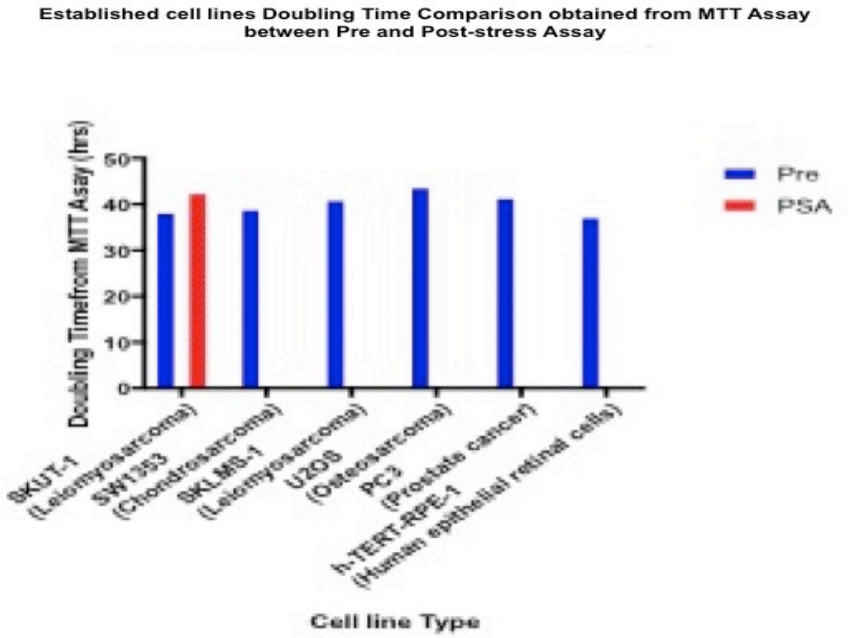


Figure 5. 3: Established cell lines doubling time comparison between Pre and Post-Stress Assay, and Cell Growth, evaluated by MTT Proliferation Assay previously.

Cells were passaged as mentioned before in the previous MTT analysis (chapter 4). Data is representative of experiments done in triplicate on three different passages (N=3). The analysis was done using Graph Pad® Prism software (version 7) and the doubling time calculated by using the same software and using Exponential Growth Equation as well.

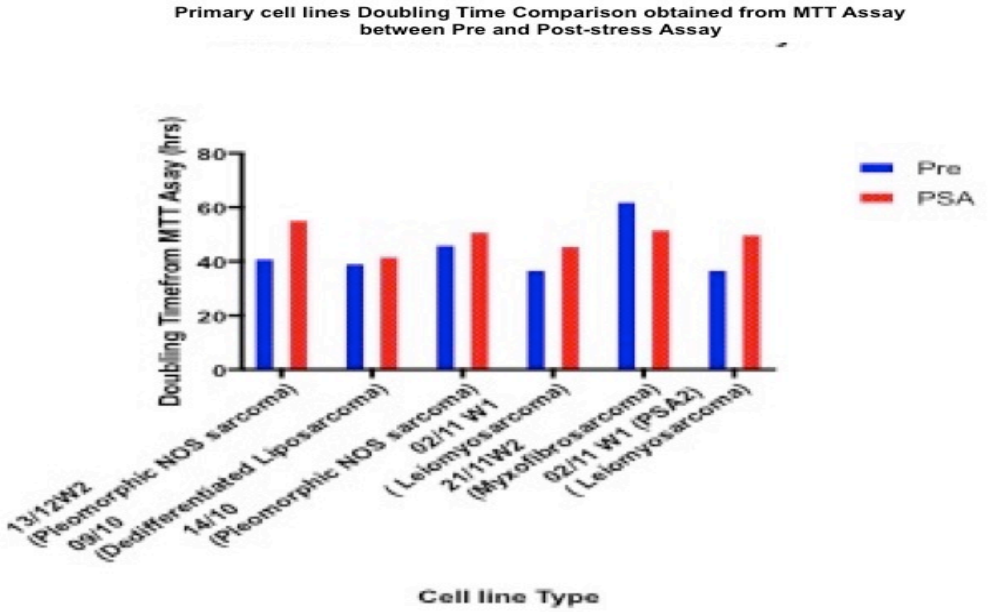


Figure 5. 4: Comparison of Sheffield derived cell lines doubling time between Pre and Post-Stress Assay, Cell Growth evaluated by MTT Proliferation Assay previously.

Cells were passaged as mentioned before in the previous MTT analysis. Data is representative of experiments done in triplicate on three different passages (N=3). The analysis was done using Graph Pad® Prism software (version 7) and the doubling time calculated by using the same software and using Exponential Growth Equation as well.

5.2.2 Clonogenic Assay (Pre vs. Post Stress Assay)

The clonogenic assay (see section 2.2.6) was performed only on cell lines that survived the stress assay (section 4.2.3). Colonies and clonal efficiencies were counted on three independent experiments for each cell lines and an average was calculated for each result. A comparison then was made between the pre-SA and POST-SA for the cell lines to determine any changes that might occur. The results indicated that both pre and PS assay produced colonies from single cells. However, the post-stress assay appeared to show a reduction with fewer colonies produced, and this was reflected in the clonal efficiency percentages. The differences were more apparent for the Sheffield derived cell lines, when comparing the preliminary results of clonogenic assay (chapter three section 3.2.5) with these results, and cell lines with lower passage numbers tend to have lower clonal efficiency.

5.2.2.1 Clonogenic Assay for SKUT-1 POST-SA (Human Uterus Leiomyosarcoma (GIII))

The number of colonies out of 250, 500, 1000 and 2000 cells seeded were 54.7(± 2.4), 73.7 (±2.2), 126.7(±2) and 263.7(±2.3) respectively. Clonal efficacy CE based on the clonogenic assay colony results was 15.61% (± 0.341) SEM for POST-SA. Figure 5.5, Figure 5.6, Figure 5.7 and Figure 5.8 show colonies number differences and CE percentages between Pre-SA and POST-SA for this cell line in-group and individually.

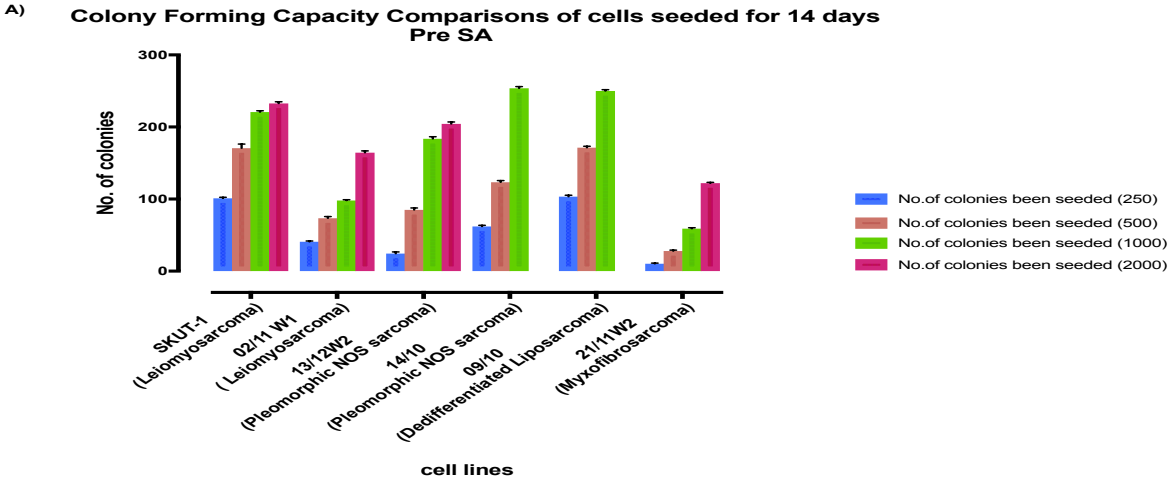


Figure 5. 5: A): A bar chart representing the comparison of the colony-forming capacity of cells seeded for 14 days pre-stress assay in different densities for established and Sheffield derived cell lines.

The numbers of colonies shown were for commercial cell line (SKUT-1) and some selected primary cell lines Pre-SA. Cell lines were seeded in different densities (250, 500, 1000, and 2000) cells per Petri dishes. **SKUT-1** Number of colonies out of 250, 500, 1000 and 2000 cells seeded were 101(± 1.53), 170 (±5.6), 220.7(±1.7) and 232.7(±2.3) respectively, (N=3). **02/11W1** was 40.7(± 1.2), 73 (±2.4), 98(±1.2) and 164(±2.6) respectively, (N=3). **13/12W2** was 24(±2.3), 85(±2.7), 184(±2.7) and 204(±2.6) colonies respectively, (N=3). **14/10** was 62(±1.5), 123(±2.4) and 253(±2.3) colonies respectively, (N=3). **09/10** was 103(±2), 171(±1.9) and 250(±1.7) colonies respectively, (N=3). **21/11W2** was 10(±0.8), 28(±1.2), 59(±1.2) and 122(±1.2) colonies respectively, (N=3). N=3 SEM for all cell lines been tested. Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

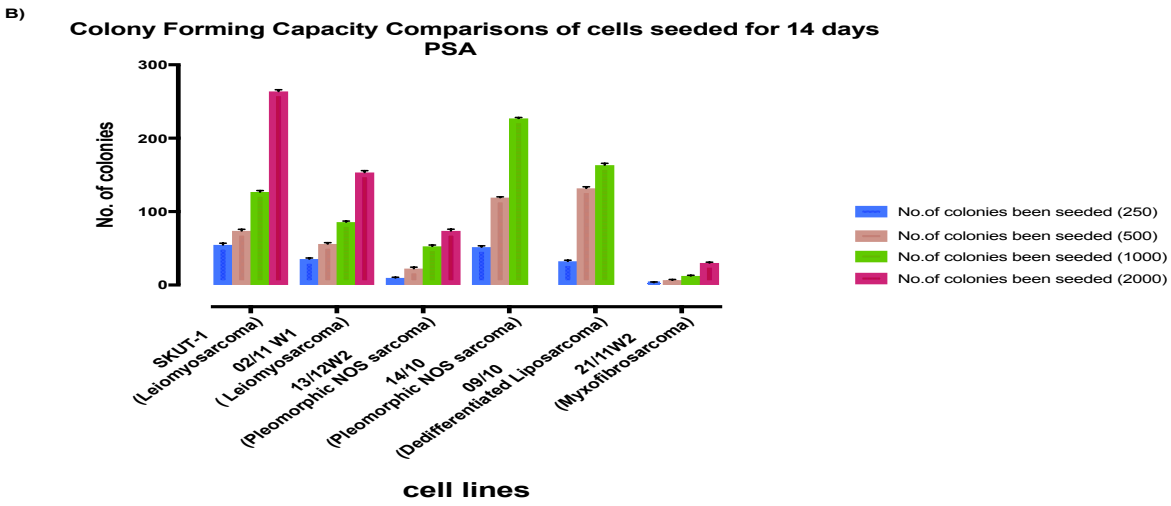


Figure 5. 6: B): A bar chart representing the comparison of the colony-forming capacity of cells seeded for 14 days post-stress assay at different densities for the established line and Sheffield derived cell lines.

The numbers of colonies shown were for one commercial cell line and some selected primary cell lines Post-SA. Cell lines were seeded in different densities (250, 500, 1000, and 2000) cells per Petri dishes. **SKUT-1** Number of colonies out of 250, 500, 1000 and 2000 cells seeded were 54.7(± 2.4), 73.7 (±2.2), 126.7(±2) and 263.7(±2.3) respectively, (N=3). **02/11W1** was 35(± 1.5), 56 (±1.7), 85.7(±1.5) and 153(±2.4) respectively, (N=3). **13/12W2** was 9.7(±0.8), 22(±2), 52(±1.8) and 73.7(±2) colonies respectively, (N=3). **14/10** was 51.7(±1.7), 119(±1.2) and 227(±1.2) colonies respectively, (n=3). **09/10** was 32(±1.5), 132(±2.2) and 163(±2.4) colonies respectively, (N=3). **21/11W2** was 3(±0.9), 7(±0.8), 12(±0.8) and 30(±1.2) colonies respectively, (N=3). N=3 SEM for all cell lines tested. Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7). N.B (14/10 and 09/10 cell lines at 2000 cells seeded Petri dish could not be counted due to the overgrowth.

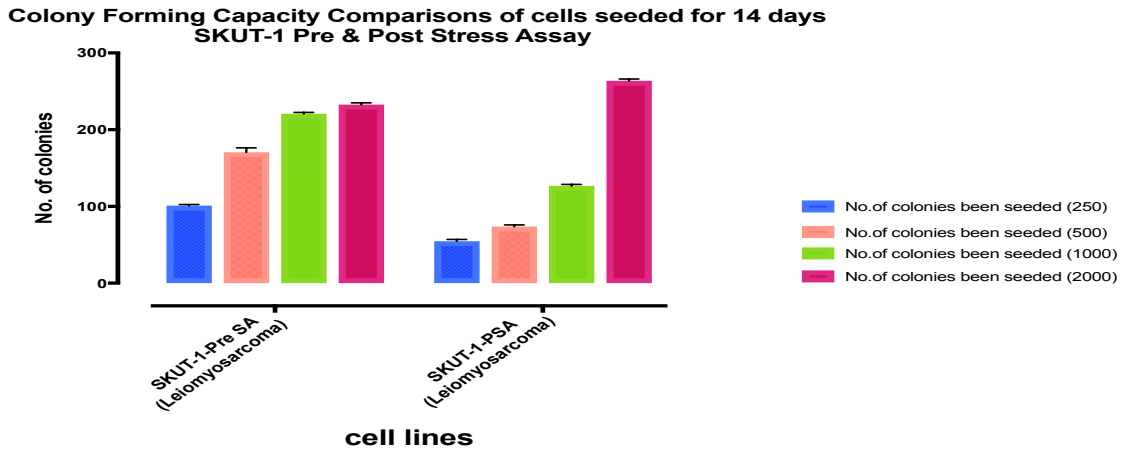


Figure 5. 7: A bar chart representing the comparison of the colony-forming capacity of cells seeded for 14 days pre and post-stress assay at different densities for **SKUT-1.**

Cell lines were seeded in different densities (250, 500, 1000, and 2000) cells per Petri dishes for both. **SKUT-1 Pre-SA** Number of colonies out of 250, 500, 1000 and 2000 cells seeded were 101(± 1.53), 170 (±5.6), 220.7(±1.7) and 232.7(±2.3), respectively, (N=3). **SKUT-1 POST-SA** colonies were 54.7(± 2.4), 73.7 (±2.2), 126.7(±2) and 263.7(±2.3), respectively, (N=3). Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

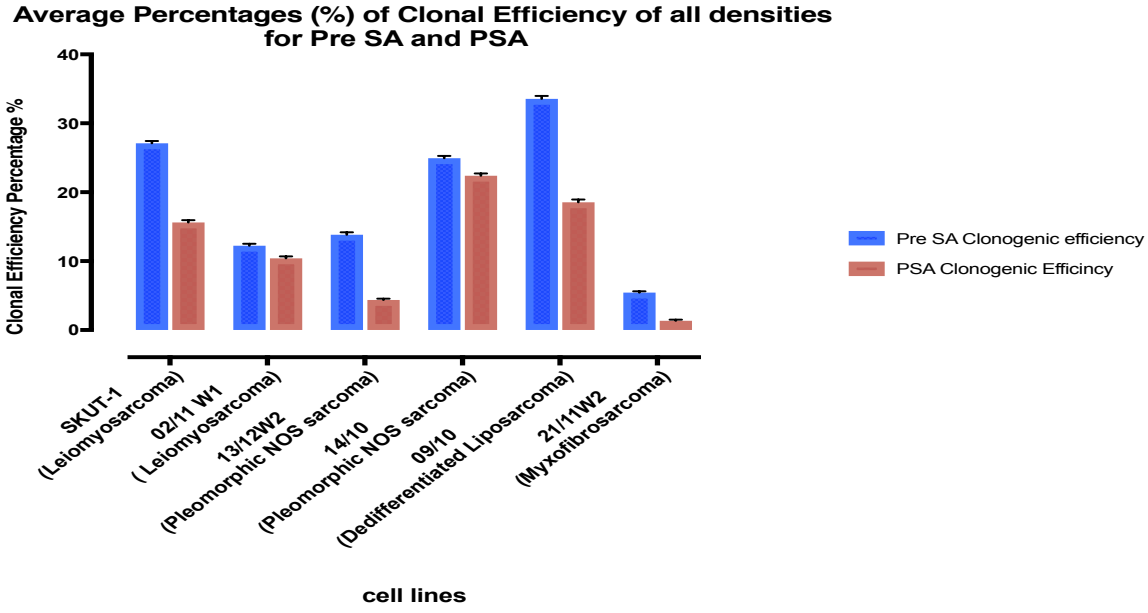


Figure 5. 8: A bar chart representing the comparison of average percentages for the clonal efficiency **(CE)** of all densities for the different cell lines that survived the stress assay **(commercial and Sheffield derived)**.

Cell lines were seeded in different densities (250, 500, 1000, and 2000) cells per petri dishes. **SKUT-1 Pre-SA** CE was 27.15% (± 0.32) (N=3) and **POST-SA** CE was 15.61% (± 0.341) (N=3). **02/11W1 Pre-SA** CE was 12.2%(± 0.3) (N=3) and **POST-SA** CE was 10.39% (± 0.298) (N=3). **13/12W2 Pre-SA** CE was 13.8%(±0.4) (N=3) and **POST-SA** CE was 4.373% (± 0.169) (N=3). **14/10 Pre-SA** CE was 25%(±0.3) (N=3) and **POST-SA** CE was 22.4%(± 0.33) (N=3). **09/10 Pre-SA** CE was 33.5%(±0.4) (N=3) and **POST-SA** CE was 18.5% (± 0.41) (N=3). **21/11W2 Pre-SA** CE was 5%(±0.1) (N=3) and **POST-SA** CE was 1.4% (± 0.17) (N=3). N=3 SEM for all cell lines tested. Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

5.2.2.2 Clonogenic Assay for STS (13/12W2 POST-SA) Pleomorphic NOS Sarcoma

For 13/12W2 POST-SA cell line the number of colonies out of 250, 500, 1000 and 2000 cells seeded were 9.7(±0.8), 22(±2), 52(±1.8) and 73.7(±2) colonies respectively. CE based on the clonogenic assay colonies results was 4.373% (± 0.169) SEM. Figures 5.5, 5.6, 5.8 and 5.9 show colony number differences and CE percentages between Pre-SA and POST-SA.

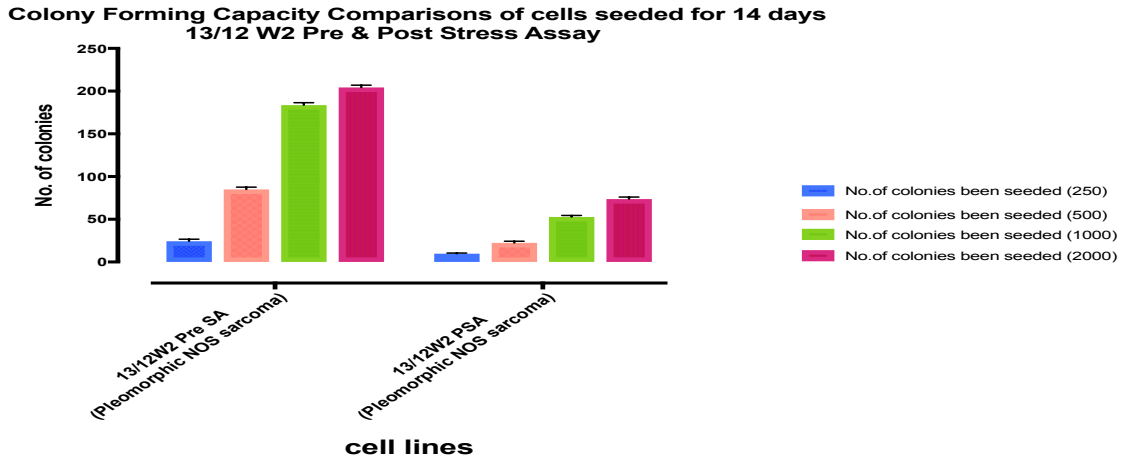


Figure 5. 9: A bar chart representing the comparison of the colony-forming capacity of cells seeded for 14 days pre and post-stress assay at different densities for a Sheffield derived cell line **13/12W2**.

Cell lines were seeded at different densities (250, 500, 1000, and 2000) cells per petri dishes. **13/12W2 Pre-SA** number of colonies out of 250, 500, 1000 and 2000 cells seeded were 24(±2.3), 85(±2.7), 184(±2.7) and 204(±2.6), respectively (N=3). **13/12W2 POST-SA** colonies were 9.7(±0.8), 22(±2), 52(±1.8) and 73.7(±2), respectively (N=3). Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

5.2.2.3 Clonogenic Assay for STS (09/10 POST-SA) Dedifferentiated Sarcoma

The number of colonies out of 250, 500 and 1000 cells seeded were 32(±1.5), 132(±2.2) and 163(±2.4) colonies respectively. Due to overgrowth 2000 colonies was uncountable. CE based on the clonogenic assay results was 18.5% (± 0.41) SEM. Figures 5.5, 5.6, 5.8 and 5.10 show colony number differences and CE percentages between Pre-SA and POST-SA.

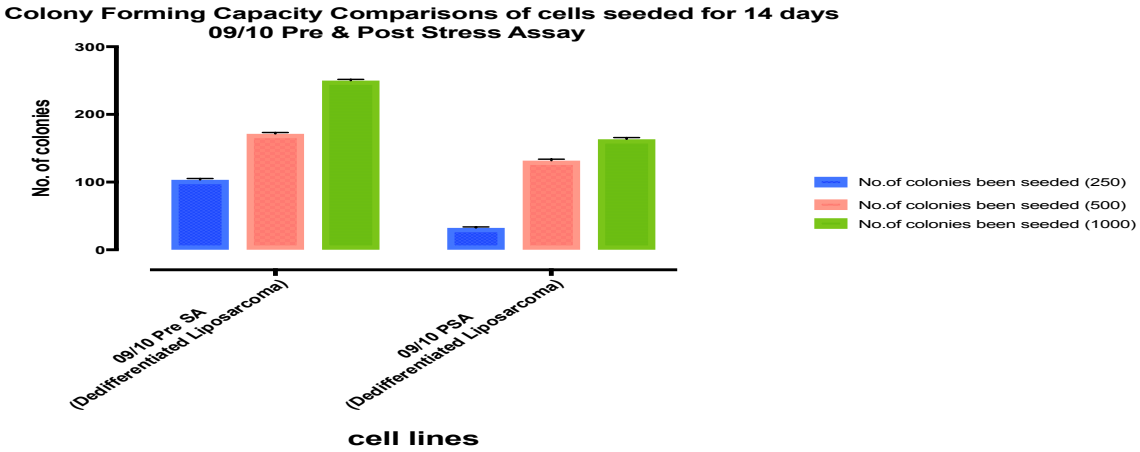


Figure 5. 10: A bar chart representing the comparison of the colony-forming capacity of cells seeded for 14 days pre and post-stress assay at different densities for Sheffield derived cell line **09/10**.

Cell lines were seeded at different densities (250, 500, and 1000) cells per petri dishes. **09/10 Pre-SA** the number of colonies out of 250, 500 and 1000 cells seeded were 103(±2), 171(±1.9) and 250(±1.7), respectively (N=3). **09/10 POST-SA** colonies were 32(±1.5), 132(±2.2) and 163(±2.4), respectively (N=3). Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

5.2.2.4 Clonogenic Assay for STS (14/10 POST-SA) Pleomorphic NOS Sarcoma

The number of cells been seeded in each dish were 250, 500 and 1000 cells and colonies out of seeded cells were 51.7(±1.7), 119(±1.2) and 227(±1.2) colonies respectively. Due to the overgrowth of the 2000 petri dish colonies were uncountable. CE based on the clonogenic assay results was calculated at 22.4% (± 0.33) SEM. Figures 5.5, 5.6, 5.8 and 5.11 show colony number differences and CE percentages between Pre-SA and POST-SA.

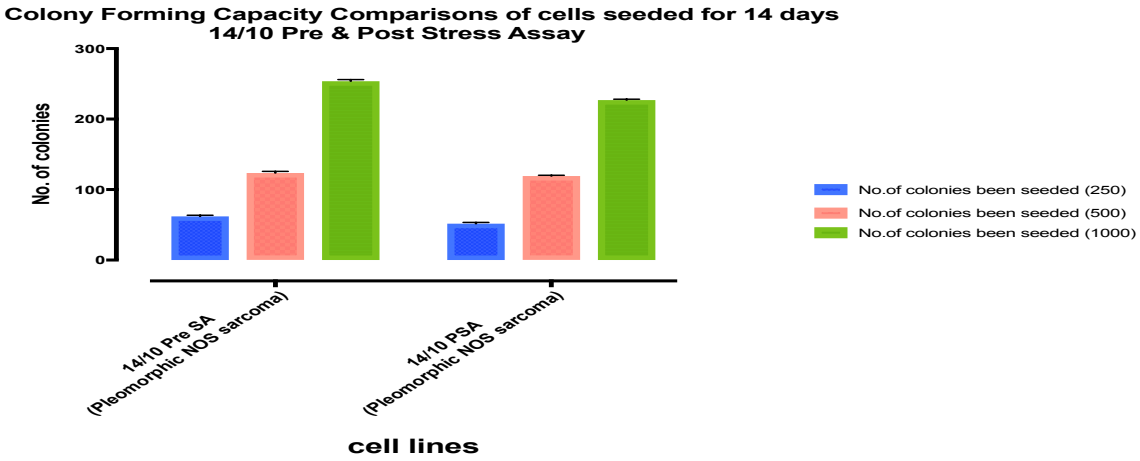


Figure 5. 11: A bar chart representing the comparison of the colony-forming capacity of cells seeded for 14 days pre and post-stress assay at different densities for Sheffield derived cell line 14/10.

Cell lines were seeded at different densities (250, 500, and 1000) cells per petri dishes. **14/10 Pre-SA** number of colonies out of 250, 500 and 1000 cells seeded were 62(±1.5), 123(±2.4) and 253(±2.3), respectively (N=3). **14/10 POST-SA** colonies were 51.7(±1.7), 119(±1.2) and 227(±1.2), respectively (N=3). Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

5.2.2.5 Clonogenic Assay for STS (02/11W1 POST-SA) Leiomyosarcoma

The number of cells seeded in each dish were 250, 500, 1000 and 2000 cells and colonies out of seeded cells were 35 (±1.5), 56 (±1.7), 85.7(±1.5) and 153 (±2.4) respectively. CE based on the clonogenic assay results was calculated at 10.39% (± 0.298) SEM. Figure 5.5, 5.6, 5.8 and 5.12 show colony number differences and CE percentages between Pre-SA and POST-SA.

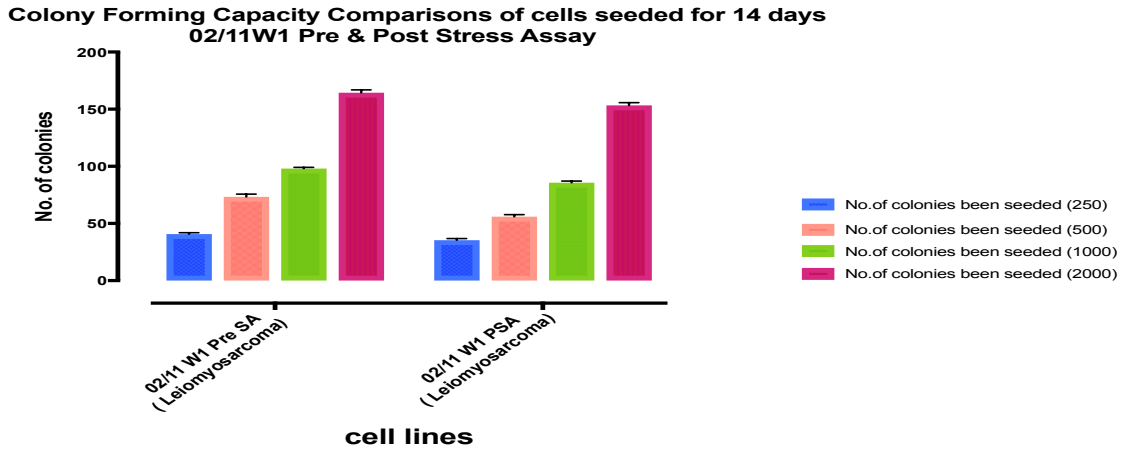


Figure 5. 12: A bar chart representing the comparison of the colony-forming capacity of cells seeded for 14 days pre and post-stress assay at different densities for Sheffield derived cell line **02/11W1**.

Cell lines were seeded at different densities (250, 500, 1000, and 2000) cells per petri dishes. **02/11W1 Pre-SA** number of colonies out of 250, 500, 1000 and 2000 cells seeded were 40.7(± 1.2), 73 (±2.4), 98(±1.2) and 164(±2.6) respectively (N=3). **02/11W1 POST-SA** colonies were 35(± 1.5), 56 (±1.7), 85.7(±1.5) and 153(±2.4) respectively (N=3). Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

5.2.2.6 Clonogenic Assay for STS (21/11W2 POST-SA) Myxofibrosarcoma

The number of cells seeded in each dish were 250, 500, 1000 and 2000 cells and colonies out of seeded cells were 3(±0.9), 7(±0.8), 12(±0.8) and 30(±1.2) respectively. CE based on the clonogenic assay results was calculated at 1.4% (± 0.17) SEM. Figure 5.5, 5.6, 5.8 and 5.13 show colony number differences and CE percentages between Pre-SA and POST-SA. This cell line was the clearly the lowest compared to the rest of the other cell lines for both colonies counted and CE.

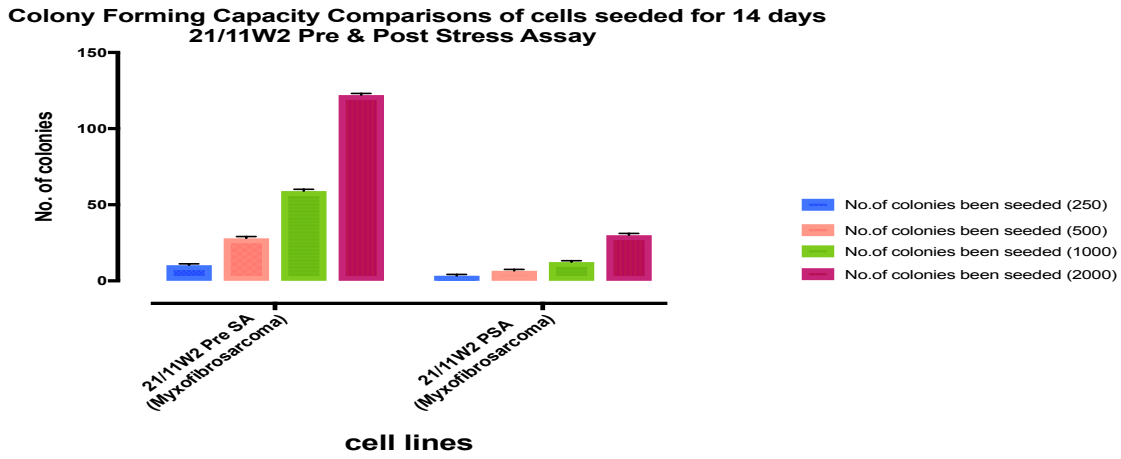


Figure 5. 13: A bar chart representing the comparison of the colony forming-capacity of cells seeded for 14 days pre and post-stress assay at different densities for Sheffield derived cell line **21/11W2**.

Cell lines were seeded at different densities (250, 500, 1000, and 2000) cells per petri dishes. **21/11W2 Pre-SA** number of colonies out of 250, 500, 1000 and 2000 cells seeded were 10(±0.8), 28(±1.2), 59(±1.2) and 122(±1.2) respectively (N=3). **21/11W2 POST-SA** colonies were 3(±0.9), 7(±0.8), 12(±0.8) and 30(±1.2) respectively (N=3). Data is representative of experiments done in triplicate on three different passages. Mean and SEM was done using Graph Pad® Prism software (version 7).

In summary, at this stage of the project the differences between pre and post stress cell line populations seem to be showing a consistent pattern when the results of the MTT and clonogenic assay are considered. Cells after the stress assay were found to have slower proliferation taking longer to become confluent, around 3-4 days longer, and the colony forming-capacity and CE was also significantly less than the pre-stressed cell lines.

5.2.3 aCGH Array (Pre vs. Post Stress Assay)

In this section array CGH was performed on cell lines that survived the stress assay, to provide an estimate of global differences in the genetics of pre and post stressed populations. Array CGH was performed as described in section 2.2.20. Firstly, the pre and post-stress cell lines were compared against control reference DNA, and secondly, the pre and post lines were compared directly against each other to identify any differences in global genetic changes.

5.2.3.1 SKUT-1 (Human Uterus Leiomyosarcoma (GIII))

Array CGH was performed using SKUT-1's DNA extracted from cells at passage 94 for pre-SA and passage 98 for POST-SA against non-sex match commercial DNA. Then they were tested against each other using SKUT-1 Pre-SA cell line's DNA as reference. Both pre-SA and POST-SA showed identical partial amplification in chromosomes 5p and 8q, and some minor insignificant deletions and amplifications on other chromosomes. Pre-SA DNA against POST-SA DNA was found to be an exact match (Figure 5.14).

5.2.3.2 STS (13/12W2) Pleomorphic NOS Sarcoma

DNA was extracted from cells at passage 18 for pre-SA and passage 21 for POST-SA against sex match commercial DNA. Then they were tested against each other, using 13/12W2 pre-SA cell line's DNA as reference. Pre-SA cells showed aberrations in almost all chromosomes. However, POST-SA cells showed some remarkable differences; with some of the deletions or gains lost. These changes affected 1q, 2q, 3q, 4q, 8(p and q), 11q, 12p, 13q, 17(p and q), 18q, 19(p and q) and 22q. When pre-SA and POST-SA cells were tested against each other, the major genomic regions found to be significant were in 1q, 2q, 4(p and q), 8(p and q), 9(p and q), 12(p and q), 14(p and q), 18(p and q), 19q and 22q. There were also, some minor insignificant deletions and amplifications found on other chromosomes Figure 5.15.

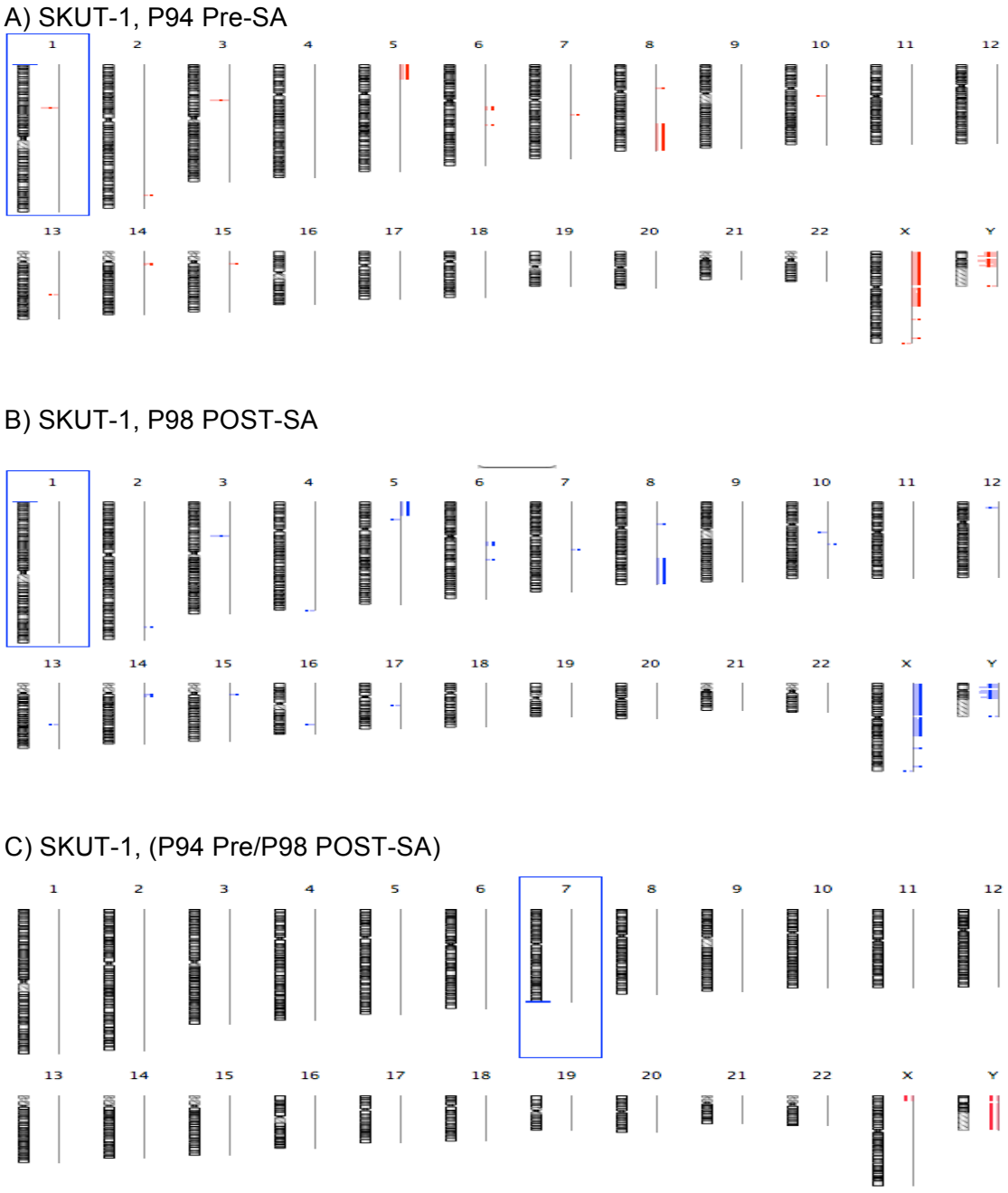


Figure 5. 14: Array CGH ideograms of chromosomal aberration **SKUT-1** comparing pre-SA and POST-SA.

A: Genomic ideogram view exhibits chromosomal aberrations for SKUT-1 pre-SA at passage 94 showing, 5p and 8q amplification. **B:** Genomic ideogram represents chromosomal aberrations for SKUT-1 POST-SA at passage 98, showing 5p and 8q amplification. **C:** The whole genomic ideogram shows no significant differences for pre-SA and POST-SA when tested against each other, using pre-SA as reference DNA. Aberrant areas are represented as blue or red colour bars, and the black vertical line (zero lines) express the mean log ratio on the chromosome. Left colour regions identify deletions and right colour regions for amplification (gain). All images were obtained from Agilent Genomic workbench version 7.0.4

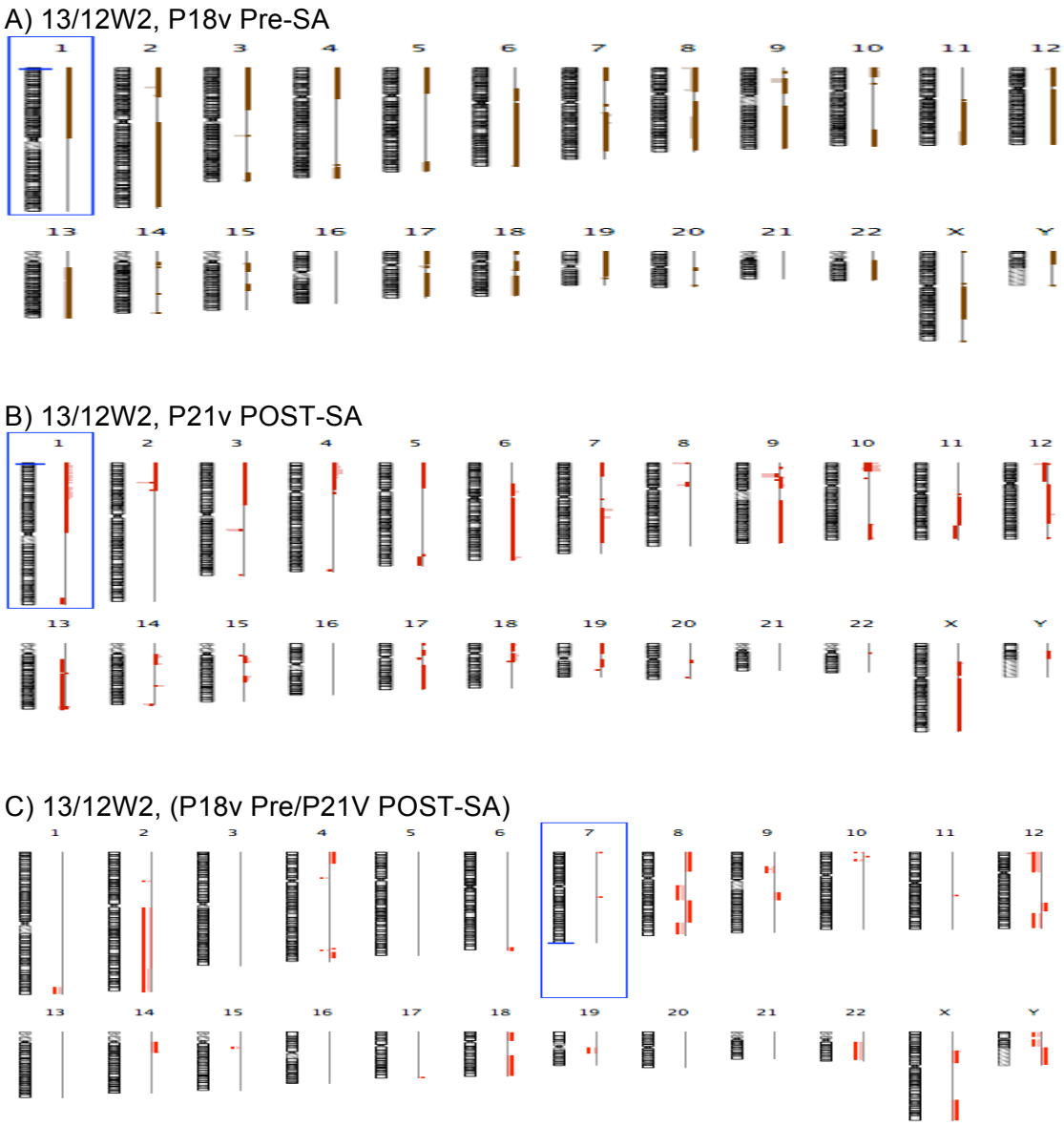


Figure 5. 15: Array CGH ideograms of chromosomal aberration in primary cell line **13/12W2** comparing pre-SA and POST-SA.

A: Genomic ideogram view exhibits chromosomal aberrations for 13/12W2 pre-SA at passage 18 showing abnormalities on all the chromosomes. **B:** Genomic ideogram view represents chromosomal aberrations for 13/12W2 POST-SA at passage 21 showing differences of 12 chromosomes compared to pre-SA cells. **C:** whole genomic ideogram shows the main aberrant areas for pre-SA and POST-SA when tested against each other using pre-SA as reference DNA. Aberrant areas are represented as brown or red colour bars and the black vertical line (zero lines) express the mean log ratio on the chromosome. Left colour regions exhibit deletions and right for amplification (gain). All images were obtained from Agilent Genomic workbench version 7.0.4

5.2.3.3 STS (09/10) Dedifferentiated Sarcoma

DNA was extracted from cells at passage 82 for pre-SA and passage 84 for POST-SA against sex match commercial DNA. Pre-SA cells showed aberrations in almost all chromosomes. However, POST-SA cells showed close similarity in their profile compared

to pre-SA cells profile even in complex aberrations for example those on 9p. There were however some difference and these changes mainly affected chromosomal regions 2q, 15q and 18q. Comparison of the aCGH of the pre-SA and the POST-SA showed some minor insignificant deletions and amplification in some chromosomes Figure 5.16.

5.2.3.4 STS (14/10) Pleomorphic NOS Sarcoma

DNA was extracted from cells at passage 112 for pre-SA and passage 114 for POST-SA. Pre-SA cells showed high instability with aberrations of all chromosomes. POST-SA cells showed some close similarity in their profile to the pre-SA profile for some regions, but there were clear differences, for example those on chromosomes 2, 11 and 14. Comparison of the aCGH of the pre-SA and the POST-SA cells when they tested against each other showed no significant deletions or amplifications Figure 5.17.

5.2.3.5 STS (02/11W1) Leiomyosarcoma

DNA was extracted from cells at passage 65 for pre-SA and passage 67 for POST-SA. Pre-SA cells showed aberrations in almost all chromosomes and POST-SA had a similar profile even for complex aberrations, for example those on 9p and 11p and q. In addition, there were some differences mainly changes found for 4q, 7q, 8p and 17p. Comparison of the aCGH of the pre-SA and the POST-SA cells when they tested against each other showed changes in 7q, 12p and 18q. and insignificant changes found in terms of deletions and amplification for some chromosomes Figure 5.18.

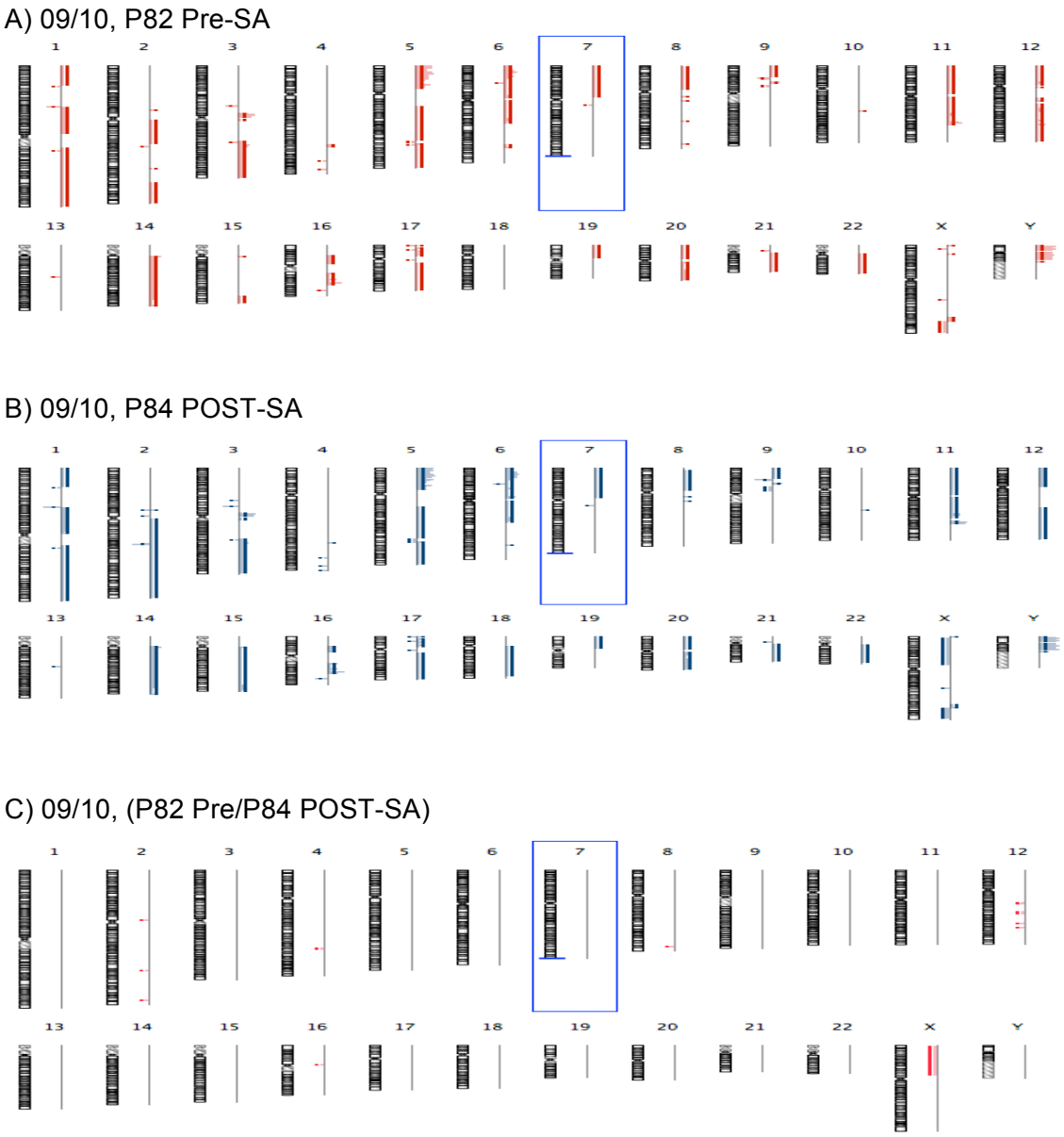


Figure 5. 16: Array CGH ideograms of chromosomal aberration for **09/10** comparing pre-SA and POST-SA.

A: Genomic ideogram view exhibits chromosomal aberrations for 09/10 pre-SA at passage 82, showing abnormalities on all chromosomes. **B:** Genomic ideogram view represents chromosomal aberrations for 09/10 POST-SA at passage 84 showing, with differences compared to pre-SA cells of 3 chromosomes. **C:** whole genomic ideogram shows the main aberrant areas for pre-SA and POST-SA when tested against each other using pre-SA as reference DNA. Aberrant areas are represented as dark blue or red colour bars and the black vertical line (zero lines) express the mean log ratio on the chromosome. Left colour regions signify deletions and right are for amplifications (gain). All images were obtained from Agilent Genomic workbench version 7.0.4

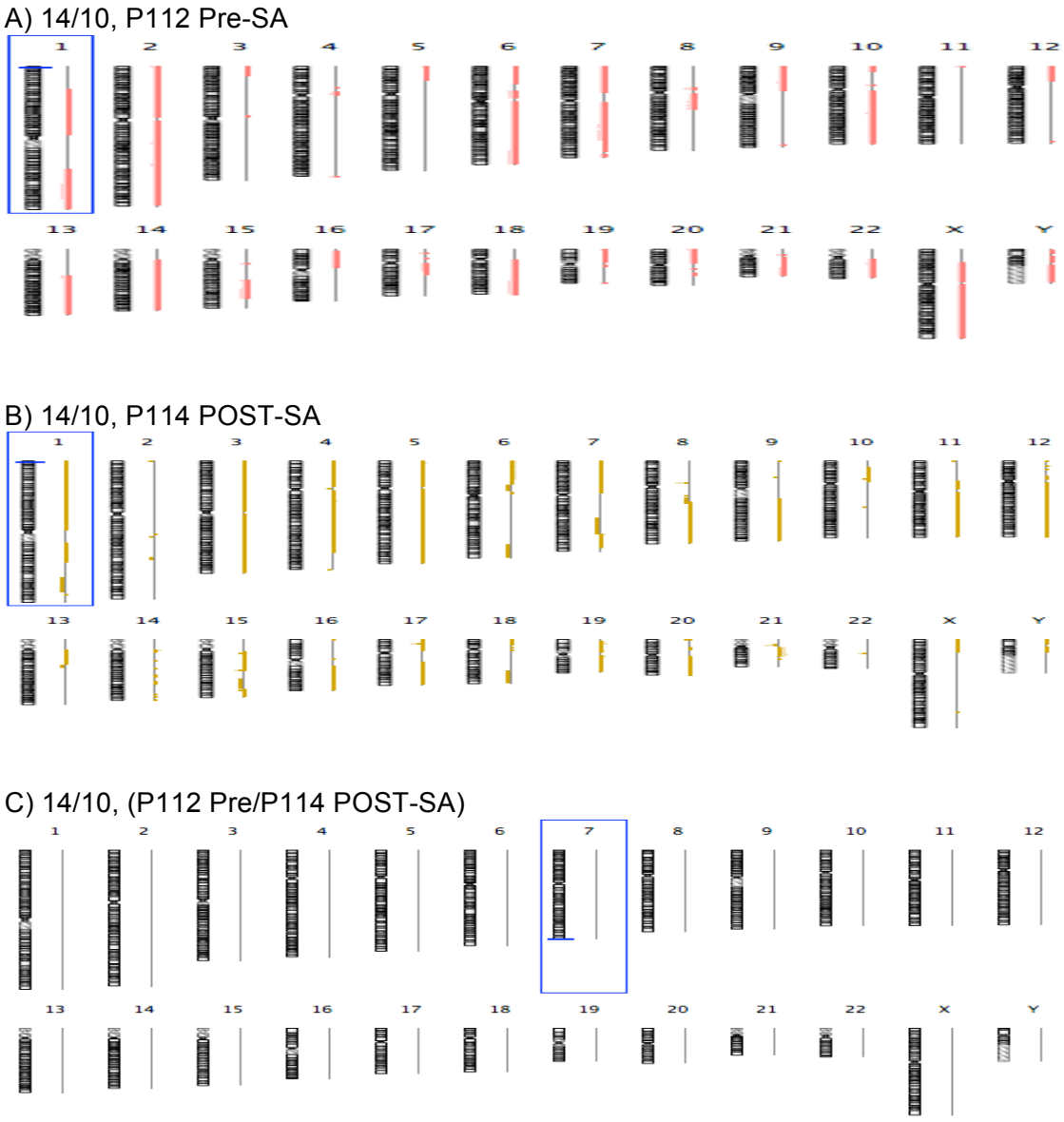


Figure 5. 17: Array CGH ideograms of chromosomal aberration of **14/10** comparing pre-SA and POST-SA.

A: Genomic ideogram view exhibits chromosomal aberrations for 14/10 pre-SA at passage 112, showing abnormalities of all chromosomes. **B:** Genomic ideogram represents chromosomal aberrations for 14/10 POST-SA at passage 114, showing differences compared to pre-SA cells of all chromosomes. **C:** whole genomic ideogram shows no main significant aberration areas for pre-SA and POST-SA when tested against each other using pre-SA as reference DNA. Aberrant areas are represented as dark yellow or red colour bars and the black vertical line (zero lines) express the mean log ratio on the chromosome. Left colour regions indicate deletions and right side are for amplifications (gain). All images were obtained from Agilent Genomic workbench version 7.0.4

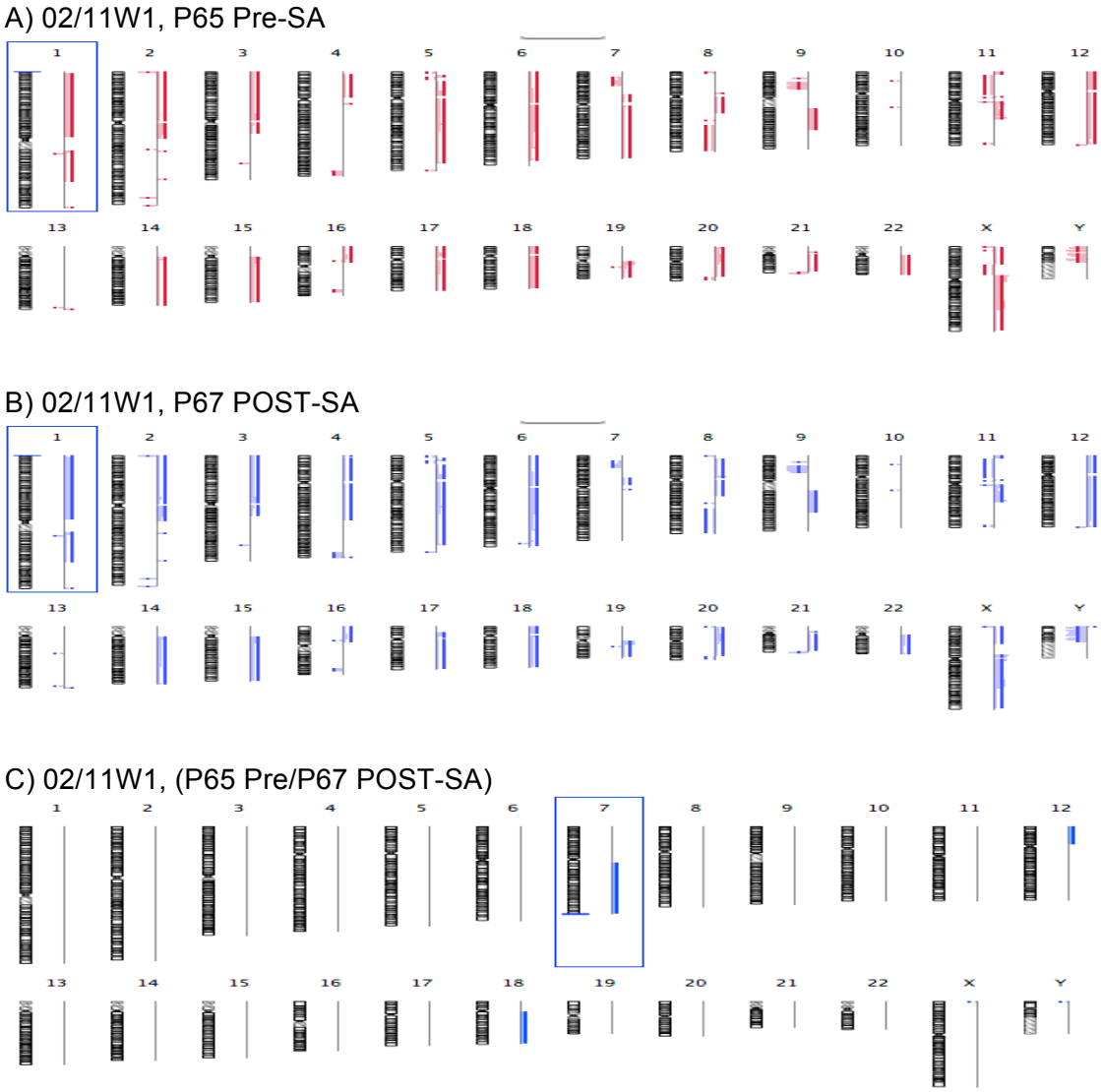


Figure 5. 18: Array CGH ideograms of chromosomal aberration for **02/11W1** comparing pre-SA and POST-SA.

A: Genomic ideogram view exhibits chromosomal aberrations for 02/11W1 pre-SA at passage 65, showing abnormalities of all chromosomes. **B:** Genomic ideogram represents chromosomal aberrations for 02/11W1 POST-SA at passage 67, showing differences compared to pre-SA cells for 4 chromosomes. **C:** whole genomic ideogram shows the main aberrant areas for pre-SA and POST-SA when tested against each other using pre-SA as reference DNA, mainly for chromosomal regions 7q, 12p and 18q. Aberrant areas are represented as blue or red colour bars and the black vertical line (zero lines) express the mean log ratio on the chromosome. Left colour regions indicate deletions and the right coloured regions identify amplifications (gain). All images were obtained from Agilent Genomic workbench version 7.0.4

5.2.3.6 STS (21/11W2) Myxofibrosarcoma

DNA was extracted from cells at passage 56 for pre-SA and passage 58 for POST-SA. Pre-SA cells showed aberrations in almost all chromosomes, however POST-SA cells had a different profile. There were differences in most genomic regions and were noted on almost all chromosomes except chromosome 21. Comparison of the aCGH on the pre-SA and the POST-SA cells when tested against each other showed no significant differences Figure 5.19.

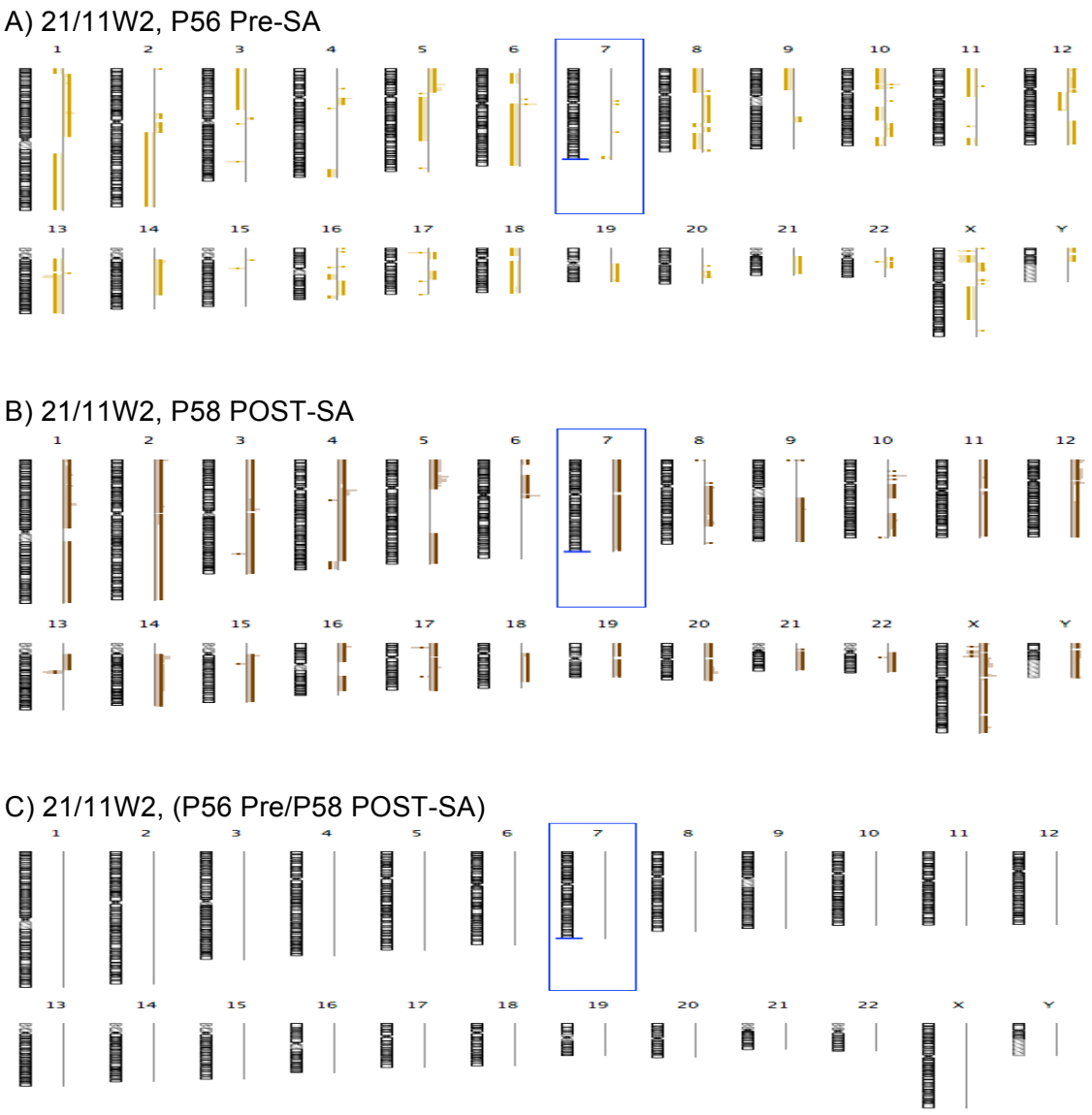


Figure 5. 19: Array CGH ideograms of chromosomal aberrations for **21/11W2** comparing pre-SA and POST-SA.

A: Genomic ideogram view exhibits chromosomal aberrations for 21/11W2 pre-SA at passage 56, showing abnormalities on all chromosomes. **B:** Genomic ideogram represents chromosomal aberrations for 21/11W2 POST-SA at passage 58, showing differences compared to pre-SA cells on almost all chromosomes. **C:** whole genomic ideogram shows no significant aberrations for pre-SA against POST-SA when tested against each other using pre-SA as reference DNA. Aberrant areas are represented as dark yellow or brown colour bars and the black vertical line (zero lines) express the mean log ratio on the chromosome. Left colour regions identify deletions and right colours are for amplification (gain). All images were obtained from Agilent Genomic workbench version 7.0.4.

5.2.3.7 Array CGH Results Discussion

Sarcomas are known for their genetic instability that results from dysfunction of DNA repair. Chromosomal instability is often the main form of genetic instability in sarcomas and is manifested as changes in the structure and number of chromosomes (Martin et al., 1998, Amant et al., 2001, Verelst et al., 2004, Rucinska et al., 2005, Nakagawa et al., 2006, Ferreira et al., 2008, Nakano and Takahashi, 2018, Doyle et al., 2019, Liu et al., 2021). Array CGH is a powerful tool to detect copy number changes of chromosomes (gaining or losses) and it is useful to sarcoma classification even with the complex karyotypes that sarcomas have (Burns et al., 2020). For instance, the combination of aCGH and IHC was able to define three different molecular subtypes of leimyosarcoma, one of these subtypes was found to have a distinct genomic aberrations and gene expression pattern that was linked to muscle differentiation (Beck et al., 2010, Lazar et al., 2017). Furthermore, IHC markers for the five most expressed genes for one of the groups was highly correlation with improved survival (Beck et al., 2010). Moreover, Skubitz et al., 2008, identified two major subsets of malignant fibrous histiocytoma, a form of undifferentiated pleomorphic sarcoma, as having distinct gene expression profiles that classified subgroups of pleomorphic STS for development of metastasis (Skubitz et al., 2012). These studies show the impact of aCGH in combination with biomarkers and other methods (FISH, RT-PCR next generation sequencing) to identify these rearrangements and to detect translocations; in order to enhance sarcomas diagnosis classification and prognosis particularly for complicated cases.

A summary of the CGH results is presented below in Table 5.2, showing the main aberrations on each chromosomes for pre, post, and when tested against each other; each cell line has its own signature. Commercial cell line SKUT-1 had no differences for the pre and Post-SA results, with them being almost identical, possibly due to prolonged culture. The Sheffield derived primary cell lines on the other hand showed some interesting results, with aberrations that differ from each other and differences after the stress assay. Furthermore only two of the lines had differences when the pre and Post-SA were tested against each other.

Cell lines & Cancer Type	Main Chromosomal abnormality Pre-SA cell lines	Main Chromosomal abnormality Post-SA cell lines	Main Chromosomal abnormality Pre against Post-SA cell lines
SKUT-1 Human uterus leiomyosarcoma (GIII) cell line	Instability (Aberrations in some chr.)	No significant deletions or amplification	No significant deletions or amplification
13/12W2 Pleomorphic NOS sarcoma	Instability (Aberrations in almost all chr.)	1q, 2q, 3q, 4q, 8(p and q), 11q, 12p, 13q, 17(p and q), 18q, 19(p and q) and 22q	1q, 2q, 4(p and q), 8(p and q), 9(p and q), 12(p and q), 14(p and q), 18(p and q), 19q and 22q
09/10 Dedifferentiated Liposarcoma	Instability (Aberrations in almost all chr.)	2q, 15q and 18q	Minor insignificant deletions and amplification
14/10 Pleomorphic Sarcoma NOS	Instability (Aberrations in almost all chr.)	Almost all chr. q arm and chr.2, 3, 4, 5, 6 and 7 (p&q). Clear differences seen in chr. 2, 11 and 14 (p&q)	No significant deletions or amplification
02/11 W1 Leiomyosarcoma	Instability (Aberrations in almost all chromosomes)	9p and 11(p &q), 4q, 7q, 8p and 17p	7q, 12p and 18q
21/11W2 Myxofibrosarcoma	Instability (Aberrations in almost all chr.)	Instability (Aberrations in almost all chr.), With different profile from pre-stress	No significant deletions or amplification

Table 5. 2: Summary of the aCGH results showing the main differences between pre and post-stress assay cell lines

Chr.=chromosome.

In Figure 5.20 an overall comparison of pre and post stress assay for chromosomal aberrations of all cell lines is presented. Both pre and post-stress assay cell lines showed deletion and gain for all the chromosomes, reflecting the instability of sarcoma in general. Overall it can be seen that most deletions happened on the pre-SA whilst most of the gains were found in the Post-SA, and there were some interesting results when they were tested against each other. The most frequently aberrant regions were for chromosomes 1, 2, 4, 6, 7, 8, 9, 12, 14, 18, 19 and 22, but the most significant aberrations were noticed in 2q, 7 (p/q), 8(p/q), 12(p/q), 18 (p/q) and 22 q. It is possible that these regions are directly correlated with selection through the stress assay and may play a role in stemness properties before adaptation to culture. Interestingly the aCGH results for Post-SA for Sheffield derived cell lines had a closer resemblance to the aCGH profiles performed on early passage numbers when the cell lines were first established (based on data from

previous research done by Dr Abdulazeez Salawu (data not shown here). For further information table 1.2 in chapter one shows common chromosomal translocations known in sarcoma.

Figure 5. 20: Combined Array CGH ideograms of chromosomal aberration for all cell lines tested in this study, comparing pre-SA and POST-SA and showing the most frequently aberrant regions.

A: Genomic ideogram for chromosomal aberrations of all pre-SA cell lines, all chromosomes have abnormalities. **B:** Genomic ideogram view of chromosomal aberrations for all POST-SA cell lines with clear differences compared to pre-SA cells on almost all chromosomes. **C:** Genomic ideogram indicating the most common aberrations for pre-SA and POST-SA when tested against each other using pre-SA as reference DNA. Aberrant areas are represented as red or green colour bars and the black vertical line (zero lines) express the mean log ratio on the chromosome. Left colour regions (green) are deletions and the right side are for amplification/gain (red). All images were obtained from Agilent Genomic workbench version 7.0.4.

Leiomyosarcoma (LMS) have characteristics consistent with smooth muscle differentiation, and spindle shape cells. There are no constant genetic aberrations, however frequent losses of the tumour suppressor genes RB1 (13q) and the phosphatase and tensin homolog (PTEN) at 10q have been identified in the literature (El-Rifai et al., 1998, Ul-Hassan et al., 2009, Salawu et al., 2012). Copy number variation has also been found for other tumour suppressor genes such as TP53 and cadherin-1 (CDH1) (Agaram et al., 2016). According to the Genome Atlas consortium, analysis conducted on 206 STS patient sample using genome sequencing, DNA methylation, messenger RNA and microRNA revel that LMS and uterine LMS (uLMS) were similar to each other when compared to other STS subtypes. In this study, there was no specific evidence to suggest that the regions harbouring these relevant tumour suppressor genes were implicated in the post stress assay survival of LMS. In it however possible that RB1 may be relevant, as there appears to be a focal deletion of the region in the post stress cells compared to the line before (5.18). Unfortunately, there was not time to conduct a deeper interrogation of the data to confirm this observation and whether cells with RB1 deletion are specifically able to survive prolonged stress. It is also possible that if RB1 deletion is an early change driving LMS development, its greater evidence in the post stress populations may correlate to a earlier less culturally adapted population, as has been suggested by the comparison with the early passages of the cell lines (data not shown) but some information was published as part of the cell line publication (Salawu et al., 2016).

Myxofibrosarcoma MFS, is a common STS subtype, and like most STS they can have a high level of genomic instability, but there are also variations within the subtype, with some MFS having an apparently normal karyotype whilst other have highly complex ones. The aCGH data shown in Figure 5.19 demonstrates how unstable they can be. Studies have suggested that many tumours suppressor genes may be important to MFS reflecting the high level of variability for this sarcoma. Specifically loss of chromosome 9, with deletion of the methylthioadenosine phosphorylase (MTAP) and CDKN2A/CDKN2B genes (9q21.3) has been linked to increased myxofibrosarcoma aggressiveness (Barretina et al., 2010, Li et al., 2014, Ogura et al., 2018). There was no evidence from this study to suggest this region may be important, as there were so many differences between the pre and post stress populations. It is therefore difficult to suggest any region that may be important in this context specifically as only one MFS was studied here. A more detailed analysis is required to see if there are any associations, but this was unfortunately not possible in the time frame.

Liposarcoma LPS, is also a common STS sarcoma subtype, and originate from malignant transformed adipocyte progenitor cells (Lee et al., 2018), with the most common subtypes being well-differentiated (WDLPS) and dedifferentiated liposarcoma (DDLPS). There are also rare subtypes called myxoid liposarcoma (MLPS) and pleomorphic liposarcoma (PLPS). WDLPS is known as aggressive with no metastatic potential. While DDLPS is considered as a more aggressive subtype prone to recurrence and increased metastatic potential. However, both WDLPS and DDLPS share similar genetic aberrations profiles and have amplifications of 12q21-15. This region includes the MDM2 proto-oncogene (MDM2) and cyclin dependent kinase 4 (CDK4) oncogenes (Kanojia et al., 2015). Although sharing this aberration there are in addition clinical phenotyping differences between WDLPS and DDLPS, since DDLPS contain higher copy numbers of chromosome 12 and higher numbers of somatic copy number variations and fusion transcripts (Beird et al., 2018). Moreover, MLPS is characterized with a translocation t(12,16)(q13;p11) that produces a fusion transcription factor (FUS RNA binding protein) and in common with t(12,22)(q13;q12) the EWS binding protein-1 (EWSR1), these two transcription factors share roles in DNA damage and in combination with transcript-3 (DDIT3) on chromosome 12 can inhibit adipocyte differentiation (Powers et al., 2010). In this study only one liposarcoma was investigated (STS 9/10) and as seen in figure (5.16) the array profile for the pre and post stressed populations were fairly closely matched. Both populations had clear evidence of amplification for chromosome 12 that included the MDM2 region, and had been reported previously (salawu et al). This abnormality seems certainly to be a driver of liposarcomas, and is clearly retained in the post stress survival population, but it is difficult to conclude whether it relates to the ability to survive. It was however noted that the post stress survival population did seem to have some focal deletions of chromosome 12q that were not present earlier, so there seems to have been in part some selective process. Whether this is relevant requires further more detailed analysis and also confirmation with other cell lines.

Finally Pleomorphic Sarcoma NOS are a relatively unstudied subtype but they are known to have very high levels of instability (Widemann and Italiano, 2018, Zheng et al., 2019). In this study there were variations in the level of instability (see figures 5.15, 5.17 and table 5.2) and it was not possible to clearly relate any findings, or to suggest any regions of interest for post stress survival.

To conclude, as only small numbers from each subtype of sarcoma was studied it was not possible to identify specific genetic changes associated with these subtypes that may be

related to enhance survival and stemness. Overall the findings as mentioned above our may suggest regions of 2q, 7 (p/q), 8(p/q), 12(p/q), 18 (p/q) and 22q might be areas of interest for further investigation, in combination with other methods. A more detailed analysis of the data is required, but was not possible during this investigation, and as yet there is no clear evidence for any copy number change that may be related to enhanced stemness.

5.2.4 STS Gating Strategy - Phase One Screening for CSC Markers (Gating All Cells/Pre-Stress Assay)

This stage was discussed earlier in section 3.2.4. (CD44, CD133, ALDH, CD24, CD90, CD71 and CD34 antibodies were used for both single expression and co-expression on the cell lines tested. The experimental analysis and results were discussed in detail in chapter three. Table 3.1 in chapter 3 section 3.2.4 summarized the expressions of every single marker, co-expression and gating percentages for each cell lines out of 100% live cells.

5.2.5 STS Gating Strategy - Phase Two Screening for CSC Markers (Gating Large Cells Only / Pre-Stress Assay)

In this stage, analysis of the same commercial and Sheffield derived cell lines was undertaken as in stage one (section 3.2.4), but the focus was on one distinct population (large cells) instead of gating for all cells. The reasoning behind this analysis was based on the observation that only large cells appeared to have the ability to resist long and hard conditions as mentioned in sections 4.2.3, 4.2.3.3 and 4.3, see also, figures 4.8 and 4.13. In addition, co-expression of markers was assessed for pre and post-stress assay, and the co-expression areas are demonstrated in the dot plot in the upper right (UR) (Figure 5.21). The analysis was also expanded to include the new markers suggested as possible markers for CSC (CD90, CD71, CD24 and CD34). Table 5.3 summarizes the expressions of every single marker as well as gating percentages for each cell lines out of 100% live cells as discussed previously (Chapter 3). Only SKUT-1 dot plots are shown as an example of the gating strategy.

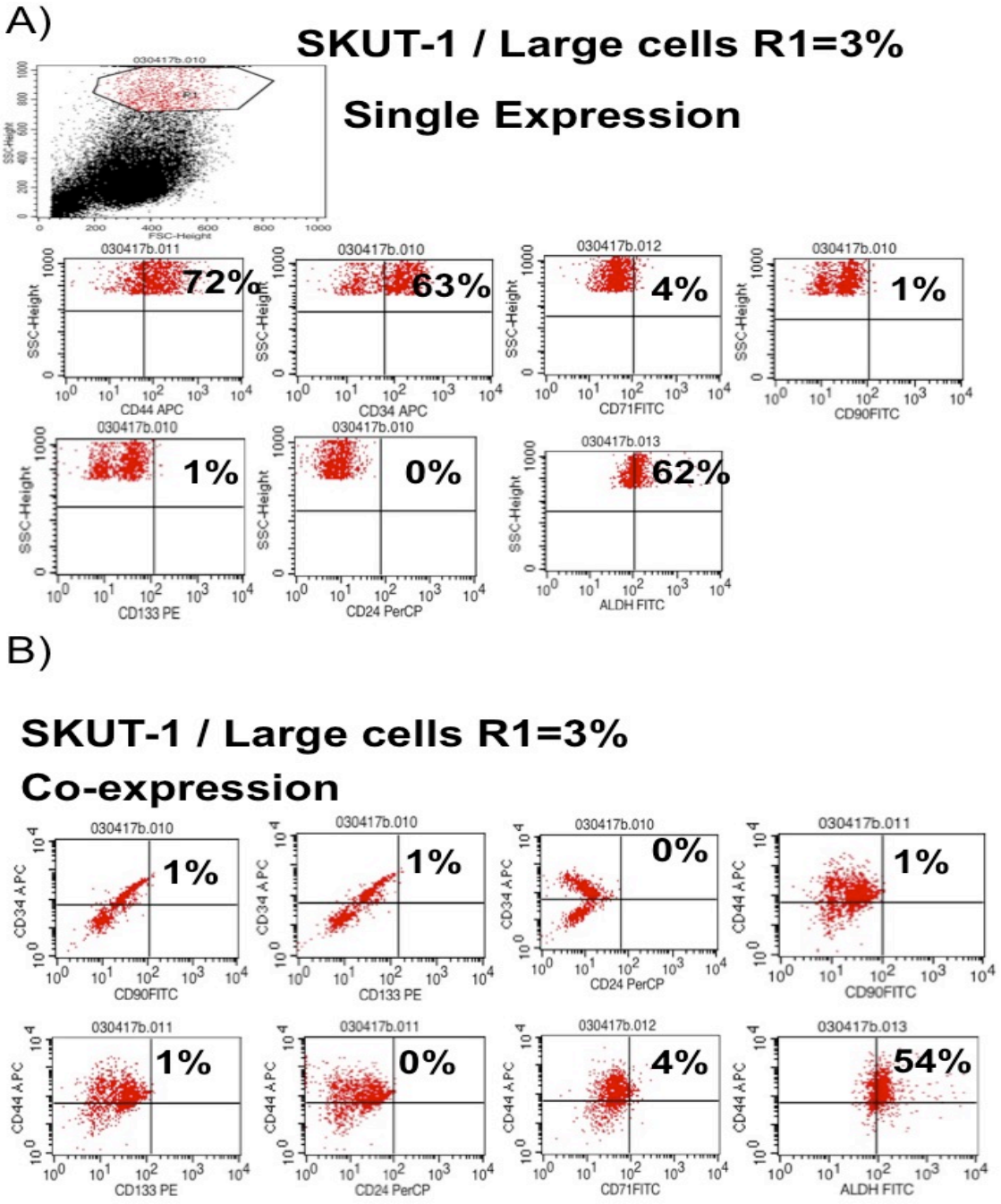


Figure 5. 21: These figures show the dot plot analysis of different CD markers expression and co-expression on **SKUT-1** Pre-SA cell line using flow cytometry staining by gating large cells only.

The expression was based on one distinct cell population (gating for large cells only). The CD44 mean expression was 71.7% ± 0.3% (n=3 SEM); CD34 mean expression was 62.7% ±0.3 (n=3 SEM); CD71=3.7% ± 0.3% (n=3 SEM) and ALDH mean activity was 61.3% ±0.7 (n=3 SEM). Co-expression was CD44/ALDH=54.3% ±0.3 and CD44/71=3.7% ±0.3. The data corresponds to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculated was done using Graph Pad® Prism software (version 7).

5.2.5.1 Flow cytometry Screening of Pre-SA Cell Lines Gating for Large Cells Only

Gating percentages were between (26%-1%) for all the cell lines. The expression results of all cell lines for CD44 was almost the same as previously tested in stage one and two in chapter 3 section 3.2.3 and 3.2.4. ALDH activity however showed higher, brighter and more constant positive activity results than previously (68%-100%). Furthermore, co-expression of CD133 and CD90 showed increased positivity in STS 09/10 around 6%; and 1% for SKUT-1 and 02/11W1. CD71 expression also was found to be brighter and the highest result was observed for 13/12W2 (65%) but was negative on SW1353, SKLMS-1, U2OS and h--TERT-RPE-1, but notably SKUT-1 showed 4% whereas previously it was negative (section 3.2.4.1 and table 3.1). CD34 was also variable again with a stronger expression than reported previously; the highest result was found on SKUT-1 (63%) but it was again negative for SKLMS-1, h-TERT-RPE-1, and also 14/10. CD24 showed no significant outcome because it was negative on all cell lines tested. Co-expression for CD90, CD133, CD71 and ALDH with CD44 was performed, and although CD34 and CD44 markers had the same fluorochrome dye colours (APC, hence impossible to stain them in the same test tube), hypothetically as all cell lines were highly positive for CD44 it can be assumed that there is a similar positive co-expression for these two markers. As was shown in section (2.2.18), the co-expression panel was limited due to fluorochrome dyes Figure 5.21 and Table 5.3.

Cell line	(Gated Cells) Large cells out of 100% gate	CD44	ALDH	CD71	CD34	CD133	CD90	CD24	CD44 / ALDH	CD71 / CD44	CD24 / CD34	CD90 / CD34	CD90 / CD44	CD133/ CD44
SKUT-1 (Leiomyosarcoma)	3%	**72% ******	**62% ******	**4% ***	**63% ******	**1% ***	**1% ***	0% (N)	**54% ******	4% *	0% (N)	**1% ***	**1% ***	**1% ***
SW1353 (Chondrosarcoma)	26%	**67% ******	0% (N)	**1% ***	**12% ****	0% (N)	0% (N)	0% (N)	0% (N)	**1% ***	0% (N)	0% (N)	0% (N)	0% (N)
SKLMS-1 (Leiomyosarcoma)	4%	**98% *******	**98% *******	0% (N)	0% (N)	0% (N)	0% (N)	0% (N)	**98% *******	0% (N)	0% (N)	0% (N)	0% (N)	0% (N)
U2OS (Osteosarcoma)	7%	**100% *******	**89% ******	0% (N)	**4% ***	0%(N)	0% (N)	0% (N)	**89% ******	0% (N)	0% (N)	0% (N)	0% (N)	0% (N)
PC3 (Prostate cancer)	18%	**94% *******	**100% *******	**11% ****	**28% *****	0% (N)	0% (N)	0% (N)	**94% *******	**11% ****	0% (N)	0% (N)	0% (N)	0% (N)
h-TERT-RPE-1 (Human epithelial retinal cells)	4%	**99% *******	**99% *******	0% (N)	**1% ***	0% (N)	0% (N)	0% (N)	**99% *******	0% (N)	0% (N)	0% (N)	0% (N)	0% (N)
13/12W2 (Pleomorphic NOS sarcoma)	3%	**100% *******	**98% *******	**65% ******	**28% *****	0% (N)	0% (N)	0% (N)	**91% *******	**65% ******	0% (N)	0% (N)	0% (N)	0% (N)
09/10 (Dedifferentiated Liposarcoma)	2%	**100% *******	**98% *******	**64% ******	**59% ******	**6% ****	**6% ****	0% (N)	**98% *******	**64% ******	0% (N)	0% (N)	**6% ****	**6% ****
14/10 (Pleomorphic NOS sarcoma)	1%	**98% *******	**100% *******	**42% *****	**1% ***	0% (N)	**1% ***	0% (N)	**99% *******	**42% *****	0% (N)	**1% ***	**1% ***	0% (N)
02/11 W1 (Leiomyosarcoma)	4%	**99% *******	**99% *******	**43% *****	**17% *****	1% *	**1% ***	0% (N)	**99% *******	**42% *****	0% (N)	**1% ***	**1% ***	**1% ***
21/11W2 (Myxofibrosarcoma)	7%	**100% *******	**97% *******	**11% ****	**71% ******	0% (N)	0% (N)	0% (N)	**97% *******	**10% ****	0% (N)	0% (N)	0% (N)	0% (N)
02/11 W1 (POST-SA2) (Leiomyosarcoma)	4%	**99% *******	**99% *******	**42% *****	**16% *****	**1% ***	**1% ***	0% (N)	**99% *******	**41% *****	0% (N)	**1% ***	**1% ***	**1% ***

Table 5. 3: Gating strategy phase **2.**A summary of CD markers expression and gating percentages out of 100% for Pre-SA cell lines based on gating large cells only. The gated cell percentages (large cells gate) were out of total gating events 10,000 events (cells) determent by the flow cytometry.

N/A = not available, N= 0% Negative, *(1%-5% = very weak (negative), **(6%-15%) = weak, ***(16%-49% = moderate, ****(50%-89 = strong, *****(90%-100% = very strong.

5.2.6 Phase Three Gating Strategy - Screening for CSC Markers by Gating All Cells of Post-Stress Assay Cell Lines

In this stage only cell lines that survived the stress assay were assessed (one commercial and 6 Sheffield derived cell lines) and all cells were gated. Single expression and co-expression analysis was performed, and Table 5.4 summarizes the expression of each marker, co-expression of markers and the gating percentages for each cell line out of 100% live cells. The analysis was performed to compare the outcome with pre stress assay cells lines, considering both all cells and when gating for large cells only.

5.2.6.1 Flow cytometry Screening of Post Stress Assay Cell Lines Gating for All Cells

Gating percentages were above 72% for all the cell lines. For all cell lines the expression results of CD44 and CD133 were almost the same as previously in phase one (Table 3.1). ALDH activity however showed variability and less activity than in phase one, highest was on 02/11W1POST-SA2 (99%) and lowest on SKUT-1 (12%), and the ALDH activity had dropped for SKUT-1, 14/10 and 02/11W1 compared with previous Pre-SA results (see Table 3.1). The extra markers, such as CD24 and CD90 showed no significant change because as before they were negative on all cell lines. Moreover, CD34 was also variable and weaker than phase one, except for 21/11W2 (63%) as the highest. CD71 expression results were also variable and positivity was observed for Sheffield cell lines only, highest was on 02/11W1POST-SA2. Co-expression for CD71 and ALDH with CD44 was also found Table 5.4.

Cell line	POST-SA (Gating All Alive Cells) Out of 100% gate	CD44	ALDH	CD71	CD34	CD133	CD90	CD24	CD44 / ALDH	CD71 / CD44	CD24 / CD34	CD90 / CD34	CD90 / CD44	CD133/ CD44
SKUT-1 (Leiomyosarcoma)	74%	**74% ******	**12% ****	0% (N)	**7% ****	0% (N)	0% (N)	0% (N)	**12% ****	0% (N)	0% (N)	0% (N)	0% (N)	0% (N)
13/12W2 (Pleomorphic NOS sarcoma)	85%	**94% *******	**52% ******	**7% ****	**2% ***	0% (N)	0% (N)	0% (N)	**52% ******	**7% ****	0% (N)	0% (N)	0% (N)	0% (N)
09/10 (Dedifferentiated Liposarcoma)	86%	**94% *******	**32% *****	**30% *****	**9% *****	0% (N)	0% (N)	0% (N)	**32% *****	**30% *****	0% (N)	0% (N)	0% (N)	0% (N)
14/10 (Pleomorphic NOS sarcoma)	82%	**91% *******	**25% *****	**4% ***	**7% ****	0% (N)	0% (N)	0% (N)	**24% *****	**4% ***	0% (N)	0% (N)	0% (N)	0% (N)
02/11 W1 (Leiomyosarcoma)	84%	**96% *******	**52% ******	**12% ****	**5% ***	0% (N)	0% (N)	0% (N)	**50% ******	**11% ****	0% (N)	0% (N)	0% (N)	0% (N)
21/11W2 (Myxofibrosarcoma)	72%	**98% *******	**53% ******	**11% ****	**63% ******	0% (N)	0% (N)	0% (N)	**53% ******	**11% ****	0% (N)	0% (N)	0% (N)	0% (N)
02/11 W1 (POST-SA2) (Leiomyosarcoma)	87%	**99% *******	**99% *******	**27% *****	0% (N)	0% (N)	0% (N)	0% (N)	**99%*******	**26% *****	0% (N)	0% (N)	0% (N)	0% (N)

Table 5. 4: Gating strategy - phase three. A summary of CD marker expression and gating percentages out of 100% for Post-SA cell lines based on gating all cells. The gated cell percentages (all cells gate) were out of total gating events 10,000 events (cells) by flow cytometry.

N/A = not available, N= 0% Negative, *(1%-5% = very weak (negative), **(6%-15%) = weak, ***(16%-49% = moderate, ****(50%-89 = strong, *****(90%-100% = very strong.

5.2.7 STS Gating Strategy - Phase Four screening for CSC Markers by Gating Large Cells Only on Post-SA Cell Lines

In this stage only cell lines that survived the stress assay were assessed. The same methods were used (section 2.2.7 and Section 2.2.18), but the strategy was to gate only the large cells. Table 5.3 summarizes the expressions of each marker and the gating percentages for each cell line out of 100% live cells. Gating percentages were between (4%-8%) for all the cell lines. The expression results of CD44 were almost the same as previously tested (section 3.2.3, 3.2.4 and 5.2.6) for all cell lines. ALDH activity however for all cell lines was consistently higher, brighter and more positive (85%-100%), than previously shown when gating all cells (section 5.2.6). CD133 and CD90 showed weak expressions (1%-3%) except 21/11W2, which was negative. CD71 expression was found to be brighter; the highest results were observed on 09/10 and 02/11W1POST-SA2 (59%) and notably, 1% on SKUT-1. CD34 was also variable but had stronger expression than previously, the highest result was found on SKUT-1 (58%) and negative/weak expression on 02/11W1POST-SA2 (2%). CD24 was negative on all cell lines. Co-expression for CD90, CD133, CD71 and ALDH with CD44 was also detected Table 5.5. Some negative co-expression results were not included in the table. Figures 5.2 to 5.29) summarize the expression and co-expression differences of CD markers between pre and Post-SA cell lines.

Cell line	POST-SA (Gating Large Cells) Out of 100% gate	CD44	ALDH	CD71	CD34	CD133	CD90	CD24	CD44 / ALDH	CD71 / CD44	CD24 / CD34	CD90 / CD34	CD90 / CD44	CD133/ CD44
SKUT-1 (Leiomyosarcoma)	5%	**91% *******	**89% ******	**2% ***	**58% ******	**1% ***	0% (N)	0% (N)	**88% ******	**2% ***	0% (N)	0% (N)	0% (N)	**1% ***
13/12W2 (Pleomorphic NOS sarcoma)	4%	**99% *******	**99% *******	**37% *****	**12% ****	0% (N)	**2% ***	0% (N)	**99% *******	**37% *****	0% (N)	**2% ***	**2% ***	0% (N)
09/10 (Dedifferentiated Liposarcoma)	8%	**100% *******	**85% ******	**59% ******	**17% *****	**1% ***	**1% ***	0% (N)	**85% ******	**58% ******	0% (N)	**1% ***	**1% ***	**1% ***
14/10 (Pleomorphic NOS sarcoma)	7%	**97% *******	**93% *******	**20% *****	**11% ****	0% (N)	**1% ***	0% (N)	**91% *******	**19% *****	0% (N)	**1% ***	**1% ***	0% (N)
02/11 W1 (Leiomyosarcoma)	7%	**98% *******	**98% *******	**43% *****	**19% *****	**1% ***	**3% ***	0% (N)	**98% *******	**42% *****	0% (N)	**3% ***	**3% ***	**1% ***
21/11W2 (Myxofibrosarcoma)	7%	**100% *******	**90% *******	**27% *****	**82% ******	0% (N)	0% (N)	0% (N)	**90% *******	**24% *****	0% (N)	0% (N)	0% (N)	0% (N)
02/11 W1 (POST-SA2) (Leiomyosarcoma)	7%	**100% *******	**100% *******	**59% ******	2% *	0% (N)	**1% ***	0% (N)	**100% *******	**59% ******	0% (N)	**1% ***	**1% ***	0% (N)

Table 5. 5: Gating strategy - phase four. A summary of CD marker expression and gating percentages out of 100% for Post-SA cell lines based on gating large cells only. The gated cell percentages (large cells gate) were out of total gating events 10,000 events (cells) determent by the flow cytometry.

N/A = not available, N= 0% Negative, *(1%-5% = very weak (negative), **(6%-15%) = weak, ***(16%-49% = moderate, ****(50%-89 = strong, *****(90%-100% = very strong.

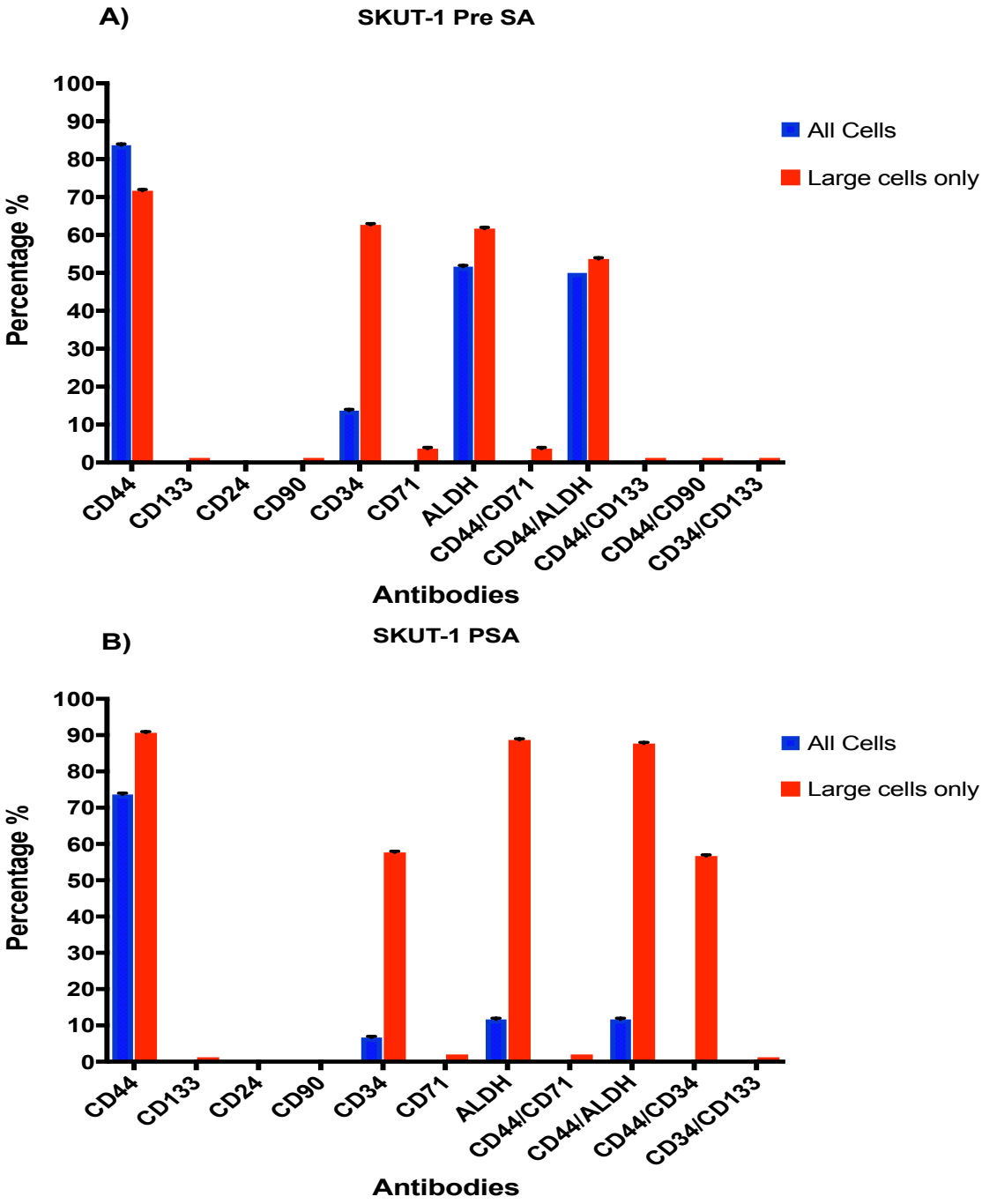


Figure 5. 22: SKUT-1 cell line. A) Pre-SA. **B)** Post-SA.

A comparison of different CD marker expression gating for all cell or large cells only, for cell line **SKUT-1** Pre and Post-SA populations using flow cytometry. The expression was based on one distinct cell population of each analysis. Averages and calculations for each marker are found in the previous dot plot graphs. The data corresponds to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculation was done using Graph Pad® Prism software (version 7).

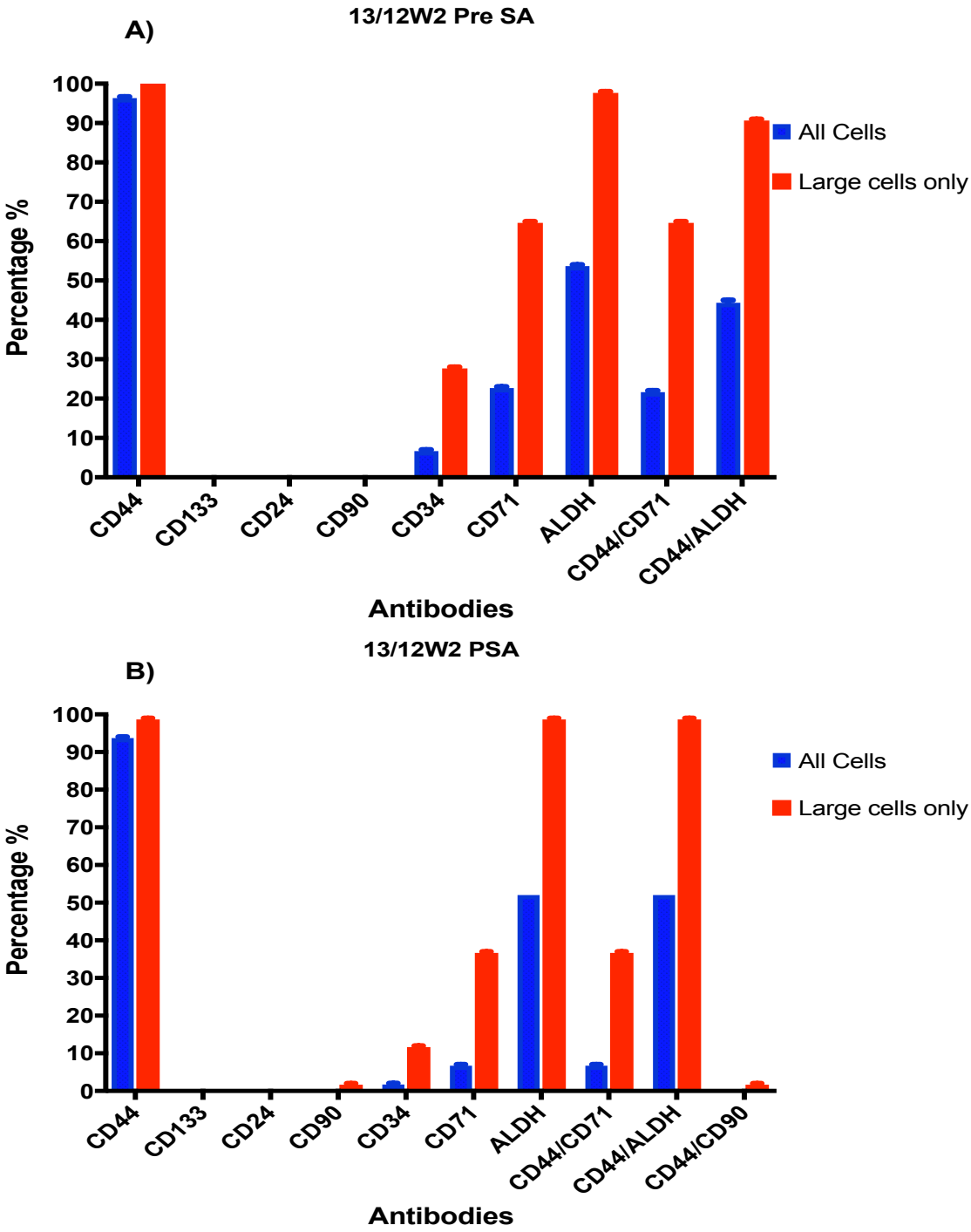


Figure 5. 23: 13/12W2 cell line. A) Pre-SA. **B)** Post-SA.

A comparison of different CD marker expression gating for all cells or large cells only, for cell line **13/12W2** Pre and Post-SA using flow cytometry. The expression was based on one distinct cell population of each analysis. Averages and calculations for each marker are found in the previous dot plot graphs. The data corresponds to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculation was done using Graph Pad® Prism software (version 7).

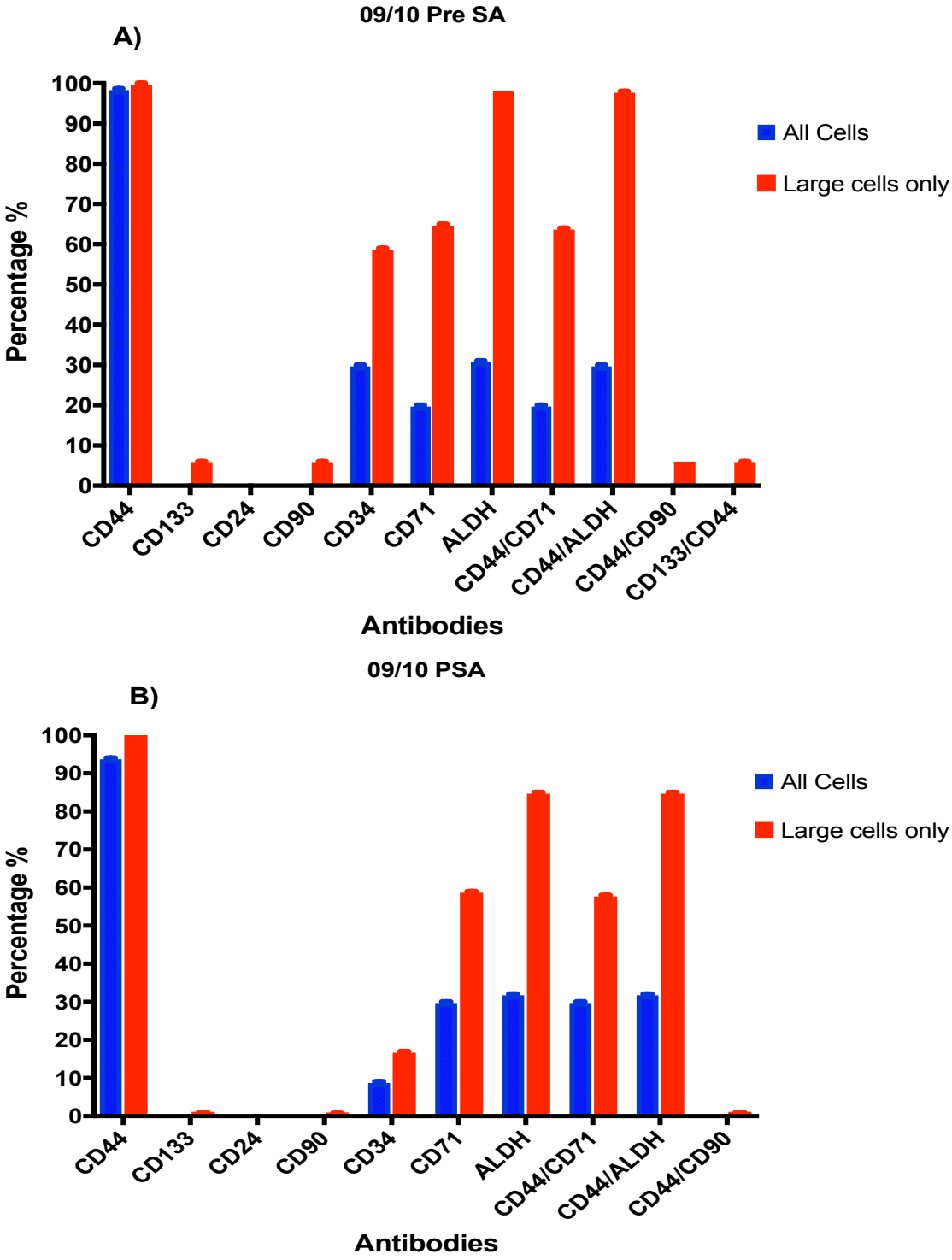


Figure 5. 24: 09/10 cell line. A) Pre-SA. **B)** Post-SA.

A comparison of different CD marker expression gating for all cell or large cells only, for cell line **09/10** Pre and Post-SA populations using flow cytometry. The expression was based on one distinct cell population of each analysis. Averages and calculations for each marker are found in the previous dot plot graphs. The data corresponds to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculation was done using Graph Pad® Prism software (version 7).

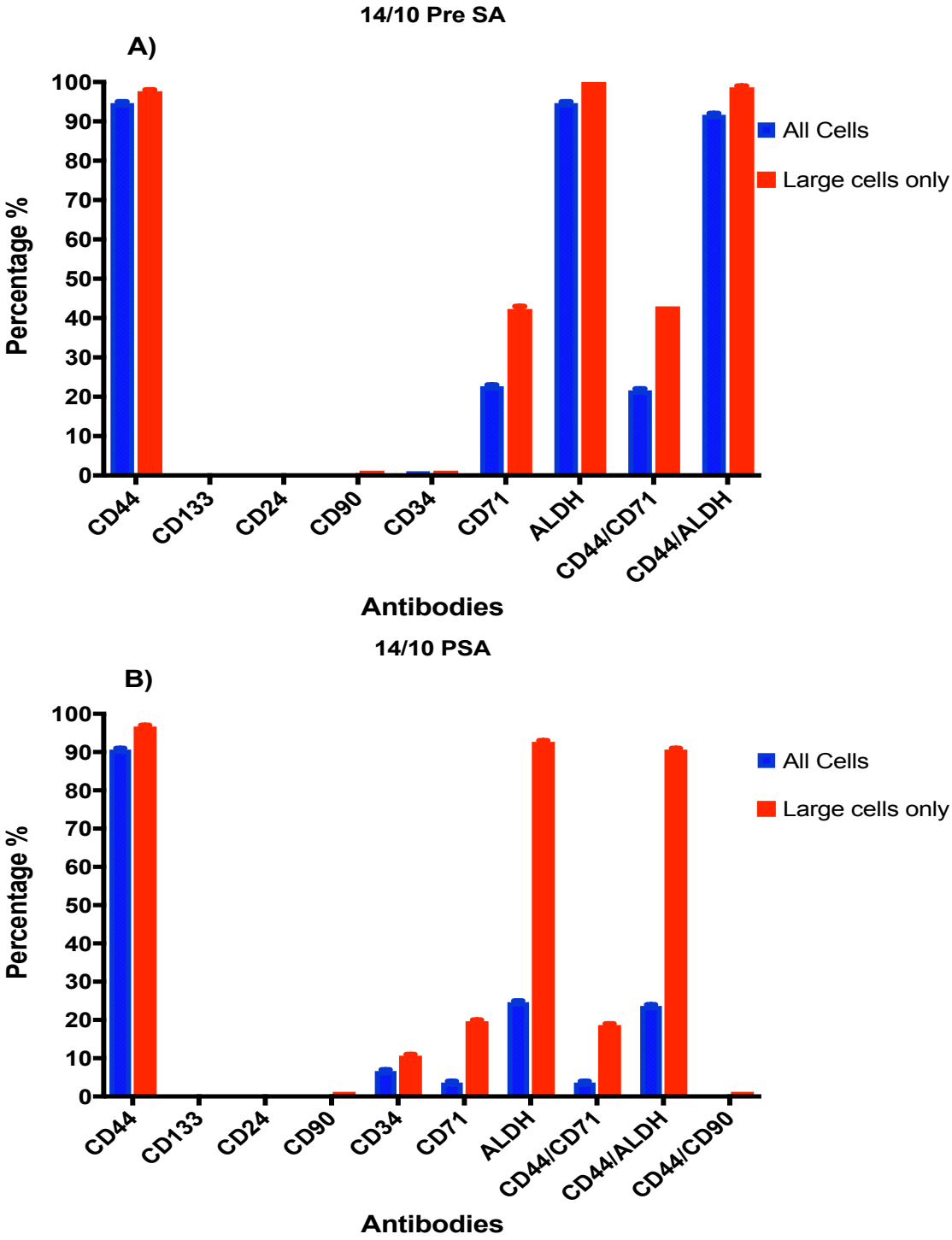


Figure 5. 25: 14/10 cell line. A) Pre-SA. **B)** Post-SA.

A comparison of different CD marker expression for gating all cells and large cells only, for cell line **14/10** Pre and Post-SA populations using flow cytometry. The expression was based on one distinct cell population of each analysis. Averages and calculations for each marker are found in the previous dot plot graphs. The data corresponds to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculation was done using Graph Pad® Prism software (version 7).

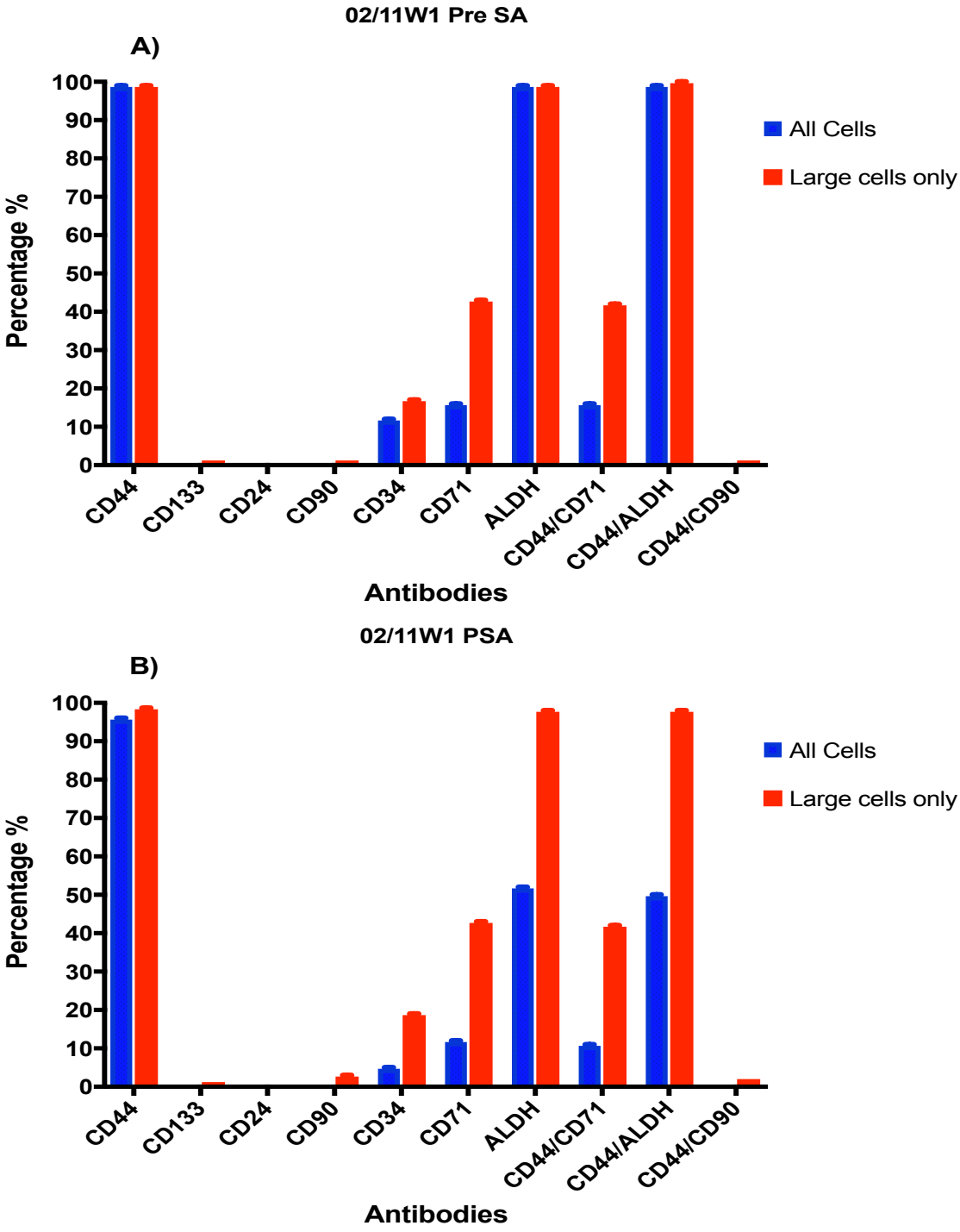


Figure 5. 26: 02/11W1 cell line. A) Pre-SA. **B)** Post-SA.

A comparison of different CD marker expression gating for all cells and large cells only, for cell line **02/11W1** Pre and Post-SA populations using flow cytometry. The expression was based on one distinct cell population of each analysis. Averages and calculations for each marker are found in the previous dot plot graphs. The data corresponds to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculation was done using Graph Pad® Prism software (version 7).

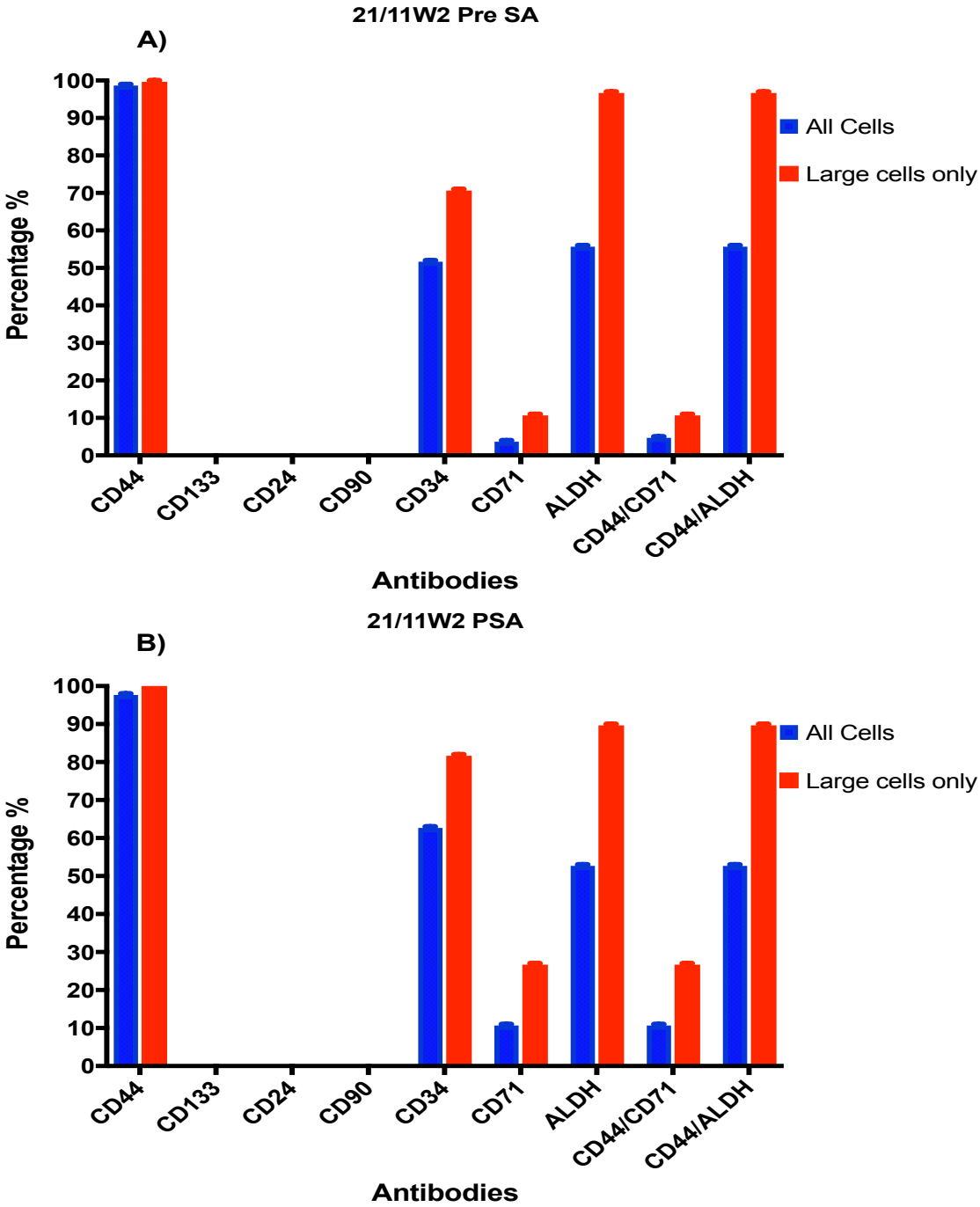


Figure 5. 27: 21/11W2 cell line. A) Pre-SA. **B)** Post-SA.

A comparison of different CD markers expression gating for all cells and large cells only, for cell line **21/11W2** Pre and Post-SA populations using flow cytometry. The expression was based on one distinct cell population of each analysis. Averages and calculations for each marker are found in the previous dot plot graphs. The data corresponds to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculation was done using Graph Pad® Prism software (version 7).

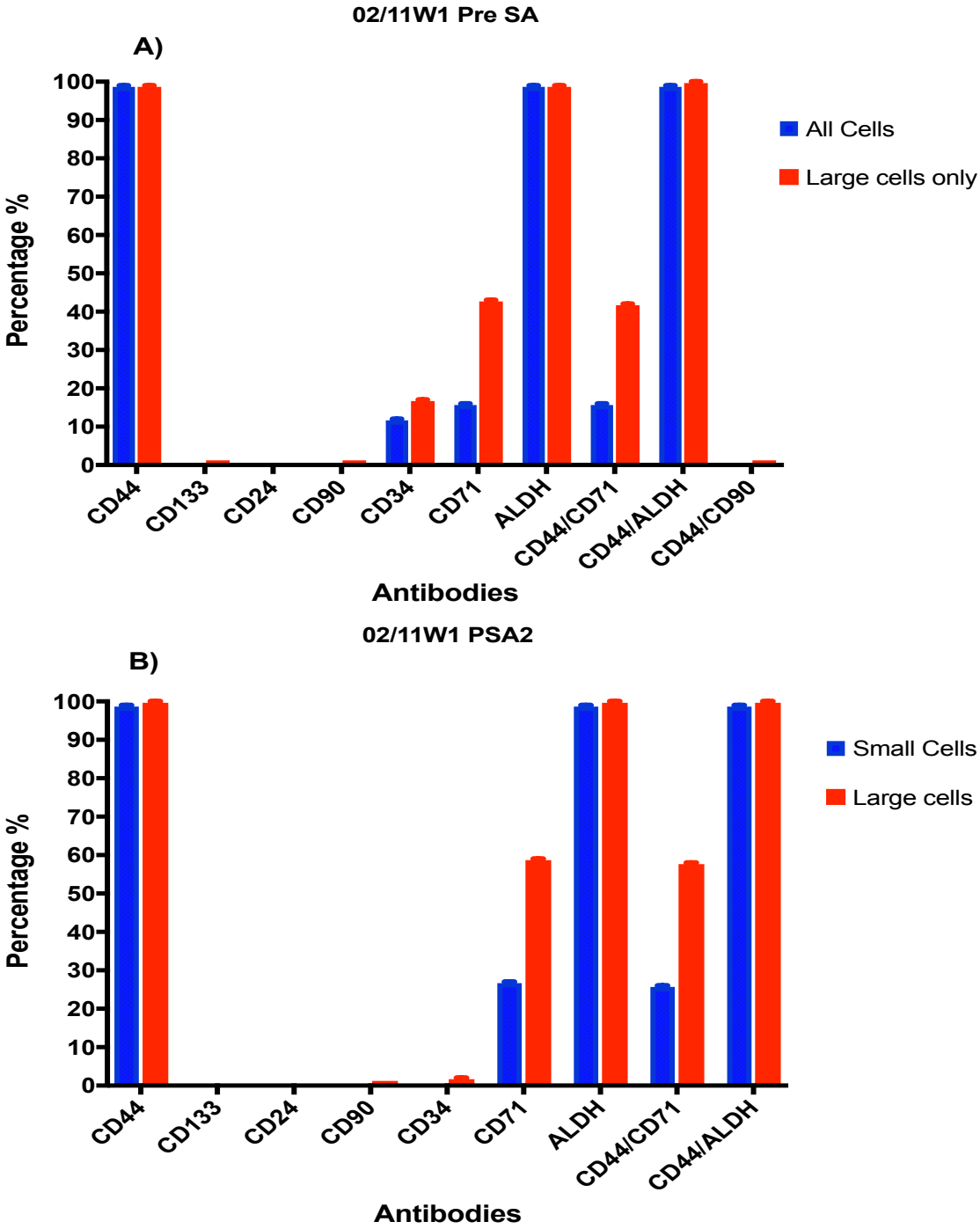


Figure 5. 28: 02/1W1 cell line. A) Pre-SA. **B)** Post-SA.

A comparison of different CD marker expression gating for all cells and large cells only, for cell line **02/11W1** Pre and Post-SA populations using flow cytometry. The expression was based on one distinct cell population of each analysis. Averages and calculations for each marker are found in the previous dot plot graphs. The data corresponds to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculation was done using Graph Pad® Prism software (version 7).

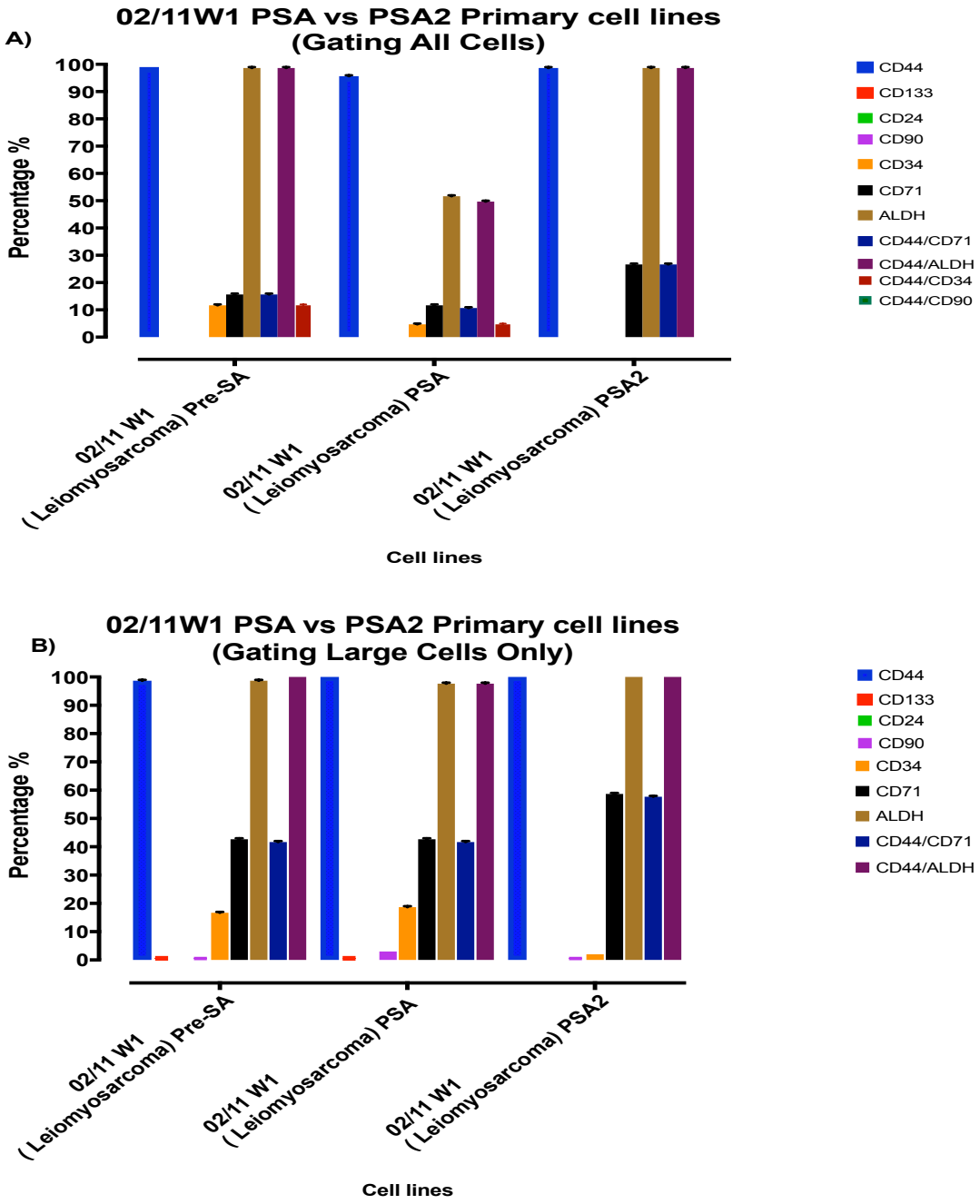


Figure 5. 29: Comparison for **02/11W1** cell line Pre-SA, Post-SA and Post-SA2 antibody expression. **A)** Gating all cells. **B)** Gating Large cells only**.**

A comparison of CD marker expression gating all cells and large cells only for gates, **for 02/11W1** for Pre Post stress assay phase two and three populations using flow cytometry. 02/11W2 Post-SA2 was the population of cells surviving the first SA and then recovered after another round of stress. The expression was based on one distinct cell population of each analysis. Averages and calculations for each marker are found in the previous dot plot graphs. The data corresponds to mean ± SEM from three independent experiments unless otherwise stated. SEM fitting and calculation was done using Graph Pad® Prism software (version 7).

5.2.8 Preliminary Analysis of Post-SA Cell Line 13/12W2 with New Potential Stem Cell Markers (CD19, CD154 and CD117)

These extra markers CD19, CD117 and CD154 have not been tested before on different sarcoma subtypes. CD117 stains haemopoietic adult stem cells and osteogenic progenitor cells, and co-expression is a possible indicator for osteosarcoma (Adhikari et al., 2010, He et al., 2012). CD34 and CD19 also identify haemopoietic adult stem cells and may have value for some sarcoma subtypes (Harris et al., 2009, Abbaszadegan et al., 2017, Brown et al., 2017, Skoda and Veselska, 2018). In addition, CD154 (CD40L) was found positive (low level) in sphere forming cells (tumour sphere) from renal carcinoma which was rich in CSC and found to express on haematopoietic and non-haematopoietic cells. Also, CD154 is co-stimulation molecule of the tumour necrosis factor family (TNF), and involve in other pathological process related to the immune system development (Seijkens et al., 2010, Shen et al., 2018), a panel was constructed to cover these new markers, also considering their co-expression with markers already tested, especially those not highly expressed. Unfortunately, due to technical challenges (difficulty in finding the right conjugate colours) and time limits, the analysis could not performed as planned on all the cell lines that survived the stress assay. Therefore as an initial test only 13/12W2 was selected for this study, and it was found that CD154 and CD19 were negative (0%), but interestingly CD117 showed 6% positivity (weak) and was co-expressed at 4% with CD71. Furthermore, the cells were then analyzed by gating only the large cells and CD19 and CD154 were again negative, 1% for both. Notably, there was however a large increase in expression for CD117 at 36% and correspondingly co-expression with CD71 was also increased to around 10% positivity (Figure 5.30 and Figure 5.31). This experiment was performed only on one cell line due to the nature of this experiment, and the time limit, and although these results are interesting they are preliminary and so further modifications and adjustments would be ideal before repeating in order to achieve more reliability.

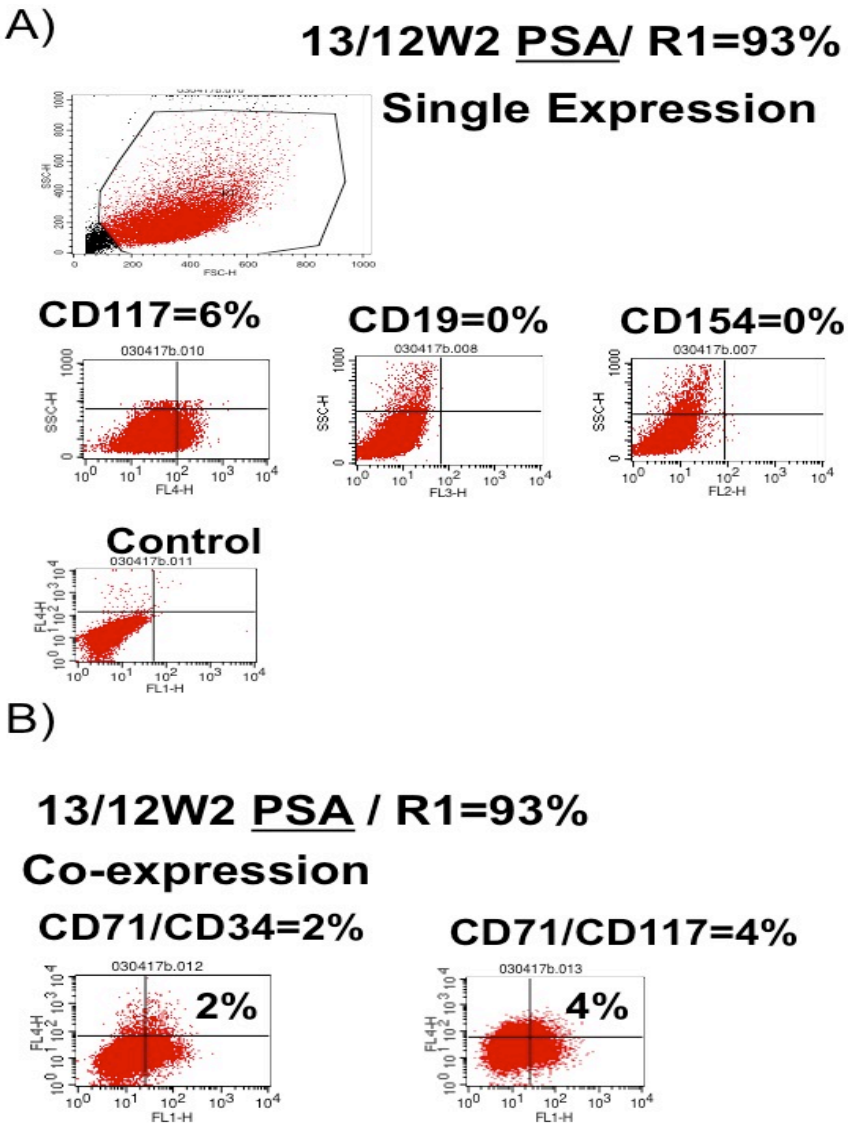


Figure 5. 30: These figures show the dot plot analysis of newly tested CD markers expression and co-expression on a Post-SA 13/12W2 using flow cytometry.

The expression was based on one distinct cell population. The CD117 expression was 6%; CD19 and CD154 expression was 0%, CD34 expression was 2% and CD71 expression was 7%. Co-expression was CD71/CD34=2%%, and CD71/117=4%. Data presented was from only one experiment; due to the time limit technical issues. Data percentages were analysed and fitted by flow cytometry dot plot software.

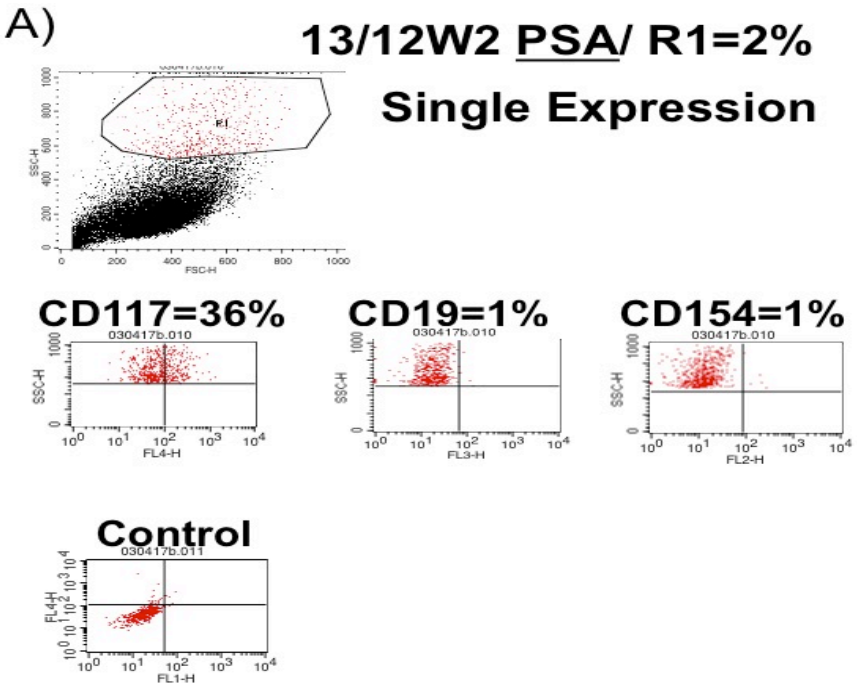


Figure 5. 31: These figures show the dot plot analysis of new tested CD markers expression and co-expression on Post-SA 13/12W2 POST-SA whilst gating large cells only using flow cytometry staining.

The expression was based on one distinct cell population. The CD117 expression was 36%, CD19 and CD154 expression was 1%, CD34 expression was 12% and CD71 expression was 37%. Co-expression was CD71/CD34=12%, and CD71/117=10%. Data presented was from only one experiment; due to the time limit and technical issues. Data percentages were analysed and fitted by flow cytometry dot plot software.

5.2.9 Statistical Analysis Using Linear Regression and Correlations

In chapter three and in this chapter (sections from 5.2.4 to 5.2.7) the expressions and co-expressions of potential CSC markers were investigated (Al-Hajj *et al*., 2003, Adhikari *et al*., 2010, Cruz *et al*., 2012, Joshua *et al*., 2012, Malanchi et al., 2012, Mannelli and Gallo, 2012, Trapasso and Allegra, 2012, Yang et al., 2015, Yuce *et al*., 2011). The expression of each marker was displayed as a dot plot individually or when co-expressed with other markers, and two gating strategies were applied on pre-SA and Post-SA cell lines. In order to better understand the relationship, statistical analysis of all antibodies tested on the sarcoma cell lines in this study was performed using linear regression (see section 2.2.24). It was hoped that this analysis could better explain some of the findings and differences when gating large cell only as opposed to all cells, and would assist if finding if any antibodies are correlated and linear to each other representing the same state. Initially, the focus was on cell lines that survived the stress assay, but the other cell lines were included later in the analysis and the linear regression analysis was also performed on PC3 as the positive control and h-TERT as the negative control. The overall findings of these results detailed below showed that strong correlations were detected between the new markers used in this study and the more standard ones CD44, CD133 and ALDH. Interestingly, these correlations were however diluted when gating all cells instead of gating only large cells. Also, the correlations of these markers were variable and so each cell line had it's own signature. The results were consistent with the previous gating analysis results of all cells and large cells only in sections 5.2.4, 5.2.5, 5.2.6 and 5.2.7. It is however important to remember that only single expression of the CD markers was performed in this preliminary statistical analysis. Pre and Post-SA expression levels were treated as one set of data, one as all cells gate and the second as large cells only. The correlations for pre and Post-SA separately and against each other (to look for any differences between them) in terms of all cells gate and large cells gate only showed stronger correlations in Post-SA large cells gates (data not shown here). However, pre and Post-SA percentages were treated as one set when the correlations analysis was carried out as shown below. The co-expression analysis was not carried out due to time limits and the enormous amount of data that would be generated and the difficulty to analyze and understand especially given that antibodies conjugation (colour) in co-expression limited the ability to do complete co expression analysis, so the decision was made to leave the in-depth analysis for future work and focus at this study on the single expression only.

5.2.9.1 SKUT-1 (Human Uterus Leiomyosarcoma (GIII))

SKUT-1 Strong Positive Correlations (R^2 from 1-0.8)

By selecting all gates data based on the R squared value reveals that CD34 correlates strongly with CD71, CD71 with CD133 and CD34 with CD133, respectively 0.8, 0.8 and 0.98. While gating large cells only, there are strong correlations between CD44 with ALDH; CD44 with CD34 and ALDH with CD34, R^2 results were 0.99, 0.95 and 0.9, respectively (Figure 5.32).

SKUT-1 Medium Positive Correlations (R^2 from 0.7-0.5)

Based on all gates R^2 results was 0.6 for ALDH with CD34. Gating large cells only found medium correlations for CD44 with CD71, ALDH with CD71 and CD34 with CD71. R^2 were 0.6, 0.6 and 0.5, respectively (Figure 5.32).

SKUT-1 Weak Positive Correlations (R^2 from 0.4-0)

Based on all gates CD44 weakly correlated with ALDH, CD44 with CD34, CD44 with CD71, ALDH with CD71 and CD133 with CD90 and R^2 values were 0.4, 0.02, 0.4 and 0.3, respectively. No week correlations were detected when gating for large cells (Figure 5.32).

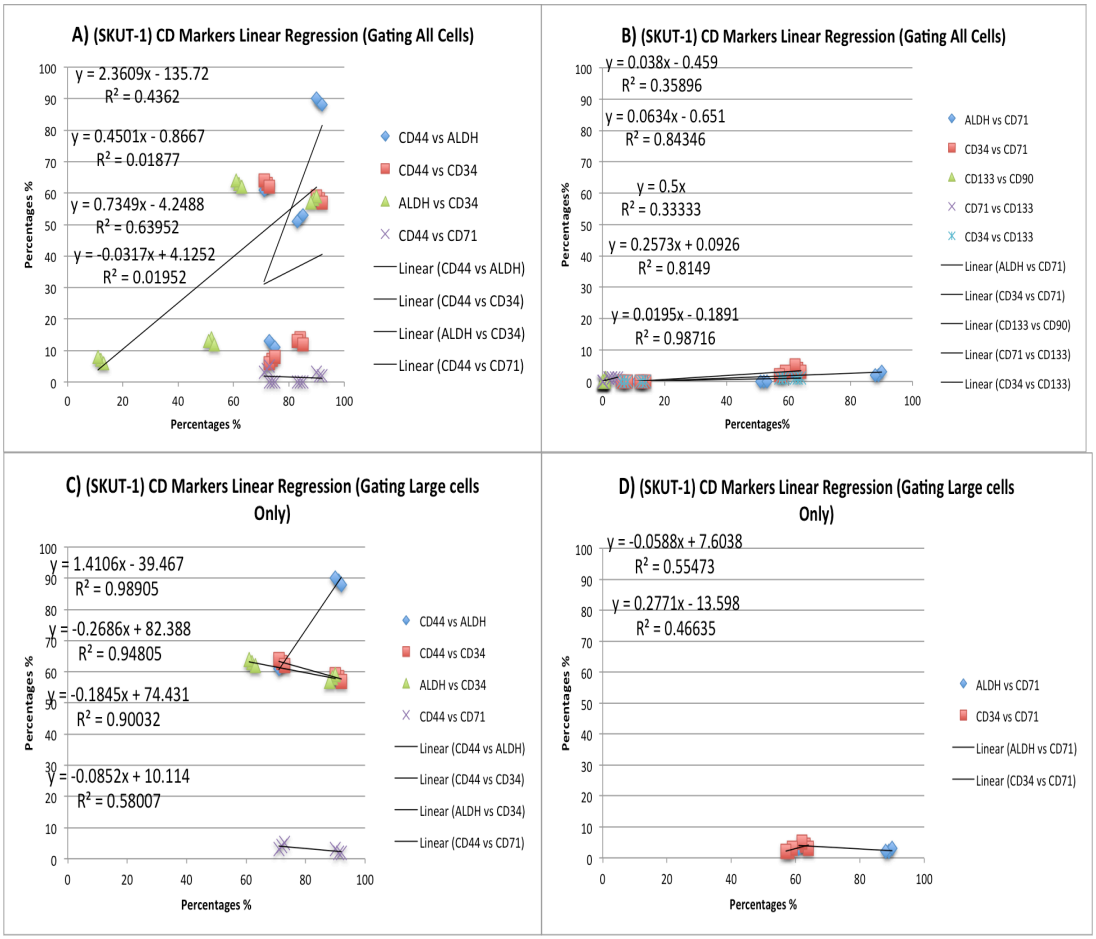


Figure 5. 32: Linear regression analysis of correlations between the expression of different CD markers (percentages) for **SKUT-1** cell line**.** Data represent correlations for Pre and Post-SA single expression results as they treated as one set to roll out (all gates) and (large cells only) differences and the possible dilution of the CD marker expressions between the two gates.

A) and B) show correlations based on all gates, while **C) and D)** show correlations based on gating large cells only. Data were based on the R squared value (**R²).** All calculations were done using Microsoft Excel (version 2011)..

5.2.9.2 STS (13/12W2) Pleomorphic NOS Sarcoma

13/12W2 Strong Positive Correlations (R² from 1-0.8)

Gating for all cells showed strong correlations for CD44 with ALDH, CD34, and CD71, R² values were 0.9, 0.8 and 0.8. ALDH correlation with CD71 was also strong =0.96.

Gating for large cells only similarly had strong correlations for CD44 with ALDH, CD34 and CD71, R² values were 0.8, 0.99 and 0.996, respectively. ALDH also correlated strongly with CD34 and CD71, R² values were 0.8 and 0.8, respectively. In addition, CD34 was found to be strongly correlated with CD71, R² was 0.97 (Figure 5.33).

13/12W2 Medium Positive Correlations (R^2 from 0.7-0.5)

Medium correlations were detected only when gating for all cells and CD34 correlated with ALDH and CD71, and R^2 values were 0.6 and 0.7 respectively. No medium correlations were detected when gating for just large cells, and it is important that these medium correlations found when gating for all cells were strong correlations on large cells only as mentioned above (Figure 5.33).

13/12W2 Weak Positive Correlations (R^2 from 0.4-0)

No weak correlations were detected on both gates (all gates and large cells) for this cell line (Figure 5.33).

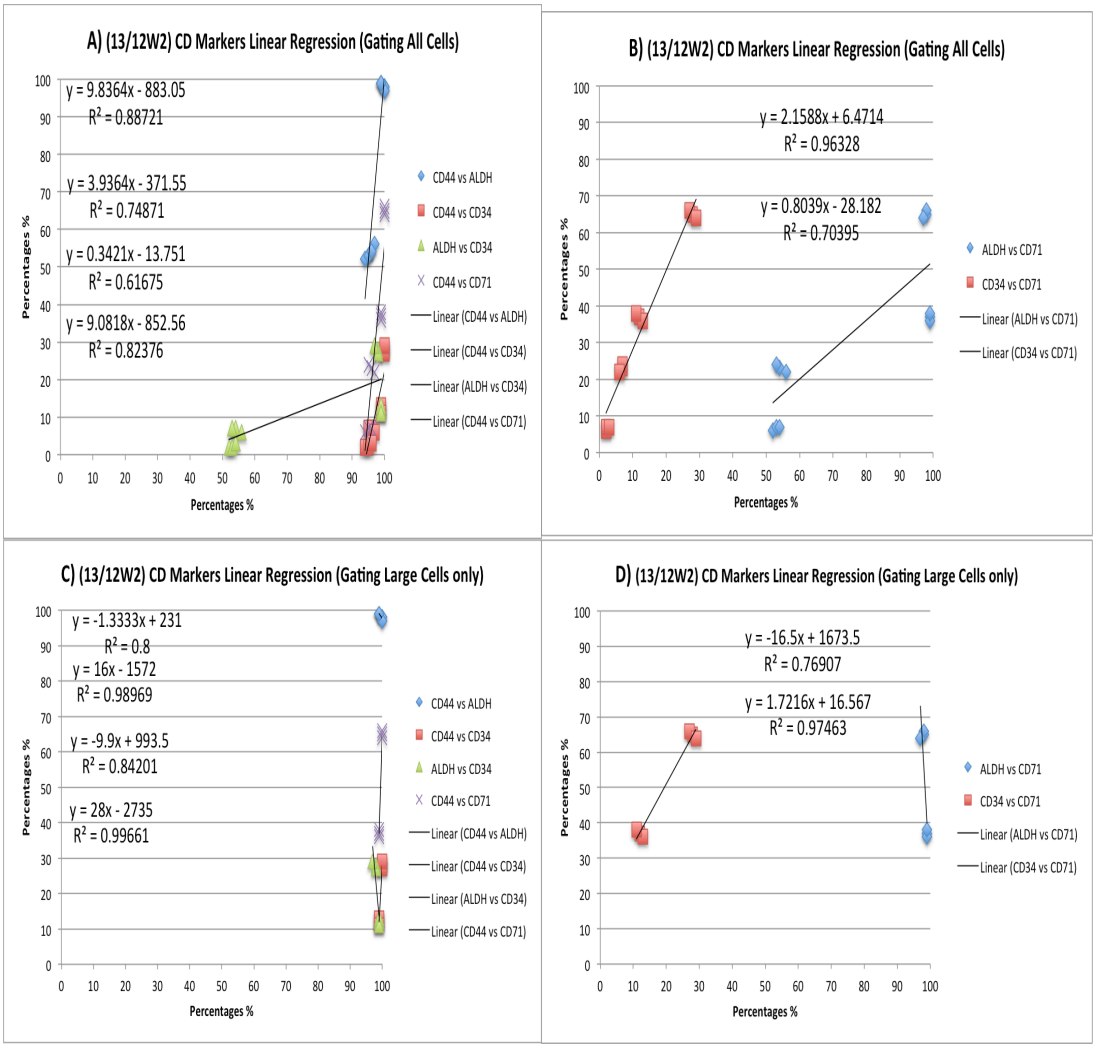


Figure 5. 33: Linear regression analysis of correlations between the expression of different CD markers (percentages) for **13/12W2** cell line. Data represent correlations for Pre and Post-SA single expression results as they treated as one set to roll out (all gates) and (large cells only) differences and the possible dilution of the CD marker expressions between the two gates.

A) and B) show correlations based on all gates, while **C) and D)** show correlations based on gating large cells only. Data were based on the R squared value (**R^2).** All calculations were done using Microsoft Excel (version 2011).

5.2.9.3 STS (09/10) Dedifferentiated Sarcoma

09/10 Strong Positive Correlations (R^2 from 1-0.8)

Gating all cells had strong correlations between ALDH and CD71 and CD133 with CD90 R^2 values were 0.97 and 1 respectively. Gating large cells only showed strong correlations for ALDH with CD34 and CD71 R^2 values were 0.99 and 0.9 respectively. Also CD71 was correlated with CD34 and CD133 R^2 values were 0.9 and 0.8 respectively and CD133 correlated with CD90 R^2 value was 1 (Figure 5.34).

09/10 Medium Positive Correlations (R^2 from 0.7-0.5)

Gating all cells produced medium correlations for CD44 with ALDH and CD71 with CD133, R^2 values were 0.5 and 0.6 respectively. No medium correlations were detected when gating for large cells only as they were already strongly correlated (Figure 5.34).

09/10 Weak Positive Correlations (R^2 from 0.4-0)

No weak correlations were detected on both gates (all gates and large cells only gate) for this cell line (Figure 5.34).

5.2.9.4 STS (14/10) Pleomorphic NOS Sarcoma

14/10 Strong Positive Correlations (R^2 from 1-0.8)

Gating all cells produced strong correlations between CD44 and ALDH with a 0.9 R^2 value. Remarkably when gating just large cells strong correlations were found for all antibodies, ALDH was correlated with CD34 and CD71, R^2 values were 0.9 and 0.95 respectively and CD34 with CD71 $R^2 = 0.99$ (Figure 5.35).

14/10 Medium Positive Correlations (R^2 from 0.7-0.5)

Medium correlations for ALDH with CD34 and CD71 had R^2 values of 0.7 when gating for all cells and no correlations were found for large cells gates (Figure 5.35).

14/10 Weak Positive Correlations (R^2 from 0.4-0)

There were weak correlations when gating for all cells results between CD44 with CD34, CD44 with CD71, CD34 with CD71 and R^2 values were 0.02, 0.1 and 0.3. Large cells only gating had weak correlations for CD44 with ALDH, CD44 with CD34 and CD44 with CD71 with R^2 values of 0.3, 0.1 and 0.2 respectively (Figure 5.35).

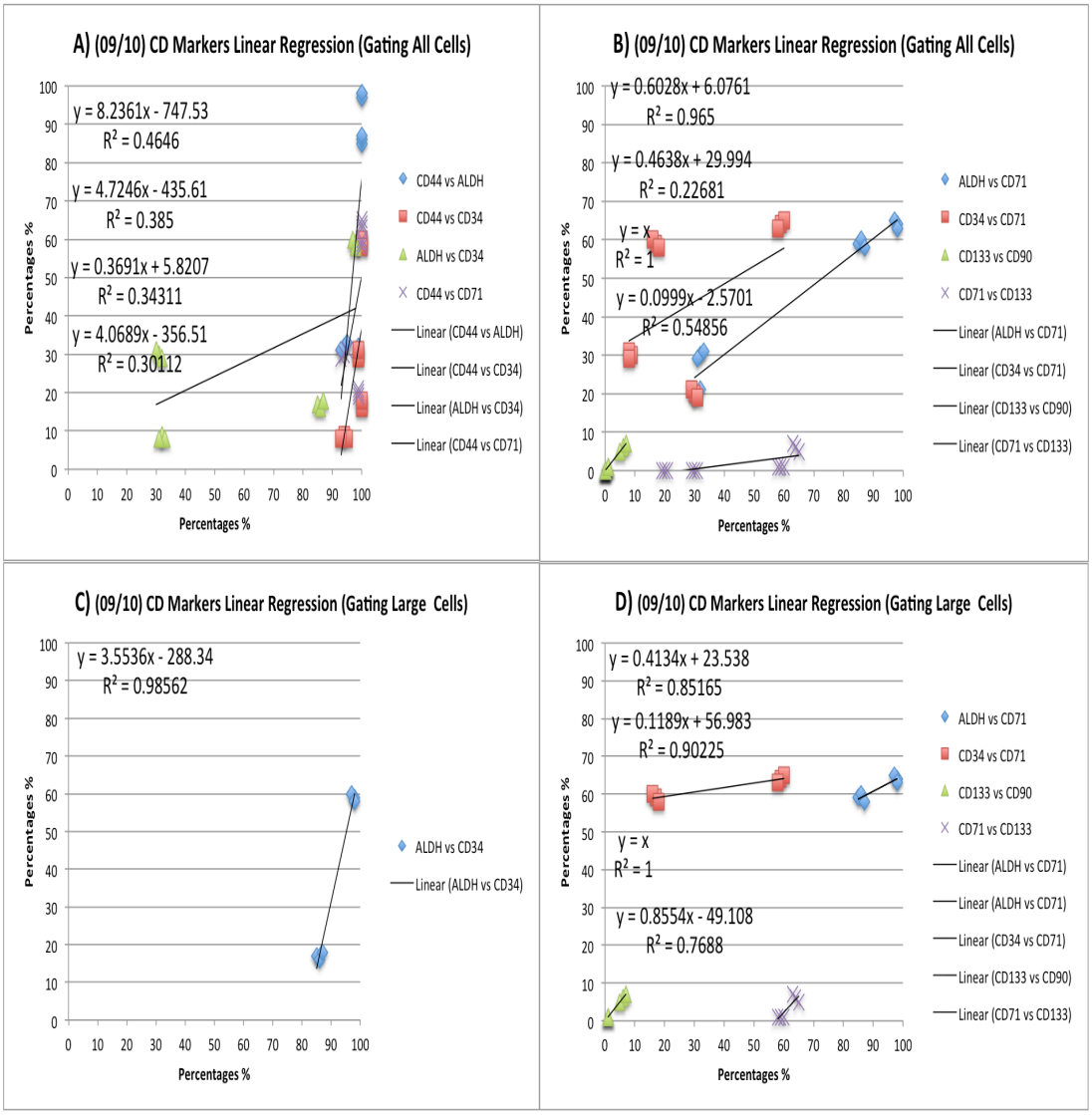


Figure 5. 34: Linear regression analysis of correlations between the expression of different CD markers (percentages) for **09/10** cell line**.** Data represent correlations for Pre and Post-SA single expression results as they treated as one set to roll out (all gates) and (large cells only) differences and the possible dilution of the CD marker expressions between the two gates.

A) and B) show correlations based on all gates, while **C) and D)** show correlations based on gating large cells only. Data were based on the R squared value (**R²).** All calculations were done using Microsoft Excel (version 2011).

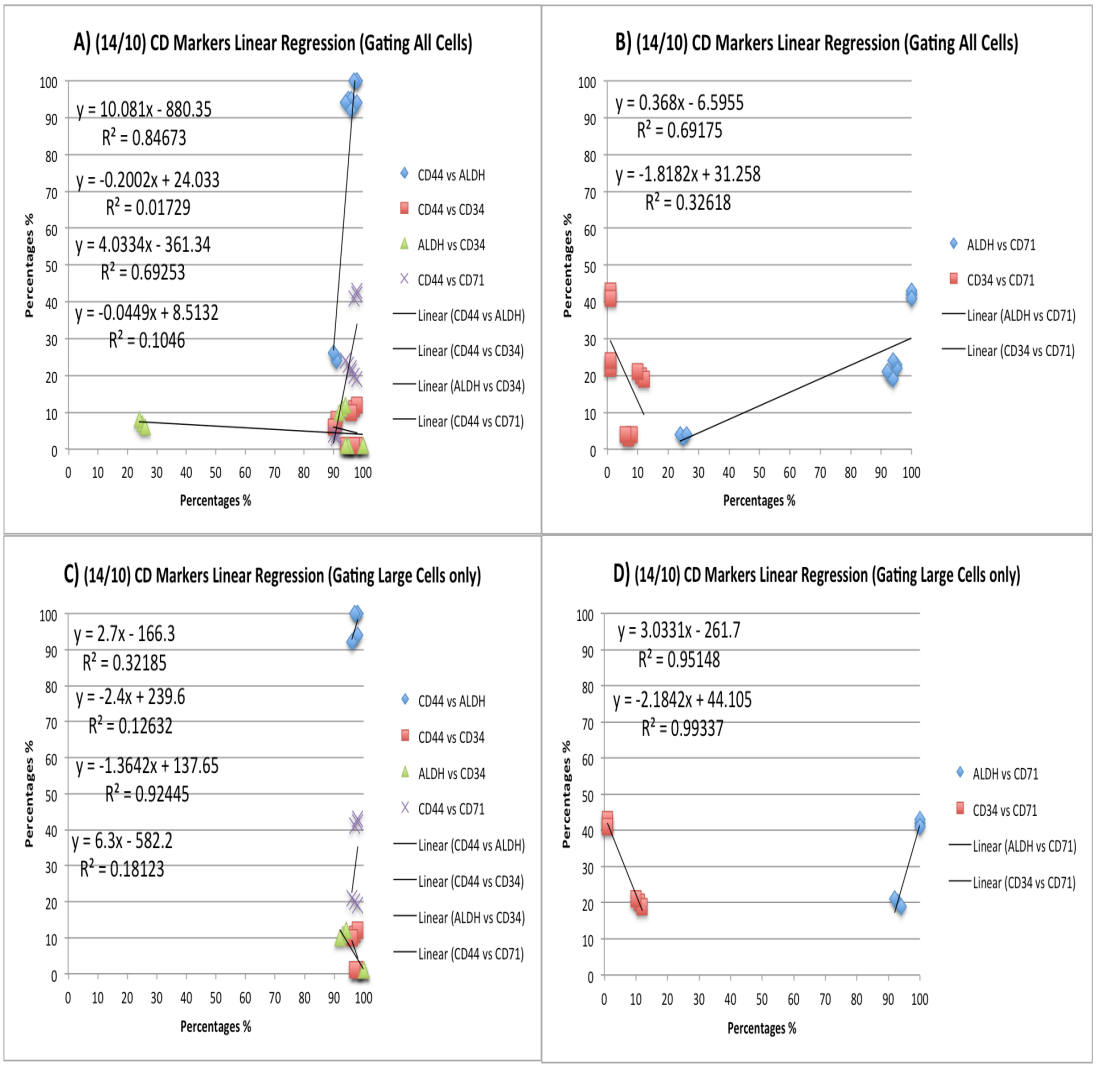


Figure 5. 35: Linear regression analysis of correlations between the expression of different CD markers (percentages) for **14/10 cell line.** Data represent correlations for Pre and Post-SA single expression results as they treated as one set to roll out (all gates) and (large cells only) differences and the possible dilution of the CD marker expressions between the two gates.

A) and B) show correlations based on all gates, while **C) and D)** show correlations based on gating large cells only. Data were based on the R squared value (**R^2).** All calculations were done using Microsoft Excel (version 2011).

5.2.9.5 STS (02/11W1) Leiomyosarcoma

02/11W1 Strong Positive Correlations (R^2 from 1-0.8)

No strong correlations were found when gating for all cells. However, just gating large cells had strong correlations for all antibodies, CD44 was correlated with ALDH, CD44 with CD34, ALDH with CD34, CD44 with CD71, ALDH with CD71 and CD34 with CD71, R^2 values were 1, 0.8, 0.8, 0.8, 0.8, 0.97, respectively (Figure 5.36).

02/11W1 Medium Positive Correlations (R^2 from 0.7-0.5)

The only medium correlation found was when gating for all cells and that was between CD133 and CD90 with a value of 0.6 R^2 (Figure 5.36).

02/11W1 Weak Positive Correlations (R^2 from 0.4-0)

When gating for all cells weak correlations were found for CD44 with ALDH, CD44 with CD34, ALDH with CD34, CD44 with CD71, ALDH with CD71, CD34 with CD71 and CD71 with CD133, R^2 values were 0.4, 0.01, 0.3, 0.04, 0.3, 0.01 and 0.2 respectively. In addition, CD133 with CD90 was also weak with R^2 value of 0.2 (Figure 5.36).

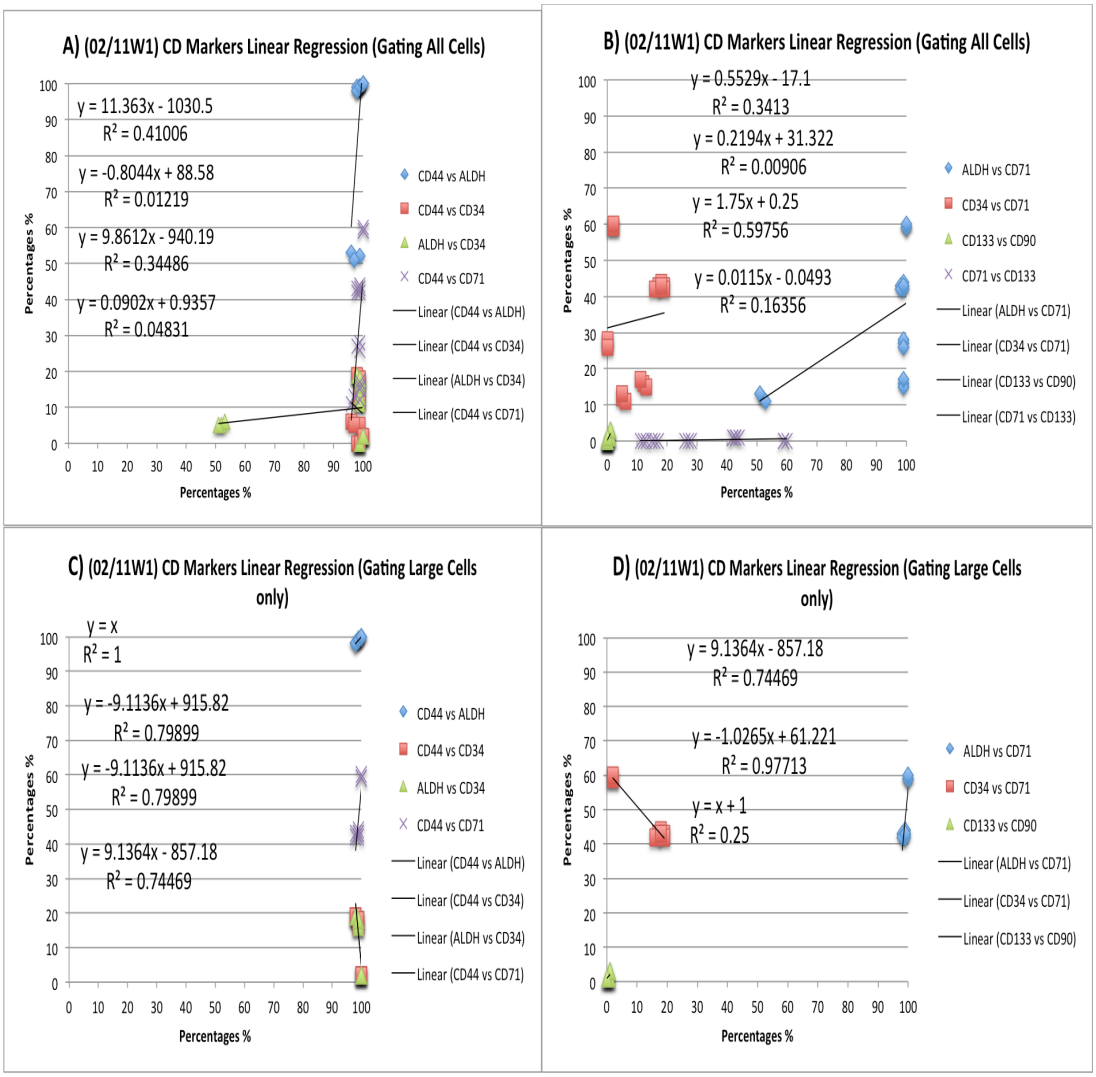


Figure 5. 36: Linear regression analysis of correlations between the expression of different CD markers (percentages) for **02/11W1** cell line**.** Data represent correlations for Pre and Post-SA single expression results as they treated as one set to roll out (all gates) and (large cells only) differences and the possible dilution of the CD marker expressions between the two gates.

A) and B) show correlations based on all gates, while **C) and D)** show correlations based on gating large cells only. Data were based on the R squared value (**R^2).** All calculations were done using Microsoft Excel (version 2011).

5.2.9.6 STS (21/11W2) Myxofibrosarcoma

21/11W2 Strong Positive Correlations (R^2 from 1-0.8)

When gating for all cells strong correlations were found for CD44 with ALDH and CD34 with CD71 with R^2 equals of 0.8 and 0.9 respectively. On the other hand gating for just large cells had strong correlations between ALDH with CD34, ALDH with CD71 and CD34 with CD71 with R^2 values of 0.9, 0.9 and 0.97 respectively (Figure 5.37).

21/11W2 Medium Positive Correlations (R^2 from 0.7-0.5)

The only medium correlation found when gating for all cells and was between ALDH with CD34 with a 0.6 R^2 value **(**Figure 5.37).

21/11W2 Weak Positive Correlations (R^2 from 0.4-0)

Weak correlations were detected between CD44 with CD34, ALDH with CD71 and CD44 with CD71 with 0.4, 0.3 and 0.3 R^2 values respectively but only when gating for all cells. No weak correlations were observed in the large cells gates (Figure 5.37).

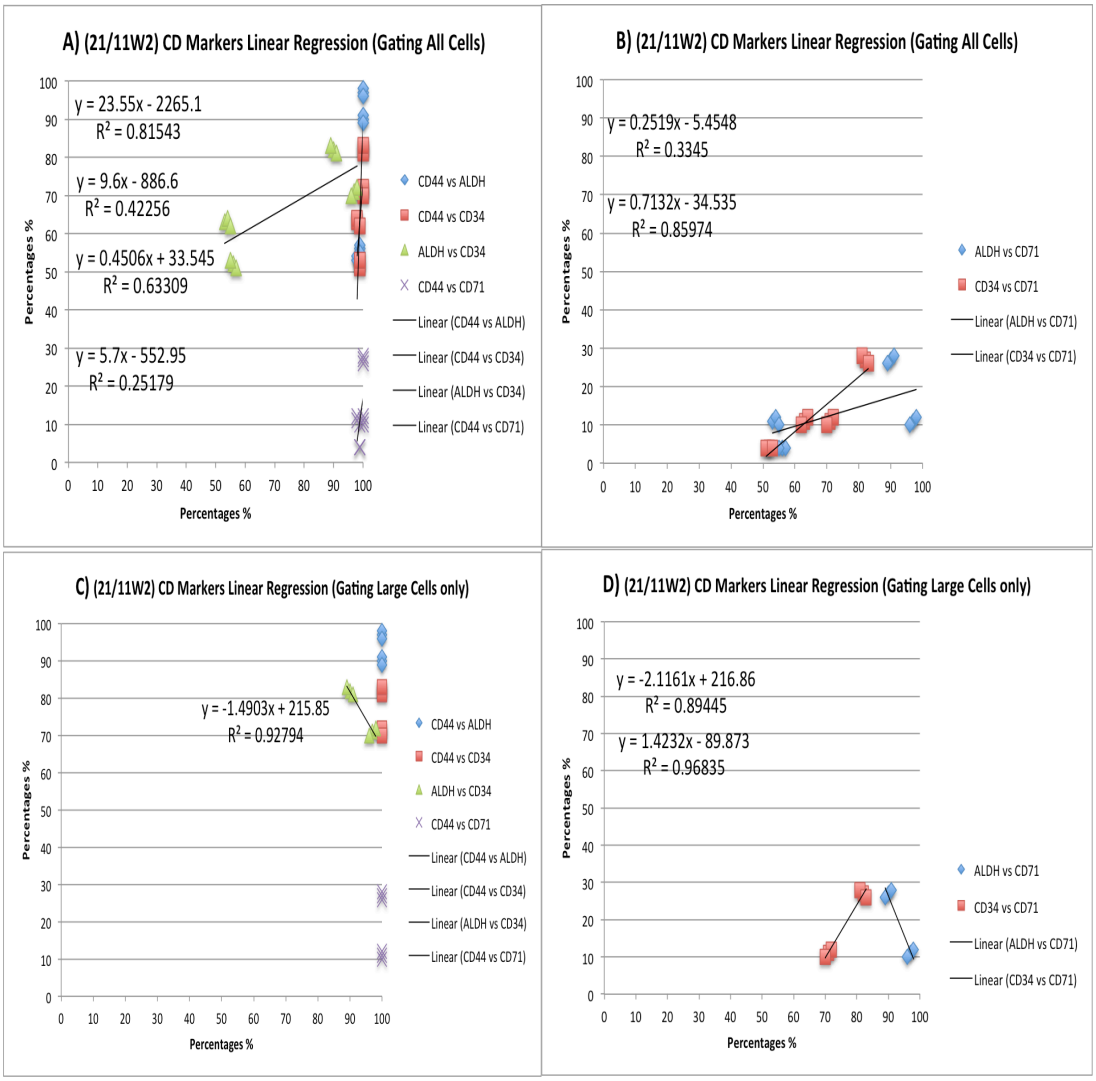


Figure 5. 37: Linear regression analysis of correlations between the expression of different CD markers (percentages) (percentages) for 21/11W2 cell line. Data represent correlations for Pre and Post-SA single expression results as they treated as one set to roll out (all gates) and (large cells only) differences and the possible dilution of the CD marker expressions between the two gates.

A) and B) show correlations based on all gates, while **C) and D)** show correlations based on gating large cells only. Data were based on the R squared value (**R^2).** All calculations were done using Microsoft Excel (version 2011).

5.2.9.7 Overview of Correlations for All Sarcoma Cell Lines

It is important to bear in mind there was a consistent pattern across the sarcoma cell lines to have more strong correlations when just gating for just large cells. Furthermore, often correlations that were weak when gating for all cells were seen to be strong when just the large cells were gated for, and this was a consistent pattern for individual sarcoma cell lines.

Strong Positive Correlations for All Sarcoma Cell Lines (R^2 from 1-0.8)

When gating for all cells the only strong correlations found across all sarcoma cell lines was between CD34 with CD71 with a 0.8 R^2 value. However, when gating just large cells strong correlations were found between CD133 with CD90 and CD44 with ALDH, 0.8 R^2 values for both (Figure 5.38).

All Sarcoma Cell Lines Medium Positive Correlations (R^2 from 0.7-0.5)

No medium correlations were detected on both gates (all gates and large cells) (Figure 5.38).

All Sarcoma Cell Lines Weak Positive Correlations (R^2 from 0.4-0)

When considering all sarcoma cell lines together there were mostly weak correlations. Gating for all cells had weak correlations for CD44 with ALDH, CD44 with CD34, ALDH with CD34, CD44 with CD71, ALDH with CD71, CD133 with CD90 and CD71 with CD133; R^2 values were 0.4, 0.0, 0.03, 0.2, 0.3, 0.0 and 0.2 respectively. Large cell only gating also displayed weak correlations for ALDH with CD71, CD44 with CD34, CD44 with CD71, CD34 with CD71 and CD71 with CD133; with R^2 values of 0.2, 0.01, 0.3, 0.02 and 0.1 respectively. No correlation was detected for ALDH VS CD34 (Figure 5.38).

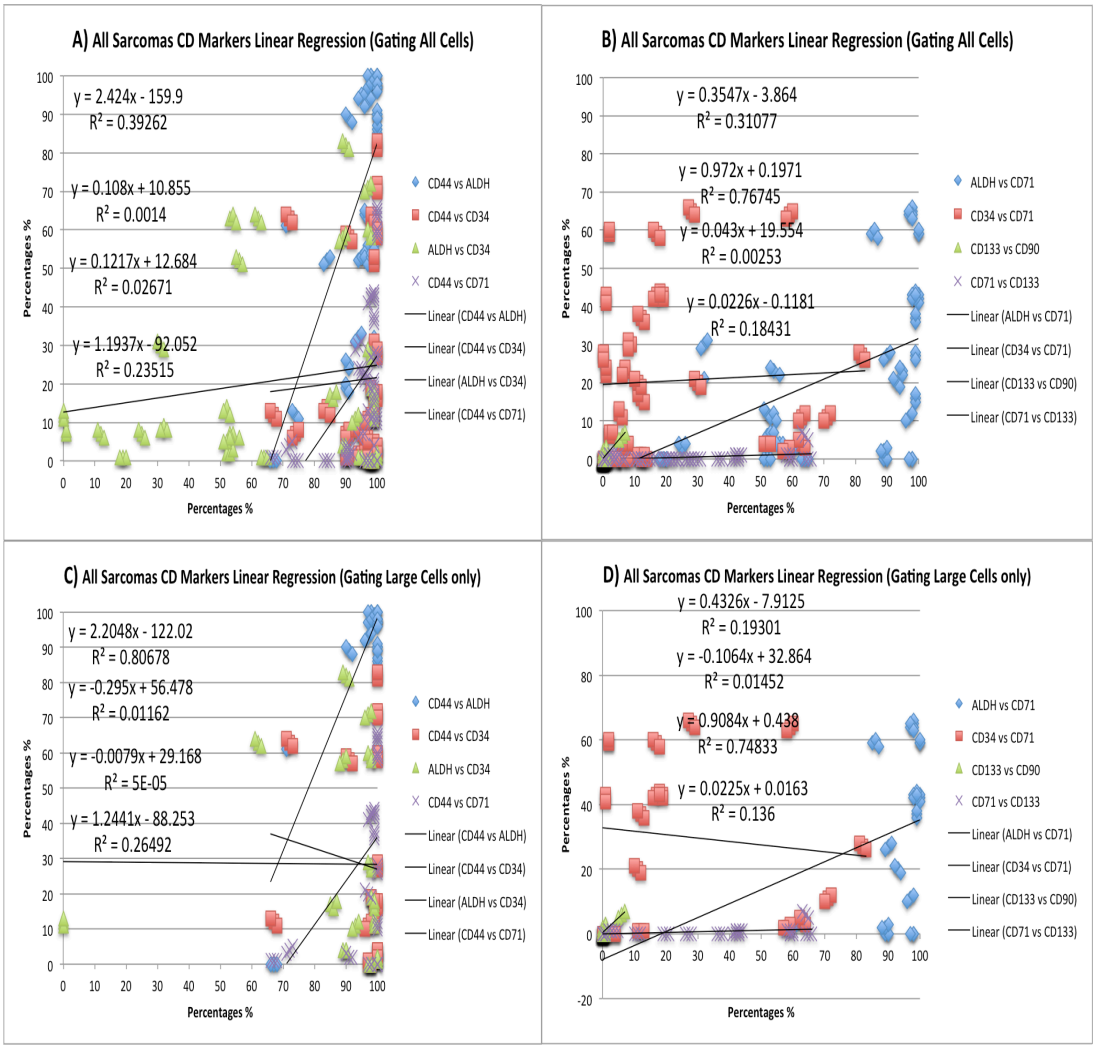


Figure 5. 38: Linear regression analysis of correlations between the expression of different CD markers (percentages) for all sarcoma Pre-SA and Post-SA cell lines**.** Data represent correlations for Pre and Post-SA single expression results as they treated as one set to roll out (all gates) and (large cells only) differences and the possible dilution of the CD marker expressions between the two gates.

A) and B) show correlations based on all gates, while **C) and D)** show correlations based on gating large cells only. Data were based on the R squared value (**R²).** All calculations were done using Microsoft Excel (version 2011).

5.2.9.8 Prostate Cancer Cell Line (PC3)

Gating for all cells had only one strong correlation between ALDH with CD34; the R² value was 0.9. Interestingly the large cells gates analysis also only had one strong correlation between CD44 with CD71 and the R² value was 1. No medium correlations were detected on both gates (all gates and large cells). Gating for all cells had weak correlations for CD44 with ALDH, CD44 with CD34 and CD44 with CD71; and R² values were 0.0, 0.01 and 0.05 respectively. Large cells only gating found weak correlations for CD44 with

ALDH, CD44 with CD34 and ALDH with CD34; R^2 values were 0.3, 0.3 and 0.3 respectively (Figure 5.39).

5.2.9.9 Human Epithelial Retinal Cells (hTERT-RPE-1)

The only strong correlation detected was between CD44 with ALDH; the R^2 value was 0.97 and this was gating for all cells. No correlations were measured when gating for in large cells only, as there were no large cells to gate (Figure 5.39).

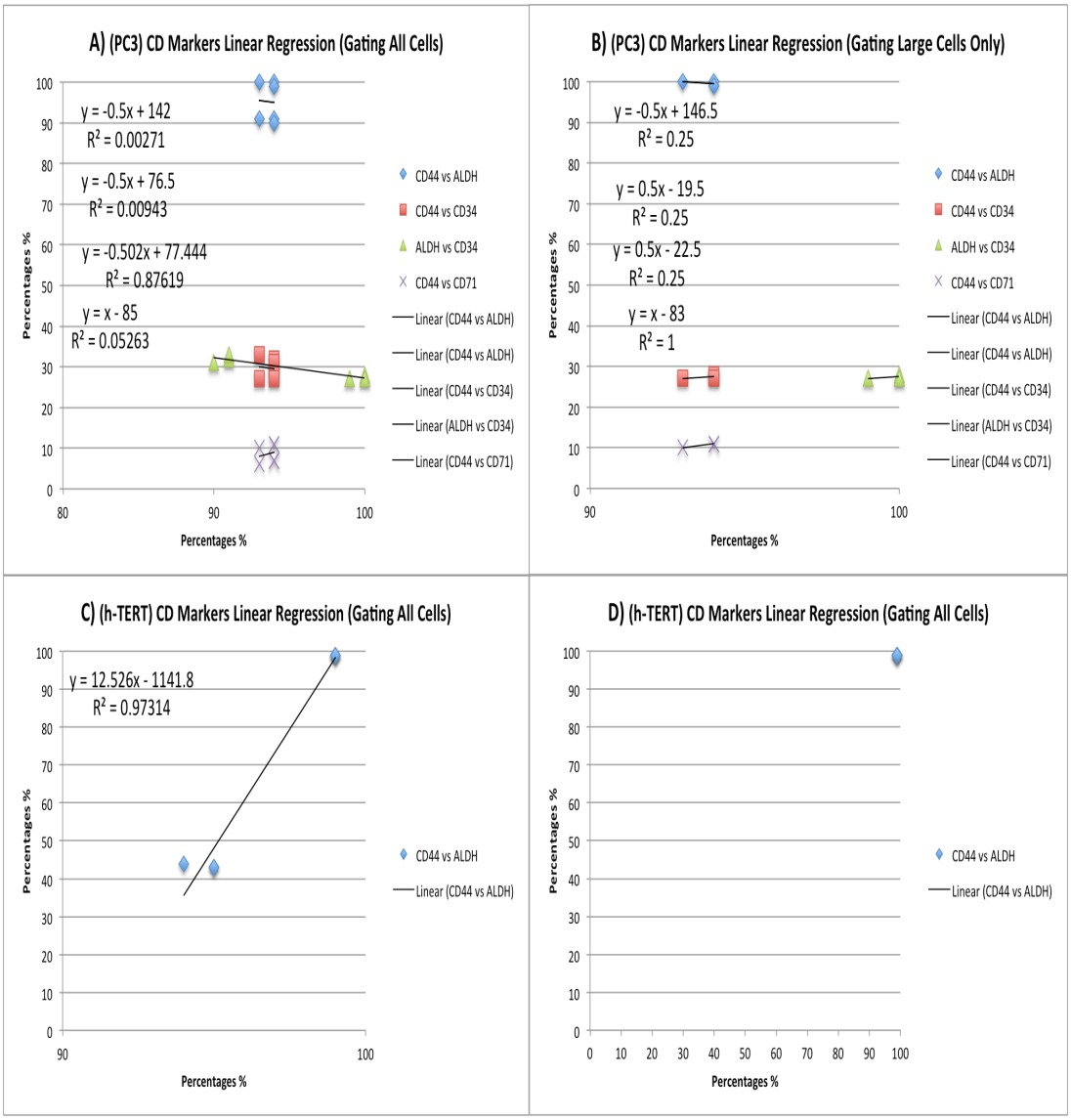


Figure 5. 39: Linear regression analysis of correlations between the expression of different CD markers (percentages) for **PC3 and h-TERT Pre-SA** cell lines (both used as a control for this study).

A) and B) show correlations for PC3 based on all gates and large cells only, while **C) and D)** show correlations for h-TERT based on gating all and large cells only, respectively. Data were based on the R squared value (**R^2).** All calculations were done using Microsoft Excel (version 2011).

In conclusion, linear regression correlation analysis was performed to try to better understand their relationship and indicate how reliably they may be able to identify a CSC subpopulation. Linear regression as a statistical method can be used to assess the coefficient of determination and correlation coefficient between two variables; if there are any associations, this descriptive method aims to show data (independent and dependent variables) not linear to each other and convert into linear relation. The variables in our case were the known CSC and the suggested CSC markers. However, these correlations do not mean that these results were final and conclusive in this preliminary stage of analysis. Undoubtedly, more in depth statistical analysis is needed to have a better understanding. Due to the enormous amount of data that will be generated, the time limit and other reasons mentioned at the beginning of this section a full analysis could not be performed in this PhD study. In general, more strong correlations were found when just gating for large cells only, but there was variability between different sarcoma cell lines, and this may suggest that certain potential CSC markers are of relevance for some sarcoma cell types only. The same outcome was found when this analysis was applied to combined analysis of all sarcoma cell lines and PC3. Also, it suggested that the relevance of the new markers used in this study could be considered as a new tool in detecting CSC subpopulations. The analysis also showed (data not included) a notable difference between Pre and Post-SA sarcoma cell lines when gating for large cells only between CD34, CD44, CD71 and ALDH with R^2 values of 1. In other words POST-SA had stronger correlations between these markers, and the CD44 and ALDH correlations were increased, which could indicate the same cell state. Also, Post-SA populations gated for large cells only had stronger relations (R^2 =1) between CD44, ALDH and CD34 on SKUT-1.

Ultimately, the importance of using the more commonly known CSC markers and more newly identified markers in terms of detecting CSC populations is very dependent on correctly identifying and gating for the relevant population. Here is suggested that gating for large cells is associated with increased expression of many of these putative markers. As there large cells were observed to be the ones that gave rise to recovered populations Post-SA it is suggestive that the enrichment for these markers correlates with the ability to survive, and potentially suggests these are putative CSC. A finding that is also supported by the observation that the Post-SA population had comparably higher expression and more associations when large cells only were gated. In addition, these remarks also reflect the observation of the shift from medium and weak R^2 values to a strong correlation when gating for large cells was applied as summarized in Table 5.6.

Cell line	Strong R^2 Value (0.8-1) Single expression	Medium R^2 Value (0.5-0.7) Single expression	Weak R^2 Value (0-0.4) Single expression
SKUT-1 All gates	CD34 vs. CD71 CD71 vs. CD133 CD34 vs. CD133	ALHD vs. CD34	CD44 vs. ALDH CD44 vs. CD34 CD44 vs. CD71 ALDH vs. CD71 CD133 vs. CD90
SKUT-1 Gating Large cells	CD44 vs. ALDH CD44 vs. CD34 ALDH vs. CD34	CD44 vs. CD71 ALDH vs. CD71 CD34 vs. CD71	-
13/12W2 All gates	CD44 vs. ALDH CD44 vs. CD34 CD44 vs. CD71 ALDH vs. CD71	CD34 vs. ALDH CD34 vs. CD71	-
13/12W2 Gating Large cells	CD44 vs. ALDH CD44 vs. CD34 CD44 vs. CD71 ALDH vs. CD34 ALDH vs. CD71 CD34 vs. CD71	-	-
09/10 All gates	ALDH vs. CD71 CD133 vs. CD90	CD44 vs. ALDH CD44 vs. CD71	-
09/10 Gating Large cells	ALDH vs. CD34 ALDH vs. CD71 CD71 vs. CD34	-	-
14/10 All gates	CD44 vs. ALDH	ALDH vs. CD34 ALDHC vs. D71	CD44 vs. CD34 CD44 vs. CD71 CD34 vs. CD71
14/10 Gating Large cells	ALDH vs. CD34 ALDH vs. CD71 CD71 vs. CD34	-	CD44 vs. ALDH CD44 vs. CD34 CD44 vs. CD71
02/11W1 All gates	-	CD133 vs. CD90	CD44 vs. ALDH CD44 vs. CD34 ALDH vs. CD34 CD44 vs. CD71 ALDH vs. CD71 CD34 vs. CD71 CD71 vs. CD133 CD133 vs. CD90
02/11W1 Gating Large cells	CD44 vs. ALDH CD44 vs. CD34 ALDHCD34 CD44 vs. CD71 ALDH vs. CD71 CD34 vs. CD71	-	-
21/11W2 All gates	CD44 vs. ALDH CD34 vs. CD71	ALDH vs. CD34	CD44 vs. CD34 ALDHC vs. D71 CD44 vs. CD71
21/11W2 Gating Large cells	ALDH vs. CD34 ALDH vs. CD71 CD34 vs. CD71	-	-

All sarcoma cell lines All gates	CD34 vs. CD71	-	CD44 vs. ALDH CD44 vs. CD34 ALDH vs. CD34 CD44 vs. CD71 ALDH vs. CD71 CD133 vs. CD90 CD71 vs. CD133
All sarcoma cell lines Gating Large cells	CD133 vs. CD90 CD44 vs. ALDH	-	ALDH vs. CD71 CD44 vs. CD34 CD44 vs. CD71 CD34 vs. CD71 CD71 vs. CD133
PC3 All gates	ALDH vs. CD34	-	CD44 vs. ALDH CD44 vs. CD34 CD44 vs. CD71
PC3 Gating Large cells	CD44 vs. CD71	-	CD44 vs. ALDH CD44 vs. CD34 CD34 vs. ALDH
h-TERT-RPE-1 All gates	CD44 vs. ALDH	-	-
h-TERT-RPE-1 Gating Large cells	-	-	-

Table 5. 6: Summary of the results of linear regression performed in this study, showing the correlation between different CD markers for established and Sheffield sarcoma cell lines that survived the stress assay. In addition, PC3 and h-TERT-RPE-1 also used cancer (positive control) and non-cancer (negative control). The data were transformed into linear relation divided into three R^2 value groups (strong, medium and weak). All calculations were done using Microsoft Excel (version 2011). *All gates = all cells gated +large cells gated (two different gating strategies).

5.2.10 Clonogenic Assay Performed Post-sorting for CD44 and ALDH Co-expression Using Pre and Post-Stress Populations

In this preliminary experiment the markers time limitations restricted the number or pre and Post-SA lines that could be tested so only two lines were studied, the commercial SKUT1 and 02/11W1. Equally a restricted number of antibodies were tested and CD44, CD34, CD90, CD24 and ALDH were selected. The suggested panel is given below.

- Unlabeled (for each cell line).
- Controls (for each cell lines and each colour).
- CD90/CD34/CD24/CD44.
- ALDH/CD34/CD24/CD44.

There was however an immediate complication, because the number of cells to be sorted would be expected to be present only in very low numbers (2.2.19) and the flow machine took much longer than expected to sort the co-expression. The results are therefore less reliable for this initial study and following advice by the lead FACS technician it was decided to sort based only on CD44 and ALDH co-expression. Table 5.7 is based on the findings of the previous studies of CD marker expression (see sections 5.2.4 to 5.2.7), and explains the percentages of each cell line gate and the CD markers expression in each gate. As shown below CD34 and CD90 gates were between 7% and 3%, which is considered as being very low and hard to isolate (Figure 5.40), on and based the previous (sections 5.2.4 and 5.2.7) the gates that supposedly enriched for CD90 and CD34 were in a different area. This experiment was performed only once due to the nature of this experiment and the time limit and needs to be repeated with some modifications in order to achieve a better understanding.

Cell line	CD44	ALDH	CD34	CD90
SKUT-1 Pre-SA (Leiomyosarcoma) Gating All cells **(78%)**	84%	52%	14%	0%
SKUT-1 Pre-SA (Leiomyosarcoma) Gating large cells **(3%)**	72%	62%	63%	1%
SKUT-1 POST-SA (Leiomyosarcoma) Gating All cells **(74%)**	74%	12%	7%	0%
SKUT-1 POST-SA (Leiomyosarcoma) Gating large cells **(5%)**	91%	89%	58%	0%
02/11W1 Pre-SA (Leiomyosarcoma) Gating All cells **(25%)**	99%	99%	12%	0%
02/11W1 Pre-SA (Leiomyosarcoma) Gating large cells **(4%)**	99%	99%	17%	1%
02/11W1 POST-SA (Leiomyosarcoma) Gating All cells **(84%)**	96%	52%	5%	0%
02/11W1 POST-SA (Leiomyosarcoma) Gating large cells **(7%)**	98%	98%	19%	3%

Table 5. 7: Summary of CD marker expression for **SKUT-1** and **02/11W1** (Pre-SA and POST-SA) when gating for all cells or large cells only.

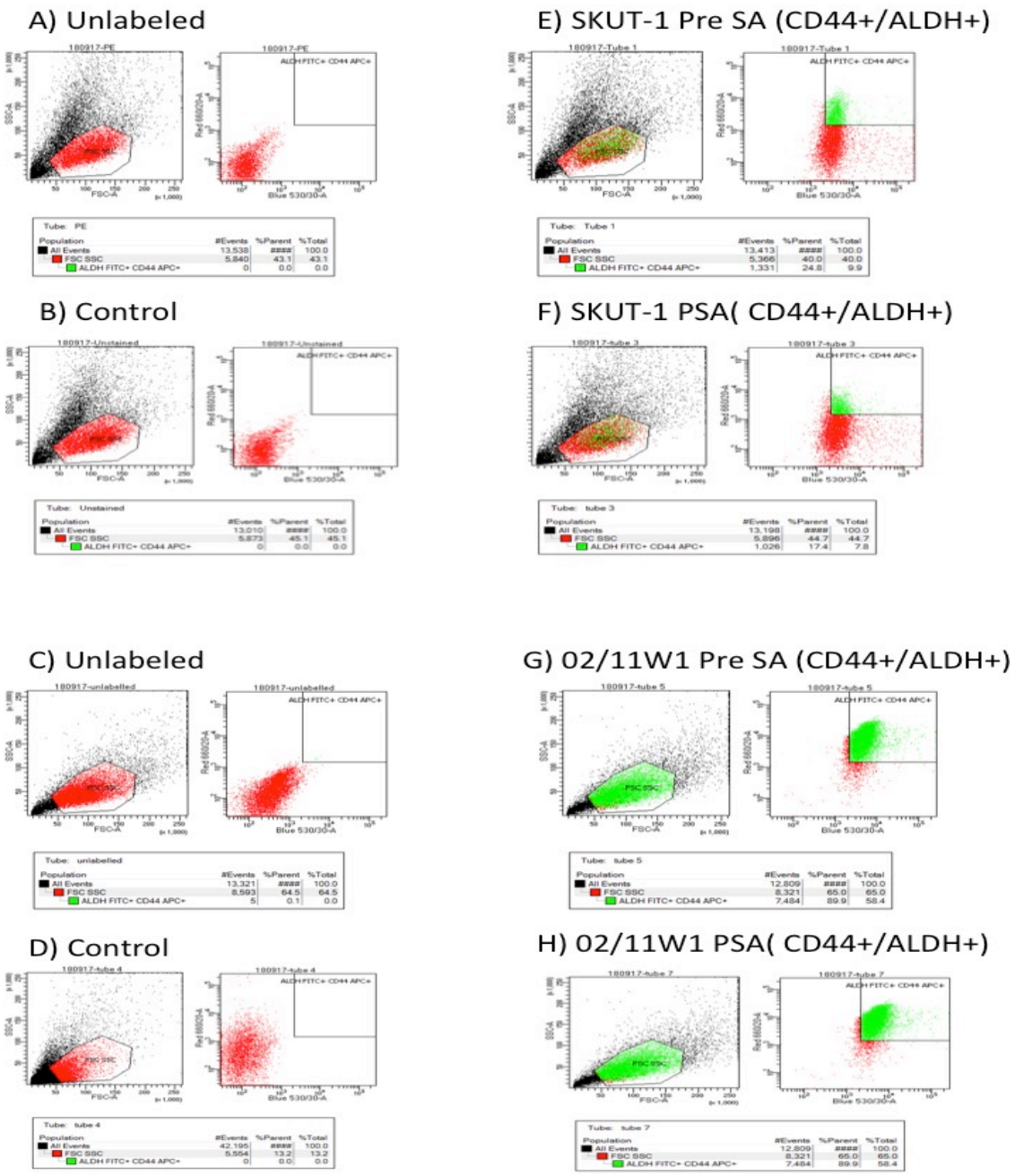


Figure 5. 40: Dot plots show the gating strategy used to sort **SKUT-1** and **02/11W1** pre and Post-SA based on CD44 and ALDH positivity (gating all cells and only co-expression cells were sorted).

Sorting was conducted prior to the clonogenic assay experiment. **E) SKUT-1 Pre-SA** number of cells sorted was (406,356 cells) and **F) SKUT-1 Post-SA** was (633,534 cells). While **G) 0211W1 Pre-SA** was (93,741 cells) and **H) 02/111W Post-SA** was (317,824 cells). Sorting was done using flow cytometry sorter BD FACSDiva 8.0.1

SKUT-1 (Pre-SA and POST-SA)

Cell lines were sorted and the number of cells post-sorting was 406,356 cells for SKUT-1 Pre-SA and 633,534 cells for SKUT-1 Post-SA. Then the isolated cells were kept in media and transported to the hood in preparation for the clonogenic assay; (extra precautions

were taken to avoid contamination). Cell lines were then seeded at different densities (250, 500, 1000, 2000 and 3000 cells) per Petri dish. SKUT-1 Pre-SA unsorted colonies numbers were 101, 171, 221 and 233 out of 250, 500, 1000, 2000 and 3000 cells seeded respectively. The SKUT-1 pre-SA sorted colony numbers counted were 24, 50, 99, 162 and 220. SKUT-1 Post-SA unsorted colonies numbers were 55, 74, 127 and 264 and SKUT-1 Post-SA sorted colonies numbers were 45, 109, 142, 266 and 334. There was a clear difference in the colony forming ability between unsorted Pre and Post-SA (Figure 5.41). Unsorted Pre-SA cells formed between 100 – 230 regardless of the cell seeding density. Sorting based on CD44 and ALDH reduced the number of colonies for pre- SA at all densities and was similar to Post-SA at the lower densities. Post-SA however had more colonies arising from the higher densities and this was increased for the sort population

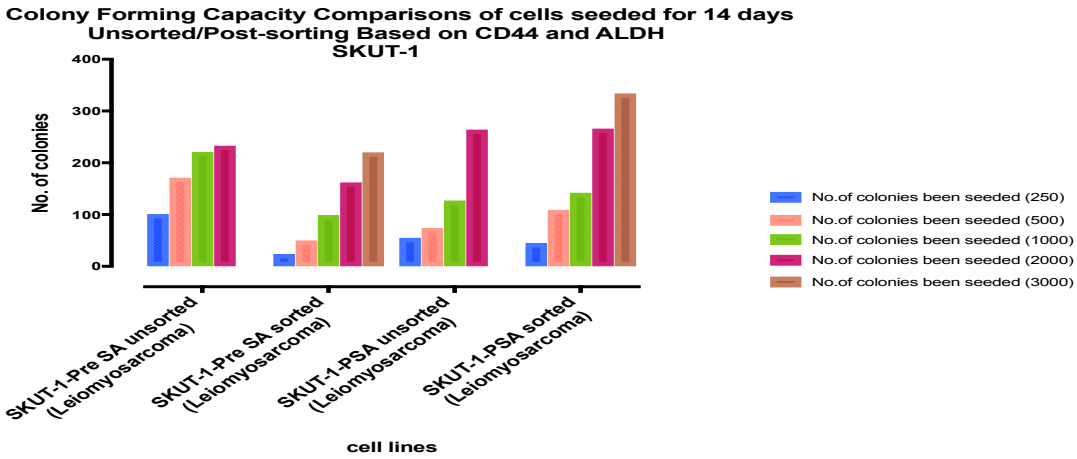


Figure 5. 41: The colony-forming capacity of SKUT-1 pre and post-stress populations comparing unsorted with sorted cells based on the co-expression of CD44 and ALDH. The flow cytometry sorter BD FACSDiva 8.0.1 was used to sort.

Cell lines were seeded at different densities (250, 500, 1000 and 2000 cells). Using 3000 created issue with counting, as it was uncountable in the petri dish after seeding the cells in some experiments, the cells were confluent. **SKUT-1 Pre-SA unsorted,** colony numbers were 101, 171, 221 and 233, and **SKUT-1 pre-SA sorted,** were 24, 50, 99 and 162, respectively. **SKUT-1 Post-SA unsorted** colony numbers were 55, 74, 127 and 264 and **SKUT-1 Post-SA sorted,** colonies were 45, 109, 142 and 266. This experiment was performed only once. Diagrams were done using Graph Pad® Prism software (version 7).

02/11W1 (Pre-SA and POST-SA)

Cell lines were sorted and the number of cells post-sorting was 93,741 cells for 02/11W1 Pre-SA and 317,824 cells for 02/11W1 Post-SA. Then the isolated cells were placed in media and transported to the hood in preparation for clonogenic assay (extra precautions were taken to avoid any contamination). Cell lines then were seeded at different densities (250, 500, 1000, 2000 and 3000 cells) per Petri dish. 02/11W1 Pre-SA unsorted colonies were 41, 73, 98 and 164 out of 250, 500, 1000 and 2000 cells seeded and for 02/11W1 Pre-SA sorted the number of colonies were 50, 94, 162 and 209 respectively. 02/11W1 Post-SA unsorted were 35, 56, 86 and 153 and for the sorted 55, 99, 180, 265 and 334. Interestingly, there were clear differences in the colony forming ability of Pre and Post based on co-expression of CD44 and ALDH with an increase in the colony forming ability post sorting (Figures 5.42). The pattern was more consistent here but did mirror the overall trend see for SKUT-1

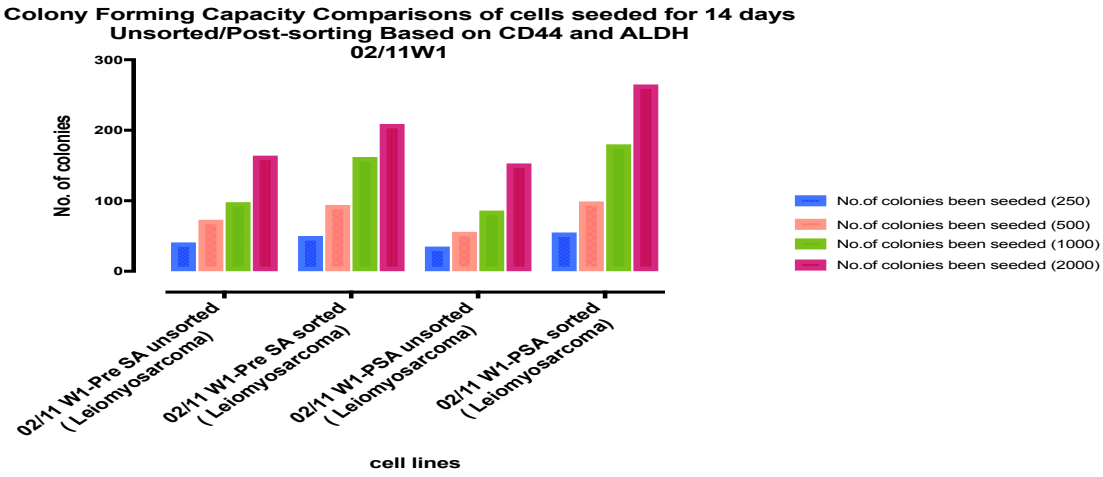


Figure 5. 42: The colony-forming capacity of cells seeded for 14 days for pre and Post-SA **02/11W1** and comparing the ability when sorted **for** CD44 and ALDH using flow cytometry sorter BD FACSDiva 8.0.1

Cell lines were seeded in different densities (250, 500, 1000 and 2000 cells). **02/11W1 Pre-SA unsorted,** numbers of colonies were 41, 73, 98 and 164 and for **02/11W1 pre-SA sorted,** the number of colonies out of 250, 500, 1000 and 2000 cells seeded were 50, 94, 162 and 209 respectively. **02/11W1 Post-SA unsorted** colonies numbers were 35, 56, 86 and 153 and for **02/11W1 Post-SA sorted** 55, 99, 180, 265 and 334. This experiment was performed only once. Diagrams were done using Graph Pad® Prism software (version 7).

5.3 DISCUSSION

The stress assay exploited the essential nutritional requirement for all cells to metabolize and survive and capitalized on the Warburg effect, defined as the network of metabolic alterations that occur to neoplastic cells (regulated by genetic and/or epigenetic mutations) (Pacini and Borziani, 2014). These changes happen to all neoplastic cells whether epithelial or mesenchymal and affect glycolysis, decrease the mitochondrial functions and oxidative phosphorylation and collectively are called the Warburg effect (Warburg et al., 1927).

Stem cells are governed by different conditions related to the microenvironment and share common features such as low oxidative phosphorylation, reactive oxygen species (ROS), increased glycolysis and the intracellular ATP level is reduced (Siggins et al., 2008). These metabolic features have also been identified in adult stem cells and embryonic stem cells, and when these cells start to differentiate these metabolic features change, with the recovery of oxidative phosphorylation and mitochondrial biogenesis and the elevation of ROS. Chen et al. investigate these changes in hESCs, in myotubes and myoblasts during differentiation (Chen et al., 2008). Glucose oxidation as a result of glycolysis happened in aerobic conditions, leading to partial pressure of O_2 inside the cells and this low oxygenation as seen with cancers is related to Warburg effect (Tennant et al., 2010).

In cancers the imbalance in the ratio of NADH/NAD^+ as a result of energy metabolism with glycolysis is important to maintain NADH storage and prevent lactic acid production (Kim and Dang, 2006). Excessive production of lactic acid is as a result of pH decreases and will promote neo-angiogenesis through hypoxia-inducible factor-1 (HIF-1) stimulation (Goetze et al., 2011). Elevation of the lactate dehydrogenase-5 (LDH-5) isoform is correlated with most cancers and inhibition of LDH-5 affects proliferation (Li et al., 2012). Furthermore, neoplastic cells have high expression of an enzyme protein NADPH/NADH-oxidase (NOX) that is regulated by the rat sarcoma oncogene. Increased activity of both enzymes in cancer cells forms large amounts of superoxide anion and hydrogen peroxide (Kawahara and Lambeth, 2007) and NOX activity is necessary to maintain the strong glycolytic flux and adjust alterations of NADH/NAD^+ ratio and is responsible for increasing ROS (Lu et al., 2012). It is important to bear in mind that EMT is associated to the Warburg effect and to citrate synthase deregulation (Lin et al., 2012). In malignancy there is a link between EMT and intracellular ATP levels and the reactivation of specific ATP levels activates p53 and suppresses EMT (Lin et al., 2012). All these observations

suggest that the Warburg effect plays a role in the undifferentiated state of the cells, and as such stemness with hypoxic conditions is particularly employed to investigate and isolate CSC. In osteosarcoma cell putative CSC $CD133^{+ve}$ cells were sorted under hypoxic conditions and had a high level of ATP and LDH and low glucose uptake compared to ATP levels. In addition, both $CD133^{+/-ve}$ cells had high levels of ATP and LDH and less glucose uptake, meaning that metabolism uses another fuel to maintain cells (Koka et al., 2018). Investigation of these cells revealed that there was also expression of other stem cell markers, including c-Myc, SOX2, Oct4 and TERT with HIF-1α, and other markers such as CD44 (as metastasis marker) and ABCG2 (as a drug resistance marker) were also highly expressed. Most importantly, the $CD133^{+ve}$ CSC cells under normoxic condition did not show the Warburg effect and a higher proliferation rate was observed for $CD133^{+/-ve}$ under hypoxic condition (Koka et al., 2018). All these complex Warburg effect interactions open a new avenue to understand CSC roots and the changes that happened to the cells post-stress assay.

5.3.1 Does The Stress Assay Select for Potential CSC by Identifying Cells Capable of Recovery?

In this study the cells recovered after the stress assay were compared with the parental non-stressed version of the cell line. A number of trends were apparent, and it was seen that Post-SA cell lines consistently had slower proliferation, that is increased doubling times, (figures 5.2, 5.3, 5.4 and table 5.1), although this was not highly significant. Furthermore, Post-SA cell lines had lower clonogenicity with less clonal efficiency (figures from 5.5 to 5.13) a finding that was highly significant for some cell lines such as the myxofibrosarcoma (figure 5.13 and 5.8). These observations at first seem at odds when CSC are considered as driving cancer development, it might therefore be expected that these recovered Post-SA lines would have higher proliferative rates with greater clonogenicity and clonal efficiency. So, it is therefore possible that these recovered populations are not CSC but instead are quiescent cells amongst the heterogeneous population of the cell line? If so as quiescent cells it would be reasonable to expect that growth rate, clonogenicity and clonal efficiency may be less as the cells slowly recover (Zeuner et al., 2014, Lin et al., 2015, Alves et al., 2018). However, Otte et al. in their study found changes as part of extended culture, with observations of MSC in long-term culture for more than 190 days compared to umbilical cord-MSC (UC-MSC) (Otte et al., 2013). Identical doubling and cell cycle results were seen until passage 10 by both Mesenchymal

stem cells (MSC) and UC-MSC, but thereafter changes ensued and it seemed that here the absence of umbilical cord tissue microenvironment resulted in the MSC having a reduction in population doubling, cell cycle, greater senescence and absent MSC CD markers, suggesting that cells lost their stemness ability.

An alternative theory is that these Post-SA cell lines are more reminiscent of the original tumour population, and as potentially less heterogeneous they are consequently less likely to have shown drift specifically through adaption to culture. Certainly in contrast to Otte et al., there is evidence that suggests that cell lines grown in culture change and adapt and have increased proliferation and clonogenicity (Jiang et al., 2012, Froelich et al., 2013, Danisovic et al., 2017, Stepniewski et al., 2019). These changes sometimes happened after a few passages, Danisovic et al. found changes in morphology, proliferation kinetics and cell cycle after long-term culture (30 passages) of adipose tissue derived stem cells (hADSCs). Furthermore, the cell –to- cell extracellular interactions were lost, and mesenchymal markers were negative, such as CD34, CD45 and CD20. Significantly they notice that the expression started to decrease after passage 25 (Danisovic et al., 2017). This observation is consistent with other studies, with MSC from bone marrow losing their immunophenotye after only the 12^{th} passage (Pogozhykh et al., 2015) and tonsil derived MSC after the 15^{th} passage (Yu et al., 2014). Other transformations were also reported by Danisovic, with morphological differences observed after passage 7; cells become larger and there was a reduction of telomerase activity after long culture (Danisovic et al., 2017). That said, the long-term modifications of cells as they adapt to culture and the development of increased heterogeneity are poorly understood, but there does seem to be consistency for a loss of putative stem cell markers over extended culture (Jiang et al., 2012, Froelich et al., 2013, Burton and Faragher, 2015, Danisovic et al., 2017, Stepniewski et al., 2019).

Here, the primary angiosarcomas cell lines (sections 3.2.2.15- 3.2.2.18 and table 3.3) only grew for a limited period of time and rarely reach more than passage 6, and were very slow in terms of proliferation. Furthermore they also had much lower CE (section 3.2.5). In contrast the higher passage number of the Sheffield derived cell lines correlated to greater clonal efficiency and proliferation. (Figure 3.12, tables 3.3 and 3.2) These observations may indeed suggest that Post-SA cell lines are more like the original tumour when the cells were first established in the media. Certainly there was increased CD marker expression for the post -SA cell lines; suggesting that the Sheffield derived lines had lost some expression for putative CSC markers (as mentioned above). Potentially more important was the observation from the aCGH results (section 5.2.3) that suggested the

genetic changes of the recovered cell lines more closely matched the original tumours, supporting the theory of genetic drift over time in culture (Wagner, 2012, Nickkholgh et al., 2014, Stepniewski et al., 2019, Halliwell et al., 2020). How quickly this drift happens may vary with some phenotypic changes, such as morphological, occurring quickly since cells that survived the stress assay were larger in size (chapter 4), but reverted to the size of the cells before the stress assay. These observations all seemingly suggesting that cultural adaption for some aspects can ensue quickly. It could therefore be that extended time in culture dilutes any potential CSC population that is present within the original tumour, and hence the established cell lines derived in the 1970's would be highly unlikely to retain such a population as indeed was found in this study (sections 5.2.1, 5.2.2, 5.2.3 and 5.2.6).

5.3.2 If The Stress Assay Does Enrich for CSC What Markers Could Be of Value for Sarcomas?

In this study cell lines that survived the stress assay were investigated for potential differences to those of the Pre-SA parental population in order to see if the assay could serve as a biological method to enrich and identify sarcoma CSC. In chapter 3 the use of these potential CSC markers failed to provide evidence for their relevance to sarcoma. Here following analysis of the Post-SA population and comparison with the Pre-SA cell line, it was possible to develop a hierarchy for sarcoma CSC based on the CD markers analysed in this study (Figure 5.43), accounting for observations that large cells seem to initiate post survival and thus gating for them.

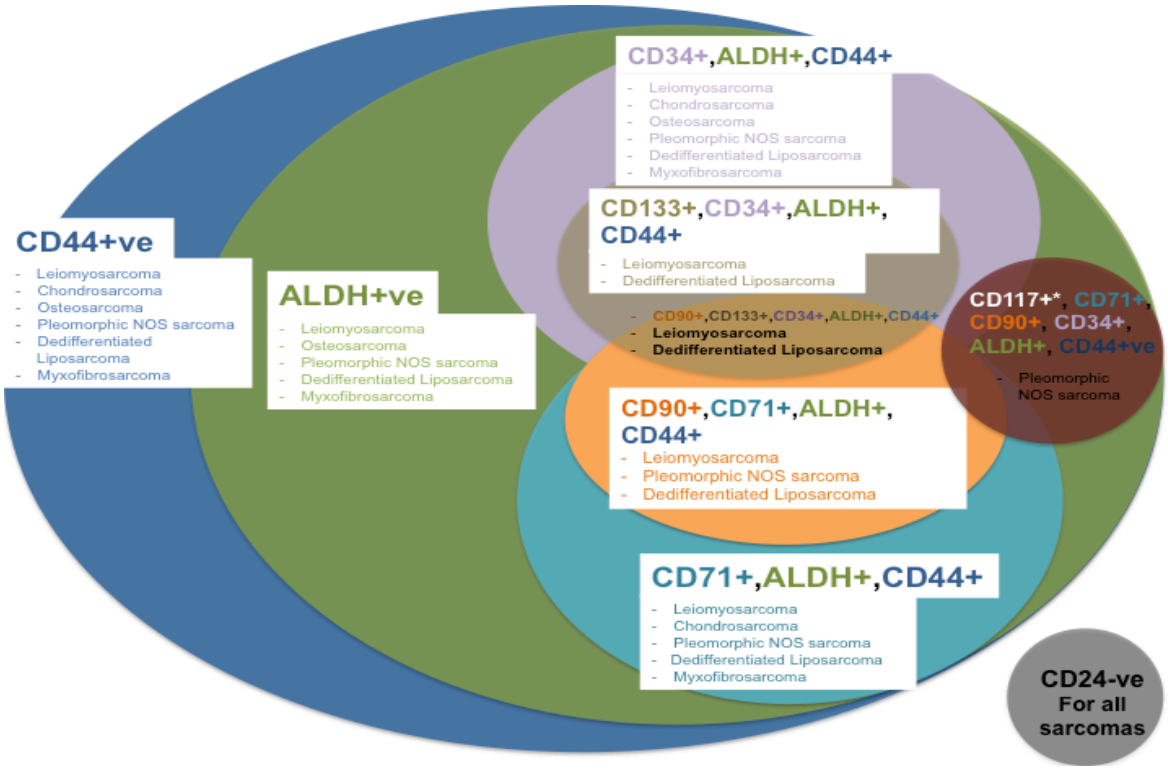


Figure 5. 43: A possible hierarchy in order to detect and identify a sarcoma CSC subpopulation based on single and co-.expression of potential CSC markers. The basis comes from the findings of this study considering the heterogeneity of sarcoma and the possibility to enrich by selective gating for large cells and is supported by published studies. Each colour represents a CD marker and is based only on the strategy of gating for large cells.

In general Post-SA cells and particularly when gating for large cells showed a greater expression of putative CSC markers that had only been expressed at low levels in the parental population (table5.5, and figures from 5.22 to 5.28). As found earlier CD44 and ALDH positivity was highly expressed and consistent for all subtypes of sarcoma and thus the findings here suggest that used on their own they have no relevance to the identification of sarcoma CSC; although could be considered a pan CSC marker for sarcomas to be used in combination and for hierarchal analysis. CD44 is the most common marker investigated in the literature for CSC and it increased expression is often associated with poor survival (Wei et al., 2016, Yang et al., 2016, Abbaszadegan et al., 2017, Najafi et al., 2019b, Sonbol et al., 2019, Atashzar et al., 2020,). Equally higher expression of ALDH1 in single expression studies is thought to associate with poor survival (Liu et al., 2015, Horimoto et al., 2016, Zhao et al., 2018), as does CD133 used in combination with other markers (He et al., 2012, Zambo et al., 2016). High expression of ALDH has also been considered to identify more tumourigenic sarcoma cells (Awad et al., 2010, Honoki et al., 2010, Lohberger et al., 2012b, Nakahata et al., 2015, Martins-Neves et al., 2016). Due to the small number of sarcomas studied here it was not possible to draw such conclusions.

The results of ALDH in this study also need careful consideration when interpreting them, because of the different isoforms that can be related to CSC phenotype, and ALDH activity could indicate heterogeneity, as has been found in osteosarcoma (Honoki et al., 2010). The variability for the expression of ALDH as found in this study (Sections 3.2.3, 3.2.4.2 and 5.2.6.1) is possibly related to heterogeneity or the effect of culture, since all angiosarcoma in this study only had low ALDH expression ranging from 4% to 7% (figure 3.6). This could be subtype specific associations, but the later passages of the other sheffield sarcoma derived cell lines had higher ALDH expression. Equally this may be cultural adaption either through selection of subpopulations or increased heterogeneity, but alternatively the constituents could promote ALDH activity over prolonged culture. It is of interest that most sarcoma reported to have high ALDH expression are associated with the overexpression of the transcription factor Sox2 (Wang et al., 2011, Lohberger et al., 2012b, Nakahata et al., 2015, Martins-Neves et al., 2016a). Sox2 (embryonic stem cell pluripotency factor) is known for playing an important role in the maintenance of the stem cell state and determination of cell fate, and high levels indicate poor prognosis and tumour recurrence (Wang et al., 2011, Lohberger et al., 2012b, Nakahata et al., 2015, Martins-Neves et al., 2016). In sarcoma Sox2 is also known for CSC phenotype maintenance and other factors such as Nanog and Oct4 in association with Sox2 also play the same role (Wuebben and Rizzino, 2017). Sox2 is also suggested to be acting as an oncogene as well (Skoda et al., 2016, Wuebben and Rizzino, 2017). As this study was started at a point when there was little information available on possible CSC markers for sarcoma, particularly STS, the assessment centered on those markers commonly of value in more mainstream cancers. There are now more studies and the relationship between CSC markers and sarcomas are seen in Figure 5.44 and Table 5.8. It is however important to bear in mind that much of this work comes from the study of bone sarcomas, and STS may have an entirely different pattern. Furthermore, Synovial sarcomas are translocation driven unlike STS which are characterized by their high instability and confirmed by this study (figures from 5.14 to 5.19).

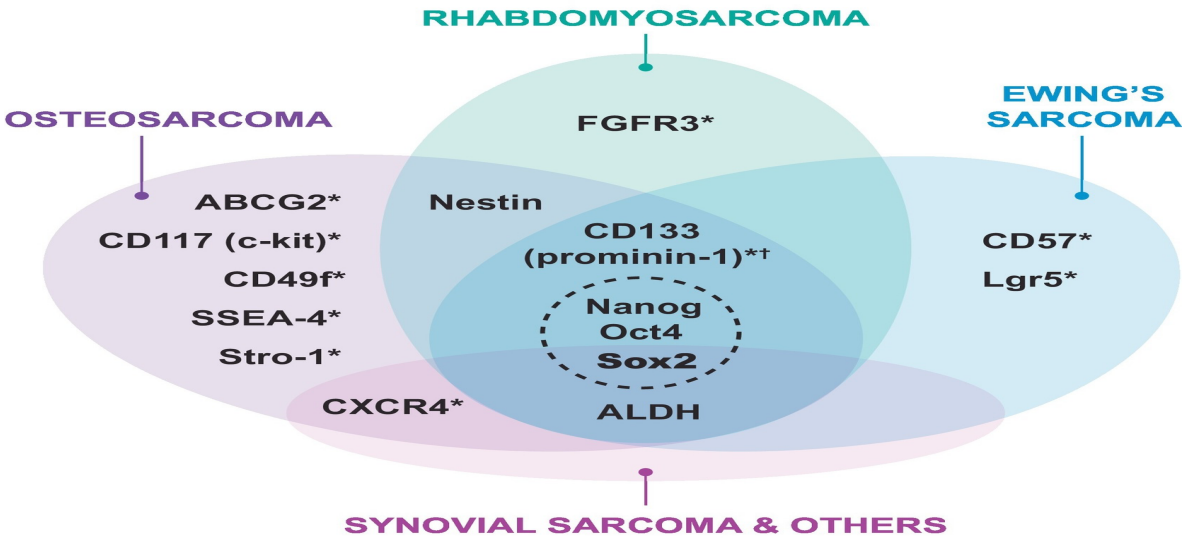


Figure 5. 44: A diagram showing overlapping of CSC markers in different sarcomas. Note the central position of core embryonic stem cell pluripotency factors (dashed circle), out of which Sox2 is shared among all reviewed sarcoma subtypes. * Cell surface (membrane) proteins; † proteins that are not well understood with complex subcellular localization. Adapted from (Skoda and Veselska, 2018).

CSC markers & some sarcoma subtype	Supporting evidence	Opposing evidence
Osteosarcoma		
ABCG	(Tirino et al., 2008, Adhikari et al., 2010, Martins-Neves et al., 2016b)	(Skoda et al., 2016, Zhang et al., 2015)
ALDH	(Honoki et al., 2010, Martins-Neves et al., 2016a)	
CD49f	(Ying et al., 2013)	(Penfornis et al., 2014)
CD117 (c-kit)	(Adhikari et al., 2010)	(Yang et al., 2011, Zhang et al., 2015)
CD133	(Tirino et al., 2008, Veselska et al., 2008)	(Tirino et al., 2011, Yang et al., 2011, Saini et al., 2012, (Zhang et al., 2015, Skoda et al., 2016)
CXCR4	(Adhikari et al., 2010)	
Nanog	(Tirino et al., 2011, Wang et al., 2011)	(Skoda et al., 2016)
Nestin	(Veselska et al., 2008, Tirino et al., 2011)	(Skoda et al., 2016)
Oct4	(Tirino et al., 2011, Wang et al., 2011)	(Skoda et al., 2016)
Sox2	(Tirino et al., 2011) (Wang et al., 2011, Basu-Roy	

	et al., 2012, Martins-Neves et al., 2016a, Skoda et al., 2016, Wang et al., 2017)	
SSEA-4	(Zhang et al., 2015)	
Stro-1	(Adhikari et al., 2010)	(Zhang et al., 2015)
Ewing's sarcoma		
ALDH	(Awad et al., 2010)	
CD57	(Wahl et al., 2010)	(Leuchte et al., 2014)
CD133	(Suva et al., 2009, Riggi et al., 2010)	(Jiang et al., 2010, Leuchte et al., 2014, Skoda et al., 2016)
Lgr5	(Yu et al., 2021)	
Nanog	(Suva et al., 2009, Riggi et al., 2010)	(Skoda et al., 2016)
Oct4	(Suva et al., 2009, Riggi et al., 2010)	(Skoda et al., 2016)
Sox2	(Riggi et al., 2010, Ban et al., 2011, Ren et al., 2016a, Skoda et al., 2016)	
Rhabdomyosarcoma		
ALDH	(Nakahata et al., 2015)	
CD133	(Sana et al., 2011, Pressey et al., 2013)	(Hirotsu et al., 2009, Skoda et al., 2016)
FGFR3	(Hirotsu et al., 2009)	
Nanog	(Walter et al., 2011)	(Skoda et al., 2016)
Nestin	Sana et al., 2011	(Skoda et al., 2016)
Oct4	(Walter et al., 2011)	(Skoda et al., 2016)
Sox2	(Walter et al., 2011, Nakahata et al., 2015, Skoda et al., 2016, Slemmons et al., 2017)	
Synovial sarcoma & others		
ALDH	(Lohberger et al., 2012b)	
CXCR4	(Kimura et al., 2016)	
Sox2	Lohberger et al., 2012b, (Kadoch and Crabtree, 2013)	

Table 5. 8: The most common CSC markers (single expression) considered of relevance to sarcomas subtypes. Amended and adapted from (Skoda and Veselska, 2018).

As indicated in Figures 5.43 and 5.44 some of the putative CSC markers appear subtype specific and do not overlap with other markers. The overlapping of markers reflects their location, with those of the cytoplasm demonstrating overlapping, but not for markers expressed on the surface, a finding that has been reported previously but could indicate the ability to identify CSC in different subtypes of sarcomas (Skoda and Veselska, 2018).

At the opposing end the most dedifferentiated and potentially pluripotent subtype, pleomorphic NOS, were enriched for all putative CSC markers. The findings suggest that it is possible that these cells in pleomorphic NOS are more primitive cells because of their dedifferentiated status, and thus could inherently hold CSC properties and hence will be inclined to express CSC markers. The finding however that the population capable of regeneration post stress for all sarcoma subtypes (or when gating for large cells) has increased putative CSC expression, suggests that the number of cells are increased using this strategy, and that this maybe a way of enriching for CSC. Some markers were of particular interest for example CD117, relevant to hematopoietic stem cells and known to stain mesenchymal cells (Foster et al., 2018, Bellio et al., 2019, Hatina et al., 2019, Li et al., 2019), showed interesting results when co-expressed with CD71 and CD34 and may offer a strategy for CSC selection.

CD71 was positive only on Sheffield derived cell lines, and although there are no studies for CD71 usage in any of these sarcoma subtypes, the findings here could link CD71 expression to primary cell lines only and not the established cell lines that are more adapted to the culture. By comparing the results of CD71 in this study between pre and Post-SA gating large cells, the expression level of CD71 in cell lines (14/10, 13/12W2 and 09/10) Post-SA was lower by few percentages (between 5%-29%). These results appear to suggest that the stress assay does not enrich for CSC or that CD71 is not a useful marker for sarcoma. However, the 21/11W2 cell line Post-SA had, double the expression, from 11% to 27% and markers unsurprisingly may be subtype specific. CD117 expression (pilot study section 5.2.8) showed some promising results in terms of co-expressing with CD34 and CD71 on 13/12W2, and the expression was increased when large cells were gated in this pilot study. Although not conclusive enrichment for CSC markers was consistently found in this study post stress, and markers such as CD71 and CD117 in combination or by themselves may be of value to the identification of CSC in sarcomas.

5.4 SUMMARY

In this study, sarcoma cell lines pre and post-stress assay were screened using a variety of assays to explore the differences and see if the stress assay could provide a possible method of enriching for sarcoma CSC. The findings showed significant differences and a small subpopulation was identified that could contain cells that possess CSC characteristics; based on the findings in chapter four (0.0003% to 0.121%) percentage survival out of 1 million cells, which is considered to be very low and from FACS (markers

expression) around 1%-3% positivity staining putative markers out of 4%-8% gated large cells which is also very low. FACS, results and CD markers co-expression showed the importance of not relying on single markers to investigate and isolate these cells. The stress populations were however seemingly enriched for CSC markers and this could be further enhanced by gating for the larger cells that were observed to initiate colony development post stress. In particular CD117 showed promising potential results in this pilot study as it was co-expressed with other monoclonal antibodies. MTT, clonogenic and linear regression results support the findings of this possible CSC subpopulation and aCGH showed notable differences in cell lines between pre and post-stress assay. Some regions may be of particular relevance and require further study (2q, 7(p/q), 8(p/q), 12(p/q), 18 (p/q) and 22 q), and more in depth interrogation of the data to fully explore this aspect of the study.

CHAPTER SIX

FINAL DISCUSSION

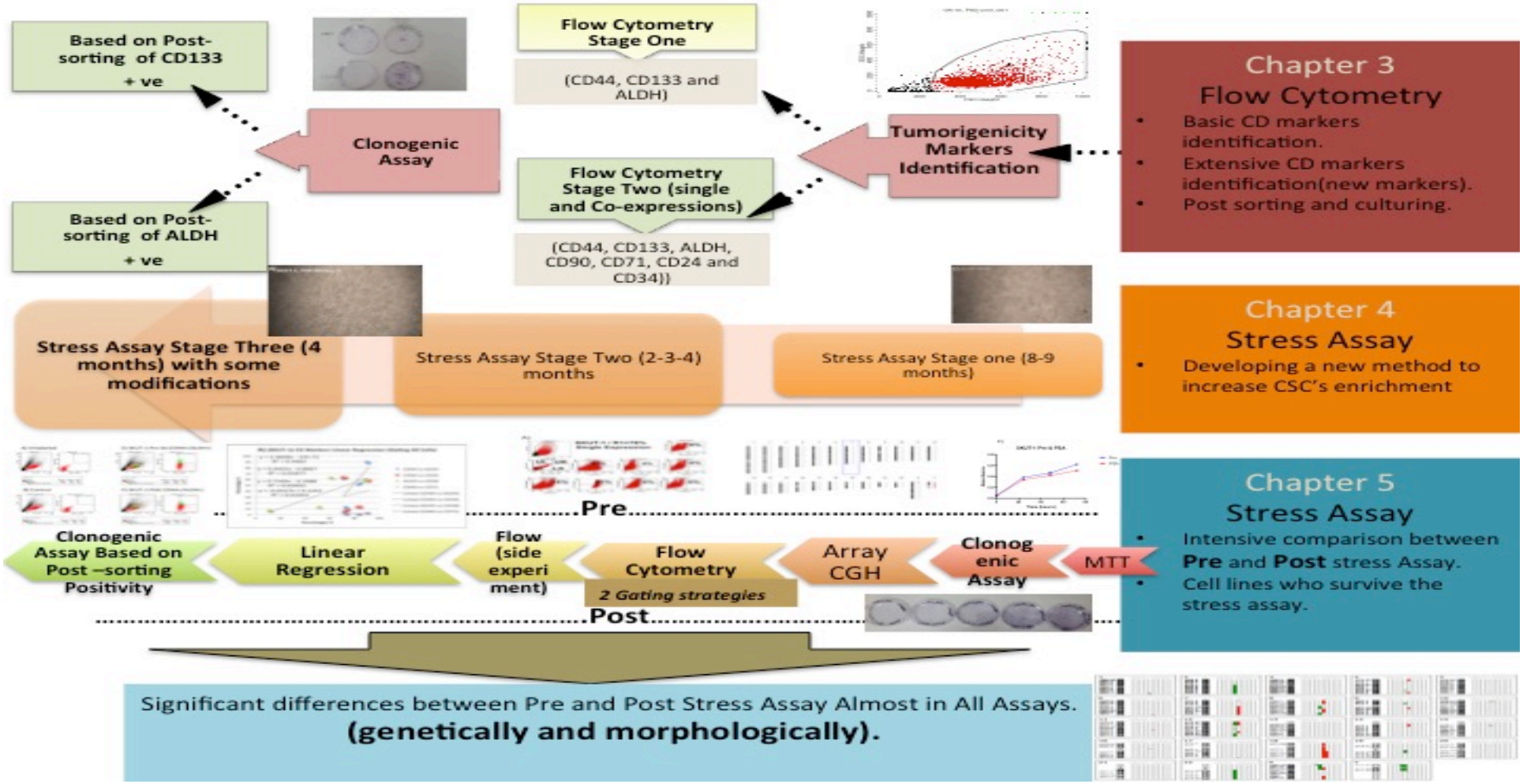


Figure 6. 1: A summary of the approach used in this PhD study, and the methodology for each aspect of the investigation research.

6. FINAL DISCUSSION

6.1 STUDY SUMMARY TO APPROACH AND OBJECTIVE

There are many different sarcoma subgroups (Jundt, 2018) but overall they account for around 1% of all tumours and specifically account for 15% of pediatric tumours. In terms of therapy sarcomas are a challenge, as they can be highly aggressive, relapsing and metastasizing (Dancsok et al., 2017, Sandler et al., 2019). The CSC subpopulation is a group of cells within tumours that have the property of self-renewal and differentiation to both progenitor cells and more mature cells forming a heterogeneous population. These cells can initiate and be responsible for tumour recurrence, progression and metastasize, and are known for their chemo-radiotherapy resistance. These stemness properties are governed by many pathways that could be detected by biomarkers. This PhD tries to explore the presence of this subpopulation and what implication and impact their identification may have to patient treatment (Dick, 2008, Bomken et al., 2010, Fanali et al., 2014, Baba and Akashi, 2015).

There has been intensive study of CSC and their importance for epithelial cancers, but far less is known about CSC in sarcoma, or their existence, and most work has been on pediatric bone sarcomas (Todorova, 2014, Genadry et al., 2018, Skoda and Veselska, 2018,,Hatina et al., 2019b, Martinez-Delgado et al., 2020). This study looked to reliably identify CSC in STS acting as a starting point for future investigation to target and eradicate them. A summary of the approach and methodology used in this PhD is illustrated in Figure 6.1. Initial work focused on the use of markers that have been shown as useful CSC markers for other cancers (chapter 3). As the use of markers had not been successful, a different approach was taken to see if CSC populations could be identified in sarcomas through the development of a functional assay, to isolate and enrich for CSC (chapter 4). Following the so called stress assay, the next step was to compare the outcome of surviving post recovery cells lines with their matched non-stressed parental cell lines, to see what differences existed and whether the findings were consistent with the enrichment for CSC.

6.2. ARE THERE RELIABLE CSC MARKERS FOR SARCOMA?

One of the biggest challenges to the identification of CSC in sarcoma is the sheer number of subtypes. Most work has been done on a small subset of pediatric bone cancers and even then there are conflicting findings CD133 and CD117 were suggested as CSC markers in osteosarcoma and CD133 in Ewing's sarcoma but not all investigations could

confirm their potential (Jiang et al., 2010, Yang et al., 2011). The initial approach at the start of this investigation many years ago was to focus on those markers considered at the time as most relevant to cancers in general, and specifically to sarcomas, although as mentioned most of this work centered on bone cancers.

Tumor	Markers	References
Colon	$CD44^+$, $CD133^+$, $CD166^+$, $CD24^+$, $EpCAM^+$, ESA^+, $ALDH1^+$	Botchkina (2013); Tseng et al. (2015)
Esophagus	$CD44^+$, $CD24^+$, $CD133^+$, $ABCG2^+$, $CXCR4^+$, $ALDH1^+$	Qian et al. (2016)
Stomach	$CD44^+$, $CD44V8\text{-}10^+$, $CD133^+$, $CD24^+$, $CD54^+$, $CD90^+$, $CD49f^+$, $CD71^+$, $EpCAM^+$, $ALDH1^+$	Brungs et al. (2016)
Pancreas	$CD44^+/CD24^+$, $CD133^+$, ESA^+, $ALHD1^+$	Li et al. (2007); Zhan, Xu, Wu, Zhang, and Hu (2015)
Breast	$CD44^+/CD24^-$, $ALDH1^+$	Carrasco et al. (2014)
Brain	$CD44^+$, $CD133^+$	Jackson, Hassiotou, and Nowak, (2015)
Lung	$CD44^+$, $CD133^+$, $CD166^+$, $ALDH1^+$	Lundin and Driscoll (2013)
Ovarian	$CD44^+$, $CD133^+$, $CD24^+$, $CD117^+$, $EpCAM^+$, $ALDH^+$	Zhan, Wang, and Ngai (2013)
Prostate	$CD44^+/CD24^-$, $CD133^+$, $\alpha2\beta1^{high}$, $ALDH1^+$	Collins, Berry, Hyde, Stower, and Maitland (2005); Sharpe, Beresford, Bowen, Mitchard, and Chalmers (2013)

Liver	$CD44^+$, $CD133^+$, $CD90^+$, $CD13^+$, $EpCAM^+$	Sun, Luo, Liu, and Song (2016)
Melanoma	$CD20^+$, $CD133^+$, $CD271^+$	Lang, Mascarenhas, and Shea (2013)
HNSCC	$CD44^+$, $CD133^+$, $ALDH^+$	Krishnamurthy and Nor (2012)
AML	$CD34^{+/-}$, $CD38^{+/-}$, $CD90^{-/+}$, $CD123^+$, $CD45RA^+$, $CD33^+$, $CD13^+$, $CD44^+$, $CD96^+$, $CD47^+$, $CD32^+$, $CD25^+$, $CLL\text{-}1^+$, $TIM3^+$	Horton and Huntly (2012)
MM	$CD138^-$, $CD19^+$, $CD27^+$	Matsui et al. (2008)

Table 6. 1: Overview of suggested markers for cancer stem cells for different cancers. Adapted from (Abbaszadegan et al., 2017).

In this study PC3 a reliable prostate cell line used for many CSC studies was used as a positive control and a normal retinal cell line as the negative. The most studied markers are CD44, CD133 and ALDH. As CD44 has been identified as a marker for mesenchymal stem cells its high expression in sarcomas is not altogether surprising (Ullah et al., 2015). CD44 appears to be a pan-cancer antibody and was the most commonly investigated marker in literature, but even for CD44 there are conflicting findings on colorectal cancer that indicate is not a pan-CSCs marker, and similar issue also apply to CD133 and ALDH (Al-Hajj et al., 2003, Hernandez-Vargas et al., 2011, Karamboulas and Ailles, 2013, Fanali et al., 2014, Woodward et al., 2014). Despite the possible controversy it was still important to exclude /confirm their importance in this investigation. CD44 may have a value as shown here for hierarchal studies (figure 5.43) and ALDH may relate to cultural adaption, either increases heterogeneity and or proliferation (sections 3.3.4.2, 3.3.3 and 5.3.2), whilst CD133 may be of relevance to subtypes of sarcoma (figure 5.43). For other markers less is known and the pattern is more variable such as CD71 (Villanueva-Toledo et al., 2014), but there can be consistency with most cell lines being negative for CD24 (Aigner et al., 1997, Karahan et al., 2006, Phillips et al., 2006, Gao et al., 2010, Yeung et al., 2010, Lu et al., 2011, Stratford et al., 2011, Tirino *et al*., 2011, Malanchi et al., 2012, Yang et al., 2014, Rao and Mohammed, 2015), as indeed was found in this study (table 5.4 and 5.5). Certainly, as suggested here CD44 has no clear value but CD34, CD71,

CD133 and potentially ALDH may have some impact, depending on subtype or as part of the enrichment for CSC as was discussed and investigated in chapters three and five. Pilot data also suggest that other markers specifically CD117 are of interest for sarcoma (section 5.2.8) Overall, this study suggest there is substantial variability for the relevance of CSC markers to STS, and the expression of these markers in the normal cell line also calls into question the reliability of these markers. It is however important to bear in mind that the retinal normal cell line was not indeed entirely normal, since the cells had become immortalized through hTERT, and as TERT is a putative CSC marker (El-Badawy et al., 2018), it may be expected that this could also impact on the expression of other markers associated with CSC and hence account for some of the findings as presented here.

This study found interesting results for CD71 and the positivity of CD71 noted for Sheffield cell lines has not been reported before. It was of interest that this was not expressed by the commercial lines. Also, the data on CD117 from the pilot study may serve to identify these 2 markers of relevance to STS, specifically the subtypes such as NOS. However as NOS are highly dedifferentiated the question arises as to whether the marker identifies CSC, or just associates with this subtype as part of the deregulation exhibited by them. CD71 protein is the transferrin receptor, and regulates through iron accumulation the stemness of cancer derived CSC like cells (Xiao et al., 2020). As part of recovery from the stress assay the uptake of iron would be essential and hence CD71 may be a critical marker for CSC ability to recover, or it could still relate to quiescent cells (Fryknas et al., 2016). The transferrin receptor is located at chromosome 3q29, and in this study there was no indication to specifically suggest that the region was important, but comparison of all STS did suggest that the region was certainly retained Post-SA and possibly amplified (figures 5.15, 5.16, 5.17, 5.20 and table 5.2). CD117 protein (mast stem cell growth factor receptor) is a transmembrane tyrosine kinase growth factor receptor, and strong staining is found in gastrointestinal stromal sarcomas (GISTS) (Parfitt et al., 2008, Novelli et al., 2010, Riddle et al., 2011, Yulianti and Hernowo, 2015). Although not specifically related to other STS its expression may therefore be part of a recovery process and therefore identify it as a useful marker. The gene is located at 4q12, and although greater interrogation of the data is required there was evidence to suggest that at the very least this region was retained if not enriched in the Post-SA cell lines (figure 5.20 and table 5.2). Ultimately further work is required, but these markers show potential as CSC for STS.

6.3 IS THE STRESS ASSAY A USEFUL FUNCTIONAL APPROACH TO ENRICH AND ISOLATE CSC IN SARCOMA?

The stress assay was designed to be of value for all STS, since chemotherapy targets rapid cell division and non-activated CSCs are slow in general; therefore it might not be effective on those types of cells (Kim et al., 2015, Kurtova et al., 2015). Also, sarcomas with the many subtypes would not necessarily respond in a similar manner. Radiotherapy also increases stemness properties for non-stem cancer cells by upregulation of stemness pathways and resulting in CSCs that resist radiotherapy treatment (Ghisolfi et al., 2012, Kim et al., 2015, Kurtova et al., 2015). This approach was also considered, but again different sarcoma subtypes have variable responses.

Certainly, cell starvation (the stress assay) as used here increases the number of functional assays that could be employed to investigate CSC. The results of this study are encouraging and the findings of this study provides clear evidence that the assay identifies a population capable of recovery; but whether this is a CSC population or a quiescent population is unclear. The number of recoverable cells from a million seeded ranged from 3 – 149 cells per million (section 4.2.3.3 and table 4.2) is in order with the reported frequencies of CSC (Tirino et al., 2011, Malanchi et al., 2012a, Veselska et al., 2012) and suggests that the assay identifies the CSC population within sarcomas. The enrichment for CSC markers found in this study in the Post-SA cell lines may also tend to indicate that the functional assay can identify CSC. Equally many of the markers that were enriched, CD71 for example, are also potentially of relevance to quiescent cells. The observation from the aCGH that recovered populations match more closely the original uncultured STS, leads to the theory that these recoverable cells become diluted over extended culture and where thus absent from commercial lines derived in the 1970's. Again evidence for both enrichment of CSC but equally quiescent cells, since as discussed before, CSCs tend to grow slowly when they have not activated in a quiescent stage of their cell cycle (Zeuner et al., 2014, Lin et al., 2015, Alves et al., 2018). This inactive CSC state may explain why the Post-SA populations had slower proliferative rates, and lower CE, but again it could just be quiescent cells. Tumour initiating or innate resistance pathways such as the notch, JAK/STAT and PI3K/ Akt, and Wnt/β-catenin would be worth investigating for these cells post-stress assay. Moreover, these pathways are also involved in the epithelial-mesenchymal transitions (EMT) and mesenchymal-epithelial transitions (MET) seen in metastasis (Yao et al., 2011, Fabregat et al., 2016). However, because of tumour heterogeneity, the involvement may vary within different tumour types (Keysar and Jimeno, 2010, Chen et al., 2013). Ultimately at this stage it is

unclear whether the functional assay developed here is of value for the identification of CSC, potentially mimicking tumour relapse, it does however offer interesting avenues for the future.

6.4 STUDY LIMITATIONS

One major constraint to this study was the time taken for cells to be set up and recover from the stress assay, and specifically due to infections a whole set of data was lost. Although the findings of this study serve as a compelling start for future investigations of CSC in sarcoma, repeat analysis would help iron out some of the issues. It is also important to bear in mind that for some subtypes of STS there was only one line investigated, for example myxofibrosarcoma. Therefore, although findings show some consistency for STS as a whole it is difficult to estimate what impact subtype variability may have. Other limitations arose because of the use of dyes to label in combination the markers for the co expression studies, which meant not all combinations could be fully investigated and restricted the analysis. There were also substantial personal issues that impacted on aspects of the study, meaning certain areas could not be explored in depth. In particular, the linear regression investigation and the data from the initial aCGH studies. It would certainly be of value to undertake further exploration and analysis of the data obtained here.

6.5 FUTURE WORK

This study showed some potential and interesting results regarding the enrichment and isolation of a CSC subpopulation in STS. It can only however be considered as a starting point. Monoclonal antibodies such as CD71 and CD34, CD117 were of potential interest as identified in the pilot study performed. This study needs to be repeated and additional lines included. Furthermore, the normal method of analyzing CD markers in the dot plot platform and for the enriched CSC's populations were improved during this investigation. In future studies this method could be expanded so as to construct the dot plot platform prior to reading the samples, and hence use it as a filtering method specifically to detect only those cells with potentially positive markers by isolating and sorting. The functional stress assay could then be performed to see if increased numbers are retrievable.

Also, a full linear regression analysis should also be undertaken on all the findings of this study (co-expression) in order to provide a strong basis for future work. A more intensive analysis of the aCGH comparing the pre and Post-SA cell lines should be performed, and

the information gained could again inform future investigations, possibly identifying new targets that could be used a potential CSC markers for STS. Furthermore as additional potential markers are identified, a FACS study could be constructed to give more insight. For example, markers like (SSEA3, SSEA4, TRA-1-81, TRA-1-60s, AA11, BE12, BF4, DA9, B159, TRA-2-49, CC9, CHB, AG10 and EF12) (Enver et al., 2005, Wright et al., 2011, Skoda and Veselska, 2018). Finally it would be of value to explore whether the Post-SA cell lines after repeated culturing have dilution of the putative CSC's population as hypothesized from the findings of the commercial sarcoma lines, and whether they can give rise to a more heterogeneous cell line.

The findings demonstrated in this PhD study are promising and encouraging for more future research in sarcoma. They are very much a starting point.

REFERENCES

References

ABBASZADEGAN, M. R., BAGHERI, V., RAZAVI, M. S., MOMTAZI, A. A., SAHEBKAR, A. & GHOLAMIN, M. 2017. Isolation, identification, and characterization of cancer stem cells: A review. *Journal of Cellular Physiology,* 232**,** 2008-2018.

ABRAMSON, D. H., ELLSWORTH, R. M., KITCHIN, F. D. & TUNG, G. 1984. 2ND NONOCULAR TUMORS IN RETINOBLASTOMA SURVIVORS - ARE THEY RADIATION-INDUCED. *Ophthalmology,* 91**,** 1351-1355.

ACHARYA, S. & KALA, P. S. 2019. Role of CD71 in acute leukemia- An immunophenotypic marker for erythroid lineage or proliferation? *Indian Journal of Pathology and Microbiology,* 62**,** 418-422.

ADEWUMI, O., AFLATOONIAN, B., AHRLUND-RICHTER, L., AMIT, M., ANDREWS, P. W., BEIGHTON, G., BELLO, P. A., BENVENISTY, N., BERRY, L. S., BEVAN, S., BLUM, B., BROOKING, J., CHEN, K. G., CHOO, A. B. H., CHURCHILL, G. A., CORBEL, M., DAMJANOV, I., DRAPER, J. S., DVORAK, P., EMANUELSSON, K., FLECK, R. A., FORD, A., GERTOW, K., GERTSENSTEIN, M., GOKHALE, P. J., HAMILTON, R. S., HAMPL, A., HEALY, L. E., HOVATTA, O., HYLLNER, J., IMREH, M. P., ITSKOVITZ-ELDOR, J., JACKSON, J., JOHNSON, J. L., JONES, M., KEE, K., KING, B. L., KNOWLES, B. B., LAKO, M., LEBRIN, F., MALLON, B. S., MANNING, D., MAYSHAR, Y., MCKAY, R. D. G., MICHALSKA, A. E., MIKKOLA, M., MILEIKOVSKY, M., MINGER, S. L., MOORE, H. D., MUMMERY, C. L., NAGY, A., NAKATSUJI, N., O'BRIEN, C. M., OH, S. K. W., OLSSON, C., OTONKOSKI, T., PARK, K. Y., PASSIER, R., PATEL, H., PATEL, M., PEDERSEN, R., PERA, M. F., PIEKARCZYK, M. S., PERA, R. A. R., REUBINOFF, B. E., ROBINS, A. J., ROSSANT, J., RUGG-GUNN, P., SCHULZ, T. C., SEMB, H., SHERRER, E. S., SIEMEN, H., STACEY, G. N., STOJKOVIC, M., SUEMORI, H., SZATKIEWICZ, J., TURETSKY, T., TUURI, T., VAN DEN BRINK, S., VINTERSTEN, K., VUORISTO, S., WARD, D., WEAVER, T. A., YOUNG, L. A., ZHANG, W. D. & INT STEM CELL, I. 2007. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nature Biotechnology,* 25**,** 803-816.

ADHIKARI, A. S., AGARWAL, N., WOOD, B. M., PORRETTA, C., RUIZ, B., POCHAMPALLY, R. R. & IWAKUMA, T. 2010. CD117 and Stro-1 Identify Osteosarcoma Tumor-Initiating Cells Associated with Metastasis and Drug Resistance. *Cancer Research,* 70**,** 4602-4612.

AGARAM, N. P., ZHANG, L., LELOARER, F., SILK, T., SUNG, Y. S., SCOTT, S. N., KUK, D., QIN, L. X., BERGER, M. F., ANTONESCU, C. R. & SINGER, S. 2016. Targeted Exome Sequencing Profiles Genetic Alterations in Leiomyosarcoma. *Genes Chromosomes & Cancer,* 55**,** 124-130.

AIGNER, S., STHOEGER, Z. M., FOGEL, M., WEBER, E., ZARN, J., RUPPERT, M., ZELLER, Y., VESTWEBER, D., STAHEL, R., SAMMAR, M. & ALTEVOGT, P. 1997. CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. *Blood,* 89**,** 3385-3395.

AIKAWA, Y., KATSUMOTO, T., ZHANG, P., SHIMA, H., SHINO, M., TERUI, K., ITO, E., OHNO, H., STANLEY, E. R., SINGH, H., TENEN, D. G. & KITABAYASHI, I. 2010. PU.1-mediated upregulation of CSF1R is crucial for leukemia stem cell potential induced by MOZ-TIF2. *Nature Medicine,* 16**,** 580-U115.

AKBARZADEH, M., MAROUFI, N. F., TAZEHKAND, A. P., BASTANI, S., SAFDARI, R., FARZANE, A., FATTAHI, A., NEJABATI, H. R., NOURI, M. & SAMADI, N. 2019. Current approaches in identification and isolation of cancer stem cells. *Journal of Cellular Physiology,* 234**,** 14759-14772.

AL-HAJJ, M., WICHA, M. S., BENITO-HERNANDEZ, A., MORRISON, S. J. & CLARKE, M. F. 2003. Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 3983-3988.

ALBA-CASTELLON, L., BATLLE, R., FRANCI, C., FERNANDEZ-ACENERO, M. J., MAZZOLINI, R., PENA, R., LOUBAT, J., ALAMEDA, F., RODRIGUEZ, R., CURTO, J., ALBANELL, J., MUNOZ, A., BONILLA, F., CASAL, J. I., ROJO, F. & DE HERREROS, A. G. 2014. Snail1 Expression Is Required for Sarcomagenesis. *Neoplasia,* 16**,** 413-421.

ALVES, C. P., DEY-GUHA, I., KABRAJI, S., YEH, A. C., TALELE, N. P., SOLE, X., CHOWDHURY, J., MINO-KENUDSON, M., LODA, M., SGROI, D., BORRESEN-DALE, A. L., RUSSNES, H. G., ROSS, K. N. & RAMASWAMY, S. 2018. AKT1(low) Quiescent Cancer Cells Promote Solid Tumor Growth. *Molecular Cancer Therapeutics,* 17**,** 254-263.

AMANT, F., DORFLING, C. M., DREYER, L., VERGOTE, I., LINDEQUE, B. G. & VAN RENSBURG, E. J. 2001. Microsatellite instability in uterine sarcomas. *International Journal of Gynecological Cancer,* 11**,** 218-223.

AMARAVADI, R. K. & THOMPSON, C. B. 2007. The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clinical Cancer Research,* 13**,** 7271-7279.

AN, X. L. & CHEN, L. X. 2018. Flow Cytometry (FCM) Analysis and Fluorescence-Activated Cell Sorting (FACS) of Erythroid Cells. *In:* LLOYD, J. A. (ed.) *Erythropoiesis: Methods and Protocols.* Totowa: Humana Press Inc.

ARRIGONI, G. & DOGLIONI, C. 2004. Atypical lipomatous tumor: molecular characterization. *Current Opinion in Oncology,* 16**,** 355-358.

ASSOU, S., LE CARROUR, T., TONDEUR, S., STROM, S., GABELLE, A., MARTY, S., NADAL, L., PANTESCO, V., REME, T., HUGNOT, J. P., GASCA, S., HOVATTA, O., HAMAMAH, S., KLEIN, B. & DE VOS, J. 2007. A meta-analysis of human embryonic stem cells transcriptome integrated into a web-based expression atlas. *Stem Cells,* 25**,** 961-973.

ATASHZAR, M. R., BAHARLOU, R., KARAMI, J., ABDOLLAHI, H., REZAEI, R., POURRAMEZAN, F. & MOGHADDAM, S. H. Z. 2020. Cancer stem cells: A review from origin to therapeutic implications. *Journal of Cellular Physiology,* 235**,** 790-803.

ATAY, S., WILKEY, D. W., MILHEM, M., MERCHANT, M. & GODWIN, A. K. 2018. Insights into the Proteome of Gastrointestinal Stromal Tumors-Derived Exosomes Reveals New Potential Diagnostic Biomarkers. *Molecular & Cellular Proteomics,* 17**,** 495-515.

AWAD, O., YUSTEIN, J. T., SHAH, P., GUL, N., KATURI, V., O'NEILL, A., KONG, Y., BROWN, M. L., TORETSKY, J. A. & LOEB, D. M. 2010. High ALDH Activity

Identifies Chemotherapy-Resistant Ewing's Sarcoma Stem Cells That Retain Sensitivity to EWS-FLI1 Inhibition. *Plos One,* 5.

BABA, E. & AKASHI, K. 2015. The fundamental concept of cancer stem cell and the progress in cancer stem cell research. *Nihon rinsho. Japanese journal of clinical medicine,* 73**,** 721-5.

BALARAJAN, R. & ACHESON, E. D. 1984. SOFT-TISSUE SARCOMAS IN AGRICULTURE AND FORESTRY WORKERS. *Journal of Epidemiology and Community Health,* 38**,** 113-116.

BALLINGER, M. L., GOODE, D. L., RAY-COQUARD, I., JAMES, P. A., MITCHELL, G., NIEDERMAYR, E., PURI, A., SCHIFFMAN, J. D., DITE, G. S., CIPPONI, A., MAKI, R. G., BROHL, A. S., MYKLEBOST, O., STRATFORD, E. W., LORENZ, S., AHN, S. M., AHN, J. H., KIM, J. E., SHANLEY, S., BESHAY, V., RANDALL, R. L., JUDSON, I., SEDDON, B., CAMPBELL, I. G., YOUNG, M. A., SARIN, R., BLAY, J. Y., O'DONOGHUE, S. I., THOMAS, D. M. & INT SARCOMA KINDRED, S. 2016. Monogenic and polygenic determinants of sarcoma risk: an international genetic study. *Lancet Oncology,* 17**,** 1261-1271.

BAN, J., JUG, G., MESTDAGH, P., SCHWENTNER, R., KAUER, M., ARYEE, D. N. T., SCHAEFER, K. L., NAKATANI, F., SCOTLANDI, K., REITER, M., STRUNK, D., SPELEMAN, F., VANDESOMPELE, J. & KOVAR, H. 2011. Hsa-mir-145 is the top EWS-FLI1-repressed microRNA involved in a positive feedback loop in Ewing's sarcoma. *Oncogene,* 30**,** 2173-2180.

BAO, S. D., WU, Q. L., SATHORNSUMETEE, S., HAO, Y. L., LI, Z. Z., HJELMELAND, A. B., SHI, O., MCLENDON, R. E., BIGNER, D. D. & RICH, J. N. 2006. Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Research,* 66**,** 7843-7848.

BARRETINA, J., TAYLOR, B. S., BANERJI, S., RAMOS, A. H., LAGOS-QUINTANA, M., DECAROLIS, P. L., SHAH, K., SOCCI, N. D., WEIR, B. A., HO, A., CHIANG, D. Y., REVA, B., MERMEL, C. H., GETZ, G., ANTIPIN, Y., BEROUKHIM, R., MAJOR, J. E., HATTON, C., NICOLETTI, R., HANNA, M., SHARPE, T., FENNELL, T. J., CIBULSKIS, K., ONOFRIO, R. C., SAITO, T., SHUKLA, N., LAU, C., NELANDER, S., SILVER, S. J., SOUGNEZ, C., VIALE, A., WINCKLER, W., MAKI, R. G., GARRAWAY, L. A., LASH, A., GREULICH, H., ROOT, D. E., SELLERS, W. R., SCHWARTZ, G. K., ANTONESCU, C. R., LANDER, E. S., VARMUS, H. E., LADANYI, M., SANDER, C., MEYERSON, M. & SINGER, S. 2010. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. *Nat Genet,* 42**,** 715-721.

BASU-ROY, U., SEO, E., RAMANATHAPURAM, L., RAPP, T. B., PERRY, J. A., ORKIN, S. H., MANSUKHANI, A. & BASILICO, C. 2012. Sox2 maintains self renewal of tumor-initiating cells in osteosarcomas. *Oncogene,* 31**,** 2270-2282.

BAYLIN, S. B. & JONES, P. A. 2011. A decade of exploring the cancer epigenome - biological and translational implications. *Nature Reviews Cancer,* 11**,** 726-734.

BECK, A. H., LEE, C. H., WITTEN, D. M., GLEASON, B. C., EDRIS, B., ESPINOSA, I., ZHU, S., LI, R., MONTGOMERY, K. D., MARINELLI, R. J., TIBSHIRANI, R., HASTIE, T., JABLONS, D. M., RUBIN, B. P., FLETCHER, C. D., WEST, R. B. &

VAN DE RIJN, M. 2010. Discovery of molecular subtypes in leiomyosarcoma through integrative molecular profiling. *Oncogene,* 29**,** 845-854.

BECKINGSALE, T. B. & SHAW, C. 2017. Epidemiology of bone and soft-tissue sarcomas. *Orthopaedics and trauma,* 31**,** 144-150.

BEIRD, H. C., WU, C. C., INGRAM, D. R., WANG, W. L., ALIMOHAMED, A., GUMBS, C., LITTLE, L., SONG, X. Z., FEIG, B. W., ROLAND, C. L., ZHANG, J. H., BENJAMIN, R. S., HWU, P., LAZAR, A. J., FUTREAL, P. A. & SOMAIAH, N. 2018. Genomic profiling of dedifferentiated liposarcoma compared to matched well-differentiated liposarcoma reveals higher genomic complexity and a common origin. *Cold Spring Harbor Molecular Case Studies,* 4.

BEKAII-SAAB, T. & EL-RAYES, B. 2017. Identifying and targeting cancer stem cells in the treatment of gastric cancer. *Cancer,* 123**,** 1303-1312.

BELAYNEH, R. & WEISS, K. 2020. The Role of ALDH in the Metastatic Potential of Osteosarcoma Cells and Potential ALDH Targets. *In:* KLEINERMAN, E. S. & GORLICK, R. (eds.) *Current Advances in the Science of Osteosarcoma: Research Perspectives: Tumor Biology, Organ Microenvironment, Potential New Therapeutic Targets, and Canine Models, 2nd Edition.* Cham: Springer International Publishing Ag.

BELINSKY, M. G., RINK, L., CAI, K. Q., CAPUZZI, S. J., HOANG, Y., CHIEN, J., GODWIN, A. K. & VON MEHREN, M. 2015. Somatic loss of function mutations in neurofibromin 1 and MYC associated factor X genes identified by exome-wide sequencing in a wild-type GIST case. *Bmc Cancer,* 15.

BELLIO, C., DIGLORIA, C., FOSTER, R., JAMES, K., KONSTANTINOPOULOS, P. A., GROWDON, W. B. & RUEDA, B. R. 2019. PARP Inhibition Induces Enrichment of DNA Repair-Proficient CD133 and CD117 Positive Ovarian Cancer Stem Cells. *Molecular Cancer Research,* 17**,** 431-445.

BEN-DAVID, U., SIRANOSIAN, B., HA, G., TANG, H., OREN, Y., HINOHARA, K., STRATHDEE, C. A., DEMPSTER, J., LYONS, N. J., BURNS, R., NAG, A., KUGENER, G., CIMINI, B., TSVETKOV, P., MARUVKA, Y. E., O'ROURKE, R., GARRITY, A., TUBELLI, A. A., BANDOPADHAYAY, P., TSHERNIAK, A., VAZQUEZ, F., WONG, B., BIRGER, C., GHANDI, M., THORNER, A. R., BITTKER, J. A., MEYERSON, M., GETZ, G., BEROUKHIM, R. & GOLUB, T. R. 2018. Genetic and transcriptional evolution alters cancer cell line drug response. *Nature,* 560**,** 325-+.

BENSON, E. K., MUNGAMURI, S. K., ATTIE, O., KRACIKOVA, M., SACHIDANANDAM, R., MANFREDI, J. J. & AARONSON, S. A. 2014. p53-dependent gene repression through p21 is mediated by recruitment of E2F4 repression complexes. *Oncogene,* 33**,** 3959-3969.

BHARTI, R., DEY, G. & MANDAL, M. 2016. Cancer development, chemoresistance, epithelial to mesenchymal transition and stem cells: A snapshot of IL-6 mediated involvement. *Cancer Letters,* 375**,** 51-61.

BHATTACHARYA, B., MIURA, T., BRANDENBERGER, R., MEJIDO, J., LUO, Y. Q., YANG, A. X., JOSHI, B. H., GINIS, I., THIES, R. S., AMIT, M., LYONS, I., CONDIE, B. G., ITSKOVITZ-ELDOR, J., RAO, M. S. & PURI, R. K. 2004. Gene

expression in human embryonic stem cell lines: unique molecular signature. *Blood,* 103**,** 2956-2964.

BHOWMICK, N. A., NEILSON, E. G. & MOSES, H. L. 2004. Stromal fibroblasts in cancer initiation and progression. *Nature,* 432**,** 332-337.

BLAIR, A., HOGGE, D. E. & SUTHERLAND, H. J. 1998. Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)/CD71(-)/HLA-DR. *Blood,* 92**,** 4325-4335.

BLAIR, A. & SUTHERLAND, H. J. 2000. Primitive acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo lack surface expression of c-kit (CD 117). *Experimental Hematology,* 28**,** 660-671.

BODNAR, A. G., OUELLETTE, M., FROLKIS, M., HOLT, S. E., CHIU, C. P., MORIN, G. B., HARLEY, C. B., SHAY, J. W., LICHTSTEINER, S. & WRIGHT, W. E. 1998. Extension of life-span by introduction of telomerase into normal human cells. *Science,* 279**,** 349-352.

BOIKOS, S. A., PAPPO, A. S., KILLIAN, J. K., LAQUAGLIA, M. P., WELDON, C. B., GEORGE, S., TRENT, J. C., VON MEHREN, M., WRIGHT, J. A., SCHIFFMAN, J. D., RAYGADA, M., PACAK, K., MELTZER, P. S., MIETTINEN, M. M., STRATAKIS, C., JANEWAY, K. A. & HELMAN, L. J. 2016. Molecular Subtypes of KIT/PDGFRA Wild-Type Gastrointestinal Stromal Tumors A Report From the National Institutes of Health Gastrointestinal Stromal Tumor Clinic. *Jama Oncology,* 2**,** 922-928.

BOMKEN, S., FISER, K., HEIDENREICH, O. & VORMOOR, J. 2010. Understanding the cancer stem cell. *British Journal of Cancer,* 103**,** 439-445.

BONNET, D. 2005. Cancer stem cells: lessons from leukaemia. *Cell Proliferation,* 38**,** 357-361.

BONNET, D. & DICK, J. E. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine,* 3**,** 730-737.

BROOKS, M. D., BURNESS, M. L. & WICHA, M. S. 2015. Therapeutic Implications of Cellular Heterogeneity and Plasticity in Breast Cancer. *Cell Stem Cell,* 17**,** 260-271.

BROWN, H. K., TELLEZ-GABRIEL, M. & HEYMANN, D. 2017. Cancer stem cells in osteosarcoma. *Cancer Letters,* 386**,** 189-195.

BUONO, R. & LONGO, V. D. 2018. Starvation, Stress Resistance, and Cancer. *Trends in Endocrinology and Metabolism,* 29**,** 271-280.

BURGER, J. A. & PELED, A. 2009. CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers. *Leukemia,* 23**,** 43-52.

BURKHARDT, J. K., HOFSTETTER, C. P., SANTILLAN, A., SHIN, B. J., FOLEY, C. P., BALLON, D. J., GOBIN, Y. P. & BOOCKVAR, J. A. 2012. Orthotopic glioblastoma stem-like cell xenograft model in mice to evaluate intra-arterial delivery of bevacizumab: From bedside to bench. *Journal of Clinical Neuroscience,* 19**,** 1568-1572.

BURKHART, D. L. & SAGE, J. 2008. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nature Reviews Cancer,* 8**,** 671-682.

BURNINGHAM, Z., HASHIBE, M., SPECTOR, L. & SCHIFFMAN, J. D. 2012. The epidemiology of sarcoma. *Clinical sarcoma research,* 2**,** 14-14.

BURNS, J., WILDING, C. P., JONES, R. L. & HUANG, P. H. 2020. Proteomic research in sarcomas - current status and future opportunities. *Seminars in Cancer Biology,* 61**,** 56-70.

BURTON, D. G. A. & FARAGHER, R. G. A. 2015. Cellular senescence: from growth arrest to immunogenic conversion. *Age,* 37.

CAI, S. B., PU, Y. G., ZHAO, F. F., WANG, H. Y., CAI, W. J., GAO, J. & LI, Y. P. 2017. MiR-34a-5p promotes the multi-drug resistance of osteosarcoma by targeting the CD117 gene (vol 7, pg 28420, 2016). *Oncotarget,* 8**,** 60723-60723.

CARINCI, F., STABELLINI, G., CALVITTI, M., PELUCCHI, S., TARGA, L., FARINA, A., PEZZETTI, F. & PASTORE, A. 2002. CD44 as prognostic factor in oral and oropharyngeal squamous cell carcinoma. *Journal of Craniofacial Surgery,* 13**,** 85-89.

CATTIN, S., RAMONT, L. & RUEGG, C. 2018. Characterization and In Vivo Validation of a Three-Dimensional Multi-Cellular Culture Model to Study Heterotypic Interactions in Colorectal Cancer Cell Growth, Invasion and Metastasis. *Frontiers in Bioengineering and Biotechnology,* 6**,** 14.

CESARE, A. J. & REDDEL, R. R. 2010. Alternative lengthening of telomeres: models, mechanisms and implications. *Nature Reviews Genetics,* 11**,** 319-330.

CHAU, C. M., DENG, Z., KANG, H. & LIEBERMAN, P. M. 2008. Cell cycle association of the retinoblastoma protein Rb and the histone demethylase LSD1 with the Epstein-Barr virus latency promoter Cp. *Journal of Virology,* 82**,** 3428-3437.

CHEN, C. T., SHIH, Y. R. V., KUO, T. K., LEE, O. K. & WEI, Y. H. 2008. Coordinated changes of mitochondrial biogenesis and antioxidant enzymes during osteogenic differentiation of human mesenchymal stem cells. *Stem Cells,* 26**,** 960-968.

CHEN, K., HUANG, Y.-H. & CHEN, J.-L. 2013. Understanding and targeting cancer stem cells: therapeutic implications and challenges. *Acta Pharmacologica Sinica,* 34**,** 732-740.

CHEN, Y., GUO, Y. F., YANG, H. S., SHI, G. D., XU, G. H., SHI, J. G., YIN, N. & CHEN, D. Y. 2015. TRIM66 overexpresssion contributes to osteosarcoma carcinogenesis and indicates poor survival outcome. *Oncotarget,* 6**,** 23708-23719.

CHEN, Y. T., ZHANG, K. S., LI, Y. & HE, Q. 2017. Estrogen-related receptor a participates transforming growth factor-beta(TGF-beta) induced epithelial-mesenchymal transition of osteosarcoma cells. *Cell Adhesion & Migration,* 11**,** 338-346.

CHENG, N., CHYTIL, A., SHYR, Y., JOLY, A. & MOSES, H. L. 2008. Transforming Growth Factor-beta Signaling-Deficient Fibroblasts Enhance Hepatocyte Growth

Factor Signaling in Mammary Carcinoma Cells to Promote Scattering and Invasion. *Molecular Cancer Research,* 6**,** 1521-1533.

CIDRE-ARANAZ, F., GRUNEWALD, T. G. P., SURDEZ, D., GARCIA-GARCIA, L., LAZARO, J. C., KIRCHNER, T., GONZALEZ-GONZALEZ, L., SASTRE, A., GARCIA-MIGUEL, P., LOPEZ-PEREZ, S. E., MONZON, S., DELATTRE, O. & ALONSO, J. 2017. EWS-FLI1-mediated suppression of the RAS-antagonist Sprouty 1 (SPRY1) confers aggressiveness to Ewing sarcoma. *Oncogene,* 36**,** 766-776.

CLARKE, M. F. & FULLER, M. 2006. Stem cells and cancer: Two faces of eve. *Cell,* 124**,** 1111-1115.

CLAY, M. R., TABOR, M., OWEN, J. H., CAREY, T. E., BRADFORD, C. R., WOLF, G. T., WICHA, M. S. & PRINCE, M. E. 2010. SINGLE-MARKER IDENTIFICATION OF HEAD AND NECK SQUAMOUS CELL CARCINOMA CANCER STEM CELLS WITH ALDEHYDE DEHYDROGENASE. *Head and Neck-Journal for the Sciences and Specialties of the Head and Neck,* 32**,** 1195-1201.

COLE, A. J., FAYOMI, A. P., ANYAECHE, V. I., BAI, S. & BUCKANOVICH, R. J. 2020. An evolving paradigm of cancer stem cell hierarchies: therapeutic implications. *Theranostics,* 10**,** 3083-3098.

COLLADO, M. & SERRANO, M. 2010. SENESCENCE Senescence in tumours: evidence from mice and humans. *Nature Reviews Cancer,* 10**,** 51-57.

COLLINS, A. T., BERRY, P. A., HYDE, C., STOWER, M. J. & MAITLAND, N. J. 2005a. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Research,* 65**,** 10946-10951.

COLLINS, C. A., OLSEN, I., ZAMMIT, P. S., HESLOP, L., PETRIE, A., PARTRIDGE, T. A. & MORGAN, J. E. 2005b. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell,* 122**,** 289-301.

COMBA, P., ASCOLI, V., BELLI, S., BENEDETTI, M., GATTI, L., RICCI, P. & TIEGHI, A. 2003. Risk of soft tissue sarcomas and residence in the neighbourhood of an incinerator of industrial wastes. *Occupational and Environmental Medicine,* 60**,** 680-683.

CORLESS, C. L. 2014. Gastrointestinal stromal tumors: what do we know now? *Modern Pathology,* 27**,** S1-S16.

CRAWFORD, H. C., DI MAGLIANO, M. P. & BANERJEE, S. 2019. Signaling Networks That Control Cellular Plasticity in Pancreatic Tumorigenesis, Progression, and Metastasis. *Gastroenterology,* 156**,** 2073-2084.

CREE, I. A., GLAYSHER, S. & HARVEY, A. L. 2010. Efficacy of anti-cancer agents in cell lines versus human primary tumour tissue. *Current Opinion in Pharmacology,* 10**,** 375-379.

CRONIN, K. A., LAKE, A. J., SCOTT, S., SHERMAN, R. L., NOONE, A. M., HOWLADER, N., HENLEY, S. J., ANDERSON, R. N., FIRTH, A. U., MA, J. M., KOHLER, B. A. & JEMAL, A. 2018. Annual Report to the Nation on the Status of Cancer, part I: National cancer statistics. *Cancer,* 124**,** 2785-2800.

CRUZ, M. H., SIDEN, A., CALAF, G. M., DELWAR, Z. M. & YAKISICH, J. S. 2012. The stemness phenotype model. *ISRN oncology,* 2012**,** 392647-392647.

DALERBA, P., CHO, R. W. & CLARKE, M. F. 2007. Cancer stem cells: Models and concepts. *Annual Review of Medicine,* 58**,** 267-284.

DANCSOK, A. R., ASLEH-ABURAYA, K. & NIELSEN, T. O. 2017. Advances in sarcoma diagnostics and treatment. *Oncotarget,* 8**,** 7068-7093.

DANISOVIC, L., ORAVCOVA, L., KRAJCIOVA, L., NOVAKOVA, Z. V., BOHAC, M., VARGA, I. & VOJTASSAK, J. 2017. Effect of long-term culture on the biological and morphological characteristics of human adipose tissue-derived stem cells. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society,* 68**,** 149-158.

DAO, M. A. & NOLTA, J. A. 2000. CD34: To select or not to select? That is the question. *Leukemia,* 14**,** 773-776.

DARENA, G., CASCAVILLA, N., MUSTO, P., GRECO, M., DIMAURO, L., CARELLA, A. M., DELLOIACONO, N. & CAROTENUTO, M. 1996. Flow cytometric characterization of CD34(+) hematopoietic progenitor cells in mobilized peripheral blood and bone marrow of cancer patients. *Haematologica,* 81**,** 216-223.

DE PAIVA, A. C. G., DE ABREU, M. & DE SOUZA, M. P. 2018. Undifferentiated pleomorphic sarcoma. *Anais Brasileiros De Dermatologia,* 93**,** 154-155.

DELLA PORTA, M. G., MALCOVATI, L., INVERNIZZI, R., TRAVAGLINO, E., PASCUTTO, C., MAFFIOLI, M., GALLI, A., BOGGI, S., PIETRA, D., VANELLI, L., MARSEGLIA, C., LEVI, S., AROSIO, P., LAZZARINO, M. & CAZZOLA, M. 2006. Flow cytometry evaluation of erythroid dysplasia in patients with myelodysplastic syndrome. *Leukemia,* 20**,** 549-555.

DESANTIS, C. E., MILLER, K. D., DALE, W., MOHILE, S. G., COHEN, H. J., LEACH, C. R., SAUER, A. G., LEMAL, A. & SIEGEL, R. L. 2019. Cancer statistics for adults aged 85 years and older, 2019. *Ca-a Cancer Journal for Clinicians,* 69**,** 452-467.

DESCHLER, B. & LUEBBERT, M. 2006. Acute myeloid leukemia: Epidemiology and etiology. *Cancer,* 107**,** 2099-2107.

DI FIORE, R., SANTULLI, A., FERRANTE, R. D., GIULIANO, M., DE BLASIO, A., MESSINA, C., PIROZZI, G., TIRINO, V., TESORIERE, G. & VENTO, R. 2009. Identification and Expansion of Human Osteosarcoma-Cancer-Stem Cells by Long-Term 3-Aminobenzamide Treatment. *Journal of Cellular Physiology,* 219**,** 301-313.

DICK, J. E. 2005. Acute myeloid leukemia stem cells. *Hematopoietic Stem Cells V,* 1044**,** 1-5.

DICK, J. E. 2008. Stem cell concepts renew cancer research. *Blood,* 112**,** 4793-4807.

DINAVAHI, S. S., BAZEWICZ, C. G., GOWDA, R. & ROBERTSON, G. P. 2019. Aldehyde Dehydrogenase Inhibitors for Cancer Therapeutics. *Trends in Pharmacological Sciences,* 40**,** 774-789.

DIRKS, P. B. 2005. Brain tumor stem cells. *Biology of Blood and Marrow Transplantation,* 11**,** 12-13.

DOHERTY, M. R., SMIGIEL, J. M., JUNK, D. J. & JACKSON, M. W. 2016. Cancer Stem Cell Plasticity Drives Therapeutic Resistance. *Cancers,* 8**,** 13.

DOYLE, L. A., NOWAK, J. A., NATHENSON, M. J., THORNTON, K., WAGNER, A. J., JOHNSON, J. M., ALBRAYAK, A., GEORGE, S. & SHOLL, L. M. 2019. Characteristics of mismatch repair deficiency in sarcomas. *Modern Pathology,* 32**,** 977-987.

DRAPER, J. S., PIGOTT, C., THOMSON, J. A. & ANDREWS, P. W. 2002. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *Journal of Anatomy,* 200**,** 249-258.

DUENSING, A., MEDEIROS, F., MCCONARTY, B., JOSEPH, N. E., PANIGRAHY, D., SINGER, S., FLETCHER, C. D. M., DEMETRI, G. D. & FLETCHER, J. A. 2004. Mechanisms of oncogenic KIT signal transduction in primary gastrointestinal stromal tumors (GISTs). *Oncogene,* 23**,** 3999-4006.

EL BEAINO, M., ARAUJO, D. M., LAZAR, A. J. & LIN, P. P. 2017. Synovial Sarcoma: Advances in Diagnosis and Treatment Identification of New Biologic Targets to Improve Multimodal Therapy. *Annals of Surgical Oncology,* 24**,** 2145-2154.

EL-BADAWY, A., GHONEIM, N. I., NASR, M. A., ELKHENANY, H., AHMED, T. A., AHMED, S. M. & EL-BADRI, N. 2018. Telomerase reverse transcriptase coordinates with the epithelial-to-mesenchymal transition through a feedback loop to define properties of breast cancer stem cells. *Biology Open,* 7.

EL-NAGGAR, A. M., VEINOTTE, C. J., CHENG, H. W., GRUNEWALD, T. G. P., NEGRI, G. L., SOMASEKHARAN, S. P., CORKERY, D. P., TIRODE, F., MATHERS, J., KHAN, D., KYLE, A. H., BAKER, J. H., LEPARD, N. E., MCKINNEY, S., HAJEE, S., BOSILJCIC, M., LEPRIVIER, G., TOGNON, C. E., MINCHINTON, A. I., BENNEWITH, K. L., DELATTRE, O., WANG, Y. Z., DELLAIRE, G., BERMAN, J. N. & SORENSEN, P. H. 2015. Translational Activation of HIF1 alpha by YB-1 Promotes Sarcoma Metastasis. *Cancer Cell,* 27**,** 682-697.

EL-RIFAI, W., SARLOMO-RIKALA, M., KNUUTILA, S. & MIETTINEN, M. 1998. DNA copy number changes in development and progression in leiomyosarcomas of soft tissues. *American Journal of Pathology,* 153**,** 985-990.

ENGELHOLM, S. A., VINDELOV, L. L., SPANGTHOMSEN, M., BRUNNER, N., TOMMERUP, N., NIELSEN, M. H. & HANSEN, H. H. 1985. GENETIC INSTABILITY OF CELL-LINES DERIVED FROM A SINGLE HUMAN SMALL CELL-CARCINOMA OF THE LUNG. *European Journal of Cancer & Clinical Oncology,* 21**,** 815-824.

ENVER, T., SONEJI, S., JOSHI, C., BROWN, J., IBORRA, F., ORNTOFT, T., THYKJAER, T., MALTBY, E., SMITH, K., ABU DAWUD, R., JONES, M., MATIN, M., GOKHALE, P., DRAPER, J. & ANDREWS, P. W. 2005. Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. *Human Molecular Genetics,* 14**,** 3129-3140.

F. FARSHADPOUR , M. S. B. A. J. H. S., A.N.M. WYMENGA , & R. OTTER , H. J. H. 2005. Soft tissue sarcoma: why not treated? . *Critical Reviews in Oncology/Hematology,* 54**,** 77-83.

FABIAN, A., BAROK, M., VEREB, G. & SZOELLOSI, J. 2009. Die Hard: Are Cancer Stem Cells the Bruce Willises of Tumor Biology? *Cytometry Part A,* 75A**,** 67-74.

FABREGAT, I., MALFETTONE, A. & SOUKUPOVA, J. 2016. New Insights into the Crossroads between EMT and Stemness in the Context of Cancer. *Journal of Clinical Medicine,* 5.

FANALI, C., LUCCHETTI, D., FARINA, M., CORBI, M., CUFINO, V., CITTADINI, A. & SGAMBATO, A. 2014. Cancer stem cells in colorectal cancer from pathogenesis to therapy: Controversies and perspectives. *World Journal of Gastroenterology,* 20**,** 923-942.

FARID, M. & NGEOW, J. 2016. Sarcomas Associated With Genetic Cancer Predisposition Syndromes: A Review. *Oncologist,* 21**,** 1002-1013.

FEARON, E. R. & VOGELSTEIN, B. 1990. A GENETIC MODEL FOR COLORECTAL TUMORIGENESIS. *Cell,* 61**,** 759-767.

FENG, Z. M. & GUO, S. M. 2016. Tim-3 facilitates osteosarcoma proliferation and metastasis through the NF-kappa B pathway and epithelial-mesenchymal transition. *Genetics and Molecular Research,* 15**,** 9.

FERNANDO, A., GLAYSHER, S., CONROY, M., PEKALSKI, M., SMITH, J., KNIGHT, L. A., DI NICOLANTONIO, F. & CREE, I. A. 2006. Effect of culture conditions on the chemosensitivity of ovarian cancer cell lines. *Anti-Cancer Drugs,* 17**,** 913-919.

FERREIRA, B. I., ALONSO, J., CARRILLO, J., ACQUADRO, F., LARGO, C., SUELA, J., TEIXEIRA, M. R., CERVEIRA, N., MOLARES, A., GOMEZ-LOPEZ, G., PESTANA, A., SASTRE, A., GARCIA-MIGUEL, P. & CIGUDOSA, J. C. 2008. Array CGH and gene-expression profiling reveals distinct genomic instability patterns associated with DNA repair and cell-cycle checkpoint pathways in Ewing's sarcoma. *Oncogene,* 27**,** 2084-2090.

FIALKOW, P. J. 1972. Use of genetic markers to study cellular origin and development of tumors in human females. *Advances in cancer research,* 15**,** 191-226.

FIALKOW, P. J. 1990. STEM-CELL ORIGIN OF HUMAN MYELOID BLOOD-CELL NEOPLASMS. *Pathology of the Hematopoietic Stem Cell : Quantative Pathology, Molecular Pathology: Pathologie Der Haematopoietischen Stammzelle : Quantative Pathologie, Molekularpathologie,* 74**,** 43-47.

FIALKOW, P. J., MARTIN, P. J., NAJFELD, V., PENFOLD, G. K., JACOBSON, R. J. & HANSEN, J. A. 1981. EVIDENCE FOR A MULTISTEP PATHOGENESIS OF CHRONIC MYELOGENOUS LEUKEMIA. *Blood,* 58**,** 158-163.

FIEDOROWICZ, M., BARTNIK, E., SOBCZUK, P., TETERYCZ, P. & CZARNECKA, A. M. 2018. Molecular biology of sarcoma. *Oncology in Clinical Practice,* 14**,** 307-330.

FINK, D. J. 1979. CANCER OVERVIEW. *Cancer Research,* 39**,** 2819-2821.

FIORETTI, F., TAVANI, A., GALLUS, S., NEGRI, E., FRANCESCHI, S. & LA VECCHIA, C. 2000. Menstrual and reproductive factors and risk of soft tissue sarcomas. *Cancer,* 88**,** 786-789.

FLETCHER, C. D. M. 2014. The evolving classification of soft tissue tumours - an update based on the new 2013 WHO classification. *Histopathology,* 64**,** 2-11.

FONSECA, I., PEREIRA, T., ROSA-SANTOS, J. & SOARES, J. 2001. Expression of CD44 isoforms in squamous cell carcinoma of the border of the tongue: A correlation with histological grade, pattern of stromal invasion, and cell differentiation. *Journal of Surgical Oncology,* 76**,** 115-120.

FOSTER, B. M., ZAIDI, D., YOUNG, T. R., MOBLEY, M. E. & KERR, B. A. 2018. CD117/c-kit in Cancer Stem Cell-Mediated Progression and Therapeutic Resistance. *Biomedicines,* 6**,** 19.

FRANCESCANGELI, F., PATRIZII, M., SIGNORE, M., FEDERICI, G., DI FRANCO, S., PAGLIUCA, A., BAIOCCHI, M., BIFFONI, M., VITIANI, L. R., TODARO, M., DE MARIA, R. & ZEUNER, A. 2012. Proliferation State and Polo-Like Kinase1 Dependence of Tumorigenic Colon Cancer Cells. *Stem Cells,* 30**,** 1819-1830.

FRIDBORG, H., JONSSON, E., NYGREN, P. & LARSSON, R. 1999. Relationship between diagnosis-specific activity of cytotoxic drugs in fresh human tumour cells ex vivo and in the clinic. *European Journal of Cancer,* 35**,** 424-432.

FROELICH, K., MICKLER, J., STEUSLOFF, G., TECHNAU, A., TIRADO, M. R., SCHERZED, A., HACKENBERG, S., RADELOFF, A., HAGEN, R. & KLEINSASSER, N. 2013. Chromosomal aberrations and deoxyribonucleic acid single-strand breaks in adipose-derived stem cells during long-term expansion in vitro. *Cytotherapy,* 15**,** 767-781.

FRYKNAS, M., ZHANG, X. N., BREMBERG, U., SENKOWSKI, W., OLOFSSON, M. H., BRANDT, P., PERSSON, I., D'ARCY, P., GULLBO, J., NYGREN, P., SCHUGHART, L. K., LINDER, S. & LARSSON, R. 2016. Iron chelators target both proliferating and quiescent cancer cells. *Scientific Reports,* 6.

FU, W. L., LI, J., CHEN, G., LI, Q., TANG, X. & ZHANG, C. H. 2015. Mesenchymal Stem Cells Derived from Peripheral Blood Retain Their Pluripotency, but Undergo Senescence During Long-Term Culture. *Tissue Engineering Part C-Methods,* 21**,** 1088-1097.

GALLI, R., BINDA, E., ORFANELLI, U., CIPELLETTI, B., GRITTI, A., DE VITIS, S., FIOCCO, R., FORONI, C., DIMECO, F. & VESCOVI, A. 2004. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Research,* 64**,** 7011-7021.

GAO, M. Q., CHOI, Y. P., KANG, S., YOUN, J. H. & CHO, N. H. 2010. CD24(+) cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. *Oncogene,* 29**,** 2672-2680.

GARGALLO, P., YANEZ, Y., JUAN, A., SEGURA, V., BALAGUER, J., TORRES, B., OLTRA, S., CASTEL, V. & CANETE, A. 2020. Review: Ewing Sarcoma Predisposition. *Pathology & Oncology Research,* 26**,** 2057-2066.

GENADRY, K. C., PIETROBONO, S., ROTA, R. & LINARDIC, C. M. 2018. Soft Tissue Sarcoma Cancer Stem Cells: An Overview. *Frontiers in Oncology,* 8.

GHISOLFI, L., KEATES, A. C., HU, X., LEE, D.-K. & LI, C. J. 2012. Ionizing Radiation Induces Stemness in Cancer Cells. *Plos One,* 7.

GISINA, A. M., LUPATOV, A. Y., KARALKIN, P. A., MAINOVSKAYA, O. A., PETROV, L. O., SIDOROV, D. V., YARYGIN, V. N. & YARYGIN, K. N. 2011. Detection of Minor Subpopulations of Colorectal Adenocarcinoma Cells Expressing Cancer Stem Cell Markers. *Bulletin of Experimental Biology and Medicine,* 151**,** 234-238.

GOETZE, K., WALENTA, S., KSIAZKIEWICZ, M., KUNZ-SCHUGHART, L. A. & MUELLER-KLIESER, W. 2011. Lactate enhances motility of tumor cells and inhibits monocyte migration and cytokine release. *International Journal of Oncology,* 39**,** 453-463.

GOKHALE, P. J., AU-YOUNG, J. K., DADI, S., KEYS, D. N., HARRISON, N. J., JONES, M., SONEJI, S., ENVER, T., SHERLOCK, J. K. & ANDREWS, P. W. 2015. Culture Adaptation Alters Transcriptional Hierarchies among Single Human Embryonic Stem Cells Reflecting Altered Patterns of Differentiation. *Plos One,* 10**,** 13.

GRIMER, R., JUDSON, I., PEAKE, D. & SEDDON, B. 2010. Guidelines for the Management of Soft Tissue Sarcomas. *Sarcoma***,** 1-15.

GROSSE-GEHLING, P., FARGEAS, C. A., DITTFELD, C., GARBE, Y., ALISON, M. R., CORBEIL, D. & KUNZ-SCHUGHART, L. A. 2013. CD133 as a biomarker for putative cancer stem cells in solid tumours: limitations, problems and challenges. *Journal of Pathology,* 229**,** 355-378.

GRUNEWALD, T. G. P., ALONSO, M., AVNET, S., BANITO, A., BURDACH, S., CIDRE-ARANAZ, F., DI POMPO, G., DISTEL, M., DORADO-GARCIA, H., GARCIA-CASTRO, J., GONZALEZ-GONZALEZ, L., GRIGORIADIS, A. E., KASAN, M., KOELSCHE, C., KRUMBHOLZ, M., LECANDA, F., LEMMA, S., LONGO, D. L., MADRIGAL-ESQUIVEL, C., MORALES-MOLINA, A., MUSA, J., OHMURA, S., ORY, B., PEREIRA-SILVA, M., PERUT, F., RODRIGUEZ, R., SEELING, C., AL SHAAILI, N., SHAABANI, S., SHIAVONE, K., SINHA, S., TOMAZOU, E. M., TRAUTMANN, M., VELA, M., VERSLEIJEN-JONKERS, Y. M. H., VISGAUSS, J., ZALACAIN, M., SCHOBER, S. J., LISSAT, A., ENGLISH, W. R., BALDINI, N. & HEYMANN, D. 2020. Sarcoma treatment in the era of molecular medicine. *Embo Molecular Medicine,* 12**,** 33.

GU, W., YEO, E., MCMILLAN, N. & YU, C. 2011. Silencing oncogene expression in cervical cancer stem-like cells inhibits their cell growth and self-renewal ability. *Cancer Gene Therapy,* 18**,** 897-905.

GURZU, S., TURDEAN, S., KOVECSI, A., CONTAC, A. O. & JUNG, I. 2015. Epithelial-mesenchymal, mesenchymal-epithelial, and endothelial-mesenchymal transitions in malignant tumors: An update. *World Journal of Clinical Cases,* 3**,** 393-404.

HALLIWELL, J., BARBARIC, I. & ANDREWS, P. W. 2020. Acquired genetic changes in human pluripotent stem cells: origins and consequences. *Nature Reviews Molecular Cell Biology,* 21**,** 715-728.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of Cancer: The Next Generation. *Cell,* 144**,** 646-674.

HARRIS, M. A., YANG, H., LOW, B. E., MUKHERJEE, J., GUHA, A., BRONSON, R. T., SHULTZ, L. D., ISRAEL, M. A. & YUN, K. 2009. Cancer stem cells are enriched in the side population cells in a mouse model of glioma (vol 68, pg 10051, 2008). *Cancer Research,* 69**,** 6005-6005.

HASSAN, K. A., WANG, L., KORKAYA, H., CHEN, G. A., MAILLARD, I., BEER, D. G., KALEMKERIAN, G. P. & WICHA, M. S. 2013. Notch Pathway Activity Identifies Cells with Cancer Stem Cell-like Properties and Correlates with Worse Survival in Lung Adenocarcinoma. *Clinical Cancer Research,* 19**,** 1972-1980.

HATINA, J., BOESCH, M., SOPPER, S., KRIPNEROVA, M., WOLF, D., REIMER, D., MARTH, C. & ZEIMET, A. G. 2019a. Ovarian Cancer Stem Cell Heterogeneity. *In:* BIRBRAIR, A. (ed.) *Stem Cells Heterogeneity in Cancer.* Cham: Springer International Publishing Ag.

HATINA, J., KRIPNEROVA, M., HOUFKOVA, K., PESTA, M., KUNCOVA, J., SANA, J., SLABY, O. & RODRIGUEZ, R. 2019b. Sarcoma Stem Cell Heterogeneity. *Stem Cells Heterogeneity - Novel Concepts,* 1123**,** 95-118.

HE, A., QI, W., HUANG, Y., FENG, T., CHEN, J., SUN, Y., SHEN, Z. & YAO, Y. 2012. CD133 expression predicts lung metastasis and poor prognosis in osteosarcoma patients: A clinical and experimental study. *Experimental and Therapeutic Medicine,* 4**,** 435-441.

HECHT, F. 1987. ON THE ORIGINS OF CANCER GENETICS AND CYTOGENETICS. *Cancer Genetics and Cytogenetics,* 29**,** 187-190.

HENDERSON, T., CHEN, M. Y., DARROW, M. A., LI, C. S., CHIU, C. L., MONJAZEB, A. M., MURPHY, W. J. & CANTER, R. J. 2018. Alterations in cancer stem-cell marker CD44 expression predict oncologic outcome in soft-tissue sarcomas. *Journal of Surgical Research,* 223**,** 207-214.

HERNANDEZ-VARGAS, H., OUZOUNOVA, M., LE CALVEZ-KELM, F., LAMBERT, M.-P., MCKAY-CHOPIN, S., TAVTIGIAN, S. V., PUISIEUX, A., MATAR, C. & HERCEG, Z. 2011. Methylome analysis reveals Jak-STAT pathway deregulation in putative breast cancer stem cells. *Epigenetics,* 6**,** 429-440.

HESS, D. A., WIRTHLIN, L., CRAFT, T. P., HERRBRICH, P. E., HOHM, S. A., LAHEY, R., EADES, W. C., CREER, M. H. & NOLTA, J. A. 2006. Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. *Blood,* 107**,** 2162-2169.

HIROTSU, M., SETOGUCHI, T., MATSUNOSHITA, Y., SASAKI, H., NAGAO, H., GAO, H., SUGIMURA, K. & KOMIYA, S. 2009. Tumour formation by single fibroblast growth factor receptor 3-positive rhabdomyosarcoma-initiating cells. *British Journal of Cancer,* 101**,** 2030-2037.

HOANG, N. T., ACEVEDO, L. A., MANN, M. J. & TOLANI, B. 2018. A review of soft-tissue sarcomas: translation of biological advances into treatment measures. *Cancer Management and Research,* 10**,** 1089-1114.

HONOKI, K., FUJII, H., KUBO, A., KIDO, A., MORI, T., TANAKA, Y. & TSUJIUCHI, T. 2010. Possible involvement of stem-like populations with elevated ALDH1 in sarcomas for chemotherapeutic drug resistance. *Oncology Reports,* 24**,** 501-505.

HORIMOTO, Y., ARAKAWA, A., SASAHARA, N., TANABE, M., SAI, S., HIMURO, T. & SAITO, M. 2016. Combination of Cancer Stem Cell Markers CD44 and CD24 Is Superior to ALDH1 as a Prognostic Indicator in Breast Cancer Patients with Distant Metastases. *Plos One,* 11.

HUMPHREY, G., HAZEL, D. L., MACLENNAN, K. & LEWIS, I. 1999. Expression of CD44 by rhabdomyosarcoma: a new prognostic marker? *British Journal of Cancer,* 80**,** 918-921.

HWANG-VERSLUES, W. W., KUO, W. H., CHANG, P. H., PAN, C. C., WANG, H. H., TSAI, S. T., JENG, Y. M., SHEW, J. Y., KUNG, J. T., CHEN, C. H., LEE, E., CHANG, K. J. & LEE, W. H. 2009. Multiple Lineages of Human Breast Cancer Stem/Progenitor Cells Identified by Profiling with Stem Cell Markers. *Plos One,* 4**,** 11.

IKUSHIMA, H. & MIYAZONO, K. 2010. TGF beta signalling: a complex web in cancer progression. *Nature Reviews Cancer,* 10**,** 415-424.

JAISWAL, S., JAMIESON, C. H. M., PANG, W. W., PARK, C. Y., CHAO, M. P., MAJETI, R., TRAVER, D., VAN ROOIJEN, N. & WEISSMAN, I. L. 2009. CD47 Is Upregulated on Circulating Hematopoietic Stem Cells and Leukemia Cells to Avoid Phagocytosis. *Cell,* 138**,** 271-285.

JANSEN-LANDHEER, M. L. E. A., KRIJNEN, P., OOSTINDIER, M. J., KLOOSTERMAN-BOELE, W. M., NOORDIJK, E. M., NOOIJ, M. A., STEUP, W. H., TAMINIAU, A. H. M., VREE, R., HOGENDOORN, P. C. W., TOLLENAAR, R. A. E. M. & GELDERBLOM, H. 2009. Improved diagnosis and treatment of soft tissue sarcoma patients after implementation of national guidelines: A population-based study. *Ejso,* 35**,** 1326-1332.

JAYACHANDRAN, A., DHUNGEL, B. & STEEL, J. C. 2016. Epithelial-to-mesenchymal plasticity of cancer stem cells: therapeutic targets in hepatocellular carcinoma. *Journal of Hematology & Oncology,* 9**,** 12.

JEMAL, A., BRAY, F., CENTER, M. M., FERLAY, J., WARD, E. & FORMAN, D. 2011. Global cancer statistics. *CA: a cancer journal for clinicians,* 61**,** 69-90.

JIANG, L. L., LIU, T. Q. & SONG, K. D. 2012. Growth Characteristics of Human Adipose-Derived Stem Cells During Long Time Culture Regulated by Cyclin A and Cyclin D1. *Applied Biochemistry and Biotechnology,* 168**,** 2230-2244.

JIANG, X. H., GWYE, Y., RUSSELL, D., CAO, C., DOUGLAS, D., HUNG, L., KOVAR, H., TRICHE, T. J. & LAWLOR, E. R. 2010. CD133 expression in chemo-resistant Ewing sarcoma cells. *Bmc Cancer,* 10**,** 12.

JO, V. Y. & FLETCHER, C. D. M. 2014. WHO classification of soft tissue tumours: an update based on the 2013 (4th) edition. *Pathology,* 46**,** 95-104.

JOSHUA, B., KAPLAN, M. J., DOWECK, I., PAI, R., WEISSMAN, I. L., PRINCE, M. E. & AILLES, L. E. 2012. FREQUENCY OF CELLS EXPRESSING CD44, A HEAD

AND NECK CANCER STEM CELL MARKER: CORRELATION WITH TUMOR AGGRESSIVENESS. *Head and Neck-Journal for the Sciences and Specialties of the Head and Neck,* 34**,** 42-49.

JUNDT, G. 2018. Updates to the WHO classification of bone tumours. *Pathologe,* 39**,** 107-116.

JUNTTILA, M. R. & EVAN, G. I. 2009. p53-a Jack of all trades but master of none. *Nature Reviews Cancer,* 9**,** 821-829.

KADOCH, C. & CRABTREE, G. R. 2013. Reversible Disruption of mSWI/SNF (BAF) Complexes by the SS18-SSX Oncogenic Fusion in Synovial Sarcoma. *Cell,* 153**,** 71-85.

KAHLERT, U. D., JOSEPH, J. V. & KRUYT, F. A. E. 2017. EMT- and MET-related processes in nonepithelial tumors: importance for disease progression, prognosis, and therapeutic opportunities. *Molecular Oncology,* 11**,** 860-877.

KANOJIA, D., NAGATA, Y., GARG, M., LEE, D. H., SATO, A., YOSHIDA, K., SATO, Y., SANADA, M., MAYAKONDA, A., BARTENHAGEN, C., KLEIN, H. U., DOAN, N. B., SAID, J. W., MOHITH, S., GUNASEKAR, S., SHIRAISHI, Y., CHIBA, K., TANAKA, H., MIYANO, S., MYKLEBOST, O., YANG, H., DUGAS, M., MEZA-ZEPEDA, L. A., SILBERMAN, A. W., FORSCHER, C., TYNER, J. W., OGAWA, S. & KOEFFLER, H. P. 2015. Genomic landscape of liposarcoma. *Oncotarget,* 6**,** 42429-42444.

KARAHAN, N., GUNEY, M., ORAL, B., KAPUCUOGLU, N. & MUNGAN, T. 2006. CD24 expression is a poor prognostic marker in endometrial carcinoma. *European Journal of Gynaecological Oncology,* 27**,** 500-504.

KARAMBOULAS, C. & AILLES, L. 2013. Developmental signaling pathways in cancer stem cells of solid tumors. *Biochimica Et Biophysica Acta-General Subjects,* 1830**,** 2481-2495.

KASPER, B., GIL, T., D'HONDT, V., GEBHART, M. & AWADA, A. 2007. Novel treatment strategies for soft tissue sarcoma. *Critical Reviews in Oncology Hematology,* 62**,** 9-15.

KAWAHARA, T. & LAMBETH, J. D. 2007. Molecular evolution of Phox-related regulatory subunits for NADPH oxidase enzymes. *Bmc Evolutionary Biology,* 7.

KEDES, D. H., OPERSKALSKI, E., BUSCH, M., KOHN, R., FLOOD, J. & GANEM, D. 1996. The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): Distribution of infection in KS risk groups and evidence for sexual transmission. *Nature Medicine,* 2**,** 918-924.

KEYSAR, S. B. & JIMENO, A. 2010. More than Markers: Biological Significance of Cancer Stem Cell-Defining Molecules. *Molecular Cancer Therapeutics,* 9**,** 2450-2457.

KIKUSHIGE, Y., SHIMA, T., TAKAYANAGI, S., URATA, S., MIYAMOTO, T., IWASAKI, H., TAKENAKA, K., TESHIMA, T., TANAKA, T., INAGAKI, Y. & AKASHI, K. 2010. TIM-3 Is a Promising Target to Selectively Kill Acute Myeloid Leukemia Stem Cells. *Cell Stem Cell,* 7**,** 708-717.

KIM, J.-K., JEON, H.-Y. & KIM, H. 2015. The molecular mechanisms underlying the therapeutic resistance of cancer stem cells. *Archives of Pharmacal Research,* 38**,** 389-401.

KIM, J. W. & DANG, C. V. 2006. Cancer's molecular sweet tooth and the Warburg effect. *Cancer Research,* 66**,** 8927-8930.

KIM, M. H., OH, J. E., PARK, S., KIM, J. H., LEE, K. Y., BAI, S. J., SONG, H., HWANG, H. J., KIM, D. W. & YOO, Y. C. 2019. Tramadol use is associated with enhanced postoperative outcomes in breast cancer patients: a retrospective clinical study with in vitro confirmation. *British Journal of Anaesthesia,* 123**,** 865-876.

KIM, S. K. & PARK, Y. K. 2016. Ewing sarcoma: a chronicle of molecular pathogenesis. *Human Pathology,* 55**,** 91-100.

KIMURA, T., WANG, L., TABU, K., TSUDA, M., TANINO, M., MAEKAWA, A., NISHIHARA, H., HIRAGA, H., TAGA, T., ODA, Y. & TANAKA, S. 2016. Identification and analysis of CXCR4-positive synovial sarcoma-initiating cells. *Oncogene,* 35**,** 3932-3943.

KOKA, P., MUNDRE, R., RANGARAJAN, R., CHANDRAMOHAN, Y., SUBRAMANIAN, R. & DHANASEKARAN, A. 2018. Uncoupling Warburg effect and stemness in CD133(+ve) cancer stem cells from Saos-2 (osteosarcoma) cell line under hypoxia. *Molecular Biology Reports,* 45**,** 1653-1662.

KOKKO, L.-L., HURME, S., MAULA, S.-M., ALANEN, K., GRENMAN, R., KINNUNEN, I. & VENTELA, S. 2011. Significance of site-specific prognosis of cancer stem cell marker CD44 in head and neck squamous-cell carcinoma. *Oral Oncology,* 47**,** 510-516.

KOSHIJI, M., KAGEYAMA, Y., PETE, E. A., HORIKAWA, I., BARRETT, J. C. & HUANG, L. E. 2004. HIF-1 alpha induces cell cycle arrest by functionally counteracting Myc. *Embo Journal,* 23**,** 1949-1956.

KRUPKOVA, O., JR., LOJA, T., ZAMBO, I. & VESELSKA, R. 2010. Nestin expression in human tumors and tumor cell lines. Minireview. *Neoplasma,* 57**,** 291-298.

KURTOVA, A. V., XIAO, J., MO, Q., PAZHANISAMY, S., KRASNOW, R., LERNER, S. P., CHEN, F., ROH, T. T., LAY, E., HO, P. L. & CHAN, K. S. 2015. Blocking PGE(2)-induced tumour repopulation abrogates bladder cancer chemoresistance. *Nature,* 517**,** 209-U224.

LAHAT, G., ZHANG, P. Y., ZHU, Q. S., TORRES, K., GHADIMI, M., SMITH, K. D., WANG, W. L., LAZAR, A. J. & LEV, D. 2011. The expression of c-Met pathway components in unclassified pleomorphic sarcoma/malignant fibrous histiocytoma (UPS/MFH): a tissue microarray study. *Histopathology,* 59**,** 556-561.

LAPIDOT, T., SIRARD, C., VORMOOR, J., MURDOCH, B., HOANG, T., CACERESCORTES, J., MINDEN, M., PATERSON, B., CALIGIURI, M. A. & DICK, J. E. 1994. A CELL INITIATING HUMAN ACUTE MYELOID-LEUKEMIA AFTER TRANSPLANTATION INTO SCID MICE. *Nature,* 367**,** 645-648.

LAVON, N., ZIMERMAN, M. & ITSKOVITZ-ELDOR, J. 2018. Scalable Expansion of Pluripotent Stem Cells. *In:* MARTIN, U., ZWEIGERDT, R. & GRUH, I. (eds.)

Engineering and Application of Pluripotent Stem Cells. Cham: Springer International Publishing Ag.

LAZAR, A. J., MCLELLAN, M. D., BAILEY, M. H., MILLER, C. A., APPELBAUM, E. L., CORDES, M. G., FRONICK, C. C., FULTON, L. A., FULTON, R. S., MARDIS, E. R., SCHMIDT, H. K., WONG, W., WILSON, R. K., YELLAPANTULA, V., RADENBAUGH, A. J., HOADLEY, K. A., HAYES, D. N., PARKER, J. S., WILKERSON, M. D., AUMA, J. T., BALU, S., BODENHEIMER, T., HOYLE, A. P., JEFFERYS, S. R., JONES, C. D., LEHMANN, K. V., MENG, S. W., MIECZKOWSKI, P. A., MOSE, L. E., PEROU, C. M., ROACH, J., SENBABAOGLU, Y., SHI, Y., SIMONS, J. V., SKELLY, T., SOLOWAY, M. G., TAN, D. H., VELUVOLU, U., DAVIS, I. J., HEPPERLA, A. J., BROHL, A. S., KASAIAN, K., MUNGALL, K., SADEGHI, S., BARTHEL, F. P., VERHAAK, R., HU, X., CHIBON, F., CHERNIACK, A. D., SHIH, J., BEROUKHIM, R., MEYERSON, M., CIBULSKIS, C., GABRIEL, S. B., SAKSENA, G., SCHUMACHER, S. E., GAO, Q. S., WONG, W. H., WYCZALKOWSKI, M., BOWLBY, R., ROBERTSON, A. G., ALLY, A., BALASUNDARAM, M., BROOKS, D., CARLSEN, R., CHUAH, E., DHALLA, N., HOLT, R. A., JONES, S. J., LEE, D., LI, I., MA, Y., MARRA, M. A., MAYO, M., MOORE, R. A., MUNGALL, A. J., SCHEIN, J. E., SIPAHIMALANI, P., TAM, A., THIESSEN, N., WONG, T., DANILOVA, L., COPE, L., BAYLIN, S. B., BOOTWALLA, M. S., LAI, P. H., LAIRD, P. W., MAGLINTE, D. T., VAN DEN BERG, D. J., WEISENBERGER, D. J., WRANGLE, J., DRILL, E., SHEN, R. L., IYPE, L., REYNOLDS, S. M., SHMULEVICH, I., YAU, C., ARMENIA, J., LIU, E. M., BENZ, C., et al. 2017. Comprehensive and Integrated Genomic Characterization of Adult Soft Tissue Sarcomas. *Cell,* 171**,** 950-+.

LEE, A. T. J., THWAY, K., HUANG, P. H. & JONES, R. L. 2018. Clinical and Molecular Spectrum of Liposarcoma. *Journal of Clinical Oncology,* 36**,** 151-+.

LEE, W. W. 2018. Heterogeneity Does Matter for Tumor Characterization. *Nuclear Medicine and Molecular Imaging,* 52**,** 167-167.

LEUCHTE, K., ALTVATER, B., HOFFSCHLAG, S., POTRATZ, J., MELTZER, J., CLEMENS, D., LUECKE, A., HARDES, J., DIRKSEN, U., JUERGENS, H., KAILAYANGIRI, S. & ROSSIG, C. 2014. Anchorage-independent growth of Ewing sarcoma cells under serum-free conditions is not associated with stem-cell like phenotype and function. *Oncology Reports,* 32**,** 845-852.

LI, B. C., LI, L., LI, X. Y., WANG, Y. Y., XIE, Y. W., LIU, C. X. & LI, F. 2015. Undifferentiated pleomorphic sarcoma with co-existence of KRAS/PIK3CA mutations. *International Journal of Clinical and Experimental Pathology,* 8**,** 8563-8567.

LI, C. F., FANG, F. M., KUNG, H. J., CHEN, L. T., WANG, J. W., TSAI, J. W., YU, S. C., WANG, Y. H., LI, S. H. & HUANG, H. Y. 2014. Downregulated MTAP expression in myxofibrosarcoma: A characterization of inactivating mechanisms, tumor suppressive function, and therapeutic relevance. *Oncotarget,* 5**,** 11428-11441.

LI, G., GAO, J., TAO, Y. L., XU, B. Q., TU, Z. W., LIU, Z. G., ZENG, M. S. & XIA, Y. F. 2012. Increased pretreatment levels of serum LDH and ALP as poor prognostic factors for nasopharyngeal carcinoma. *Chinese Journal of Cancer,* 31**,** 197-206.

LI, Z. Z., CZECHOWICZ, A., SCHECK, A., ROSSI, D. J. & MURPHY, P. M. 2019. Hematopoietic chimerism and donor-specific skin allograft tolerance after non-

genotoxic CD117 antibody-drug-conjugate conditioning in MHC-mismatched allotransplantation. *Nature Communications,* 10**,** 7.

LIAN, Q. Z., LYE, E., YEO, K. S., TAN, E. K. W., SALTO-TELLEZ, M., LIU, T. M., PALANISAMY, N., EL OAKLEY, R. M., LEE, E. H., LIM, B. & LIM, S. K. 2007. Derivation of clinically compliant MSCs from CD105+, CD24-differentiated human ESCs. *Stem Cells,* 25**,** 425-436.

LIM, S. C. & OH, S. H. 2005. The role of CD24 in various human epithelial neoplasias. *Pathology Research and Practice,* 201**,** 479-486.

LIN, C. C., CHENG, T. L., TSAI, W. H., TSAI, H. J., HU, K. H., CHANG, H. C., YEH, C. W., CHEN, Y. C., LIAO, C. C. & CHANG, W. T. 2012. Loss of the respiratory enzyme citrate synthase directly links the Warburg effect to tumor malignancy. *Scientific Reports,* 2.

LIN, H. H., LEE, H. W., LIN, R. J., HUANG, C. W., LIAO, Y. C., CHEN, Y. T., FANG, J. M., LEE, T. C., YU, A. L. & CHANG, H. C. 2015. Tracking and Finding Slow-Proliferating/Quiescent Cancer Stem Cells with Fluorescent Nanodiamonds. *Small,* 11**,** 4394-4402.

LIN, J., KIM, D., TSE, H. T., TSENG, P., PENG, L. L., DHAR, M., KARUMBAYARAM, S. & DI CARLO, D. 2017. High-throughput physical phenotyping of cell differentiation. *Microsystems & Nanoengineering,* 3**,** 7.

LIU, J., JIN, J., YANG, Y., HAN, W. K., YU, W., SHEN, C., ZHANG, N., DU, P., ZHAO, Q., WANG, S. & TANG, X. X. 2015. Prognostic Impact of ALDH1 Expression in Transitional Cell Carcinoma of the Renal Pelvis. *Anticancer Research,* 35**,** 4829-4836.

LIU, Q., BAO, Q. Y., XU, Y. Q., FU, Y. C., JIN, Z. J., WANG, J., ZHANG, W. B. & SHEN, Y. H. 2021. MCM4 Is a Novel Biomarker Associated With Genomic Instability, BRCAness Phenotype, and Therapeutic Potentials in Soft-Tissue Sarcoma. *Frontiers in Cell and Developmental Biology,* 9.

LIU, Q. H., NGUYEN, D. H., DONG, Q. H., SHITAKU, P., CHUNG, K., LIU, O. Y., TSO, J. L., LIU, J. Y., KONKANKIT, V., CLOUGHESY, T. F., MISCHEL, P. S., LANE, T. F., LIAU, L. M., NELSON, S. F. & TSO, C. L. 2009. Molecular properties of CD133+glioblastoma stem cells derived from treatment-refractory recurrent brain tumors. *Journal of Neuro-Oncology,* 94**,** 1-19.

LIU, W., QIAO, R. H., WANG, D. M., HUANG, X. W., LI, B. & WANG, D. 2016. UHRF1 promotes human osteosarcoma cell invasion by downregulating the expression of E-cadherin in an Rb1-dependent manner. *Molecular Medicine Reports,* 13**,** 315-320.

LIU, X., TAFTAF, R., KAWAGUCHI, M., CHANG, Y.-F., CHEN, W., ENTENBERG, D., ZHANG, Y., GERRATANA, L., HUANG, S., PATEL, D. B., TSUI, E., ADORNO-CRUZ, V., CHIRIELEISON, S. M., CAO, Y., HARNEY, A. S., PATEL, S., PATSIALOU, A., SHEN, Y., AVRIL, S., GILMORE, H. L., LATHIA, J. D., ABBOTT, D. W., CRISTOFANILLI, M., CONDEELIS, J. S. & LIU, H. 2019. Homophilic CD44 Interactions Mediate Tumor Cell Aggregation and Polyclonal Metastasis in Patient-Derived Breast Cancer Models. *Cancer Discovery,* 9**,** 96-113.

LIU, X. L., DING, J. & MENG, L. H. 2018. Oncogene-induced senescence: a double edged sword in cancer. *Acta Pharmacologica Sinica,* 39**,** 1553-1558.

LOBO, N. A., SHIMONO, Y., QIAN, D. & CLARKE, M. F. 2007. The biology of cancer stem cells. *Annual Review of Cell and Developmental Biology,* 23**,** 675-699.

LOBRY, C., OH, P. & AIFANTIS, I. 2011. Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think. *Journal of Experimental Medicine,* 208**,** 1931-1935.

LOBRY, C., OH, P., MANSOUR, M. R., LOOK, A. T. & AIFANTIS, I. 2014. Notch signaling: switching an oncogene to a tumor suppressor. *Blood,* 123**,** 2451-2459.

LOHBERGER, B., RINNER, B., STUENDL, N., ABSENGER, M., LIEGL-ATZWANGER, B., WALZER, S. M., WINDHAGER, R. & LEITHNER, A. 2012a. Aldehyde Dehydrogenase 1, a Potential Marker for Cancer Stem Cells in Human Sarcoma. *Plos One,* 7**,** 10.

LOHBERGER, B., RINNER, B., STUENDL, N., ABSENGER, M., LIEGL-ATZWANGER, B., WALZER, S. M., WINDHAGER, R. & LEITHNER, A. 2012b. Aldehyde Dehydrogenase 1, a Potential Marker for Cancer Stem Cells in Human Sarcoma. *Plos One,* 7.

LU, W. Q., HU, Y. M., CHEN, G., CHEN, Z., ZHANG, H., WANG, F., FENG, L., PELICANO, H., WANG, H., KEATING, M. J., LIU, J. S., MCKEEHAN, W., WANG, H. M., LUO, Y. D. & HUANG, P. 2012. Novel Role of NOX in Supporting Aerobic Glycolysis in Cancer Cells with Mitochondrial Dysfunction and as a Potential Target for Cancer Therapy. *Plos Biology,* 10.

LU, X. Q., DENG, Q., LI, H. X. & SUO, Z. H. 2011. Altered characteristics of cancer stem/initiating cells in a breast cancer cell line treated with persistent 5-FU chemotherapy. *Experimental and Therapeutic Medicine,* 2**,** 821-826.

LUCKEY, C. J., BHATTACHARYA, D., GOLDRATH, A. W., WEISSMAN, I. L., BENOIST, C. & MATHIS, D. 2006. Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 3304-3309.

LUGOWSKA, I., TETERYCZ, P., MIKULA, M., KULECKA, M., KLUSKA, A., BALABAS, A., PIATKOWSKA, M., WAGRODZKI, M., PIENKOWSKI, A., RUTKOWSKI, P. & OSTROWSKI, J. 2018. IDH1/2 Mutations Predict Shorter Survival in Chondrosarcoma. *Journal of Cancer,* 9**,** 998-1005.

LUKENDA, A., DOTLIC, S., VUKOJEVIC, N., SARIC, B., VRANIC, S. & ZARKOVIC, K. 2016. Expression and prognostic value of putative cancer stem cell markers CD117 and CD15 in choroidal and ciliary body melanoma. *Journal of Clinical Pathology,* 69**,** 234-239.

MACKALL, C. L., MELTZER, P. S. & HELMAN, L. J. 2002. Focus on sarcomas. *Cancer Cell,* 2**,** 175-178.

MAJETI, R., PARK, C. Y. & WEISSMAN, I. L. 2007. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Blood,* 110**,** 663A-663A.

MALANCHI, I., PEINADO, H., KASSEN, D., HUSSENET, T., METZGER, D., CHAMBON, P., HUBER, M., HOHL, D., CANO, A., BIRCHMEIER, W. & HUELSKEN, J. 2008. Cutaneous cancer stem cell maintenance is dependent on beta-catenin signalling. *Nature,* 452**,** 650-U12.

MALANCHI, I., SANTAMARIA-MARTINEZ, A., SUSANTO, E., PENG, H., LEHR, H.-A., DELALOYE, J.-F. & HUELSKEN, J. 2012a. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature,* 481**,** 85-U95.

MALANCHI, I., SANTAMARIA-MARTINEZ, A., SUSANTO, E., PENG, H., LEHR, H. A., DELALOYE, J. F. & HUELSKEN, J. 2012b. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature,* 481**,** 85-U95.

MANNELLI, G. & GALLO, O. 2012. Cancer stem cells hypothesis and stem cells in head and neck cancers. *Cancer Treatment Reviews,* 38**,** 515-539.

MARTIN, S. S., HURT, W. G., HEDGES, L. K., BUTLER, M. G. & SCHWARTZ, H. S. 1998. Microsatellite instability in sarcomas. *Annals of Surgical Oncology,* 5**,** 356-360.

MARTINEZ-DELGADO, P., LACERENZA, S., OBRADOR-HEVIA, A., LOPEZ-ALVAREZ, M., MONDAZA-HERNANDEZ, J. L., BLANCO-ALCAINA, E., SANCHEZ-BUSTOS, P., HINDI, N., MOURA, D. S. & MARTIN-BROTO, J. 2020. Cancer Stem Cells in Soft-Tissue Sarcomas. *Cells,* 9**,** 21.

MARTINS-NEVES, S. R., CORVER, W. E., PAIVA-OLIVEIRA, D. I., VAN DEN AKKER, B., BRIAIRE-DE-BRUIJN, I. H., BOVEE, J., GOMES, C. M. F. & CLETON-JANSEN, A. M. 2016a. Osteosarcoma Stem Cells Have Active Wnt/beta-catenin and Overexpress SOX2 and KLF4. *Journal of Cellular Physiology,* 231**,** 876-886.

MARTINS-NEVES, S. R., PAIVA-OLIVEIRA, D. I., WIJERS-KOSTER, P. M., ABRUNHOSA, A. J., FONTES-RIBEIRO, C., BOVEE, J., CLETON-JANSEN, A. M. & GOMES, C. M. F. 2016b. Chemotherapy induces sternness in osteosarcoma cells through activation of Wnt/beta-catenin signaling. *Cancer Letters,* 370**,** 286-295.

MENG, Q. T., REN, C. L., WANG, L. P., ZHAO, Y. J. & WANG, S. J. 2015. Knockdown of ST6Gal-I inhibits the growth and invasion of osteosarcoma MG-63 cells. *Biomedicine & Pharmacotherapy,* 72**,** 172-178.

MING, G. L. & SONG, H. J. 2005. Adult neurogenesis in the mammalian central nervous system. *Annual Review of Neuroscience,* 28**,** 223-250.

MIRAGLIA, S., GODFREY, W., YIN, A. H., ATKINS, K., WARNKE, R., HOLDEN, J. T., BRAY, R. A., WALLER, E. K. & BUCK, D. W. 1997. A novel five-transmembrane hematopoietic stem cell antigen: Isolation, characterization, and molecular cloning. *Blood,* 90**,** 5013-5021.

MOGHBELI, M., MOGHBELI, F., FORGHANIFARD, M. M. & ABBASZADEGAN, M. R. 2014. Cancer stem cell detection and isolation. *Medical Oncology,* 31**,** 7.

MOORE, K. J., HUBBARD, A. K., WILLIAMS, L. A. & SPECTOR, L. G. 2020. Childhood cancer incidence among specific Asian and Pacific Islander populations in the United States. *International Journal of Cancer,* 147**,** 3339-3348.

MORRISON, S. J. & KIMBLE, J. 2006. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature,* 441**,** 1068-1074.

MORRISON, S. J., QIAN, D., JERABEK, L., THIEL, B. A., PARK, I. K., FORD, P. S., KIEL, M. J., SCHORK, N. J., WEISSMAN, I. L. & CLARKE, M. F. 2002. A genetic determinant that specifically regulates the frequency of hematopoietic stem cells. *Journal of Immunology,* 168**,** 635-642.

NA, J., FURUE, M. K. & ANDREWS, P. W. 2010. Inhibition of ERK1/2 prevents neural and mesendodermal differentiation and promotes human embryonic stem cell self-renewal. *Stem Cell Research,* 5**,** 157-169.

NAJAFI, M., FARHOOD, B. & MORTEZAEE, K. 2019a. Cancer stem cells (CSCs) in cancer progression and therapy. *Journal of Cellular Physiology,* 234**,** 8381-8395.

NAJAFI, M., MORTEZAEE, K. & MAJIDPOOR, J. 2019b. Cancer stem cell (CSC) resistance drivers. *Life Sciences,* 234**,** 11.

NAKAGAWA, Y., NUMOTO, K., YOSHIDA, A., KUNISADA, T., OHATA, H., TAKEDA, K., WAI, D., POREMBA, C. & OZAKI, T. 2006. Chromosomal and genetic imbalances in synovial sarcoma detected by conventional and microarray comparative genomic hybridization. *Journal of Cancer Research and Clinical Oncology,* 132**,** 444-450.

NAKAHATA, K., UEHARA, S., NISHIKAWA, S., KAWATSU, M., ZENITANI, M., OUE, T. & OKUYAMA, H. 2015. Aldehyde Dehydrogenase 1 (ALDH1) Is a Potential Marker for Cancer Stem Cells in Embryonal Rhabdomyosarcoma. *Plos One,* 10**,** 16.

NAKANO, K. & TAKAHASHI, S. 2018. Translocation-Related Sarcomas. *International Journal of Molecular Sciences,* 19**,** 19.

NICKKHOLGH, B., MIZRAK, C., VAN DAALEN, S. K. M., KORVER, C. M., SADRI-ARDEKANI, H., REPPING, S. & VAN PELT, A. M. M. 2014. Genetic and epigenetic stability of human spermatogonial stem cells during long-term culture. *Fertility and Sterility,* 102**,** 1700-U531.

NICOLAZZO, C. & GRADILONE, A. 2015. Significance of Circulating Tumor Cells in Soft Tissue Sarcoma. *Analytical Cellular Pathology,* 2015**,** 5.

NIETO, M. A. 2013. Epithelial Plasticity: A Common Theme in Embryonic and Cancer Cells. *Science,* 342**,** 708-+.

NOTTA F, D. S., LAURENTI E, POEPPL A, JURISICA I, DICK JE. 2011. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science,* 333**,** 218- 221.

NOVELLI, M., ROSSI, S., RODRIGUEZ-JUSTO, M., TANIERE, P., SEDDON, B., TOFFOLATTI, L., SARTOR, C., HOGENDOORN, P. C. W., SCIOT, R., VAN GLABBEKE, M., VERWEIJ, J., BLAY, J. Y., HOHENBERGER, P., FLANAGAN, A. & DEI TOS, A. P. 2010. DOG1 and CD117 are the antibodies of choice in the diagnosis of gastrointestinal stromal tumours. *Histopathology,* 57**,** 259-270.

NUNUKOVA, A., NERADIL, J., SKODA, J., JAROS, J., HAMPL, A., STERBA, J. & VESELSKA, R. 2015. Atypical nuclear localization of CD133 plasma membrane

glycoprotein in rhabdomyosarcoma cell lines. *International Journal of Molecular Medicine,* 36**,** 65-72.

OGURA, K., HOSODA, F., ARAI, Y., NAKAMURA, H., HAMA, N., TOTOKI, Y., YOSHIDA, A., NAGAI, M., KATO, M., ARAKAWA, E., MUKAI, W., ROKUTAN, H., KAWAI, A., TANAKA, S. & SHIBATA, T. 2018. Integrated genetic and epigenetic analysis of myxofibrosarcoma. *Nature Communications,* 9.

OHKUMA, M., HARAGUCHI, N., ISHII, H., MIMORI, K., TANAKA, F., KIM, H. M., SHIMOMURA, M., HIROSE, H., YANAGA, K. & MORI, M. 2012. Absence of CD71 Transferrin Receptor Characterizes Human Gastric Adenosquamous Carcinoma Stem Cells. *Annals of Surgical Oncology,* 19**,** 1357-1364.

OLEMPSKA, M., EISENACH, P. A., AMMERPOHL, O., UNGEFROREN, H., FANDRICH, F. & KALTHOFF, H. 2007. Detection of tumor stem cell markers in pancreatic carcinoma cell lines. *Hepatobiliary & Pancreatic Diseases International,* 6**,** 92-97.

ONG, C. S., TRAN, E., NGUYEN, T. T. T., ONG, C. K., LEE, S. K., LEE, J. J., NG, C. P., LEONG, C. & HUYNH, H. 2004. Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expressions. *Oncology Reports,* 11**,** 727-733.

OSANYINGBEMI-OBIDI, J., DOBROMILSKAYA, I., ILLEI, P. B., HANN, C. L. & RUDIN, C. M. 2011. Notch Signaling Contributes to Lung Cancer Clonogenic Capacity In Vitro but May Be Circumvented in Tumorigenesis In Vivo. *Molecular Cancer Research,* 9**,** 1746-1754.

OSKARSSON, T., BATLLE, E. & MASSAGUE, J. 2014. Metastatic Stem Cells: Sources, Niches, and Vital Pathways. *Cell Stem Cell,* 14**,** 306-321.

OTTE, A., BUCAN, V., REIMERS, K. & HASS, R. 2013. Mesenchymal Stem Cells Maintain Long-Term In Vitro Stemness During Explant Culture. *Tissue Engineering Part C-Methods,* 19**,** 937-948.

OUYANG, Y. R., LI, H., BU, J., LI, X. Y., CHEN, Z. Y. & XIAO, T. 2016. Hypoxia-inducible factor-1 expression predicts osteosarcoma patients' survival: a meta-analysis. *International Journal of Biological Markers,* 31**,** E229-E234.

PACINI, N. & BORZIANI, F. 2014. Cancer Stem Cell Theory and the Warburg Effect, Two Sides of the Same Coin? *International Journal of Molecular Sciences,* 15**,** 8893-8930.

PARFITT, J. R., RODRIGUEZ-JUSTO, M., FEAKINS, R. & NOVELLI, M. R. 2008. Gastrointestinal Kaposi's sarcoma: CD117 expression and the potential for misdiagnosis as gastrointestinal stromal tumour. *Histopathology,* 52**,** 816-823.

PATTABIRAMAN, D. R. & WEINBERG, R. A. 2014. Tackling the cancer stem cells - what challenges do they pose? *Nature Reviews Drug Discovery,* 13**,** 497-512.

PEIPER, M., SATO, T., ZURAKOWSKI, D., EISENBERGER, C., HEINECKE, A., HOSCH, S. & KNOEFEL, W. T. 2004. CD44s expression is associated with improved survival in soft tissue sarcoma. *Anticancer Research,* 24**,** 1053-1056.

PENFORNIS, P., CAI, D. Z., HARRIS, M. R., WALKER, R., LICINI, D., FERNANDES, J. D. A., ORR, G., KOGANTI, T., HICKS, C., INDURU, S., MEYER, M. S., KHOKHA, R., BARR, J. & POCHAMPALLY, R. R. 2014. High CD49f expression is associated with osteosarcoma tumor progression: a study using patient-derived primary cell cultures. *Cancer Medicine,* 3**,** 796-811.

PETROVA, V., ANNICCHIARICO-PETRUZZELLI, M., MELINO, G. & AMELIO, I. 2018. The hypoxic tumour microenvironment. *Oncogenesis,* 7**,** 13.

PHI, L. T. H., SARI, I. N., YANG, Y. G., LEE, S. H., JUN, N., KIM, K. S., LEE, Y. K. & KWON, H. Y. 2018. Cancer Stem Cells (CSCs) in Drug Resistance and their Therapeutic Implications in Cancer Treatment. *Stem Cells International,* 2018**,** 16.

PHILLIPS, T. M., MCBRIDE, W. H. & PAJONK, F. 2006. The response of CD24(-/low)/CD44(+) breast cancer-initiating cells to radiation. *Jnci-Journal of the National Cancer Institute,* 98**,** 1777-1785.

PIANO, M. A., BRUNELLO, A., CAPPELLESSO, R., DEL BIANCO, P., MATTIOLO, A., FRITEGOTTO, C., MONTINI, B., ZAMUNER, C., DEL FIORE, P., RASTRELLI, M., SOMMARIVA, A., DE SALVO, G. L., MONTESCO, M. C., ROSSI, C. R., ZAGONEL, V. & CALABRO, M. L. 2020. Periostin and Epithelial-Mesenchymal Transition Score as Novel Prognostic Markers for Leiomyosarcoma, Myxofibrosarcoma, and Undifferentiated Pleomorphic Sarcoma. *Clinical Cancer Research,* 26**,** 2921-2931.

PINEDO, H. M. & VERWEIJ, J. 1986. THE TREATMENT OF SOFT-TISSUE SARCOMAS WITH FOCUS ON CHEMOTHERAPY - A REVIEW. *Radiotherapy and Oncology,* 5**,** 193-205.

POGOZHYKH, O., POGOZHYKH, D., NEEHUS, A. L., HOFFMANN, A., BLASCZYK, R. & MULLER, T. 2015. Molecular and cellular characteristics of human and non-human primate multipotent stromal cells from the amnion and bone marrow during long term culture. *Stem Cell Research & Therapy,* 6.

POTTER, J. W., JONES, K. B. & BARROTT, J. J. 2018. Sarcoma-The standard-bearer in cancer discovery. *Critical Reviews in Oncology Hematology,* 126**,** 1-5.

POWERS, M. P., WANG, W. L., HERNANDEZ, V. S., PATEL, K. S., LEV, D. C., LAZAR, A. J. & LOPEZ-TERRADA, D. H. 2010. Detection of myxoid liposarcoma-associated FUS-DDIT3 rearrangement variants including a newly identified breakpoint using an optimized RT-PCR assay. *Modern Pathology,* 23**,** 1307-1315.

PRESSEY, J. G., HAAS, M. C., PRESSEY, C. S., KELLY, V. M., PARKER, J. N., GILLESPIE, G. Y. & FRIEDMAN, G. K. 2013. CD133 Marks a Myogenically Primitive Subpopulation in Rhabdomyosarcoma Cell Lines That Are Relatively Chemoresistant but Sensitive to Mutant HSV. *Pediatric Blood & Cancer,* 60**,** 45-52.

PUCA, F., COLAMAIO, M., FEDERICO, A., GEMEI, M., TOSTI, N., BASTOS, A. U., DEL VECCHIO, L., PECE, S., BATTISTA, S. & FUSCO, A. 2014. HMGA1 silencing restores normal stem cell characteristics in colon cancer stem cells by increasing p53 levels. *Oncotarget,* 5**,** 3234-3245.

QUAIL, D. F., TAYLOR, M. J. & POSTOVIT, L. M. 2012. Microenvironmental Regulation of Cancer Stem Cell Phenotypes. *Current Stem Cell Research & Therapy,* 7**,** 197-216.

RAJARAJAN, A., STOKES, A., BLOOR, B. K., CEDER, R., DESAI, H., GRAFSTROM, R. C. & ODELL, E. W. 2012. CD44 Expression in Oro-Pharyngeal Carcinoma Tissues and Cell Lines. *Plos One,* 7.

RAO, C. V. & MOHAMMED, A. 2015. New insights into pancreatic cancer stem cells. *World Journal of Stem Cells,* 7**,** 547-555.

RATAJCZAK, M. Z., BUJKO, K., MACK, A., KUCIA, M. & RATAJCZAK, J. 2018. Cancer from the perspective of stem cells and misappropriated tissue regeneration mechanisms. *Leukemia,* 32**,** 2519-2526.

RAY-COQUARD, I. & LE CESNE, A. 2012. A role for maintenance therapy in managing sarcoma. *Cancer Treatment Reviews,* 38**,** 368-378.

REN, C. M., REN, T. T., YANG, K., WANG, S. D., BAO, X., ZHANG, F. & GUO, W. 2016a. Inhibition of SOX2 induces cell apoptosis and G1/S arrest in Ewing's sarcoma through the PI3K/Akt pathway. *Journal of Experimental & Clinical Cancer Research,* 35.

REN, Z. W., LIANG, S. L., YANG, J. L., HAN, X. X., SHAN, L. L., WANG, B. Y., MU, T. Y., ZHANG, Y. Q., YANG, X. L., XIONG, S. B. & WANG, G. W. 2016b. Coexpression of CXCR4 and MMP9 predicts lung metastasis and poor prognosis in resected osteosarcoma. *Tumor Biology,* 37**,** 5089-5096.

REYA, T., MORRISON, S. J., CLARKE, M. F. & WEISSMAN, I. L. 2001. Stem cells, cancer, and cancer stem cells. *Nature,* 414**,** 105-111.

RICKEL, K., FANG, F. & TAO, J. N. 2017. Molecular genetics of osteosarcoma. *Bone,* 102**,** 69-79.

RIDDLE, N. D., GONZALEZ, R. J., BRIDGE, J. A., ANTONIA, S. & BUI, M. M. 2011. A CD117 and CD34 Immunoreactive Sarcoma Masquerading as a Gastrointestinal Stromal Tumor: Diagnostic Pitfalls of Ancillary Studies in Sarcoma. *Cancer Control,* 18**,** 152-159.

RIGGI, N., SUVA, M.-L., DE VITO, C., PROVERO, P., STEHLE, J.-C., BAUMER, K., CIRONI, L., JANISZEWSKA, M., PETRICEVIC, T., SUVA, D., TERCIER, S., JOSEPH, J.-M., GUILLOU, L. & STAMENKOVIC, I. 2010. EWS-FLI-1 modulates miRNA145 and SOX2 expression to initiate mesenchymal stem cell reprogramming toward Ewing sarcoma cancer stem cells. *Genes & Development,* 24**,** 916-932.

RUCINSKA, M., KOZLOWSKI, L., PEPINSKI, W., SKAWRONSKA, M., JANICA, J. & WOJTUKIEWICZ, M. Z. 2005. High grade sarcomas are associated with microsatellite instability (chromosom 12) and loss of heterozygosity (chromosom 2). *Medical Science Monitor,* 11**,** BR65-BR68.

SAINI, V., HOSE, C. D., MONKS, A., NAGASHIMA, K., HAN, B. N., NEWTON, D. L., MILLIONE, A., SHAH, J., HOLLINGSHEAD, M. G., HITE, K. M., BURKETT, M. W., DELOSH, R. M., SILVERS, T. E., SCUDIERO, D. A. & SHOEMAKER, R. H. 2012. Identification of CBX3 and ABCA5 as Putative Biomarkers for Tumor Stem Cells in Osteosarcoma. *Plos One,* 7.

SALAWU, A., FERNANDO, M., HUGHES, D., REED, M. W. R., WOLL, P., GREAVES, C., DAY, C., ALHAJIMOHAMMED, M. & SISLEY, K. 2016. Establishment and molecular characterisation of seven novel soft-tissue sarcoma cell lines. *British Journal of Cancer,* 115**,** 1058-1068.

SALAWU, A., UL-HASSAN, A., HAMMOND, D., FERNANDO, M., REED, M. & SISLEY, K. 2012. High quality genomic copy number data from archival formalin-fixed paraffin-embedded leiomyosarcoma: optimisation of universal linkage system labelling. *PLoS One,* 7**,** e50415.

SANA, J., ZAMBO, I., SKODA, J., NERADIL, J., CHLAPEK, P., HERMANOVA, M., MUDRY, P., VASIKOVA, A., ZITTERBART, K., HAMPL, A., STERBA, J. & VESELSKA, R. 2011. CD133 expression and identification of CD133/nestin positive cells in rhabdomyosarcomas and rhabdomyosarcoma cell lines. *Analytical Cellular Pathology,* 34**,** 303-318.

SANDLER, G., YOKOI, A. & HAYES-JORDAN, A. 2019. An update in the management of pediatric sarcoma. *Current Opinion in Pediatrics,* 31**,** 368-377.

SANEI, B., KEFAYAT, A., SAMADI, M., GOLI, P., SANEI, M. H., GHAHREMANI, F. & AMOUHEIDARI, A. 2018. Undifferentiated Pleomorphic Sarcoma of Pancreas: A Case Report and Review of the Literature for the Last Updates. *Case Reports in Medicine***,** 6.

SANNINO, G., MARCHETTO, A., KIRCHNER, T. & GRUNEWALD, T. G. P. 2017. Epithelial-to-Mesenchymal and Mesenchymal-to-Epithelial Transition in Mesenchymal Tumors: A Paradox in Sarcomas? *Cancer Research,* 77**,** 4556-4561.

SBARAGLIA, M. & DEI TOS, A. P. 2019. The pathology of soft tissue sarcomas. *Radiologia Medica,* 124**,** 266-281.

SCADDEN, D. T. 2006. The stem-cell niche as an entity of action. *Nature,* 441**,** 1075-1079.

SCHATTON, T., FRANK, N. Y. & FRANK, M. H. 2009. Identification and targeting of cancer stem cells. *Bioessays,* 31**,** 1038-1049.

SCHWARTZ, L., SUPURAN, C. T. & ALFAROUK, K. O. 2017. The Warburg Effect and the Hallmarks of Cancer. *Anti-Cancer Agents in Medicinal Chemistry,* 17**,** 164-170.

SEIJKENS, T., ENGEL, D., TJWA, M. & LUTGENS, E. 2010. The role of CD154 in haematopoietic development. *Thrombosis and Haemostasis,* 104**,** 693-701.

SEO, A. N., LEE, H. J., KIM, E. J., JANG, M. H., KIM, Y. J., KIM, J. H., KIM, S.-W., RYU, H. S., PARK, I. A., IM, S.-A., GONG, G., JUNG, K. H., KIM, H. J. & PARK, S. Y. 2016. Expression of breast cancer stem cell markers as predictors of prognosis and response to trastuzumab in HER2-positive breast cancer. *British Journal of Cancer,* 114**,** 1109-1116.

SHEN, A. D., ZHANG, Y. Q., YANG, H. G., XU, R. S. & HUANG, G. W. 2012. Overexpression of ZEB1 relates to metastasis and invasion in osteosarcoma. *Journal of Surgical Oncology,* 105**,** 830-834.

SHEN, C. J., HU, G. Y., ZHANG, S. R., AO, X., ZHOU, Q. L., XIAO, P. & ZHONG, Y. 2018. Immunophenotypic characterization of sphere-forming cells derived from the human renal cell carcinoma cell line 786-0. *American Journal of Translational Research,* 10**,** 3978-+.

SHIPITSIN, M., CAMPBELL, L. L., ARGANI, P., WERERNOWICZ, S., BLOUSHTAIN-QIMRON, N., YAO, J., NIKOLSKAYA, T., SEREBRYISKAYA, T., BEROUKHIM, R., HU, M., HALUSHKA, M. K., SUKUMAR, S., PARKER, L. M., ANDERSON, K. S., HARRIS, L. N., GARBER, J. E., RICHARDSON, A. L., SCHNITT, S. J., NIKOLSKY, Y., GELMAN, R. S. & POLYAK, K. 2007. Molecular definition of breast tumor heterogeneity. *Cancer Cell,* 11**,** 259-273.

SHUKLA, G., KHERA, H. K., SRIVASTAVA, A. K., KHARE, P., PATIDAR, R. & SAXENA, R. 2017. Therapeutic Potential, Challenges and Future Perspective of Cancer Stem Cells in Translational Oncology: A Critical Review. *Current Stem Cell Research & Therapy,* 12**,** 207-224.

SIEGEL, R., NAISHADHAM, D. & JEMAL, A. 2013. Cancer statistics, 2013. *Ca-a Cancer Journal for Clinicians,* 63**,** 11-30.

SIGGINS, R. W., ZHANG, P., WELSH, D., LECAPITAINE, N. J. & NELSON, S. 2008. Stem Cells, Phenotypic Inversion, and Differentiation. *International Journal of Clinical and Experimental Medicine,* 1**,** 2-21.

SILVA, I. A., BAI, S., MCLEAN, K., YANG, K., GRIFFITH, K., THOMAS, D., GINESTIER, C., JOHNSTON, C., KUECK, A., REYNOLDS, R. K., WICHA, M. S. & BUCKANOVICH, R. J. 2011. Aldehyde Dehydrogenase in Combination with CD133 Defines Angiogenic Ovarian Cancer Stem Cells That Portend Poor Patient Survival. *Cancer Research,* 71**,** 3991-4001.

SKODA, J., NUNUKOVA, A., LOJA, T., ZAMBO, I., NERADIL, J., MUDRY, P., ZITTERBART, K., HERMANOVA, M., HAMPL, A., STERBA, J. & VESELSKA, R. 2016. Cancer stem cell markers in pediatric sarcomas: Sox2 is associated with tumorigenicity in immunodeficient mice. *Tumor Biology,* 37**,** 9535-9548.

SKODA, J. & VESELSKA, R. 2018. Cancer stem cells in sarcomas: Getting to the sternness core. *Biochimica Et Biophysica Acta-General Subjects,* 1862**,** 2134-2139.

SKOTTMAN, H., MIKKOLA, M., LUNDIN, K., OLSSON, C., STROMBERG, A. M., TUURI, T., OTONKOSKI, T., HOVATTA, O. & LAHESMAA, R. 2005. Gene expression signatures of seven individual human embryonic stem cell lines. *Stem Cells,* 23**,** 1343-1356.

SKUBITZ, K. M., FRANCIS, P., SKUBITZ, A. P. N., LUO, X. H. & NILBERT, M. 2012. Gene expression identifies heterogeneity of metastatic propensity in high-grade soft tissue sarcomas. *Cancer,* 118**,** 4235-4243.

SKUBITZ, K. M., PAMBUCCIAN, S., MANIVEL, J. C. & SKUBITZ, A. P. N. 2008. Identification of heterogeneity among soft tissue sarcomas by gene expression profiles from different tumors. *Journal of Translational Medicine,* 6**,** 13.

SLEMMONS, K. K., CROSE, L. E. S., RIEDEL, S., SUSHNITHA, M., BELYEA, B. & LINARDIC, C. M. 2017. A Novel Notch-YAP Circuit Drives Stemness and

Tumorigenesis in Embryonal Rhabdomyosarcoma. *Molecular Cancer Research,* 15**,** 1777-1791.

SMITH, J., STEWART, B. J., GLAYSHER, S., PEREGRIN, K., KNIGHT, L. A., WEBER, D. J. & CREE, I. A. 2010. The effect of pentamidine on melanoma ex vivo. *Anti-Cancer Drugs,* 21**,** 181-185.

SOMARELLI, J. A., SHETLER, S., JOLLY, M. K., WANG, X. Y., DEWITT, S. B., HISH, A. J., GILJA, S., EWARD, W. C., WARE, K. E., LEVINE, H., ARMSTRONG, A. J. & GARCIA-BLANCO, M. A. 2016. Mesenchymal-Epithelial Transition in Sarcomas Is Controlled by the Combinatorial Expression of MicroRNA 200s and GRHL2. *Molecular and Cellular Biology,* 36**,** 2503-2513.

SONBOL, M. B., AHN, D. H. & BEKAII-SAAB, T. 2019. Therapeutic Targeting Strategies of Cancer Stem Cells in Gastrointestinal Malignancies. *Biomedicines,* 7.

SOREIDE, K., SANDVIK, O. M., SOREIDE, J. A., GILJACA, V., JURECKOVA, A. & BULUSU, V. R. 2016. Global epidemiology of gastrointestinal stromal tumours (GIST): A systematic review of population-based cohort studies. *Cancer Epidemiology,* 40**,** 39-46.

STEPNIEWSKI, J., FLORCZYK-SOLUCH, U., SZADE, K., BUKOWSKA-STRAKOVA, K., CZAPLA, J., MATUSZCZAK, S., JAROSZ-BIEJ, M., LANGRZYK, A., TOMCZYK, M., RUMIENCZYKS, I., KULECKA, M., MIKULA, M., OSTROWSKI, J., JAZWA-KUSIOR, A., ZEMBALA, M., JOZKOWICZ, A., ZEMBALA, M. O. & DULAK, J. 2019. Transcriptomes of human mesenchymal cells isolated from the right ventricle and epicardial fat differ strikingly both directly after isolation and long-term culture. *Esc Heart Failure,* 6**,** 351-361.

STRATFORD, E. W., CASTRO, R., WENNERSTROM, A., HOLM, R., MUNTHE, E., LAUVRAK, S., BJERKEHAGEN, B. & MYKLEBOST, O. 2011. Liposarcoma Cells with Aldefluor and CD133 Activity have a Cancer Stem Cell Potential. *Clinical sarcoma research,* 1**,** 8-8.

SUGIHARA, E. & SAYA, H. 2013. Complexity of cancer stem cells. *International Journal of Cancer,* 132**,** 1249-1259.

SUN, B., ZHAO, S., ZHOU, C., YAN, Q. & WANG, H. 2010. Detection of PD4, CD44, PCNA protein and its clinical significance in human laryngeal carcinoma. *Lin chuang er bi yan hou tou jing wai ke za zhi = Journal of clinical otorhinolaryngology, head, and neck surgery,* 24**,** 817-9.

SUN, Y. M., WANG, H. B., LIU, M., LIN, F. G. & HUA, J. 2015. Resveratrol abrogates the effects of hypoxia on cell proliferation, invasion and EMT in osteosarcoma cells through downregulation of the HIF-1 alpha protein. *Molecular Medicine Reports,* 11**,** 1975-1981.

SUN, Y. N., MAO, X. Y., FAN, C. F., LIU, C., GUO, A. Y., GUAN, S., JIN, Q. X., LI, B., YAO, F. & JIN, F. 2014. CXCL12-CXCR4 axis promotes the natural selection of breast cancer cell metastasis. *Tumor Biology,* 35**,** 7765-7773.

SUNG, H., FERLAY, J., SIEGEL, R. L., LAVERSANNE, M., SOERJOMATARAM, I., JEMAL, A. & BRAY, F. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca-a Cancer Journal for Clinicians,* 71**,** 209-249.

SUVA, M.-L., RIGGI, N., STEHLE, J.-C., BAUMER, K., TERCIER, S., JOSEPH, J.-M., SUVA, D., CLEMENT, V., PROVERO, P., CIRONI, L., OSTERHELD, M.-C., GUILLOU, L. & STAMENKOVIC, I. 2009. Identification of Cancer Stem Cells in Ewing's Sarcoma. *Cancer Research,* 69**,** 1776-1781.

TANG, D. G. 2012. Understanding cancer stem cell heterogeneity and plasticity. *Cell Res,* 22**,** 457-472.

TARHRIZ, V., BANDEHPOUR, M., DASTMALCHI, S., OULADSAHEBMADAREK, E., ZARREDAR, H. & EYVAZI, S. 2019. Overview of CD24 as a new molecular marker in ovarian cancer. *Journal of Cellular Physiology,* 234**,** 2134-2142.

TARIN, D. 2011. Cell and tissue interactions in carcinogenesis and metastasis and their clinical significance. *Seminars in Cancer Biology,* 21**,** 72-82.

TAYLOR, B. S., BARRETINA, J., MAKI, R. G., ANTONESCU, C. R., SINGER, S. & LADANYI, M. 2011. Advances in sarcoma genomics and new therapeutic targets. *Nature Reviews Cancer,* 11**,** 541-557.

TEICHER, B. A. 2012. Searching for molecular targets in sarcoma. *Biochemical Pharmacology,* 84**,** 1-10.

TENNANT, D. A., DURAN, R. V. & GOTTLIEB, E. 2010. Targeting metabolic transformation for cancer therapy. *Nature Reviews Cancer,* 10**,** 267-277.

THANKAMONY, A. P., SAXENA, K., MURALI, R., JOLLY, M. K. & NAIR, R. 2020. Cancer Stem Cell Plasticity - A Deadly Deal. *Frontiers in Molecular Biosciences,* 7**,** 16.

THOMPSON, L. D. R. & FRANCHI, A. 2018. New tumor entities in the 4th edition of the World Health Organization classification of head and neck tumors: Nasal cavity, paranasal sinuses and skull base. *Virchows Archiv,* 472**,** 315-330.

THWAY, K. 2009. Pathology of Soft Tissue Sarcomas. *Clin Oncol (R Coll Radiol),* 21**,** 695-705.

TINHOFER, I., SAKI, M., NIEHR, F., KEILHOLZ, U. & BUDACH, V. 2014. Cancer stem cell characteristics of circulating tumor cells. *International Journal of Radiation Biology,* 90**,** 622-627.

TIRINO, V., DESIDERIO, V., D'AQUINO, R., DE FRANCESCO, F., PIROZZI, G., GALDERISI, U., CAVALIERE, C., DE ROSA, A. & PAPACCIO, G. 2008. Detection and Characterization of CD133(+) Cancer Stem Cells in Human Solid Tumours. *Plos One,* 3.

TIRINO, V., DESIDERIO, V., PAINO, F., DE ROSA, A., PAPACCIO, F., FAZIOLI, F., PIROZZI, G. & PAPACCIO, G. 2011. Human primary bone sarcomas contain CD133(+) cancer stem cells displaying high tumorigenicity in vivo. *Faseb Journal,* 25**,** 2022-2030.

TODOROVA, R. 2014. Ewing's Sarcoma Cancer Stem Cell Targeted Therapy. *Current Stem Cell Research & Therapy,* 9**,** 46-62.

TOMITA, H., TANAKA, K., TANAKA, T. & HARA, A. 2016. Aldehyde dehydrogenase 1A1 in stem cells and cancer. *Oncotarget,* 7**,** 11018-11032.

TORO, J. R., TRAVIS, L. B., WU, H. J., ZHU, K., FLETCHER, C. D. M. & DEVESA, S. S. 2006. Incidence patterns of soft tissue sarcomas, regardless of primary site, in the Surveillance, Epidemiology and End Results program, 1978-2001: an analysis of 26,758 cases. *International Journal of Cancer,* 119**,** 2922-2930.

TRAPASSO, S. & ALLEGRA, E. 2012. Role of CD44 as a marker of cancer stem cells in head and neck cancer. *Biologics : targets & therapy,* 6**,** 379-83.

TRUCCO, M. & LOEB, D. 2012. Sarcoma Stem Cells: Do We Know What We Are Looking for? *Sarcoma***,** 291705-Article No.: 291705.

TU, S.-M. & LIN, S.-H. 2012. Prostate Cancer Stem Cells. *Clinical Genitourinary Cancer,* 10**,** 69-76.

UL-HASSAN, A., SISLEY, K., HUGHES, D., HAMMOND, D. W., ROBINSON, M. H. & REED, M. W. R. 2009. Common genetic changes in leiomyosarcoma and gastrointestinal stromal tumour: implication for ataxia telangiectasia mutated involvement. *International Journal of Experimental Pathology,* 90**,** 549-557.

ULLAH, I., SUBBARAO, R. B. & RHO, G. J. 2015. Human mesenchymal stem cells - current trends and future prospective. *Bioscience Reports,* 35**,** 18.

ULLMANN, P., NURMIK, M., BEGAJ, R., HAAN, S. & LETELLIER, E. 2019. Hypoxia- and MicroRNA-Induced Metabolic Reprogramming of Tumor-Initiating Cells. *Cells,* 8**,** 16.

VAN HOECK, A., TJOONK, N. H., VAN BOXTEL, R. & CUPPEN, E. 2019. Portrait of a cancer: mutational signature analyses for cancer diagnostics. *Bmc Cancer,* 19**,** 14.

VERELST, S. J. G., HANS, J., HANSELMANN, R. G. & WIRBEL, R. J. 2004. Genetic instability in primary leiomyosarcoma of bone. *Human Pathology,* 35**,** 1404-1412.

VESELSKA, R., HERMANOVA, M., LOJA, T., CHLAPEK, P., ZAMBO, I., VESELY, K., ZITTERBART, K. & STERBA, J. 2008. Nestin expression in osteosarcomas and derivation of nestin/CD133 positive osteosarcoma cell lines. *Bmc Cancer,* 8.

VESELSKA, R., SKODA, J. & NERADIL, J. 2012. Detection of cancer stem cell markers in sarcomas. *Klinicka onkologie : casopis Ceske a Slovenske onkologicke spolecnosti,* 25 Suppl 2**,** 2S16-20.

VIALE, A., DE FRANCO, F., ORLETH, A., CAMBIAGHI, V., GIULIANI, V., BOSSI, D., RONCHINI, C., RONZONI, S., MURADORE, I., MONESTIROLI, S., GOBBI, A., ALCALAY, M., MINUCCI, S. & PELICCI, P. G. 2009. Cell-cycle restriction limits DNA damage and maintains self-renewal of leukaemia stem cells. *Nature,* 457**,** 51-U52.

VICENTE-DUENAS, C., ROMERO-CAMARERO, I., COBALEDA, C. & SANCHEZ-GARCIA, I. 2013. Function of oncogenes in cancer development: a changing paradigm. *Embo Journal,* 32**,** 1502-1513.

VIEYRA, D. S., ROSEN, A. & GOODELL, M. A. 2009. Identification and Characterization of Side Population Cells in Embryonic Stem Cell Cultures. *Stem Cells and Development,* 18**,** 1155-1166.

VILLANUEVA-TOLEDO, J., PONCIANO-GOMEZ, A., ORTIZ-SANCHEZ, E. & GARRIDO, E. 2014. Side populations from cervical-cancer-derived cell lines have stem-cell-like properties. *Molecular Biology Reports,* 41**,** 1993-2004.

VONHOFF, D. D. 1988. HUMAN-TUMOR CLONING ASSAYS - APPLICATIONS IN CLINICAL ONCOLOGY AND NEW ANTINEOPLASTIC AGENT DEVELOPMENT. *Cancer and Metastasis Reviews,* 7**,** 357-371.

WAGNER, W. 2012. Implications of long-term culture for mesenchymal stem cells: genetic defects or epigenetic regulation? *Stem Cell Research & Therapy,* 3.

WAHL, J., BOGATYREVA, L., BOUKAMP, P., ROJEWSKI, M., VAN VALEN, F., FIEDLER, J., HIPP, N., DEBATIN, K. M. & BELTINGER, C. 2010. Ewing's sarcoma cells with CD57-associated increase of tumorigenicity and with neural crest-like differentiation capacity. *International Journal of Cancer,* 127**,** 1295-1307.

WALTER, D., SATHEESHA, S., ALBRECHT, P., BORNHAUSER, B. C., D'ALESSANDRO, V., OESCH, S. M., REHRAUER, H., LEUSCHNER, I., KOSCIELNIAK, E., GENGLER, C., MOCH, H., BERNASCONI, M., NIGGLI, F. K., SCHAFER, B. W. & GRP, C. W. S. S. 2011. CD133 Positive Embryonal Rhabdomyosarcoma Stem-Like Cell Population Is Enriched in Rhabdospheres. *Plos One,* 6.

WANG, J. H., GONG, C., GUO, F. J., ZHOU, X., ZHANG, M. S., QIU, H., CHAO, T. F., LIU, Y., QIN, L. & XIONG, H. H. 2020. Knockdown of STIP1 inhibits the invasion of CD133-positive cancer stem-like cells of the osteosarcoma MG63 cell line via the PI3K/Akt and ERK1/2 pathways. *International Journal of Molecular Medicine,* 46**,** 2251-2259.

WANG, J. Y., PAN, Y., WAN, Y., WANYAN, Z. X., WANG, Z. T., TAO, Q. S. & ZHAI, Z. M. 2021. SENEX gene promotes cell proliferation by activating RB/E2F pathway in diffuse large B-cell lymphoma cells. *Biocell,* 45**,** 933-942.

WANG, L., PARK, P., ZHANG, H., LA MARCA, F. & LIN, C.-Y. 2011. Prospective identification of tumorigenic osteosarcoma cancer stem cells in OS99-1 cells based on high aldehyde dehydrogenase activity. *International Journal of Cancer,* 128**,** 294-303.

WANG, S. J., WONG, G., DE HEER, A.-M., XIA, W. & BOURGUIGNON, L. Y. W. 2009. CD44 Variant Isoforms in Head and Neck Squamous Cell Carcinoma Progression. *Laryngoscope,* 119**,** 1518-1530.

WANG, Z., TAN, M. Y., CHEN, G., LI, Z. & LU, X. B. 2017. LncRNA SOX2-OT is a novel prognostic biomarker for osteosarcoma patients and regulates osteosarcoma cells proliferation and motility through modulating SOX2. *Iubmb Life,* 69**,** 867-876.

WARBURG, O., WIND, F. & NEGELEIN, E. 1927. The metabolism of tumors in the body. *Journal of General Physiology,* 8**,** 519-530.

WAZIR, U., ORAKZAI, M., MARTIN, T. A., JIANG, W. G. & MOKBEL, K. 2019. Correlation of TERT and Stem Cell Markers in the Context of Human Breast Cancer. *Cancer Genomics & Proteomics,* 16**,** 121-127.

WEI, H. Y., FU, P. F., YAO, M. Y., CHEN, Y. M. & DU, L. L. 2016. Breast cancer stem cells phenotype and plasma cell-predominant breast cancer independently indicate poor survival. *Pathology Research and Practice,* 212**,** 294-301.

WIDEMANN, B. C. & ITALIANO, A. 2018. Biology and Management of Undifferentiated Pleomorphic Sarcoma, Myxofibrosarcoma, and Malignant Peripheral Nerve Sheath Tumors: State of the Art and Perspectives. *Journal of Clinical Oncology,* 36**,** 160-+.

WILLEMS, S. M., DEBIEC-RYCHTER, M., SZUHAI, K., HOGENDOORN, P. C. W. & SCIOT, R. 2006. Local recurrence of myxofibrosarcoma is associated with increase in tumour grade and cytogenetic aberrations, suggesting a multistep tumour progression model. *Modern Pathology,* 19**,** 407-416.

WONG, J. C. & FISCUS, R. R. 2015. Resveratrol at Anti-angiogenesis/Anticancer Concentrations Suppresses Protein Kinase G Signaling and Decreases IAPs Expression in HUVECs. *Anticancer Research,* 35**,** 273-281.

WOODWARD, W. A., KRISHNAMURTHY, S., LODHI, A., XIAO, L., GONG, Y., CRISTOFANILLI, M., BUCHHOLZ, T. A. & LUCCI, A. 2014. Aldehyde Dehydrogenase1 Immunohistochemical Staining in Primary Breast Cancer Cells Independently Predicted Overall Survival But Did Not Correlate with the Presence of Circulating or Disseminated Tumors Cells. *Journal of Cancer,* 5**,** 360-367.

WORCH, J., MATTHAY, K. K., NEUHAUS, J., GOLDSBY, R. & DUBOIS, S. G. 2010. Ethnic and Racial Differences in Patients With Ewing Sarcoma. *Cancer,* 116**,** 983-988.

WRIGHT, A., ANDREWS, N., BARDSLEY, K., NIELSEN, J. E., AVERY, K., PEWSEY, E., JONES, M., HARLEY, D., NIELSEN, A. R., MOORE, H., GOKHALE, P., RAJPERT-DE MEYTS, E., ANDREWS, P. W., WALSH, J. & HARRISON, N. J. 2011. Mapping the stem cell state: eight novel human embryonic stem and embryonal carcinoma cell antibodies. *International Journal of Andrology,* 34**,** E175-E188.

WUEBBEN, E. L. & RIZZINO, A. 2017. The dark side of SOX2: cancer - a comprehensive overview. *Oncotarget,* 8**,** 44917-44943.

XIAO, C., FU, X., WANG, Y. T., LIU, H., JIANG, Y. F., ZHAO, Z. Y. & YOU, F. M. 2020. Transferrin receptor regulates malignancies and the stemness of hepatocellular carcinoma-derived cancer stem-like cells by affecting iron accumulation. *Plos One,* 15.

XIE, X. Y., HIONA, A., LEE, A. S., CAO, F., HUANG, M., LI, Z. J., CHERRY, A., PEI, X. T. & WU, J. C. 2011. Effects of Long-Term Culture on Human Embryonic Stem Cell Aging. *Stem Cells and Development,* 20**,** 127-138.

XU, C. H., INOKUMA, M. S., DENHAM, J., GOLDS, K., KUNDU, P., GOLD, J. D. & CARPENTER, M. K. 2001. Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotechnology,* 19**,** 971-974.

YADAV, A. K. & DESAI, N. S. 2019. Cancer Stem Cells: Acquisition, Characteristics, Therapeutic Implications, Targeting Strategies and Future Prospects. *Stem Cell Reviews and Reports,* 15**,** 331-355.

YAMORI, T. 2003. Panel of human cancer cell lines provides valuable database for drug discovery and bioinformatics. *Cancer Chemotherapy and Pharmacology,* 52**,** S74-S79.

YANG, C. H., WANG, H. L., LIN, Y. S., KUMAR, K. P. S., LIN, H. C., CHANG, C. J., LU, C. C., HUANG, T. T., MARTEL, J., OJCIUS, D. M., CHANG, Y. S., YOUNG, J. D. & LAI, H. C. 2014a. Identification of CD24 as a Cancer Stem Cell Marker in Human Nasopharyngeal Carcinoma. *Plos One,* 9**,** 14.

YANG, F., CAO, L. L., SUN, Z. J., JIN, J., FANG, H. H., ZHANG, W. W. & GUAN, X. X. 2016. Evaluation of Breast Cancer Stem Cells and Intratumor Stemness Heterogeneity in Triple-negative Breast Cancer as Prognostic Factors. *International Journal of Biological Sciences,* 12**,** 1568-1577.

YANG, J., MANI, S. A. & WEINBERG, R. A. 2006. Exploring a new twist on tumor metastasis. *Cancer Research,* 66**,** 4549-4552.

YANG, J. W., CHANG, E., CHERRY, A. M., BANGS, C. D., OEI, Y., BODNAR, A., BRONSTEIN, A., CHIU, C. P. & HERRON, G. S. 1999. Human endothelial cell life extension by telomerase expression. *Journal of Biological Chemistry,* 274**,** 26141-26148.

YANG, M., YAN, M., ZHANG, R., LI, J. & LUO, Z. J. 2011. Side population cells isolated from human osteosarcoma are enriched with tumor-initiating cells. *Cancer Science,* 102**,** 1774-1781.

YANG, M. H., IMRALI, A. & HEESCHEN, C. 2015a. Circulating cancer stem cells: the importance to select. *Chinese Journal of Cancer Research,* 27**,** 437-449.

YANG, P., WANG, G., HUO, H. J., LI, Q., ZHAO, Y. & LIU, Y. H. 2015b. SDF-1/CXCR4 signaling up-regulates survivin to regulate human sacral chondrosarcoma cell cycle and epithelial-mesenchymal transition via ERK and PI3K/AKT pathway. *Medical Oncology,* 32**,** 7.

YANG, T., RYCAJ, K., LIU, Z. M. & TANG, D. G. 2014b. Cancer Stem Cells: Constantly Evolving and Functionally Heterogeneous Therapeutic Targets. *Cancer Research,* 74**,** 2922-2927.

YANG, Z. F., HO, D. W., NG, M. N., LAU, C. K., YU, W. C., NGAI, P., CHU, P. W. K., LAM, C. T., POON, R. T. P. & FAN, S. T. 2008. Significance of CD90(+) cancer stem cells in human liver cancer. *Cancer Cell,* 13**,** 153-166.

YAO, D., DAI, C. & PENG, S. 2011. Mechanism of the Mesenchymal-Epithelial Transition and Its Relationship with Metastatic Tumor Formation. *Molecular Cancer Research,* 9**,** 1608-1620.

YE, X., TAM, W. L., SHIBUE, T., KAYGUSUZ, Y., REINHARDT, F., EATON, E. N. & WEINBERG, R. A. 2015. Distinct EMT programs control normal mammary stem cells and tumour-initiating cells. *Nature,* 525**,** 256-+.

YEUNG, T. M., GANDHI, S. C., WILDING, J. L., MUSCHEL, R. & BODMER, W. F. 2010. Cancer stem cells from colorectal cancer-derived cell lines. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 3722-3727.

YI, X. J., ZHAO, Y. H., QIAO, L. X., JIN, C. L., TIAN, J. & LI, Q. S. 2015. Aberrant Wnt/beta-catenin signaling and elevated expression of stem cell proteins are associated with osteosarcoma side population cells of high tumorigenicity. *Molecular Medicine Reports,* 12**,** 5042-5048.

YIN, A. H., MIRAGLIA, S., ZANJANI, E. D., ALMEIDAPORADA, G., OGAWA, M., LEARY, A. G., OLWEUS, J., KEARNEY, J. & BUCK, D. W. 1997. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood,* 90**,** 5002-5012.

YING, M., LIU, G., SHIMADA, H., DING, W., MAY, W. A., HE, Q., ADAMS, G. B. & WU, L. 2013. Human osteosarcoma CD49f(-) CD133(+) cells: impaired in osteogenic fate while gain of tumorigenicity. *Oncogene,* 32**,** 4252-4263.

YLIPAA, A., HUNT, K. K., YANG, J. L., LAZAR, A. J. F., TORRES, K. E., LEV, D. C., NYKTER, M., POLLOCK, R. E., TRENT, J. & ZHANG, W. 2011. Integrative Genomic Characterization and a Genomic Staging System for Gastrointestinal Stromal Tumors. *Cancer,* 117**,** 380-389.

YU, W., XIE, C. R., CHEN, F. C., CHENG, P., YANG, L. & PAN, X. Y. 2021. LGR5 enhances the osteoblastic differentiation of MC3T3-E1 cells through the Wnt/beta-catenin pathway. *Experimental and Therapeutic Medicine,* 22.

YU, Y., PARK, Y. S., KIM, H. S., KIM, H. Y., JIN, Y. M., JUNG, S. C., RYU, K. H. & JO, I. 2014. Characterization of long-term in vitro culture-related alterations of human tonsil-derived mesenchymal stem cells: role for CCN1 in replicative senescence-associated increase in osteogenic differentiation. *Journal of Anatomy,* 225**,** 510-518.

YUCE, I., BAYRAM, A., CAGLI, S., CANOZ, O., BAYRAM, S. & GUNEY, E. 2011. The role of CD44 and matrix metalloproteinase-9 expression in predicting neck metastasis of supraglottic laryngeal carcinoma. *American Journal of Otolaryngology,* 32**,** 141-146.

YULIANTI, H. & HERNOWO, B. S. 2015. Correlation between Ki-67 Immunoexpression and the Tumor Aggressivity Risk in Gastrointestinal Stromal Tumor. *Majalah Kedokteran Bandung-Mkb-Bandung Medical Journal,* 47**,** 231-236.

ZAJAC, A., KROL, S. K., RUTKOWSKI, P. & CZARNECKA, A. M. 2021. Biological Heterogeneity of Chondrosarcoma: From (Epi) Genetics through Stemness and Deregulated Signaling to Immunophenotype. *Cancers,* 13.

ZAMBO, I., HERMANOVA, M., ZAPLETALOVA, D., SKODA, J., MUDRY, P., KYR, M., ZITTERBART, K., STERBA, J. & VESELSKA, R. 2016. Expression of nestin, CD133 and ABCG2 in relation to the clinical outcome in pediatric sarcomas. *Cancer Biomarkers,* 17**,** 107-116.

ZENG, Y. A. & NUSSE, R. 2010. Wnt Proteins Are Self-Renewal Factors for Mammary Stem Cells and Promote Their Long-Term Expansion in Culture. *Cell Stem Cell,* 6**,** 568-577.

ZEUNER, A., FRANCESCANGELI, F., CONTAVALLI, P., ZAPPARELLI, G., APUZZO, T., ERAMO, A., BAIOCCHI, M., DE ANGELIS, M. L., BIFFONI, M., SETTE, G., TODARO, M., STASSI, G. & DE MARIA, R. 2014. Elimination of quiescent/slow-proliferating cancer stem cells by Bcl-X-L inhibition in non-small cell lung cancer. *Cell Death and Differentiation,* 21**,** 1877-1888.

ZHANG, W., DING, M. L., ZHANG, J. N., QIU, J. R., SHEN, Y. H., DING, X. Y., DENG, L. F., ZHANG, W. B. & ZHU, J. 2015. mTORC1 Maintains the Tumorigenicity of SSEA-4(+) High-Grade Osteosarcoma. *Scientific Reports,* 5.

ZHAO, W. G., ZANG, C. X., ZHANG, T. T., LI, J., LIU, R. J., FENG, F. B., LV, Q. L., ZHENG, L., TIAN, J. H. & SUN, C. G. 2018. Clinicopathological characteristics and prognostic value of the cancer stem cell marker ALDH1 in ovarian cancer: a meta-analysis. *Oncotargets and Therapy,* 11**,** 1821-1831.

ZHENG, B. Q., ZHANG, S. R., CAI, W. L., WANG, J., WANG, T., TANG, N., SHI, Y. Q., LUO, X. Y. & YAN, W. J. 2019. Identification of Novel Fusion Transcripts in Undifferentiated Pleomorphic Sarcomas by Transcriptome Sequencing. *Cancer Genomics & Proteomics,* 16**,** 399-408.

ZHENG, M., JIANG, J., TANG, Y. L. & LIANG, X. H. 2013. Oncogene and non-oncogene addiction in inflammation-associated cancers. *Future Oncology,* 9**,** 561-573.

ZHOU, L., SHENG, D. D., WANG, D., MA, W., DENG, Q. D., DENG, L. & LIU, S. L. 2019. Identification of cancer-type specific expression patterns for active aldehyde dehydrogenase (ALDH) isoforms in ALDEFLUOR assay. *Cell Biology and Toxicology,* 35**,** 161-177.

ZOLLER, M. 2011. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nature Reviews Cancer,* 11**,** 254-267.

ZWEIGERDT, R., OLMER, R., SINGH, H., HAVERICH, A. & MARTIN, U. 2011. Scalable expansion of human pluripotent stem cells in suspension culture. *Nature Protocols,* 6**,** 689-700.

APPENDICES

APPENDIX 1

This table shows the commercial sarcoma cell lines were used in this study all assays were performed Pre and Post Stress Assay.

No.	Cell line	STS Subtype	Gender	Age	Date Set Up in Culture	Cloning Assay	Flow CD44	Flow CD133	Flow ALDH	Flow CD71	Flow CD90	Flow CD24	Flow CD34	Stress Assay	aCGH	MTT
1	U2OS	Osteosarcoma	F	15 Years	Established in 1964	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2	SK-LMS-1	leiomyosarcoma	F	43 years	1971	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
3	SW1353	chondrosarcoma	F	72 years	1977	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
4	SKUT-1	Human uterus leiomyosarcoma (GIII) cell line	F	75 years	1972	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

APPENDIX 2. This table shows the primary sarcoma cell lines were used in this study. Not all assays were performed on them.

No.	Column1	STS No.	STS Subtype	Tissue Type	Bate of Birth	Age	Ethnicty	Gender	Date of Presentation	Date of Diagnosis	Tumour Site	Tumour Size	Grade
1	Clone 1	STS 02 / 11 w1	Leiomyosarcoma	Both Fresh & FFPE	18-Nov-48	6 5	Caucasian	F	22/07/10	N/A	Vagina	50	3
	Clone 2	STS 02 /11 ws											
2	One clone	STS 14 / 10	Pleomorphic Sarcoma NOS	Both Fresh & FFPE	07/12/56	5 7	Caucasian	F	24/06/10	10/08/10	left adductor compartment	230mm	3
3	Clone 1	STS 09/10											
	Clone 2	STS 09 / 10 w2	Dedifferentiated Liposarcoma	Fresh	21/01/42	7 0	Caucasian	F		19/03/10	Retroperitoneal	300 X 230 X 150	3
4	One clone	STS 10/12 W1 P4	Fibroblastic Reticular Cell Sarcoma	?	27/08/39	73 Year	N/A	M		N/A	N/A	N/A	N/A
5	Clone 1	STS 13/12 WS P3	Pleomorphic NOS sarcoma	FFPE	02/09/32	80 year	N/A	M		N/A	Lower limb	N/A	N/A
	Clone 2	STS 13/12 W2 P8											
	Clone 3	STS 13/12 W1											
	Clone 4	STS 13/12 W2 P7V											
6	One clone	STS 20/11 P21	Dedifferentiated Liposarcoma	Fresh	24/07/41	70 Year	N/A	F		N/A	Lower limb/ Left thigh	N/A	3
7	One clone	STS 15/12 P2	myxofibrosarcoma	Fresh	29/08/25	87 year	N/A	M		N/A	N/A	N/A	N/A
8	One clone	STS 16/12 W1 P3	Dedifferentiated Liposarcoma waiting 2 grow/ infected	?	10/08/30		N/A	M		N/A	N/A	N/A	N/A
9	One clone	STS 21/11	Myxofibrosarcoma	Fresh	13/04/38		N/A	M		16/09/11	Upper limb/ Left arm	N/A	3
10	One clone	STS 09/11	Pleomorphic Sarcoma NOS	Both Fresh & FFPE	26/06/44		N/A	F		15/05/11	Right thigh	N/A	3
11	Clone 1	STS 06/11 P96	Pleomorphic Sarcoma NOS	Fresh	22/01/1939 ??	7 4	Caucasian	M	07/01/11	19/01/11	Lower limb/ Right thigh	170mm	3

	Clone 2	STS 06/11 W2 P82											
	Clone 3	STS 06/11 WS P93											
	Clone 4												
14	One clone	STS 11/11	Leiomyosarcoma	Both Fresh & FFPE	?	?	?	M	?	?	Intraabdominal	95	3
15	One clone	STS 18/11	Leiomyosarcoma	Both Fresh & FFPE	?	?	?	F	?	?	Ischirectal fossa	85	1
16	One clone	STS 01/11	Mlignant Periphereal Nerve Sheath Tumour	Fresh	?	?	?	F	?	?	?	?	?
17	One clone	STS 08/11	Myxofibrosarcoma	Fresh	?	?	?	M	?	?	Lower limb	105	3
18	One clone	STS 16/10	Myxofibrosarcoma	Fresh	?	?	?	M	?	?	Lower limb	35	3
19	One clone	STS 07/10	Pleomorphic liposarcoma	Fresh	?	?	?	M	?	?	Lower limb	190	?
20	One clone	STS 03/11	Pleomorphic liposarcoma	Fresh	?	?	?	M	?	?	Pelvic	?	?
21	One clone	STS 04/11	Pleomorphic NOS	Fresh	?	?	?	F	?	?	?	?	?
22	One clone	STS 15/11	Pleomorphic NOS	Fresh	?	?	?	F	?	?	?	?	?
23	One clone	STS 06/10	ALT/WDLPS	Fresh	?	?	?	M	?	?	Trunk	280	1
24	One clone	STS 11/10	ALT/WDLPS	Fresh	?	?	?	M	?	?	Trunk	62	1
25	One clone	STS 14/11	ALT/WDLPS	Fresh	?	?	?	M	?	?	Retroperitoneal	195	1
26	One clone	STS 19/10	ALT/WDLPS	Fresh	?	?	?	F	?	?	Limb	105	1
27	One clone	STS 12/10	Alveolar Rhabdomyosarcoma	Fresh	?	?	?	M	?	?	Limb	42	3
28	One clone	STS 01/11	Alveolar Soft Part Sarcoma	Fresh	?	?	?	F	?	?	Limb	92	?
29	One clone	STS 15/10	Alveolar Soft Part Sarcoma	Fresh	?	?	?	M	?	?	Trunk	38	3
30	One clone	STS 05/10	Angiosarcoma	Fresh	?	?	?	F	?	?	Trunk	70	3
31	One clone	STS 10/11	Angiosarcoma	Fresh	?	?	?	M	?	?	Trunk	120	3
32	One clone	STS 21/10	Angiosarcoma	Fresh	?	?	?	M	?	?	Limb	60	3
33	One clone	STS 17/11	Dedifferentiated Liposarcoma	Fresh	?	?	?	M	?	?	Retroperitoneal	80	3
34	One clone	STS 19/11	Dedifferentiated	Fresh	?	?	?	F	?	?	Retroperiton	?	3

			Liposarcoma								eal		
35	One clone	STS 18/10	Dedifferentiated Liposarcoma	Fresh	?	?	?	F	?	?	Retroperiton eal	210	3
36	One clone	STS 13/11	Ewings' Sarcoma	Fresh	?	?	?	M	?	?	Pelvis	?	?
37	One clone	STS 02/10	Extraskeletal Myxoid	Fresh	?	?	?	M	?	?	?	?	?
38	One clone	STS 17/10	Solitary Fibrous Tumour	Fresh	?	?	?	F	?	?	Lower limb	?	?
39	One clone	STS 04/10	Synovial Sarcoma	Fresh	?	?	?	F	?	?	Trunk	40	3

APPENDIX 3 This table shows the primary sarcoma cell lines were used in this study. Not all assays were performed on them.

No.	STS No.	Treatment	Relevent Previous/Patient Medical History (PMHx)	Status	Date of Death	Surgery Date	Date of Metastases	R0	**Date of Metastases**	Date Set Up in Culture
1	STS 02 / 11 w1	N/A		Alive	N/A	18/01/11	N/A	R1	N/A	Jan-11
	STS 02 /11 ws									
2	STS 14 / 10	Adjuvant radiotherapy after surgery, 60 gy in 30 fractions then 6 gy in 3 fractions.		Alive	N/A	05/08/10	N/A	R1	N/A	Aug-10
3	STS 09/10									
	STS 09 / 10 w2	N/A		Dead	13/06/12	10/06/10	N/A	R0	N/A	Jun-10
4	STS 10/12 W1 P4	N/A		?	?	11/10/12	?	?	?	Oct-12
5	STS 13/12 WS P3	N/A		Alive	N/A	18/10/12	N/A	R1	N/A	Oct-12
	STS 13/12 W2 P8									
	STS 13/12 W1									
	STS 13/12 W2 P7V									
6	STS 20/11 P21	Neoadjuvant radiotherapy before surgery, before taking the sample		Alive	N/A	27/10/11	13/01/13	R1	13/01/13	Oct-11
7	STS 15/12 P2	N/A		?	?	26/10/12	N/A	?	N/A	Oct-12
8	STS 16/12 W1 P3	N/A		?	?	13/11/12	N/A	?	N/A	Nov-12
9	STS 21/11	N/A		Alive	N/A	01/11/11	24/08/12	R1	24/08/12	Nov-11
10	STS 09/11	N/A		Dead	26/09/11	N/A	N/A	R1	N/A	?
11	STS 06/11 P96	N/A	Renal cell carcinoma	Dead	15/02/2013	17/02/11	N/A	R1	N/A	Feb-11
	STS 06/11 W2 P82									
	STS 06/11 WS P93									

14	STS 11/11	?	?	Alive	N/A	?	01/01/12	R1	01/01/12	?
15	STS 18/11	?	?	Alive	N/A	?	N/A	R1	N/A	?
16	STS 01/11	?	?	Alive	N/A	?	N/A	R1	N/A	?
17	STS 08/11	?	?	Alive	N/A	?	N/A	R1	N/A	?
18	STS 16/10	?	?	Alive	N/A	?	N/A	R0	N/A	?
19	STS 07/10	?	?	Dead	11/07/11	?	08/01/11	R2	08/01/11	?
20	STS 03/11	?	?	Dead	16/08/2011	?	?	R0	?	?
21	STS 04/11	?	?	Alive	N/A	?	?	R1	?	?
22	STS 15/11	?	?	Dead	29/03/2012	?	N/A	R1	N/A	?
23	STS 06/10	?	?	Alive	N/A	?	N/A	R1	N/A	?
24	STS 11/10	?	?	Alive	N/A	?	N/A	R1	N/A	?
25	STS 14/11	?	?	Alive	N/A	?	N/A	R2	N/A	?
26	STS 19/10	?	?	Alive	N/A	?	N/A	R1	N/A	?
27	STS 12/10	?	?	Alive	N/A	?	N/A	R0	N/A	?
28	STS 01/11	?	?	Alive	N/A	?	03/01/11	R1	03/01/11	?
29	STS 15/10	?	?	Alive	N/A	?	N/A	R0	N/A	?
30	STS 05/10	?	?	Alive	N/A	?	N/A	R1	N/A	?
31	STS 10/11	?	?	Alive	N/A	?	01/01/13	R0	01/01/13	?
32	STS 21/10	?	?	Alive	N/A	?		R0		?
33	STS 17/11	?	?	Dead	11/01/11	?	N/A	R2	N/A	?
34	STS 19/11	?	?	Alive	N/A	?	?	R1	?	?
35	STS 18/10	?	?	Alive	N/A	?	?	R1	?	?
36	STS 13/11	?	?	Alive	N/A	?	?	R1	?	?
37	STS 02/10	?	?	Alive	N/A	?	?	R1	?	?
38	STS 17/10	?	?	Alive	N/A	?	?	R1	?	?
39	STS 04/10	?	?	Alive	N/A	?	?	R0	?	?

APPENDIX 4. Table was made to monitor the work flow and experiments.

2ed RUN										
Cell lines	**MTT**		**Stress Assay** **1million (1x10^6) cells will be seeded**							**DNA Extraction**
			Stress Assay (3 flasks) **Maximum viable number** **(Cell counted)** **(3 flasks)**							
			Photograph 1-Leave the flasks for 24-72 hrs after they fully confluent. 2- change the media before counting.							
	Early passage	Late passage	Seeding day	7th, 14th, 21th day(every week)	100% confluent day	Dying day	Dead day	After re-feed dead cells 7th day and every week		
06/11WS										
09/10										
02/14										
02/11W1										
07/13										
21/11W2										
2/14										
13/12W2										
14/10										
SKLMS-1										
U2OS										
SW1353										
SKUT-1										
PC3										
H-tert										