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Circulating Fibroblast Precursors in Soft Tissue Sarcoma Metastasis

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by

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Abstract

Soft tissue sarcomas (STS) are rare, malignant tumors of mesenchymal origin that manifest in the connective tissues, including muscle, adipose and deep skin tissue, nerves, and joint tissue. Complications from primary or recurrent sarcomas often lead to increased morbidity, but the most lethal aspect of sarcomas is their propensity for hematogenous dissemination leading to metastatic disease. After development of distant metastases, the median survival for patients with STS is merely 11 to 15 months[1,2]. Therefore, gaining a better understanding of the metastatic environment is essential to developing new therapies. The tumor microenvironment has been implicated as an essential component for metastatic progression and one of the most prominent cell types of the tumor microenvironment is the cancer-associated fibroblast (CAF)[3,4]. CAFs promote tumor progression by stimulating angiogenesis, cancer cell growth, invasion and metastasis, and through evasion of the immune response[5,6]. Studies clearly show the importance of CAFs in tumor advancement; however, their origins have not been conclusively determined, although multiple origins have been proposed[7–12]. Studies previously conducted in our lab have demonstrated that hematopoietic stem cells (HSCs) are a novel source for CAFs and their precursors in the blood, circulating fibroblast precursors (CFPs)[13]. Our lab has further shown that murine CFPs are present in peripheral blood, found *in vivo* in the tumor stroma, increase with tumor development, and contribute to tumor growth, suggesting that CFPs play a direct role in promoting the progression of solid tumors[14]. Additionally, studies from our lab revealed a HSC origin for human CFPs[15]. The function of human CFPs in tumor metastasis, specifically STS, however, has yet to be established.

Therefore, the goal of this study was to evaluate the peripheral blood of patients with STS in order to characterize CFPs in these patients and determine

their role in promoting STS metastatic progression. In this study, peripheral blood was collected from patients with STS and from normal control subjects. Our data has shown that CFPs are present within the peripheral blood of patients with STS and normal control subjects and that these cells display markers of fibroblasts, including collagen 1, alpha smooth muscle actin, and vimentin. Our data have also shown that although there is no significant difference in average number of CFPs present within the peripheral blood of STS patients compared to normal control, evaluating individual results indicated that patients with metastatic disease who are not on chemotherapeutic treatment may have increased numbers of CFPs. We further showed that CFPs from patients with STS contribute to sarcoma cell proliferation, migration, and invasion *in vitro* and that *in vitro* generated tumor-exposed CFPs showed enhanced ability to contribute to sarcoma invasion and displayed an altered cytokine profile when compared to control CFPs. Our data indicate CFPs may play an important role in tumor progression and that through further analysis, it may be discovered that these differences are subtype, tumor stage, and treatment dependent.

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List of Abbreviations

αSMA- alpha smooth muscle actin

ATCC- American Type Culture Collection

BM- Bone Marrow

CAF- Cancer-Associated Fibroblast

CCR2- Chemokine Receptor Type 2

CD45- Leukocyte Common Antigen

CDK4- Cyclin-dependent Kinase 4

CFP- Circulating Fibroblast Precursor

Col1- Collagen 1

CML- Chronic Myelogenous Leukemia

Cq- quantification cycle

CT- Computerize Tomography

CXCR4- Chemokine Receptor Type 4

Cy3- Cyanine 3

DDR2- Discoidin Domain Receptor 2

ECM- Extracellular Matrix

EGF- Epidermal Growth Factor

EGFP- Enhanced Green Fluorescent Protein

ELISA- Enzyme-Linked Immunosorbent Assay

EMMPRIN- Extracellular Matrix Metalloproteinase Inducer

FGF- Fibroblast Growth Factor

FISH- Fluorescent In Situ Hybridization

FITC- Fluorescin Isothiocyanate

FNCLCC- Fédération Nationale des Centres de Lutte Contre le Cancer

HFF1- Human Foreskin Fibroblast cell line

HGF- Hepatocyte Growth Factor

HPF- High Powered Field

HSC- Hematopoietic Stem Cell

HT-1080- Human Fibrosarcoma cell line

IGF- Insulin-like Growth Factor

IHC- Immunohistochemical

IL1-β- Interleukin-1 Beta

IL-6- Interleukin 6

IL-8- Interleukin 8

MCP1- Monocyte Chemoattractant Protein 1

MDM2- Mouse Double Minute 2 Homolog

MEMα- Modified Eagle's Medium alpha

MMP- Matrix Metalloproteinase

MPNST- Malignant Peripheral Nerve Sheath Tumor

MRI- Magnetic Resonance Imaging

MSC- Mesenchymal Stem/Stromal Cell

NAd- Non-adherent cells

NCCN- National Comprehensive Cancer Network

NF1- Neurofibromin 1

PBS- Phosphate Buffered Saline

PET- Positron Emission Tomography

PFS- Progression-Free Survival

Pt- Patient

RB1- Retinoblastoma 1

RT- Radiation Therapy

RT-qPCR- Reverse Transcription quantitative Real-time Polymerase Chain Reaction

SDF-1- Stromal-Derived Cell Factor 1 (also known as CXCL12)

STS- Soft Tissue Sarcoma

TGFβ- Transforming Growth Factor Beta

TNM- Tumor, Lymph Nodes, Metastasis

TP53- Tumor Protein p53

uPA- Urokinase-type Plasminogen Activator

uPAR- Urokinase Receptor

Serpin E1- Serpin Peptidase inhibitor, clade E, member 1 (plasminogen activator inhibitor, type I)

TME- Tumor Microenvironment

VEGF- Vascular Endothelial Growth Factor

Vim- Vimentin

Acknowledgements

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Chapter 1: Introduction

Section 1: Soft Tissue Sarcoma

1.1 Epidemiology and Etiology

Soft Tissue Sarcomas (STS) are rare, malignant tumors of mesenchymal origin. They constitute 12% of pediatric cancers, but only 1% of all adult malignant neoplasms, with a slight male predominance[16,17]. Three-fourths of STSs can be classified as either undifferentiated pleomorphic sarcoma, liposarcoma, leiomyosarcoma, myxofibrosarcoma, synovial sarcoma, or malignant peripheral nerve sheath tumor (MPNST)[18]. It is estimated that 11,930 new cases of STS will be diagnosed in 2015, along with 4,870 deaths[19]. The incidence according to age varies: embryonal rhabdomyosarcoma occurs predominately in the pediatric population, while synovial sarcoma occurs mostly in young adults, and undifferentiated pleomorphic sarcoma, liposarcoma, leiomyosarcoma, and myxofibrosarcoma tend to occur in the elderly. In similarity with other malignant tumors, the rate of adult STS increases with age- the median diagnosis being around 65 years[17].

Although the etiology of many soft tissue sarcomas is unknown, chemical carcinogens, radiation, viral infection and immunodeficiency, or genetic susceptibility have been associated with tumor development. Some studies have indicated there is increased incidence of STS in those exposed to chlorophenols, phenoxyacetic herbicides, and dioxin[20,21]. Other reports suggest breast cancer patients exposed to radiation treatments are at increased risk of developing post-irradiation sarcoma[22]. Additionally, patients with the retinoblastoma gene (*RB1*) germline mutation are at elevated risk for STS (leiomyosarcoma, malignant fibrous histiocytoma, rhabdomyosarcoma, and liposarcoma) with statistically significant increases in STS in

patients treated with radiation, with the majority of STS occurring in the radiation field after exposure[23]. Viral infection and immunodeficiency play a prominent role in the development of Kaposi sarcoma, which is clinically linked to Human Herpes Virus 8, a gamma-herpes virus found to contain genes involved in evading the normal immune response[24,25]. Genetic susceptibility is also known to be associated with elevated risk of STS. Li-Fraumeni syndrome, an autosomal dominant disease caused by germline mutations of the *TP53* tumor suppressor gene predisposes individuals to the development of primary tumors at a young age. A study of 200 Li-Fraumeni syndrome family members revealed that 17% of their primary cancers were diagnosed as STS[26]. As stated previously, the retinoblastoma gene (*RB1*) is also associated with increased risk of STS. Neurofibromatosis 1, an autosomal dominant disease caused by mutations in the *NF1* gene has been linked to certain STSs, including malignant peripheral nerve sheath tumors, of which 50% have the *NF1* mutation[27].

1.2 Histopathology

Soft Tissue Sarcomas (STS) are an extremely diverse group of tumors- according to the World Health Organization they comprise more than 100 different histological subtypes[17]. STSs are classified according to their presumed origins and the diagnosis of STS is based upon morphology along with immunohistochemical (IHC) staining. In addition to IHC staining, Fluorescence In Situ Hybridization (FISH) is often used to identify subtypes of STS containing chromosomal translocations, which one third of all STSs are found to harbor (**Table 1.1**).

Table 1.1 Soft Tissue Sarcoma Translocations

Diagnosis	Chromosomal Abnormality	Genes Involved
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(1;13)(p36;q14) t(2;2)(p23;q36) t(X;2)(q13;q36)	PAX3-FKHR PAX7-FKHR PAX3–NCOA1 PAX3–FOXO4
Alveolar soft part sarcoma	t(X;17)(p11.2;q25)	TFE3-ASPL
Clear cell sarcoma	t(12;22)(q13;q12) t(2;22)(q33;q12)	EWS-ATF1 EWS1R–CREB1
Congenital fibrosarcoma/ Congenital mesoblastic nephroma	t(12;15)(p13;q25)	ETV6-NTRK3
Ewing's sarcoma/ Peripheral primitive neuroectodermal tumor	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(17;22)(q12;q12) t(2;22)(q33;q12) t(16;21)(p11;q22)	EWS-FLI1 EWS-ERG EWS-ETV1 EWS-FEV EWS-E1AF FUS-ERG
Low grade fibromyxoid sarcoma	t(7;16)(q33;p11) t(11;16)(p11;p11)	FUS-CREB3L2 FUS-CREB3L1
Myxoid chondrosarcoma	t(9;22)(q22;q12) t(9;15)(q22;q21) t(9;17)q22;q11)	EWS-CHN TFC12-CHN TAF2N-CHN
Myxoid liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12)	TLS-CHOP EWS-CHOP
Synovial Sarcoma	t(X;18)(p11;q11) t(X;18)(p11;q11) t(X;18)(p11;q11) t(X;20)(p11;q13)	SSX1-SYT SSX2-SYT SSX4-SYT SSX1-SS181

Common IHC stains include desmin to identify rhabdomyosarcoma and leiomyosarcoma (as one of multiple markers). S100 positivity correlates with cells arising from the neural sheath and Factor VII identifies tumors of endothelial origin. Cytokeratin staining can distinguish between synovial sarcoma and epithelioid sarcoma (both stain positive) and fibrosarcoma (stains negative). While some markers are more characteristic than others, it is often necessary to examine a spectrum of markers in order to determine histologic subtype.

As stated above, the most frequent subtypes of STS include: undifferentiated sarcoma, liposarcoma, leiomyosarcoma, myxofibrosarcoma, synovial sarcoma, and malignant peripheral nerve sheath tumor, all of which have distinct characteristics. Undifferentiated sarcoma, previously called malignant fibrous histiocytoma, is the most common subtype of STS. This subtype refers to sarcomas that lack specific lines of differentiation and may be further divided into pleomorphic, round cell, and spindle cell variants. Liposarcomas are believed to be derived from precursors of adipocytes and are commonly found in the extremities and retroperitoneum. The three main subgroups of liposarcoma are well-differentiated/dedifferentiated, myxoid/round cell, and pleomorphic. Myxoid liposarcomas typically reveal a FUS-CHOP or TLS-CHOP translocation[28,29]. Well-differentiated/dedifferentiated almost invariably express diffuse nuclear MDM2 and/or CDK4, whereas pleomorphic liposarcomas are negative for expression[30]. Leiomyosarcomas show pure smooth-muscle differentiation and are found in the retroperitoneum, veins, and uterus. Synovial sarcomas histologically resemble synovial cells, however their origin is unknown. Synovial sarcomas are characterized by translocations of the SYT gene on chromosome 18 to SSX1, SSX2, or SSX4 on the X chromosome[31]. Lastly, MPNST are commonly found in the trunk, extremities, head, and neck. Diagnosis is difficult due to varied histomorphology and although S100 positivity is often found, it is not uniformly expressed.

1.3 Clinical Presentation

STS manifests in the connective tissues (striated skeletal and smooth muscle, tendons, adipose tissue, fibrous tissue, blood vessels, peripheral nerves, and the lining of the joints) and often presents as a gradually enlarging, painless mass. While some patients complain of pain or edema in an extremity due to compression by the tumor

mass, it is rare for a patient to present with constitutional symptoms of fever or weight loss. STSs most prevalently occur in the extremities (75%), followed by the trunk wall (10%), and the retroperitoneum (10%). These tumors have the capacity to become quite large, particularly in the thigh and retroperitoneum, and growth rates vary depending upon tumor aggressiveness. Their common pattern of spread is hematogenous, predominately to the lung, whereas spread to regional lymph nodes is infrequent, excluding a few histological subtypes (rhabdomyosarcoma, synovial sarcoma, epithelioid sarcoma, clear cell sarcoma, and the vascular sarcomas)[32,33].

1.4 Diagnosis, Grading, and Staging

Due to their rarity and usually asymptomatic presentation, STSs tend to be initially misdiagnosed, exacerbating their malignant potential. Further causation of diagnostic and therapeutic challenges is the heterogeneity of these tumors, which are comprised of more than 100 different histological subtypes[17]. Because STS is rare and often unsuspected, multiple diagnostic tests should be performed to verify presence of STS. To distinguish between a benign soft tissue mass and STS, the United Kingdom Department of Health published criteria for urgent referral of patients with soft tissue lesions. The criteria includes a soft tissue mass greater than 5 cm, painful lump, lump that is increasing in size, a lump of any size that is deep to the muscle fascia, or recurrence of a lump after previous excision[34]. After referral, imaging techniques should be performed to assess tumor location and size in order to aid in surgical planning. MRI is preferred for imaging masses of the extremities, head, trunk, and neck, while CT is useful for imaging abdominal, visceral, or retroperitoneal masses. A core needle biopsy or incisional biopsy of the tumor should also be performed for histological examination to aid in determination of STS subtype and grade. The less invasive method of a core

needle biopsy is preferred and has shown efficacy for accurately determining the subtype of STS in 88% of patients, whereas, incisional biopsy is often employed when more tumor tissue is needed for the use of molecular analysis, flow cytometry, or cytogenetics[35]. Furthermore, PET imaging, can distinguish benign tumors from sarcomas, having the greatest sensitivity for high grade tumors. Lastly, due to the propensity for STS lung metastasis, a chest CT scan is recommended after diagnosis of STS to discern whether pulmonary nodules are present. Similarly, patients with known angiosarcoma should undergo brain imaging due to this tumor's propensity for metastatic spread to the central nervous system.

Tumor grade, which should only be applied to untreated primary STSs, is indicative of how likely a tumor is to grow and disseminate and thus it is an important factor when planning treatment regimens. The two most predominant systems for grading STSs are those proposed by the National Cancer Institute and the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC). The FNCLCC is more precisely defined and has revealed greater efficacy, therefore it is the preferred system. However, grading is not valuable for the prognosis of all STS subtypes. Subtypes excluded from grading are MPNST, angiosarcoma, alveolar soft part sarcoma, extraskeletal myxoid chondrosarcoma, clear cell sarcoma, and epithelioid sarcoma. Three prognostic factors are used for defining tumor grade according to FNCLCC: tumor differentiation, mitotic count, and tumor necrosis (**Table 1.2**).

Table 1.2 FNCLCC Histological Grading System

Histological Parameter	Definition
Tumor Differentiation	• Score 1: Sarcomas closely resembling normal adult mesenchymal tissue and potentially difficult to distinguish from the counterpart benign tumor (e.g. well-differentiated liposarcoma, well-differentiated leiomyosarcoma) • Score 2: Sarcomas for which histological typing is certain (e.g. myxoid liposarcoma, myxofibrosarcoma) • Score 3: Embryonal and undifferentiated sarcomas, synovial sarcomas, sarcomas of doubtful type
Mitotic Count (1 HPF measures 0.1734 mm^2)	• Score 1: 0-9 mitoses per 10 HPF • Score 2: 10-19 mitoses per 10 HPF • Score 3: >19 mitoses per 10 HPF
Tumor Necrosis	• Score 0: No necrosis • Score 1: <50% tumor necrosis • Score 2: ≥50% tumor necrosis
Histological Grade	• Grade 1: total score 2, 3 • Grade 2: total score 4, 5 • Grade 3: total score 6, 7, 8

While tumor grade is indicative of tumor aggressiveness, tumor stage determines the size of the primary tumor and the extent to which the tumor has already spread. The staging system most commonly used for STS is the TNM system created by the International Union for Cancer and the American Joint Committee on Cancer (AJCC)[36]. The TNM staging system incorporates four factors: tumor size and depth (T), lymph node involvement (N), distant metastasis (M), and histologic grade (G) (**Table 1.3**). STSs of the extremities and trunk are staged with the TNM system; however, an alternate system should be used for STSs of the retroperitoneum as other factors including histology, metastasis, and resection margins are more prognostic.

Table 1.3 AJCC's TNM Staging System

Primary Tumor (T)	TX	Primary tumor cannot be assessed			
	T0	No evidence of primary tumor			
	T1 • T1a • T1b	Tumor 5 cm or less in greatest dimension • Superficial tumor • Deep tumor			
	T2 • T2a • T2b	Tumor more than 5 cm in greatest dimension • Superficial tumor • Deep tumor			
Regional Lymph Nodes (N)	NX	Regional lymph nodes cannot be assessed			
	N0	No regional lymph node metastasis			
	N1	Regional lymph node metastasis			
Distant Metastasis (M)	M0	No distant metastasis			
	M1	Distant metastasis			
Histologic Grade (G)	GX	Grade cannot be assessed			
	G1	Grade 1			
	G2	Grade 2			
	G3	Grade 3			
Anatomic Stage/Prognostic Groups	Stage IA	T1a	N0	M0	G1, GX
		T1b	N0	M0	G1, GX
	Stage IB	T2a	N0	M0	G1, GX
		T2b	N0	M0	G1, GX
	Stage IIA	T1a	N0	M0	G2, G3
		T1b	N0	M0	G2, G3
	Stage IIB	T2a	N0	M0	G2
		T2b	N0	M0	G2
	Stage III	T2a, T2b	N0	M0	G3
		Any T	N1	M0	Any G
	Stage IV	Any T	Any N	M1	Any G

1.5 Treatment

The goal of any treatment plan is to minimize tumor recurrence, while maintaining function and quality of life for the patient. Due to STS heterogeneity, treatment plans vary based on subtype and extent of disease. According to the National Comprehensive Cancer Network (NCCN) guidelines, surgical resection is an essential component of treatment for primary low grade STS tumors of the extremities and trunk wall[37]. Efforts to achieve a complete resection with negative margins of at least 1 cm should be made, but if tumor margins are close, the addition of adjuvant radiation therapy is recommended. When primary tumors are intermediate to high grade, greater than 5 cm in size, or deep, the addition of radiation therapy is also recommended. For patients with high grade primary tumors, the NCCN recommends resection alone or along with the following: pre-operative radiation therapy, post-operative radiation therapy, neoadjuvant chemotherapy, or adjuvant chemotherapy.

Radiation therapy (RT) dose is dependent upon tissue location and tolerance. Newer techniques, including intensity modulated RT, intraoperative RT, and brachytherapy have demonstrated improved outcomes in STS patients[38–40]. Intensity modulated RT is beneficial for its ability to reduce exposure of normal tissue surrounding the tumor to high dose radiation, whereas intraoperative RT is a process in which radiation treatment occurs during surgery. Brachytherapy is the use of radioactive seeds, which are implanted into the tumor bed through the use of catheters and can include low-dose rate, fractionated high-dose rate, or intraoperative high-dose rate therapy.

Although the value of chemotherapy remains a subject of considerable debate, distinct STS histologies have shown chemosensitivity to certain regimens, thus, chemotherapy should be prescribed based upon STS subtype and location. The pediatric Ewing sarcomas and alveolar/embryonal rhabdomyosarcomas are routinely

treated with neoadjuvant chemotherapy (vincristine, cyclophosphamide, doxorubicin) due to their high risk for metastasis and sensitivity to chemotherapy. Synovial sarcoma is another chemosensitive tumor, with progression-free survival (PFS) response rates for combination therapy of ifosfamide and doxorubicin showing 58% in one study[41]. For patients with leiomyosarcoma, gemcitabine and docetaxel combination therapy has shown PFS rates of 16%[42]. Yondelis, a more recently developed synthetic chemotherapeutic drug originally derived from the marine tunicate ecteinascidia turbinata, has shown PFS rates of up to 51% in patients with liposarcoma and leiomyosarcoma[43].

Current treatment for patients with metastatic disease often comprises resection, radiation therapy, and/or chemotherapy. First line chemotherapeutic treatments include ifosfamide (alkylating agent) and doxorubicin (anthracyclin antibiotic), which have proven the most active against metastatic soft tissue sarcomas, yet still maintain paltry response rates observed in only 15-35% of patients[44,45]. Single agent chemotherapy regimens that should be considered are doxorubicin, epirubicin, and ifosfamide, which have shown response rates in metastatic STS[46,47]. Combination chemotherapy regimens consist of AIM (adriamycin, ifosfamide, and mesna); MAID (mesna, adriamycin, ifosfamide, and dacarbazine); gemcitabine with either docetaxel, vinorelbine, or dacarbazine; and doxorubicin along with dacarbazine. For many patients with metastatic soft tissue sarcoma, chemotherapy is used with palliative intent- to reduce tumor burden, slow disease progression, and improve symptoms. While some studies have shown enhanced PFS, no studies have shown significant overall survival improvement and potential benefits may be offset by treatment-related toxicities, especially upon use of combination chemotherapy[48–50]. Yet, molecular pathogenesis has provided key insight towards developing novel chemotherapeutic treatments. For example, patients with ckit

positive gastrointestinal stromal tumors have shown 70% PFS rates for 12 months after treatment with Imatinib, a tyrosine kinase inhibitor known to inhibit ckit, depicting increased effectiveness of targeted therapies[51]. The effectiveness of targeted therapies in comparison to alkylating or intercalating agents reinstates the need to uncover specific molecular markers that can be utilized to develop more compelling chemotherapeutic agents. Additionally, because STS chemotherapeutic agents exhibit varied PFS rates, it is a challenge for physicians to identify which patients will benefit from treatment, thus, markers to assess chemotherapeutic efficacy in patients with certain types of STS must be determined. These prognostic markers may then identify which patients will benefit from potential chemotherapeutic regimens, while sparing those who will not benefit from possible toxicity-related treatment problems.

1.6 Clinical Outcomes

Multiple studies have shown that tumor size, stage, and histologic grade are important indicators of clinical outcome for patients with STS[52–54]. In one study of over 1000 patients with STS of the extremities, it was found that being over the age of 50 and having positive resected margins predicted local recurrence, while tumor grade and size were the main predictors of distant recurrence and survival[53]. Other factors associated with survival include age, histologic subtype, and anatomic site[55].

For STS patients who are successfully treated of their primary tumor, approximately 25% will go on to develop metastatic disease and once the primary tumor exceeds 5 cm in size, is deep to the fascia, and intermediate or high grade, the incidence of metastasis increases to 40-50%[56,57]. Furthermore, approximately 10% of patients have detectable metastases at primary tumor diagnosis and the majority of patients with metastatic disease are incurable[17]. Thus, metastasis, via hematogenous dissemination, remains

one of the most lethal aspects of soft tissue sarcomas and constitutes the greatest obstacle towards effective treatment and positive clinical outcomes.

Section 2: Tumor Microenvironment in Metastasis

2.1 Tumor Microenvironment (TME)

The TME is pivotal to cancer progression, as it establishes a permissive and supportive environment for the cancer cell. It consists of extracellular matrix components (collagen, elastin, fibrilin, fibronectin, and proteoglycans), cellular components (immune cells, endothelial cells, adipocytes, fibroblasts), nerves, and vasculature all within close proximity to the malignant cancer cells.

Dvorak first observed that the tumor microenvironment contains striking similarities to that of an active wound and, thus, described tumors as "wounds that do not heal"[58]. During the normal wounding process, quiescent fibroblasts undergo activation to become myofibroblasts, producing alpha-smooth muscle actin (α-SMA), an important component of the cytoskeleton involved in cell contractility and motility[59]. Additionally, these activated fibroblasts promote angiogenesis and secrete a complex extracellular matrix (ECM) to further assist in repairing the damaged tissue. After completing reparation of the wound, activated fibroblasts undergo programmed cell death and are eliminated from the granulation tissue, whereas during tumor progression, fibroblasts in the TME are consistently present in the tumor stroma, remain perpetually activated, and are not removed by apoptosis[60].

2.2 Cancer-Associated Fibroblasts (CAFs)

The most prominent cell type of the TME is the cancer-associated fibroblast (CAF)[34]. CAFs are mature, activated tissue fibroblasts associated with the solid tumor, which promote tumor progression by stimulating angiogenesis, cancer cell growth, invasion and metastasis, and through evasion of the immune response[56]. They are activated by tumor cell secretion and stromal cell secretion of TGF-β1, which has been

shown to mediate α-SMA production, a marker of activated fibroblasts[61]. Through production of chemokines and growth factors such as fibroblast growth factor 2 (FGF-2), stromal-derived cell factor 1 (SDF-1), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF), CAFs implement a direct effect upon promotion of growth in the surrounding epithelium and upon angiogenesis in the tumor stroma[62]. CAFs are also an important source of tumor inflammatory cytokines such as interleukin-1β (IL1-β)[63]. They participate in remodeling of the extracellular matrix by secretion of collagen type 1 and fibronectin, ECM proteins correlated with tumor stroma stiffening and enhanced metastatic potential[64,65]. Tumor stroma stiffening, which is associated with more efficient TGF-β activation, may create a positive feedback loop through enhancing fibroblast activation and ECM synthesis, leading to increased matrix rigidity[66,67]. CAFs are additionally a source for matrix-metalloproteinases (MMP)-2,-3, and -9, which degrade the ECM, potentiating tumor cell migration, invasion, and metastasis, and creating a pathway for angiogenesis[62]. Furthermore, CAFs secrete chemokines exploited by tumor cells to aid in their metastatic potential. During hypoxic conditions, tumor cells increase CXCR4 expression, enabling them to escape to a normoxic environment by migration towards SDF1/CXCL12-expressing CAFs; these SDF1/CXCL12-expressing CAFs additionally recruit endothelial-progenitor cells from the bone marrow that differentiate into tumor-associated vascular endothelial cells, which lay the foundation for angiogenic growth[68,69]. CAFs have also been shown to be involved in chemotherapeutic resistance. With the secretion of ECM proteins, CAFs contribute to tumor ECM rigidity, causing increased interstitial fluid pressures, resulting in diminished chemotherapeutic delivery to the tumor[64]. Additionally, ECM rigidity has been associated with TGF-β1 tumorigenic functions, including EMT and cell motility[70]. Other studies have shown that activated

fibroblasts are capable of preventing apoptosis of melanoma cells following cisplatin treatment[71]. Factors secreted by CAFs, including HGF, have been implicated in promoting chemotherapeutic resistance by inhibiting drug induced apoptosis[72]. Studies clearly show the importance of CAFs in tumor advancement; however, their origin has not been conclusively determined.

2.3 HSC-Derived Circulating Fibroblast Precursors

Various origins for cancer-associated fibroblasts (CAFs) have been proposed, including resident tissue fibroblasts, smooth muscle cells, epithelial to mesenchymal transition, endothelial cells, and bone marrow-derived mesenchymal stromal cells [7–12]. Yet, our studies, based upon a murine single hematopoietic stem cell transplantation model have demonstrated an additional, novel hematopoietic stem cell (HSC) source for CAFs and their precursors in the blood, circulating fibroblast precursors (CFPs)[13].

The HSC transplant method performed by our group is essential for the identification of HSC-derived cells in various murine disease models. In our transplant method, bone marrow is obtained from mice that ubiquitously express enhanced green fluorescent protein (EGFP) on a B6.SJL-*Ptprc*^{b} *Pep3*^{b}/BoyJ (C57BL/6-CD45.2) background. The EGFP+ bone marrow then undergoes flow cytometric cell sorting using markers of HSCs (Lineage-, Sca-1+, ckit^{hi}, CD34-, side population), with Lineage- denoting cells that are negative for markers of B cells, T cells, granulocytes, and erythrocytes. Single cells are sorted into each well of a 96 well plate and undergo short term culture. Because the majority of HSCs remain in G0 for up to 3-4 days after initiation of cell culture, we select clones consisting of 20 or fewer cells after one week incubation (as clones consisting of greater than 20 cells are likely hematopoietic progenitor cells that do not remain quiescent for the initial 3-4 days in culture)[73]. This

clonal population of putative HSCs recovered from the wells is then intravenously injected into a black syngeneic B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ (C57BL/6-CD45.1) mouse (in order to distinguish donor from recipient, which can be done by examining presence or absence of EGFP or CD45.1 expression) whose bone marrow has been ablated through lethal irradiation. After 2 months, the mice are checked for multilineage engraftment. The defining property of a HSC is in its ability to repopulate the bone marrow and reconstitute hematopoiesis following transplantation, thus the presence of multilineage engraftment unequivocally indicates an HSC origin for any EGFP+ cell in our transplanted mice.

Our group employed this HSC transplant method to reveal that EGFP+ cells derived from the bone marrow of clonally engrafted mice gave rise to fibroblasts *in vitro* and expressed procollagen 1-α1, discoidin domain receptor 2 (DDR2, receptor for collagen), vimentin, and fibronectin mRNA, all markers of fibroblasts[74]. In murine melanoma and Lewis lung carcinoma tumor models, our group previously identified HSC-derived EGFP+ cells that incorporated into the tumor stroma and gave rise to cells phenotypically resembling fibroblasts in the tumor capsule[13]. Further analysis revealed these cells expressed type 1 procollagen and α-SMA, indicating that these HSC-derived cells in the tumor stroma were indeed fibroblasts, specifically activated fibroblasts[13]. Our group then went on to examine whether the circulating precursors to these fibroblasts homed to and were present within the tumor stroma. CFPs were found *in vivo* in the tumor stroma, most interestingly within the tumor blood vessels and it was determined that these CFPs were of HSC origin and could be defined as $CD45^{+}$ $DDR2^{+}$ cells expressing markers associated with the monocyte lineage[14]. CFP number increased in correlation with tumor progression and CFPs were shown to migrate in response to tumor-conditioned media[14]. Additionally, CFPs were found to express chemokine

receptor type 2 (CCR2), a receptor for monocyte chemoattractant protein 1 (MCP-1), and it was further found that CFP migration was MCP-1 dependent[14]. This research clearly revealed a HSC origin for murine CFPs and suggested their role in tumor progression; however, the identification of human HSC-derived CFPs had yet to be studied.

To examine the origin of human CFPs, our group obtained peripheral blood from human subjects, then cultured and stained the cells for fibroblast markers. It was found that peripheral blood mononuclear cells stained positive for αSMA, pro-collagen type 1, vimentin, and 5B5, all markers of fibroblasts[15]. A subsequent study was performed on peripheral blood obtained from three female subjects transplanted with GCSF-mobilized stem cells from male donors[15]. Non-adherent mononuclear cells derived from the peripheral blood of the female subjects were cultured and fluorescence in situ hybridization (FISH) analysis revealed the presence of both X and Y chromosomes in all cultured cells, indicating the donor origin of CFPs. Although this study revealed the donor origin of human CFPs, further evidence was needed to unequivocally show an HSC origin for CFPs. An additional study was performed on the peripheral blood of two untreated patients with Philadelphia chromosome positive chronic myelogenous leukemia (CML). The Philadelphia chromosome occurs when chromosomes 9 and 22 translocate, resulting in the fusion of the BCR and C-ABL genes. FISH analysis for the BCR/ABL translocation on cultured non-adherent mononuclear cells revealed the presence of the BCR-ABL translocation. Because CML is a clonal disorder of the HSC, the presence of the BCR-ABL translocation strongly indicates a HSC origin for human CFPs.

Chapter 2: Rationale, Hypothesis, and Specific Aims

Rationale

Our studies, based upon a murine single hematopoietic stem cell transplantation model have demonstrated that hematopoietic stem cells (HSCs) are a novel source for cancer-associated fibroblasts (CAFs) and their blood precursors, circulating fibroblast precursors (CFPs). Our research has shown that murine CFPs ($CD45^+$ $DDR2^+$) are present in the peripheral blood and increase with tumor burden, implicating their importance in tumor progression. Our lab has also uncovered an HSC origin for human CFPs. However, the identification and characterization of human CFPs in cancer, specifically soft tissue sarcoma, has yet to be established. Preliminary evidence from our lab suggests we are capable of isolating CFPs from human peripheral blood and once cultured, CFPs exhibit markers of fibroblasts.

Cancer-associated fibroblasts (CAFs), potentially derived from circulating fibroblast precursors (CFPs), are mature tissue fibroblasts associated with the solid tumor and have been shown to promote tumor progression by stimulating angiogenesis, cancer cell growth, invasion and metastasis, and through evasion of the immune response. CAFs implement these effects by production of various growth factors (such as VEGF, a potent angiogenesis inducer), matrix metalloproteinases (such as MMP9 which degrades collagen and fibronectin, allowing the tumor cell to transverse the basement membrane), and through production of chemokines and cytokines. Our lab has shown that CFPs, a precursor to CAFs, increase with tumor burden, are present in the tumor stroma, and migrate in response to tumor conditioned media, suggesting that CFPs may play a direct role in promoting the progression and metastasis of solid tumors. Additionally, no studies have investigated the role of CAFs in soft tissue

sarcoma progression. We are therefore interested in uncovering whether CFP-derived fibroblasts play a functional role in soft tissue sarcoma metastasis through production of specific chemokine, cytokine, and growth factors involved in promoting the proliferation, migration, and invasion of sarcoma cells.

Hypothesis

HSC-derived CFPs are present in patients with soft tissue sarcoma and promote soft tissue sarcoma tumor cell proliferation, migration, and invasion *in vitro*.

Specific Aims

Aim 1: **To isolate, characterize, and quantify circulating fibroblast precursors (CFPs) in patients with soft tissue sarcoma through analysis of hematopoietic and fibroblastic marker expression.** Mononuclear cells were isolated via ultra-density centrifugation from peripheral blood and utilized immediately or cultured for growth of fibroblasts and examined for presence of hematopoietic and fibroblastic markers by immunofluorescence, flow cytometry, and quantitative real-time polymerase chain reaction.

Aim 2: **To determine the role of CFP-derived fibroblasts from patients with soft tissue sarcoma in promoting proliferation, migration, and invasion of sarcoma cells.** The ability of CFP-derived fibroblasts to promote proliferation was assessed via Cyquant Direct Cell assay and ability to promote migration and invasion was assessed via transwell assays. CFPs were tested for production of chemokine, cytokine, and growth factors associated with metastatic progression (i.e. ability to promote proliferation, migration, and invasion of sarcoma cells) via antibody array.

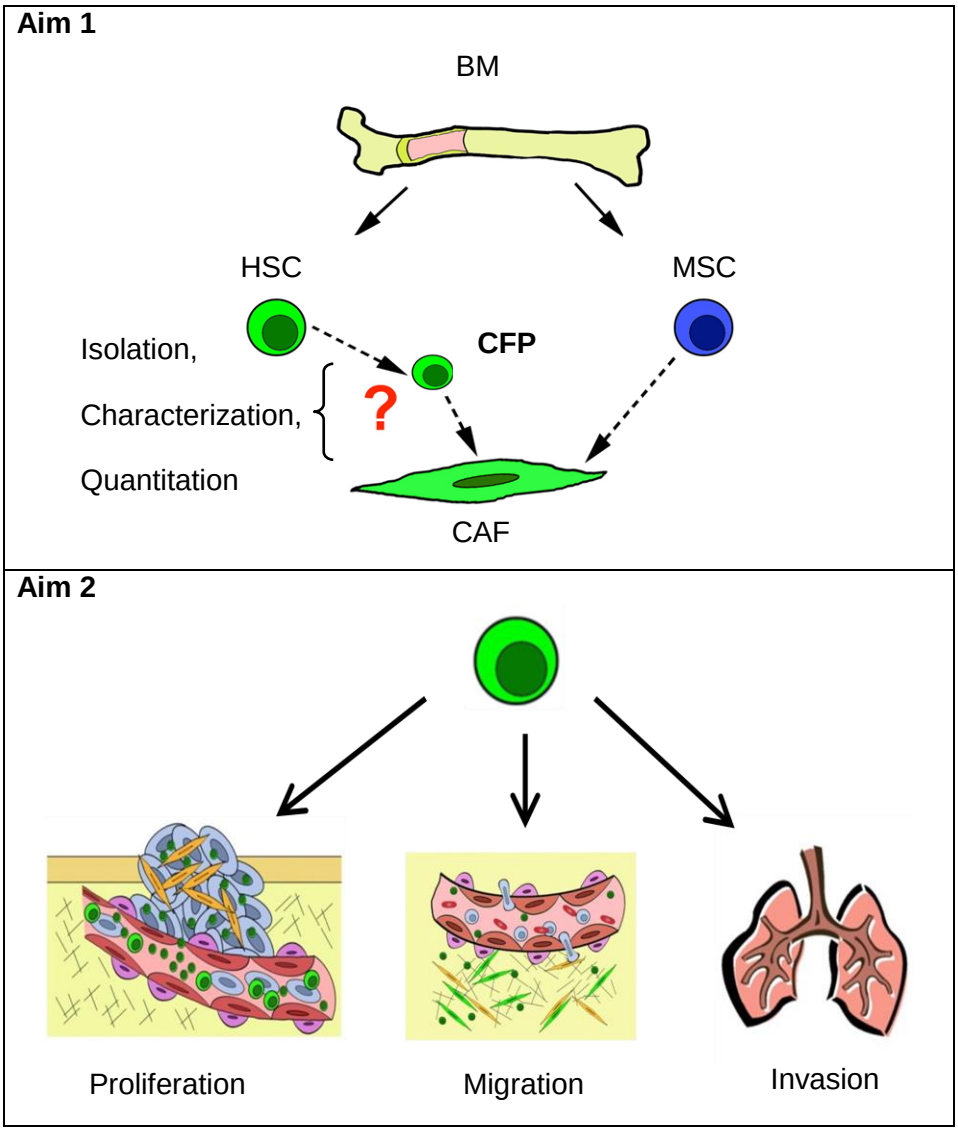


Figure 1. Specific Aims. Aim 1: To isolate, characterize, and quantify circulating fibroblast precursors (CFPs) in patients with soft tissue sarcoma through analysis of hematopoietic and fibroblastic marker expression. Aim 2: To determine the role of CFP-derived fibroblasts from patients with soft tissue sarcoma in promoting proliferation, migration, and invasion of sarcoma cells.

Chapter 3: Significance

90% of all cancer deaths are due to metastatic disease, making it the single most significant challenge to management of patients with malignant neoplasms. Patients with metastatic soft tissue sarcomas have a five year survival rate of 16% and only 15-35% of patients respond to standard chemotherapeutic regimens[44,45,75]. Although many chemotherapeutic agents target the tumor cell, few agents target the tumor stroma even though it is an essential component of the tumor, providing a supportive and permissive habitat for tumor progression. Cancer-associated fibroblasts (CAFs) are the most abundant cell type of the tumor microenvironment and promote metastasis through production of factors that support tumor cell growth, vascularization, migration and invasion, escape from immune response, and chemotherapeutic drug resistance[62]. While multiple origins for CAFs have been proposed, our group has shown a hematopoietic stem cell (HSC)-derived circulating fibroblast precursor (CFP) origin for CAFs[13]. This HSC-derived CFP origin for CAFs is especially pertinent in the realm of soft tissue sarcomas that have a predilection towards hematogenous dissemination. Studies have demonstrated the ability of bone marrow derived cells to colonize pre-metastatic sites prior to tumor cell colonization, further implicating a role of HSC-derived cells from the bone marrow in metastatic progression[65]. Our research will elucidate the effects of HSC-derived CFPs on soft tissue sarcoma metastasis, potentially identifying novel factors responsible for disease progression. Furthermore, once CFPs are implicated in soft tissue sarcoma metastasis, they may serve as unique therapeutic targets. Additionally, the known HSC-origin of CFPs may allow us to target CFPs at an even earlier state, depleting all possible HSC-derived CFPs that have the potential to differentiate into CAFs within the tumor microenvironment.

Conclusively, the key to uncovering new therapies for treating advanced cancers, such as metastatic soft tissue sarcoma, lies in the enhanced understanding of their metastatic environment. Our studies will result in a better understanding of human tumor metastasis, specifically through CFP marker expression profiling and discovery of the role of CFPs in promoting sarcoma tumor cell proliferation, migration, and invasion. Our studies may then lead to the identification of unique CFP-based therapies for soft tissue sarcoma metastasis inhibition.

Chapter 4: Specific Aim 1

To isolate, characterize, and quantify CFPs in patients with soft tissue sarcoma through analysis of hematopoietic and fibroblastic marker expression.

Section 1: Introduction

Previous studies in our lab, based upon a murine single hematopoietic stem cell transplantation model have demonstrated that hematopoietic stem cells (HSCs) are a novel source for cancer-associated fibroblasts (CAFs) and their blood precursors, circulating fibroblast precursors (CFPs)[13]. Our lab's research has shown that murine CFPs ($CD45^+$ $DDR2^+$) are present in the peripheral blood and increase with tumor burden[14]. Our lab has also uncovered a HSC origin for human CFPs[15]. However, the identification and characterization of human CFPs in cancer, specifically soft tissue sarcoma, has yet to be established. CFP classification is based upon expression of markers that recognize both their hematopoietic origins and fibroblastic traits, thus we define CFPs as peripheral blood cells expressing the hematopoietic cell marker CD45 (common leukocyte antigen) along with the fibroblast marker, discoidin domain receptor 2 (DDR2, collagen receptor). Collagen type I, another marker of fibroblasts was also examined. Because CFPs have the potential to differentiate into activated CAFs in the tumor microenvironment, further examination of markers of activated fibroblasts was conducted; these included vimentin and alpha-smooth muscle actin (α-SMA).

Section 2: Hypothesis

We hypothesized that CFPs were present in the peripheral blood of patients with soft tissue sarcoma and displayed hematopoietic and fibroblastic marker expression profiles.

Section 3: Materials and Methods

Cell Culture of Human Peripheral Blood

Peripheral blood (9-15 mL) was drawn from patients with soft tissue sarcoma and from control subjects (All Cells #WB001-NH) without any known malignancies and collected in sodium heparin-coated vacutainer tubes (BD #367878). Mononuclear cells were isolated via density centrifugation with Lympholyte H (Cedarlane #CL5010) and either frozen for later analysis or plated on a T25 tissue culture treated flask (Corning #430639) and incubated at 37°C in free gas exchange at 5% CO_2. After a 20 hour incubation period, non-adherent HSC-derived mononuclear cells were removed and plated on 4-well fibronectin-coated tissue culture plates (Corning #354559) for 14-21 days at a concentration of $6x10^5$ cells per well in 1 mL of Minimum Essential Medium α (MEMα) (Life Technologies #12561) supplemented with 20% fetal bovine serum (Atlanta Biologicals, lot #6005C) and penicillin streptomycin in order to promote the differentiation of CFPs into fibroblasts (CFP-derived fibroblasts). The rationale for obtaining the non-adherent population of mononuclear cells is derived from the concept that two types of stem cells are present in the bone marrow- hematopoietic stem cells (HSCs) and mesenchymal stem/stromal cells (MSCs). MSCs are characterized by their ability to adhere to plastic, thus, we obtain the non-adherent population in order to enrich for HSC-derived cells[76].

Cell Culture of HT-1080 and HFF-1 cells

The human HT-1080 fibrosarcoma and HFF-1 foreskin fibroblast cell lines were purchased from American Type Culture Collection (ATCC). HT-1080 cells (ATCC #CCL-121) were cultured in Eagle's Minimum Essential Medium (ATCC #30-2003) supplemented with 10% Fetal Bovine Serum (ATCC #30-2020) and HFF-1 cells (ATCC

#SCRC-1041) were cultured in Dulbecco's Modified Eagle's Medium (ATCC #30-2002) supplemented with 15% Fetal Bovine Serum (ATCC #30-2020). Both cell lines were incubated at 37°C in free gas exchange at 5% CO_2 and allowed to grow to confluency before splitting. Cell lines were used below passage 8.

Immunofluorescence Staining

Antibodies were first titrated and tested on cultured HFF-1 fibroblast cells in order to optimize our staining methods for fibroblast markers (**Figure 2**).

After 14-21 days culture, CFP-derived fibroblasts were washed in phosphate buffered saline (PBS), then fixed with 4% paraformaldehyde and stored in 0.05% sodium azide until ready for staining. Cells were then washed 3 times with PBS. 0.02% Triton X was added for 20 minutes, then cells were again washed 3 times with PBS. Cells were then blocked for 1 hour in 5% donkey serum in 3% bovine serum albumin/PBS. Primary antibodies for alpha smooth muscle actin (αSMA), Collagen 1, and Vimentin (**Table 4.1**) were added and incubated overnight at 4°C. Cells were washed 3 times with PBS and secondary antibodies for Cyanine 3 (Cy3) and Fluorescin Isothiocyanate (FITC) (**Table 4.1**) were added for 1 hour, incubated at room temperature and protected from light. Cells were washed 3 times with PBS, then Hoechst 33342 (Invitrogen #H3570) diluted 1:25000 in PBS was added for 8 minutes. Fluorogel (Electron Microscopy Sciences #17985-10) was then added before slides were coverslipped. Slides stained with Secondary only were used as controls for non-specific antibody staining. Epifluorescence and differential contrast (DIC) microscopy was conducted using a Nikon Eclipse 90i microscope with NIS Elements AR3.2 Software. Images were processed using Adobe Photoshop CS2 (Adobe Systems, Inc., San Jose, CA).

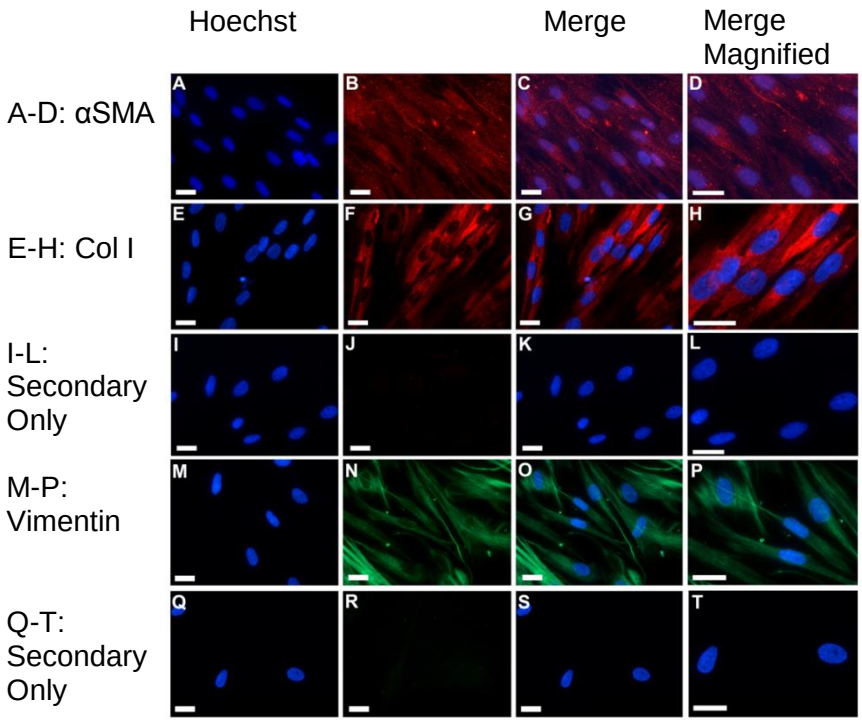


Figure 2. Immunohistochemical standardization of staining for fibroblast markers. Optimization of antibody staining for activated fibroblast markers, including αSMA (red; A-D), Col I (red; E-H), and Vimentin (green; M-P), with cultured human foreskin fibroblast (HFF-1) cells. Nuclei were stained with Hoechst dye (blue). Secondary only control staining for αSMA/Col I (I-L) and Vimentin (Q-T). Mag bars in A-T = 25 µm.

Table 4.1 Antibodies Used for Immunofluorescence Staining

Antibody	Company	Catalog #	Lot #	Concentration
Rabbit αSMA	Abcam	ab15734	GR110540-1	1:50 in PBS
Mouse Collagen 1	Abcam	ab90395	GR117081-1	1:50 in PBS
Rabbit Collagen 1	Abcam	ab34710	GR33585-2	1:50 in PBS
Mouse Vimentin	Abcam	ab8979	GR39704	1:50 in PBS
Donkey anti Rabbit-Cy3	Jackson	715-165-152	109623	1:100 in PBS
Donkey anti Mouse-FITC	Jackson	711-096-150	106884	1:100 in PBS

Flow Cytometry Staining

Antibodies were first titrated to determine optimal staining concentrations for flow cytometric analysis.

After mononuclear cells were isolated from peripheral blood via density centrifugation with Lympholyte H (Cedarlane #CL5010), they were washed, then resuspended in cell culture freeze media with DMSO (Millipore #S002-5F), stored at -80°C overnight, then placed in liquid Nitrogen storage until analysis. When ready for analysis, cells were quickly thawed in a 37°C water bath, washed, then resuspended in growth media and allowed to recover for 1 hour in an incubator at 37°C in free gas exchange at 5% CO_2. Cells were then counted with Trypan Blue for viability, washed, and allocated into tubes for staining. Cells were resuspended in 100 µL phosphate buffered saline (PBS), then 11 µL Human AB Serum (10% of cell suspension volume) was added to block non-specific antibody binding. After 1 minute incubation, cells were stained with CD45 and Discoidin Domain Receptor 2 (DDR2) antibodies (**Table 4.2**) and incubated for 20 minutes on ice, protected from light. After washing, APC secondary antibody was added and cells were incubated for 20 minutes on ice, protected from light. Live/Dead Fixable Cell Stain (Invitrogen #L-23102) was used according to manufacturer's protocol. Cells were acquired on BD LSR Fortessa Analytic Flow Cytometer. Positive staining and gating strategy were determined by comparison to isotype control or fluorescence minus one controls. Data were analyzed using FlowJo software.

Table 4.2 Antibodies Used for Flow Cytometry

Antibody	Company	Catalog #	Lot #	Clone #
Mouse IgG1k CD45-BV510	Becton Dickenson	563204	3172808	H130
Mouse IgG2B DDR2	R&D Systems	MAB25381	CACM0112051	290804
Rat anti Mouse IgG2B APC	R&D Systems	F0133	ACKM0113121	332723

Quantitative Real-time PCR

Total RNA from cells was isolated using Trizol reagent (Life Technologies #15596) in combination with the RNeasy Mini Kit (Qiagen #74104). 0.5 µg total RNA was reverse transcribed in a 20 µL reaction using the iScript cDNA synthesis kit (Bio-Rad #170-8890). Quantitative Real-time PCR was performed with 2.5 µL of a 1:20 dilution of reverse-transcribed cDNA, along with SsoAdvanced Universal Probes Supermix (Bio-Rad #172-5284) and primer-probe combinations (**Table 4.3**) designed through the Universal ProbeLibrary Design Center (Roche) in a 10 µL reaction in the Light Cycler 96 (Roche). Cycling conditions were conducted according to the manufacturer's protocol. Reactions were run in triplicate for each cDNA sample, along with non-reverse transcriptase and negative controls. Relative gene expression was quantified based on Cq value measured against standard curves for each primer-probe set. Data were normalized to HPRT reference gene.

Table 4.3 Primer Sequences for RT-qPCR

Gene	GenBank Accession #	Primer Sequence 5' to 3'	Probe
CD45	Y00062.1	F: agtcaaagttattgttatgctgacaga R: tgctttccttctccccagta	54
Coll1A1	NM_000088.3	F: gggattccctggacctaaag R: ggaacacctcgctctcca	67
Vimentin	ENST00000224237.5	F: gaccagctaaccaacgacaaa R: gaagcatctcctcctgcaat	39
αSMA	NM_001141945.1	F: ctgttccagccatccttcat R: tcatgatgctgttgtaggtggt	58
DDR2	NM_006182.2	F: ggagagttcttccgagacca R: aggacaaatggctggttgag	16
CD14	NM_000591.2	F: gttcggaagacttatcgaccat R: acaaggttctggcgtggt	74
Coll5	NM_000093.3	F: cctggatgaggaggtgtttg R: cggtggtccgagacaaag	42
CD11b	J03925.1	F: ggcatccgcaaagtggta R: ggatcttaaaggcattctttcg	9
HPRT	ENST00000298556.7	F: gaccagtcaacaggggacat R: gtgtcaattatatcttccacaatcaag	22

Human Consent

Peripheral blood was obtained after informed consent was given and subjects were enrolled into our Institutional Review Board approved study (CTO 101576) through the MUSC Hollings Cancer Center. All subjects consented were over 18 years of age and had soft tissue sarcoma, either metastatic or non-metastatic.

Statistical Data Analysis

Data are presented as mean ± standard deviation of the value. To compare variables between 2 groups, the two-tailed unpaired Student t-test was performed in Microsoft Excel, with significance determined as $p<0.05$.

Section 4: Results

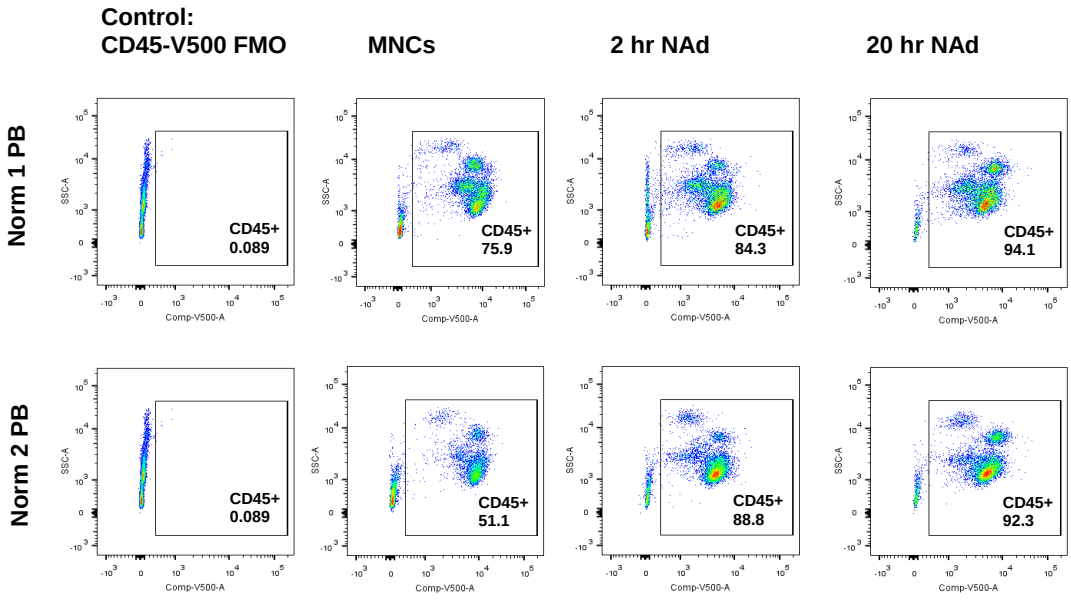
Enrichment of HSC-derived cells from Human Peripheral Blood

Bone marrow contains two types of stem cells: the hematopoietic stem cell (HSC) and the mesenchymal stem/stromal cell (MSC). Previous dogma held that HSCs gave rise exclusively to blood lineage cells, while MSCs were capable of giving rise to mesenchymal cell types including adipocytes, endothelial cells, chondrocytes, osteoblasts/osteocytes, fibroblasts, and microglial cells[77–83]. Our lab has shown that HSCs are also capable of giving rise to mesenchymal cells- adipocytes, osteochondrogenic cells, fibroblasts/myofibroblasts, and microglial and perivascular cells of the brain[74,84–87]. Therefore, we are particularly interested in the plasticity and potential of HSC-derived cells. Thus, it is imperative for our studies to examine populations enriched for HSC-derived cells in order to diminish MSC contamination and its corresponding effects. As one of the characteristics of a MSC is adherence to plastic, we obtained the non-adherent population of cells from peripheral blood and cultured these for our studies in order to enrich for an HSC-derived population[76]. To determine whether our non-adherence culture method was optimal towards enriching for cells of an HSC origin in the peripheral blood, flow cytometric analysis of expression of CD45 (common leukocyte antigen), a known marker of hematopoietic cells, was performed.

Peripheral blood was obtained from normal control subjects, then mononuclear cells (MNCs) were isolated via density centrifugation. MNCs were saved for flow cytometric analysis or plated on a T25 tissue culture treated flask and incubated at 37°C in free gas exchange at 5% CO_2. After either a 2 hour or 20 hour incubation period, the non-adherent MNCs were removed from culture for flow cytometric analysis. As shown in **Figure 3**, obtaining the non-adherent population of MNCs is sufficient for enriching for $CD45^+$ HSC-derived cells and this enrichment increases with increasing culture time

from 2 hrs to 20 hrs. Thus, by isolating and culturing the non-adherent population of peripheral blood MNCs, we selected for an enriched population of HSC-derived cells to be used in our studies.

A.



B.

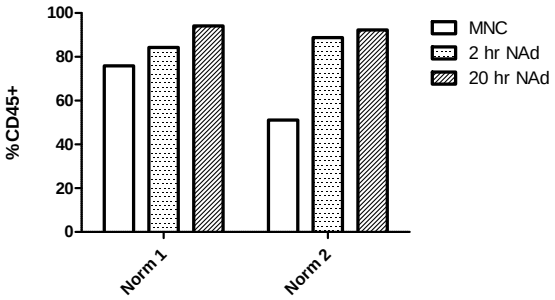
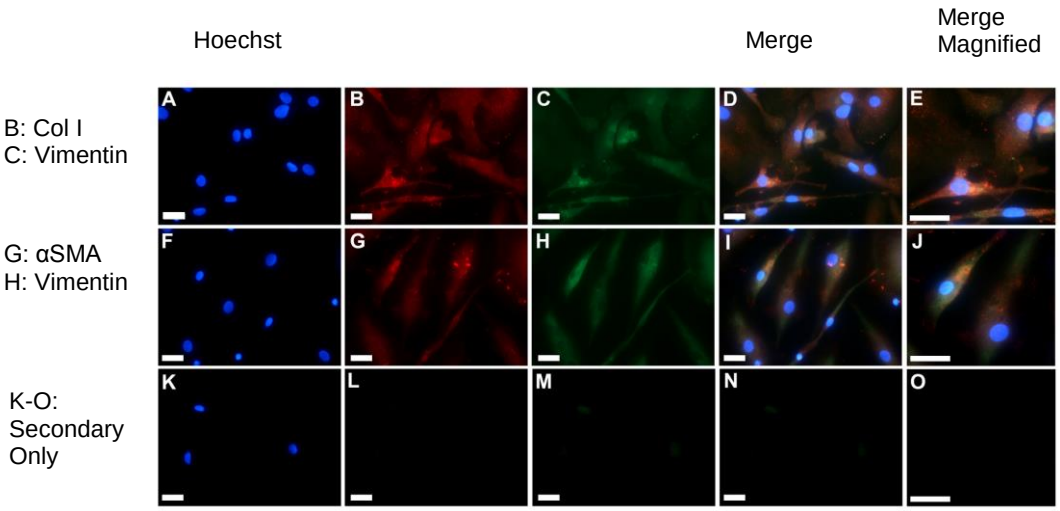


Figure 3. Enrichment of hematopoietic-derived cells from the NAd population of MNCs. (A) Total peripheral blood (PB) mononuclear cells (MNCs) compared to the non-adherent (NAd) population of MNCs derived after culture for either 2 hours (2 hr NAd) or 20 hours (20 hr NAd) from two different normal control subjects (Norm 1 PB and Norm 2 PB) were stained for CD45, the common leukocyte antigen, and analyzed via flow cytometry. CD45 FMO was used as a control for gating strategy. (B) Graphical depiction of flow cytometric data from each normal control subject.

Characterization of Cultured CFPs from Human Peripheral Blood

In order to determine if our isolation and culture methods were sufficient for obtaining fibroblasts from human peripheral blood (CFP-derived fibroblasts), we cultured the non-adherent population of MNCs, shown to be enriched for hematopoietic-derived cells, from normal control subjects (n=3) for 14-21 days then performed immunohistochemical analysis for the following fibroblast markers: collagen I (Col I), vimentin, and alpha smooth muscle actin (αSMA) (Representative data shown in **Figure 4A**). Because there is no single specific fibroblast marker, we chose a panel of fibroblast markers to examine whether our cultured cells were indeed displaying a fibroblastic phenotype. We then performed the same immunohistochemical analysis on patients with metastatic STS (n=3) in order to determine whether CFP-derived fibroblasts were also present in the peripheral blood of patients with STS tumor burden (Representative data shown in **Figure 4B**). Our data demonstrate the presence and feasibility of isolating CFPs from human peripheral blood, in both normal controls and patients with STS, and show that once cultured, CFPs were positive for Col I, Vimentin, and αSMA, all markers of fibroblasts.

A.



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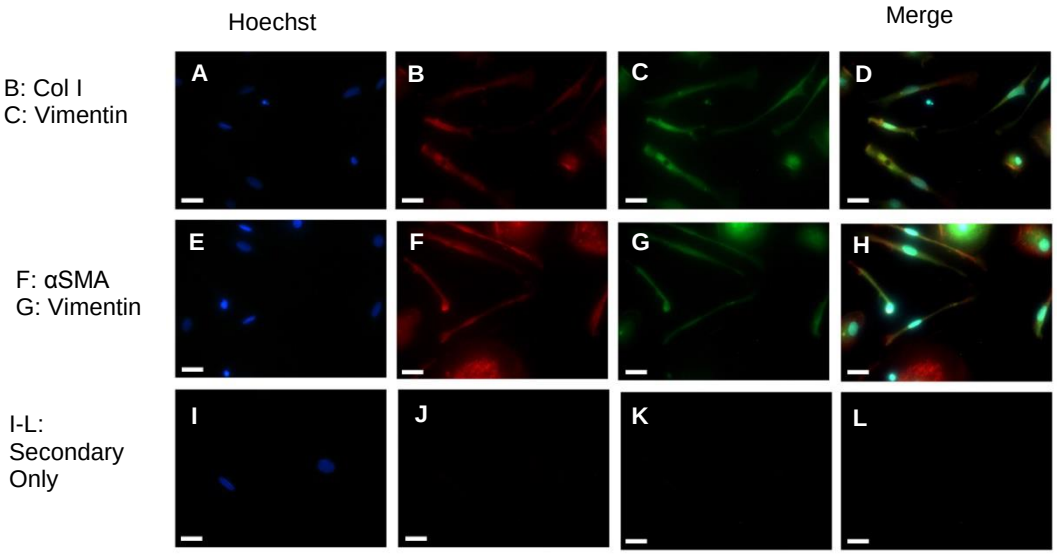


Figure 4. Immunohistochemical analysis of cultured CFPs. (A) Representative image of CFPs cultured from the peripheral blood of a normal control subject. Cells were stained with antibodies for Col I (red; B), Vimentin (green; C,H), and αSMA (red; G). Nuclei were stained with Hoechst dye (blue). Merged images for Col I and Vimentin (D,E) and αSMA and Vimentin (I,J). Secondary only control staining for Col I/ αSMA (L) and Vimentin (M). (B) Representative image of CFPs cultured from the peripheral

blood of a patient with metastatic STS (leiomyosarcoma). Cells were stained with antibodies for Col I (red; B), Vimentin (green; C,G), and αSMA (red; F). Nuclei were stained with Hoechst dye (blue). Merged images for Col I and Vimentin (D) and αSMA and Vimentin (H). Secondary only control staining for Col I/ αSMA (J) and Vimentin (K). Mag bars = 25 μm.

After confirming our ability to isolate and culture CFP-derived fibroblasts from the peripheral blood of normal control subjects and patients with STS, we aimed to further characterize these cultured CFPs through examination of mRNA expression levels for genes associated with a fibroblast-like phenotype via RT-qPCR (**Figure 5**). Our data indicate that we indeed isolated CFPs from both STS patients and control subjects, as they revealed mRNA expression of the following hematopoietic and fibroblastic markers: CD45, DDR2, Col1, Vimentin, and αSMA. Our data further indicated differences between STS patients and control subjects as CD45, Collagen I (Col1), and Vimentin expression was decreased, while DDR2 expression was increased in CFPs from patients with STS as compared to CFPs from normal control subjects. No significant difference in αSMA expression was found between the two cohorts.

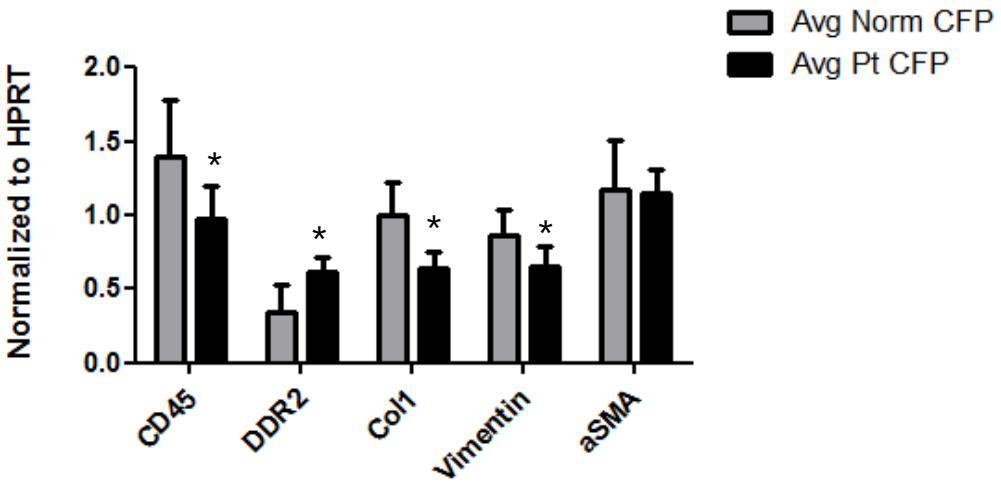


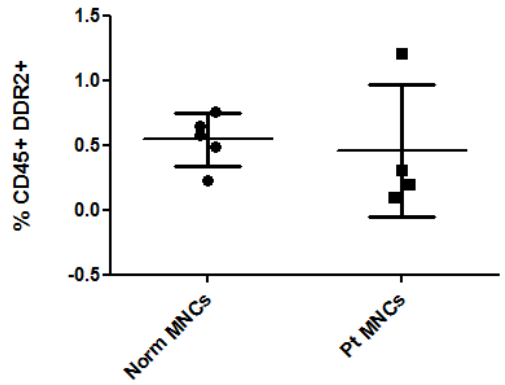
Figure 5. RT-qPCR characterization of CFPs from patients with STS

RT-qPCR of mRNA expression levels in cultured CFPs from patients with STS (Pt CFP) and normal control subjects (Norm CFP) normalized to HPRT. Each bar represents averaged expression for each group with n=4. *P<0.05 compared to Norm CFP.

Quantification of CFPs in Peripheral Blood of Normal Control Subjects and STS Patients

We then sought to determine whether CFP numbers varied in the circulation of patients with STS compared to normal control subjects without known malignancies. Peripheral blood MNCs were isolated (without culture), then stained with antibodies for CD45 (common leukocyte antigen) and discoidin domain receptor 2 (DDR2, a receptor for collagen) and analyzed via flow cytometry (**Figure 6**). We define our CFPs as $CD45^+$ $DDR2^+$, as these two markers indicate both the hematopoietic origin and fibroblastic phenotype of our cell population. Flow cytometric analysis of five normal control subjects without known malignancies was compared to four STS patients, three of whom had metastatic disease (**Table 4.4**). Flow cytometric analysis revealed no significant difference in the average percentage of CFPs present within the peripheral blood of patients with STS (mean ± standard deviation: 0.54 ± 0.20) compared to normal control subjects (mean ± standard deviation: 0.45 ± 0.51). However, when examining individual data points it is apparent that patient 1, who had metastatic STS without treatment, had the highest percentage of $CD45^+$ $DDR2^+$ CFPs with a 2.3 fold increase compared to average normal control. Patient 1 showed an increased percentage of CFPs over two other patients (patient 3, 4) who also had metastatic STS, but were undergoing chemotherapeutic treatment (with Yondelis). It was also noted that the patient without evidence of metastatic disease (patient 2) had a lower percentage of CFPs than either of the patients with metastasis or normal controls.

A.



B.

Sample	% $CD45^+$ $DDR2^+$
Norm 1	0.58
Norm 2	0.23
Norm 3	0.65
Norm 4	0.76
Norm 5	0.49
Pt 1	*1.21*
Pt 2	0.096
Pt 3	0.31
Pt 4	0.20

Figure 6. CFP quantification in human peripheral blood. Peripheral blood (PB) mononuclear cells (MNCs) were isolated from normal control subjects (Norm MNCs) and patients with Soft Tissue Sarcoma (Pt MNCs) and stained with antibodies for CD45 and DDR2. The percentage of $CD45^+$ $DDR2^+$ cells (CFPs) was analyzed via flow cytometry. (A) Summary of flow cytometric data is shown as % $CD45^+$ $DDR2^+$. (B) % CFPs shown for each subject.

Table 4.4 Patient Profiles for Figure 6

Patient	Age	Diagnosis	Current Treatment
1	61	Metastatic Synovial Sarcoma	None
2	66	Pleiomorphic Undifferentiated Sarcoma *without evidence of metastases*	None
3	72	Metastatic Angiosarcoma	Yondelis
4	52	Metastatic Synovial Sarcoma	Yondelis

Section 5: Discussion

Our lab previously demonstrated that HSCs are a novel source for CAFs and their blood precursors, CFPs, and that murine CFPs ($CD45^+$ $DDR2^+$) were present in the peripheral blood and increased with tumor burden[13,14]. However, the identification and characterization of human CFPs in cancer, specifically soft tissue sarcoma, had not been established. Thus, the goal of this study was to isolate, characterize, and quantify CFPs in patients with STS. Our data revealed that our isolation technique for obtaining the non-adherent population of MNCs was sufficient for enriching for $CD45^+$ HSC-derived cells and that this enrichment was enhanced with increasing culture time (from 2 hrs to 20 hrs). Immunohistochemical analysis of the cultured non-adherent MNC population from the peripheral blood of patients with metastatic STS and normal control subjects revealed CFPs were present in this fraction of human peripheral blood as these cultured cells displayed fibroblastic marker expression for collagen 1 (Col I), alpha smooth muscle actin (αSMA), and vimentin. Fibroblasts are known to secrete Col I, an important protein found in the extracellular matrix (ECM) and to express vimentin, a member of the intermediate filament family. αSMA is implicated in cell contractility and motility and fibroblast expression of αSMA has been associated with a myofibroblast or activated phenotype and is upregulated by TGF-β, particularly in the tumor environment[88,89].

RT-qPCR data revealed differences between STS patients and normal control subjects in mRNA expression of hematopoietic and fibroblastic markers. The decrease in CD45 expression in CFPs from patients with STS as compared to normal control subjects is of interest as studies have shown that CD45 is downregulated as fibrocytes (a cell type similar to that of CFPs) differentiate[90,91]. This may indicate that CFPs from patients with STS are further differentiated compared to CFPs from normal control

subjects. The increase in DDR2 expression in patients with STS compared to control subjects is also of interest as studies have shown that fibroblast spreading and migration is dependent upon DDR2 [92–94]. Additionally the decrease in Col1 expression, a ligand for DDR2, may cause DDR2 on the CFPs to not be saturated with collagen via autocrine signaling, potentially freeing DDR2 to bind to collagen at sites further away (such as the lung- a common site of STS metastasis), aiding in CFP migration. Thus, our RT-qPCR data may suggest that CFPs from patients with STS display a more differentiated and migratory phenotype compared to normal control subjects.

Flow cytometric analysis of $CD45^+$ $DDR2^+$ cells revealed no significant difference in the average percentage of CFPs present within the peripheral blood of patients with STS compared to normal control subjects. However, it was found that patient 1 who had metastatic STS without treatment showed the highest percentage of $CD45^+$ $DDR2^+$ CFPs than any of the subjects, with a 2.3 fold increase compared to average normal control. Patients with metastatic STS were shown to have a higher percentage of CFPs compared to a patient with non-metastatic STS (patient 2). Patient 2, without evidence of metastatic disease, showed a lower percentage of CFPs than patients with metastatic disease and even that of normal control subjects. Given that this was the only non-metastatic patient in this data set, it remains to be determined if this CFP profile would hold for other patients without evidence of metastatic disease. If so, it may indicate that CFPs are a unique marker for the metastatic phase of disease. Of further interest is that patient 1 showed an increased percentage of CFPs over two other patients (patient 3, 4) who also had metastatic STS, but were undergoing chemotherapeutic treatment. This may suggest chemotherapeutic treatment functions in part by suppressing the CFP population, reinforcing the idea of an important role for CFPs in metastatic disease. This data may also implicate percentage of CFPs as a biomarker for response to

chemotherapeutic treatment in metastatic disease. Given the small number of samples, further analysis of patients with metastatic STS receiving chemotherapeutic treatment should be compared to patients without treatment in order to determine if this trend is applicable to the majority of STS patients and whether this trend is associated with certain STS subtypes. This knowledge would be especially efficacious in the treatment of patients with STS because chemotherapeutic agents have exhibited varied response rates, making it a challenge for physicians to identify which patients will benefit from treatment.

Chapter 5: Specific Aim 2

To determine the role of CFP-derived fibroblasts from patients with soft tissue sarcoma in promoting proliferation, migration, and invasion of sarcoma cells.

Section 1: Introduction

Cancer-associated fibroblasts (CAFs), some of which are potentially derived from circulating fibroblast precursors (CFPs), are mature tissue fibroblasts associated with the solid tumor and have been shown to promote tumor progression by stimulating angiogenesis, cancer cell growth, invasion and metastasis, and through evasion of the immune response. CAFs implement these effects by production of various growth factors (such as VEGF, a potent angiogenesis inducer), matrix metalloproteinases (such as MMP9 which degrades collagen and fibronectin, allowing the tumor cell to transverse the basement membrane), and through production of chemokines and cytokines. Our lab has shown that CFPs, a precursor to CAFs, contribute to tumor growth and migrate in response to monocyte chemoattractant protein-1 (MCP-1), suggesting that CFPs may play a direct role in promoting the progression and metastasis of solid tumors. We, therefore, aimed to test the hypothesis that CFP-derived fibroblasts play a functional role in soft tissue sarcoma metastasis through production of specific chemokine, cytokine, and growth factors involved in promoting the proliferation, migration, and invasion of sarcoma cells.

Section 2: Hypothesis

We hypothesized that CFP-derived fibroblasts, through production of specific chemokine, cytokine, and growth factors promoted proliferation, migration, and invasion of sarcoma cells.

Section 3: Materials and Methods

Cell Culture of Human Peripheral Blood

Peripheral blood (9-15 mL) was drawn from patients with soft tissue sarcoma and from control subjects (All Cells #WB001-NH) without any known malignancies and collected in sodium heparin-coated vacutainer tubes (BD #367878). Mononuclear cells were isolated via density centrifugation with Lympholyte H (Cedarlane #CL5010) and plated on a T25 tissue culture treated flask (Corning #430639) and incubated at 37°C in free gas exchange at 5% CO_2. After a 20 hour incubation period, non-adherent HSC-derived mononuclear cells were removed and plated on 4-well fibronectin-coated tissue culture plates (Corning #354559) for 14-21 days at a concentration of $6x10^5$ cells per well in 1 mL of Minimum Essential Medium α (MEMα) (Life Technologies #12561) supplemented with 20% fetal bovine serum (Atlanta Biologicals, lot #6005C) and penicillin streptomycin in order to promote the differentiation of CFPs into fibroblasts (CFP-derived fibroblasts). Cells were then gently washed 3 times in MEMα and serum starved for 24 hours in 0.5 mL MEMα per well ($6x10^5$cells/ well). CM was collected and filtered to remove debris, then used for proliferation, migration, and invasion experiments (**Figure 7**).

In vitro Generation of Tumor-Exposed CFPs

HT-1080 Conditioned Media (CM) was obtained by culturing $3x10^6$ HT-1080 cells in a T75 tissue culture plate (Corning #353136), then cells were gently washed 3 times in MEMα to remove residual growth media, and cells were serum starved in 10 mL MEMα (Life Technologies #12561) for 24 hours. After 24 hours, HT-1080 CM was collected and filtered to remove debris.

Peripheral blood was obtained from control subjects (All Cells #WB001-NH) without any known malignancies and collected in sodium heparin-coated tubes. Mononuclear cells were isolated via density centrifugation with Lympholyte H (Cedarlane #CL5010) and plated on a T25 tissue culture treated flask (Corning #430639) and incubated at 37°C in free gas exchange at 5% CO_2. After a 20 hour incubation period, non-adherent HSC-derived mononuclear cells were removed and plated on 4-well fibronectin-coated tissue culture plates (Corning #354559) at a concentration of $6x10^5$ cells per well in 1 mL of either MEMα (Life Technologies #12561) supplemented with 20% fetal bovine serum (Atlanta Biologicals, lot #6005C) and penicillin streptomycin (*Normal Control*) or HT-1080 CM supplemented with 20% fetal bovine serum (Atlanta Biologicals, lot #6005C) and penicillin streptomycin (*Tumor-Exposed*) and cultured for growth of CFP-derived fibroblasts. Normal Control and Tumor-Exposed CFP-derived fibroblasts were cultured in their designated media for up to 15 days with media renewal every 3 days after the first week of culture. Normal Control and Tumor-Exposed cells were then gently washed 3 times in MEMα and serum starved for 24 hours in 0.5 mL MEMα per well ($6x10^5$ cells/well). CM was then collected and filtered to remove debris, then used for proliferation, invasion, and cytokine array experiments as an *in vitro* model of Tumor-Exposed CFPs (**Figure 8, 9**).

Proliferation Assay

$1x10^3$ HT-1080 (human fibrosarcoma) cells were plated in triplicate in 150 µL of CM in individual wells of a 96-well tissue culture treated plate (Corning #3799). Proliferation was measured using CyQuant Direct Cell Proliferation Assay (Invitrogen #C35011) according to manufacturer's protocol. Fluorescence was read at 480nm

excitation and 535nm emission on a BioTek Synergy HT microplate reader using Gen 5 software.

Migration and Invasion Assay

Prior to migration assay, inserts (Costar #3422 transwell permeable support 8.0µm polycarbonate membrane) were coated with fibronectin (5 µg/mL) and incubated 1 hour at 37°C, then air dried. 600 µL of conditioned media was placed in the bottom of individual wells (with 3 replicate wells) of a 24-well non-tissue culture treated plate (Corning #351147), then 1.5×10^4 HT-1080 (human fibrosarcoma) cells in 100 µL αMEM were placed in each insert.

Prior to invasion assay, invasion chambers (BD #354480 BioCoat BD Matrigel Invasion Chamber, 8.0 µm PET Membrane) were allowed to come to room temperature. Then inserts were rehydrated for 2 hours at 37°C in warm DMEM. After rehydration, 750 µL of conditioned media was placed in bottom of individual wells (with each sample plated in triplicate wells) of a 24-well non-tissue culture treated plate (Corning #351147), then 5.0×10^4 HT-1080 cells in 500 µL αMEM was placed in each insert.

After 8 hours (migration) or 22 hours (invasion) incubation at 37°C in free gas exchange at 5% CO_2, inserts were removed and the top of each insert was gently scrubbed with a q-tip to remove cells that have not invaded through the membrane. Inserts were then stained with the Diff-Quik stain set (Siemens #B4132-1A). 10 high powered fields were counted on each insert using a Nikon Eclipse Ti-U microscope with NIS-Elements BR3.2 software.

Cytokine Array

Levels of cytokines in CM were measured with the Human XL Cytokine Array Kit (R&D Systems #ARY022) according to manufacturer's protocol with the use of 500 µL CM in 1 mL Array Buffer 6 for each membrane. Membranes were then exposed to autoradiography film (MidSci #EBNU2) and average pixel density for each cytokine was analyzed using ImageJ software (NIH).

Human Consent

Peripheral blood was obtained after informed consent was given and subjects were enrolled into our Institutional Review Board approved study (CTO 101576) through the MUSC Hollings Cancer Center. All subjects consented were over 18 years of age and had soft tissue sarcoma, either metastatic or non-metastatic.

Statistical Data Analysis

Data are presented as mean ± standard deviation of the value. To compare variables between 2 groups, the two-tailed unpaired Student t-test was performed, with significance determined as $p<0.05$.

Section 4: Results

CFP-derived fibroblasts from patients with STS promote HT-1080 fibrosarcoma proliferation, migration, and invasion

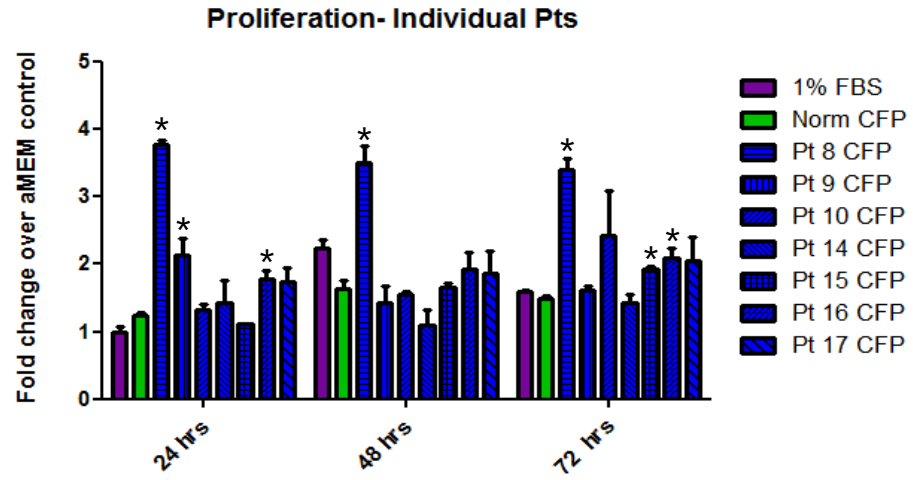
HSC-derived CFPs are a potential source for CAFs, which are known to promote tumor cell proliferation, migration, and invasion, all essential elements of metastatic progression[95–97]. Although CAFs have not been investigated in STS progression or metastasis, given our identification of CFPs in patients with STS, we sought to investigate the functionality of these cells, particularly in relation to their ability to support tumor advancement. In order to examine whether CFP-derived fibroblasts from patients with STS promote tumor cell progression, conditioned media (CM) was collected from cultured CFP-derived fibroblasts from either patients with STS or normal control subjects and assays were performed to analyze the ability of conditioned media from CFP-derived fibroblasts to promote HT-1080 (human fibrosarcoma) cell proliferation, migration, and invasion. Due to the rarity and heterogeneity of STS, our studies were performed on CFP-derived fibroblasts from patients with various types of STS. As there are not cell lines for most types of STS, we utilized the HT-1080 cell line from ATCC as a standard cell line in each of our assays. Diagnosis and treatment status of patients is found in **Table 5.1**.

One of the hallmarks of cancer progression is the ability of a tumor cell to sustain proliferation[97]. Tumor cells continually proliferate through production of growth factor ligands, resulting in autocrine stimulation or through secretion of factors to normal cells in the surrounding environment, which may then respond to the tumor by supplying it with growth factors[95]. Our studies found that CFPs from patients with STS were capable of promoting HT-1080 tumor cell proliferation over αMEM (**Figure 7A**). CFPs from one patient in particular, patient 8, who had metastatic myxoid liposarcoma, significantly

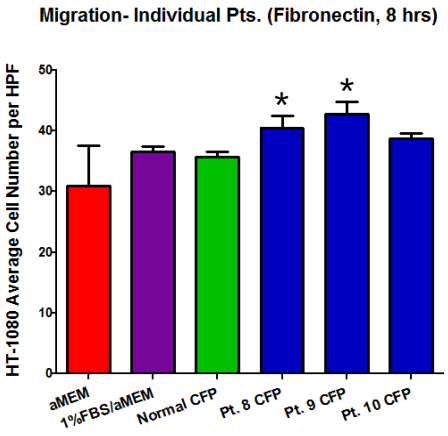
enhanced HT-1080 tumor cell proliferation over normal control subjects during all 3 time points. CFPs from patient 15 and 16, who also had metastatic myxoid liposarcoma showed significant increases in HT-1080 proliferation after 72 hours, potentially suggesting CFPs from patients with the myxoid liposarcoma subtype of STS have enhanced ability to promote sarcoma tumor cell proliferation.

Another hallmark of tumor progression is the activation of invasion and metastasis[97]. This requires chemotactic migration of tumor cells, along with the ability of the tumor cell to protrude its cell membrane while remodeling and degrading the extracellular matrix (ECM)[98]. During invasion, matrix degrading proteases, either produced by the tumor cell or recruited inflammatory cells present in the tumor microenvironment, produce MMPs that play an important role in tumor escape[97]. In order to examine whether CFPs were capable of enhancing sarcoma migration and invasion, transwell assays were performed using CFP conditioned media (CM) as a chemoattractant. Our data indicate that CFPs from two of the three patients with STS promoted HT-1080 tumor cell migration over normal control (**Figure 7B**). Furthermore, CM from CFPs from all patients with STS showed increased HT-1080 invasion over αMEM. We found enhanced HT-1080 invasion toward CFP CM in 4 out of 9 patients with STS over normal control subjects (**Figure 7C**, asterisks).

A.



B.



C.

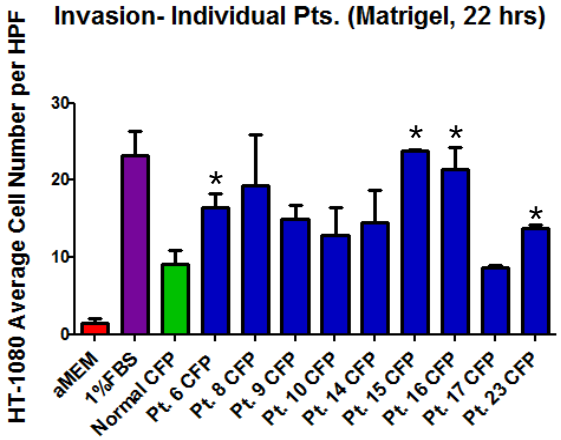


Figure 7. CFPs from patients with STS promote HT-1080 sarcoma cell proliferation, migration, and invasion. Conditioned media (CM) from cultured CFP-derived fibroblasts from patients (Pt) with STS (individual results) and normal (Norm) control subjects (averaged, n=4) was examined *in vitro* for ability to promote HT-1080 fibrosarcoma proliferation after 24, 48 and 72 hours (A), ability to promote HT-1080 migration (B) through 0.8µm pore fibronectin coated transwells and invasion (C) through 0.8µm pore matrigel coated transwells. 1% FBS served as positive control. For

proliferation assay, fold change over αMEM is presented. * P<0.05 compared to normal CFP (A,B,C). Note in C, all patients showed significant increase (p<0.05) over αMEM control.

Table 5.1 Patient Profiles for Figure 7

Patient #	Age	Diagnosis	Current Treatment
6	42	Metastatic Uterine Leiomyosarcoma	Yondelis
8	44	Metastatic Myxoid Liposarcoma	Yondelis
9	44	Metastatic Mixed Mullerian tumor	Yondelis
10	48	Metastatic Synovial Sarcoma	Temsirolimus
14	41	Metastatic Pleomorphic Sarcoma	Yondelis
15	52	Metastatic Myxoid Liposarcoma	Yondelis
16	48	Metastatic Myxoid Liposarcoma	Yondelis
17	55	Metastatic Leiomyosarcoma	Yondelis
23	79	Metastatic Spindle Cell Sarcoma	None

In vitro tumor-exposed CFPs promote HT-1080 fibrosarcoma invasion and display altered cytokine profiles compared to control CFPs.

Our *in vitro* data with CM from patients with STS suggests CFP-derived fibroblasts promote HT-1080 proliferation, migration, and invasion over αMEM, although not all patient CFPs show a significant difference in ability to do so compared to normal control CFPs (**Figure 7**). A complication of these studies was the use of the HT-1080 fibrosarcoma cell line for assays with STS patients that had other types of STS, meaning the HT-1080 fibrosarcoma tumor cell type used in the previous assays did not match the tumors of the individual STS patients. The rarity and heterogeneity (over 100 different subtypes) of STS means there are few cell lines available and cell lines have not been created for each STS subtype. Thus, we aimed to create an *in vitro* culture method for generating tumor-exposed CFPs (an *in vitro* analogue of CFPs derived from patients with STS) that could then be used in proliferation and invasion assays with tumor cells of the same subtype to which the CFPs were exposed to.

Peripheral blood was obtained from normal control subjects without any known malignancies and cultured for growth of CFP-derived fibroblasts in either αMEM supplemented with 20% fetal bovine serum and penicillin streptomycin (normal control) or HT-1080 CM supplemented with 20% fetal bovine serum and penicillin streptomycin (tumor-exposed). Normal control and tumor-exposed CFP-derived fibroblasts were cultured in their designated media for up to 15 days with media renewal every 3 days after the first week of culture. CM was then collected from normal control and tumor-exposed CFPs for use in proliferation, invasion, and cytokine array assays (**Figure 8**). Because CFPs are present in the peripheral blood, our chosen method for generating *in vitro* tumor-exposed CFPs was co-culture of CFPs with tumor CM, rather than direct cell-to-cell contact with tumor cells as may be important for studies of tumor-stroma

interactions. Exposure to tumor CM is therefore a better model for our studies in order to study CFP response to tumor secreted proteins in the peripheral blood.

Our data indicated that tumor-exposed CFP CM promoted HT-1080 tumor cell proliferation over αMEM and 1% FBS positive control, although not significantly over normal control CFP CM. Tumor-exposed CFP CM was further found to promote HT-1080 invasion over normal control CFPs (2.25 fold increase), implicating that CFPs may play a more important role in tumor invasiveness through production of matrix degrading proteins than through secretion of growth factors involved in tumor cell proliferation.

Cytokine array analysis revealed an altered cytokine profile of tumor-exposed CFP CM compared to normal control CFP CM (**Figure 9**). Factors found to be increased more than 2 fold in tumor-exposed CFP CM included CD14, Complement Factor D, EMMPRIN, CXCL5, IL-8, MCP-1, MIF, Serpin E1, and uPAR, with MCP-1 having the highest expression. Additionally, RANTES, although lowly expressed in control CFPs, was found to be decreased in tumor-exposed CFP CM. Our cytokine array data suggest CFP exposure to tumor secreted factors found in HT-1080 CM alters the CFP expression profile. Tumor crosstalk between cells of the stroma and cells in circulation is known to play an important role in tumor progression. Our data implicate this crosstalk is causing enhanced expression in CFPs of proteins important to tumor advancement.

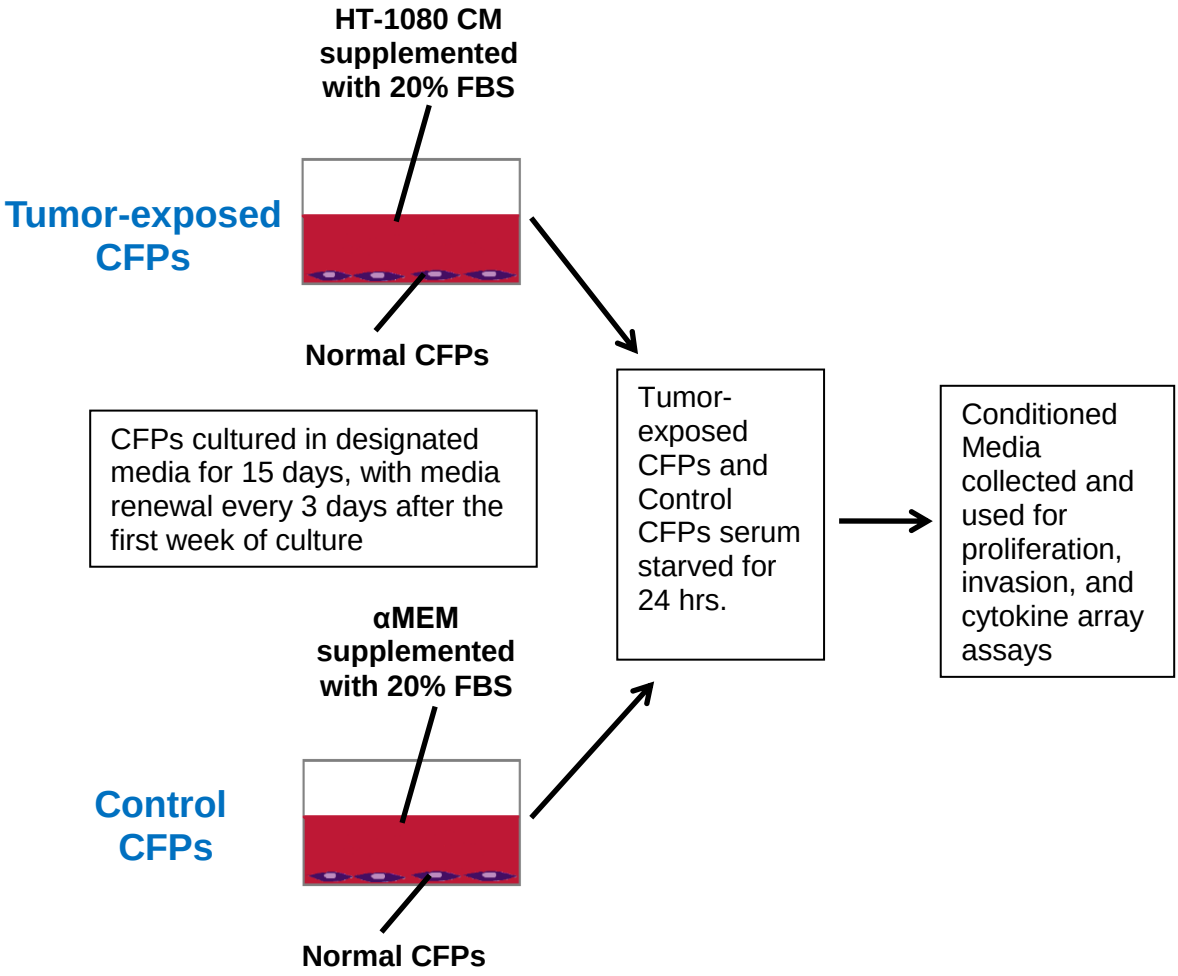


Figure 8. *In vitro* generation of tumor-exposed CFPs. Tumor-exposed CFPs were generated *in vitro* by culturing normal CFPs in HT-1080 fibrosarcoma conditioned media (CM) supplemented with 20% FBS for 15 days with media renewal every 3 days after the first week of culture. Control CFPs were cultured in αMEM supplemented with 20% FBS for 15 days with media renewal every 3 days after the first week of culture. Media was then removed from cells, and cells were serum starved for 24 hours to obtain conditioned media to be used in subsequent assays.

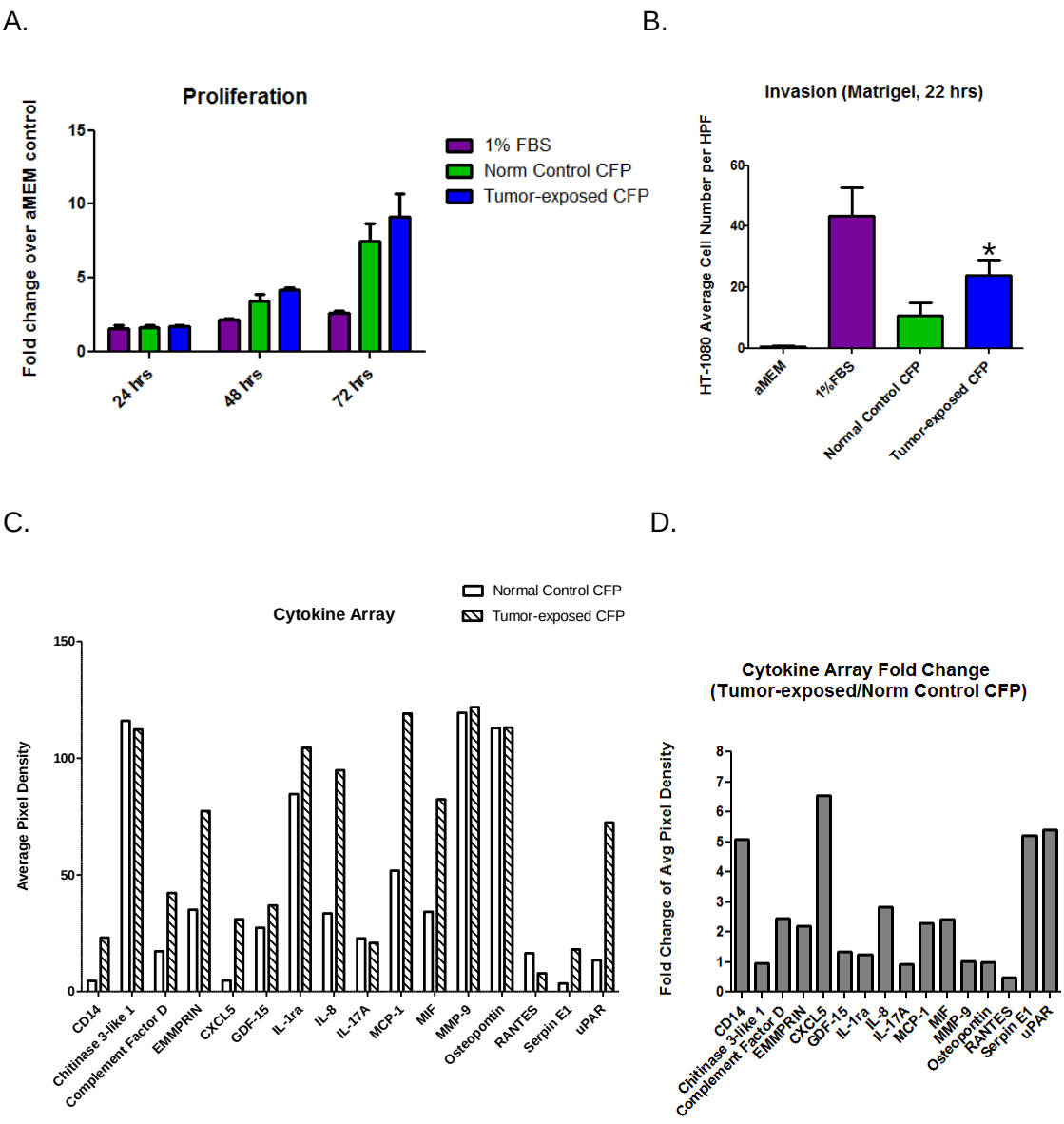


Figure 9. *In vitro* generated tumor-exposed CFPs promote invasion of tumor cells along with an altered cytokine profile compared to control CFPs. Conditioned media (CM) was collected from normal control CFP-derived fibroblasts (Norm CFP) or from norm CFP-derived fibroblasts exposed to HT-1080 CM (Tumor-exposed CFP) and examined *in vitro* for ability to promote HT-1080 fibrosarcoma proliferation after 24, 48 and 72 hours (A) and ability to promote HT-1080 invasion (B) through 0.8µm pore

fibronectin matrigel coated transwells after 22 hours. 1% FBS served as positive control. CM from Norm Control CFP and Tumor-exposed CFPs was subjected to cytokine array analysis and average pixel density was analyzed by ImageJ software (C). Fold change of average pixel density of Tumor-exposed CFP over Norm CFP CM was then assessed (D). * $P<0.05$ compared to normal control CFP CM (A,B).

Section 5: Discussion

Cancer-associated fibroblasts (CAFs), have been shown to promote tumor progression in numerous tumor types, although they have not be investigated in STS. CAFs accelerate tumor progression by stimulating angiogenesis, cancer cell growth, invasion and metastasis, and through evasion of the immune response. CAFs implement these effects by production of various growth factors, matrix metalloproteinases (such as MMP9 which degrades collagen and fibronectin, allowing the tumor cell to transverse the basement membrane), and through production of chemokines and cytokines. Our lab has shown that CFPs, a precursor to CAFs, contribute to tumor growth and migrate in response to monocyte chemoattractant protein-1 (MCP-1), suggesting that CFPs may play a direct role in promoting the progression and metastasis of solid tumors[14]. It was, therefore, the goal of our study to test whether CFP-derived fibroblasts play a functional role in soft tissue sarcoma metastasis through production of specific chemokine, cytokine, and growth factors involved in promoting the proliferation, migration, and invasion of sarcoma cells.

Our data suggest that CFPs from both patients with STS and normal control subjects promoted sarcoma tumor cell proliferation, migration, and invasion over αMEM (control media). CFPs from some patients with STS further showed enhanced ability to promote tumor cell proliferation, migration, and invasion over normal control CFPs. CFPs from the three patients with metastatic myxoid liposarcoma further displayed enhanced ability to promote HT-1080 proliferation over normal control subjects, with one of these patients (patient 8) showing an appreciable enhancement even over other STS patients. Because we often receive a limited amount of peripheral blood from patients, not all patients had enough cells to conduct proliferation, migration, and invasion assays. However, CFPs for patients 8, 9, and 10 were able to be analyzed for ability to promote

HT-1080 proliferation, migration, and invasion. Patient 8, with metastatic myxoid liposarcoma and Yondelis treatment showed increased ability to promote proliferation and migration over normal controls. Patient 9, with metastatic mixed mullerian tumor, showed increased ability to promote migration over normal controls, whereas patient 10, with metastatic synovial sarcoma and Temsirolimus treatment did not show a significant increase over normal control subjects in any assay. Acquiring more patients with varying STS subtypes and chemotherapeutic treatments would allow us to analyze whether these effects are subtype or treatment related and allow us to determine whether these results correlate with patient prognosis.

A complication of our studies using the HT-1080 fibrosarcoma cell line for proliferation, migration, and invasion assays was that STS patients had STS subtypes that did not match that of the HT-1080 fibrosarcoma tumor cell being used in the previous assays. Thus, we aimed to create an *in vitro* culture method for generating tumor-exposed CFPs. Our *in vitro* generated tumor-exposed CFPs displayed an increased potential to promote tumor proliferation and invasion, with invasive ability being significant over normal control CFPs. The *in vitro* generated tumor-exposed CFPs revealed altered cytokine profiles compared to normal control CFPs with many proteins of interest in regards to tumor progression increased over that of normal control CFPs. Cytokines including EMMPRIN, MCP-1, IL-8, uPAR, and Serpin E1 showed increased expression in tumor-exposed CFPs compared to normal control CFPs. EMMPRIN is known to induce MMP synthesis by both tumor cells and fibroblasts, potentiating tumor cell invasiveness in the tumor microenvironment and leading to the induction of angiogenesis through stimulation of VEGF production[99]. The induction of MMP synthesis through EMMPRIN may correlate with our invasion studies where tumor-exposed CFP ability to promote tumor invasion was significantly increased compared to control CFPs.

EMMPRIN has further been implicated as a prognostic marker for osteosarcoma when combined with MMP expression[100]. MCP-1 was found to be an important cytokine involved in trafficking of HSC-derived CFPs in our lab's previous murine tumor studies[14]. These studies revealed that CFP migration toward tumor conditioned media was MCP-1 dependent and it was shown that MCP-1 inhibition blocked tumor progression *in vivo*. IL-8 is a proinflammatory cytokine and its expression has been shown to correlate with tumorigenicity, angiogenesis, and metastasis in numerous *in vivo* tumor models[101–103]. UPAR plays an important role in tumorigenicity, ECM degradation, and angiogenesis[104]. Studies have demonstrated that co-detection of uPAR in tumor tissue and serum correlated with poor prognosis in patients with STS[105]. Serpin E1 is an inhibitor of tissue plasminogen activator and urokinase (uPA, an ECM degrading protease). Human fibrosarcoma cells transfected with Serpin E1 cDNA and then injected into the tail vein of mice led to increased number and incidence of lung colonization[106]. Furthermore, it was found that UPAR and Serpin E1 are induced in migratory cells and focalize to the leading edge where they function in matrix restructuring and migration[107]. Therefore, our data may indicate that after exposure to tumor cells, CFPs have enhanced abilities to function in migration and matrix restructuring. Overall, our data suggest CFPs from patients with STS promote tumor progression and *in vitro* generated tumor-exposed CFPs have enhanced potential to promote tumor progression in relation to normal control CFPs.

Chapter 6: Conclusions and Future Directions

Soft tissue sarcomas (STS) are rare, heterogeneous malignant tumors of mesenchymal origin that manifest in the connective tissues. The most lethal aspect of sarcomas is their propensity for hematogenous dissemination leading to metastatic disease. After development of distant metastases, the median survival for patients with STS is merely 11 to 15 months[1,2]. Therefore, gaining a better understanding of the metastatic environment is essential to developing new therapies.

The tumor microenvironment has been implicated as an essential component for metastatic progression and one of the most prominent cell types of the tumor microenvironment is the cancer-associated fibroblast (CAF)[3,4]. Studies clearly show the importance of CAFs in tumor advancement; however, their origins have not been conclusively determined[7–12]. Our lab previously demonstrated a novel hematopoietic stem cells (HSCs) source for CAFs and their blood precursors, circulating fibroblast precursors (CFPs)[13]. Our lab further revealed that murine CFPs were present in peripheral blood, found *in vivo* in the tumor stroma, and increased with increasing tumor burden, potentially implicating CFPs in the progression of solid tumors[14]. Additionally, our lab revealed a HSC origin for human CFPs[15]. The function of human CFPs in tumor metastasis, specifically STS, however, had yet to be established. It was therefore, the goal of this study to isolate and evaluate the peripheral blood of patients with STS in order to characterize CFPs in these patients and determine their role in promoting STS tumor progression.

Our data indicated that our isolation technique for obtaining the non-adherent population of MNCs was sufficient for enriching for $CD45^+$ HSC-derived cells and that this enrichment was enhanced with increasing culture time (from 2 hrs to 20 hrs). Immunohistochemical analysis of the cultured non-adherent MNC population from the peripheral blood of patients with metastatic STS and normal control subjects revealed

CFPs were present in this fraction of human peripheral blood, displaying fibroblastic marker expression for collagen 1 (Col I), alpha smooth muscle actin (αSMA), and vimentin. In order to quantify CFPs in the peripheral blood of patients with STS, flow cytometric analysis of $CD45^+$ $DDR2^+$ cells (CFPs) was performed. Although there was no significant difference in the average percentage of CFPs present within the peripheral blood of patients with STS compared to normal control subjects, changes in percentage of CFPs were observed between individual STS patients with different treatment regimens and disease stages. The patient with metastatic disease without chemotherapeutic treatment showed significant increase in percentage of CFPs over other patients and control. Furthermore, patients with metastatic STS were shown to have a higher percentage of CFPs compared to a patient with non-metastatic STS. This data may suggest chemotherapeutic treatment functions in part by suppressing the CFP population and may also implicate percentage of CFPs as a biomarker for response to chemotherapeutic treatment.

We then went on to test whether CFP-derived fibroblasts played a functional role in STS metastasis through the promotion of sarcoma cell proliferation, migration, and invasion via production of specific chemokine, cytokine, and/or growth factors. Our data suggest that CFPs from both patients with STS and normal control subjects promoted tumor cell proliferation, migration, and invasion over αMEM (control media). CFPs from some patients with STS further showed enhanced ability to promote tumor cell proliferation, migration, and invasion over normal control CFPs. CFPs from patients with metastatic myxoid liposarcoma (n=3) further displayed enhanced ability to promote HT-1080 proliferation over normal control subjects, with one of these patients (patient 8) showing an appreciable enhancement even over other STS patients.

In vitro generated tumor-exposed CFPs additionally displayed an increased potential to promote tumor proliferation and invasion, with invasive ability being significant over normal control CFPs. *In vitro* generated tumor-exposed CFPs revealed altered cytokine profiles compared to normal control CFPs with increased expression of cytokines including EMMPRIN, MCP-1, IL-8, MIF, and uPAR, all known to play important roles in tumor advancement. Together, our data demonstrate that CFPs promote the invasive/metastatic potential of STS cells and suggest this is affected, at least in part, by paracrine factors.

Future directions for the continued characterization of CFPs in patients with STS should include increased STS patient accrual in order to identify differences in percentage of CFPs based upon subtype, tumor stage (metastatic vs. non-metastatic), and treatment. This knowledge will further enhance our understanding of the role of CFPs in tumor progression and allow us to determine whether percentage of CFPs in the peripheral blood correlates with STS subtype, tumor stage, and or treatment and if this may be a predictor of patient response to chemotherapy. This knowledge would be especially efficacious in the treatment of patients with STS because chemotherapeutic agents have exhibited varied response rates, making it a challenge for physicians to identify which patients will benefit from treatment. Thus, it may spare patients who would not respond to chemotherapy from possible toxicity-related side effects of treatment. Additionally, it would be beneficial to examine STS tumor sections in primary tumor and metastatic sites and identify presence of CFPs to determine whether CFP numbers at the tumor site correspond with numbers of circulating CFPs in the peripheral blood of patients with STS and whether these numbers are indicative of prognosis. Tumor samples from patients with STS, as examined through Oncomine data, have been shown to express DDR2 (one of the markers of a CFP), potentially implicating it as

an important factor in this disease, justifying DDR2 as a focus for future studies[108–113]. Further *in vitro* analysis of CFP function may also be performed to assess cytokine role in tumor progression. For example, we saw increased EMMPRIN, MCP-1, IL-8, MIF, and uPAR expression in our *in vitro* tumor-exposed CFPs compared to control CFPs and through specifically blocking these cytokines *in vitro*, we may determine their role in promoting sarcoma cell proliferation, migration, and invasion, potentially identifying factors that could be blocked in the clinical setting during therapeutic treatment.

The end goal of these studies is improved outcome for patients with metastatic STS. We hope that by better characterizing CFPs and their functional roles in patients with STS, we may identify markers of disease progression that will assist clinicians in determining which patients will possibly benefit from chemotherapeutic treatment, while sparing those who will not benefit from possible toxicity-related side effects of tumor treatment.

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